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Copper Chaperone In Streptococcus Mutans Biofilm Formation And Dispersion Of Biofilms By Small Molecules

Sandra Stephanie Garcia
University of Alabama at Birmingham

COPPER CHAPERONE IN *STREPTOCOCCUS MUTANS* BIOFILM FORMATION AND DISPERSION OF BIOFILMS BY SMALL MOLECULES

by

SANDRA STEPHANIE GARCIA

HUI WU, COMMITTEE CHAIR
WILLIAM H. BENJAMIN, JR
CHRISTIAN MELANDER
SUZANNE MICHALEK
MOON NAHM

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ABSTRACT

Dental caries, commonly known as tooth decay, is characterized by the destruction of the tooth's hydroxyapatite, the primary mineral of enamel. Tooth decay is caused by the combination of poor salivary flow, frequent dietary sugar intake, poor dental hygiene, and the prevalence of cariogenic bacteria. Exposure to sucrose promotes *Streptococcus mutans* to form a cariogenic biofilm, which is the major risk factor for dental caries. Currently there are no anticaries therapies that selectively target *S. mutans* biofilms. In this study, we identified small molecule 3F1 which was capable of selectively dispersing *S. mutans* biofilms and utilized it to determine whether selective targeting of *S. mutans* biofilms could effectively prevent dental caries and preserve the oral microbiome.

We identified small molecule 3F1 capable of selectively dispersing *S. mutans* biofilms *in vitro*. Dispersal by 3F1 was independent of known factors essential for biofilm development. In a rat caries study, treatment of rat molars for 4 weeks with 3F1 controlled *S. mutans* and prevented dental caries while preserving the oral microbiome. These results indicate that a *S. mutans* biofilm specific small molecule is a viable therapy to prevent dental caries without disturbing the oral microbiome.

In order to persist in the oral cavity and cause disease, *S. mutans* must survive against toxic metal ions and antagonistic commensal species. Toxic levels of copper may result from amalgam and dietary intake. Imperative to survival against copper is the copper-resistance operon *copYAZ*. Evidence from *copYAZ* operon homologues in bacterial pathogens suggests that the components may play a role in fitness and virulence in *S. mutans*. Although the intact *copYAZ* operon has been implicated in copper-independent processes, the role of individual genes of the *copYAZ* operon in virulence has not been previously investigated. In this study, we elucidated the role of individual genes in the *copYAZ* operon in *S. mutans* virulence.

In this study we demonstrated that only CopZ and not the CopYAZ operon plays a role in biofilm formation, expression and secretion of glucosyltransferases, and competitiveness against oral commensal species. Elucidation of the mechanism of *copZ* on the role of biofilm formation and competitiveness may lead to the identification of novel virulence-regulatory pathways that are amenable to drug discovery.

Keywords: Dental caries, copper, microbiome

DEDICATION

This work is dedicated to my mother, Sandra, father, Edgar, and brother, Alex, because they have fervently believed in my goals and ambitions since the days I played with dolls and drew masterpieces in crayon. Specifically, I would like to dedicate this work to my mother, whose support made it possible for me to attend the college of my choice and chase my wildest dreams.

I would also like to dedicate this work to my dearest fiancé, Will who has shown me nothing but love, support, and snacks to make it through the PhD program, and has also filled my life with adventure.

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LIST OF ABBREVIATIONS

AgI/II	Antigen I/II
BM	Biofilm Media
BrpA	Biofilm Regulatory Protein A
CPD	Caries-Promoting Diet
CLSM	Confocal Laser Scanning Microscopy
eDNA	Extracellular DNA
Gtfs	Glucosyltransferases
qPCR	Quantitative Polymerase Chain Reaction
qRT-PCR	Qualitative Real Time Polymerase Chain Reaction
WT UA159	Wild-type *Streptococcus mutans* UA159

INTRODUCTION

DENTAL CARIES

Dental caries, or tooth decay, is characterized by the demineralization of the enamel. Despite the multitude of preventative therapies against dental caries, this disease continues to be the most prevalent chronic disease worldwide. Dental caries affects approximately 42% of children aged 2-11 with prevalence increasing among children of poor economic status and Hispanic or Black families (Dye *et al.*, 2012). Among adults, prevalence of dental caries increases with age. Approximately 90% of adults and seniors have had decay in their permanent teeth (Dye *et al.*, 2012).

While limited decay can be reversed by proper dental hygiene, extensive tooth decay is irreversible and must be professionally treated. Common treatment for dental caries includes dental restoration with metal-based amalgam or other dental materials. If left untreated, dental caries may result in severe infections, tooth loss by extraction, or other costly dental services. Untreated tooth decay may lead to the infection of the pulp, which is a result of a deep lesion reaching the pulp and nerves of the tooth. Infection of the pulp can lead to lengthy and painful endodontic treatment.

Conventional prevention methods against dental caries include flossing and tooth brushing with products commonly composed of remineralizing ingredients, which may also cause broad-spectrum killing of bacteria, e.g., fluoride or essential oils. Despite the availability of potent preventative therapeutics, dental caries continues to be the most prevalent infectious disease worldwide, suggesting current therapies are inadequate in preventing caries. Proper prevention of dental caries is dependent on elucidating the different factors that lead to disease. Dental caries is a multifactorial disease caused by

the right combination of a susceptible tooth surface, salivary flow, a sugary diet, and the colonization and persistence of cariogenic bacteria. Cariogenic species *Streptococcus mutans* is the most associated species with dental caries. *Streptococcus mutans* is a Gram positive bacterium that is first transmitted to erupting teeth in the infant by their mother or guardian. After initial attachment of *S. mutans* to the tooth surface, exposure to dietary sugars enables *S. mutans* to form a tenacious biofilm, allowing *S. mutans* to persist in the oral cavity. Within the biofilm, *S. mutans* can metabolize sucrose and secrete lactic acid into the local milieu. Frequent exposure to sucrose results in repeated lactic acid challenge and eventually the demineralization of enamel.

THE ORAL MICROBIOTA & DENTAL CARIES

The human microbiome is composed of aggregates of microorganisms colonizing the surfaces and deep tissues of the body. Locations such as the gut, skin, urogenital tract, and oral cavity are each colonized by their own microbiomes which have a functional role in promoting health (Fujimura *et al.*, 2010, Grice & Segre, 2011). These specialized microbiomes are distinguished by the diversity and composition of microbes within the community. While the composition of the microbiome is generally similar across geographical locations, it can differ between individuals, age, and race (Lif Holgerson *et al.*, 2015, Consortium, 2012). Disrupting the normal or native flora by unspecific treatment, an autoimmune disease, or an unhealthy diet can cause microbial dysbiosis which can have detrimental consequences. In the gut, destruction of the normal flora by antibiotic treatment may make the gut susceptible to a *Clostridium difficile* infection, causing diarrheal illness (Johanesen *et al.*, 2015). The autoimmune disease rheumatoid

arthritis causes gut and oral microbial dysbiosis, an effect which is reversible with disease-modifying anti-rheumatic drugs (Zhang *et al.*, 2015). Dietary intake can also play a major role in the composition of the microbiome. Distinct microbiota compositions were shown in mice when fed a low fat diet and then switched to a high fat diet regardless of host genotype, suggesting that diet is the dominating factor of the microbiome structure in the gut (Carmody *et al.*, 2015).

The oral microbiome is composed of hundreds of bacterial species which live on teeth, gums, tongue, and inside of the cheek. Located at the origin of saliva production and entrance of food and drink, oral bacteria inhabiting the oral cavity have evolved to withstand the environmental stress of the oral cavity and efficiently acquire nutrients from the saliva and diet. At the phylum-level, the human oral microbiota is predominantly composed of *Firmicutes*, *Bacteriodetes*, *Proteobacteria*, *Actinobacteria*, *Spirochaetes*, and *Fusobacteria* (Dewhirst *et al.*, 2010). At the genus-level, the oral cavity is predominantly composed of *Streptococcus* including commensal species *Streptococcus gordonii* and *Streptococcus sanguinis*. During active disease such as dental caries, the composition may be similar to a healthy microbiome, but the differential abundance of a particular species may be indicative of disease. The overall phylogenetic composition does not change dramatically between health and disease (Yang *et al.*, 2012, Adler *et al.*, 2013, Peterson *et al.*, 2013). The same species were present in both healthy and caries-active plaque although cariogenic species *S. mutans* was more abundant in caries-active samples (Peterson *et al.*, 2013). Likewise, in salivary samples from healthy and caries-active adults, there were no caries-specific taxa detected (Yang *et al.*, 2012). While the composition of caries microbiomes and healthy microbiomes were similar

besides the differential abundance of *S. mutans*, genetic diversity of the microbiome increased with disease. Healthy oral microbiomes are less genetically diverse (Gross *et al.*, 2010). Caries microbiomes are more diverse and variable (Yang *et al.*, 2012). Therefore, the increased abundance of *S. mutans* may cause increased diversity which helps convert the microbiome into a caries-microbiome.

The increase of *S. mutans*, the establishment of a caries-microbiome, and disease may be influenced by dietary intake. Plaque composition in ancient humans demonstrates how diet can promote the prevalence of particular pathogens. The phylum-level composition between Neolithic age, industrial revolution humans, and modern humans was virtually similar. Plaque isolated from hunters and gatherers, whose diets were rich in meat and vegetables, were predominated by the periodontal pathogen *Porphorymonas gingivalis* while *S. mutans* was virtually undetected. However, plaque examined from humans during the industrial revolution was predominated by cariogenic pathogen *S. mutans* (Adler *et al.*, 2013). The rise in *S. mutans* predominance in plaque coincides with the production and popularization of processed sugars such as in cereals. A sugary diet has in fact been weighed more heavily than *S. mutans* in causing disease due to the necessity for the prolonged exposure of free sugars for the increase of tooth decay over time (Sheiham & James, 2015, Sohn *et al.*, 2006, Heller *et al.*, 2001, Pekkala *et al.*, 2002).

A comprehensive study of *S. mutans* biofilms, the influence of diet, dental caries, and anticaries therapeutics on the oral microbiota has not been conducted. Currently, oral microbiome studies in rats have been used for investigation into changes due to antibiotics or dietary nitrate (Manrique *et al.*, 2013, Hyde *et al.*, 2014). Changes in the

oral microbiome throughout the rat caries model have not been previously characterized. Characterizing the rat oral microbiota throughout the rat caries study could elucidate the importance of *S. mutans* and the influence of a sugary diet on the composition of the oral microbiota in health and disease. Sampling the rat salivary oral microbiome would be ideal since saliva is non-invasive and the composition of the salivary microbiota can distinguish between caries-active and healthy humans (Yang *et al.*, 2012).

STREPTOCOCCUS MUTANS VIRULENCE

The oral microbiome is inhabited by hundreds of different bacterial species, including cariogenic species *S. mutans*. In order for *S. mutans* to survive in the oral cavity and cause disease, *S. mutans* must first successfully maintain homeostasis against detrimental metal ions in the saliva and outcompete antagonistic oral commensal species. There are naturally abundant metal ions such as copper in the saliva. The level of copper can increase with dietary intake and leakage from dental restoration material such as copper-based amalgams (Brune, 1986). To combat lethal copper concentrations, the copper-resistance operon *copYAZ* recognizes copper and transports it outside of the cell (Vats & Lee, 2001). The predicted function of *S. mutans* copper chaperone CopZ is to bind cytoplasmic copper and transport it to copper-binding receptors such as cytochrome C (Karlin, 1993). *S. mutans* colonization is also challenged by antagonistic oral commensal species such as *Streptococcus gordonii* and *Streptococcus sanguinis* (Kreth *et al.*, 2008). To inhibit commensal species growth, *S. mutans* secretes acid and some strains secrete antimicrobial peptides. *S. mutans* UA159 secretes mutacins IV, V, and VI which

have been shown to be necessary for inhibition of *Streptococcus gordonii* and *Streptococcus sanguinis* (Merritt & Qi, 2012).

To successfully colonize the tooth surface, *S. mutans* attaches to the tooth surface to initiate biofilm formation. Initial attachment to the salivary pellicle is mediated by *S. mutans* cell-surface associated protein Antigen I/II (AgI/II) (Ahn *et al.*, 2008). Although AgI/II is not necessary for sucrose-dependent colonization of *S. mutans*, mutants lacking AgI/II had decreased cariogenicity in rats (Crowley *et al.*, 1999). Upon exposure to sucrose, *S. mutans* utilizes glucosyltransferases (Gtfs), particularly GtfB and GtfC, to extracellularly metabolize dietary sugars such as lactose and sucrose to produce sticky glucans. Glucans are utilized to form the glucan matrix which mediates adherence to the tooth surface and protection of microcolonies from environmental stress. GtfB produces insoluble glucans (α-1,3 linked) which are necessary for rigidity of the biofilm. GtfC produces a mixture of insoluble and water-soluble (α-1,6 linked) glucans necessary for surrounding microcolonies and mediating attachment to the tooth surface (Bowen & Koo, 2011, Hanada & Kuramitsu, 1988, Xiao *et al.*, 2012). Glucan-binding proteins (Gbps) facilitate adherence to the glucan matrix and therefore promote biofilm formation (Lynch *et al.*, 2007). Mutants lacking Gtfs or Gbps are defective in biofilm formation and are less virulent in rats (Lynch *et al.*, 2013, Munro *et al.*, 1991). Essential to forming the structured glucan matrix adherent to both microcolonies and the tooth surface is extracellular DNA (eDNA). eDNA forms nano-fibers which are actively released by membrane vesicles during biofilm formation, providing a scaffold for the glucan matrix (Liao *et al.*, 2014). An example of the *S. mutans* biofilm and its composition can be seen in Figure 1.

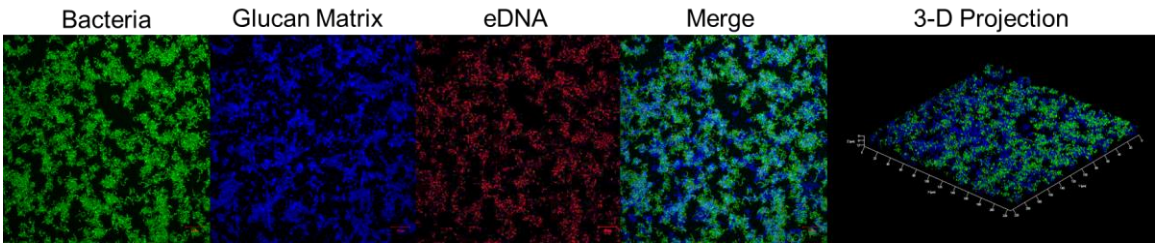

Figure 1. The structure and composition of the *Streptococcus mutans* biofilm. The *S. mutans* biofilm was formed in chemically-defined media supplemented with 1% sucrose on a 96-well polystyrene plate. Clustering of bacteria (green) into microcolonies associated with the glucan matrix (blue) and eDNA (red).

S. mutans virulence is controlled by various regulatory networks. ComE regulates mutacin production, necessary for competitiveness, through the competence stimulatory peptide (Reck *et al.*, 2015). Two-component regulatory system VicKR affects Gtfs and GbpB gene expression, plays a role in bacteriocin production, and is necessary for proper biofilm formation (Senadheera *et al.*, 2005). LuxS-mediated quorum sensing affects initial biofilm formation and is involved in acid and oxidative stress responses (He *et al.*, 2015, Wen & Burne, 2004). Biofilm regulatory protein A (BrpA) regulates acid and oxidative stress and is necessary for proper biofilm accumulation due to decreased GbpD expression (Wen *et al.*, 2006). Secondary messenger cyclic-di-AMP has been shown to be essential for regulation of GtfB expression and *S. mutans* biofilm formation (Peng *et al.*, 2015). Interestingly, the copper-resistance operon *copYAZ* has been implicated in modulating key virulence factors. Copper resistance plays a major role in virulence of other pathogens such as *Mycobacterium tuberculosis*, *Staphylococcus aureus*, and *Streptococcus pneumoniae* (Baker *et al.*, 2010, Shafeeq *et al.*, 2011, Wolschendorf *et al.*, 2011). Indeed, strains lacking the *copYAZ* operon have decreased GtfB expression,

impaired acid tolerance, and defective oxidative stress response (Singh *et al.*, 2015). However the mechanism of the *copYAZ* operon-mediated regulation of *S. mutans* virulence factors is unknown.

SMALL MOLECULE INHIBITORS AGAINST BIOFILM

Various bacterial pathogens establish a biofilm on or within host tissues in order to cause clinical manifestations of disease. Respiratory pathogen *Streptococcus pneumoniae* commonly asymptomatically colonizes the nasopharynx; however, the biofilm can disperse and cause pneumonia, otitis media, or meningitis (Chao *et al.*, 2014). *Pseudomonas aeruginosa* forms a persistent biofilm in the thick mucus lining of lungs of cystic fibrosis patients, often leading to fatal infections (Sousa & Pereira, 2014). Ingestion of contaminated food or water can lead to the formation of *Vibrio cholerae* biofilms on the intestinal lining and cause severe diarrheal illness and dehydration (Silva & Benitez, 2016). On the tooth surface, cariogenic *S. mutans* forms biofilm and persists in dental plaque. Frequent exposure of the *S. mutans* biofilm to sucrose can lead to the localized demineralization of the tooth's enamel.

Characteristically, biofilms are difficult to target and destroy due to their resistance to environmental stress and conventional antibiotics and protection against detrimental molecules by the exopolymeric matrix. With the rise in antibiotic resistance and increase in chronic infections, it is best to utilize alternative approaches to inhibiting biofilm formation or destroying established biofilms such as vaccines, antimicrobial peptides, and small molecules. Vaccines can potentially inhibit colonization and

subsequent biofilm formation if they are able to promote a high immune response. Vaccines containing the polysaccharide capsule serotypes of *Streptococcus pneumoniae* selectively prevent colonization of vaccine capsule types. Caveats associated with these vaccines are the lack of response in children with a non-conjugant polysaccharide vaccine and the inability to protect against non-vaccine serotypes (Gaviria-Agudelo *et al.*, 2016, Ahman *et al.*, 1998). Alternatively, potent antimicrobial peptides may effectively get rid of pathogenic species. Cationic peptide 1037 inhibited biofilm formation by *Pseudomonas aeruginosa* and *Listeria monocytogenes* by altering flagella-related gene expression (de la Fuente-Nunez *et al.*, 2012). While targeting virulence factors to inhibit biofilm is a potent strategy, peptide 1037 lacked species specificity, caused cell death in biofilms, and its efficacy *in vivo* was not explored.

Small molecules can also be potent inhibitors of chronic biofilms. The effective use of small molecules against biofilms has been shown for *Staphylocccous aureus, Pseudomonas aeruginosa, Vibrio cholerae, Candida albicans,* and oral biofilm. Sortase A inhibitors effectively block Sortase A binding to its substrates in *S. aureus*. These small molecules protected animals from lethal challenge, but were not species specific (Zhang *et al.*, 2014). In another approach, effective small molecules may be synthesized to mimic the structure of quorum sensing molecules. Quorum sensing inhibitors for *Pseudomonas aeruginosa* controls pyocyanin production, inhibits pathogenesis, and prevents biofilm formation. However, these small molecules were only effective at extraordinarily high concentrations of 100µM (O'Loughlin *et al.*, 2013). Small molecules may also be made to mimic secondary messenger Cyclic-di-GMP. Cyclic-di-GMP inhibitors effectively inhibit *Vibrio cholerae* biofilm formation, but do not induce dispersal. Since cyclic-di-GMP is

conserved across various species of bacteria, a structural analogue was found to also inhibit *Pseudomonas aeruginosa* biofilm (Sambanthamoorthy *et al.*, 2012). Another small molecule approach involves using derivatives of natural products. These derivatives are the most diverse in structure and in mode(s) of action. Small molecules can make ideal therapeutic candidates due to their stability, potency, diversity in structure and mode of action, and independence of immune responses. Additionally, strains resistant to small molecule treatments rarely occur, allowing for repeated use. Grape seed extract has selective antimicrobial activity against the oral species *Fusobacterium nucleatum* and *Streptococcus oralis* when in a 5-species biofilm (Munoz-Gonzalez *et al.*, 2014). However, it was not as effective as broad spectrum antimicrobial chlorhexidine to control dental plaque formation (Munoz-Gonzalez *et al.*, 2014). Development of a small molecule inhibitor against established biofilms may be the best therapeutic approach.

ANTICARIES THERAPEUTICS

S. mutans biofilms are irreversibly attached and must be mechanically removed by scraping or brushing. If not removed, the cariogenic biofilm can mature and continuously secrete acid which locally demineralizes the enamel. Conventional prevention methods against cariogenic biofilms and dental caries involve products containing fluoride, essential oils, or chlorhexidine. Fluoride and essential oils are important for remineralizing the enamel, but also display broad-spectrum antimicrobial activity. Despite the availability of preventative therapeutics, dental caries continues to be the most prevalent infectious disease worldwide.

Therapies selectively targeting pathogenic species *S. mutans* may better preserve the oral microbiota and effectively prevent dental caries. Vaccines against *S. mutans* colonization were unsuccessful for decades due to poor immune responses including salivary antibody production (Childers *et al.*, 2002). Recent vaccine strategies include potent adjuvants to better induce a mucosal immune response. An intranasal vaccination with the AgI/II antigen conjugated to cholera toxin inhibited oral colonization of *S. mutans* in mice (Saito *et al.*, 2001). A DNA vaccine targeting AgI/II containing recombinant flagella protein from *Salmonella* as an adjuvant inhibited *S. mutans* colonization in rats and prevented extensive decay when administered intranasally (Shi *et al.*, 2012). While these vaccines were effective against a specific strain of *S. mutans*, neither study demonstrated protection against different strains of *S. mutans* after immunization. Additionally, the potential cost to produce and maintain these vaccines would make it difficult to distribute to poor endemic areas. Alternatively, antimicrobial peptides are simple to generate and cost effective. Novel antimicrobial peptide C16G2 specifically targets *S. mutans* strains. However, the mechanism of C16G2 is dependent on inducing cell death in planktonic cells by disrupting the cell membrane (Kaplan *et al.*, 2011). While potent, C16G2 is not an ideal therapy since it does not target biofilm and the treatment with C16G2 has not been shown to preserve the oral microbiome *in vivo* (Guo *et al.*, 2015). Alternatively, extracts and derivatives of natural products have been examined for their utility against biofilms. Tt-farnesol, small molecule derivative of propolis, combined with fluoride inhibits *S. mutans* glucan production and biofilm accumulation by regulating expression of *gtfB* and *sloA* (Falsetta *et al.*, 2012). Mulberry leaf extract specifically targets *S. mutans* and reduces biomass at different maturation

time points by inducing cell death (Islam *et al.*, 2008). Proanthocyanidins isolated from cranberry extracts decreased the amount of insoluble glucans in *S. mutans* biofilms leading to inhibition of biofilm formation and dental caries incidence in rats (Koo *et al.*, 2010).

While some of the aforementioned therapies may seem potent, ideally an effective therapy against *S. mutans* biofilms should be simple to produce, non-cytotoxic, species specific, biofilm specific, soluble, and potent. To identify an ideal therapeutic, a previous study, Liu *et al.* (2011), utilized a small molecule library derived from marine sponge product bromoageliferin to identify a small molecule fulfilling these criteria. Bromoageliferin is a large compound that exhibits both antibiofilm and antibacterial properties against both gram negative and gram positive bacterial species (Huigens *et al.*, 2008). The chemical structure of bromoageliferin is shown in Figure 2. A potent structural motif from bromoageliferin was identified as 2-aminoimidazole. 2-aminoimidazole conjugates have been shown to inhibit and disperse biofilms of pathogenic species of *Staphylococcus aureus* and *Pseudomonas aeruginosa* (Rogers & Melander, 2008). In Liu *et al.* (2011), a library composed of novel small molecules containing the 2-aminoimidazole scaffold was constructed for the purpose of identifying a small molecule capable of selectively inhibiting *S. mutans* biofilm formation. Several small molecules were identified that were capable of inhibiting *S. mutans* biofilm formation by reducing gene expression of *gtfB* and *vicR*. These small molecules were effective in selectively inhibiting *S. mutans* in a mixed species biofilm. Although considerable research has been devoted to developing therapies inhibiting *S. mutans*

biofilm formation, rather less attention has been paid to dispersing established *S. mutans* biofilms.

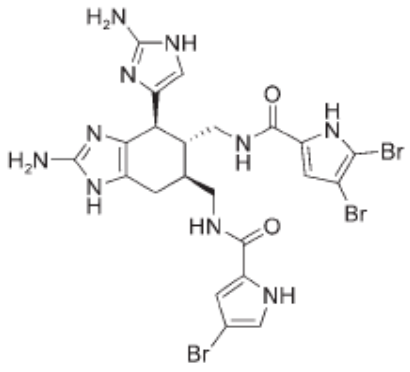

Figure 2. The chemical structure of marine sponge product, bromoageliferin. Structural motif 2-aminoimidazole was used to construct hundreds of small molecules, including small molecule 3F1, in Liu *et al.* (2011).

In this study, we repurposed the library of 2-aminoimidazole conjugates in Liu *et al.* (2011) to identify a small molecule capable of selectively dispersing established *S. mutans* biofilms. *S. mutans* biofilm maintenance and maturation processes are largely unknown. High-throughput quantitative proteomics identified approximately 70 proteins that are unique to biofilm maturation, suggesting that there are pathways involved in maintenance and maturation that are unique compared to initial biofilm formation (Klein *et al.*, 2012).

Although it is known that *S. mutans* biofilms and the frequent exposure to sucrose leads to dental caries, a therapy selective for *S. mutans* biofilms and its efficacy in

preventing dental caries *in vivo* has not been previously shown. Various factors are involved in *S. mutans* biofilms, including the copper-resistance operon *copYAZ* which has shown to be involved in GtfB expression. However, the individual role of the *copYAZ* operon components in virulence is unknown. In this study, we focus on the identification and efficacy of a *S. mutans* biofilm specific small molecule on controlling *S. mutans* biofilms to prevent dental caries and preserve the oral microbiome, and focus on elucidating the individual roles of *copY*, *copA*, and *copZ* in *S. mutans* biofilm formation and competitiveness.

SELECTIVE TARGETING OF *STREPTOCOCCUS MUTANS* BIOFILMS *IN VITRO* AND *IN VIVO*

by

SANDRA S. GARCIA, MEGHAN BLACKLEDGE, CHRISTIAN MELANDER, SUZANNE MICHALEK, LINGKAI SU, TRAVIS PTACEK, PETER EIPERS, CASEY MORROW, ELLIOT LEFKOWITZ, AND HUI WU

Format adapted for dissertation

ABSTRACT

Dental caries is a costly disease characterized by the demineralization of the tooth's enamel. Influential in disease outcome are host factors, dietary intake, and cariogenic bacteria. Cariogenic species *Streptococcus mutans* metabolizes sucrose to initiate biofilm formation on the tooth surface. Utilizing sucrose, *S. mutans* can rapidly produce lactic acid which degrades the tooth's enamel. Persistence of the *S. mutans* biofilm in the oral cavity can lead to tooth decay. To date, there are no therapies specific for *S. mutans* biofilms shown to prevent dental caries and not disturb the oral microbiome. Our study identified and utilized a small molecule capable of selectively targeting *S. mutans* biofilms to investigate the different components of dental caries on disease outcome and impact on oral microbiome.

We used an established library of 2-aminoimidazole conjugates and a biofilm dispersion assay to identify a small molecule specific for *S. mutans* biofilm. At 5 μM, 3F1 dispersed approximately half of the established *S. mutans* biofilm, but did not disperse biofilms formed by the commensal species *Streptococcus sanguinis* or *Streptococcus gordonii.* 3F1 dispersed *S. mutans* biofilms independently of biofilm-essential factors antigenI/II and glucosyltransferases. 3F1 treatment effectively prevented dental caries by controlling *S. mutans* in a rat caries model. Specific treatment of *S. mutans* by 3F1 did not perturb the oral microbiota. Moreover, we found that the diversity and composition of the oral microbiome was driven by daily dietary sucrose intake rather than *S. mutans* prevalence or disease outcome. The impact of the high sucrose diet on the oral microbiome was made evident through this study. Our study demonstrates that selective targeting of *S. mutans* biofilms by our small molecule was able to effectively

prevent dental caries in vivo without affecting the overall oral microbiota, suggesting that microbe specific treatment is a viable strategy for preventing disease.

INTRODUCTION

Dental caries is a detrimental disease characterized by the demineralization of the enamel tooth surface. Dental caries results from the right combination of a susceptible tooth surface, salivary flow, frequent sucrose intake, poor dental hygiene, and the colonization and persistence of cariogenic bacteria. Cariogenic species *Streptococcus mutans* extracellularly metabolizes dietary sucrose into biofilm-essential glucans via glucosyltransferases (Gtf). As a by-product of sucrose metabolism, *S. mutans* secretes lactic acid, leading to the local demineralization of the tooth's enamel. Due to the importance of sucrose in the persistence of *S. mutans* and development of disease, it is no surprise that the prevalence of *S. mutans* has been highly associated with a sugary diet. The prevalence of *S. mutans* in plaque from ancient humans correlated with the introduction of processed sugar (Adler *et al.*, 2013). However, selective targeting of *S. mutans* biofilms on disease prevention and the interaction of *S. mutans* biofilms and dietary sucrose on the oral microbiome has not been shown. We selectively targeted cariogenic biofilms to demonstrate experimentally that selective reduction of *S. mutans* biofilms *in vivo*, despite of daily dietary sucrose intake can preserve the microbiome and result in less dental caries.

In this study, we identified and utilized an *S. mutans* biofilm specific small molecule to investigate if this mode of action is efficient in preventing dental caries and preserving the oral microbiome *in vivo*. An early study designed an antimicrobial peptide

C16G2 that selectively target *S. mutans*, however it is cytotoxic and not specific for biofilms which are important in *S. mutans* virulence (Eckert *et al.*, 2006). Here we identify small molecule 3F1 that is capable of selectively dispersing established *S. mutans* biofilms. The selective dispersal phenotype of 3F1 *in vitro* translated to less dental caries *in vivo*, despite daily sucrose intake. Control of *S. mutans* by our small molecule did not perturb the oral microbiota. Daily ---Overall, we demonstrate that selective targeting of *S. mutans* biofilms by our small molecule can effectively prevent dental caries without disturbing the oral microbiome.

MATERIALS AND METHODS

Strains

S. mutans UA159 (WT UA159) was used as the model organism. GtfB::Kan^R and GtfC::Kan^R mutant strains were kindly provided by Dr. Robert A. Burne (University of Florida, Gainesville, FL). Mutant strain AntigenI/II::Tet^R was kindly provided by Dr. L. Jeannine Brady (University of Florida, Gainesville, FL). All *Streptococcus mutans* strains, *Streptococcus gordonii Challis*, and *Streptococcus sanguinis* SK36 were grown in Todd-Hewitt broth (THB; BD Biosciences, Franklin Lakes, NJ) at 37^{o}C with 5% CO_2.

Biofilm Dispersion Assay

Single colonies of *S. mutans* were grown in THB overnight. Overnight cultures were diluted into fresh THB and grown to an OD_{470}0.5-0.6. Bacterial cultures were then

diluted into pre-warmed biofilm media, a chemically-defined media containing 1% sucrose (Liu *et al.*, 2011). Aliquots (200µl) of biofilm media inoculated with bacteria were plated into 96-well polystyrene plates and sealed (Thermo Fisher Scientific, Waltham, MA). Biofilms were formed statically at 37^{o}C with 5% CO_2. Six hours after initial biofilm development, biofilms were gently washed twice with 200 µl Phosphate-Buffered Saline (PBS) to remove planktonic cells. Small molecules from a 10 mM stock in Dimethyl sulfoxide (DMSO) were diluted in biofilm media and added on top of the established biofilm and incubated for 14 h. For comparison, identical volumes of DMSO were added to untreated wells. To quantitate biomass, the loosely adhered cells were washed off and the biofilm was stained with 0.1% crystal violet for 15 min. Crystal violet was then solubilized in 30% acetic acid and values read at OD_{562}.

Imaging Biofilms with Confocal Laser Scanning Microscopy

For confocal laser scanning microscopy (CLSM), 500 nM Cascade Blue-dextran conjugated dye (Molecular Probes, Eugene, OR) was added to biofilm media prior to biofilm development for visualization of the glucan matrix and biofilms were grown on 8-well ibiTreat slides (ibidi #80826, Martinsried, Germany) (Xiao *et al.*, 2012). For visualization of cells and extracellular DNA, 1 µM of Syto-9 (Molecular Probes) and 0.67 µM Propidium Iodide (Sigma-Aldrich, St. Louis, MO), respectively, were diluted into PBS and added to each well. All images were taken on a Zeiss Confocal Microscope with a 63X oil immersion lens (UAB High Resolution Imaging Facility).

Rat Caries Model

The *in vivo* effect of 3F1 on *S. mutans* biofilm and cariogenicity was assessed in gnotobiotic rats using a modification of a previously described method (Palmer *et al.*, 2012, Crowley *et al.*, 1999). Fischer 344 rats were bred and maintained in trexler isolators. At approximately 20 days old, 18 pups were removed from isolators and randomly assigned into 3 groups of 6 animals each: 100 µM small molecule 3F1, 250 ppm of fluoride (positive control), or no treatment (negative control). All animals were provided an antibiotic cocktail of sulfamethoxazole trimethoprim oral in their drinking water (10 ml/473 ml) and sterile rat chow *ad libitum* for 2 days. Animals were then infected with *S. mutans* UA159 by swabbing the oral cavity of each rat with a swab saturated with an overnight culture of *S. mutans* UA159 daily for three consecutive days, and provided a caries-promoting diet (CPD)-Teklad Diet 305 containing 5% sucrose (Harlan Laboratories, Inc., Indianapolis, IN) and sterile drinking water *ad libitum*. Colonization of *S. mutans* UA159 was confirmed by oral swab samples on THB agar plates. For treatment, a solution of fluoride (250 ppm) or the inhibitor (100 µM) was applied to the surfaces of the rat molars for 30 sec with the aid of a camel hair brush. Rats were treated twice daily for 4 weeks beginning 10 days post infection. Brushes were cleaned between uses. Animals were weighed weekly to monitor for signs of toxicity. Following treatment, all animals were sacrificed and their mandibles excised for plaque analysis and scoring for caries by the method of Keyes (Keyes, 1958).

Microbiome Analysis

For microbiome analysis of the rat oral cavity throughout the study, saliva swabs were collected from each rodent at the following time points: after removal from isolator, after antibiotic treatment, after *S. mutans* UA159 inoculation, and end of study. Briefly, the mouth of each rat was quickly and gently rubbed with a sterile swab so as to not disturb the plaque. Swab samples were stored at -80^{o}C. DNA was extracted from swab samples with the ZR Fecal DNA Miniprep Kit (Zymo Research, Irvine, CA). The V4 region of the 16SrRNA gene was amplified and then sequenced on an Illumina Miseq as previously discussed (Kumar *et al.*, 2014, Daft *et al.*, 2015). Sequences were analyzed using the Quantitative Insight into Microbial Ecology (QIIME) suite v1.7 (Caporaso *et al.*, 2010) and a QIIME wrapper called QWRAP (Kumar *et al.*, 2014). Sequences were assigned to operational taxonomic units (OTUs) using uclust at 97% similarity. OTUs were assigned taxonomic groups using the Ribosomal Database Project (RDP) classifier (Wang *et al.*, 2007) and the May 2013 of the Greengenes 16S rRNA sequence database (DeSantis *et al.*, 2006). Distance matrices generated by clustering were used to generate principle components analysis (PCoA) plots. To determine whether samples clustered differently by treatment and/or time point, samples were grouped by treatment and/or time point and the distance matrices were tested using the PERMANOVA test for significant differences in clustering ($p<0.05$). To determine the differences in taxonomic frequency underlying differences in clustering, OTUs were grouped by phyla, classes, orders, families, and genera and tested for significant differences in frequency between groups using the Kurskal-Wallis test ($p<0.05$ after FDR correction).

qPCR

S. mutans UA159 specific primers (Table 1) were used to determine *S. mutans* CFUs in microbiome samples. SYBR Green Supermix (Biorad, Hercules, CA) was used to perform real-time quantitative PCR (qPCR) as described in Childers *et al.* (2011).

RESULTS

Small molecule 3F1 disperses *S. mutans* biofilm *in vitro*

To determine whether selective targeting of *S. mutans* biofilms could effectively prevent dental caries, we first needed to identify a therapy capable of selectively targeting *S. mutans* biofilms. Small molecules present ideal therapeutic candidates due to their stability, potential membrane permeability, activity at low concentrations, less toxicity, difficulty in rise of resistant clones, and may be less prone to modification or cleavage than antimicrobial peptides (Pierce *et al.*, 2015, Worthington *et al.*, 2012). 2-aminoimidazole conjugates derived from marine sponge product bromoageliferin have been shown to be potent and versatile against biofilm formation and biofilm dispersion across phylum and genera (Rogers *et al.*, 2010, Rogers & Melander, 2008). A previous study from our group utilized a library of 2-aminoimidazole conjugates and a biofilm formation assay with *S. mutans* and successfully identified small molecules capable of selectively inhibiting *S. mutans* biofilm formation (Liu *et al.*, 2011). We utilized this library and a biofilm dispersion assay to identify a small molecule capable of selectively dispersing established *S. mutans* biofilms. We only pursued small molecules that met the following criteria: low minimum dispersion concentration (MDC50, or concentration of

the small molecule needed to disperse approximately 50% of biofilm), little to no cytotoxicity, and selectivity to *S. mutans* biofilms. We identified one small molecule capable of selectively dispersing *S. mutans* biofilms at 5 μM when compared to biofilms treated with DMSO. Small molecule 3F1 or *N*-(4-(2-amino-1*H*-imidazol-4-yl)phenyl)cyclopropanecarboxamide (Fig. 1A) dispersed biofilms at an MDC of 5 μM (3F1, Fig. 1B). Dispersion of the biofilm led to increased turbidity in the media (Fig. 1B). To determine whether the structure of 3F1 was specific for the dispersal phenotype, structural analogue 3F2 (Fig. 1A) was tested for activity. 3F2 did not disperse *S. mutans* biofilms (3F2, Fig. 1C), suggesting that dispersal activity was specific to the structure of 3F1. To determine whether 3F1 was inhibiting cell growth thereby reducing biofilms, planktonic cells were treated with 5 μM 3F1 for 14 h and viable cells were enumerated. CFUs recovered from 3F1 treated cells were similar to CFUs recovered from cells treated with DMSO (Fig. 1D), suggesting that biofilm dispersal by 3F1 was not due to growth inhibition. We further investigated whether 3F1 was selective for *S. mutans* biofilms. Six hour biofilms formed by oral commensal species *Streptococcus sanguinis* or *Streptococcus gordonii* were treated with 5 μM 3F1 or DMSO. 3F1 did not disperse or negatively impact biofilms formed by oral commensal species at this concentration (Fig. 1E). Biofilms formed by the mutans streptococci member *Streptococcus sobrinus* were also unaffected by 3F1 activity (data not shown), implicating pathogen specificity of 3F1.

Essential to *S. mutans* biofilm formation and maintenance are antigen I/II (AgI/II) and glucans produced by glucosyltransferases (Gtfs) (Ahn *et al.*, 2008, Xiao *et al.*, 2012). Because of the role of AgI/II and Gtfs, particularly GtfB and GtfC, in initial bacterial attachment and biofilm development, we reasoned that 3F1 could be targeting AgI/II,

GtfB, or GtfC. Biofilms of single-gene mutants of AgI/II, GtfB, or GtfC were treated with 5 μM 3F1. Mutants of AgI/II, GtfB, or GtfC were similarly sensitive to 3F1 treatment as the parent strain (Fig. 2A). We further investigated the mechanism of 3F1 by visualizing the *S. mutans* biofilm after 3F1 treatment. The *S. mutans* biofilm is a 3-dimensional complex composed mainly of bacterial microcolonies, glucan matrix, and extracellular DNA (eDNA). DMSO, 3F1, and 3F2 treated biofilms exhibited similar fluorescent levels of glucan matrix (Fig. 2B), suggesting 3F1 treatment did not impact the glucan matrix. Since glucans are produced by GtfB and GtfC, our microscopy results corroborated our results in Fig. 2A. All treated biofilms also exhibited similar eDNA fluorescence (eDNA, Fig. 2B). However, the fluorescence of syto9 stained microcolonies was visibly decreased in biofilm treated with 3F1 compared to DMSO or 3F2 (3F1 bacteria, Fig. 2A), suggesting that the decrease in biomass (Fig. 1B) is due to less cells in the biofilm due to dispersion by 3F1. These results suggest that 3F1 disperses the *S. mutans* biofilm independent of AgI/II, Gtfs, glucans, and eDNA.

Selective targeting of *S. mutans* by small molecule 3F1 treatment prevents dental caries and does not perturb the oral microbiome

The oral cavity is a unique environment of saliva, teeth, the occasional food and drink, and hundreds of bacterial species. To examine the activity of our small molecule *in vivo*, we utilized a rat caries model. The rat caries model is a well-established model used to study the efficacy of anticaries therapies due to the controlled environment and diet, rapid caries progression, and an established caries scoring system for rat molars (Larson

et al., 1977). However, the oral microbiota throughout the study has yet to be investigated. Specific-pathogen free rats were equally randomized into one of three groups depending on treatment: 3F1, Fluoride, or no treatment. All rats were infected with *S. mutans* UA159 and fed a caries-promoting diet (CPD, 5% sucrose) prior to starting treatment. After 4 weeks of treatment, the severity of enamel lesions were significantly reduced in all locations for the *S. mutans*-specific inhibitor and fluoride groups compared to the no treatment group (Caries, $p<0.001$, Table 3). *S. mutans* CFUs recovered from the dental plaque of animals treated with 3F1 or fluoride were decreased compared to the no treatment group (CFUs, Table 3). All animals gained weight similarly, suggesting the treatments were not toxic (Weight, Table 3). Collectively, these results demonstrate that selective control of *S. mutans* by 3F1 treatment prevented dental caries similar to fluoride despite of daily sugar intake.

We next investigated whether selective targeting of *S. mutans* by small molecule 3F1 disturbed the oral microbiota. We sampled the salivary microbiome at various time points throughout the rat caries study. Salivary samples are ideal because they are non-invasive and the microbiomes between caries-active and healthy humans are distinguishable (Yang *et al.*, 2012). Saliva samples were taken from all rats at initial removal from isolators (Native), after antibiotic treatment (After Abx), *S. mutans* UA159 inoculation and concurrent start of a caries-promoting diet (Sm+CPD), and end of study (End). Beta-diversity of groups over time was compared by principal component analysis (PCoA) plots (Fig.3A). Notably, groups clustered together by time rather than treatment ($p<0.001$). At the end of the study, all animal microbiomes are closely clustered together regardless of treatment ($p>0.05$). Clusters did not change depending on sex or caging

(data not shown). To investigate whether treatment perturbed the composition of the rat oral microbiota, we first compared phylogenetic composition of the microbiota at different time points. The major phyla detected throughout the study were *Firmicutes*, *Bacteroides*, *Actinobacteria*, *Proteobacteria*, *Fusobacteria*, *Thermi*, *Cyanobacteria*, *Deferribacteres*, *Tenericutes*, and *Verrucomicrobia* (Fig. 3B). *TM7* and *Gemmatimonadetes* were only significantly enriched at the end of the study in the no treatment group ($p<0.031$ and $p<0.042$, respectively) and 3F1 treated group ($p<0.031$ and $p<0.042$, respectively) (End, Fig. 3B). At the genus level, the native or healthy oral rat microbiome was predominated by *Clostridium* and to a lesser extent, *Enterococcus* and *Stenotrophomonas* (Native, Fig. 3C). Treatment with antibiotics abolished *Clostridium*, seemingly providing *Enterococcus* and commensal *Streptococci* an opportunity to colonize rat mouths (After Abx, Fig. 3C). Inoculation with *S. mutans* and the concurrent start of a caries-promoting diet undoubtedly led to the dramatic increase in the *Streptococcus* genus (Sm+CPD, Fig. 3C). After 4 weeks of treatment and CPD, microbiomes were characterized by *Streptococcus* and many low abundance genera (End, Fig. 3C). Genera were not significantly altered by treatments (Sm+CPD to End, Fig. 3C). At species-level, we utilized *S. mutans* specific primers to quantitate *S. mutans* CFUs in our 'end' salivary microbiome samples. There were less *S. mutans* CFUs detected in 3F1 and fluoride treated rats compared to the no treatment control (Fig. 3D). We suspect that these values are lower than CFUs recovered from plaque since these are salivary samples and some cells are also probably lost during DNA extraction. Nonetheless, this validates our microbiome data. Overall, our microbiome data suggest that *S. mutans* biofilm specific small molecule 3F1 selectively reduced *S. mutans* without perturbing the

microbial community structure. Furthermore, our results show that reduction of *S. mutans* and dental caries outcome did not affect the microbiome composition or diversity, suggesting that under our conditions, daily intake of a caries-promoting diet is the major key factor in shaping the community structure of the microbiota.

DISCUSSION

We identified small molecule 3F1 which dispersed established *S. mutans* biofilms at 5μM without causing defects in viability. 3F1 preserved the biofilms formed by oral commensal species *S. sanguinis* or *S. gordonii* (Fig.1E). The mechanism of 3F1 was independent of biofilm-essential AgI/II, GtfB, and GtfC (Fig. 2A). In the rat caries model, selective targeting of *S. mutans* biofilms by 3F1 effectively reduced the number of *S. mutans* in plaque and prevented tooth decay despite daily sugar intake (Table 2). Treatment with 3F1 did not perturb the oral microbiota (Fig. 3A-C). A caries-promoting diet strongly influenced the microbial community structure, regardless of treatment or disease outcome. Overall, our data show that selective targeting of *S. mutans* biofilms by small molecule 3F1 treatment can effectively prevent dental caries and preserve the microbiome while dietary sucrose is the key factor in shifting the oral microbial community structure.

Small molecule 3F1 selectively dispersed *S. mutans* biofilms *in vitro* independent of factors essential for biofilm formation, selectively controlled *S. mutans* in the dental plaque, and prevented dental caries in an *in vivo* rat caries study. Increased *S. gordonii* biofilms by 3F1 treatment *in vitro* may be beneficial in controlling *S. mutans in vivo* since *S. gordonii* antagonizes *S. mutans* with hydrogen peroxide production (Kreth *et al.*,

2008). Unsurpsingly, the mechanism of 3F1 is not dependent on AgI/II, Gtfs, glucans, and eDNA since they are most associated with initial development of *S. mutans* biofilms and proteins involved in maintenance and maturation are largely unknown (Bowen & Koo, 2011, Crowley *et al.*, 1999). Reduction in *S. mutans* CFUs after treatment with 3F1 *in vivo* (Table 2) corroborates with results from other rat caries studies where slight reductions in *S. mutans* CFUs recovered resulted in less dental caries (Falsetta *et al.*, 2012, Branco-de-Almeida *et al.*, 2011).

The oral microbiome is a unique subset of bacteria which can vary between individuals and can be influenced by pathogens, diet, treatment, and disease. One of the novel aspects of our study is the characterization of the oral microbiome throughout the rat caries model. We demonstrated a clear distinction between healthy (prior to Sm+CPD) and caries-active microbiomes (after Sm+CPD) in beta-diversity (Fig. 3A) and genus composition (Fig. 3C). Phylum-level comparison of groups from initial infection of *S. mutans* to after 4 weeks of treatment showed that selective treatment of *S. mutans* by 3F1 did not perturb the rat oral microbiome (Fig. 3A-C). Overall, our data suggests our *S. mutans* biofilm specific therapy is a novel viable method for preventing caries and preserving the oral microbiome. Species selectivity against *S. mutans* or preservation of the oral microbiome *in vivo* has not been previously shown. Derivatives or extracts from natural products such as cranberries, green tea, tea tree oil, garlic or hop polyphenols are able to reduce or inhibit the oral biofilm but do not specifically target *S. mutans* (Koo *et al.*, 2010, Houshmand *et al.*, 2013, Thomas *et al.*, 2015, Tagashira *et al.*, 1997). Thus far, to our knowledge, there has been one *S. mutans* specific therapy; synthetic antimicrobial peptide C16G2. Although C16G2 specifically kills *S. mutans*, in an *in vitro* oral biofilm

model, C16G2 treatment reduced *S. mutans*, indiscriminately eliminated other oral species, and increased the presence of other oral *Streptococci*, overall leading to a drastic shift of the structure of the microbiota (Guo *et al.*, 2015).

Treatment with 3F1 or fluoride reduced *S. mutans* and dental caries, but the beta-diversity and overall composition of the oral microbiome were similar between all groups. This suggested that daily intake of a caries-promoting diet was the major driver of microbiota diversity and composition. The minimal changes in the microbiome after fluoride treatment are corroborated by studies in humans (Reilly *et al.*, 2016, Koopman *et al.*, 2015). However these studies do not take diet into consideration, which we show here that a sugary intake can significantly impact the composition of the microbiome despite of treatment and *S. mutans*. The influence of the frequent ingestion of sucrose such as carbonated drinks or pastries, rather than the prevalence of *S. mutans,* has been shown to correlate with the dysbiosis of the oral microbiome (Bernabe *et al.*, 2016, Tian *et al.*, 2015, Rudney *et al.*, 2015). The direct relationship between dietary intake and the microbial community structure has also been shown for the gut microbiome. Dietary intake of either low-fat or high-fat diets were more influential than host genotype in the abundance of bacteria in the gut microbiota (Carmody *et al.*, 2015).

In this study, we identified novel small molecule 3F1 capable of selectively dispersing *S. mutans* biofilms independent of well-studied biofilm-related factors AgI/II, GtfB, and GtfC. The *S. mutans* specificity of 3F1 controlled *S. mutans* in a rat caries model and prevented dental caries without perturbing the oral microbiota. The dysbiosis of the oral microbiome was mainly attributed to daily sugar intake and not *S. mutans* prevalence. To our knowledge, we are the first to demonstrate selective targeting the

cariogenic biofilms to prevent dental caries and characterize the rat oral microbiome throughout the rat caries model. These results demonstrate that a therapy aimed specifically at *S. mutans* can effectively prevent dental caries and preserve the microbiome. Our data provide scientific basis for developing a complete anticaries therapy that is selective for *S. mutans* and does not interact with the microbiome, and takes in consideration the impact of the diet.

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Table 1. qPCR *S. mutans* UA159 specific primers.

Primer set	Sequence	Source
S. mutans UA159 GtfB	F-5'-CAAAATGGTATTATGGCTGTCG R-5'-GCTTAGATGTCACTTCGGTTG	This Study

Table 2. Summary of results from the rat caries study.

	No treatment	Small Molecule 3F1	Fluoride
Mean caries score by location (E)	Buccal: 13.0±0.9 Sulcal: 20.3±0.7 Proximal: 3.5±0.4	Buccal: 6.0±0.4* Sulcal: 12.0±0.4* Proximal: 0*	Buccal: 6.5±0.7* Sulcal: 11.5±0.7* Proximal: 0*
Weight (g)	171±14	158±16	157±11
CFUs recovered *S. mutans* UA159 ($x10^6$)	8.2±1.2	6.1±1.1	6.8±1.1

n=6, *=$p<0.001$ compared to no treatment

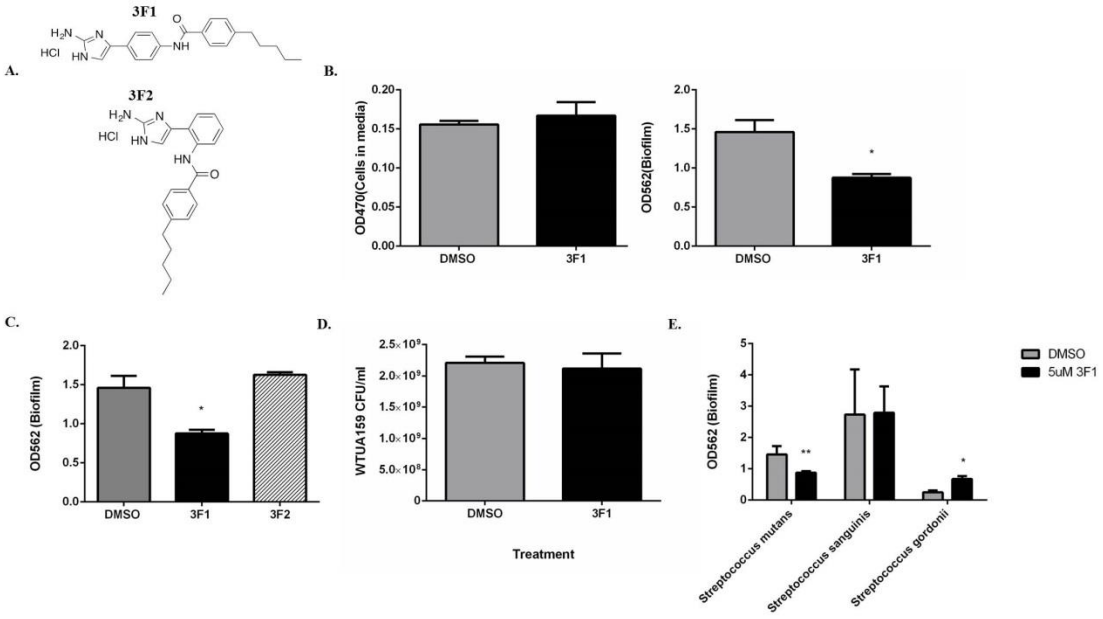

Figure 1. Small molecule 3F1 selectively disperses *Streptococcus mutans* biofilms *in vitro*.

A. Chemical structure of small molecule 3F1 and 3F1 structural analogue, 3F2. B. *S. mutans*UA159 biofilms were formed for 6 h. Biofilms were then washed and treated with DMSO5µM 3F1, or 5µM 3F2 for 14 h. After 14 h, cells released into the media were measured at OD_{470}. Loosely adhered cells were then washed off and remaining biomass quantitated by crystal violet staining at OD_{562}. C. *S. mutans* UA159 biofilms were formed for 6 h, washed, and treated with DMSO5µM 3F1, or 5µM 3F2 for 14 h. Loosely adhered cells were then washed off and remaining biomass quantitated by crystal violet staining at OD_{562}. D. Planktonic *S. mutans* UA159 (WT UA159) cells were treated for 14 h with DMSO or 5µM 3F1. E. Oral commensal species, *S. sanguinis* and *S. gordonnii* were allowed to form biofilms for 6 h before treatment with DMSO or 5µM 3F1 for 14 h. Bars represent the mean of 3 independent experiments. Error bars represent standard error. **=$p<0.01$, *= $p<0.05$.

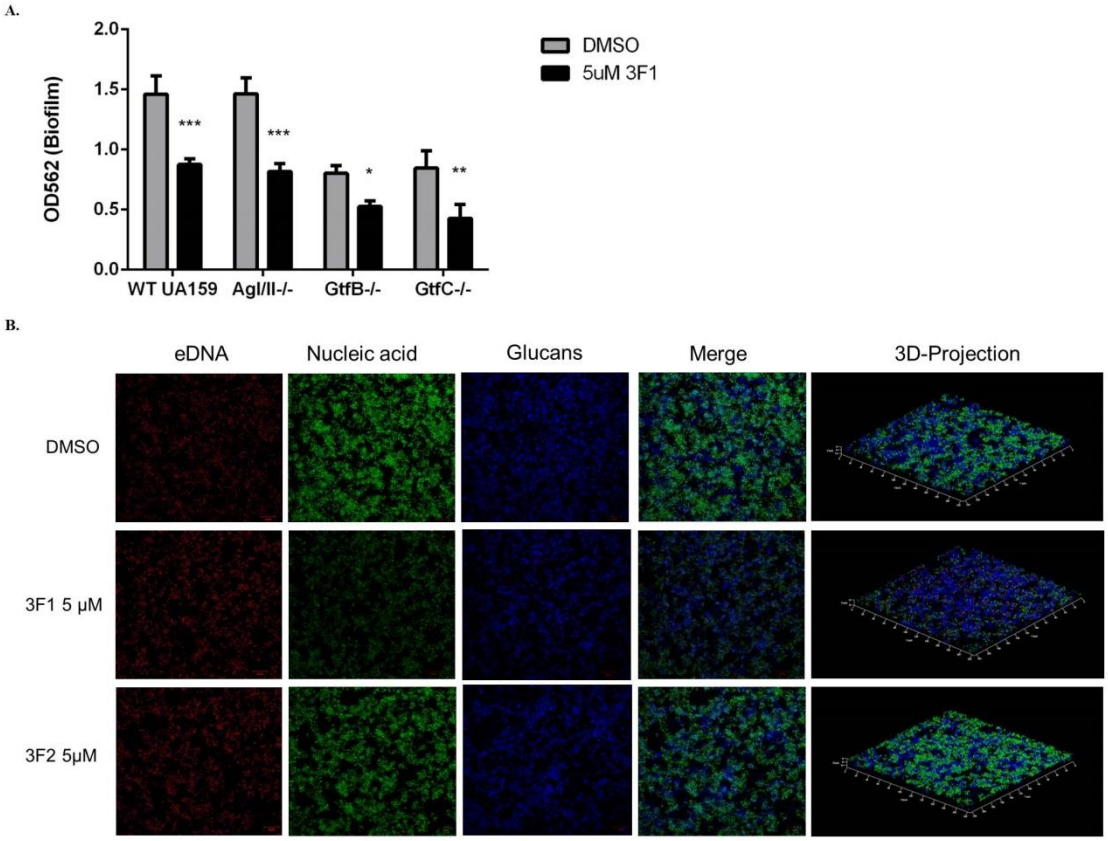

Figure 2. Small molecule 3F1 disperses *S. mutans* biofilm independent of known biofilm-related factors.

A. WT UA159, and mutants lacking AgI/II, GtfB, or GtfC were allowed to form biofilms for 6 h. Biofilms were then washed and treated with DMSO or 5μM 3F1 for 14 h. Loosely adhered cells were then washed off and remaining biomass quantitated by crystal violet staining at OD_{562}. B. Confocal laser scanning microscopy images of WT UA159 biofilm treated with DMSO or 5μM 3F1. Bacterial cells (green), extracellular DNA (red), and glucans (blue) were visualized by CLSM after the dispersion assay. Representative images are shown.

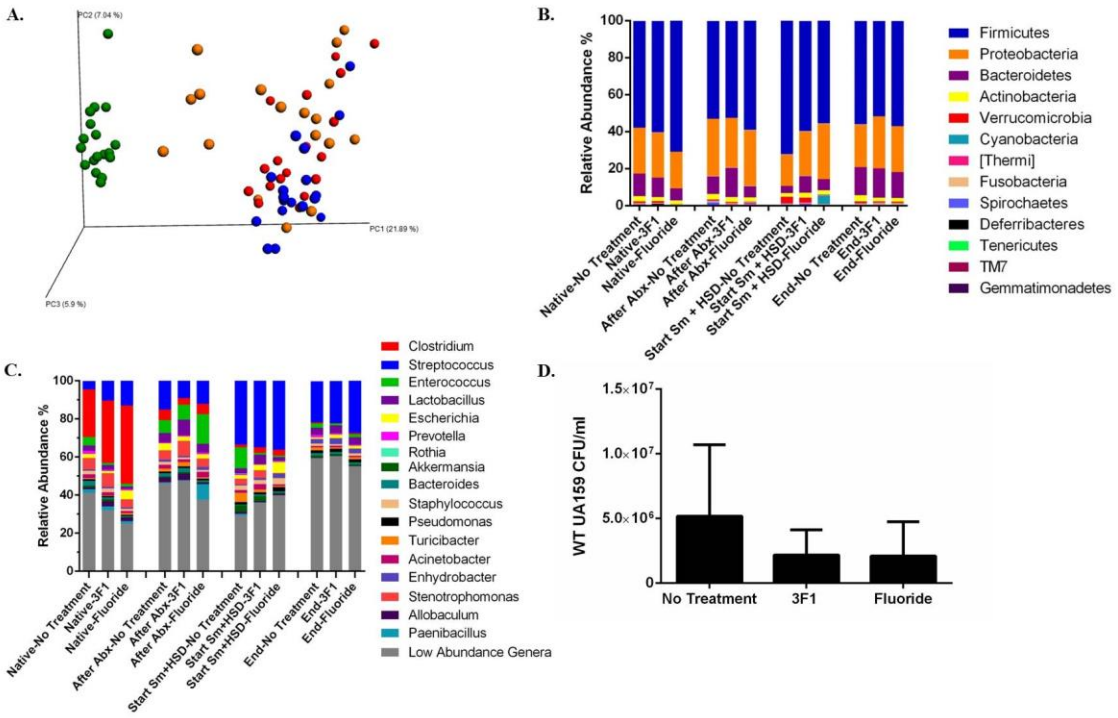

Figure 3. 3F1 treatment does not perturb the microbiome.

Oral microbiome samples were obtained from individual rats at the following time points: native, after antibiotics, after inoculation of *S. mutans* and start of high-sucrose diet, and at the end of the study. The microbiota between groups at different time points was analyzed for diversity and composition. A. PCoA plots of beta-diversity of all animals in groups over time; native (blue), after antibiotics (red), infect with *S. mutans* and start CPD (orange), and end (green). B. Phyla composition in all groups. n=6. C. Genus-level composition of all groups. n=6. D. qPCR of WT UA159 recovered from microbiome samples at the end of the study.

STREPTOCOCCUS MUTANS COPPER CHAPERONE, COPZ, IS CRITICAL FOR BIOFILM FORMATION AND COMPETITIVENESS

by

SANDRA S. GARCIA, QIN DU, AND HUI WU

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SUMMARY

The oral cavity is a dynamic environment characterized by hundreds of bacterial species, saliva, and an influx of nutrients and metal ions such as copper. While there is a physiologic level of copper in the saliva, the oral cavity is often challenged with an influx of copper ions. At high concentrations copper is toxic and must therefore be strictly regulated by pathogens in order to persist and cause disease. The cariogenic pathogen *Streptococcus mutans* manages excess copper using the *copYAZ* operon that encode negative DNA-binding repressor (CopY), P1-ATPase copper exporter (CopA), and copper chaperone (CopZ). These hypothetical roles of the *copYAZ* operon in regulation and copper transport to receptors led us to investigate their contribution to *S. mutans* virulent properties.

Mutants defective in the copper chaperone C*opZ*, but not CopY or CopA, were impaired in biofilm formation and competitiveness against commensal Streptococci. Characterization of the CopZ mutant biofilm revealed a decreased secretion of glucosyltransferases and reduced expression of mutacin genes. These data suggest that the function of *copZ* on biofilm and competitiveness is independent of copper resistance and CopZ is a global regulator for biofilm and other virulence factors. Further characterization of CopZ may lead to the identification of new biofilm pathways.

INTRODUCTION

Streptococcus mutans is an oral pathogen associated with the development of dental caries. Crucial to *S. mutans* cariogenicity is the ability to form a tenacious, well-structured, three-dimensional biofilm. To establish a cariogenic biofilm and consequently cause disease, *S. mutans* must first manage to survive environmental stress encountered within the oral cavity which harbors antagonistic bacteria, but also host-derived compounds or antimicrobials and metal ions such as copper. In the oral cavity *S. mutans* is continuously exposed to copper, however, *S. mutans* has been reported to be resistant to copper at high concentrations (i.e. approximately 64 mg/L), in comparison to bacteria that dwell in niches that contain no copper (Vats & Lee, 2001). The physiologic concentration of copper in saliva ranges from 0.2 to 7.05 mg/L (Duggal et al., 1991), however, copper concentrations in the oral cavity can fluctuate depending on intake of dietary copper and the presence of metal alloy dental restorative materials which may leak copper ions into the surrounding milieu (Orstavik, 1985). Under physiologic conditions copper is used as a cofactor for enzymes such as cytochrome C oxidase (Karlin, 1993). At elevated concentrations however, abundant free copper is toxic and commonly used as an antimicrobial against *S. mutans* (Evans et al., 1986, Drake et al., 1993). Therefore, it is necessary for *S. mutans* to regulate excess copper to survive and consequently establish a cariogenic biofilm.

In *S. mutans* UA159, copper resistance is mediated by operon *copYAZ* (Vats & Lee, 2001, Ajdic et al., 2002). Although sensitivity to copper increases without *copYAZ,* functional annotation of *S. mutans* CopY, CopA, and CopZ is based on extensive studies of the *Enterococcus hirae copYABZ* operon (Solioz & Stoyanov, 2003). The *S. mutans*

copYAZ operon is predicted to encode a negative repressor (CopY) of the operon that allows for transcription upon copper transfer from direct binding with CopZ, a P1-ATPase whose sole function is to export excess copper ions out of the cell for copper resistance (CopA), and a small protein that tightly binds and delivers copper to CopY to presumably positively regulate *copYAZ* (CopZ) (Cobine et al., 1999, Vats & Lee, 2001). Due to the function of CopYAZ to recognize, bind, and transport copper, and the connection between copper and virulence in other bacterial pathogens (Baker et al., 2010, Cobine et al., 1999, Wolschendorf et al., 2011), we sought to characterize the role of CopYAZ in *S. mutans* virulence traits and competitiveness.

In this study, we determined copper chaperone CopZ indeed binds to copper and established a role for CopZ in the regulation of *S. mutans* virulence factors. Distinct from *copY* or *copA* mutants, *S. mutans* copper chaperone mutants were defective in biofilm formation and competitiveness against commensal Streptococci. Elucidation of the new role of *copZ* could identify novel regulatory pathways in *S. mutans*.

METHODS

Bacterial strains and cultures

All strains and plasmids used in this study are listed in Table 1. *Streptococcus mutans* UA159 (wild-type, WT UA159), serotype C, was used as the model organism. Single gene mutants of *copY* (ΔcopY), *copA* (ΔcopA), and *copZ* (ΔcopZ) were made through allelic exchange with a kanamycin cassette as described (Lau et al., 2002, Wen et al., 2015). In brief, the gene and flanking regions were amplified and inserted into pGEM T-easy vector (Promega, Madison, WI). Since the *copY* and *copA* genes overlap, ΔcopY and

ΔcopA were created by replacing domains 1-123aa and 2-742aa, respectively, with a kanamycin cassette. In contrast, the *copZ* gene is downstream of both *copY* and *copA*, therefore, the intact gene of ΔcopZ (1-67aa) was replaced by a kanamycin cassette (Vats & Lee, 2001). Transformants were recovered following the pGEM protocol. In brief, transformants were screened on X-gal and IPTG plates. White colonies were chosen and checked for insertion by PCR. pGEM plasmids containing the amplified regions were extracted and used for inverse PCR with primers listed in Table 2. Inverse PCR products and the kanamycin cassette were digested with KpnI. Digested products were ligated and resulting plasmids were transformed into *E. coli*. Plasmids containing the kanamycin cassette in place of the gene of interest were transformed into *S. mutans* UA159 to replace the kanamycin cassette with the gene in the chromosome. Primers used to make mutants are listed in Table 2. All mutants were examined by PCR for correct placement of the kanamycin cassette in the chromosome. For the ΔcopZ complement strain (ΔcopZ/copZpVPT), ΔcopZ was transformed with a shuttle plasmid, pVPT(erythromycin resistance), expressing *copZ* . All *S. mutans* strains, *Streptococcus gordonii* DL1, and *Streptococcus sanguinis* SK36 were grown statically in Todd-Hewitt media (THB) (BD Biosciences, Franklin Lakes, NJ) at 37^{o}C under 5% CO_2.

Biofilm assay

Biofilms were formed similar to Liu et al. (2011). In brief, overnight cultures were diluted into fresh THB and grown to exponential phase. Cultures were diluted to $O.D._{470}$ 0.01 into 10mL of pre-warmed chemically-defined biofilm media containing 1% sucrose as the carbohydrate source (Loo et al., 2000). Doubled inoculum of ΔcopZ was added to

biofilm media to create a biofilm with biomass similar to wild-type, labelled as ΔcopZ(double) in Fig.3B to compensate for decreased biomass of ΔcopZ. Biofilms were grown statically for 18 hours in 96-well polystyrene plates. To measure biomass loosely adhered cells were gently washed off and the biofilm was stained with 0.1% crystal violet for 15 minutes and solubilized in 30% acetic acid. Biomass was quantified using $O.D._{562}$.

Protein Expression & Isothermal Titration Calorimetry

The *copZ* (SMU_427) coding region from *S. mutans* UA159 was cloned into the pET28a-hisSUMO vector in *E. coli* BL21 Gold (DE3) cells to make the copZpET28a-hisSUMO expression plasmid. Primers used to make the construct are listed in Table 2. Recombinant protein hisSUMO-CopZ was expressed and purified similar to Zhang et al. (2014). For Isothermal Titration Calorimetry, 1mM CopZ and 10mM of $CuSO_4$, $MgSO_4$, $ZnSO_4$, or $NiSO_4$ were each diluted in water and were loaded on a Microcal Automated Isothermal Titration Calorimetry ITC 200 in the Center for Biophysical Sciences and Engineering, UAB. The sample cell was filled with 400uL CopZ protein and the injection syringe was loaded with 150uL metal solution. Data was collected from 25 injections at 20^oC, and analyzed using Origin 7 Microcal Software (OriginLab, Northampton, MA).

Protein extraction

Five milliliter cultures of each strain were grown to the same OD_{470} in THB. Cells were pelleted and the supernatants were mixed with 1.5mL of 100% Molecular Grade Ethanol (Fisher Scientific, Pittsburgh, PA) and frozen overnight at -80^oC. Supernatants were thawed out, spun down, and pellets containing proteins were collected. Equal amounts of

cells were lysed by vortexing with glass beads and boiled for 10 minutes at 95^{o}C. Proteins were separated on SDS-PAGE gels for coomassie staining and western blotting.

Inhibition assay

Inhibition assays were performed similar to the interference assays described in Scoffield and Wu (2015). *S. mutans* strains were grown to early exponential phase and plated on THB agar plates. Plates were incubated overnight at 37^{o}C under 5% CO_2. *Streptococcus sanguinis* or *Streptococcus gordonii* cultures in early exponential phase were spotted immediately adjacent to the established *S. mutans* colony and incubated overnight. Inhibition of *S. sanguinis* or *S. gordonii* growth was visually observed and imaged using Gel Logic 100 Imaging System.

Confocal Laser Scanning Microscopy

For confocal laser scanning microscopy (CLSM), cultures were diluted in biofilm media+1% sucrose containing 500nM dextran-conjugated cascade (Ex: 400/Em: 420, Molecular Probes, Invitrogen, Life Technologies, Grand Island, NY). Dextran-conjugated dye has been shown to help visualize the extracellular polysaccharide matrix in *S. mutans* (Koo *et al.*, 2010, Peng *et al.*, 2015). Biofilms were grown on ibiTreat 8 well µ-slides (ibidi #80826, Martinsried, Germany) for imaging. After 18 hours, biofilms were gently dip washed 3X in sterile 1XPBS. Bacteria in biofilms were visualized with 1µM Syto9 green (Ex: 485/Em: 495, Molecular Probes, Invitrogen, Life Technologies, Grand Island, NY). Biofilms were imaged at 63X magnification using a Zeiss Laser Scanning

Microscope LSM 710 Confocal Microscope at the UAB High Resolution Imaging Shared Facility.

Qualitative Real Time PCR

RNA was extracted from late exponential phase cultures using the Direct-zol kit (Zymo Research, Irvine, CA). Residual DNA was digested using RQ1 DNase (Promega). RNA was purified with the miniRNAeasy kit (Qiagen, Venlo, Limberg), and converted into cDNA using the iScript cDNA Synthesis kit (Bio-rad, Hercules, CA). cDNA was then used for qRT-PCR with iQ SYBR Green Supermix (Bio-rad). Primers used are shown in Table 3.

Statistical analysis

All experiments were repeated three times. Biomass and qRT-PCR results are presented as mean ± SEM and statistical significance determined by one-way ANOVA. Samples were considered statistically significant if the difference has a p-value of <0.05. Analysis of biomass and gene expression data was done using GraphPad Prism 6 (GraphPad Software, San Diego, CA).

RESULTS

CopZ plays a role in proper biofilm formation

Recently, the intact *copYAZ* operon of *Streptococcus mutans* has been implicated in cariogenic biofilm properties such as acid tolerance and competence (Singh et al., 2015). To investigate which gene(s) of the *copYAZ* operon could be attributed to biofilm

formation, we constructed single-gene mutants (ΔcopY, ΔcopA, and ΔcopZ). Mutants were allowed to form biofilms in biofilm media containing 1% sucrose for 18 hours. At the end of 18 hours, biomass was quantitated by crystal violet staining. Compared to the parent strain (WT UA159), the ΔcopZ biofilm had significantly less biomass, while loss of *copY* and *copA* did not affect biofilm formation ($p<0.05$, Fig. 1). Proper biofilm formation could be restored by complementation of *copZ* (Fig. 1, ΔcopZ/copZpVPT). All strains grew similarly in biofilm media for 18 hours, suggesting the biofilm defect of ΔcopZ was not due to reduced cell growth (data not shown).

We first wanted to demonstrate the function of *S. mutans* CopZ as a copper-binding chaperone. Copper chaperones display strong and specific binding to copper (Urvoas *et al.*, 2004, Kihlken *et al.*, 2002). Purified CopZ protein and divalent cations $CuSO_4$, $ZnSO_4$, $MgSO_4$, and $NiSO_4$ were used for isothermal titration calorimetry (ITC). CopZ bound copper with high affinity, as evidenced by the best fit line (Fig. 2). In contrast, while there seems to be some binding activity of CopZ to $ZnSO_4$, $MgSO_4$, and $NiSO_4$, this binding occurred at a much lower affinity. This data demonstrates the selective binding of CopZ and copper and validates the function of CopZ as a copper chaperone.

We reasoned that a mutation in *copZ* may result in an altered protein profile, which could potentially be involved in regulating biofilm formation. To investigate this, we examined intracellular and secreted protein patterns in cells and culture media, respectively, of WT UA159, ΔcopZ, and ΔcopZ/copZpVPT. Extracted proteins were separated on an SDS-PAGE gel for comparison. The top two bands (approximately 150kDa) were reduced in the *copZ* mutant secreted protein profile. Since these bands

correlated with the size of glucosyltransferases (Gtfs), the bands were probed with a GtfB antibody (Nakano & Kuramitsu, 1992). ΔcopY and ΔcopA protein profiles were similar to the parent strain. In contrast, there were drastically less Gtfs in the cell and secreted into the supernatant of ΔcopZ (Fig. 3A). Gtf production and secretion were restored in by complementation (ΔcopZ/copZpVPT, Fig. 3A). Gtfs, particularly GtfB and GtfC, extracellularly metabolize sucrose into mostly insoluble glucans which are essential for providing a rigid scaffold for the development of a tenacious, cariogenic biofilm (Nakano & Kuramitsu, 1992, Bowen & Koo, 2011). To determine whether decrease in secreted Gtf translated to the production of less glucan matrix, we examined production of the glucan matrix in biofilms using a dextran-conjugated cascade dye with confocal laser scanning microscopy (CLSM). Biofilms were grown identically to those depicted in Fig.1 except with added dextran-conjugated cascade dye and on an 8-well slide for optimal CLSM imaging. Under these conditions, we visualized composition and architecture of the biofilms with CLSM. We compared the WT UA159 to the ΔcopY, ΔcopA, and ΔcopZ mutant biofilms. Eighteen-hour biofilms of the WT UA159, ΔcopY, and ΔcopA displayed similar biofilm structure of aggregates of bacteria encased or surrounded by glucan matrix (First and fourth rows, Fig. 3B) (Xiao *et al.*, 2012). The ΔcopZ biofilm produced less glucan matrix which led to bacterial cells being more exposed, and therefore, seemed to exhibit higher intensity of fluorescence (Fig. 3B). Three-dimensional panels reflect thickness observed in Fig. 1 (last column, Fig. 3B). To demonstrate ΔcopZ biofilm phenotypes are not biomass dependent, we doubled the inoculum of ΔcopZ bacteria added to biofilm media prior to biofilm development to compensate for differences in biomass (ΔcopZ(double)) and then examined biofilms by

CLSM (Fig. S1). At 18 hours, the ΔcopZ (double) biomass was similar to WT UA159 and ΔcopZ/copZpVPT (bar graph, Fig. S1). Since both ΔcopZ and ΔcopZ (double) exhibited reduced glucan matrix and compared to the parent and complement strains, these phenotypes observed in ΔcopZ were therefore not due to reduced biomass. These data suggest *copZ*, but not *copY* or *copA*, plays an important role in proper biofilm formation in a Gtf-dependent manner.

S. mutans competitiveness dependent on CopZ

To persist in the oral cavity, *S. mutans* must out-compete commensal bacteria that produce detrimental hydrogen peroxide or other antimicrobials. As a defense mechanism, *S. mutans* secretes bacteriocins-antimicrobial peptides that inhibit growth of commensal species such as *Streptococcus sanguinis* and *Streptococcus gordonii* (Kreth et al., 2008, Hossain & Biswas, 2011). Because the *copZ* mutant biofilm was defective in biomass, composition, and architecture, we suspected that the mutant would not be able to effectively inhibit commensal species. To determine whether ΔcopZ could inhibit commensal species, strains were analyzed using an inhibition assay. Strains WT UA159, ΔcopY, ΔcopA, ΔcopZ, and ΔcopZ/copZpVPT were inoculated on THB plates first. Liquid cultures of commensal species were then plated immediately adjacent to the *S. mutans* strains. After 18 hours incubation, growth inhibition of commensal species was observed. Strains ΔcopY and ΔcopA inhibited *S. gordonii* and *S. sanguinis* similar to the parent strain. ΔcopZ showed reduced ability to inhibit *S. gordonii* or *S. sanguinis*, albeit the effect on *S. sanguinis* was modest while inhibition of *S. gordonii* was completely

abolished (Fig. 4A). This effect was restored by complementation (ΔcopZ/copZpVPT, Fig. 4A).

S. mutans UA159 secretes three bacteriocins, mutacin IV, mutacin V, and mutacin VI (Hossain & Biswas, 2011, Merritt & Qi, 2012). To validate the inhibition assay, we examined the expression of genes *nlmA* and *nlmB* (mutacin IV), *nlmC* (mutacin V), and *nlmD* (mutacin VI) in the *copZ* mutant. Expression of all four genes was significantly downregulated in ΔcopZ compared to the parent strain (Fig. 4B). Since the *com* system has been recently implicated in mediating mutacin production, we checked expression of *comCDE* (Reck *et al.*, 2015). All *com* genes were downregulated in ΔcopZ (Fig.4B). The expression for *gtfB* was downregulated in ΔcopZ, further demonstrating the decrease in Gtfs and glucans observed in ΔcopZ only (Fig. 3A & 3B). Because Biofilm Regulatory Protein A, BrpA, has been associated with biofilm, we examined the expression of *brpA* in ΔcopZ. Notably, *brpA* expression in ΔcopZ looked similar to WT UA159, suggesting that the impact of *copZ* on *S. mutans* biofilms and competitiveness is BrpA-independent. Collectively, these data suggest that CopZ plays a critical role in the expression of mutacins and consequently inhibition of commensal *Streptococci*.

DISCUSSION

Copper plays a critical role in bacterial pathogenesis, survival, stress response, and biofilm formation (Baker et al., 2010, Mitrakul et al., 2004, Shafeeq et al., 2011, Solioz & Stoyanov, 2003, Solioz et al., 2010, Wolschendorf et al., 2011). The cariogenic pathogen, *S. mutans* survives copper challenge by inducing the *copYAZ* operon and mediating copper resistance (Singh *et al.*, 2015, Vats & Lee, 2001). While *copYAZ*

counterparts in other pathogenic organisms have been shown to be necessary for virulence expression, the individual *copYAZ* genes in *S. mutans* have not been investigated for their potential roles in bacterial fitness and virulence traits. In this study, we demonstrate the importance of copper chaperone CopZ in *S. mutans* biofilm formation and competitiveness. Unlike ΔcopY and ΔcopA biofilms, the ΔcopZ biofilm had significantly decreased biomass. The defective biofilm of the *copZ* mutant was in part attributed to decreased production and secretion of biofilm-essential enzymes, GtfB and GtfC. The *copZ* mutant biofilm characterized with CLSM revealed reduced glucan matrix and exposed bacterial cells in contrast to the microcolonies enveloped by glucans in biofilms formed by WT UA159, ΔcopY, ΔcopA, and ΔcopZ/copZpVPT. Overexpression of CopZ in the complement strain (ΔcopZ/copZpVPT) resulted in increased biomass and robust glucan matrix formation, further demonstrating the crucial role of CopZ for biofilm formation. In addition, the *copZ* mutant was impaired in competitiveness against oral commensal species *S. sanguinis* and *S. gordonii*. Essential to competitiveness of *S. mutans* UA159 is the expression and secretion of mutacins. These mutacins are critical for *S. mutans* UA159 to out-compete commensal species in order to establish a biofilm in the oral cavity. The *copZ* mutant exhibited impaired ability to inhibit growth of commensal species *S. gordonii* and *S. sanguinis.* This result was further corroborated by our qRT-PCR data showing decreased expression of *S. mutans* UA159 mutacin IV,V, and VI genes.

Reduced competitiveness of the *copZ* mutant cannot be attributed to reduced competence observed in the *copYAZ* mutant since recent studies suggest that mutacin

secretion is independent of competence (Singh et al., 2015, Reck et al., 2015). Mutacin transcription and synthesis has instead been shown to be regulated by ComCDE (Reck et al., 2015). Indeed we demonstrate reduced expression of mutacin IV, V, VI and *comCDE* genes in ΔcopZ. This study first demonstrated the role of CopZ in competitiveness of *S. mutans* against commensal oral species and uncovered the underlying mechanism.

The *copYAZ* operon has previously been characterized for its role in copper resistance in *Streptococcus mutans. S. mutans* encounters an influx of copper from diet and metal dental restorations. Under copper stress, *S. mutans* induces the *copYAZ* operon for resistance against copper toxicity (Vats & Lee, 2001). The copper chaperone CopZ is unique compared to its operon counterparts; CopY, a negative repressor of *copYAZ,* and CopA, a P1-ATPase copper exporter. In the study, we also demonstrated CopZ tightly binds copper, which provided the first experimental evidence that CopZ is a copper chaperone in *S. mutans*. Due to the apparent difference among the *copYAZ* components, we investigated their contribution to *S. mutans* virulence traits.

CopZ is a copper chaperone, previously characterized for its importance in copper homeostasis in other organisms. In *Enterococcus hirae*, CopZ has been shown to bind and transport copper to CopY for transcription initiation of the *copYAZ* operon (Cobine et al., 1999). Since CopZ can bind and transport copper to regulate *copYAZ*, it is possible that CopZ may help regulate other processes. Beyond copper transport, *copZ* has been shown to affect biofilm detachment but unnecessary for biofilm formation in oral commensal *Streptococcus gordonii* (Mitrakul et al., 2004). In *S. mutans*, the *copYAZ* operon has been implicated in oxidative stress and *gtfB* transcription (Singh *et al.*, 2015). In this study, we demonstrate that CopZ is critical for biofilm formation by Gtfs and

competitiveness by mutacins. The impact of *copZ* on *S. mutans* biofilm formation and competitiveness are, to the best of our knowledge, unique to a copper chaperone. The phenotypes of the *copZ* mutant are independent of *copY* and *copA*, implicating the importance of CopZ in *S. mutans* virulence traits. The function of CopZ to bind and transport copper, and its unique role in *S. mutans* biofilm formation and competitiveness suggests CopZ may have an independent role aside from copper resistance. Further investigation into the mechanism of *copZ* on biofilm formation and competitiveness may lead to the identification of novel virulence-regulatory pathways.

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Supporting Information

Additional Supporting information may be found in the online version of this article:

Figure S1. CopZ biofilm phenotype is not due to reduced biomass.

Table1. Strains and plasmids used in this study

Strain or Plasmid	Description	Source
WT UA159	*Streptococcus mutans* UA159	This study
ΔcopY	*S. mutans* UA159 with in frame replacement with a kanamycin cassette	This study
ΔcopA	*S. mutans* UA159 with in frame replacement with a kanamycin cassette	This study
ΔcopZ	*S. mutans* UA159 with in frame replacement with a kanamycin cassette	This study
ΔcopZ/copZpVPT	*S. mutans* UA159 ΔcopZ transformed with pVPT encoding *copZ*	This study
S. gordonii	*Streptococcus gordonii* DL1	This study
S. sanguinis	*Streptococcus sanguinis SK36*	This study
pET28a-hisSUMO	Plasmid optimal for expressing soluble proteins with a His-tag in *E. coli* BL21 Gold (DE3)	This study
pVPT	*E. coli-Streptococcus* shuttle vector and expression plasmid (erythromycin)	Zhou *et al.* (2008)
copZpVPT	*S. mutans* UA159 *copZ* gene cloned into pVPT for expression of *copZ*	This study

Table 2. Primers used to make mutants or constructs

Constructs	Primers used (5'-3') Restriction enzyme sites are underlined
copY mutant	Amplify gene + flanking region: F- GAGATGTCGGTTGGCTAACCAGAC R- GAGATGGTGAGCATGATGTGTATGTCCC Inverse PCR with added KpnI site: F- GGCGCGGTACCGACATCAAAATGAGTGAAGAAG R- GGCGCCGGTACCTTTGAGCTCCTTTCATCTAC
copA mutant	Amplify gene + flanking region: F- GGAGCAGCTGTAGGTGCTGCTACTTTTTG R- CAGAGCTAAAGCTCATGGCTAGACCAG Inverse PCR with added KpnI site: F- GATCGGTACCAAAGGTCGAACCTCAGATGC R- GGGCGCGGTACCTTTTGATGTCACCTCCAAATG
copZ mutant	Amplify gene + flanking region: F- GAATATGGGGGTTGAAGTGGCCATGCTGAC R- GTGCTTGCGTAAATACCAGACTCGCATC Inverse PCR with added KpnI site: F- GGCCGGTACCGTAGGCTTCATATACCTTAA R- GCGCCGCGGTACCTGATAATTCTCCTTTAT
ΔcopZ/copZpVPT	Amplify gene with added BspHI & BamHI sites for insertion: F- GCGGGGCGCTCATGAATGGAAAAAACATATCA R- GGCGCGGATCCTTAAATTTCTGCTCCCAAT
copZpET28a-hisSUMO	Amplify gene with added BamHI & XhoI sites for insertion: F- GGCGGCGCGGATCCATGGAAAAAACATATCA R- GCGCGCTCGAGTTAAATTTCTGCTCCCAAT

Table 3. qRT-PCR Primers

Gene Name	Primer Sequences (5'-3')
nlmA	F-ATGGATACACAGGCATTTC R-TATGGGGTAACAAGAGTCC
nlmB	F-TGTCAGAAGTTTTTGGTGGA R-AGCACATCCAGCAAGAATA
nlmC	F-AGCATATGGACCAAGAAATC R-ACGTAATGGATAATGAAGCAC
nlmD	F-GAGGGTGGTGGTATGATTAGATGTG R-TCCAGACCAGCCTCCTAAAGC
comC	F-ACGAATTAGAGATTATCATTGGCGG R-CCCAAAGCTTGTGTAAAACTTCTGT
comD	F-TGATTGCTGTTACGATGGTG R-AAGTCAGAACTGGCAACAGG
comE	F-TCATACTGCCGTAGAATTCA R-AAGAATGGTCAATCAGAGGA
gtfB	F-ACACTTTCGGGTGGCTTG R-GCTTAGATGTCACTTCGGTTG
brpA	F-TACAGCATCAGTTGAGCCCG R-ACCTTGCTGATGACCTCACG

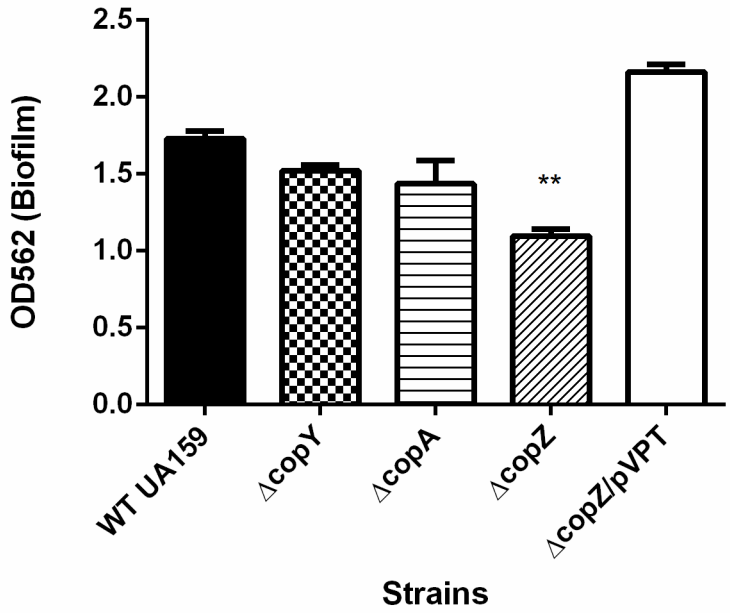

Figure 1. *S. mutans* UA159 *copZ* mutant is defective in biofilm formation. Single-gene mutants of *copYAZ* and the parent strain were allowed to form biofilms for 18 hours in biofilm media supplemented with 1% sucrose. Biomass was quantitated by crystal violet staining and reading at $O.D._{562}$. Complement strain of ΔcopZ restored biofilm formation (ΔcopZ/copZpVPT). **=$p<0.01$. Data represents three biological replicates.

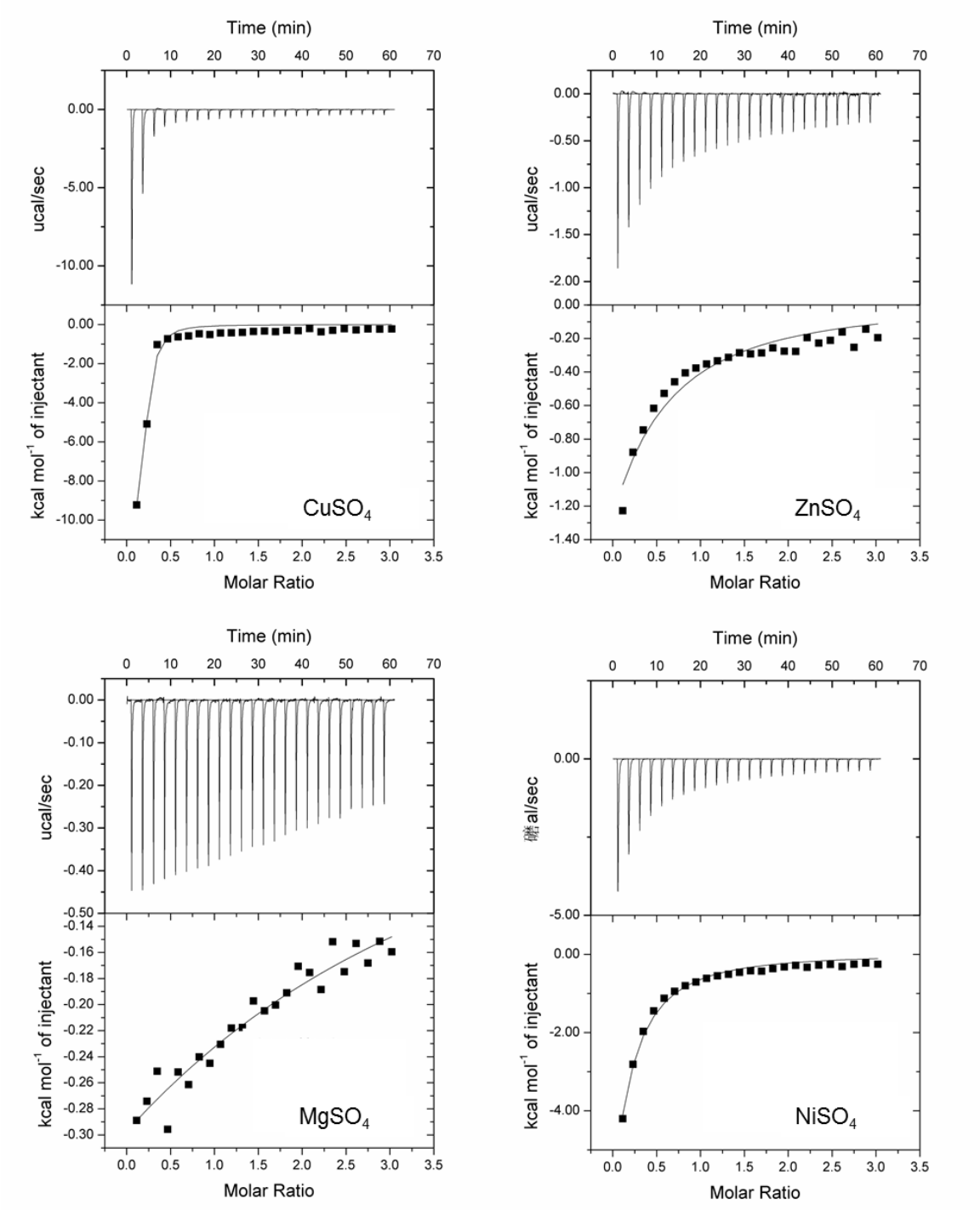

Figure 2. CopZ affinity for Copper.

ITC curves of purified CopZ protein and free copper, zinc, magnesium, or nickel binding.

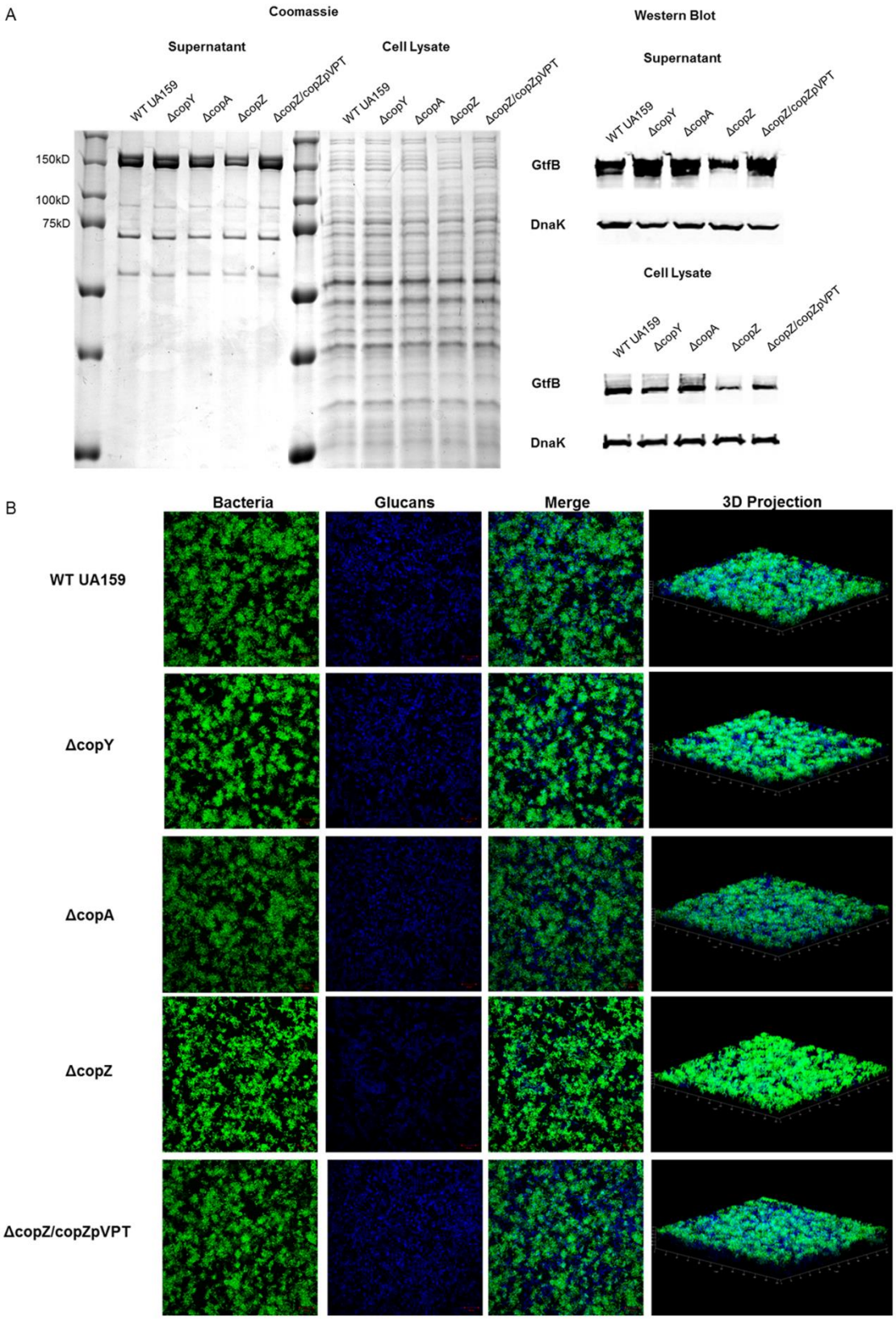

Figure 3. CopZ mutant is deficient in Gtf protein levels and production of glucan matrix.

(A) Protein profiling of *S. mutans*. Proteins were extracted from supernatants and cells of WT UA159, ΔcopY, ΔcopA, ΔcopZ, and ΔcopZ/copZpVPT cultures grown to the same OD_{470}. Extracted proteins from supernatants and cell lysates were run on SDS-PAGE gels and stained with coomassie (left) or probed with the GtfB or DnaK (for loading control) antibodies for western blot (right). (B) Biofilms of *S. mutans*. Biofilms of WT UA159, ΔcopY, ΔcopA, ΔcopZ, and ΔcopZ/copZpVPT were grown for 18 hours. All images are maximum intensity projections or Three-dimensional projections of bacteria (green) and glucan matrix (blue). Biomass of biofilms imaged by CLSM is plotted. Results are representative of three independent experiments.

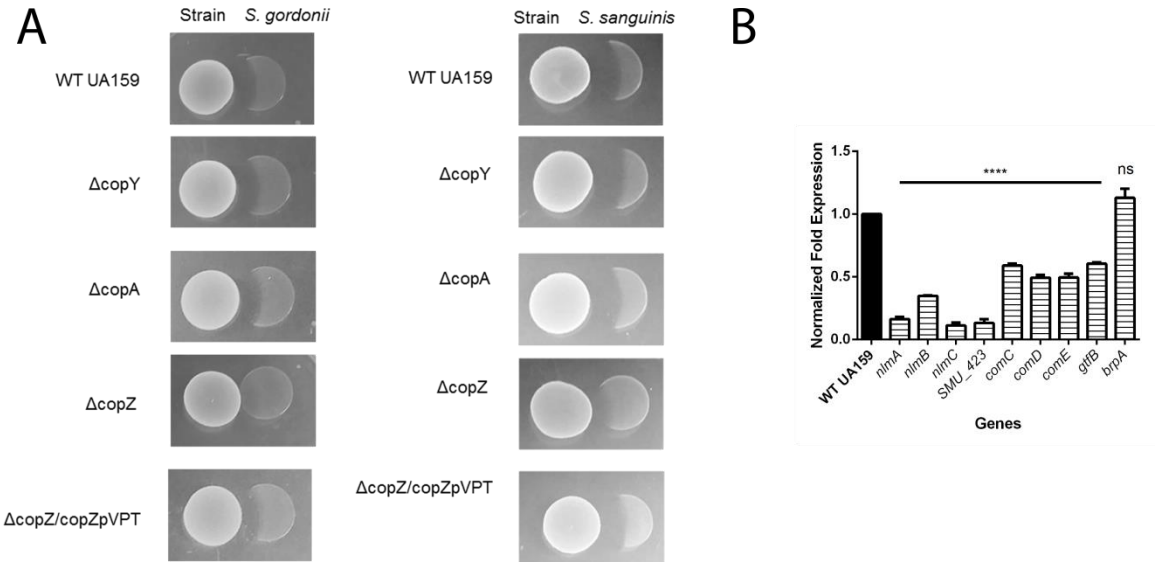

Figure 4. CopZ mutant is defective in inhibiting commensal Streptococci.

(A) Plate inhibition assays. WT UA159, ΔcopY, ΔcopA, ΔcopZ, and ΔcopZ/copZpVPT were inoculated first. Commensals *S. gordonii* and *S. sanguinis* were plated second and growth inhibition was assessed the following day. (B) Expression of mutacin genes is reduced in the CopZ mutant. RNA was extracted from late exponential phase cultures of WT UA159 and ΔcopZ. Expression of mutacin genes were analyzed by qRT-PCR and normalized by comparison to the expression of 16S. Results are representative of three biological replicates. ****=p-value≤0.0001.

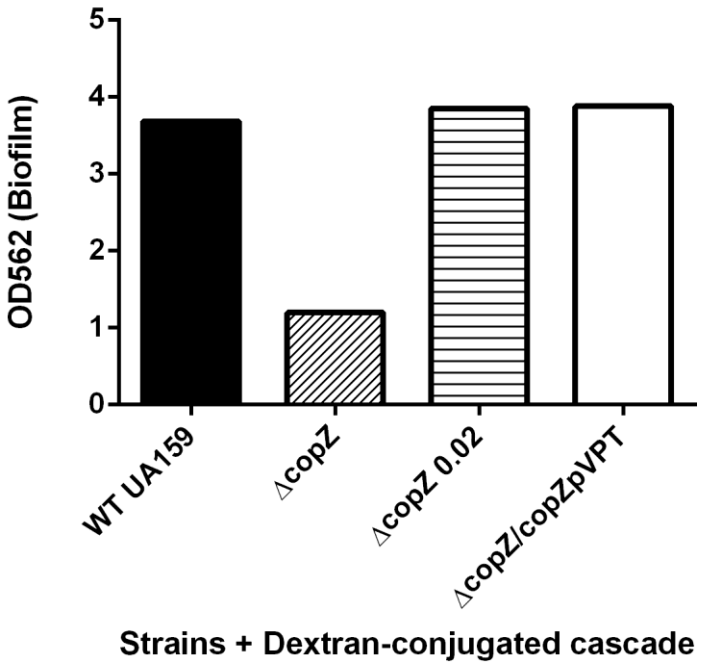

Figure S1. CopZ biofilm phenotype is not due to reduced biomass.

SUMMARY AND DISCUSSION

Dental caries is a costly disease characterized by the demineralization of the enamel, otherwise known as tooth decay. The etiologic agent of dental caries is *Streptococcus mutans.* Utilization of dietary sugars by *S. mutans* promotes biofilm formation and production of lactic acid, leading to the dental caries. Besides *S. mutans*, there are hundreds of different bacterial species inhabiting the oral cavity. Furthermore, some species interact with *S. mutans*, some of which are advantageous in the control of pathogenic *S. mutans*. However, there are currently no anticaries therapies aimed at specifically targeting *S. mutans* biofilms in order to preserve the beneficial oral microbiome.

In our first study, we identified a novel small molecule 3F1 that is capable of selectively dispersing *S. mutans* biofilms to prevent dental caries and preserve the oral microbiome. Small molecule 3F1 dispersed approximately half of the *S. mutans* biofilm. In contrast, 3F1 did not negatively impact biofilms formed by oral commensal species *Streptococcus sanguinis* or *Streptococcus gordonii*. Dispersal by 3F1 was independent of known factors essential for initial biofilm development. In an *in vivo* rat caries model, selective targeting of *S. mutans* biofilm by 3F1 reduced *S. mutans* and effectively prevented dental caries despite daily sucrose intake. Moreover, selective reduction of *S. mutans* biofilms did not perturb the microbiome. Further characterization of the oral

microbiome implicated the significant impact of daily sucrose intake in the maintenance of diversity, and composition of the oral microbiome.

The strengths of our study include utilizing a small molecule and a well-established rat caries model. Small molecules are generally simple to synthesize, soluble, and stable. Small molecule 3F1 did not cause *S. mutans* cell death. Antimicrobial peptide C16G2 specifically killed *S. mutans* in a single species model, however in an *in vitro* oral biofilm, C16G2 eliminated various species (Guo *et al.*, 2015). Non-cytotoxic activity by 3F1 may make it less likely for resistant strains to develop. In this study, we knew the chemical structures of the small molecules in the compound library. Knowing the 3F1 structure aided us in synthesizing analogues to potentially optimize potency or determine specificity of activity to 3F1. Since structural isoform 3F2 did not have any dispersal activity, the potency of 3F1 may be due to the unique structural characteristics of 3F1. Anticaries therapies derived from other natural products typically inhibit Gtf expression or glucan production by Gtfs (Falsetta *et al.*, 2012, Koo *et al.*, 2010). However, our data indicate that 3F1 does not target factors necessary for initial biofilm development such as AgI/II, Gtfs, glucans, or eDNA. Different proteins are involved in processes for initial biofilm development, maintenance, and maturation (Klein *et al.*, 2012). Small molecule 3F1 was applied after initial development of biofilms, therefore the target of 3F1 may target proteins involved in biofilm maintenance or maturation. However, these proteins and their pathways are largely unknown. To identify the protein target of 3F1, we will utilize a novel method called drug affinity responsive target stability, or DARTS. This method depends on the binding of the small molecule to the target to protect the target from degradation by proteases (Lomenick *et al.*, 2009). The advantage of this method is

the ability to use whole cell lysate in order to determine the molecular target. As an alternative, we can apply proteomics to investigate any changes in proteins when *S. mutans* biofilms are treated with 3F1. A potential mechanism involves the inhibition between bacterial cells and glucan matrix, in which we could test this interaction with purified glucan and 3F1 by ITC. Another advantage of our study was the use of the rat caries model. In this model, we could determine the effect of selective targeting of *S. mutans* in the oral cavity under dietary sucrose conditions and the impact on the oral microbiome. Current reports on anticaries therapies do not encompass the host factors, diet, and microbial communities. Previous rat caries studies executed with anticaries therapies derived from natural products have been shown to prevent dental caries, but have not been *S. mutans* specific and did not characterize the impact on the rat overall oral microbiome (Falsetta *et al.*, 2012). *S. mutans* specific antimicrobial peptide C16G2 decreased *S. mutans* in an *in vitro* oral biofilm model with saliva, but this resulted in the elimination of other species (Guo *et al.*, 2015). Peptide C16G2 has not been investigated for efficacy *in vivo* where diet plays a role. Although we demonstrated efficacy in rats, humans eat more complex diets and the human oral microbiome is much more diverse and complex (Dewhirst *et al.*, 2010). Testing our compound using a relevant *in vitro* human oral microbiome model may allow us to evaluate the selectivity of our compound. Taken together, this study demonstrates the efficacy of selectively targeting *S. mutans* biofilms in the prevention of dental caries and maintenance of the oral microbiome, despite of daily sucrose intake. Moreover, our data suggest that selectively targeting *S. mutans* biofilms is a viable therapy against dental caries.

Prior to establishing a biofilms and causing disease, *S. mutans* must first survive by tolerating acid, maintaining metal ion homeostasis, and resisting oxidative stress. As a by-product of sucrose metabolism, *S. mutans* produces lactic acid which acidifies the local milieu, dropping the pH from 7.0 to 5.5 or even 3.0 (Matsui & Cvitkovitch, 2010). To survive and proliferate in acidic conditions, *S. mutans* regulates intracellular pH via acid tolerance response, which includes increasing activity of a proton exporting ATPase (Welin-Neilands & Svensater, 2007). Metal ions, e.g. copper, are essential at low concentrations and are present at physiologic concentrations in saliva. However, excess copper ions due to leakage from copper-based amalgam or dietary intake may lead to cytotoxic levels in *S. mutans*. Commensal inhabitants of the oral cavity such as *Streptococcus gordonii* secrete H_2O_2, effectively competing against *S. mutans* (Kreth *et al.*, 2008). Critical to acid tolerance, copper homeostasis, and resistance to oxidative stress is the *S. mutans* copper-resistance operon *copYAZ* (Singh *et al.*, 2015, Vats & Lee, 2001). While the *copYAZ* operon has been implicated in protecting *S. mutans* against stress, other pathogenic species show a dependence on *copYAZ* operon homologues for complete virulence. In *S. mutans,* the role of individual genes of the copper-resistance *copYAZ* operon in virulence has not been previously shown.

We characterized the single-gene mutants of the copper-resistance operon *copYAZ* in virulence. Despite the intact *copYAZ* operon being shown to affect GtfB expression and being necessary for physiologic processes (Singh *et al.* (2015), we found that only the *copZ* mutant played a role in virulence. The *copZ* mutant, but not the *copY* or *copA* mutants, exhibited defective biofilm formation, reduced GtfB production and secretion, and impaired commensal inhibition due to decreased mutacin expression. We

confirmed the function of CopZ as a copper chaperone by expressing the protein and demonstrating high affinity copper binding activity. The apparent role of *copZ,* but not other *cop* operon counterparts in virulence suggested that copper chaperone CopZ plays a distinct role.

In a pathogen such as *Mycobacterium tuberculosis*, genes involved in copper-resistance are necessary for bacterial survival in macrophages and subsequent disease in animals (Wolschendorf *et al.*, 2011). However, infiltration of macrophages is not part of the disease process for *S. mutans*. Therefore it is still unclear why CopZ is relevant to biofilm-related processes. Copper is a necessary cofactor for metalloenzymes such as cytochrome C (Karlin, 1993). CopZ may interact with receptors to regulate fitness and virulence. Besides CopY, there are no other reported CopZ receptors in Gram positive bacteria. We may discover putative interacting receptors of CopZ by producing a recombinant CopZ protein and utilizing an affinity pull-down assay. Alternatively, the effect of CopZ on several genes may indicate its role as a global regulator. *S. mutans* lacking the *copYAZ* operon results in decreased expression of several genes involved in biofilm and competence, which were most likely due to the lack of *copZ* (Singh *et al.*, 2015). To determine whether CopZ is a global regulator, we use RNAseq to evaluate the gene expression profile of the CopZ mutant. RNAseq would be able to show the differential expression of genes in the CopZ mutant compared to the parent strain.

In this study, we report a previously unidentified role of the copper chaperone, CopZ, in *S. mutans* fitness and virulence. Elucidation of the mechanism of *copZ* on the role of biofilm formation and competitiveness may lead to the identification of novel

virulence-regulatory pathways and novel therapeutic targets for the development of anticaries therapies.

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APPENDIX

INSTITUTIONAL ANIMAL CARE AND USE COMMITTEE APPROVAL FORM

THE UNIVERSITY OF ALABAMA AT BIRMINGHAM
Institutional Animal Care and Use Committee (IACUC)

MEMORANDUM

DATE: 07-Oct-2015

TO: Michalek, Suzanne M

FROM: Robert A. Kesterson, Ph.D., Chair

Institutional Animal Care and Use Committee (IACUC)

SUBJECT: **NOTICE OF APPROVAL**

The following application was approved by the University of Alabama at Birmingham Institutional Animal Care and Use Committee (IACUC) on 07-Oct-2015.

Protocol PI: Michalek, Suzanne M

Title: Targeting Oral Biofilms with 2-Aminoimidazole/Triazole Conjugates

Sponsor: UAB DEPARTMENT

Animal Project Number (APN): IACUC-09771

This institution has an Animal Welfare Assurance on file with the Office of Laboratory Animal Welfare (OLAW), is registered as a Research Facility with the USDA, and is accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International (AAALAC).

Institutional Animal Care and Use Committee (IACUC) | Mailing Address:
CH19 Suite 403 | CH19 Suite 403
933 19th Street South | 1530 3rd Ave S
(205) 934-7692 | Birmingham, AL 35294-0019
FAX (205) 934-1188 |