ASSESSING CONTRIBUTION OF THE *STREPTOCOCCUS MUTANS CID AND LRG* OPERONS TO OXIDATIVE STRESS RESISTANCE AND COMPETENCE

By

O'NESHIA VANAE CARNEY

A THESIS PRESENTED TO THE GRADUATE SCHOOL
OF THE UNIVERSITY OF FLORIDA IN PARTIAL FULFILLMENT
OF THE REQUIREMENTS FOR THE DEGREE OF
MASTER OF SCIENCE

UNIVERSITY OF FLORIDA

2014

To my beautiful family and in remembrance of my great-grandmother

ACKNOWLEDGMENTS

First and foremost, I would like to express my deepest appreciation to my parents for always supporting me throughout my endeavors. Thank you to my brothers for always reminding me that I am their prestigious role model. I offer my sincerest gratitude to my thesis mentor, Dr. Kelly C. Rice, for supporting me throughout my research with patience and accepting me into her lab without hesitation. I attribute the level of my Master's degree to her encouragement and effort. I simply could not have wished for a better or friendlier principal investigator. I thank my lab mates April Sapp, Austin Mogen, Silvia Matzdorf, and Ieisha Collins for their continuous guidance and assisting me with my adjustment to graduate school. In addition, many thanks to Dr. Sang-Joon Ahn, Dept. Oral Biology, UF, for helpful discussions and for providing plasmid pGB-E. Finally, I thank my committee members Drs Kelly Rice, Tony Romeo, Nematollah Keyhani and the Department of Microbiology and Cell Science at the University of Florida that have provided the support and equipment I have needed to produce and complete my thesis.

TABLE OF CONTENTS

page

LIST OF TABLES

LIST OF FIGURES

Abstract of Thesis Presented to the Graduate School
of the University of Florida in Partial Fulfillment of the
Requirements for the Degree of Doctor of Philosophy

ASSESSING CONTRIBUTION OF THE *STREPTOCOCCUS MUTANS CID AND LRG* OPERONS TO OXIDATIVE STRESS RESISTANCE AND COMPETENCE

By

O'neshia Vanae Carney

August 2014

Chair: Kelly C. Rice
Major: Microbiology and Cell Science

S. mutans is a common member of the oral cavity microbiota, but when this organism predominates in dental plaque biofilm, it can cause dental cavities (caries). Published and unpublished studies from our research lab have shown that components of the *cid* and *lrg* operons can affect *S. mutans* cell death and biofilm formation. These studies also demonstrated that components of the *lrg* operon affect *S. mutans* oxidative stress resistance as well as competence. Therefore, this research addresses the hypothesis that components of the Cid/Lrg system may represent previously unrecognized modulators of the *S. mutans* oxidative stress and competence systems. Hydrogen peroxide (H_2O_2) is produced by competing streptococcal species in dental plaque biofilm, and is also a common component of commercially-available mouthwash formulations. Therefore, the sensitivity of *S. mutans* and its panel of isogenic *cid* and *lrg* mutants to this biologically relevant oxidative stress were assessed by growth curve analysis. These studies revealed that the *lrgAB, cidB* and *cidAB* mutants displayed severe growth inhibition in the presence of 1.0 mM H_2O_2. Quantitative competence assays also revealed that the *cidB* mutant displayed decreased natural competence compared to the wild-type strain. However, competence of the *cidB* mutant could be restored to wild-type levels in the presence of exogenously-added competence stimulating peptide (CSP), a *S. mutans* quorum-sensing signal

that controls expression of various competence genes. The addition of 0.25 mM H_2O_2 did not show an effect on *S. mutans* biofilm formation. The *cidB* mutant exhibited less biofilm formation when grown in biofilm media (BM) supplemented with 18 mM glucose/2 mM sucrose, relative to the wild-type strain. When the carbohydrate content in BM changed to 11 mM/10 mM sucrose, the *cidB* mutant biofilm phenotype changed from reduced biofilm to a more pronounced biofilm relative to the wild-type strain. Future research efforts will try to elucidate the mechanism behind the *cidB* mutant competence and biofilm phenotypes, assessing the impact of *cid* genes on eDNA release, and complementation of the *cidB* mutation. This research will contribute to our long-term overall goal of better understanding *S. mutans* virulence and survival in the oral cavity.

CHAPTER 1
INTRODUCTION

Streptococcus mutans Metabolism

Streptococcus mutans is a non-motile, gram-positive coccus that lives in the human oral cavity, and is considered to be the primary etiological agent in the formation of dental caries [1, 2]. This facultative anaerobe prefers low-oxygen conditions, however it can adapt to both aerobic and oxidative stress conditions, which is a critical component of its ability to colonize tooth surface and survive in plaque biofilm [3]. *S. mutans* does not contain an electron transport system, and possesses an incomplete tricarboxylic acid (TCA) cycle, the primary role of which is for synthesis of amino acid precursors [4]. *S. mutans* metabolizes a variety of carbohydrates using glycolytic/fermentative pathways, and in fact, at the time that its genome was completely sequenced, its predicted modes of sugar metabolism suggested that *S. mutans* is able to metabolize a wider variety of carbohydrates than any other Gram- positive organism [4]. Oral streptococci such as *S.mutans* have developed multiple mechanisms by which to obtain sugar from the human diet [5, 6]. Dietary intake of sugars includes but is not limited to glucose, fructose, and sucrose; however it has been reported that a high sucrose diet is associated with the virulence of *S.mutans* and development of dental caries [7]. In the absence of dietary sugar intake, *S. mutans* is also able to grow with various components of saliva as its primary carbon and nitrogen source [8]. The glycolytic pathway in *S.mutans* produces pyruvate that is then reduced by fermentation to products such as lactic acid, formate, ethanol, and acetate [4]. It has been reported that under strict anaerobic conditions *S.mutans* produces a significant amount of formate, acetate, and ethanol as well as lactate, even in the presence of excess glucose [9]. Conversely, when *S. mutans* is grown under microaerobic conditions the pyruvate formate-lyase enzyme is inactivated and lactate is exclusively produced [10]. The ability of *S. mutans* to

withstand the acidic conditions that are produced from carbohydrate fermentation is based, in large part, on the presence of a membrane-bound, acid- stable, proton-trans locating F_0F_1 ATPase that maintains the intracellular pH at 7.5 [11].

Dental Plaque Biofilm and the Formation of Dental Caries

A biofilm is an aggregation of microorganisms that attaches to a solid or liquid surface concealed by an exopolysaccharide (EPS) matrix [12], usually composed of proteins and sugars. Dental caries is a classic form of biofilm disease, initiated by changes in the oral environment that enhance the establishment of cariogenic bacteria, which are capable of converting carbohydrates to the organic acids that demineralize the tooth enamel [13]. Reports suggest that *S. mutans* isolates have a greater capacity to form biofilm than other streptococcal species that colonize the human oral cavity environment [1]. Plaque biofilm development is a dynamic multi-species process that is initiated by a host pellicle located on the surface of the tooth enamel (Figure 1-1). Proteins, antibodies, and enzymes that normally reside in the saliva bind to the host pellicle. The next steps in oral biofilm development involve the multi-species process that is initiated by early colonizers followed by the attachment of late colonizers. There are approximately 260 oral bacterial species that have been cultivated from humans, but actual diversity is approximated at 500 common species based upon sequence methods [14, 15]. During plaque biofilm development both mutualistic and antagonistic interactions occur between different species of bacteria. It has been suggested, for example, that when pioneer colonizers such as *Streptococcus gordonii* and *Streptococcus sanguinis* are present at high amounts in the oral biofilm they can antagonize *S. mutans* [3]. This occurs by *S. gordonii* and *S. sanguinis* binding to amylase, an enzyme that catalyzes the hydrolysis of starch into sugars that is present in saliva components and producing growth-inhibiting amounts of H_2O_2 as a competitive measure against *S. mutans* during the initial aerobic growth of early plaque biofilm development

[3, 16, 17]. Thus, it is hypothesized that *S. gordonii* and *S. sanguinis* exhibit antagonistic behavior by inhibiting *S. mutans* when grown aerobically [3].

Oral biofilm accumulation of *S. mutans* and certain other oral bacteria can lead to a number of diseases such as halitosis, periodontal disease, and dental caries. Various host factors, which promote the overgrowth of *S. mutans* and/or its ability to outcompete non-cariogenic bacterial species in plaque biofilm, include teeth composition such as spacing and/or overcrowding, decreased saliva flow, poor oral hygiene habits, and a diet high in sugar [18]. Deficiencies in fluorine, zinc, lead, and iron content of the enamel, teeth crowding and abnormal spacing can increase the susceptibility to caries [19, 20]. Saliva has a cleaning effect on teeth and reduced secretion (xerostomia) increases caries [21, 22]. Poor oral hygiene practices (i.e. lack of dental cleanings, daily brushing and flossing) can promote the accumulation of microorganisms and dental plaque formation on the tooth surface [23]. Intake of refined carbohydrates such as sucrose, maltose, lactose, glucose, fructose, and cooked sticky starch has also been associated with dental caries [24]. *S. mutans* is cariogenic because upon carbohydrate intake it can lower the pH in dental plaque rapidly to about pH 4 within a few minutes, due to the rapid metabolism of dietary sugars [25]. Acidogenic bacteria such as *S. mutans* are adept at surviving at low pH, and can become dominant in plaque microflora when there is an increase in acidity of dental plaque, whereas the proportion of other non-cariogenic bacteria such as *S. sanguinus* tends to decrease [26, 27]. Oral bacteria experience repeated cycles of acidification of their environment followed by neutralization periods during growth in dental plaque [25]. These repeated cycles of acidification not only promote demineralization of the tooth surface but can also cause damage to the non-cariogenic bacteria in dental plaque [25]. As mentioned above, *S. mutans* can tolerate low pH by the proton-pumping activity of its F-ATPase [28]. The acids generated through the

fermentation of sugar by microorganisms such as *S. mutans* in dental plaque lead to decalcification of the superficial layers of tooth enamel and this is what ultimately initiates dental caries [10].

According to the World Health Organization (W.H.O), dental caries is a common infectious disease affecting humans that remains untreated in many underdeveloped areas that may lead to suffering that is often relieved only by loss or extraction of the infected tooth. Reports show that dental caries is less common and less severe in developing countries of Africa; however it is anticipated that the incidence of caries will increase in several countries of that continent, due to changing living conditions and dietary habits, and inadequate exposure to fluoride [29]. Upon tooth eruption, bacterial colonization of human teeth begins [18]. Specifically, *S. mutans* is mostly transmitted to infant children from their mothers [30]. Poor diet and poor oral hygiene may result in the appearance of surface damage to the teeth as young as 6-12 months of age [31]. *S. mutans* is also associated with non-oral infections, specifically sub-acute bacterial endocarditis [32]. Dental scientists in the United States have been key players in developing ways to manage and control caries [33]. Treatment of dental caries consists of removal of the decayed regions of the tooth by operative procedures, and restoration with materials such as silver fillings, composite resin, and full metal or porcelain crowns [24]. Severe cases of dental caries involve the pulp of the tooth and endodontic treatment may be used such as root canals [24]. Sealants are another form of caries management that prevent food from collecting in molar pits and fissures and, therefore, prevent dental caries [34-36]. The World Health Organization oral health reported that dental caries can be controlled by the joint action of communities, professionals and individuals aimed at reducing the impact of sugar consumption and emphasizing the beneficial impact of fluorides administered through water fluoridation and

use of fluoride toothpastes and mouth rinses. Fluoride prevents and controls dental caries by four ways: (1) inhibits the demineralization of sound enamel, (2) enhances the remineralization (i.e., recovery) of demineralized enamel [19, 37], (3) it inhibits the process by which cariogenic bacteria metabolize carbohydrates to produce acid and, (4) affects bacterial production of adhesive polysaccharides [38].

Factors Affecting *S. mutans* Biofilm Formation (Summarized in Table 1-1)

Sucrose-Independent Adhesion

Mucins are acidic glycoproteins that are normally found in the saliva, and form what is known as the acquired enamel pellicle (AEP) [39-43]. The AEP is a shapeless membrane layer that is approximately 0.1 to 3 μM in depth, and contains sulfate and carboxyl groups that increase the net negative charge of the tooth surface [42]. Sucrose-independent adhesion refers to the ability of *S. mutans* to attach to specific components of the AEP and salivary agglutinins. This is mediated primarily by the *S. mutans* cell-surface protein antigen I/II (also known as multifunctional P1 adhesin), which is an 185 kDa surface protein [18] encoded by the *spaP* gene [44-47]. The role of antigen I/II in adhesion is based on the study of *S. mutans* adhesion to saliva-coated hydroxyapatite [48-50]. It has been reported that isogenic mutants lacking P1 (antigen I/ II) did not bind as well as the wild-type to saliva-coated hydroxyapatite, but bound similarly well as the wild-type to saliva-coated hydroxyapatite that also contained in situ synthesized glucan polymers [51].

Sucrose-Dependent Adhesion

Sucrose-dependent adhesion during *S. mutans* biofilm development is mediated primarily by its production of glycosyltransferase (GTF) enzymes and glucan-binding proteins (GBPs) [52]. GTFs are responsible for synthesis of insoluble extracellular glucan polymers that promote biofilm adhesion. An *in vitro* biofilm formation experiment comparing sucrose-

dependent adhesion of *S. mutans* wild-type, GTF-deficient mutants (GtfB, GtfC, and GtfD), and recombinant GTFs (rGTFs) demonstrated that the presence of three GTFs (GtfB, GtfC, and GtfD) are necessary for sucrose-dependent adherence of *S. mutans* [53]. In many oral streptococci, the extracellular biofilm "slime layer" produced in the presence of sucrose is comprised of glucans, which aid in biofilm adhesion and formation of dental plaque biofilm [54]. Glucans are synthesized from sucrose by the enzymatic action of one or more GTF enzymes that can produce both water- insoluble or soluble glucans [55]. In oral streptococci, the chemical structure of extracellular glucans is comprised of alpha-1,3 and alpha-1,6 glucosidic linkages [1]. Specifically, most *S. mutans* water- insoluble glucans contain different degrees of branching and mostly alpha-1,3 linkages [1]. *S. mutans* GTFs are encoded by three different glucosyltransferase genes (*gtfB*, *gtfC*, and *gtfD*) and a single fructosyltransferase gene (*ftf*).The GtfB is responsible for synthesizing insoluble glucan in α-1,3-linkages, whereas GtfC generates a mixture of soluble α-1,6-linkages and insoluble glucans, and GtfD mostly produces soluble glucans [56-58]. In general, GtfC tends to be adsorbed in the enamel within the pellicle area, while GtfB binds strongly to the bacterial surface which promotes tight cell clustering while enhancing cohesion of plaque [59]. GtfD forms a soluble, readily metabolizable extracellular glucan polysaccharide and acts as an anchor for GtfB [59]. Specifically GtfB and not GtfC is involved in the production of water- insoluble and adhesive glucan exopolysaccharides, the production of which are down-regulated when *S. mutans* is grown aerobically [60-63] [59] [64]. Four cell-surface proteins, GBPs have also been identified in *S. mutans* (GbpA, GbpB, GbpC, and GbpD), which also play a role in sucrose-dependent adhesion by binding the extracellular glucan polymers produced by the GTFs [55, 65, 66].

The Competence (com) System

Competence, also known as natural genetic transformation, is the ability of bacteria to take up exogenous free DNA from their environment [67]. The induction of genetic competence in *S. mutans* is mediated by quorum-sensing that depends on a competence-stimulating peptide (CSP) signaling molecule [68-70]. Quorum-sensing is the ability of bacteria to regulate different physiological processes in a cell-density dependent manner [71-75]. In general, quorum-sensing signal molecules have also been shown to facilitate the regulation of gene expression in bacterial biofilms [76, 77]. Often bacteria secrete quorum-sensing signals and sense them through receptors [78]. The receptors do not induce behavioral changes until the bacterial cell density has increased, which allows the signal concentrations to exceed a critical threshold [79, 80]. Bacteria respond by adopting communal behavior that can lead to community behaviors such as biofilm formation [62].

The *S. mutans* CSP is a pheromone that belongs to the class of double- glycine- type leader peptides [81, 82], and is encoded by the *comC* gene. CSP is sensed by a two-component regulatory system (TCS) comprising the sensor histidine kinase, ComD, and the response regulator, ComE [68, 83-85] as outlined in Figure 1-2 (adapted from [86]). Binding of CSP to ComD induces a phosphorylation event that is transferred to ComE, which in turn activates expression of *comX*, a gene encoding an alternative sigma factor specifically required for the synthesis of late competence proteins that are involved in DNA uptake and internalization [87-89]. Addition of synthetic CSP to non-competent cells (with an intact ComDE system) facilitates increased transformation frequencies of *S. mutans*, with approximately 1% of the total cell population transformed [81]. The *comGB* gene encodes a protein involved in DNA binding-uptake and inactivation of *comGB* resulted in complete loss of transformability, both in the presence and in the absence of synthetic CSP [90]. Furthermore, mutants with inactivated *comD*,

comE, or *comX* genes formed biofilms with a reduced biomass [91]. Studies of other bacteria have shown that growth of bacteria in biofilms can facilitate horizontal gene transfer between bacterial species by conjugation or transformation by competence [92-94], and it has been shown that *S. mutans* is more competent when grown as a biofilm relative to planktonic culture [91].

The competence-signaling system in *S. mutans* can also be triggered by another pheromone, XIP (for sigX-inducing peptide), which is a small hydrophobic peptide [81, 82, 95, 96]. The *comR* and *comS* genes encode a response regulator and XIP, respectively, and have directly linked to the expression of *sigX* [96, 97]. In brief, ComR activates the expression of the peptide precursor ComS and ComS is secreted, processed, and internalized through the peptide transporter Opp [98]. This ComR and XIP then form a complex then functions as a transcriptional activator of *sigX*, facilitating development of competence in *S. mutans* [95]. Studies have shown that deleting the *comR* gene completely inhibited *S. mutans* competence, which suggests that all regulatory systems in the presence or absence of CSP may be incorporated at the level of ComR [98].

Extracellular DNA (eDNA)

Cell lysis and the release of eDNA have been shown to be a major structural component of biofilms produced by a number of bacteria [99-103]. In *S. pneumonie*, DNA release is triggered by competence induction, which involves cell lysis regulated by the autolytic amidase LytA [104, 105] and the autolytic lysozyme LytC [104]. It has also been reported in *S. pneumoniae* that DNA release is activated by the CSP-dependent quorum-sensing system that is involved in competence development for natural transformation Thus, the autolysis induced by CSP may provide a source of DNA during competence development [105, 106]. In a study conducted by Petersen *et al.* [90] addition of synthetic *S. mutans* CSP to *S. mutans* cultures induced biofilm formation and eDNA release in the wild-type strain. Extracellular DNA is

critical during biofilm formation in the presence of a functional DNA binding-uptake system such as the *com* system [90]. However, experimental evidence showed that the *comGB* mutant (deficient in DNA binding and uptake, but not in CSP signaling) formed reduced biofilms, presumably due to its inability to bind and adhere to the eDNA [90]. Furthermore, increased eDNA-dependent biofilm adherence was observed in a *S. mutans cipI* (bacteriocin immunity protein) mutant, presumably due to increased susceptibility to bacteriocin-mediated cell death and lysis [107].

Environmental Effects on Biofilm Formation

Oxygen has been reported to have profound effects on *S. mutans* biofilm composition [60, 108, 109]. Through an RNA microarray analysis it was observed that 5% of all *S. mutans* genes are differentially expressed under aerobic versus anaerobic growth conditions, which also inhibits biofilm formation [60]. Up-regulated genes in response to aerated growth in this study included autolysis-related genes such as *atlA* and genes that encode bacteriocins, competence genes, the ClpB protease chaperone subunit [60], and the bacteriocin immunity protein (BIP) which is also up-regulated during biofilm formation [110]. *S. mutans* cells respond to oxygen by the VicK sensor kinase of a CovRS-like two-component system (TCS), and also by the AtlA autolysin pathway, that plays a major role in modulating cell surface composition [60]. It has been reported that inactivation of the gene for AtlA or VicK restored the capacity of *S. mutans* to form biofilms in the presence of oxygen [60]. The ability of *S. mutans* to form sucrose-dependent biofilms was also dramatically impaired when grown with aeration for 48 h, while an *atlA* mutant formed greater biomass of biofilms under this condition [61]. Similarly, under aerobic conditions a *vicK* mutant showed significantly increased sucrose-dependent biofilm formation [61].

Hydrogen Peroxide

Certain members of the oral streptococci have been shown to exhibit an antagonist, relationship in which they compete for adhesion-binding sites on the tooth pellicle [111]. Some oral streptococci are able to produce antimicrobial compounds such as bacteriocins and hydrogen peroxide (H_2O_2), which inhibit the competing species in order to minimize their growth [3, 112]. Furthermore, H_2O_2 has been shown to be a potent stimulator of competence and eDNA release in species that produce H_2O_2, such as *S. sanguinis*, *S. gordonii*, and *S. pneumoniae* [3, 113]. Aerobic growth conditions were shown to stimulate the H_2O_2-dependent release of heterologous DNA from mixed cultures of *S. sanguinis* and *S. gordonii* [114]. Currently, the direct effects, if any, of H_2O_2 on *S. mutans* competence, cell lysis and eDNA release have not been elucidated. However, growth of *S. mutans* under aerobic conditions stimulates the expression of competence genes [90]. Unraveling the effects of H_2O_2 on *S. mutans* biofilm formation and competence, in addition to its defenses against H_2O_2 is crucial towards achieving a better understanding of its ability to survive in the oral cavity. Table 1 summarizes factors influencing biofilm development in *S.mutans*.

cidAB* and *lrgAB

The bacterial *cidAB* and *lrgAB* operons both encode predicted membrane proteins and have been found in many bacterial, archael, and even plant genomes [115, 116]. The CidA and LrgA predicted secondary structures are similar to the bacteriophage lambda holin proteins [115, 117]. Bacteriophage holins are small membrane proteins that oligomerize in the cell membrane, where they regulate the timing and lysis of the host cell during lytic infection [118]. The lambda S holin protein functions to regulate cell death and lysis by forming large rafts that cause the cytosol to leak and also allows the phage-encoded murein hydrolase access to the cell wall of *E. coli* [118-120]. The bacterial Cid/Lrg system was first characterized in *S. aureus* and has been

shown to affect cell death and murein hydrolase activity [121, 122], antibiotic tolerance [121] [122], and biofilm development[103] in this organism. Specifically, it was reported that mutations in both the *cid* and *lrg* operons of *S. aureus* led to altered biofilm development, cell death and eDNA release [99, 103]. Expression of the *S. aureus cid* operon is positively-regulated by CidR, a LysR-type transcription factor encoded by the gene located directly upstream of *cidA* [123], whereas the *lrg* operon is positively-regulated by the LytSR two-component system encoded by the genes located directly upstream of *lrgA* [124]. The expression of both *cid* and *lrg* is responsive to by-products of glucose metabolism in *S. aureus*, which was shown to be dependent on the accumulation of acetic acid within the supernatant as a result of glucose metabolism [125]. Moreover, *cid* expression was found to be upregulated during low-oxygen growth and also in the tower structures of *S. aureus* biofilms [126]. Although the precise mechanism by which the Cid and Lrg proteins affect or regulate cell death and biofilm formation remains to be elucidated, a recent paper has confirmed that *S. aureus* CidA and LrgA are membrane proteins that oligomerize in a cysteine-dependent manner, similar to holin proteins [127].

The Cid/Lrg system has also been identified in *Pseudomonas aeruginosa,* where mutations in the *cidAB* and *lrgAB* operons were also shown to influence cell death and lysis during biofilm development [128]. Interestingly, the pathogen *Bacillus anthracis* contains four different operons with open reading frames homologous to *cidAB* and *lrgAB* of *S. aureus* [129]. In this organism, some of these *cid* and *lrg* operons appear to affect sporulation [129]. As in *S. aureus*, the *B. anthracis* LytSR two-component regulatory system plays a positive role in regulating *lrgAB* expression but had no apparent effect on *cidAB* expression, and a gene homologous to *S. aureus cidR* was required for *cidAB* expression [129, 130]. In plants, Cid/Lrg-

like proteins have also been proposed to function as effectors of cell death [131]. For example, the *Arabidopsis thaliana* plant appears to require the chloroplast inner envelope membrane protein AtLrgB (an apparent LrgA-LrgB fusion protein) for proper chloroplast development, although the molecular mechanism is unclear [132]. Furthermore, it has also been demonstrated that a *atlrgB* mutation caused spontaneous chlorotic cell death in *Arabidopsis thaliana* when grown under continuous light which suggests that AtLrgB may function against cell death in a manner similar to that proposed for LrgA/B in *S. aureus* [133].Ultimately, Cid and Lrg appear to function in both plants and bacteria as mediators of cellular destruction [131]. Based on these collective observations, it has been proposed that the Cid/Lrg system may be part of a programmed cell death (PCD) pathway that regulates cell death and lysis in developmental processes in both prokaryotes and eukaryotes [134].

In *S. mutans*, *lrgAB* and *cidAB* are dicistronic operons (an mRNA that encodes two proteins that are transcribed as demonstrated in Figure 1-3 (adapted from [135]). Although direct regulators of *S. mutans cidAB* transcription have not been identified, expression of *cidAB* has been shown to be upregulated by metabolism of excess glucose [135] and during anaerobic growth [60] and downregulated by the carbon catabolite protein A (CcpA) [135]. Expression of the *S. mutans lrgAB* operon, conversely, is repressed by growth in media containing excess glucose [126], and positively regulated by aerobic growth [61, 136] and by the LytST two-component system [135], that is encoded by the genes located immediately upstream of *lrgAB* [126, 136]. A recently-published RNA microarray analysis of a *S. mutans lytS* mutant revealed a pleiotropic effect on global gene expression, including genes involved in competence as well as oxidative stress tolerance [136]. These experiments also showed that *lytS* and *lrgAB* mutants were more sensitive to H_2O_2 stress [136]. Competence assays published by Ahn et al. [136] have

also revealed that the *lrgA* mutant is less competent in both the absence and presence of CSP. Unlike previous results with *S. aureus*, mutation of *cid* and *lrg* had no apparent effect on *S. mutans* murein hydrolase activity under standard growth conditions [135]. It was also demonstrated that growth of *S. mutans cidAB, lrgAB,* and *cidB* mutants in an aerobic incubator on BHI agar plates was completely inhibited [135], suggesting that the Cid/ Lrg system is required for efficient aerobic growth of *S. mutans*. Therefore it appears that although some aspects of Cid/Lrg are conserved among organisms, their functions may have evolved specifically to benefit the physiology and particular niche of individual organisms.

Hypothesis and Specific Aims

As discussed above, biofilm formation, oxidative stress resistance, and competence are highly inter-related virulence properties of *S. mutans* that promote survival in the oral cavity. Given that components of the *cid* and *lrg* operons have been shown to affect biofilm development in other organisms, and that components of the *lrgAB* operon have been previously shown to affect competence, oxidative stress and biofilm formation in *S. mutans*, the Cid/ Lrg system may represent an attractive target for drug development for anti-caries therapies due to the observed influences of these genes on all three phenotypes. Therefore, this research project addresses the overall hypothesis that components of the Cid/Lrg system are previously unrecognized modulators of *S. mutans* oxidative stress and competence. The specific research objectives of this project are: (1) to assess the effects of *cid* mutations on oxidative stress, competence, and biofilm formation, and (2) to investigate a role for H_2O_2 in regulating *S. mutans* competence and biofilm formation.

Table 1-1. Factors influencing biofilm development in *S.mutans*

Variable	Effect on *S. mutans* biofilm	Reference (s)
GTFs/ Gbps	+	Ahn et al., 2007; Munro et al., 1991
Sucrose- independent adhesions	+	Burne et al., 1997; Yoshida and Kuramitsu (2002)
eDNA	+	Petersen et al., 2005
Competence (CSP)	+	Petersen et al., 2005; Li et al., 2001; Li et al., 2002
Aerobic Growth	-	Ahn and Burne (2007); Ahn et al., 2007
Anaerobic Growth	+	Ahn and Burne (2007); Ahn et al., 2007
Hydrogen peroxide	?	Unclear

"+" denotes variable has a positive effect on biofilm formation, "-" denotes negative effect on biofilm formation, "?" indicates the variable has not been tested in *S. mutans*

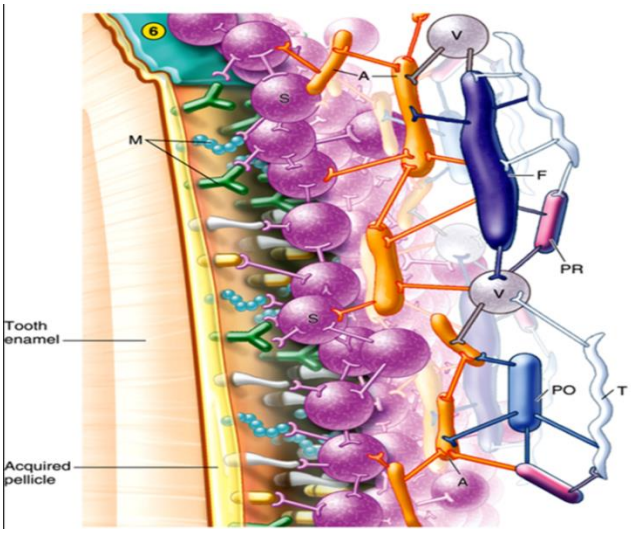


Figure 1-1. Oral biofilm development model in *S. mutans.* Starting from left to right, the first layer is showing the host pellicle that contains proteins from saliva. The second layer is where proteins, enzymes, antibodies adhere to host pellicle. The middle layer is showing the multi- species with additional colonizers which generates the final dense layer of plaque. Lastly, the initial damage of tooth enamel is shown at the top left corner. http://www.studyblue.com/#flashcard/view/1418503

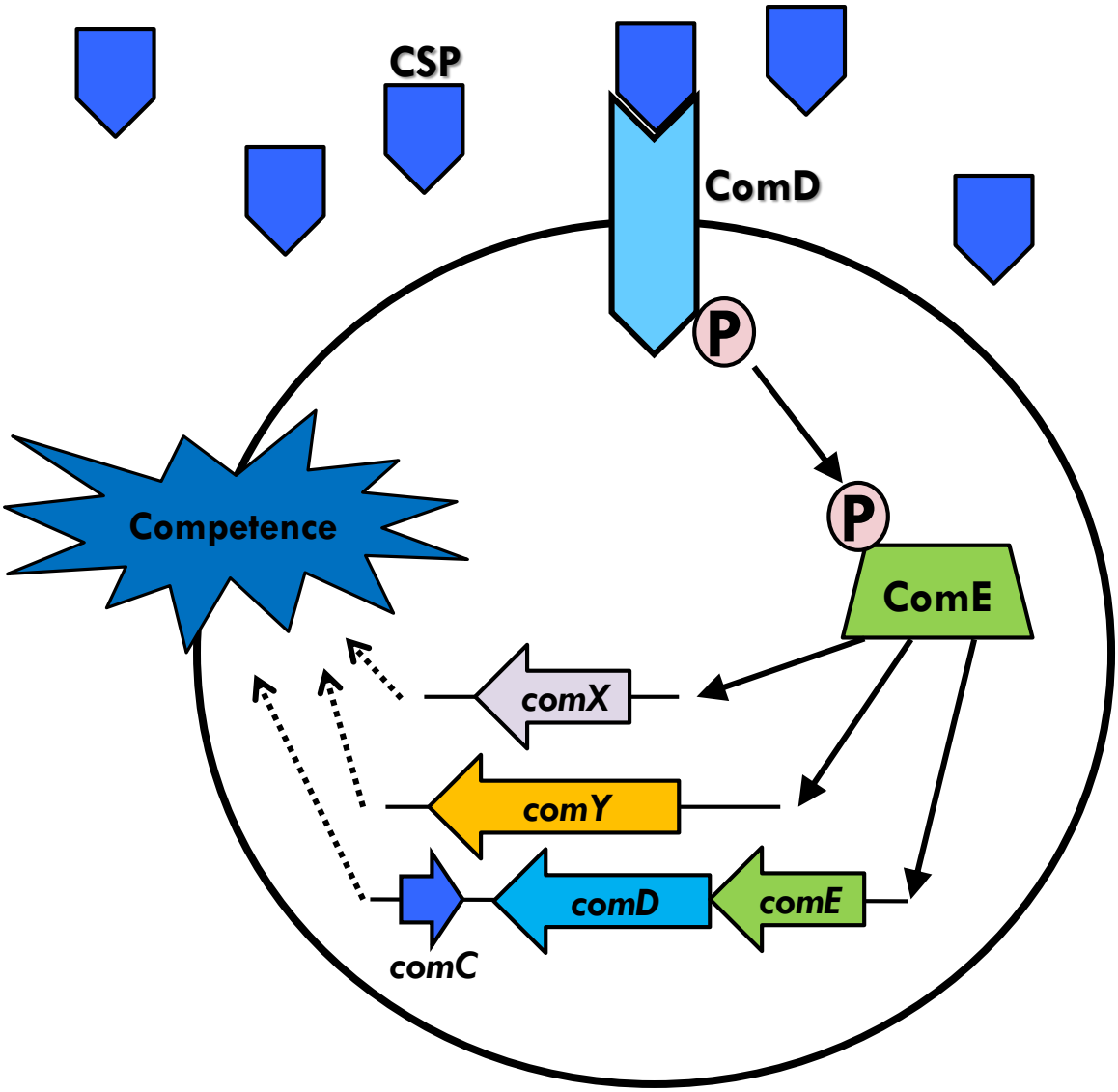


Figure 1-2. Model of *S. mutans* CSP- dependent cell signaling in genetic competence. When CSP reaches a critical density, it is detected by neighboring cells via a sensor kinase, ComD, which then phosphorylates the response regulator ComE. This initiates transcription of an alternate sigma factor (ComX) which upregulates expression of genes required for DNA uptake and recombination. Then the transcription of *comY* is initiated which upregulates expression of genes required for DNA binding uptake machinery. The *comCDE* operon functions as autofeedback mechanism to induce competence. Adapted from Ahn et al. 2006[86].

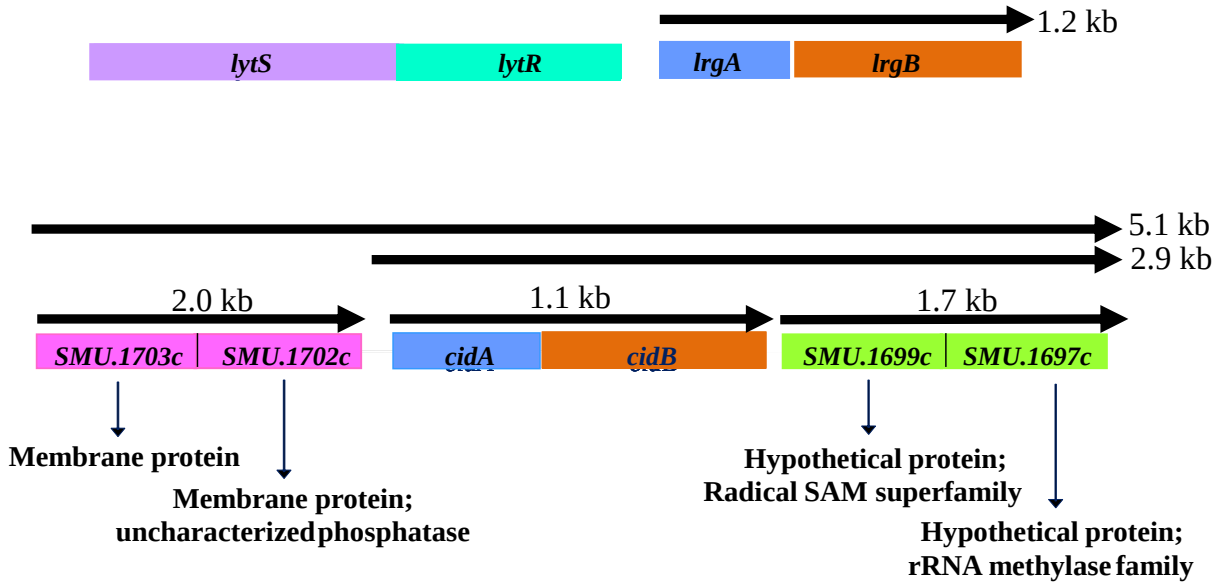


Figure 1-3. The *S. mutans cid* and *lrg* operons. The direction of RNA transcription and sizes of the RNA transcripts (previously determined by northern blotting) are indicated by the arrows above the genes, and the predicted functions are indicated below each gene. The *lytST* dicistronic operon encodes a two-component system that positively-regulates *lrgAB* expression. Figure provided by Dr. Kelly Rice, University of Florida. Adapted from Ahn et al. (2010) [135].

CHAPTER 2
MATERIALS AND METHODS

Bacterial Strains and Growth Conditions

The *Streptococcus mutans* strains and plasmids used for this study are listed in Table 2-1. For each experiment below, S. *mutans* UA159 and/or its isogenic *lrgA* (Δ*lrgA*:: NPSp^r), *lrgB* (Δ*lrgB*::NPEm^r), *lrgAB* (Δ*lrgAB*:: ΩKm^r), *cidA*(Δ*cidA*:: NPKm^r), *cidB(*Δ*cidB*:: NPKm^r), and *cidAB*(Δ*cidA*:: ΩKm^r) mutants created in [135] were streaked from frozen glycerol stocks on Brain Heart Infusion (BHI), containing selective antibiotics: kanamycin (Km) - 1000µg/ml, erythromycin (Em) - 10µg/ml, spectionmycin (Sp) - 1000 µg/ml, as appropriate. All planktonic *S. mutans* cultures were grown at 37°C in 5% CO_2 conditions. Growth media used for culturing *S. mutans* were Brain Heart Infusion (BHI) or semi-defined biofilm medium (BM) [137], containing either 18 mM glucose/ 2 mM sucrose or 11 mM glucose/10 mM sucrose, as indicated for each experiment. *E. coli* was grown in aerobic conditions (37°C, 250 RPM) in Luria- Bertani (LB) broth with erythromycin 300 µg/ml, or 500 µg/ml. Glycerol stock cultures were maintained at -80°C and were prepared by mixing equal volume of overnight with sterile 50% (vol/vol) glycerol in cryogenic tubes.

Competence Assays

To compare the ability of UA159 and isogenic *cidA*, *cidB*, and *cidAB* mutants to take up externally-added plasmid DNA, a quantitative competence assay (at least n = 3 independent experiments per strain) was performed using a previously-published protocol [138]. The following modifications listed below were made to the protocol: *S. mutans* UA159 and isogenic *cidA*, *cidB*, and *cidAB* mutant strains were grown in BHI broth for 16-18 hours at 37°C in a 5% CO_2 incubator. Overnight cultures of each strain were diluted to an OD_{600} = 0.02 in BHI, then grown in a 96-well plate to an OD_{600}= 0.15 before the addition of 81 ng plasmid DNA

(methylated or unmethylated pOri23, as indicated in the results section), with or without addition of synthetic CSP to final concentration of 0.5 µg/ml per culture. When indicated, H_2O_2 was also added to a final concentration of 0.5 mM. After 2.5 hours of further incubation at 37°C and 5% CO_2, cultures were serially diluted and plated on BHI agar with and without selective antibiotic. The colony forming units per milliliter (CFU/ml) of each culture were enumerated after 48 hours of growth at 37°C in a 5% CO_2 incubator. The transformation efficiencies were calculated as the percentage of transformants (CFU/ml on BHI + selective antibiotic) among total viable cells (CFU/ml on BHI). To test the effect of pre-treating CSP with H_2O_2 on competence, the following modifications were made: 40µl of 250 µg/ml CSP and H_2O_2 were each added to sterile water to a final concentration of 200 µg/ml and 1 mM, respectively. After15 minutes incubation, catalase was added to a final concentration of 500 µg/ml. 1 µl of this mixture was then added to 200 µl of *S. mutans* culture to achieve a final concentration of 1 µg/ml CSP, prior to the addition of plasmid DNA (pOri23) to each culture.

Hydrogen Peroxide (H_2O_2) Challenge Assays

To compare the ability of UA159, *lrgA, lrgB, lrgAB, cidA, cidB*, and *cidAB* mutants to grow in the presence of H_2O_2, a growth assay was performed on n= 3 independent experiments of each strain using a previously- published protocol [136]. In brief, UA159 (wild-type *S. mutans*) and isogenic *lrgA, lrgB, lrgAB, cidA, cidB*, and *cidAB* mutant strains were grown in BHI broth for 16-18 hours at 37°C in a 5% CO_2 incubator. The overnight cultures were diluted 40-fold into fresh BHI media. Then, H_2O_2 was added to each culture to a final concentration of 0.50 mM or 1.0 mM. Aliquots of each culture were transferred to a 48-well tissue culture plate and incubated for 22 hours at 37°C. The OD_{600} was monitored every two hours in a Biotek Synergy HT microplate reader.

Biofilm Assay

Biofilm formation was assayed by the ability of cells to adhere to the wells of a 96-well, flat-bottom microtiter plate (Costar 3596; Corning, Inc, Corning, N.Y.) using a previously-published protocol [22]. UA159 (wild-type *S. mutans*) and isogenic *cidA, cidB, and cidAB* mutant strains were grown in BHI broth for 16-18 hours at 37°C in a 5% CO_2 incubator. Overnight cultures of each strain were diluted to an $OD_{600} = 0.02$ in media ± 0.25 mM of H_2O_2. Aliquots of 200µl each diluted culture was transferred in triplicate into a two 96- well plate, which was grown for 24 hours at 37°C in a 5% CO_2 incubator. After 24 hours of growth, the supernatant was removed, and biofilms were washed once with 1X PBS buffer followed by fixation for 2 minutes in 100% (vol/vol) ethanol. Biofilms were then carefully stained with 1% (vol/vol) crystal violet for 2 minutes, and washed two times with 1X PBS (1X). The absorbance of each well at 595nm was then measured.

Complementation of *cidB* mutant

To generate a complementation plasmid for the *S. mutans cidB* mutant, a cloning strategy was employed whereby the *cidAB* forward and *cidAB* reverse primers specified in Table 2-2 were designed to amplify a 1.7 kb PCR product spanning 562 bp upstream of the *cidA* start codon through 772bp of the *cidB* stop codon. The *cidA* and *cidB* open reading frames overlap by 4 nucleotides and as such, may be subject to translational coupling. Therefore, the entire *cidAB* operon was used in this complementation strategy. A BsrG1 enzyme site was incorporated at the 5' end of each primer to facilitate eventual sub-cloning into plasmid pBGE [54], a plasmid that contains sequences targeting its recombination into the chromosomal *gtfA* gene of *S. mutans*. In order to PCR amplify and clone the *cidAB* gene into the pCR-Blunt vector (Life Technologies), AccuPrime™ *Pfx* proofreading polymerase (Life Technologies) was used in this reaction. The *cidAB* PCR product was ligated into the pCR-Blunt plasmid using the PCR Blunt cloning kit

(Life Technologies), according to the manufacturer's protocols, followed by heat-shock transformation of *E. coli*. The *E.coli* transformants were screened for the correct 1.7 kb *cidAB* insert via plasmid purification (Promega Wizard SV mini-prep kit) and restriction enzyme digestion with EcoR1, followed by agarose gel electrophoresis. Plasmid DNA clones which contained the 1.7-kb *cidAB* insert were submitted for Sanger sequencing at the UF-ICBR genomics core facility. The retrieved sequences were checked by BLAST analysis to confirm that no sequence errors were introduced, and one clone was chosen to proceed with sub-cloning into pBG-E.

To sub-clone *cidAB* into pBG-E, a restriction enzyme digestion of pCR-cidAB and pBG-E each with BsrG1 was performed. The pBG-E vector was then dephosphorylated using Antarctic phosphatase (New England BioLabs), followed by gel purification each of the 1.7 kb *cidAB* fragment and the linearized and dephosphorylated pBG-E vector using a gel purification kit (Zymo Research) according to the manufacturer's protocols. The purified *cidAB* and pBG-E were then ligated and transformed into *E. coli* by electroporation [139] . Clones containing pBG-E with *cidAB* insert were confirmed via plasmid purification and restriction enzyme digestion with BsrGI, followed by agarose gel electrophoresis. Glycerol stocks of each clone that contained the *cidAB* insert were made and stored at -80°C.

Transformation of *S. mutans* with pBG-E and pBG-cidAB

Single colonies of *S. mutans* UA159 and *cidB* mutant were each inoculated in (BHI) broth containing 5% heat-inactivated horse serum (HS) (Sigma-Aldrich). After 16 h at 37°C in 5% CO_2 without agitation, the overnight cultures was diluted 1:20 in fresh pre-warmed BHI + HS, and then incubated until the optical density at 600 nm (OD_{600}) reached about 0.2. A working stock of the *S. mutans* competence stimulating peptide (CSP) was made by dissolving 250 µg of peptide in 1 ml ddH_2O, and 2.0 µl was added to 1-ml aliquots of each culture to a final

concentration of 500 ng/ml [81]. Transforming plasmid DNA (10µl for pBG-E, 35µl for pBG-cidAB) was added to each culture and incubation was continued for 90 minutes. At the end of incubation the cell density increased and cells were centrifuged and resuspended in 200 µl BHI. Aliquots (50µl) of each transformation reaction were plated on BHI-Em agar plates. The plates were incubated for 48 h at 37C in 5% CO_2. Next, a rapid single- colony PCR was performed after strains were restreaked to screen transformants for correct plasmid as follows: A sterile loop was used to pick single colonies of each strain (UA159, UA159-pBGE, *and cidB*-pBGE) from the plates. The colony was transferred to 100µl lysis buffer (25nM Tris-HCl, pH 8.0, 50mM glucose, 150mM NaCl, 10mM EDTA) containing 1 µl of 20,000 U mutanolysin. The mixture was then incubated for 1 hour and 30 minutes at 37°C. 2 µl of 10% SDS (final concentration = 0.2% w/v)) was added and samples were heated at 95°C for 10 minutes. Each sample was used as template in 50 µl PCR reactions as follows: 5 µl of 10X PCR Buffer, 2 µl of 50 mM $MgCl_2$, 1 µl of 50X dNTP mix, 1.5 µl of *gtfA* Forward primer (10µM stock), 1.5 µl of *gtfA* Reverse primer (10µM stock), 1 µl of template DNA, 1 µl of Taq DNA polymerase, and 37 µl of sterile H_20. The amplification protocol was as follows: 95°C for 5 minutes (1 cycle); 30 cycles of: 95°C for 15 seconds, 50°C for 30 seconds, 68°C for 2 minutes, 72°C for 10 minutes and 4°C infinite hold.

Table 2-1. Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant Characteristic	Source or Reference
Escherichia coli DH5α	Competent cells that provides a high efficiency of plasmid DNA	Invitrogen
JM110	Competent cells used for preparing plasmid DNA that lacks Dam and Dcm methylation	Agilent Technologies
UA159	Wild-type	Page Caufield (University of Alabama, Birmingham)
lrgA	Δ*lrgA*:: NPSp^r	[135]
lrgB	Δ*lrgB*::NPEm^r	[135]
lrgAB	Δ*lrgAB*:: ΩKm^r	[135]
cidA	Δ*cidA*:: NPKm^r	[135]
cidB	Δ*cidB*:: NPKm^r	[135]
cidAB	Δ*cidA*:: ΩKm^r	[135]
Plasmids		
pOri23	*E.coli* derived shuttle vector containing Erm cassette; 6.0Kb	[140]
pBG-E	*E.coli* vector containing Erm cassette, and multiple-cloning site flanked by regions of the *S. mutans gtfA* gene; 5.0Kb	[99]
pCR- Blunt Vector	Zero blunt pCR Cloning kit containing a Km cassette; 3.5Kb	Invitrogen

Table 2-2. Primers and probes used in this study

Primer/ Role	Forward/ Reverse	Oligonucleotide sequence (5'-3')
cidAB	Forward – BsrG1	ccctgtacaTTTCGCAACTGTAGGTTTGCTG
	Reverse – BsrG1	ccctgtacaAAAAAGATGAGACAAAAGTGTTCCCA
gtfA	Forward-Hinc II	TGCCCTGCCTATGGTGACGCTCTACA
	Reverse – Hinc II	CAAGGGTGGTGAACTGTTTCATCGGA

CHAPTER 3
RESULTS

Effect of *cid* Mutations on Hydrogen Peroxide Resistance

To determine the effect of H_2O_2 on growth of *S. mutans* UA159 (wild-type), *lrgA, lrgB, lrgAB, cidA, cidB, and cidAB* mutants, growth curves were performed for 22 hours in the absence or presence of 0.5 mM H_2O_2 or 1.0 mM H_2O_2. As seen in Figure 3-1 (Panels A and B), when comparing the growth of the wild- type and l*rg* panel of mutants, there was not much difference in the untreated condition versus 0.5 mM treatment with H_2O_2. Likewise, the *cid* panel of mutants did not show a difference between the untreated condition versus 0.5 mM treatment with H_2O_2 (Figure 3-2 panel A and B). However, when the concentration of H_2O_2 was increased 1 mM (biologically relevant to the amount of H_2O_2 that other oral *streptococci* are thought to produce in the oral cavity [114]), the *lrgA* and *lrgB* mutant strains displayed slightly increased oxidative stress resistance compared to the wild-type strain (Figure 3-3). Furthermore, *cidB, cidAB* and *lrgAB* mutant strains displayed almost complete growth inhibition when tested with 1 mM concentration of H_2O_2 (Figure 3-3). These results suggest that in order for *S. mutans* to growth during H_2O_2-induced oxidative stress, components of both the *cid* and *lrg* operons are required.

Effect of *cid* Mutations and H_2O_2 on Competence

It has been previously demonstrated that many bacteria such as *S. mutans* are naturally competent for genetic transformation [141]. Typically natural competence occurs during early to mid-exponential phase of growth in streptococci [126, 127]. Although the effect of the *lrg* genes on competence had been previously-assessed by our lab [131], the contribution of the *cid* genes to this phenotype had not been tested. Thus, to compare the transformation efficiency of UA159 (wild-type), *cidA, cidB, and cidAB* mutants, competence assays were performed in which strains

were grown to early- exponential growth phase in the presence and absence of the competence stimulating peptide (CSP) (Figure 3-4). These competence assays were performed using methylated pOri23 plasmid isolated from *E. coli* DH5α. As expected, the wild-type was naturally competent in the absence of CSP, and the addition of exogenous CSP increased competence [136] . Although the transformation efficiencies of *cidA* and *cidAB* were comparable to the wild-type strain either in the absence and presence of CSP, the *cidB* mutant transformation efficiency could not be calculated in the absence of CSP, due to lack of any antibiotic-resistant colonies on the lowest possible dilution plate (no colonies on 10^{-1}) (Figure 3-4). However, competence was completely restored by the addition of CSP in the *cidB* mutant. This competence assay was repeated on cultures that were grown to an OD_{600} = 0.2- 03 for the *cidB* mutant, in case the competence "window" for this mutant occurred later in its exponential growth phase (Figure 3-5). However, this increased growth time did not restore competence to wild-type levels in the absence of CSP addition.

To increase the sensitivity of the competence assay, it was repeated for the wild-type and *cidB* mutant strains using pOri23 plasmid DNA isolated from *E. coli* JM110, a strain that is deficient in Dam and Dcm methylation (Agilent Technologies). In this assay, the *cidB* mutant transformation efficiency was able to be calculated, but it still showed a statistically-significant ($p < 0.001$, Student T-test) 2-log decrease in competence relative to the wild-type strain (Figure 3-6). As was observed when transforming with methylated plasmid DNA, addition of CSP to the *cidB* transformation assay fully restored the transformation efficiency of this strain to wild-type levels (Fig. 3-5). Overall, these results suggest that the *cidB* mutant is overall less competent in the absence of exogenous CSP addition.

H_2O_2 Pretreatment of CSP on Competence Assays

The effects of CSP + H_2O_2, and H_2O_2 alone on *S. mutans* wild-type competence were initially performed to determine if H_2O_2 has inhibitory effects on competence. In these initial experiments, the transformation efficiency was not detectable when cultures were treated with both CSP (200μg/ml) and H_2O_2 (1 mM) simultaneously, while H_2O_2 treatment alone did not appear to affect competence compared to the untreated culture (Figure 3-7). These results suggested that H_2O_2 could be inhibiting CSP, possibly by oxidizing or damaging amino acid residues in the CSP peptide, particularly serine since it has a polar hydroxyl group that may be accessible to oxidizing free radicals [23]. To test this theory, a follow-up experiment was performed to determine if H_2O_2 pretreatment of CSP prior to its addition to *S. mutans* culture would also affect competence (Figure 3-8). To this end, CSP was pretreated with H_2O_2, followed by addition of purified catalase to remove any trace amounts of H_2O_2. When this pre-treated CSP was added to *S. mutans*, the transformation efficiency was no different than that previously-observed with CSP treatment alone (Figure 3-7) or with CSP pre-treated with catalase (but not H_2O_2; Figure 3-8). This suggests that the previously-observed inhibitory effects of H_2O_2 + CSP simultaneous addition on competence were not due to oxidative damage of the CSP peptide. Interestingly, the simultaneous addition of both CSP and H_2O_2 directly to the culture resulted in transformation efficiencies similar to that of the untreated culture (Figure 3-8), rather than complete inhibition of competence as was previously-observed in Figure 3-7. The difference in these two results is likely explained by the fact that the dilution range plated for the CSP + H_2O_2 culture was too high in the Fig 3-7 experiment. In summary, although H_2O_2 alone does not appear to affect *S. mutans* competence, its presence somehow reverses or inhibits the ability of exogenously-added CSP to stimulate competence.

The Effects of Carbohydrates and H_2O_2 on *S.mutans* Static Biofilm Formation

Previously, biofilm assays performed by Ahn et al. [135] showed that *lrg* mutations produce stronger biofilms when grown in biofilm media supplemented with sucrose rather than glucose. The data from these biofilm assays also suggested that the sugar composition affected the biofilm phenotype of the *lrgA* and *lrgB* mutants [135]. To investigate the contribution of the *cid* genes to biofilm development in *S. mutans*, 24 hour static biofilm growth was initially assessed in UA159, *cidA, cidB,* and *cidAB* mutants grown in BM media containing 18 mM glucose and 2 mM sucrose (Figure 3-9). This model of biofilm growth primarily measures biofilm attachment by reading the OD_{600} absorbance of crystal violet staining, which is proportional to the amount of biofilm biomass attached to the bottom of the well. As demonstrated in Figure 3-9, the *cidB* mutant exhibited a statistically-significant decrease ($p < 0.05$, Tukey Test) in biofilm formation in biofilm media supplemented with 18mM of glucose and 2mM of sucrose, whereas the *cidA* and *cidAB* mutants behaved like the wild-type strain.

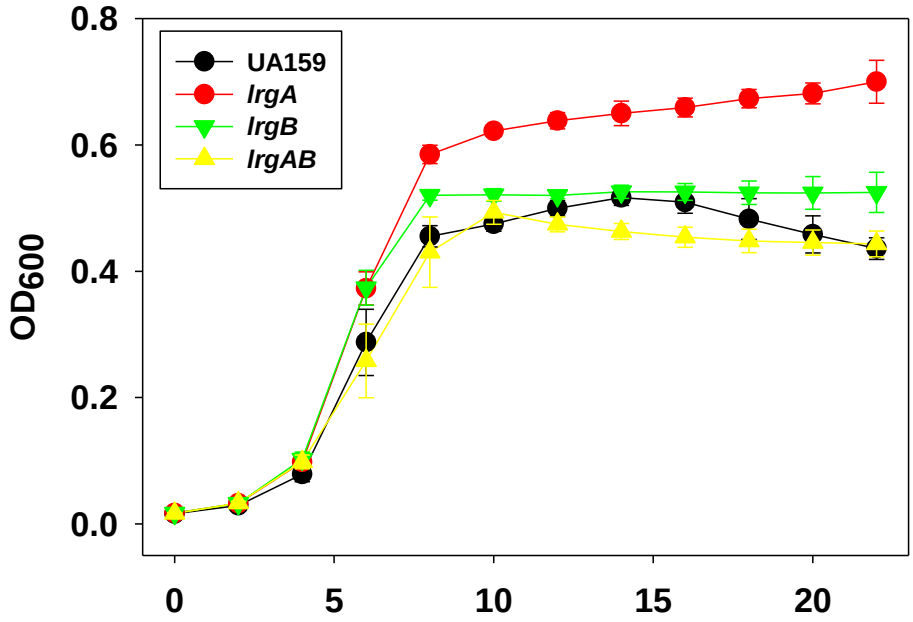
The effects of the various *cid* mutations on biofilm formation were also assessed using an 11 mM glucose/10 mM sucrose concentration, as well as different growth medias (BM verses BHI). Surprisingly, the *cidB* mutant displayed a modest, but significant ($p < 0.05$, Tukey Test) increase in biofilm compared to the wild-type strain when grown in BM or BHI containing 11 mM glucose/10 mM sucrose (Figs. 3-10 and 3-11), although this phenotype was more pronounced in the BHI media (Fig. 3-11). These results suggest that both the concentration of sugars as well as the growth media used both affected the biofilm phenotype of *cidB*. The *cidA* and *cidAB* mutants behaved like wild-type under all tested biofilm conditions. To assess the effect of H_2O_2 on biofilm formation, 0.25 mM H_2O_2 (a level not inhibitory for *S. mutans* growth) was also added at time of inoculation to the wild-type and cid mutant biofilms (Figs. 3-10 and 3-11). Although the addition of 0.25mM H_2O_2 at the time of biofilm inoculation appeared to

slightly decrease biofilm formation in both wild-type and *cid* mutant cultures, these differences were not statistically significant.

Complementation Attempt of the *cidB* Mutant

Initially, a multi-copy *S. mutans* plasmid expressing *cidAB* was used to try and complement the *cidB* mutant. Interestingly, the *cidB* mutant was not able to be transformed with this plasmid (n=3 experiments), even though this mutant strain was able to be transformed with the empty vector and also with the same vector expressing green fluorescent protein (GFP) (data not shown). Therefore, a single-copy integration vector (pBG-E) was used to clone *cidAB*. The pBG-cidAB complementation plasmid was successfully constructed (Figure. 3-12) and used to transform the *S. mutans cidB* mutant. However, initial testing of aerobic growth on BHI plates revealed that, unfortunately, integration of pBG-cidAB into the *gtfA* gene of *S. mutans* failed to restore growth of the *cidB* mutant in this assay (data not shown). Therefore, alternative complementation strategies will need to be pursued in future studies of the *cidB* mutant.

A



B

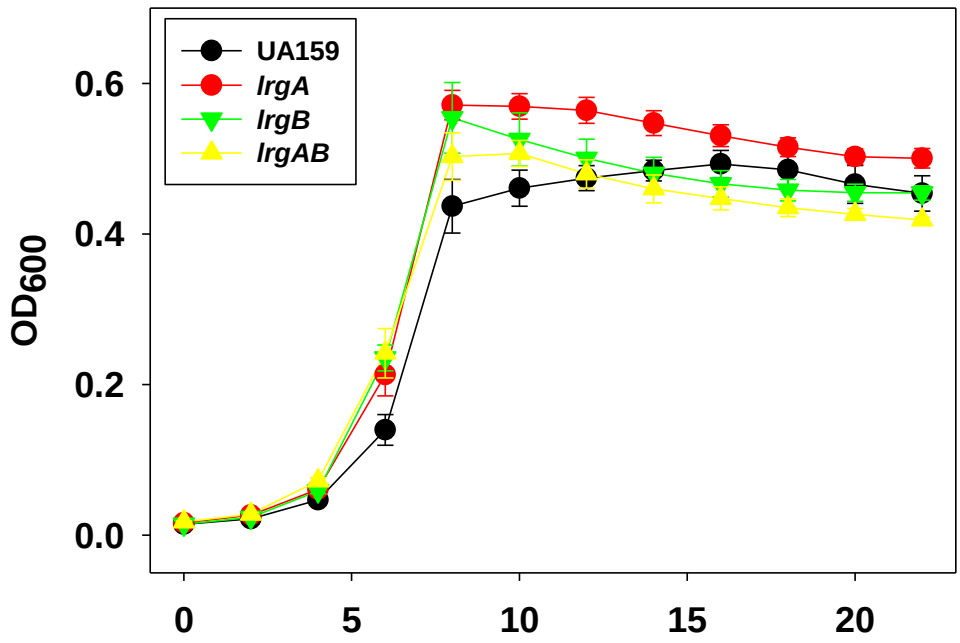
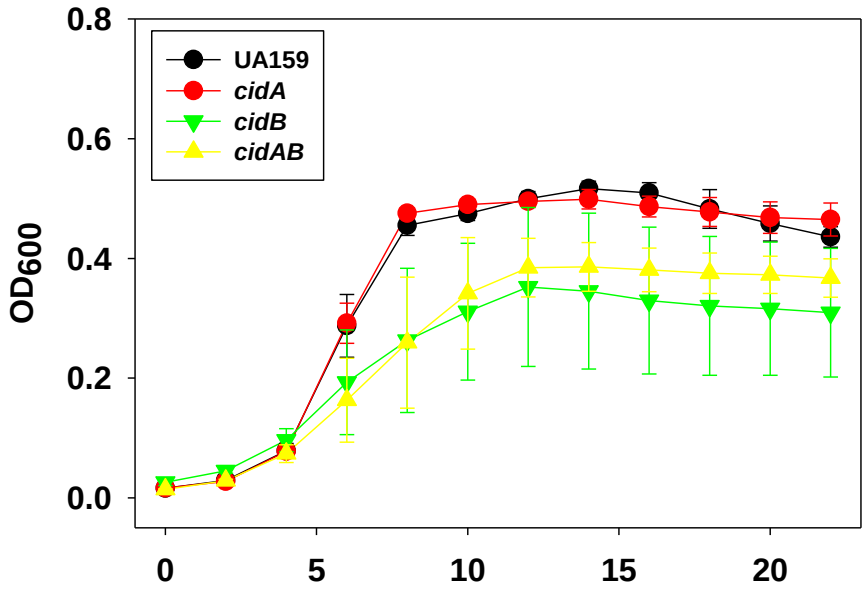


Figure 3-1. Static growth of UA159 (wild-type), *lrgA*, *lrgB*, *lrgAB* at 37 C (aerobic atmosphere) in BHI broth. A) Untreated condition B) 0.5 mM H_2O_2 Treatment. Data represents 3 independent experiments for each bacterial strain. Error bars = Standard Error of the Mean (SEM)

A



B

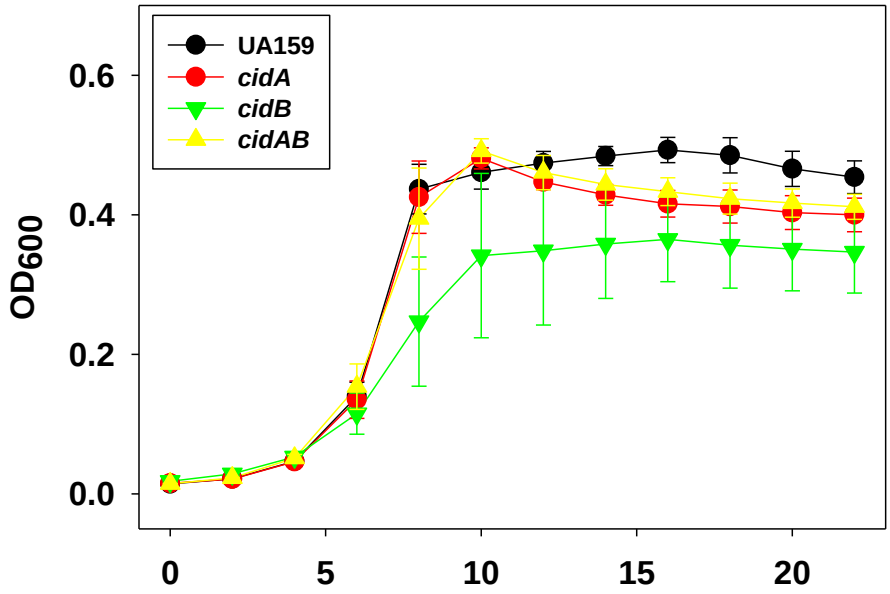


Figure 3-2. Static growth of UA159B (wild-type), *cidA*, *cidB*, *cidAB* at 37 C (aerobic atmosphere) in BHI broth. A) Untreated condition B) 0.5 mM H_2O_2 Treatment. Data represents the average of three independent experiments for each bacterial strain. Error bars = SEM

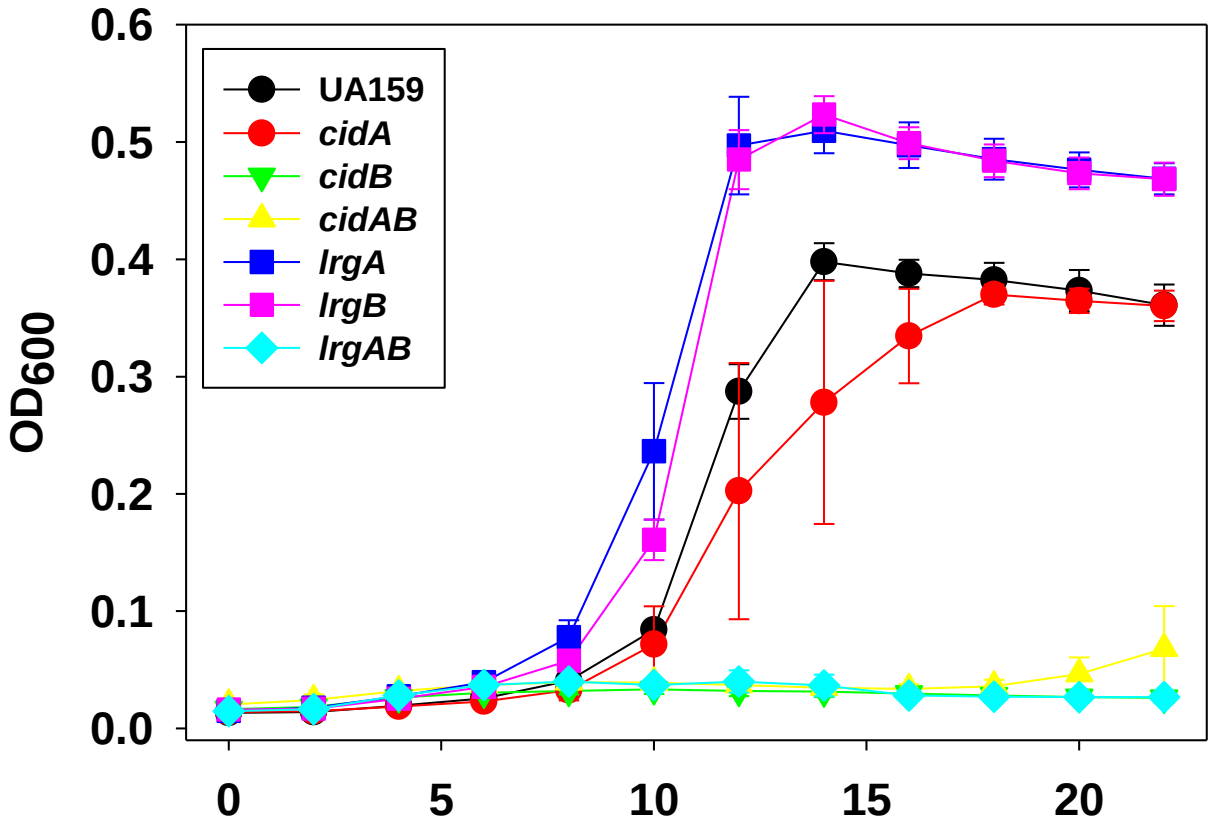


Figure 3-3. Static growth of UA159 (wild-type), *lrgA*, *lrgB*, *lrgAB*, *cidA*, *cidB*, *cidAB* challenged with 1 mM H_2O_2 at 37 C (aerobic atmosphere) in BHI broth + 1mM H_2O_2. Data represents the average of three independent experiments for each bacterial strain. Error bars = SEM

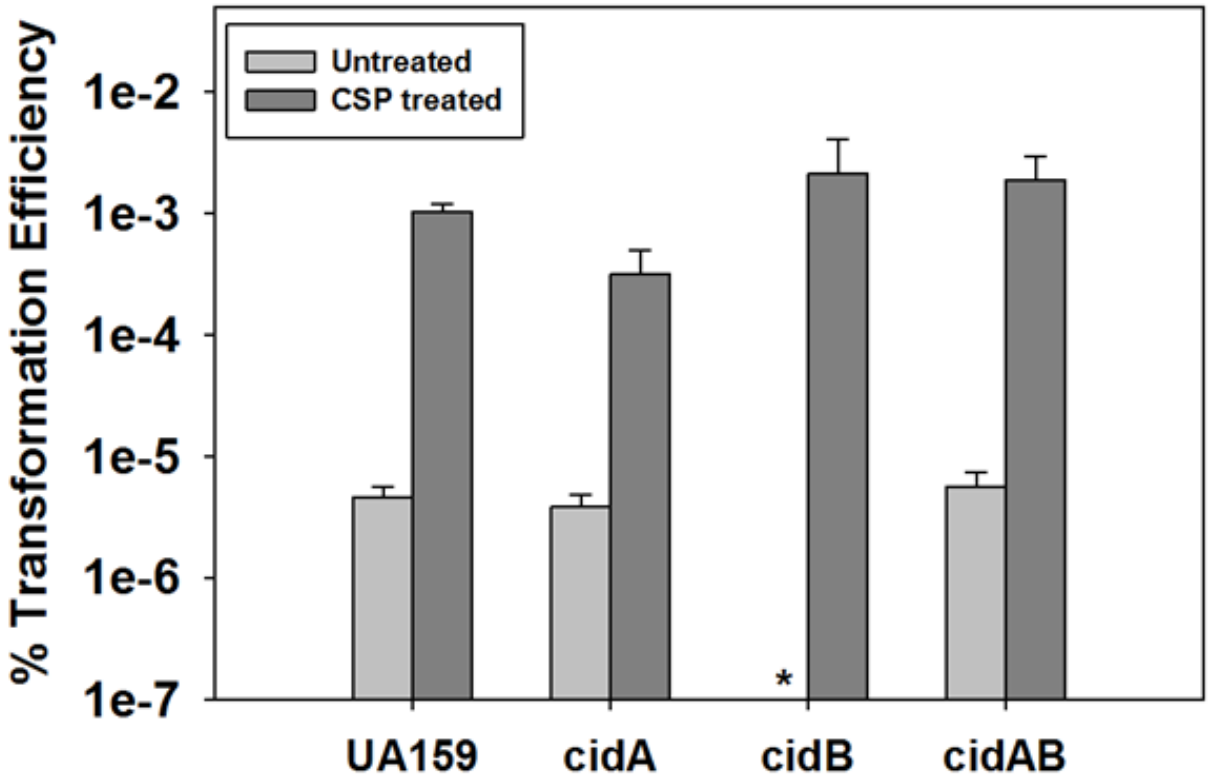


Figure 3-4. Effect of *cid* mutations on *S. mutans* competence with and without CSP addition. Results represent the average of four independent experiments for all strains except *cidAB*, n=3 independent experiments. Error bars = SEM. *no colonies detectable on 10^{-1} dilution plate. Error bars = SEM

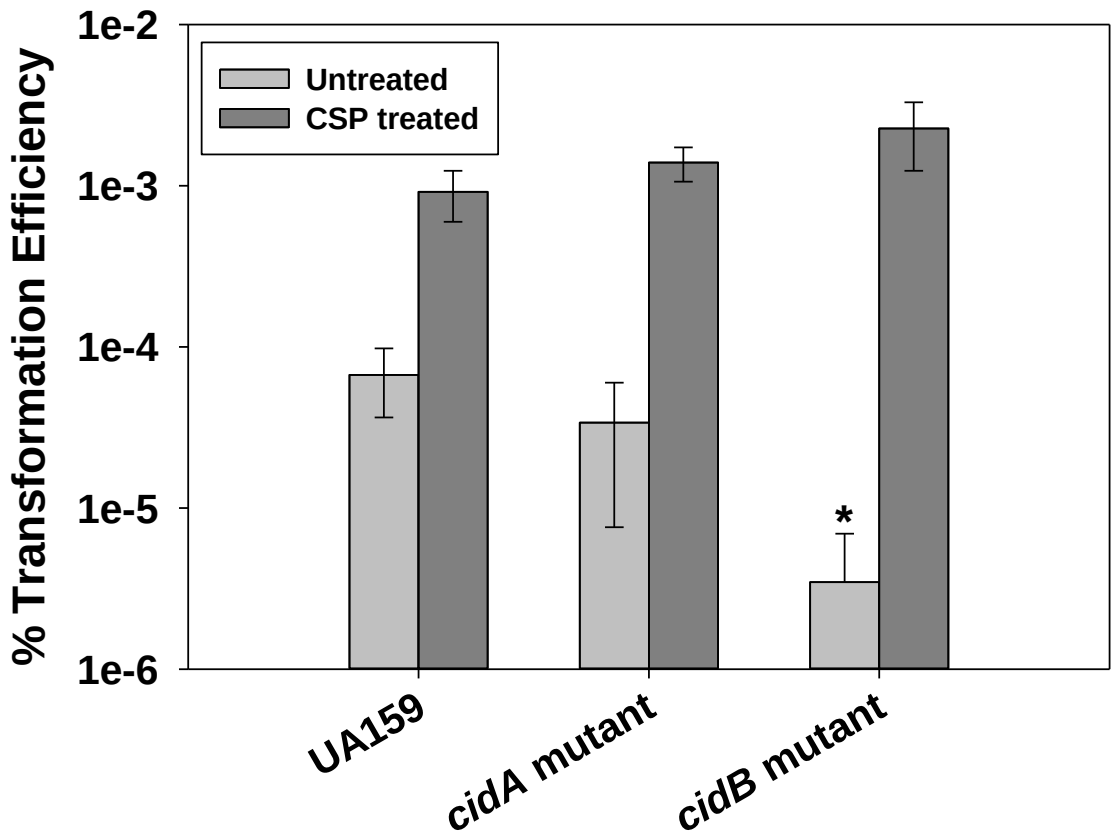


Figure 3-5. Competence assay performed on *cidB* mutant grown to an OD_{600} = 0.2-0.3. Data represents the average of three independent experiments. Error bars = SEM (*p = 0.0543, Student t-test)

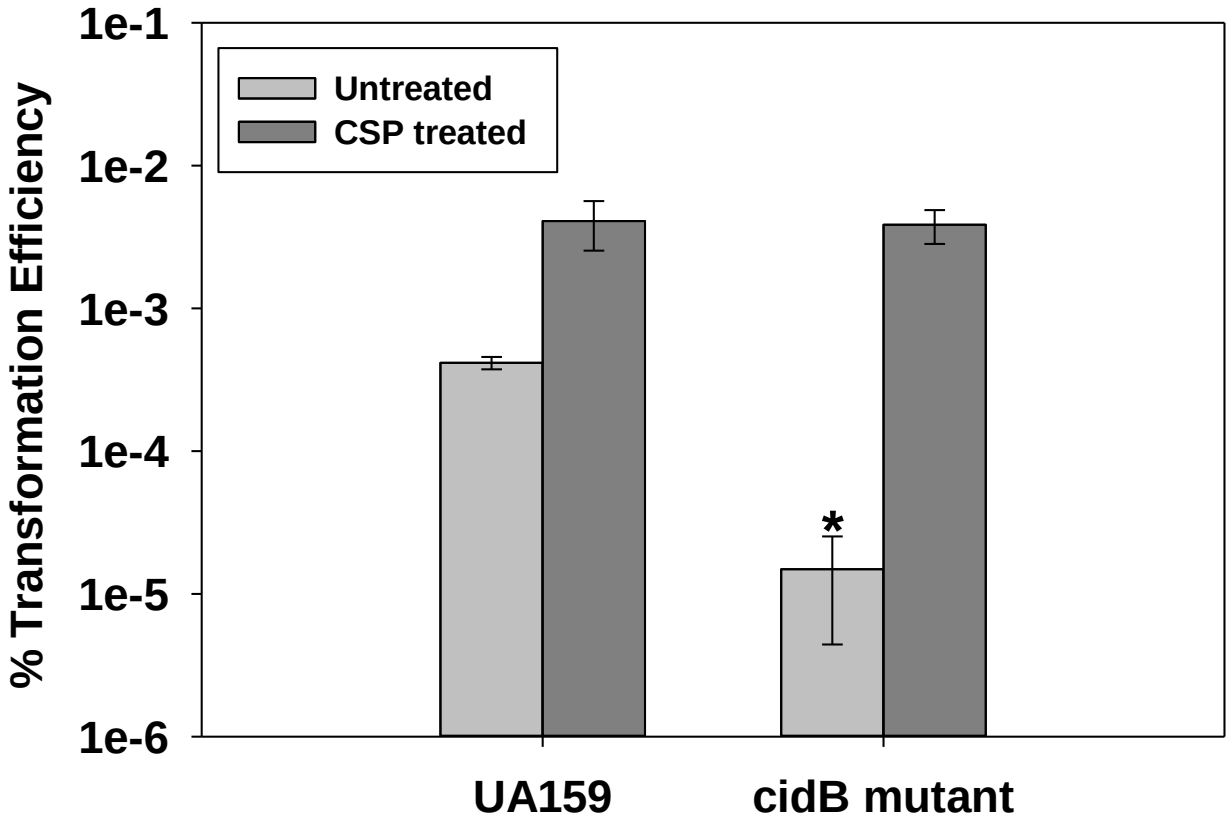


Figure 3-6. Competence Assay using unmethylated pOri23 DNA comparing UA159 (wild-type) and *cidB* mutant in the presences and absences of CSP. Data represents the average of three independent experiments per strain. Error bars = SEM. * significantly different compared to untreated UA159 ($p < 0.001$, Student t-test)

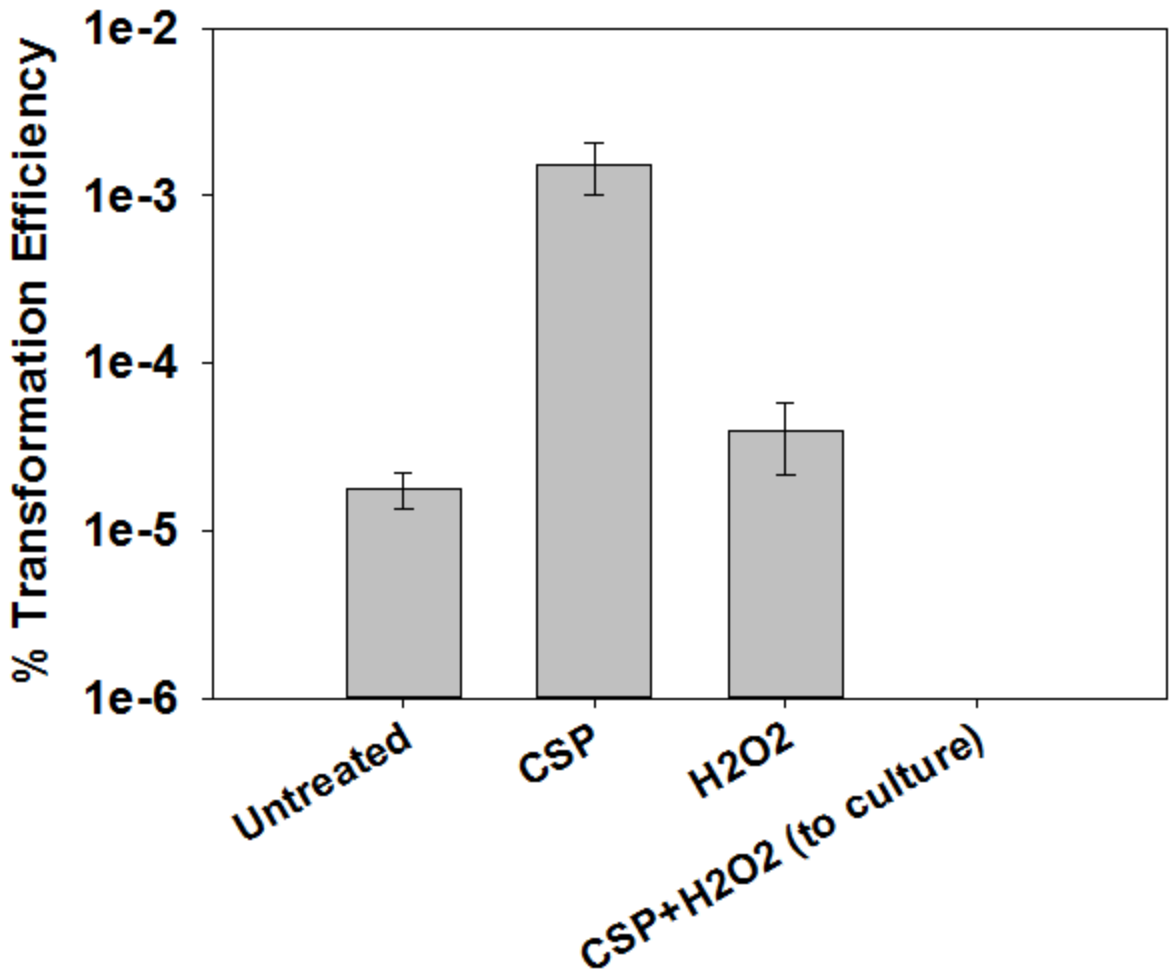


Figure 3-7. Quantitative competence assays comparing the effects of CSP (0.5 µg/ml) and H_2O_2 (0.5 mM) on *S. mutans* UA159**.** Transformation efficiency was calculated by CFU/ml BHI + selective antibiotic divided by CFU/ml on BHI multiplied by 100. Data represents the average of n=4 independent experiments, except CSP + H_2O_2 = 3 independent experiments. Error bars = SEM. *no colonies detectable on 10^{-2} dilution plate.

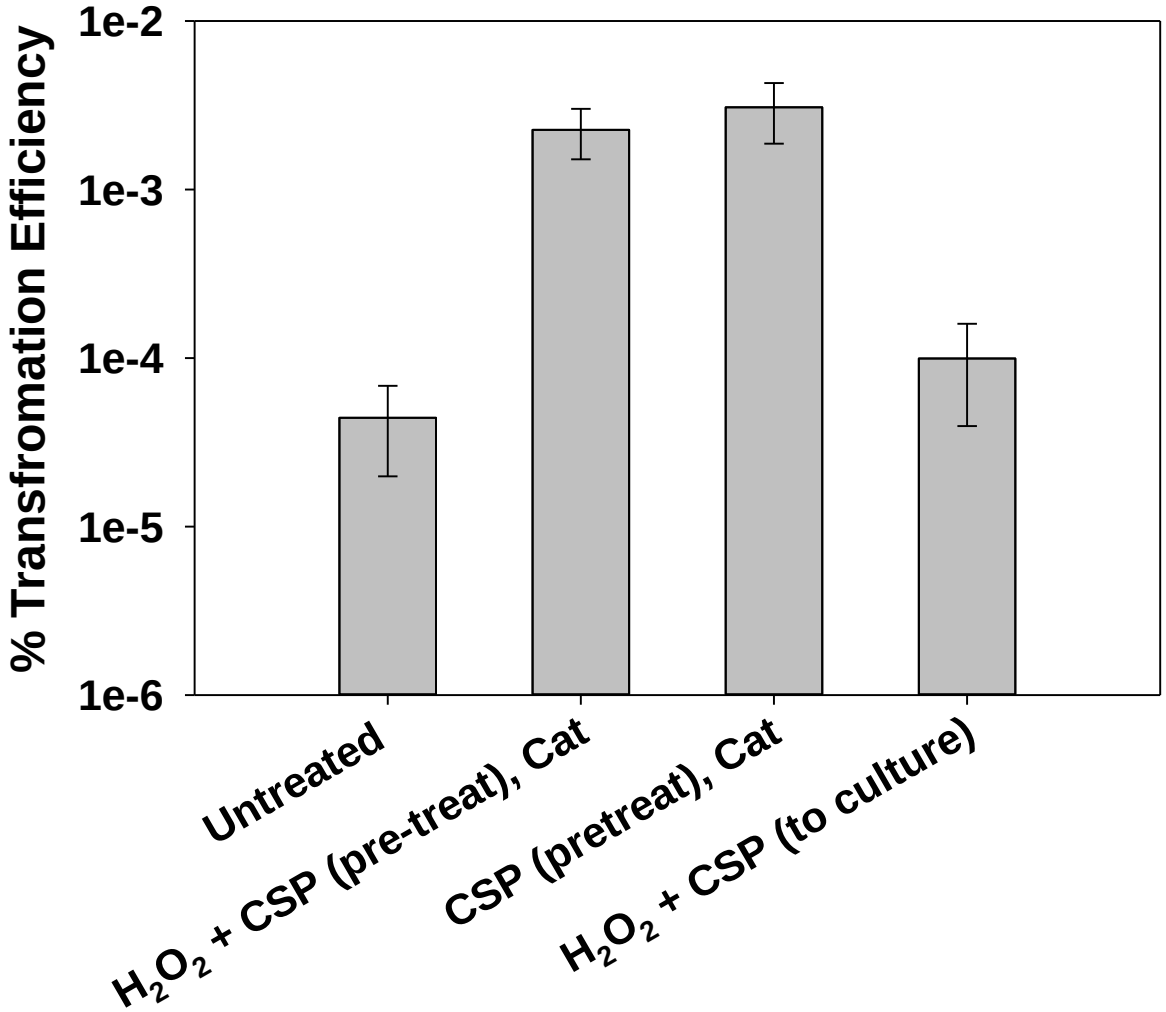


Figure 3-8. H_2O_2 pretreatment of CSP effect on competence of UA159 (wild-type). "H_2O_2 + CSP (pre-treatment), Cat" represents the pretreatment of CSP with H_2O_2 for 15 minutes followed by catalase addition, before it was added to the culture. "CSP (pretreat), Cat" refers to CSP alone (no H_2O_2 treatment) incubated for 15 minutes followed by catalase addition, before it was added to the culture. "H_2O_2 + CSP" represents the simultaneous addition of H_2O_2 and CSP directly to the culture. Transformation efficiency was calculated by CFU/ml BHI + selective antibiotic divided by CFU/ml on BHI multiplied by 100. Data represents the average of n=3 independent experiments for each test condition. Error bars = SEM.

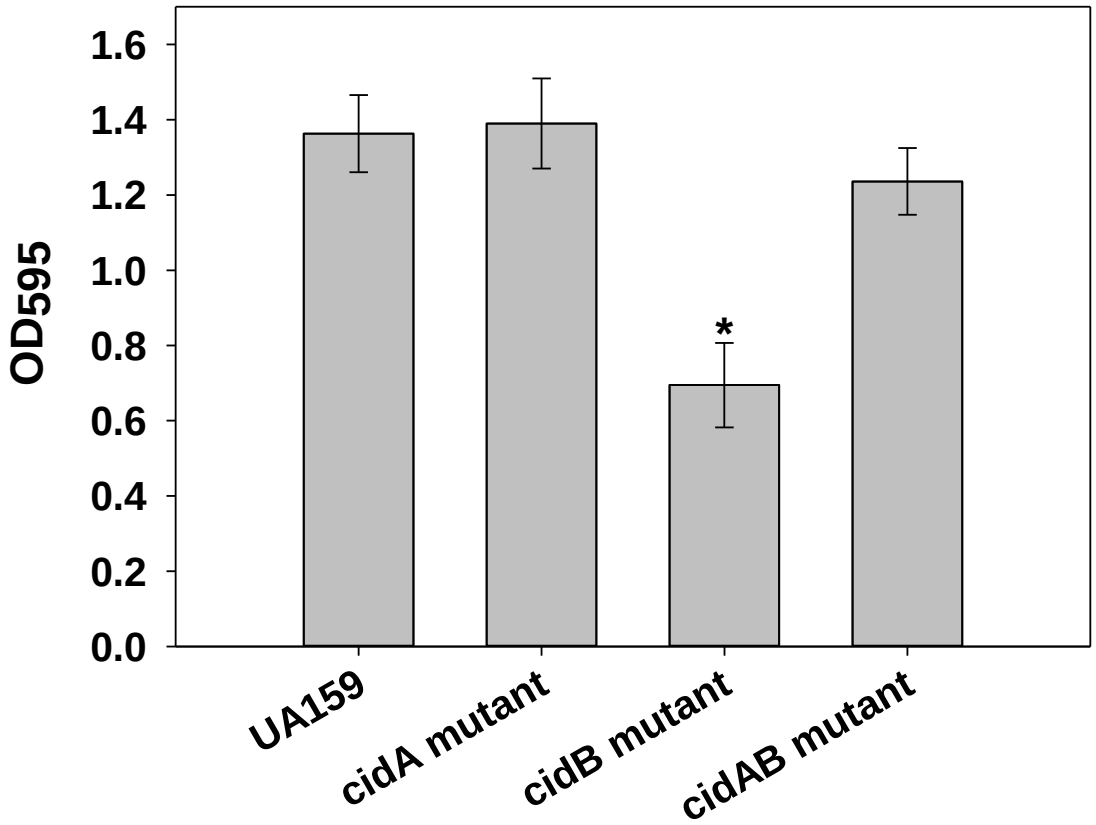


Figure 3-9. Effect of *cid* mutations on *S.mutans* biofilm grown in BM + 18 mM glucose/2 mM sucrose grown under CO_2 conditions. Data represents the average of n=3 independent experiments per bacterial strain. Error bars = SEM. *significantly different compared to untreated UA159 ($p < 0.05$, Tukey Test)

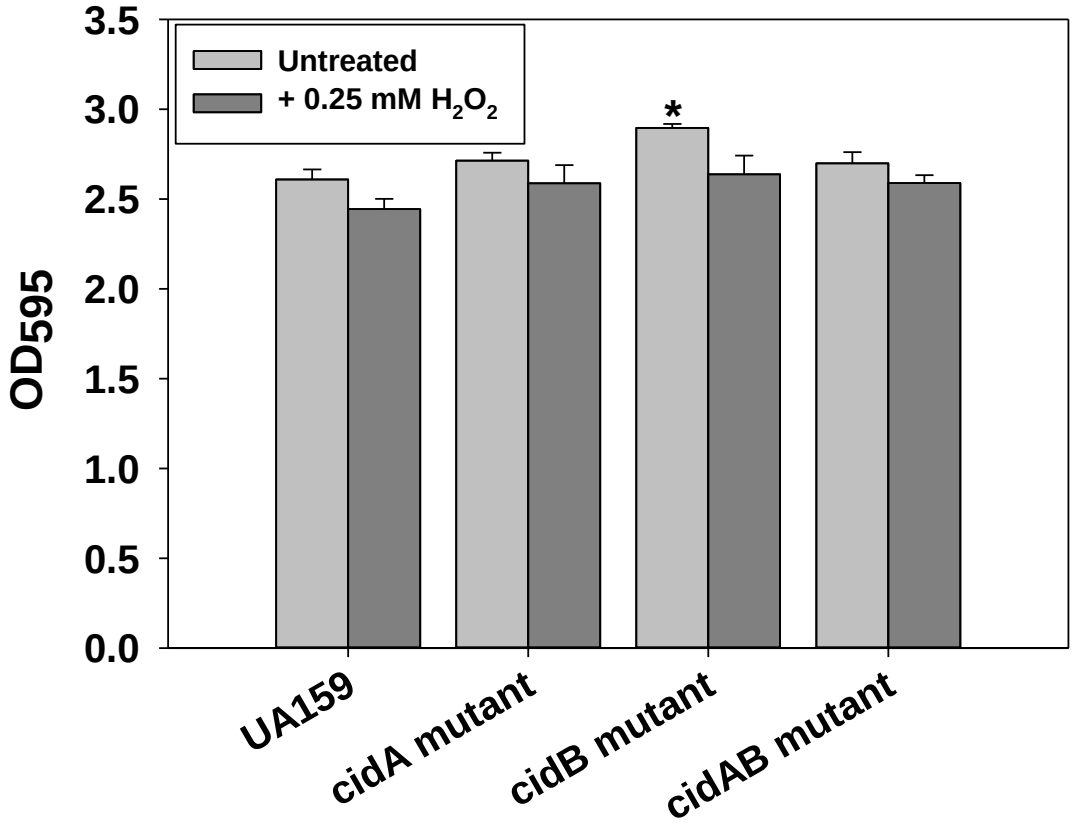


Figure 3-10. Effects of H_2O_2 and *cid* mutations on *S. mutans* biofilm formation in BM + 11 mM glucose/10 mM sucrose grown under CO_2 conditions. Data represents the average of n=3 independent experiments per bacterial strain. Error bars = SEM. *significantly different compared to untreated UA159 ($p < 0.05$, Tukey Test).

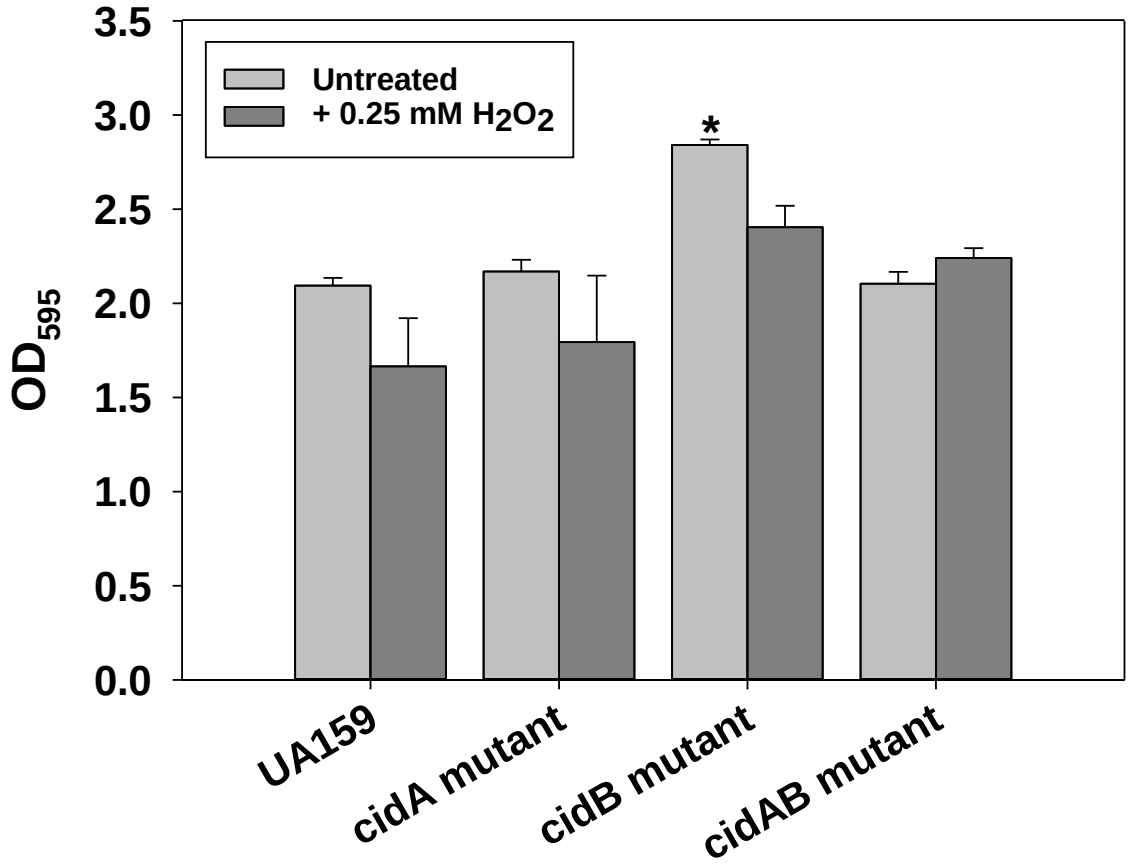


Figure 3-11. The effects of H_2O_2 and *cid* mutations on *S. mutans* biofilm formation in BHI + 11 mM glucose/10 mM sucrose grown under CO_2 conditions. Data represents the average of n=3 independent experiments per bacterial strain. Error bars = SEM. *significantly different compared to untreated UA159 ($p < 0.05$, Tukey Test)

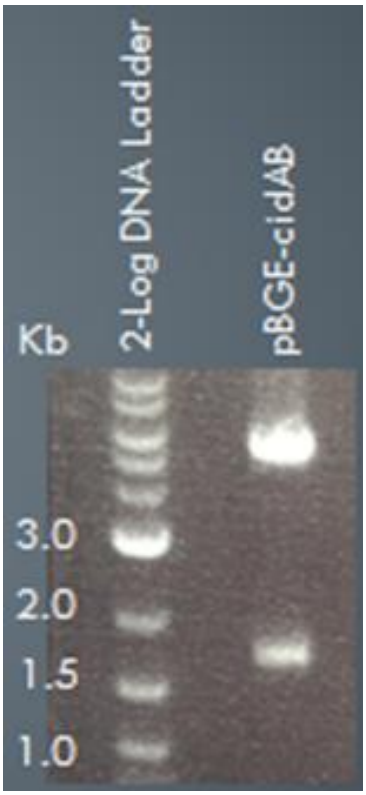


Figure 3-12. Verification of pBGE-cidAB complement cloning by BsrGI digestion and agarose gel electrophoresis. One representative experiment is depicted. For the agarose gel, Lane 1 = 2-Log ladder (New England Biolabs), Lane 2 = BsrGI-digested pBG-cidAB. The *cidAB* insert migrates at approximately 1.7-kb, and the vector backbone migrates at approximately 5-kb.

CHAPTER 4
DISCUSSION AND CONCLUSIONS

Ahn et al. [135] had previously demonstrated that *S. mutans lrgAB, cidB,* and *cidAB* mutants displayed decreased oxidative stress resistance when challenged with 10 mM paraquat, a superoxide generator. The experiments presented in this thesis confirm the sensitivity of these mutants to oxidative stress, since their growth in the presence of 1 mM H_2O_2 displayed almost complete growth inhibition relative to UA159. Although it is not known why only certain *cid* and *lrg* mutants display this sensitivity to oxidative stress, one possibility could be that functional redundancy exists between the A and B components of each operon. For example, a *lrgB* mutant may not display decreased oxidative stress resistance if the presence of CidB can compensate for loss of LrgB. Certain oral streptococcal species such as *S. gordonii* and *S. sanguinis* produce H_2O_2 which has been shown to serve as an antagonist against *S. mutans* in vitro [3], therefore determining whether the *S. mutans Cid/Lrg* system is involved in interspecies competition/ survival with these H_2O_2-producing oral streptococci would represent an important future experiment that may provide biological relevance to the Cid/Lrg system in *S. mutans*. Further evidence for a role of these proteins in oxidative stress resistance comes from a previous microarray analysis of the *S. mutans* LytST regulon [136], which revealed that some of the genes whose expression were affected by the loss of LytS were predicted to be involved in many cellular processes, including oxidative stress resistance.

Previous work in our lab had shown that a *S. mutans lrgA* mutant had a deficiency in its competence ability in both the presence and absence of CSP [136]. The effects of *cid* mutations on competence (presented in Figures 3-4, 3-5, and 3-6) were therefore assessed in this present study. Although the *cidA* and *cidAB* mutants behaved similar to the wild-type strain in both the presence and absence of CSP, the transformation efficiency of the *cidB* mutant in the absence of

CSP was not detectable with methylated DNA and was decreased by 2-logs with unmethylated DNA. It was observed while performing these assays that the *cidB* mutant sometimes took more growth time to reach an OD_{600} = 0.15 (the OD at which competence was measured in these studies) compared to the other strains. To determine whether a prolonged incubation time would restore the transformation efficiency of this strain, the *cidB* mutant was allowed to reach an OD_{600} = 0.2-0.3 prior to measuring competence (Figure 3-5). However, this modification did not restore competence to wild-type levels. Intriguingly, the addition of CSP restored competence of the *cidB* mutant to wild-type levels under all conditions tested, suggesting that this mutant may be defective in its ability to produce its own CSP. To test this theory, RNA was isolated from exponential-phase BHI cultures of the wild-type and *cidB* mutant strains, and quantitative real-time PCR was attempted to measure expression of *comC* (the CSP-encoding gene) and *comX* (sigma factor that induces expression of late competence genes). Although these experiments were unsuccessful due to various technical difficulties, it will be important to complete this work in the future to determine if lack of *comC* expression is responsible for the competence phenotype of the *cidB* mutant. As an alternative to this, the amount of CSP in culture supernatants of wild-type and *cidB* mutant cultures could be measured by mass spectrometry. Differential expression of *cidB* has not been detected in previous RNA microarray experiments examining the *S. mutans com* regulon [61], suggesting that the decreased competence observed in the *cidB* mutant may possibly be due to an indirect effect on bacterial physiology.

To determine if *cid* mutations affect *S. mutans* biofilm development, the wild-type, *cidA*, *cidB*, and *cidAB* mutants were initially grown in BM supplemented with 18 mM glucose and 2 mM sucrose and were subsequently stained with crystal violet (Figure 3-9). Again, only the *cidB* mutant had a detectable phenotype in these assays, displaying significantly less biofilm

formation than the wild-type strain. A comparable level of glucose (20 mM) has been previously-shown to stimulate high-level expression of the *cid* operon in *S. mutans* [135], thus it would expected that one or more of the *cid* mutants to display a pronounced phenotype in BM supplemented with 18 mM glucose. Interestingly, when the glucose concentration was reduced to 11 mM and the sucrose concentration was increased to 10 mM, the phenotype of the *cidB* mutant reversed, whereby it had significantly increased biofilm formation relative to the wild-type strain. It is possible that mutation of *cidB* alters the bacteria's stress response to low pH (as a result of metabolizing higher levels of glucose) or some other aspect of glucose metabolism, which in turn may affect biofilm formation under these different growth conditions. Alternatively, these different phenotypes could be a result of differential effects of the *cidB* mutation on sucrose-independent and sucrose-dependent biofilm mechanisms in *S. mutans*.

Based on of the fact that aerobic growth negatively impacts *S. mutans* biofilm formation, it would be predicted that the presence of H_2O_2 would also decrease biofilm formation [3]. In line with this hypothesis, we did observe a trend whereby the presence of sub-inhibitory levels of H_2O_2 slightly reduced biofilm formation in the wild-type and *cid* mutants. Although this observation was not statistically significant, it would still be of interest to study H_2O_2-mediated eDNA release and biofilm formation of *S. mutans* in the context of dual-species biofilm growth. Likewise, since *cid* and *lrg* mutants have been shown to affect eDNA release in other bacteria, future work will also be done to determine if H_2O_2 and/or *cid* mutations affect eDNA release in *S. mutans*. Interestingly, H_2O_2 has already been reported to stimulate competence and eDNA release phenotypes in *S. sanguinis*, *S. gordonii*, and *S. pneumoniae* [3] [113], but the effect of H_2O_2 on competence and eDNA release in *S. mutans* has not been previously published. A role for H_2O_2 in affecting *S. mutans* competence seems likely, since H_2O_2 has been reported to

stimulate competence in other oral streptococci [3] [113] and it is possible that cross-signaling could occur between H_2O_2- producing streptococci and *S. mutans*. H_2O_2- producing streptococci would most likely want to turn off *S. mutans* competence and possibly promote lysis and eDNA release, as this would potentially give the H_2O_2-producing streptococci a source of transforming DNA and/or nutrient recycling. In line with this idea, we found that H_2O_2 was able to reverse the positive effects of exogenously-added CSP on *S. mutans* competence (Fig. 3-8), and this effect did not appear to be a result of oxidative damage to the CSP peptide itself. The mechanism behind this observation is currently unknown, but will be the subject of future investigation by our research lab.

In summary, *S. mutans* is a common member of the bacterial flora in the oral cavity, but when this organism predominates dental plaque biofilm, it can cause dental cavities (caries). The *S. mutans cid* and *lrg* operons encode predicted membrane proteins that are found in many bacterial, archael, and even plant genomes. The work presented in this thesis has elaborated on a contribution of components of the *cid* and *lrg* operons to important *S. mutans* virulence traits such as biofilm formation, oxidative stress resistance, and competence [135, 136]. Although the mechanism is unclear as to how *S. mutans cid* and *lrg* genes promote oxidative stress resistance, it seems likely that targeting components of the Cid/Lrg system may eventually be viable strategy in the development of oral healthcare products to prevent caries development, given the severe growth inhibition of the *cidB*, *cidAB*, and *lrgAB* mutants in the presence of H_2O_2. For example, potential drug targets against the CidB protein could be identified by screening a small-molecule library for molecules that interact with CidB, followed by testing the candidate molecules for their ability to inhibit *S. mutans* growth in the presence of 1 mM H_2O_2.

LIST OF REFERENCES

1. Loesche WJ: **Role of Streptococcus mutans in human dental decay**. *Microbiological reviews* 1986, **50**(4):353-380.

2. Hamada S, Slade HD: **Biology, immunology, and cariogenicity of Streptococcus mutans**. *Microbiological reviews* 1980, **44**(2):331-384.

3. Kreth J, Zhang Y, Herzberg MC: **Streptococcal antagonism in oral biofilms: Streptococcus sanguinis and Streptococcus gordonii interference with Streptococcus mutans**. *Journal of bacteriology* 2008, **190**(13):4632-4640.

4. Ajdic D, McShan WM, McLaughlin RE, Savic G, Chang J, Carson MB, Primeaux C, Tian R, Kenton S, Jia H *et al*: **Genome sequence of Streptococcus mutans UA159, a cariogenic dental pathogen**. *Proceedings of the National Academy of Sciences of the United States of America* 2002, **99**(22):14434-14439.

5. Vadeboncoeur C, Pelletier M: **The phosphoenolpyruvate:sugar phosphotransferase system of oral streptococci and its role in the control of sugar metabolism**. *FEMS microbiology reviews* 1997, **19**(3):187-207.

6. Bowden GH, Hamilton IR: **Survival of oral bacteria**. *Critical reviews in oral biology and medicine : an official publication of the American Association of Oral Biologists* 1998, **9**(1):54-85.

7. Cvitkovitch DG, Gutierrez JA, Bleiweis AS: **Role of the citrate pathway in glutamate biosynthesis by Streptococcus mutans**. *Journal of bacteriology* 1997, **179**(3):650-655.

8. De Jong MH, Van der Hoeven JS: **The growth of oral bacteria on saliva**. *Journal of dental research* 1987, **66**(2):498-505.

9. Abbe K, Takahashi S, Yamada T: **Involvement of oxygen-sensitive pyruvate formate-lyase in mixed-acid fermentation by Streptococcus mutans under strictly anaerobic conditions**. *J Bacteriol* 1982, **152**(1):175-182.

10. Rosenbluh A, Rosenberg E: **Role of autocide AMI in development of Myxococcus xanthus**. *Journal of bacteriology* 1990, **172**(8):4307-4314.

11. Bender GR, Sutton SV, Marquis RE: **Acid tolerance, proton permeabilities, and membrane ATPases of oral streptococci**. *Infection and immunity* 1986, **53**(2):331-338.

12. Lemos JA, Quivey RG, Jr., Koo H, Abranches J: **Streptococcus mutans: a new Gram-positive paradigm?** *Microbiology* 2013, **159**(Pt 3):436-445.

13. Lemos JA, Burne RA: **A model of efficiency: stress tolerance by Streptococcus mutans**. *Microbiology* 2008, **154**(Pt 11):3247-3255.

14. Moore W, Moore LV: **The bacteria of periodontal diseases**. *Periodontology 2000* 1994, **5**(1):66-77.

15. Socransky SS, Haffajee AD: **Evidence of bacterial etiology: a historical perspective**. *Periodontology 2000* 1994, **5**(1):7-25.

16. Barnard JP, Stinson MW: **Influence of environmental conditions on hydrogen peroxide formation by Streptococcus gordonii**. *Infection and immunity* 1999, **67**(12):6558-6564.

17. Ryan C, Kleinberg I: **Bacteria in human mouths involved in the production and utilization of hydrogen peroxide**. *Archives of oral biology* 1995, **40**(8):753-763.

18. Banas JA: **Virulence properties of Streptococcus mutans**. *Front Biosci* 2004, **9**:1267-1277.

19. Koulourides T: **Summary of session II: fluoride and the caries process**. *J Dent Res* 1990, **69**:558.

20. Marcucci M, Bandettini M: **Dental caries in the rat in relation to the chemical composition of the teeth and diet. Variations in the diet of the Ca/P ratio obtained by changes in the phosphorus content**. *Minerva stomatologica* 1981, **30**(1):17.

21. Daniels T, Silverman Jr S, Michalski J, Greenspan J, Sylvester R, Talal N: **The oral component of Sjögren's syndrome**. *Oral Surgery, Oral Medicine, Oral Pathology* 1975, **39**(6):875-885.

22. Karmiol M, Walsh R: **Dental caries after radiotherapy of the oral regions**. *The Journal of the American Dental Association* 1975, **91**(4):838-845.

23. Rauk A, Yu D, Armstrong D: **Toward Site Specificity of Oxidative Damage in Proteins: CH and CC Bond Dissociation Energies and Reduction Potentials of the Radicals of Alanine, Serine, and Threonine Residues An ab Initio Study**. *Journal of the American Chemical Society* 1997, **119**(1):208-217.

24. CARIES D: **Oral and dental diseases: Causes, prevention and treatment strategies**. *Burden of Disease in India*:275.

25. Takahashi N, Horiuchi M, Yamada T: **Effects of acidification on growth and glycolysis of Streptococcus sanguis and Streptococcus mutans**. *Oral microbiology and immunology* 1997, **12**(2):72-76.

26. Bowden G: **Which bacteria are cariogenic in humans**. *Risk markers for Oral diseases* 1991, **1**:266-286.

27. Edwardsson S: **Microorganisms associated with dental caries**. *THYLSTRUP, A; FEJERSKOV, E Textbook of Cariology Copenhagen: Munksgaard* 1986:107-130.

28. Quivey RG, Kuhnert WL, Hahn K: **Genetics of acid adaptation in oral streptococci**. *Crit Rev Oral Biol Med* 2001, **12**(4):301-314.

29. Petersen PE, Lennon MA: **Effective use of fluorides for the prevention of dental caries in the 21st century: the WHO approach**. *Community dentistry and oral epidemiology* 2004, **32**(5):319-321.

30. Caufield P: **Dental caries--a transmissible and infectious disease revisited: a position paper**. *Pediatric dentistry* 1997, **19**(8):491.

31. Milgrom P, Riedy C, Weinstein P, Tanner A, Manibusan L, Bruss J: **Dental caries and its relationship to bacterial infection, hypoplasia, diet, and oral hygiene in 6-to 36-month-old children**. *Community dentistry and oral epidemiology* 2000, **28**(4):295-306.

32. Herzberg MC: **Oral Streptococci in health and disease**. *Streptococcal Infections: Clinical Aspects, Microbiology, and Molecular Pathogenesis* 2000:333.

33. Zero DT, Fontana M, Martinez-Mier EA, Ferreira-Zandona A, Ando M, Gonzalez-Cabezas C, Bayne S: **The biology, prevention, diagnosis and treatment of dental caries: scientific advances in the United States**. *J Am Dent Assoc* 2009, **140 Suppl 1**:25S-34S.

34. Beauchamp J, Caufield PW, Crall JJ, Donly K, Feigal R, Gooch B, Ismail A, Kohn W, Siegal M, Simonsen R: **Evidence-based clinical recommendations for the use of pit-and-fissure sealants: a report of the American Dental Association Council on Scientific Affairs**. *J Am Dent Assoc* 2008, **139**(3):257-268.

35. Hiiri A, Ahovuo-Saloranta A, Nordblad A, Makela M: **Pit and fissure sealants versus fluoride varnishes for preventing dental decay in children and adolescents**. *The Cochrane database of systematic reviews* 2010(3):CD003067.

36. Griffin SO, Oong E, Kohn W, Vidakovic B, Gooch BF, Bader J, Clarkson J, Fontana MR, Meyer DM, Rozier RG *et al*: **The effectiveness of sealants in managing caries lesions**. *J Dent Res* 2008, **87**(2):169-174.

37. Featherstone JD: **Prevention and reversal of dental caries: role of low level fluoride**. *Community dentistry and oral epidemiology* 1999, **27**(1):31-40.

38. Hamilton I: **Biochemical effects of fluoride on oral bacteria**. *Journal of dental research* 1990, **69**:660-667; discussion 682-663.

39. Gibbons R, Socransky S: **Intracellular polysaccharide storage by organisms in dental plaques: its relation to dental caries and microbial ecology of the oral cavity**. *Archives of oral biology* 1962, **7**(1):73-IN13.

40. Lie T: **Early dental plaque morphogenesis**. *Journal of periodontal research* 1977, **12**(2):73-89.

41. Newman BM, White P, Mohan SB, Cole JA: **Effect of dextran and ammonium sulphate on the reaction catalysed by a glucosyltransferase complex from Streptococcus mutans**. *Journal of general microbiology* 1980, **118**(2):353-366.

42. Rogers AH: **Bacteriocinogeny and the properties of some bacteriocins of Streptococcus mutans**. *Arch Oral Biol* 1976, **21**(2):99-104.

43. Saxton CA: **An electron microscope investigation of bacterial polysaccharide synthesis in human dental plaque**. *Arch Oral Biol* 1969, **14**(11):1275-1284.

44. Ahn SJ, Wen ZT, Brady LJ, Burne RA: **Characteristics of biofilm formation by Streptococcus mutans in the presence of saliva**. *Infection and immunity* 2008, **76**(9):4259-4268.

45. Khan AU, Islam B, Khan SN, Akram M: **A proteomic approach for exploring biofilm in Streptococcus mutans**. *Bioinformation* 2011, **5**(10):440-445.

46. Wen ZT, Yates D, Ahn SJ, Burne RA: **Biofilm formation and virulence expression by Streptococcus mutans are altered when grown in dual-species model**. *BMC microbiology* 2010, **10**:111.

47. Brady LJ, Maddocks SE, Larson MR, Forsgren N, Persson K, Deivanayagam CC, Jenkinson HF: **The changing faces of Streptococcus antigen I/II polypeptide family adhesins**. *Molecular microbiology* 2010, **77**(2):276-286.

48. Ohta H, Kato H, Okahashi N, Takahashi I, Hamada S, Koga T: **Characterization of a cell-surface protein antigen of hydrophilic Streptococcus mutans strain GS-5**. *Journal of general microbiology* 1989, **135**(4):981-988.

49. Lee SF, Progulske-Fox A, Erdos GW, Piacentini DA, Ayakawa GY, Crowley PJ, Bleiweis AS: **Construction and characterization of isogenic mutants of Streptococcus mutans deficient in major surface protein antigen P1 (I/II)**. *Infection and immunity* 1989, **57**(11):3306-3313.

50. Douglas C, Russell R: **Effect of specific antisera on adherence properties of the oral bacterium< i> Streptococcus mutans</i>**. *Archives of oral biology* 1982, **27**(12):1039-1045.

51. Bowen WH, Schilling K, Giertsen E, Pearson S, Lee SF, Bleiweis A, Beeman D: **Role of a cell surface-associated protein in adherence and dental caries**. *Infection and immunity* 1991, **59**(12):4606-4609.

52. Krzysciak W, Jurczak A, Koscielniak D, Bystrowska B, Skalniak A: **The virulence of Streptococcus mutans and the ability to form biofilms**. *European journal of clinical microbiology & infectious diseases : official publication of the European Society of Clinical Microbiology* 2014, **33**(4):499-515.

53. Ooshima T, Matsumura M, Hoshino T, Kawabata S, Sobue S, Fujiwara T: **Contributions of three glucosyltransferases to sucrose-dependent adherence of Streptococcus mutans**. *Journal of dental research* 2001, **80**(7):1672-1677.

54. Zeng L, Burne RA: **Transcriptional regulation of the cellobiose operon of Streptococcus mutans**. *Journal of bacteriology* 2009, **191**(7):2153-2162.

55. Banas JA, Vickerman MM: **Glucan-binding proteins of the oral streptococci**. *Crit Rev Oral Biol Med* 2003, **14**(2):89-99.

56. Aoki H, Shiroza T, Hayakawa M, Sato S, Kuramitsu HK: **Cloning of a Streptococcus mutans glucosyltransferase gene coding for insoluble glucan synthesis**. *Infection and immunity* 1986, **53**(3):587-594.

57. Hanada N, Kuramitsu HK: **Isolation and characterization of the Streptococcus mutans gtfC gene, coding for synthesis of both soluble and insoluble glucans**. *Infection and immunity* 1988, **56**(8):1999-2005.

58. Hanada N, Kuramitsu HK: **Isolation and characterization of the Streptococcus mutans gtfD gene, coding for primer-dependent soluble glucan synthesis**. *Infection and immunity* 1989, **57**(7):2079-2085.

59. Bowen WH, Koo H: **Biology of Streptococcus mutans-derived glucosyltransferases: role in extracellular matrix formation of cariogenic biofilms**. *Caries Res* 2011, **45**(1):69-86.

60. Ahn SJ, Wen ZT, Burne RA: **Effects of oxygen on virulence traits of Streptococcus mutans**. *J Bacteriol* 2007, **189**(23):8519-8527.

61. Ahn SJ, Burne RA: **Effects of oxygen on biofilm formation and the AtlA autolysin of Streptococcus mutans**. *Journal of bacteriology* 2007, **189**(17):6293-6302.

62. Yoshida A, Kuramitsu HK: **Multiple Streptococcus mutans Genes Are Involved in Biofilm Formation**. *Applied and environmental microbiology* 2002, **68**(12):6283-6291.

63. Ueda S, Shiroza T, Kuramitsu HK: **Sequence analysis of the< i> gtfC</i> gene from< i> Streptococcus mutans</i> GS-5**. *Gene* 1988, **69**(1):101-109.

64. Sadykov MR, Bayles KW: **The control of death and lysis in staphylococcal biofilms: a coordination of physiological signals**. *Current opinion in microbiology* 2012, **15**(2):211-215.

65. Guggenheim B: **Extracellular polysaccharides and microbial plaque**. *International dental journal* 1970, **20**(4):657.

66. Russell RR: **Glucan-binding proteins of Streptococcus mutans serotype c**. *Journal of general microbiology* 1979, **112**(1):197-201.

67. Lorenz MG, Wackernagel W: **Bacterial gene transfer by natural genetic transformation in the environment**. *Microbiological reviews* 1994, **58**(3):563-602.

68. Havarstein LS, Hakenbeck R, Gaustad P: **Natural competence in the genus Streptococcus: evidence that streptococci can change pherotype by interspecies recombinational exchanges**. *Journal of bacteriology* 1997, **179**(21):6589-6594.

69. Kleerebezem M, Quadri LE, Kuipers OP, de Vos WM: **Quorum sensing by peptide pheromones and two-component signal-transduction systems in Gram-positive bacteria**. *Molecular microbiology* 1997, **24**(5):895-904.

70. Lunsford RD, London J: **Natural genetic transformation in Streptococcus gordonii: comX imparts spontaneous competence on strain wicky**. *Journal of bacteriology* 1996, **178**(19):5831-5835.

71. Bassler BL: **How bacteria talk to each other: regulation of gene expression by quorum sensing**. *Current opinion in microbiology* 1999, **2**(6):582-587.

72. Costerton JW, Stewart PS, Greenberg EP: **Bacterial biofilms: a common cause of persistent infections**. *Science* 1999, **284**(5418):1318-1322.

73. Hassett DJ, Ma JF, Elkins JG, McDermott TR, Ochsner UA, West SE, Huang CT, Fredericks J, Burnett S, Stewart PS *et al*: **Quorum sensing in Pseudomonas aeruginosa controls expression of catalase and superoxide dismutase genes and mediates biofilm susceptibility to hydrogen peroxide**. *Molecular microbiology* 1999, **34**(5):1082-1093.

74. Otto M, Sussmuth R, Vuong C, Jung G, Gotz F: **Inhibition of virulence factor expression in Staphylococcus aureus by the Staphylococcus epidermidis agr pheromone and derivatives**. *FEBS letters* 1999, **450**(3):257-262.

75. Surette MG, Miller MB, Bassler BL: **Quorum sensing in Escherichia coli, Salmonella typhimurium, and Vibrio harveyi: a new family of genes responsible for autoinducer production**. *Proceedings of the National Academy of Sciences of the United States of America* 1999, **96**(4):1639-1644.

76. De Kievit TR, Gillis R, Marx S, Brown C, Iglewski BH: **Quorum-sensing genes in Pseudomonas aeruginosa biofilms: their role and expression patterns**. *Applied and environmental microbiology* 2001, **67**(4):1865-1873.

77. Davies DG, Parsek MR, Pearson JP, Iglewski BH, Costerton J, Greenberg E: **The involvement of cell-to-cell signals in the development of a bacterial biofilm**. *Science* 1998, **280**(5361):295-298.

78. Miller MB, Bassler BL: **Quorum sensing in bacteria**. *Annual review of microbiology* 2001, **55**:165-199.

79. de Kievit TR, Iglewski BH: **Bacterial quorum sensing in pathogenic relationships**. *Infection and immunity* 2000, **68**(9):4839-4849.

80. Parsek MR, Greenberg EP: **Acyl-homoserine lactone quorum sensing in gram-negative bacteria: a signaling mechanism involved in associations with higher organisms**. *Proceedings of the National Academy of Sciences of the United States of America* 2000, **97**(16):8789-8793.

81. Li YH, Lau PC, Lee JH, Ellen RP, Cvitkovitch DG: **Natural genetic transformation of Streptococcus mutans growing in biofilms**. *Journal of bacteriology* 2001, **183**(3):897-908.

82. Petersen FC, Fimland G, Scheie AA: **Purification and functional studies of a potent modified quorum-sensing peptide and a two-peptide bacteriocin in Streptococcus mutans**. *Molecular microbiology* 2006, **61**(5):1322-1334.

83. Berge M, Moscoso M, Prudhomme M, Martin B, Claverys JP: **Uptake of transforming DNA in Gram-positive bacteria: a view from Streptococcus pneumoniae**. *Molecular microbiology* 2002, **45**(2):411-421.

84. Cheng Q, Campbell EA, Naughton AM, Johnson S, Masure HR: **The com locus controls genetic transformation in Streptococcus pneumoniae**. *Molecular microbiology* 1997, **23**(4):683-692.

85. Pestova E, Håvarstein L, Morrison D: **Regulation of competence for genetic transformation in Streptococcus pneumoniae by an auto-induced peptide pheromone and a two-component regulatory system**. *Molecular microbiology* 1996, **21**(4):853-862.

86. Ahn SJ, Wen ZT, Burne RA: **Multilevel control of competence development and stress tolerance in Streptococcus mutans UA159**. *Infection and immunity* 2006, **74**(3):1631-1642.

87. Lee MS, Morrison DA: **Identification of a new regulator in Streptococcus pneumoniae linking quorum sensing to competence for genetic transformation**. *Journal of bacteriology* 1999, **181**(16):5004-5016.

88. Luo P, Li H, Morrison DA: **ComX is a unique link between multiple quorum sensing outputs and competence in Streptococcus pneumoniae**. *Molecular microbiology* 2003, **50**(2):623-633.

89. Luo P, Morrison DA: **Transient association of an alternative sigma factor, ComX, with RNA polymerase during the period of competence for genetic transformation in Streptococcus pneumoniae**. *Journal of bacteriology* 2003, **185**(1):349-358.

90. Petersen FC, Tao L, Scheie AA: **DNA binding-uptake system: a link between cell-to-cell communication and biofilm formation**. *Journal of bacteriology* 2005, **187**(13):4392-4400.

91. Li YH, Tang N, Aspiras MB, Lau PC, Lee JH, Ellen RP, Cvitkovitch DG: **A quorum-sensing signaling system essential for genetic competence in Streptococcus mutans is involved in biofilm formation**. *Journal of bacteriology* 2002, **184**(10):2699-2708.

92. Christensen BB, Sternberg C, Andersen JB, Eberl L, Moller S, Givskov M, Molin S: **Establishment of new genetic traits in a microbial biofilm community**. *Applied and environmental microbiology* 1998, **64**(6):2247-2255.

93. Hausner M, Wuertz S: **High rates of conjugation in bacterial biofilms as determined by quantitative in situ analysis**. *Applied and environmental microbiology* 1999, **65**(8):3710-3713.

94. Williams HG, Day MJ, Fry JC, Stewart GJ: **Natural transformation in river epilithon**. *Applied and environmental microbiology* 1996, **62**(8):2994-2998.

95. Mashburn-Warren L, Morrison DA, Federle MJ: **A novel double-tryptophan peptide pheromone controls competence in Streptococcus spp. via an Rgg regulator**. *Molecular microbiology* 2010, **78**(3):589-606.

96. Sulavik MC, Tardif G, Clewell DB: **Identification of a gene, rgg, which regulates expression of glucosyltransferase and influences the Spp phenotype of Streptococcus gordonii Challis**. *Journal of bacteriology* 1992, **174**(11):3577-3586.

97. Fontaine L, Boutry C, de Frahan MH, Delplace B, Fremaux C, Horvath P, Boyaval P, Hols P: **A novel pheromone quorum-sensing system controls the development of natural competence in Streptococcus thermophilus and Streptococcus salivarius**. *Journal of bacteriology* 2010, **192**(5):1444-1454.

98. Lemme A, Grobe L, Reck M, Tomasch J, Wagner-Dobler I: **Subpopulation-specific transcriptome analysis of competence-stimulating-peptide-induced Streptococcus mutans**. *Journal of bacteriology* 2011, **193**(8):1863-1877.

99. Rice KC, Mann EE, Endres JL, Weiss EC, Cassat JE, Smeltzer MS, Bayles KW: **The cidA murein hydrolase regulator contributes to DNA release and biofilm development in Staphylococcus aureus**. *Proceedings of the National Academy of Sciences of the United States of America* 2007, **104**(19):8113-8118.

100. Matsukawa M, Greenberg EP: **Putative exopolysaccharide synthesis genes influence Pseudomonas aeruginosa biofilm development**. *J Bacteriol* 2004, **186**(14):4449-4456.

101. van Schaik EJ, Giltner CL, Audette GF, Keizer DW, Bautista DL, Slupsky CM, Sykes BD, Irvin RT: **DNA binding: a novel function of Pseudomonas aeruginosa type IV pili**. *J Bacteriol* 2005, **187**(4):1455-1464.

102. Whitchurch CB, Tolker-Nielsen T, Ragas PC, Mattick JS: **Extracellular DNA required for bacterial biofilm formation**. *Science* 2002, **295**(5559):1487-1487.

103. Mann EE, Rice KC, Boles BR, Endres JL, Ranjit D, Chandramohan L, Tsang LH, Smeltzer MS, Horswill AR, Bayles KW: **Modulation of eDNA release and degradation affects Staphylococcus aureus biofilm maturation**. *PLoS One* 2009, **4**(6):e5822.

104. Moscoso M, Claverys JP: **Release of DNA into the medium by competent Streptococcus pneumoniae: kinetics, mechanism and stability of the liberated DNA**. *Molecular microbiology* 2004, **54**(3):783-794.

105. Steinmoen H, Teigen A, Havarstein LS: **Competence-induced cells of Streptococcus pneumoniae lyse competence-deficient cells of the same strain during cocultivation**. *Journal of bacteriology* 2003, **185**(24):7176-7183.

106. Moscoso M, Claverys JP: **Release of DNA into the medium by competent Streptococcus pneumoniae: kinetics, mechanism and stability of the liberated DNA**. *Molecular microbiology* 2004, **54**(3):783-794.

107. Perry JA, Cvitkovitch DG, Levesque CM: **Cell death in Streptococcus mutans biofilms: a link between CSP and extracellular DNA**. *FEMS microbiology letters* 2009, **299**(2):261-266.

108. Higuchi M, Shimada M, Yamamoto Y, Hayashi T, Koga T, Kamio Y: **Identification of two distinct NADH oxidases corresponding to H2O2-forming oxidase and H2O-forming oxidase induced in Streptococcus mutans**. *Journal of general microbiology* 1993, **139**(10):2343-2351.

109. Marquis R: **Oxygen metabolism, oxidative stress and acid-base physiology of dental plaque biofilms**. *Journal of industrial microbiology* 1995, **15**(3):198-207.

110. Matsumoto-Nakano M, Kuramitsu HK: **Role of bacteriocin immunity proteins in the antimicrobial sensitivity of Streptococcus mutans**. *Journal of bacteriology* 2006, **188**(23):8095-8102.

111. Nobbs AH, Zhang Y, Khammanivong A, Herzberg MC: **Streptococcus gordonii Hsa environmentally constrains competitive binding by Streptococcus sanguinis to saliva-coated hydroxyapatite**. *Journal of bacteriology* 2007, **189**(8):3106-3114.

112. Kreth J, Merritt J, Shi W, Qi F: **Competition and coexistence between Streptococcus mutans and Streptococcus sanguinis in the dental biofilm**. *Journal of bacteriology* 2005, **187**(21):7193-7203.

113. Battig P, Muhlemann K: **Influence of the spxB gene on competence in Streptococcus pneumoniae**. *J Bacteriol* 2008, **190**(4):1184-1189.

114. Itzek A, Zheng L, Chen Z, Merritt J, Kreth J: **Hydrogen peroxide-dependent DNA release and transfer of antibiotic resistance genes in Streptococcus gordonii**. *Journal of bacteriology* 2011, **193**(24):6912-6922.

115. Bayles KW: **The biological role of death and lysis in biofilm development**. *Nature Reviews Microbiology* 2007, **5**(9):721-726.

116. Bayles KW: **Are the molecular strategies that control apoptosis conserved in bacteria?** *Trends in microbiology* 2003, **11**(7):306-311.

117. Rice KC, Bayles KW: **Molecular control of bacterial death and lysis**. *Microbiology and molecular biology reviews : MMBR* 2008, **72**(1):85-109, table of contents.

118. Wang IN, Smith DL, Young R: **Holins: the protein clocks of bacteriophage infections**. *Annual review of microbiology* 2000, **54**:799-825.

119. White R, Chiba S, Pang T, Dewey JS, Savva CG, Holzenburg A, Pogliano K, Young R: **Holin triggering in real time**. *Proceedings of the National Academy of Sciences of the United States of America* 2011, **108**(2):798-803.

120. Savva CG, Dewey JS, Deaton J, White RL, Struck DK, Holzenburg A, Young R: **The holin of bacteriophage lambda forms rings with large diameter**. *Molecular microbiology* 2008, **69**(4):784-793.

121. Rice KC, Firek BA, Nelson JB, Yang SJ, Patton TG, Bayles KW: **The Staphylococcus aureus cidAB operon: evaluation of its role in regulation of murein hydrolase activity and penicillin tolerance**. *Journal of bacteriology* 2003, **185**(8):2635-2643.

122. Groicher KH, Firek BA, Fujimoto DF, Bayles KW: **The Staphylococcus aureus lrgAB operon modulates murein hydrolase activity and penicillin tolerance**. *Journal of bacteriology* 2000, **182**(7):1794-1801.

123. Yang SJ, Rice KC, Brown RJ, Patton TG, Liou LE, Park YH, Bayles KW: **A LysR-type regulator, CidR, is required for induction of the Staphylococcus aureus cidABC operon**. *Journal of bacteriology* 2005, **187**(17):5893-5900.

124. Brunskill EW, Bayles KW: **Identification of LytSR-regulated genes from Staphylococcus aureus**. *Journal of bacteriology* 1996, **178**(19):5810-5812.

125. Rice KC, Nelson JB, Patton TG, Yang SJ, Bayles KW: **Acetic acid induces expression of the Staphylococcus aureus cidABC and lrgAB murein hydrolase regulator operons**. *Journal of bacteriology* 2005, **187**(3):813-821.

126. Moormeier DE, Endres JL, Mann EE, Sadykov MR, Horswill AR, Rice KC, Fey PD, Bayles KW: **Use of microfluidic technology to analyze gene expression during Staphylococcus aureus biofilm formation reveals distinct physiological niches**. *Applied and environmental microbiology* 2013, **79**(11):3413-3424.

127. Ranjit DK, Endres JL, Bayles KW: **Staphylococcus aureus CidA and LrgA proteins exhibit holin-like properties**. *Journal of bacteriology* 2011, **193**(10):2468-2476.

128. Ma L, Conover M, Lu H, Parsek MR, Bayles K, Wozniak DJ: **Assembly and development of the Pseudomonas aeruginosa biofilm matrix**. *PLoS pathogens* 2009, **5**(3):e1000354.

129. Chandramohan L, Ahn J-S, Weaver KE, Bayles KW: **An overlap between the control of programmed cell death in Bacillus anthracis and sporulation**. *Journal of bacteriology* 2009, **191**(13):4103-4110.

130. Ahn JS, Chandramohan L, Liou LE, Bayles KW: **Characterization of CidR-mediated regulation in Bacillus anthracis reveals a previously undetected role of S-layer proteins as murein hydrolases**. *Molecular microbiology* 2006, **62**(4):1158-1169.

131. Wang J, Bayles KW: **Programmed cell death in plants: lessons from bacteria?** *Trends in plant science* 2013, **18**(3):133-139.

132. Yang Y, Jin H, Chen Y, Lin W, Wang C, Chen Z, Han N, Bian H, Zhu M, Wang J: **A chloroplast envelope membrane protein containing a putative LrgB domain related to the control of bacterial death and lysis is required for chloroplast development in Arabidopsis thaliana**. *The New phytologist* 2012, **193**(1):81-95.

133. Yamaguchi M, Takechi K, Myouga F, Imura S, Sato H, Takio S, Shinozaki K, Takano H: **Loss of the plastid envelope protein AtLrgB causes spontaneous chlorotic cell death in Arabidopsis thaliana**. *Plant & cell physiology* 2012, **53**(1):125-134.

134. Rice KC, Bayles KW: **Death's toolbox: examining the molecular components of bacterial programmed cell death**. *Molecular microbiology* 2003, **50**(3):729-738.

135. Ahn SJ, Rice KC, Oleas J, Bayles KW, Burne RA: **The Streptococcus mutans Cid and Lrg systems modulate virulence traits in response to multiple environmental signals**. *Microbiology* 2010, **156**(Pt 10):3136-3147.

136. Ahn SJ, Qu MD, Roberts E, Burne RA, Rice KC: **Identification of the Streptococcus mutans LytST two-component regulon reveals its contribution to oxidative stress tolerance**. *BMC microbiology* 2012, **12**:187.

137. Loo CY, Corliss DA, Ganeshkumar N: **Streptococcus gordonii biofilm formation: identification of genes that code for biofilm phenotypes**. *Journal of bacteriology* 2000, **182**(5):1374-1382.

138. Haldi J, Wynn W, Bentley KD, Law ML: **Dental caries in the albino rat in relation to the chemical composition of the teeth and of the diet. IV. Variations in the Ca/P ratio of the diet induced by changing the calcium content**. *The Journal of nutrition* 1959, **67**(4):645.

139. Seidman CE, Struhl K, Sheen J, Jessen T: **Introduction of plasmid DNA into cells**. *Current protocols in molecular biology* 1997:1.8. 1-1.8. 10.

140. Keyes P: **The infectious and transmissible nature of experimental dental caries: Findings and implications**. *Archives of oral biology* 1960, **1**(4):304-IN304.

141. Sadykov MR, Thomas VC, Marshall DD, Wenstrom CJ, Moormeier DE, Widhelm TJ, Nuxoll AS, Powers R, Bayles KW: **Inactivation of the Pta-AckA pathway causes cell death in Staphylococcus aureus**. *Journal of bacteriology* 2013, **195**(13):3035-3044.

BIOGRAPHICAL SKETCH

O’neshia Vanae Carney is a Miami, Florida native who graduated cum lade from Miami Norland Senior High School in 2006. Upon graduation she attended Miami Dade College where she graduated in 2009 with an Associate of Arts in biology. After completing her AA degree she transferred to the University of Florida to pursue a Bachelor of Science in biology. She then attended University of Florida and in August 2014 graduated with a Master of Science in microbiology and cell science.

After graduation O’neshia will continue to reside in Gainesville, Florida where she will work as a lab technician and prepare to gain acceptance into dental school.