The Role of MUC5B Salivary Mucin in Modulating Oral Bacterial Surface Colonization and Interspecies Competition

The role of MUC5B salivary mucin in modulating oral bacterial surface colonization and interspecies competition

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Abstract

Mucus acts as a protective barrier on wet epithelial surfaces in the body including the gastrointestinal, respiratory, and reproductive tracts. A main component of mucus is mucin, which give mucus its viscoelastic properties and is a key component of host defense at these epithelial interfaces. Altered mucin production has been linked to gastrointestinal diseases such as Crohn's disease and respiratory illnesses like cystic fibrosis, which highlights the importance of regulated mucin production.

Although the role of mucins in gastrointestinal and respiratory illnesses has been well characterized, little is known about how salivary mucins protect the oral cavity from common diseases such as dental caries. *Streptococcus mutans* is one of the primary bacteria that cause dental caries, which form when bacteria grow on tooth surfaces then produce organic acids as metabolic byproducts. The decrease in local pH leads to dissolution of tooth enamel then cavity formation. The research in this thesis uses purified human MUC5B salivary mucin to better understand how this mucin could play a role in the prevention of cavity formation. Results shown in Chapter 2 demonstrate that MUC5B significantly reduces *S. mutans* attachment and biofilm formation on glass and hydroxyapatite. In addition, MUC5B does not significantly reduce *S. mutans* viability indicating the decrease in *S. mutans* surface colonization is due to a shift in cells from the biofilm to the planktonic state. In Chapter 3, a dual-species competition

model is used to study the effects of MUC5B in a more complex environment. The two species in the model are *S. mutans* and *Streptococcus sanguinis*, which are known to compete in the oral cavity. In this study, MUC5B increases *S. mutans* and *S. sanguinis* coexistence. The reduction in interspecies competition could be caused by an increase in the relative proportion of cells in the less competitive planktonic state, which occurs in the presence of MUC5B. Taken together, the results presented in this thesis indicate that MUC5B could play a key role in protecting the oral cavity from disease and in maintaining a healthy microbiota.

Table of Contents

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List of Figures

Chapter I

Introduction

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Introduction

Mucins, the primary gel-forming component of mucus, provide a critical layer of protection on wet epithelial surfaces in the body including the gastrointestinal tract, female genital tract and respiratory tract. The importance of regulated mucin production in maintaining health is illustrated most clearly by studying the disease states that develop when mucin is dysregulated. Several studies have utilized knockout mice to more specifically characterize the changes that occur when mucins are not present. Mice lacking the Muc2 intestinal mucin showed signs of reduced health compared to wild type mice including increased inflammation in the distal colon, weight loss and mucosal thickening (Figure 1.1A and B) (1, 2). The authors postulate that thickening of the mucosa could be caused by a thinning of the mucus layer, which results in an increase in bacteria contacting the intestinal surface (Figure 1.1A) (1, 3). A separate study looking at respiratory function in Muc5b knockout mice showed that Muc5b deficient mice had significantly increased bacterial load in the lungs and middle ear leading to reduced survival compared to wild type mice (Figure 1.1C) (4). In the oral cavity, decreased salivary flow is linked to increased incidence of dental caries, which could be caused by reduced levels of salivary mucins (5, 6). On the other hand, mucin overproduction is associated with diseases such as asthma and cystic fibrosis, where increased mucus secretion leads to narrowing of airways (7–9). These findings highlight the importance of regulated mucin production, but our understanding of the precise mechanisms by which mucins provide protection in the oral cavity are not well characterized.

The goal of this thesis work is to elucidate the function MUC5B plays in protecting the oral cavity using dental caries as a disease model. *Streptococcus mutans*, one of the primary bacteria responsible for dental caries, creates cavities when it attaches to and grows

Figure 1.1

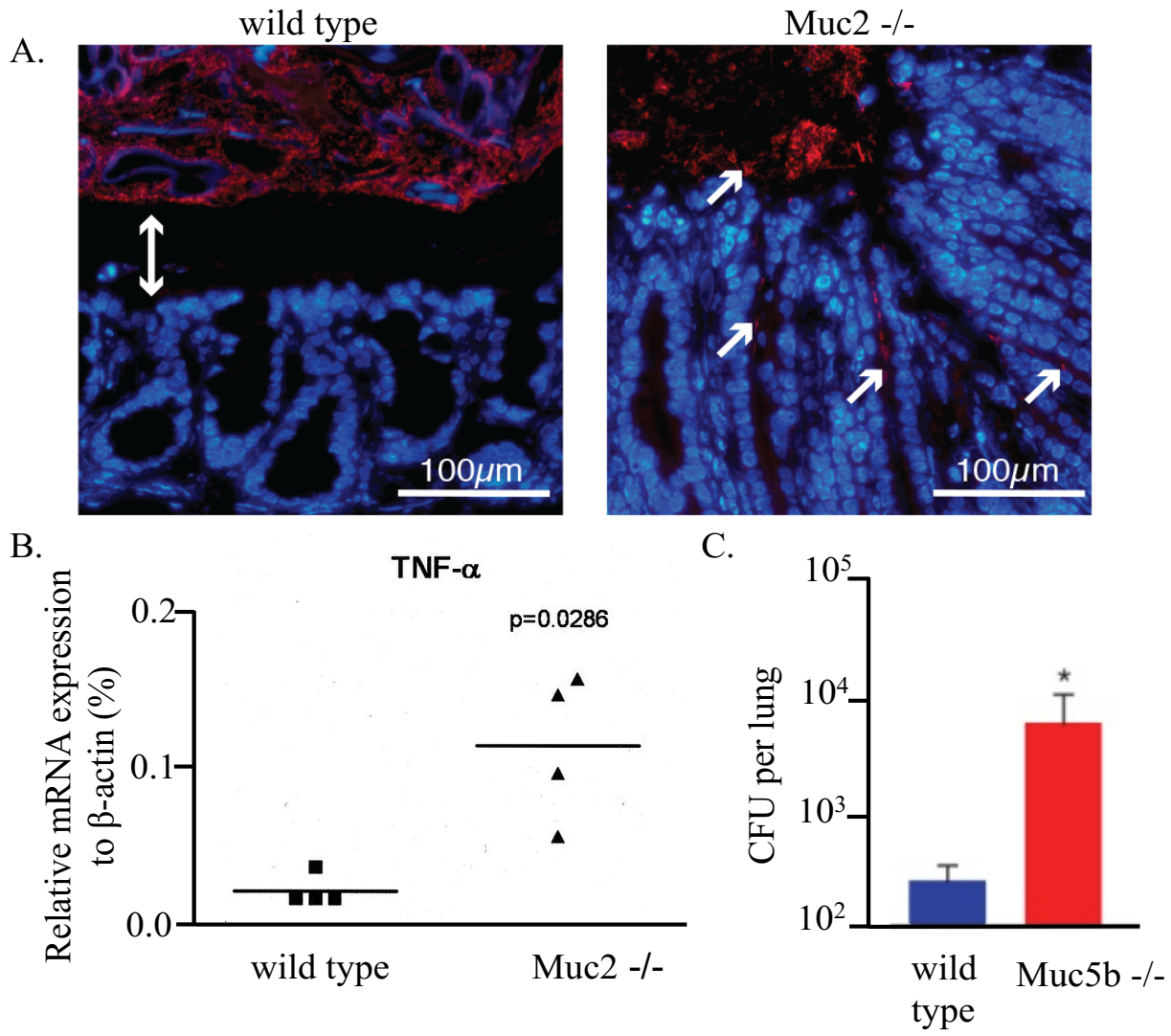

Figure 1.1 Mucin dysregulation in disease. (A) Colon section from wild type and *Muc2* -/- mice. Bacteria are stained red and tissue is counterstained with DAPI. Double arrow represents mucus layer above epithelium in wild type mice. Arrow in *Muc2* -/- mice mark bacteria. Scale bars are 100 μm. Taken from (3) with permission. Link to copyright license: https://creativecommons.org/licenses/by/4.0/legalcode. (B) TNF-alpha, an inflammatory cytokine, was significantly upregulated in the colon of *Muc2* -/- mice compared to wild type mice. P = 0.03. Taken from (1) with permission. (C) Bacterial load in the lungs of wild type and *Muc5b* -/- mice at 6 months of age. Taken from (4) with permission.

on tooth surfaces. Because surface colonization is a key step in the development of caries, I first study the effect of MUC5B on *S. mutans* attachment, biofilm formation and survival on various surfaces. Results show that MUC5B significantly reduces *S. mutans* attachment and biofilm formation without bactericidal effects, indicating that MUC5B shifts cells from the biofilm into the planktonic form. I then use a dual-species competition model with *S. mutans* and *Streptococcus sanguinis*, to determine the effect of MUC5B on interspecies interactions. In this model, MUC5B promotes *S. mutans* and *S. sanguinis* coexistence. These studies indicate that MUC5B likely plays an important role in protecting the oral cavity from disease by modulating microbial surface colonization and interspecies competition.

The following introduction will provide an overview of what is known about the structural features of salivary mucins, potential mechanisms by which salivary mucins protect the oral cavity, and methods for studying salivary mucins.

1. Introduction to salivary mucins

There are at least 20 identified mucins throughout the human body that cover wet epithelial surfaces such as the gastrointestinal tract, respiratory tract, and eyes. A summary of areas where mucins can be found in the body is given in Figure 1.2A. Each of these mucins has a unique structure that can influence its localization and function. In addition, one type of mucin can be secreted in several different locations in the body. For example MUC5B can be found in the oral cavity, lungs and female reproductive tract (10–12). This section will address structural aspects of the mucins found in the oral cavity, MUC5B, MUC7, MUC19, MUC1, and MUC4 (13).

1.1 Mucins in the oral cavity

Each of the salivary mucins MUC5B, MUC7, MUC19, MUC1, and MUC4 are composed of a unique domain structure that influences the mucins' physical properties and localization in the oral cavity (Fig. 1.2B). MUC5B is the primary gel-forming mucin in the mouth that is secreted by mucus cells in the submandibular, sublingual, palatine, and labial salivary glands (11, 14). Transcripts and glycoproteins of MUC19, another gel-forming salivary mucin, have been identified, but MUC5B is still thought to be the predominate gel-forming mucin in the oral cavity (15–17). MUC7 is also a secreted mucin that exists primarily as monomers or dimers and lacks gel-forming properties. These monomers and dimers are able to self-associate, however, to form higher order assemblies that could facilitate bacterial aggregation (18). MUC7 localization within salivary glands varies between individuals; it has been identified in mucus cells of submandibular and sublingual glands, but the presence of MUC7 in serous cells of these glands is variable (19). MUC1 and MUC4 are membrane-associated mucins that line the ducts of parotid, submandibular, and minor salivary glands (20, 21). These mucins may play a role in cell signal transduction and could form scaffolds for secreted mucins to bind (20, 22–24). Although several salivary mucins have been introduced, the following sections will focus specifically on MUC5B structure and function because it is the primary mucin studied in this research. MUC7 will be discussed briefly to emphasize the relevant differences between MUC5B and MUC7 protection in the oral cavity.

Figure 1.2

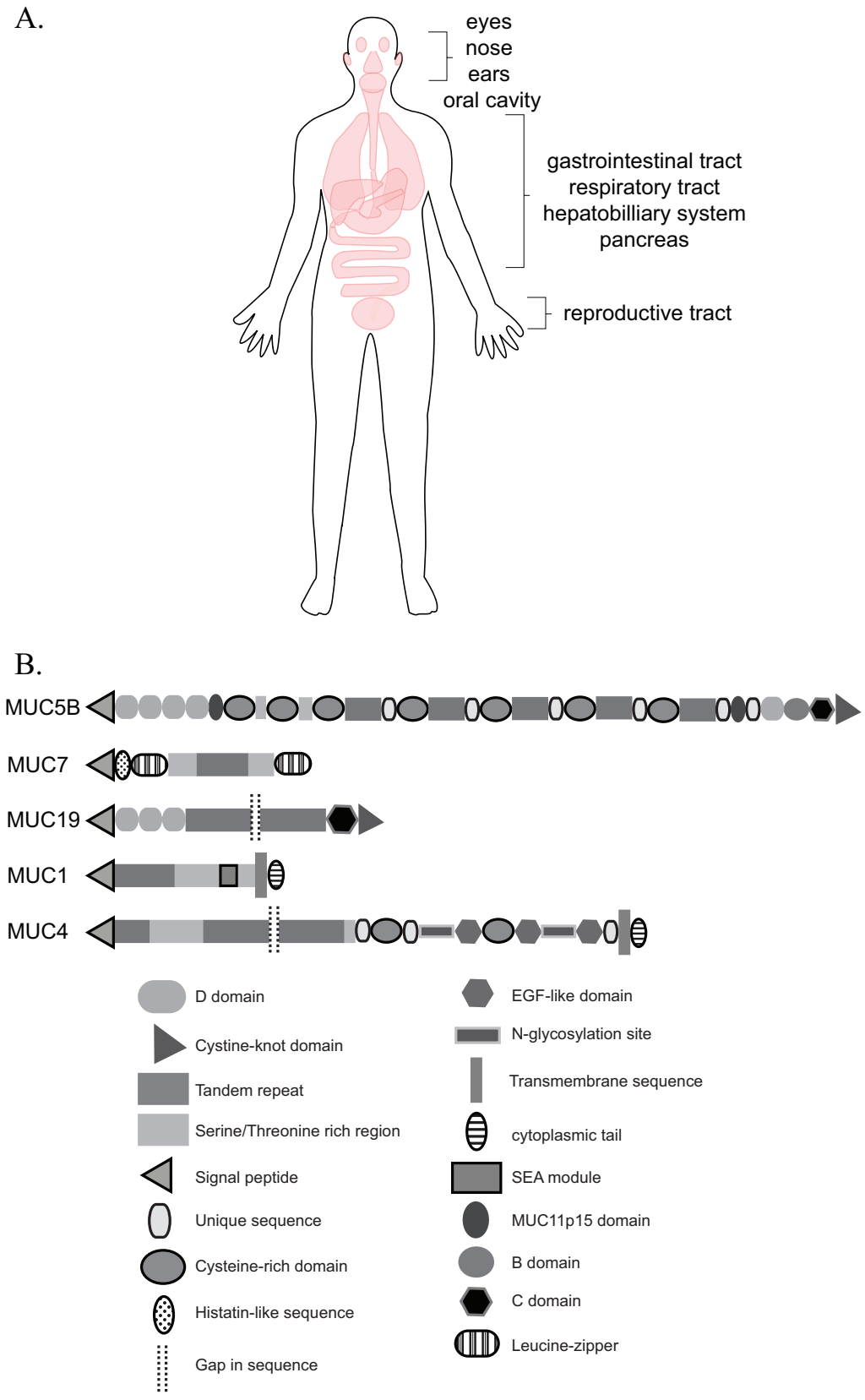
A.
eyes
nose
ears
oral cavity
gastrointestinal tract
respiratory tract
hepatobilliary system
pancreas
reproductive tract
B.
MUC5B
MUC7
MUC19
MUC1
MUC4
D domain
Cystine-knot domain
Tandem repeat
Serine/Threonine rich region
Signal peptide
Unique sequence
Cysteine-rich domain
Histatin-like sequence
Gap in sequence
EGF-like domain
N-glycosylation site
Transmembrane sequence
cytoplasmic tail
SEA module
MUC11p15 domain
B domain
C domain
Leucine-zipper

Figure 1.2 (Continued). Introduction to mucins. (A) Epithelial surfaces where mucins can be found in the body. (B) Predicted domain structures of the salivary mucins MUC5B (42, 43), MUC7 (43, 44), MUC19 (17), MUC1 (43, 45, 46) and MUC4 (47). Note that predicted domain sequences can vary based on detection method. Figure taken from (48).

1.2 MUC5B structure and secretion

MUC5B has several unique aspects of its primary sequences that determine its ability to form gels and higher order structures. MUC5B is composed of a protein backbone with glycan chains radiating outward to form a 'bottle-brush' structure, which is a common characteristic of mucins. Glycan chains account for approximately 80% of MUC5B's mass and are composed of a complex array of sugars such as fucose, galactose, and *N*-Acetylglucosamine (25, 26). There are several excellent papers that outline the composition of their glycan chains, which can be referred to for more detailed descriptions (25–28).

The MUC5B backbone is composed of approximately 5,700 amino acids and is broadly organized into the N-terminus, central glycosylated region, and C-terminus (29–31). The exact number of amino acids in the backbone varies among studies most likely because of variations in the tandem repeat region. There are several excellent reviews that further detail the structure of mucins (26, 32–38). In the endoplasmic reticulum, the C-terminal domain participates in disulfide bond formation, which links individual MUC5B monomers into dimers (39, 40). Then, in the golgi complex, polymer chains form through disulfide bond formation at the N-terminus and amino acids in the backbone are O-glycosylated (39, 40). MUC5B's central glycosylated region contains repeating units of 29 amino acids that are rich in serine and threonine (41). *N*-Acetylgalactosamine attaches to serine or threonine's hydroxyl group, which provides the foundation for O-glycosylated sugar chains (26). Several excellent reviews detail the formation

of mucin polymers and packaging within the cell (32, 33, 49). Once the packaged mucin granule is secreted, divalent calcium ions, which stabilize the folded mucin polymer within the secretory granule, are exchanged for monovalent sodium ions (30, 50). The increased osmotic pressure leads to hydration, which drives expansion of the polymers and formation of a gel (30). The expanded polymers cross-link via entanglement of glycoprotein polymer chains and/or non-covalent bonds formed by hydrophobic or carbohydrate-carbohydrate interactions (51–55). Calcium may also mediate cross-linking of MUC5B to form higher order structures (56). The resulting hydrogel coats the oral epithelium as part of the protective pellicle layer and houses a vast number of oral microbes (57, 58). MUC5B's structure and physical location in the oral cavity impact the ways in which it provides protection, which is addressed in the following section.

2. Mechanisms of protection by MUC5B

MUC5B protects the oral cavity through several different mechanisms that are influenced by its unique polymer structure. These mechanisms are summarized in Figure 1.3. First, MUC5B can interact with salivary proteins to alter their localization and retention (A), which could provide increased protection for the oral cavity. To defend the oral epithelium from potentially pathogenic microbes, MUC5B could act as a barrier (B) and/or bind microbes to facilitate their removal (C). In addition, results from this thesis demonstrate that a fourth protective mechanism is possible: MUC5B can modulate pathogenicity by facilitating bacterial dispersal (D).

Figure 1.3

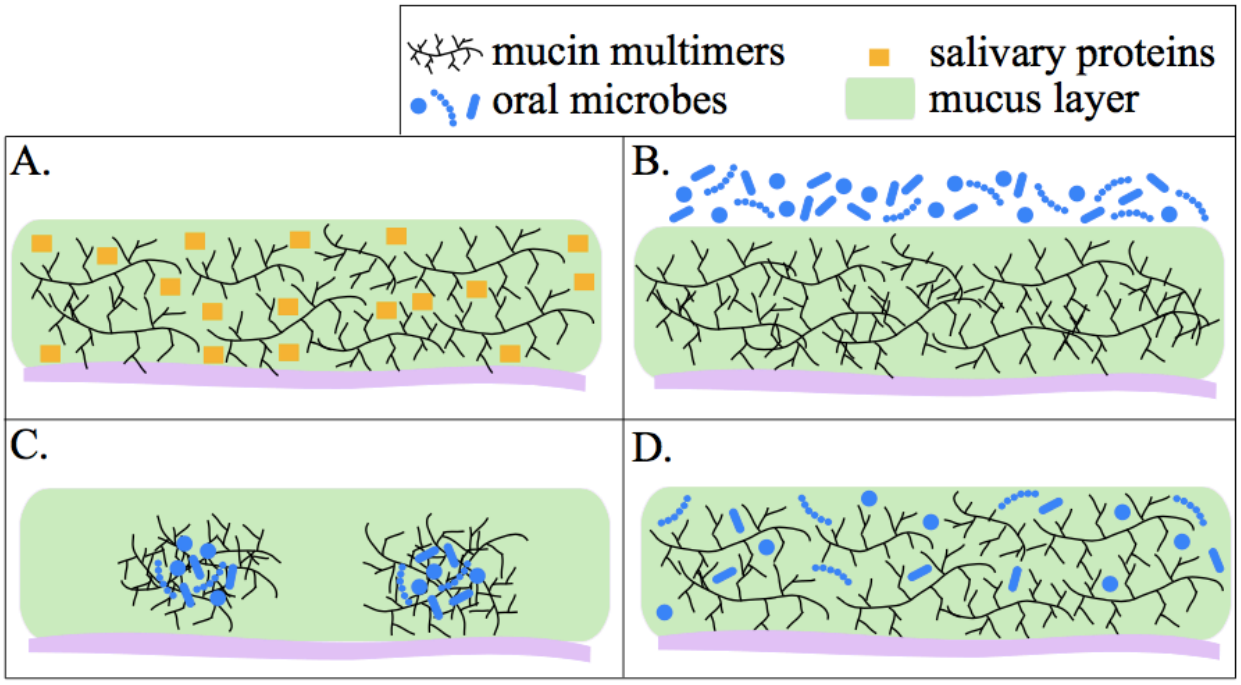

Figure 1.3 Potential mechanisms by which MUC5B protects the oral cavity from microbial colonization. (A) MUC5B could interact with protective salivary proteins to increase their retention and broaden their localization. (B) MUC5B could form a barrier that reduces bacterial contact with the epithelial surface. (C) MUC5B could agglutinate microbes, which would facilitate their removal during swallowing. (D) MUC5B could disperse bacteria through glycan-specific interactions. Selective interactions between mucins and microbes may have downstream effects on genetic regulation that reduce virulence. Schematic not drawn to scale. Adapted from (48).

2.1 Interactions between MUC5B and salivary proteins

One way MUC5B protects the oral cavity is by binding to antibacterial salivary proteins, which can influence the proteins' localization in the oral cavity, increase their retention time, and alter their biological activity (Figure 1.3A). Western blotting revealed that MUC5B forms heterotypic complexes with acidic and basic proline-rich proteins, statherins and histatin 1 (59). These proteins all have antimicrobial properties; therefore, increasing their availability in saliva could be beneficial to oral health. In some cases, salivary mucins have been shown to be

involved in sIgA binding to the mucosal pellicle, which would enhance sIgA concentration near the oral epithelium (60). MUC5B binding to this select group of salivary proteins indicates that the formation of these complexes is protein specific (59). To better understand the nature of these complexes, Iontcheva et al. show that the interaction between MUC5B and proline-rich proteins, statherins, and histatins can be dissociated using denaturing conditions, indicating that these proteins bind through hydrophobic or ionic interactions, hydrogen bonding, or van der Waals forces (59). In some cases, proline-rich proteins and statherins were also able to form bonds with MUC5B that were resistant to denaturing conditions, suggesting that covalent interactions may be involved in some types of complexes (59). Collectively, these studies indicate that MUC5B may serve as carriers for antibacterial salivary proteins to transport them throughout the oral cavity, increase their retention in the dental pellicle, and/or protect proteins from proteolytic degradation through the formation of complexes.

2.2 Mucins as barriers

The mucus layer that coats wet epithelia in the body acts as a barrier that protects the underlying tissue from mechanical damage, chemical insult, and pathogenic microbes (Figure 1.3B). In the oral cavity, MUC5B is the primary salivary mucin found in the mucus layer that coats soft and hard tissues, which is commonly referred to as the acquired pellicle (57, 58). Together with saliva, the pellicle prevents desiccation and provides lubrication that protects tissues from the forces of mastication (36, 61). One of the key roles of the mucus layer is to act as a selective barrier that allows passage of certain beneficial particles, such as gases, but not potentially harmful agents such as bacteria and acids (62). In general, microbes only directly contact wet epithelium during disease states where mucus production is dysregulated, such as

ulcerative colitis, or when the microbes have evolved specific mechanisms to subvert the barrier. In the stomach, for example, *Helicopacter pylori* increases the pH of its immediate environment by secreting urease, which reduces gastric mucus viscoelasticity and allows the pathogen to penetrate the mucus barrier and reach the gastric epithelium (63, 64). The mechanisms by which the mucus layer selects which particles and microbes can enter or pass through the barrier is an active area of research that is of great interest in the field of drug delivery. There are several excellent reviews that discuss this extensive topic (62, 65). Briefly, there are two general mechanisms by which mucins regulate the passage of particles, which include microbes. First, mucins can limit diffusion through the barrier based on the length scale of the pores formed by overlapping polymers. In general, this polymer network would allow diffusion of particles that are smaller than the pore size and exclude particles that are larger than the pore (65, 66). The biochemical properties of mucins are another way that they can impart selective diffusion characteristics to the mucus barrier (67–69). Sialic acid and sulfate residues on mucin give the polymer a net negative charge, which can exclude particles based on their chemical properties (70, 71). Mucin polymers can also participate in hydrogen bonding and hydrophilic interactions that can limit or increase the ability of particles to penetrate the mucus layer depending on the specific properties of the particles (70, 71).

2.3 Mucins binding microbes

In addition to the structural characteristics that allow mucins to form a selective barrier, mucins also contain properties that allow them to directly interact with microbes (Figure 1.3C). Early studies show that submandibular/sublingual gland saliva and salivary mucins aggregate specific strains of suspended bacteria and induce bacterial attachment to mucin-coated surfaces

(72–75). These studies primarily focus on interactions between salivary mucins and oral streptococci. Because mucins induce aggregation or surface attachment of certain bacterial species, this indicates that the bacteria recognize and bind specific glycans on the mucins, such as sialic acid and blood-group antigens (75–77). The increase in surface attachment could be beneficial because sugars in mucins' glycan chains could act as decoys for the epithelial surface (13). Importantly, many of these early studies do not distinguish between MUC5B and MUC7 salivary mucins. When these two mucins are electrophoretically separated, however, radiolabeled *Streptococcus sanguinis*, *S. sobrinus*, and *S. oralis* bind to MUC7 but not MUC5B (78). Another similar study also shows that MUC7 aggregates *S. gordonii* and promotes its adherence to surfaces whereas MUC5B has no effect on aggregation or binding (79). These findings indicate that MUC7 and MUC5B exert their protective effects through different mechanisms.

2.4 MUC5B mediated reduction in microbial pathogenicity

In contrast to MUC7, MUC5B appears to bind only a limited number of oral bacteria despite its heterogeneous glycan chains. Murray et al. tested the binding of 16 species of streptococci to MUC5B, but none of the tested strains bound this mucin (78). One explanation is that the heterogeneous glycan chains found on MUC5B would prevent binding due to the inability of bacteria to form multiple bonds or attachment points. In line with this hypothesis, *Haemophilus parainfluenzae* was shown to bind MUC5B, but this bacterium interacts with the naked peptide backbone as opposed to the glycan chains (80). *Helicobacter pylori* is another bacterium that binds MUC5B through a neutrophil-activating protein on its surface that mediates binding to sulfated glycans (81). Although few studies have shown bacteria binding to MUC5B,

there could be other oral microbes that do interact directly with MUC5B, but their interactions have not yet been characterized.

The limited number of bacteria known to bind MUC5B compared to MUC7 highlights the point that MUC5B protects the oral cavity in a unique way. The research presented in this thesis, in addition to other studies from the Ribbeck lab, indicate that mucins promote bacterial dispersal, which may have downstream effects that lead to a reduction in microbial virulence (Figure 1.3D). A recent study from the Ribbeck lab shows that Muc5ac gastric mucin reduces *Candida albicans* surface colonization by shifting cells away from the aggregated biofilm into the planktonic state (82). In addition, results show that MUC5B and Muc5ac repress *C. albicans* virulence by reducing the formation of hyphae, which are associated with host cell invasion (Figure 1.4) (82–85). *C. albicans* is the primary microbe responsible for oral candidiasis, an overgrowth of the fungus on oral tissues. The opportunistic fungus exists as part of the normal oral flora in many individuals but can become pathogenic in immune compromised individuals and lead to life-threatening systemic infection if left unchecked (86, 87). MUC5B's ability to reduce *C. albicans* virulence without killing the fungus could explain how this opportunistic pathogen can exist as part of a healthy oral microbiota without the development of overt candidiasis. MUC5B's ability to form a gel layer that guards against pathogenic microbes but does not cause bacterial killing is a unique property that contrasts with other defense proteins in saliva, such as antimicrobial peptides.

Figure 1.4

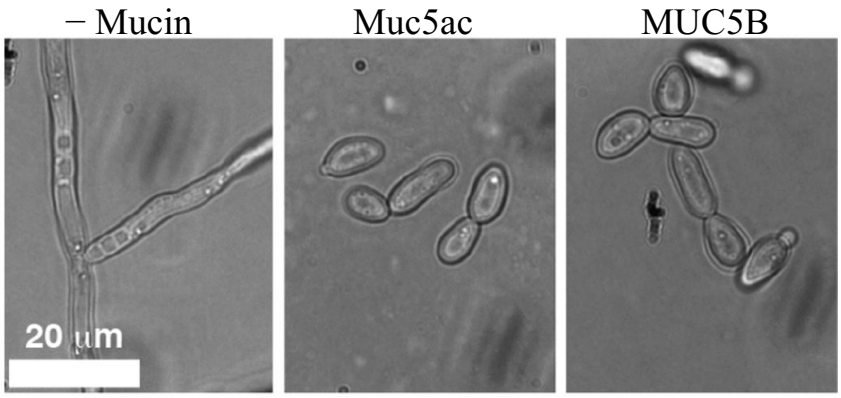

Figure 1.4 Mucins reduce *C. albicans* hyphae formation. Phase-contrast image of *C. albicans* after growth in the absence of mucins, in Muc5ac pig gastric mucin and in MUC5B human salivary mucin. Taken from (82) with permission. Link to license: https://creativecommons.org/licenses/by-nc-sa/3.0/legalcode.

3. Methods for studying salivary mucins

Because salivary mucins are relatively accessible compared to other types of mucins, such as gastric or intestinal mucin, studies using salivary mucins generally rely on purified human mucins or commercially available bovine submaxillary mucin. In contrast, studies on less accessible types of mucins commonly use animal organs for mucin purification, commercial mucin sources or mucus-secreting cell lines. Although commercially available submaxillary mucin is relatively easy to obtain compared to purification of salivary mucin from whole saliva, the properties of commercial mucin do not mimic those of purified native mucin. Specifically, commercial mucin does not maintain its gel-forming properties, which is one of the central characteristics of the mucin family of glycoproteins (63, 88). In addition, studies have shown that commercially purified mucins can be cytotoxic and do not possess the same protective properties as natively purified mucins (89). For example, commercial Muc5ac porcine gastric mucin is less efficient at reducing HPV-16 infection of HeLa cells compared to natively purified Muc5ac (89).

During the commercial purification process mucins may be proteolytically degraded, which reduces their ability to form gels and alters their chemical properties (90).

Because of the limitations of commercially available salivary mucins, I developed a protocol to purify MUC5B from human saliva. Briefly, the protocol involves collecting saliva that is enriched for sublingual and submandibular gland secretions (the primary sources of MUC5B), centrifugation to remove debris, then size exclusion chromatography. This purification protocol is relatively gentle and leaves mucins in their native form (91, 92). One drawback of this type of set up, however, is that it does not account for the other components of saliva and the mucus barrier that may function with MUC5B to protect the oral cavity. In my research, I study these purified mucins in a three-dimensional environment as opposed to a surface coating. The cell-repellent properties of mucin coatings have been studied, but these coatings may not accurately represent the environment in the oral cavity (93–95). By studying MUC5B in a three-dimensional environment, mucin polymers are fully expanded and domains can interact to form an extended gel network, which, I hypothesize, is more similar to the environment in the oral cavity (30, 34, 96, 97). By using purified human MUC5B, I am able to evaluate the effects of this mucin in a defined environment that is easily manipulated and where experimental results can be readily interpreted.

To better understand how MUC5B affects microbes to protect the oral cavity, I study MUC5B in the context of a specific disease model – dental caries. Caries is the most common chronic childhood disease and approximately 100% of adults have dental caries, therefore this model is highly relevant (98). In Chapter 2, I use purified human MUC5B mucin to study how it

affects *Streptococcus mutans* UA159 attachment and biofilm formation. Although there are several bacteria that have recently been implicated in the formation of dental caries, *S. mutans* is considered to be one of the primary cavity-causing bacteria. Results indicate that MUC5B significantly reduces *S. mutans* attachment and biofilm formation, which are key steps in the process of cavity formation. Furthermore, I show that MUC5B does not alter *S. mutans* growth rate or have bactericidal effects indicating that MUC5B reduces surface colonization by shifting cells from the biofilm into the planktonic state.

In Chapter 3, I designed an experimental dual-species model to study the effects of MUC5B on bacteria in a more complex environment. I use the model to determine the influence of MUC5B on interspecies competition using bacterial viability as a readout. The model is composed of *S. mutans* UA159 and *S. sanguinis* JFP36, which are known to compete in the oral cavity through the secretion of antimicrobial peptides and hydrogen peroxide, respectively (99–102). Results indicate that MUC5B increases coexistence of the two bacteria and shifts cells from the mixed-species biofilm into the less competitive planktonic form (103, 104). Based on these findings, **I hypothesize that MUC5B modulates oral bacteria in ways that reduce bacterial surface colonization and promote coexistence in the oral microbiota.**

When the findings of this thesis are viewed as a whole, a trend begins to emerge that hints at a general underlying mechanism of protection: MUC5B protects the oral cavity by suppressing virulent traits, such as bacterial surface attachment, without killing the organism. This mechanism of protection is very different than the classical view of mucus as a simple physical barrier overlying the epithelium. In addition, this work emphasizes that the mechanism through which MUC5B protects the oral cavity is distinct from MUC7 salivary mucins, which primarily directly bind microbes (78, 105, 106). MUC5B's ability to allow opportunistic

pathogens to exist and live within the oral microbiota as non-pathogenic residents is unprecedented by other antimicrobial proteins in saliva. This work illustrating that mucins influence bacterial surface colonization and interspecies interactions enhances our understanding of oral disease pathogenesis and could lead to novel strategies for disease prevention and treatment.

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Chapter II

Salivary Mucins Protect Surfaces from Colonization by Cariogenic Bacteria

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Abstract

Understanding how the body's natural defenses function to protect the oral cavity from the myriad of bacteria that colonize its surfaces is an ongoing topic of research that can lead to breakthroughs in treatment and prevention. This study focuses on elucidating the connection between MUC5B salivary mucins and dental caries, one of the most common oral diseases. Dental caries is predominantly caused by *Streptococcus mutans* attachment and biofilm formation on the tooth surface. Once *S. mutans* attaches to the tooth, it produces organic acids as metabolic by-products that dissolve tooth enamel, leading to cavity formation. We utilize CFU counts and fluorescence microscopy to quantitatively show that *S. mutans* attachment and biofilm formation are most robust in the presence of sucrose and that aqueous solutions of purified human MUC5B protect surfaces by acting as an antibiofouling agent in the presence of sucrose. In addition, we find that MUC5B does not alter *S. mutans* growth and decreases surface attachment and biofilm formation by maintaining *S. mutans* in the planktonic form. These insights point to the importance of salivary mucins in oral health and lead to a better understanding of how MUC5B could play a role in cavity prevention or diagnosis.

Introduction

The viscoelastic properties of mucus are attributed to mucins, large glycoproteins that play a key role in host defense and maintaining a healthy microbial environment (1–3). The diseases that result from dysregulated mucin production highlight the necessity of mucins as one of the body's key natural defenses; however, few studies have focused specifically on the connection between MUC5B salivary mucins and oral diseases. This study fills this gap in understanding by exploring the connection between purified human MUC5B and the virulence of

Streptococcus mutans (4). MUC7 is another salivary mucin, but MUC5B is the primary mucin component of the dental pellicle coating the soft and hard tissues in the oral cavity (5, 6).

S. mutans is a biofilm-forming facultative anaerobic bacterium that produces three glucosyltransferase enzymes to synthesize glucans from dietary sugar (7–9). Glucans are sticky polymers that allow the cells to attach to the tooth surface and form an extracellular matrix that protects it from host defenses and mechanical removal (10, 11). Once *S. mutans* attaches to the tooth surface, organic acids, which are produced as metabolic by-products, become concentrated within the extracellular matrix and cause a drop in pH from neutral to 5 or below. This acidic environment begins dissolving tooth enamel, leading to cavity formation, and the high tolerance of *S. mutans* for acidic environments gives it an ecological advantage. Without proper hygiene and nutritional awareness, *S. mutans* can proliferate quickly, causing serious damage to the tooth structure. *S. mutans* biofilm formation is particularly problematic in the interproximal spaces between teeth, where mechanical removal is difficult.

Because *S. mutans* attachment and biofilm formation are critical steps in cavity formation, we use CFU counts and fluorescence microscopy to quantify the effects of supplemental sugar and purified human salivary MUC5B on these key stages of disease progression. We first validate our mucin studies by showing that *S. mutans* attachment and biofilm formation are most robust in the presence of sucrose as opposed to glucose. When supplemental MUC5B is added in the presence of sucrose, however, *S. mutans* attachment and biofilm formation are significantly decreased. Although the number of surface-attached bacteria decreases in the presence of MUC5B, we show that bacterial growth is unchanged in the presence of MUC5B and the observed effects are due to increased numbers of *S. mutans* cells in the planktonic form. These findings that link MUC5B with a reduction in *S. mutans* surface colonization could significantly

impact our understanding of the pathogenesis of cavity formation and aid in the development of novel oral diagnostic methods or strategies for disease prevention.

Results and Discussion

Sucrose enhances *S. mutans* attachment and biofilm formation.

To determine the growth conditions where *S. mutans* attachment and biofilm formation are most robust, we investigated the effect of the addition of sucrose or glucose to BHI medium. The role of sucrose in enhancing *S. mutans* biofilm formation has been well established using genetic analysis and biochemical assays studying biofilm architecture (12–15). Here we characterized this phenomenon using a quantitative method that directly evaluates the number of live *S. mutans* cells attached and producing biofilm on various surfaces. *S. mutans* was inoculated into BHI containing 1% sucrose or 1% glucose. CFU counts were used to evaluate attachment at 20, 40, and 60 min and biofilm formation at 6, 18, and 24 h. Attachment was defined to occur at time points up to 60 min because the doubling time of *S. mutans* in exponential phase is approximately 1.5 h. Experiments were carried out on glass and hydroxyapatite discs because there are surface-specific effects on *S. mutans* attachment and biofilm formation (16). *S. mutans* attachment on glass and hydroxyapatite was increased by 15 and 6 times, respectively, when sucrose was present compared to when glucose was present (Figure 2.1A and C). *S. mutans* biofilm formation in the presence of sucrose was increased by 45% on glass and 8% on hydroxyapatite compared to that in the presence of glucose (Figure 2.1B and D).

Figure 2.1

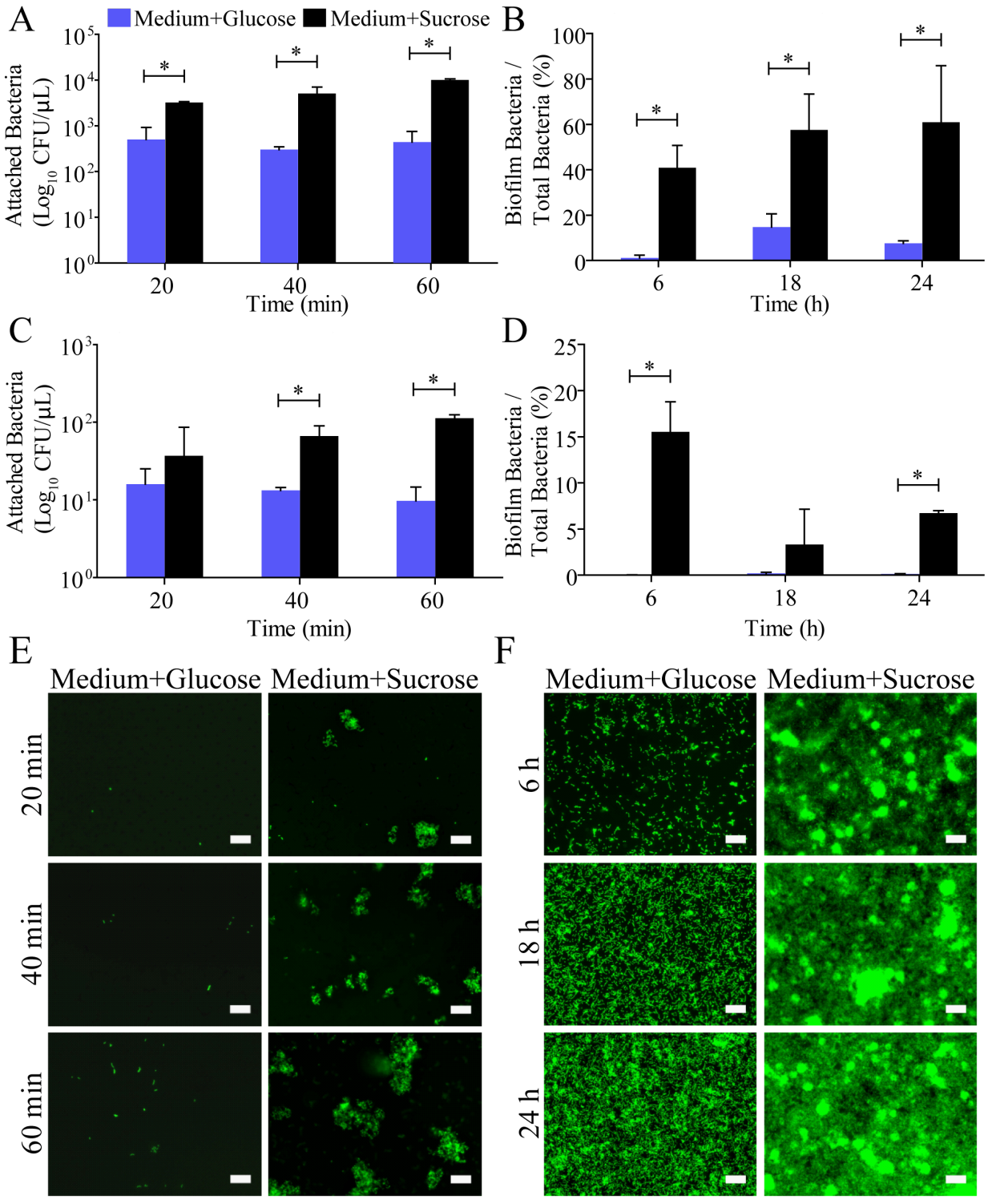
A
Medium+Glucose
Medium+Sucrose
Attached Bacteria (Log10 CFU/µL)
Time (min)
B
Biofilm Bacteria / Total Bacteria (%)
Time (h)
C
Attached Bacteria (Log10 CFU/µL)
Time (min)
D
Biofilm Bacteria / Total Bacteria (%)
Time (h)
E
Medium+Glucose
Medium+Sucrose
20 min
40 min
60 min
F
Medium+Glucose
Medium+Sucrose
6 h
18 h
24 h

Figure 2.1 (Continued). Sucrose enhances *S. mutans* attachment and biofilm formation. The levels of *S. mutans* attachment (A) and biofilm formation (B) on glass are significantly enhanced at all time points when the bacteria are grown in BHI containing 1% sucrose (Medium+Sucrose) compared to the levels achieved in BHI containing 1% glucose (Medium+Glucose). *S. mutans* attachment (C) and biofilm formation (D) on hydroxyapatite are similarly increased in the presence of sucrose, illustrating that the effect of sucrose on *S. mutans* physiology is not surface specific. Fluorescence microscopy images verify the findings of the CFU count experiments by showing an increase in *S. mutans* attachment (E) and biofilm formation (F) on glass in the presence of sucrose. *, statistically significant difference determined by Student's t test ($P<0.02$). Error bars represent SDs. Scale bars, 20 μm.

The number of *S. mutans* cells in the biofilm for each condition is represented as a fraction of the total number of bacteria in the well, because *S. mutans*' growth rate changes in the presence of sucrose compared with that in the presence of glucose (Figure 2.2).

Figure 2.2

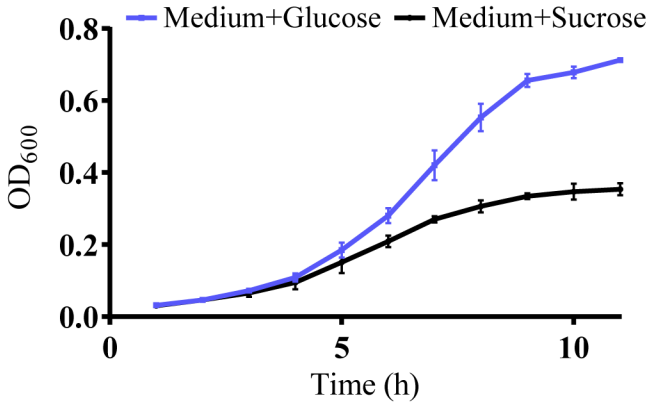

Figure 2.2 Supplemental sugar alters *S. mutans* growth. A growth curve of *S. mutans* in BHI with added 1% glucose (Medium+Glucose) or 1% sucrose (Medium+Sucrose) shows that *S. mutans*' growth rate changes based on the specific sugar present in the growth medium. Error bars represent SDs.

The increase in *S. mutans* attachment and biofilm formation in the presence of sucrose is supported by a fluorescence microscopy time series using SYTO9 nucleic acid stain to visualize *S. mutans* attachment and biofilm formation in BHI containing 1% sucrose or 1% glucose on a

glass surface (Figure 2.1E and F). Our results support those of previous studies by quantitatively showing that the addition of 1% sucrose enhanced *S. mutans* attachment and biofilm formation at all time points compared to attachment and biofilm formation in the presence of 1% glucose (12–15). There was little or no growth in BHI without a sugar source, illustrating that protein alone cannot support *S. mutans* attachment or biofilm formation. Furthermore, our results showing that sucrose enhances *S. mutans* attachment and biofilm formation were consistent on hydroxyapatite and glass surfaces, indicating that the effect is not surface specific. These findings set the groundwork for our investigation of the role of MUC5B in *S. mutans* attachment and biofilm formation. When testing the effect of MUC5B on *S. mutans* physiology, 1% sucrose was added to BHI medium to challenge the effect of MUC5B by ensuring that *S. mutans* attachment and biofilm formation are most robust.

MUC5B decreases *S. mutans* attachment and biofilm formation.

The effect of MUC5B on *S. mutans* attachment and biofilm formation was evaluated using CFU counts on various surfaces and fluorescence microscopy. *S. mutans* (10^7 bacteria) was grown in a chambered glass slide or on a hydroxyapatite disc in the presence of BHI with 1% sucrose and 0.3% MUC5B. BHI with 1% sucrose and 0.3% methylcellulose and BHI with no added polymer served as controls. Methylcellulose is a gel-forming compound that, like mucins, imparts viscosity but does not contain the complex, glycosylated structure that is characteristic of MUC5B. On glass, the addition of MUC5B to growth medium decreased *S. mutans* attachment by 88% and biofilm formation by 74% compared to the levels of attachment and biofilm formation in BHI with 1% sucrose (Figure 2.3A and B). In comparison, the addition of methylcellulose reduced *S. mutans* attachment and biofilm formation on glass by 50% and 16%, respectively (Figure 2.3A and B). When *S. mutans* was grown on hydroxyapatite discs in the

presence of MUC5B, attachment was decreased by 77% and biofilm formation was decreased by 95% compared to the levels in BHI with 1% sucrose (Figure 2.3C and D). In comparison, the presence of methylcellulose reduced *S. mutans* attachment by 27% and biofilm formation by 76% on hydroxyapatite discs (Figure 2.3C and D). There was an overall decrease in biofilm formation at 18 and 24 h due to the dissolution of the hydroxyapatite discs. By evaluating the attachment and biofilm formation of *S. mutans* in medium containing methylcellulose, which simulates an environment that has physical properties similar to those of mucins, we can better understand if MUC5B is acting as a physical barrier to attachment most likely through increased viscosity or if the observed effect is due to specific MUC5B properties. We can conclude that the latter is most likely because *S. mutans* attachment and biofilm formation in the presence of MUC5B are significantly decreased at most time points compared to the levels in the presence of methylcellulose. There are at least three potential mechanisms by which MUC5B could protect the surface from bacterial colonization: (i) MUC5B could bind or agglutinate bacteria, which would allow planktonic bacteria to be swept out of the oral cavity with salivary flow but enhance bacterial attachment to surfaces coated with MUC5B (17–26), (ii) MUC5B could have the opposite effect, where its heterogeneous glycan chains repel bacteria, thereby preventing surface attachment (4, 27–30), or (iii) MUC5B could directly downregulate *S. mutans* genes involved in attachment and biofilm formation. In our case, it appears that MUC5B is repelling *S. mutans* and/or directly influencing genetic modifications that protect the glass and hydroxyapatite surfaces from bacterial attachment and biofilm formation. The decrease in attachment caused by methylcellulose indicates that increased viscosity may also be playing some role in reducing attachment at early time points. Fluorescence microscopy experiments using SYTO9 staining confirm the findings obtained by CFU counts by showing a visually detectable decrease in the

amount of *S. mutans* on glass and that the characteristic microcolony morphology of *S. mutans* biofilms is unchanged (Figure 2.3E and F) (31, 32).

Figure 2.3

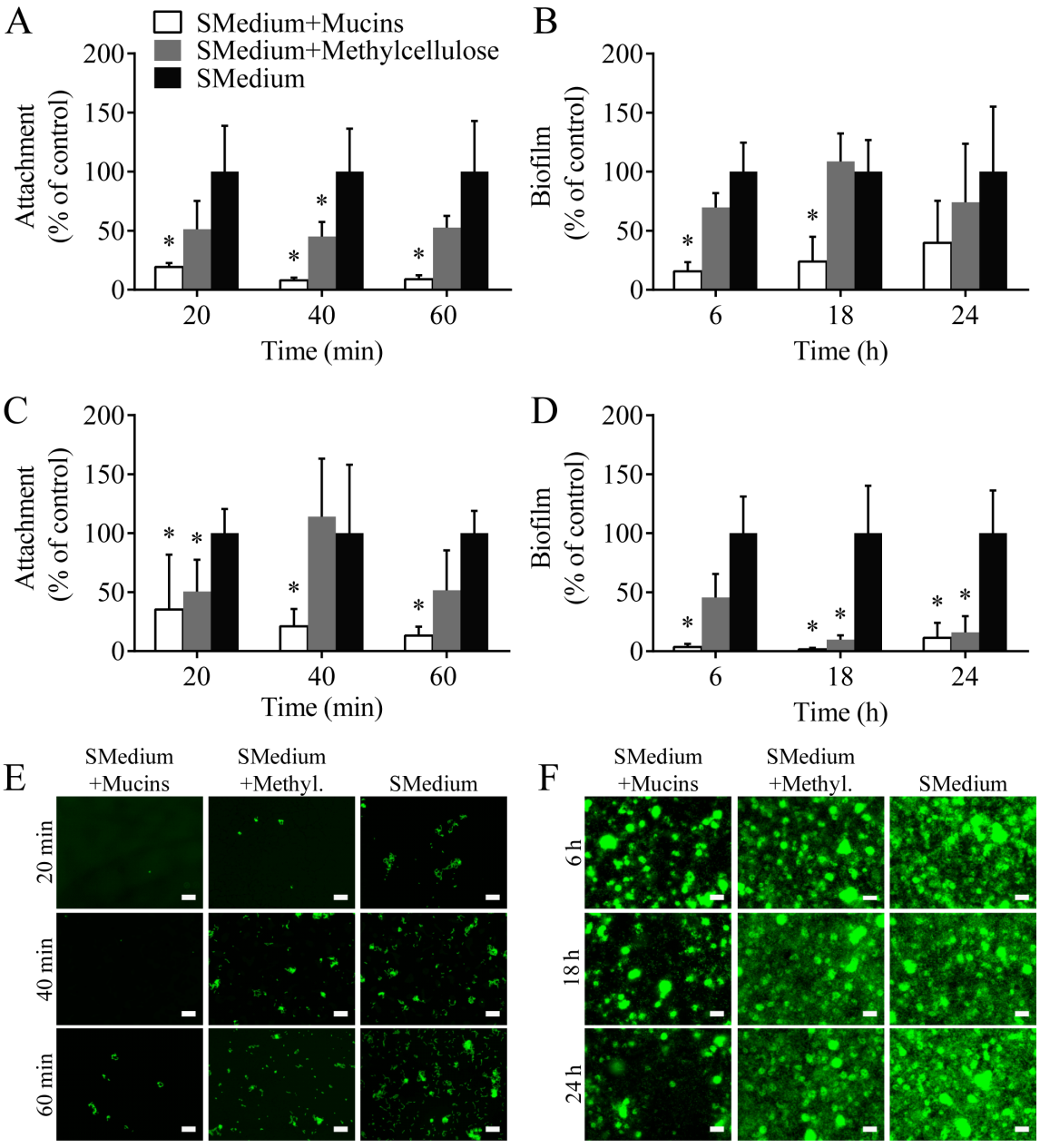

Figure 2.3 Salivary mucins reduce *S. mutans* attachment and biofilm formation. The addition of 0.3% mucins to the control medium, BHI containing 1% sucrose (SMedium), significantly reduces the levels of *S. mutans* attachment and biofilm formation on glass (A, B)

Figure 2.3 (Continued) and hydroxyapatite (C, D) compared to the levels obtained with the control consisting of BHI with 1% sucrose. Similarly, the addition of 0.3% methylcellulose to BHI with 1% sucrose reduces *S. mutans* attachment and biofilm formation; however, the effect is not significant for the majority of time points studied. Fluorescence microscopy was used to visually assess *S. mutans* attachment (E) and biofilm formation (F) on glass when the bacteria are grown in BHI with 1% sucrose and 0.3% mucins, BHI with 1% sucrose and 0.3% methylcellulose (Methyl.), and BHI with 1% sucrose. *, statistically significant difference from BHI with 1% sucrose determined by Student's *t* test ($P<0.02$). Error bars represent SDs. Scale bars, 20 μm.

MUC5B does not alter *S. mutans* growth.

To evaluate the effect of MUC5B on bacterial growth, *S. mutans* was grown in BHI medium containing 1% sucrose and 0.3% MUC5B. BHI with 1% sucrose and 0.3% methylcellulose and BHI with 1% sucrose were used as controls. These are the same media used in experiments that determine the effects of MUC5B on *S. mutans* attachment and biofilm formation. Optical density readings over the course of 12 h show that the addition of MUC5B or methylcellulose does not alter *S. mutans* growth compared to its growth in BHI containing 1% sucrose (Figure 2.4). Because the growth of *S. mutans* is unchanged by MUC5B, we can conclude that the observed decrease in *S. mutans* attachment and biofilm formation in the presence of MUC5B is not due to slower growth but, rather, is due to the intrinsic properties of the MUC5B glycoprotein.

Figure 2.4

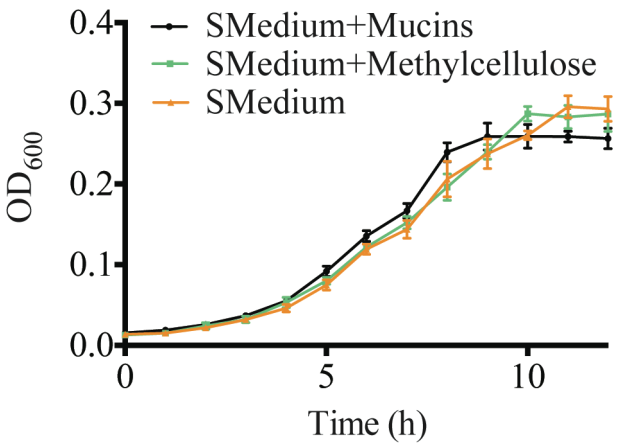

Figure 2.4 *S. mutans* growth is unaffected by the presence of salivary mucins. A growth curve of *S. mutans* in BHI with 1% sucrose (SMedium), BHI with 1% sucrose and 0.3% mucins, or BHI with 1% sucrose and 0.3% methylcellulose indicates that the presence of mucins and methylcellulose does not alter the growth of *S. mutans*. Error bars represent SDs.

MUC5B keeps *S. mutans* in planktonic form.

By quantifying the biofilm and supernatant bacteria in *S. mutans* cultures grown from 6 to 24 h, we determined the effect of MUC5B on *S. mutans* over time after stationary phase is reached. When the numbers of planktonic and biofilm *S. mutans* CFU are combined to determine the total number of viable *S. mutans* cells in a given experiment, results show that there is no significant difference between the total numbers of live bacteria in cultures containing 0.3% MUC5B and in control media (Figure 2.5). Based on these findings, we show that the reduction in *S. mutans* attachment and biofilm formation on glass and hydroxyapatite in the presence of MUC5B (Figure 2.3) is not due to bactericidal properties of MUC5B. The presence of MUC5B reduces *S. mutans* attachment and biofilm formation by maintaining bacteria in the planktonic phase. These findings point to the importance of MUC5B in establishing a healthy oral microbiota that allows species diversity but, at the same time, protects teeth from bacterial

damage.

Figure 2.5

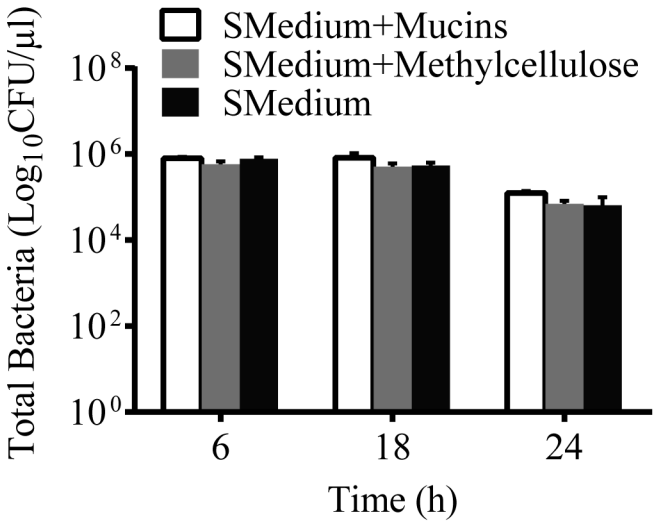

Figure 2.5 *S. mutans* survival is unaffected by salivary mucins. The graph represents the total number of viable *S. mutans* cells per well in the supernatant and biofilm in BHI with 1% sucrose (SMedium), BHI with 1% sucrose and 0.3% mucins, or BHI with 1% sucrose and 0.3% methylcellulose. Salivary mucins and methylcellulose show no bactericidal effects at time points up to 24 h. Error bars represent SDs.

Conclusion

In summary, we used CFU counts and fluorescence light microscopy to quantitatively show that *S. mutans* attachment and biofilm formation are most robust when the organism is grown in the presence of sucrose and that the addition of purified human salivary MUC5B significantly decreases *S. mutans* attachment and biofilm formation even in the presence of sucrose. We determined that MUC5B does not alter *S. mutans* growth or lead to bacterial killing over 24 h but limits biofilm formation by maintaining *S. mutans* primarily in the planktonic form (Figure 2.6). We speculate that the observed decrease in bacterial attachment and biofilm formation is due to a combination of genetic changes that decrease bacterial surface colonization and repulsion by MUC5B's heterogeneous glycans. *S. mutans* attachment and biofilm formation

are key steps in the development of dental caries; therefore, these findings have particularly important clinical implications. The presence or absence of MUC5B in the oral cavity could alter individuals' susceptibility to dental cavity formation, which could then be an easily accessible, highly predictable clinical diagnostic marker of disease. From a therapeutic standpoint, exogenous MUC5B could potentially be utilized as a treatment or preventative measure for dental caries. These findings illustrate that MUC5B may help protect teeth from cavity formation, but further studies, such as those that use RNA sequencing or other genetic profiling techniques, are needed to fully characterize the mechanism underlying the observed decrease in *S. mutans* attachment and biofilm formation.

Figure 2.6

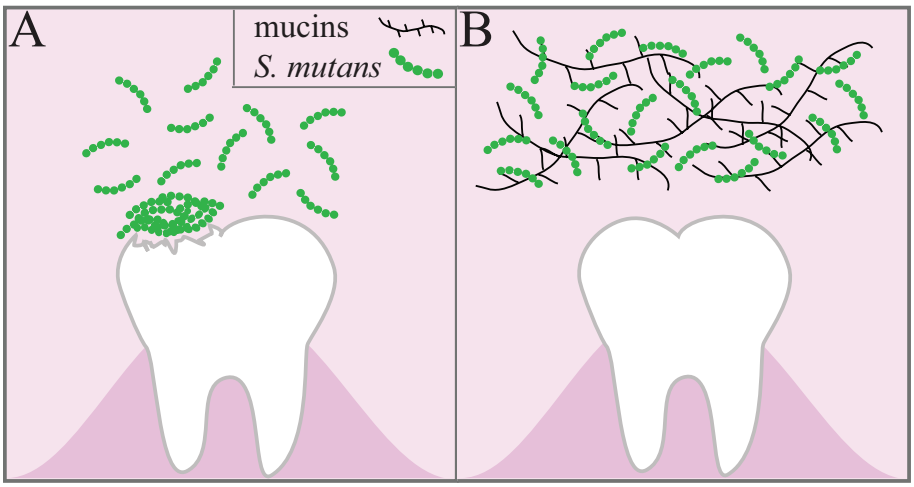

Figure 2.6 Summary of conclusions. *S. mutans* utilizes sucrose to form sticky extracellular polysaccharides that facilitate attachment to the tooth surface and subsequent biofilm formation. (A) In the biofilm, bacterial metabolism of sucrose causes a decrease in the local pH, leading to demineralization of the tooth structure. (B) The presence of mucins in sucrose-supplemented growth medium decreases *S. mutans* attachment and biofilm formation on the tooth surface by maintaining *S. mutans* in the planktonic state. Mucin illustrations represent multimers. Figure not drawn to scale.

Materials and Methods

Bacterial strains and growth conditions. The bacterial strain *Streptococcus mutans* UA159 was kindly given as a gift by Dan Smith (Forsyth Institute). For sucrose and glucose experiments, *S. mutans* was grown overnight in brain heart infusion (BHI) medium (Becton, Dickinson and Company) containing 1% (wt/vol) sucrose and BHI with 1% (wt/vol) glucose (Sigma). For experiments determining the effects of MUC5B, *S. mutans* was grown overnight in BHI with 1% sucrose. BHI with 1% sucrose and either 0.3% MUC5B or 0.3% (wt/vol) methylcellulose (Sigma) was used to resuspend *S. mutans* cells before inoculating them into the experiment. Hydroxyapatite disc (Clarkson Chromatography, Inc.) or chambered glass slide (LabTek) surfaces were used to test *S. mutans* attachment and biofilm formation. *S. mutans* was grown and incubated at 37°C with 5% CO2.

Saliva collection. Submandibular saliva was collected from 10 volunteers using a custom vacuum pump setup. Specifically, two holes were cut into the cap of a 50-ml conical tube (Falcon); the vacuum line was inserted into one hole and a small-diameter Tygon collection tube was inserted into the other hole (Saint Gobain Performance Plastics). Cotton swabs were used to absorb the volunteers' parotid gland secretions. The collection tube was used to suck up pooled unstimulated submandibular gland secretions from under the tongue. The collection vessel was kept on ice at all times. Saliva from volunteers was pooled before MUC5B purification. Protocols involving the use of human subjects were approved by Massachusetts Institute of Technology's Committee on the Use of Humans as Experimental Subjects.

MUC5B purification. Immediately after collection, saliva was diluted using 5.5 M sodium chloride containing 0.04% sodium azide so that the final concentration of sodium chloride was 0.16 M. The following antibacterial agents and protease inhibitors were then added at the

indicated final concentrations: benzamidine HCl (5 mM), dibromoacetophenone (1 mM), phenylmethylsulfonyl fluoride (1 mM), and EDTA (5 mM, pH 7) (Sigma). The mucins in the saliva were solubilized overnight by gentle stirring at 4°C. Saliva was then centrifuged at 3,800 *g* for 10 min in a swinging-bucket centrifuge to remove cellular debris. MUC5B was purified using a Bio-Rad NGC fast protein liquid chromatography (FPLC) system equipped with an XK 50 column packed with Sepharose CL-2B resin (GE Healthcare Bio-Sciences). Mucin-containing fractions were identified using a periodic acid-Schiff's reagent assay and analysis of UV absorbance at 280 nm from FPLC. Fractions were then combined, dialyzed, and concentrated using an ultrafiltration device and were then lyophilized for storage at 80°C.

Assay of CFU counts to evaluate *S. mutans* attachment and biofilm formation. To test the effects of sucrose or glucose on *S. mutans* physiology, *S. mutans* was grown to mid-exponential phase in BHI with 1% sucrose and BHI with 1% glucose, and then equal numbers of bacteria (10^7) from each culture were seeded in triplicate into wells containing glass or hydroxyapatite surfaces. For experiments testing the effect of MUC5B, *S. mutans* was grown to mid-exponential phase in BHI with 1% sucrose and then seeded in triplicate into wells containing BHI with 1% sucrose and 0.3% MUC5B or control medium. For all experiments, attachment was evaluated at 20, 40, and 60 min and biofilm formation was evaluated at 6, 18, and 24 h. Attachment was defined to occur at time points up to 1 h because the doubling time of *S. mutans* is approximately 1.5 h. Biofilm formation was defined to occur at all time points after 1 h. At the time point being evaluated, the surface was washed with phosphate buffered saline (PBS) to remove nonadherent cells, fresh PBS was added, and then adherent cells were lifted using a sterile pipette tip. The suspended bacteria were vigorously pipetted to individualize the cells. The suspension was diluted (10^{-1} to 10^{-7}) and plated on BHI agar. The numbers of CFU were counted after 24 to 36 h

of incubation. Statistically significant differences were determined using Student's *t* test, with *P* values of <0.02 considered significant.

Fluorescent staining and microscopy. To visually assess the effects of sucrose, glucose, or MUC5B on *S. mutans* attachment and biofilm formation, fluorescent SYTO9 staining with light microscopy was used (Life Technologies). *S. mutans* was grown to mid-exponential phase, then seeded into a chambered glass slide with BHI containing 1% sucrose and 0.3% MUC5B or with control medium. At the time point being evaluated, the surface was washed with PBS to remove nonadherent cells, and then 200 µl SYTO9 (0.6 µl SYTO9/200 µl Milli-Q water) was added. The biofilm was incubated with SYTO9 in the dark for 30 min. After incubation, the biofilm was washed with Milli-Q water to remove excess dye and fresh Milli-Q water was added. A Zeiss Axio Observer Z1 fluorescence inverted microscope was used for imaging. All experiments were repeated in triplicate.

Assay to evaluate *S. mutans* growth. Overnight cultures of *S. mutans* at an optical density at 600 nm (OD_{600}) of 0.05 were seeded in triplicate into a 96-well polystyrene plate containing BHI medium supplemented with 1% sucrose and 0.3% MUC5B or control medium. Bacteria were incubated at 37°C with 5% CO2. At 1-h intervals, the cultures were mixed and the OD_{600} was recorded using a microplate reader. The averages for each time point were plotted, and a comparison of *S. mutans*' growth rates in the various media was evaluated within the estimated error.

Time-kill assay. CFU counts were used to evaluate the effect of MUC5B on *S. mutans* viability at time points up to 24 h. *S. mutans* was grown to mid-exponential phase in BHI with 1% sucrose, and then equal numbers of bacteria (10^7) were seeded in triplicate into glass-bottom wells

containing BHI with 1% sucrose and 0.3% MUC5B or control medium. The cultures were incubated for 6, 18, and 24 h. At the time point being evaluated, the contents of the wells were gently mixed and then the supernatant was removed and diluted (10^{-1} to 10^{-8}). The remaining biofilm was then washed with PBS, scraped off with a sterile pipette tip, and diluted (10^{-1} to 10^{-6}). Dilutions were plated on BHI agar. The numbers of CFU were counted after 24 to 36 h of incubation to quantify the number of viable bacteria.

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Chapter III

Salivary Mucins Promote the Coexistence of Competing Oral Bacterial Species

Results presented in this chapter are in press:

Frenkel ES, Ribbeck K. Salivary mucins promote the coexistence of competing oral bacterial species. ISME J.

Abstract

Mucus forms a major ecological niche for microbiota in various locations throughout the human body, such as the gastrointestinal tract, respiratory tract and oral cavity. The primary structural components of mucus are mucin glycoproteins, which crosslink to form a complex polymer network that surrounds microbes. Although the mucin matrix could create constraints that impact inhabiting microbes, little is understood about how this key environmental factor affects interspecies interactions. In this study, we develop an experimental model using gel-forming human salivary mucins to understand the influence of mucin on the viability of two competing species of oral bacteria. We use this dual-species model to show that mucins promote the coexistence of the two competing bacteria and that mucins shift cells from the mixed-species biofilm into the planktonic form. Taken together, these findings indicate that the mucus environment could influence bacterial viability by promoting a less competitive mode of growth.

Introduction

Mucus lines wet epithelia throughout the human body and is a major ecological niche for microbiota in the respiratory tract, gastrointestinal tract and oral cavity among other locations (1–3). The mucus layer is a three dimensional hydrogel primarily composed of densely glycosylated polymers called mucins (4, 5). In the gel, mucin chains cross-link to form a network that surrounds microbes and, consequently, could create geometric and diffusive constraints for biotic and abiotic environmental factors. Little is known, however, about how these constraints influence microbial interactions, such as cell-cell communication and competition, among the vast number of organisms that live in mucus. In this study, we build upon our previous work

showing that MUC5B mucins affect intraspecies interactions by promoting dispersal of bacteria and fungi (6–8). Here, we develop an experimental model to probe the influence of gel-forming human salivary mucins on dual-species bacterial competition to understand how this matrix affects the viability of competing bacteria.

Results and Discussion

The dual-species model is composed of *Streptococcus sanguinis* JFP36 and *S. mutans* UA159, which compete in the oral cavity through the production of hydrogen peroxide and antimicrobial peptides called mutacins, respectively (9–12). In this model, the two species were inoculated sequentially to more closely mimic the natural environment of the oral cavity where surfaces are generally coated by microbes before other species attempt to colonize. When *S. mutans* was the primary colonizer, and MUC5B mucins were not present in the growth medium, viability of the secondary colonizer (*S. sanguinis*) rapidly declined, suggesting that *S. mutans* outcompetes *S. sanguinis* (Fig 3.1 A(I), (II)). In contrast, when MUC5B was present in the growth medium, the total number of viable *S. sanguinis* cells increased by 18- and 88-fold after 4 and 5 h of co-culture, respectively, compared to the control without mucin (Figure 3.1 A(II)). The same protective effect by MUC5B was observed when *S. sanguinis* was the primary colonizer. In this case, the addition of MUC5B to medium enhanced survival of both *S. sanguinis* and *S. mutans* (Figure 3.1 B(I), (II)). After 4 and 5 h of co-culture in the presence of MUC5B, the number of viable *S. sanguinis* cells increased by 9- and 94-fold, respectively, relative to the control (Figure 3.1 B(I)). *S. mutans* CFU increased by 2-, 3-, and 7-fold at 4, 5 and 6 h, respectively (Figure 3.1 B(II)).

Figure 3.1

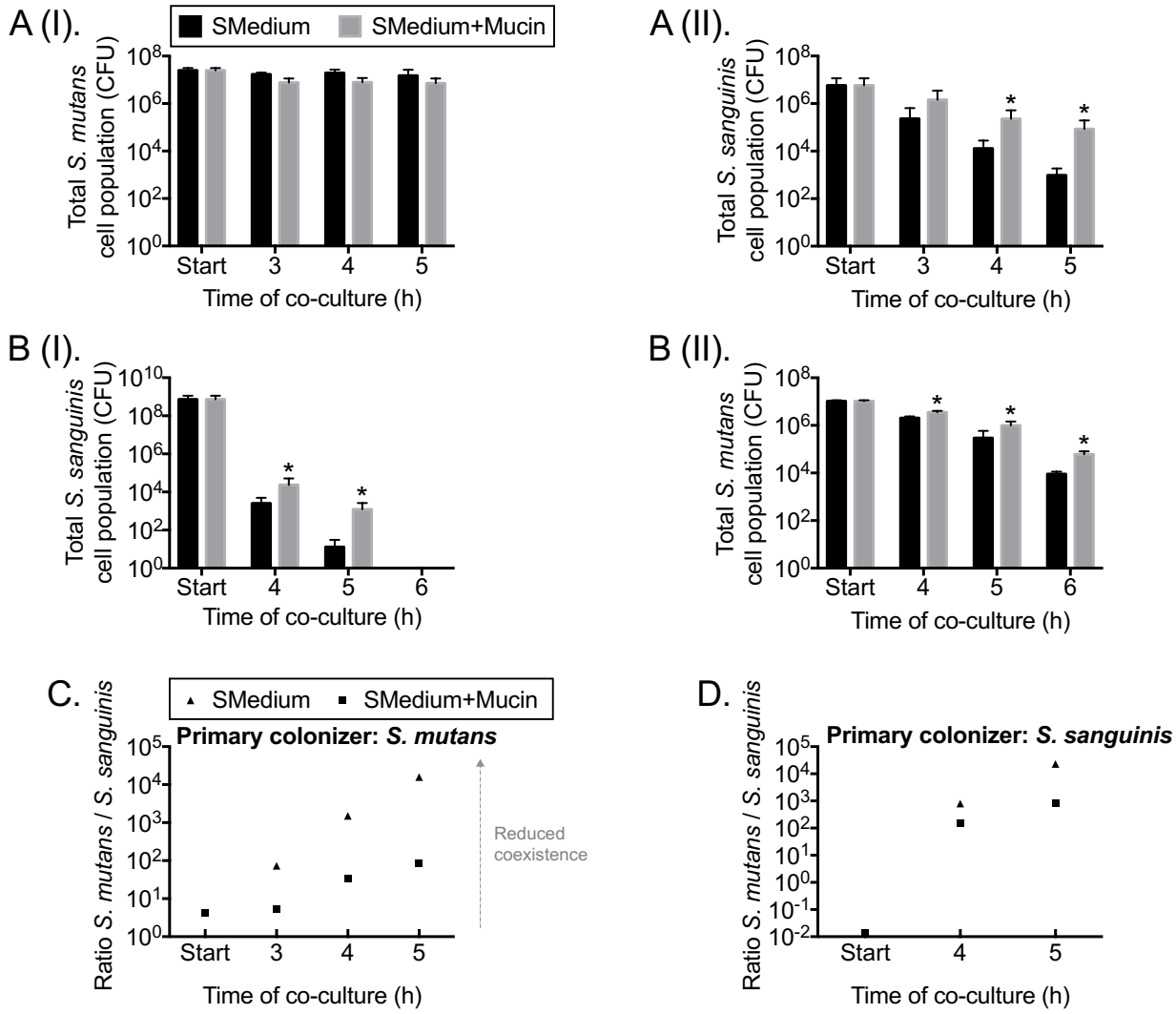

Figure 3.1 MUC5B promotes *S. mutans* and *S. sanguinis* coexistence. *S. mutans* and *S. sanguinis* viability in a dual-species experimental model containing control medium (half-strength BHI with 1% sucrose; SMedium) and control medium containing 0.4% MUC5B mucin. Viability was studied when *S. mutans* was the primary colonizer (A (I)) and *S. sanguinis* was the secondary colonizer (A (II)) and the reverse scenario where *S. sanguinis* was the primary colonizer (B (I)) and *S. mutans* was the secondary colonizer (B (II)). (C, D) The ratios of viable *S. mutans* and *S. sanguinis* when *S. mutans* was the primary colonizer (C) and when *S. sanguinis* was the primary colonizer (D) as an indication of species coexistence. *, statistically significant increase relative to the control with half-strength BHI containing 1% sucrose determined by Student's t test ($P<0.05$). Experiments were performed in triplicate and error bars represent SD of CFU between replicates.

Of note is that although MUC5B significantly enhanced *S. sanguinis* viability, there was an overall reduction in *S. sanguinis* CFU due to self-killing, which could be caused by increasing hydrogen peroxide concentrations. The same reduction in viability was observed when *S. sanguinis* was grown in monoculture, indicating that the killing was not due to *S. mutans* (Figure 3.2).

Figure 3.2

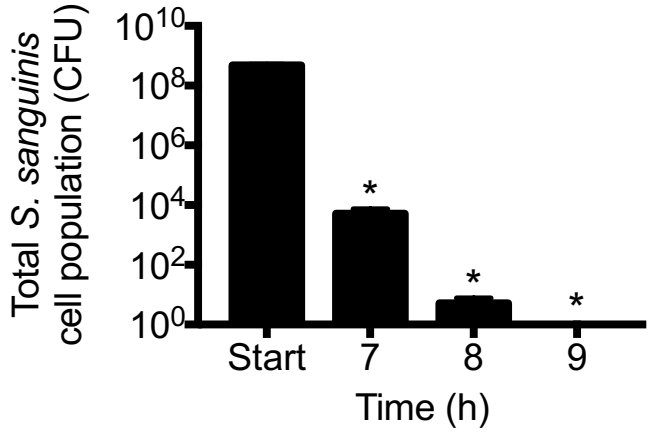

Figure 3.2 At high cell density, *S. sanguinis* viability decreases over time due to self-killing. When *S. sanguinis* is grown at high cell densities in a mono-species culture, the number of viable cells decreases over 9 hours. *, statistically significant decrease relative to the starting cell population determined by Student's t test ($P<0.05$). Experiments were performed in triplicate and error bars represent SD of CFU between replicates.

Methylcellulose, a gel-forming polymer that is commonly used to mimic the viscosity of mucus, did not have the same protective effect on *S. mutans* or *S. sanguinis* in both of these experimental models; the enhanced survival is likely not due to the addition of a polymer, which could increase viscosity or cause osmotic stress (Figure 3.3) (13, 14). Together these results show that MUC5B significantly enhances bacterial coexistence by increasing survival of at least one bacterial population compared to the control without MUC5B (Figure 3.1 C, D).

Figure 3.3

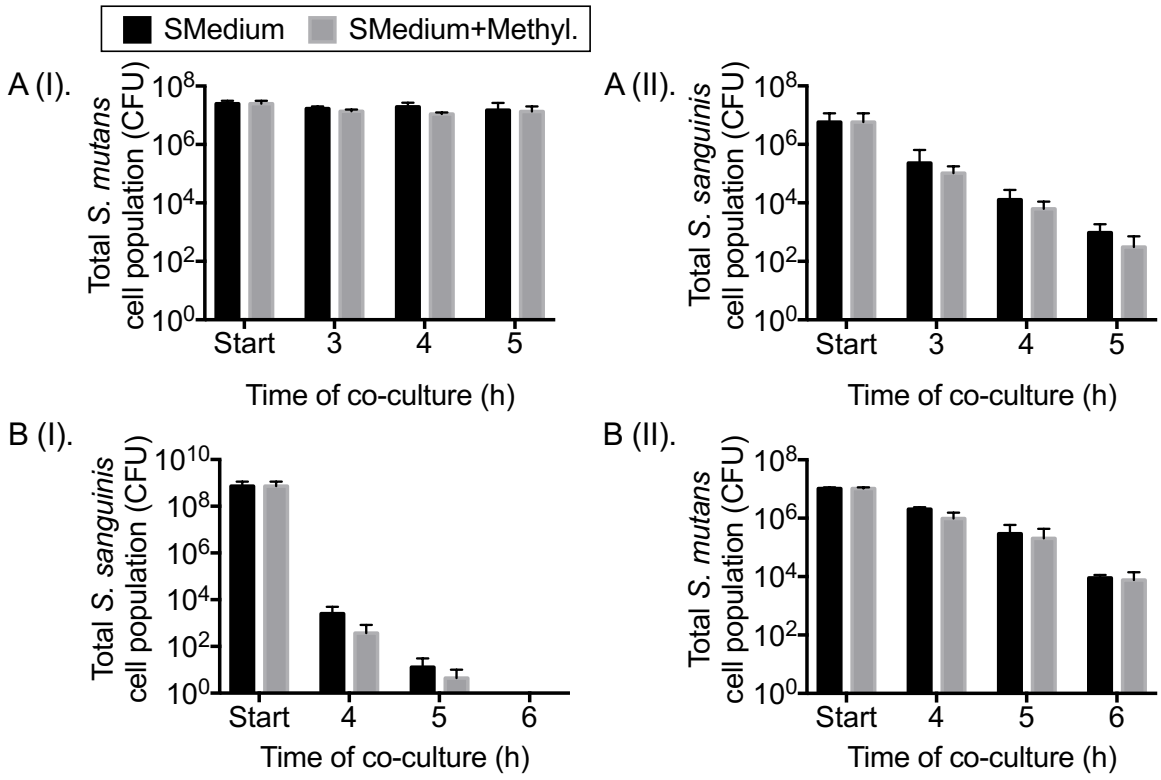

Figure 3.3. Methylcellulose does not affect *S. mutans* and *S. sanguinis* coexistence. *S. mutans* and *S. sanguinis* viability in a dual-species experimental model containing control medium (half-strength BHI with 1% sucrose; SMedium) and control medium containing 0.4% methylcellulose (Methyl.). Viability was studied when *S. mutans* was the primary colonizer (A (I)) and *S. sanguinis* was the secondary colonizer (A (II)) and the reverse scenario where *S. sanguinis* was the primary colonizer (B (I)) and *S. mutans* was the secondary colonizer (B (II)). Experiments were performed in triplicate and error bars represent SD of CFU between replicates.

To better understand how MUC5B influences bacterial viability, we studied several aspects of *S. mutans* and *S. sanguinis* growth in the presence of MUC5B. First, we determined that the observed protective effect of MUC5B was not due to increased bacterial growth; MUC5B slightly reduces or has no effect on *S. mutans* and *S. sanguinis* growth rates (Figure 3.4 A, B). In addition, *S. mutans* and *S. sanguinis* did not grow in PBS containing mucin indicating that MUC5B is not used as a nutrient source under the conditions studied (Figure 3.4 A, B).

Another way MUC5B could increase bacterial survival is by altering the cells' mode of growth, which can influence interspecies competition and cell properties such as gene regulation and cell-cell communication (15–20). Our data show that MUC5B efficiently reduces *S. mutans* and *S. sanguinis* biofilm formation in single-species cultures at 6 h and 9 h: *S. mutans* biofilm formation was reduced by 17- and 12-fold and *S. sanguinis* biofilm formation decreased 16- and 8-fold compared to the control without mucin (Figure 3.4 C(I) and (II), D (I) and (II)). Strikingly, the total *S. mutans* and *S. sanguinis* cell populations were unchanged at all time points in the presence of MUC5B (except for a slight decrease at 6 h, but the number of cells in the biofilm at this time still account for only 13% of total cells) (Figure 3.4 C(I) and (II), D (I) and (II)). This result implies that, in the presence of MUC5B, the vast majority of cells shifted into the planktonic state. Because the single cell bacterial form can be less competitive than the surface-attached state, this movement of cells away from the biofilm could be a mechanism to reduce interspecies competition. After 4 h of co-culture in the dual-species model, we found that MUC5B also reduced biofilm formation of the primary colonizer in the mixed biofilm by 19-fold for *S. mutans* and 3-fold for *S. sanguinis* relative to the control without polymer (Figure 3.4 E, F). As shown in Figure 3.1 A(I) and B(I), the total cell population in each of these cases was unaffected (*S. mutans*) or increased (*S. sanguinis*) by MUC5B, indicating a decrease in the relative proportion of biofilm cells. In the case of the secondary colonizer, there was also an overall reduction in the proportion of biofilm cells for both *S. mutans* and *S. sanguinis*; the total number of viable cells increased in the presence of MUC5B (Figure 3.1 A(II), B(II)), yet there was only a slight increase or no change in biofilm formation in the presence of MUC5B relative to the control without polymer (Figure 3.4 E, F). Taken together, these results indicate that MUC5B could enhance bacterial coexistence and possibly bacterial diversity in the oral cavity

by shifting competing species away from the biofilm and into the less competitive planktonic state, although further studies would be needed to establish this effect.

Figure 3.4

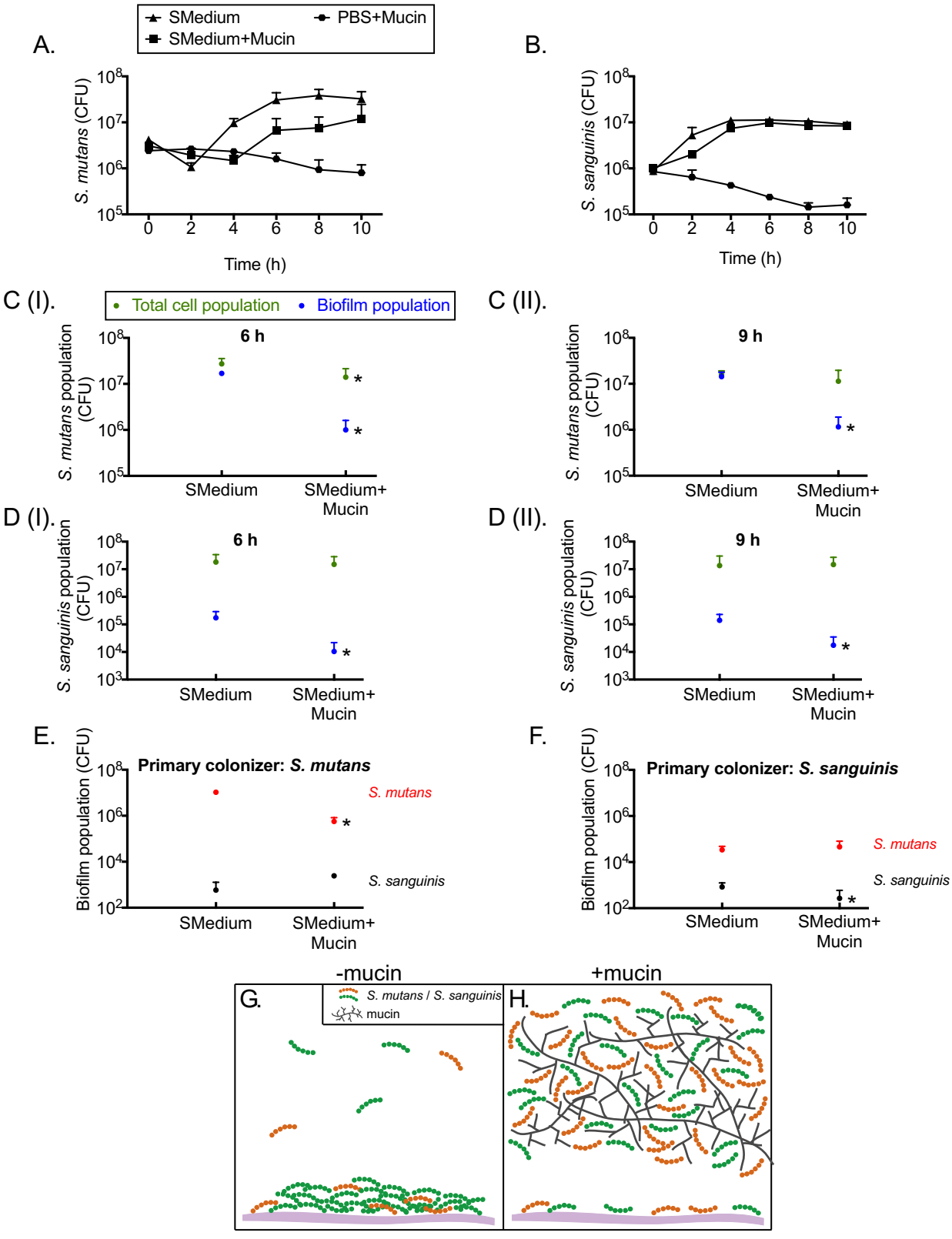
A.
SMedium
SMedium+Mucin
PBS+Mucin
S. mutans (CFU)
Time (h)
B.
S. sanguinis (CFU)
Time (h)
C (I).
Total cell population
Biofilm population
6 h
S. mutans population (CFU)
SMedium
SMedium+ Mucin
C (II).
9 h
D (I).
6 h
S. sanguinis population (CFU)
D (II).
9 h
E.
Primary colonizer: S. mutans
S. mutans
S. sanguinis
Biofilm population (CFU)
F.
Primary colonizer: S. sanguinis
-mucin
+mucin
G.
S. mutans / S. sanguinis
mucin
H.

Figure 3.4 (Continued) MUC5B reduces *S. mutans* and *S. sanguinis* surface attachment by shifting cells into the planktonic state. *S. mutans* (A) and *S. sanguinis* (B) growth in control medium (half-strength BHI with 1% sucrose; SMedium), control medium containing 0.4% MUC5B mucin, and PBS containing 0.4% MUC5B mucin. (C (I), (II)) *S. mutans* biofilm population and total cell population in mono-species cultures containing control medium and control medium with 0.4% MUC5B mucin at 6 h (C (I)) and 9 h (C (II)). (D (I), (II)) *S. sanguinis* biofilm population and total cell population in mono-species cultures containing control medium and control medium with 0.4% MUC5B mucin at 6 h (D (I)) and 9 h (D (II)). (E, F) *S. mutans* and *S. sanguinis* biofilm formation in control medium and control medium containing 0.4 % MUC5B mucin in a dual-species model after 4 h of co-culture when *S. mutans* was the primary colonizer (E) and when *S. sanguinis* was the primary colonizer (F). (G, H) Schematic illustrating a summary of conclusions. In the absence of MUC5B (G), biofilm formation of *S. mutans* and *S. sanguinis* increases and bacterial coexistence decreases compared to biofilm formation and species coexistence in the presence of mucin (H). Mucin illustrations represent multimers. Schematic not drawn to scale. *, statistically significant decrease relative to the control with half-strength BHI containing 1% sucrose determined by Student's t test ($P<0.05$). Experiments were performed in triplicate and error bars represent SD of CFU between replicates.

Conclusion

In this work, we use a dual-species bacterial model containing human MUC5B salivary mucin to understand how this prevalent environmental factor influences bacterial viability. Our results show that: 1) MUC5B promotes *S. mutans* and *S. sanguinis* coexistence, and 2) MUC5B shifts cells from the biofilm into the planktonic state (Figure 3.4 G, H). By promoting the single cell (planktonic) state, MUC5B could alter cell-cell interactions, toxin production, or other mechanisms of competition. Although this model is not as complex as the oral cavity microbiota, these findings are among the first to indicate that mucus and its primary structural component, mucins, could influence bacterial survival in a multispecies environment. Further studies are needed, however, to understand if the observed increase in bacterial survival and reduction in surface colonization in the presence of mucin are due to an indirect influence of mucin, such as altered transport of secreted factors, or a direct impact on bacterial physiology, which could change gene regulation.

Material and Methods

Bacterial strains and growth conditions. *Streptococcus mutans* UA159 was kindly given as a gift by Dr. Dan Smith (Forsyth Institute) and erythromycin-resistant *Streptococcus sanguinis* JFP36 was generously given by Dr. Todd Kitten (Virginia Commonwealth University) (9, 12). For all experiments, *S. mutans* and *S. sanguinis* were grown overnight in Brain Heart Infusion (BHI) medium (Becton, Dickinson and Company). Before inoculating an experiment, the cells were pelleted, washed with half-strength BHI containing 1% (wt/vol) sucrose (Sigma), then resuspended in half-strength BHI containing 1% sucrose. Acid-washed glass beads (425-600 μm, Sigma) were added to the resuspended bacteria to break up bacterial aggregates then the culture was vortexed for ten pulses of two seconds each. To determine the effect of methylcellulose and MUC5B, bacteria were resuspended in BHI with 1% sucrose and either 0.4% (wt/vol) MUC5B or 0.4% methylcellulose (Sigma) before inoculating them into an experiment. All experiments were performed in chambered glass slides (LabTek) at 37°C with 5% CO_2.

Saliva collection. Submandibular saliva was collected from 9 volunteers using a custom vacuum pump as previously described (7). Briefly, a vacuum line and a collection tube (Saint Gobain Performance Plastics) were inserted into a conical tube (Falcon). Unstimulated submandibular gland secretions were collected from under the tongue and the collection vessel was kept on ice at all times. Saliva from individual volunteers was combined prior to purification. Protocols involving the use of human subjects were approved by Massachusetts Institute of Technology's Committee on the Use of Humans as Experimental Subjects.

MUC5B purification. MUC5B was purified as previously described (7). Briefly, saliva was diluted using sodium chloride containing sodium azide then antibacterial agents and protease

inhibitors were added. The saliva was stirred overnight at 4°C then centrifuged at 10,000 g for 10 min in a fixed-angle centrifuge to remove cellular debris. A Bio-Rad NGC fast protein liquid chromatography (FPLC) system with a Sepharose CL-2B column (GE Healthcare Bio-Sciences) were used to purify MUC5B. Mucin-containing fractions were then dialyzed and concentrated using an ultrafiltration device. Samples were lyophilized overnight for storage at -80°C. An enzyme-linked immunosorbent assay (ELISA) confirmed that the amount of MUC7 in the purified MUC5B is negligible; in a solution of 0.4% (w/v) MUC5B, MUC7 accounted for 0.0002% of the mucin present (LifeSpan BioSciences, Inc.).

CFU count assay to quantify total cell populations in dual-species models. *S. mutans* and *S. sanguinis* were suspended in half-strength BHI with 1% sucrose, half-strength BHI with 1% sucrose and 0.4% methylcellulose, or half-strength BHI with 1% sucrose and 0.4% MUC5B. For dual-species models where *S. mutans* was the primary colonizer, *S. mutans* (10^7) was inoculated and incubated for 3 hours then *S. sanguinis* (10^6) was inoculated. For dual-species models where *S. sanguinis* was the primary colonizer, *S. sanguinis* (10^8) was inoculated, incubated for 3 hours, then *S. mutans* (10^7) was inoculated. After the secondary colonizer was inoculated, the dual-species culture was incubated until the time points indicated. At the end of the experiment a sterile pipette tip was used to scrape the biofilm, then the suspension of biofilm and supernatant cells was vortexed with glass beads. Cell suspensions were diluted then plated on BHI agar containing 10 μg/ml erythromycin to select for *S. sanguinis* and BHI agar with 1 U/ml bacitracin to select for *S. mutans*. The addition of antibiotics to agar was determined to have no significant bactericidal effect. Agar was incubated for 24-36 hours. The effect of methylcellulose and MUC5B were determined relative to the control without added MUC5B or methylcellulose.

Statistically significant differences were determined using the Student's t test, with $P<0.05$ considered significant.

***S. mutans* and *S. sanguinis* growth curve.** Overnight cultures of *S. mutans* and *S. sanguinis* were resuspended in half-strength BHI with 1% sucrose, half-strength BHI with 1% sucrose and 0.4% MUC5B, or PBS with 0.4% MUC5B. The suspensions were vortexed with glass beads to individualize cells, then approximately 10^6 cells were inoculated into wells of a 96-well polystyrene plate. At 2-hour intervals the bottom of the wells were scraped to remove any adherent cells, mixed, and an aliquot was removed, diluted and plated on BHI agar. Agar was incubated and CFU were counted after 24-36 hours.

CFU count assay to quantify biofilm and total cell populations in single-species models. *S. mutans* and *S. sanguinis* were suspended in half-strength BHI with 1% sucrose or half-strength BHI with 1% sucrose and 0.4% MUC5B. Equal numbers of CFU from each species were inoculated into chambered glass slides then incubated. After a given amount of time, the supernatant was used to gently wash the biofilm to resuspend unattached cells, then the supernatant was removed. PBS was added to the wells then the biofilm was scraped using a sterile pipette tip. The supernatant and biofilm were vortexed with glass beads to individualize cells then the suspension was diluted, plated on BHI agar and incubated for 24-36 hours. The effect of MUC5B was determined relative to the control without an added polymer. Statistically significant differences were determined using the Student's t test, with $P<0.05$ considered significant.

CFU count assay to quantify biofilm cell populations in dual-species models. *S. mutans* and *S. sanguinis* were suspended in half-strength BHI with 1% sucrose or half-strength BHI with 1%

sucrose and 0.4% MUC5B. For dual-species models where *S. mutans* was the primary colonizer, *S. mutans* (10^7) was inoculated and incubated for 3 hours then *S. sanguinis* (10^6) was inoculated. For dual-species models where *S. sanguinis* was the primary colonizer, *S. sanguinis* (10^8) was inoculated, incubated for 3 hours, then *S. mutans* (10^7) was inoculated. After the secondary colonizer was inoculated, the dual-species culture was incubated for 4 h. After 4 h of co-culture the supernatant was used to gently wash the biofilm to resuspend unattached cells, then the supernatant was removed. PBS was added to the wells then the biofilm was scraped using a sterile pipette tip. Cell suspensions were diluted then plated on BHI agar containing 10 μg/ml erythromycin to select for *S. sanguinis* and BHI agar with 1 U/ml bacitracin to select for *S. mutans*. The addition of antibiotics to agar was determined to have no significant bactericidal effect. Agar was incubated for 24-36 hours. The effect of MUC5B was determined relative to the control without MUC5B. Statistically significant differences were determined using the Student's t test, with $P<0.05$ considered significant.

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Chapter IV

Conclusions and Future Directions

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The diseases that result from dysregulated mucin production indicate that mucins play a key role in maintaining health. For example, defects in mucin production can lead to ulcerative colitis when mucins are under produced or cystic fibrosis when mucins are overproduced (1, 2). More specifically, *in vivo* studies using Muc5b knockout mice have shown that reduced production of Muc5b can lead to increased mortality due to infection (3). These examples indicate that mucins may be important for disease prevention in the oral cavity, but, prior to the work presented in this thesis, little was known about how MUC5B affects microbes to protect the oral cavity.

To understand how MUC5B could aid in the prevention of oral diseases, I study MUC5B in the context of one of the most common diseases – dental caries (4). My work focuses on two bacteria commonly found in the oral cavity, *Streptococcus mutans*, which can cause dental caries when it attaches to the tooth surface, and *S. sanguinis*, which is associated with healthy oral conditions (5, 6). Results show that MUC5B salivary mucins modulate the physiology of these bacteria in ways that reduce surface colonization and promote bacterial coexistence. The findings presented in this thesis illustrate that MUC5B likely plays a key role in protecting the oral cavity from disease and lay the foundation for future research that delves deeper into the complex relationship between mucins and the microbes that inhabit the oral cavity.

In Chapter 2, I show that **MUC5B reduces *S. mutans* attachment and biofilm formation on glass and hydroxyapatite even in the presence of sucrose. MUC5B does not alter *S. mutans* growth or have bactericidal effects, therefore the reduction in surface colonization results from a shift in biofilm cells to the planktonic state.** *S. mutans* is a biofilm-forming oral bacterium that creates dental caries when it attaches to and grows on tooth surfaces. As *S. mutans* metabolizes dietary sucrose, it produces acid as a by-product, which

decreases local pH leading to destruction of tooth surface. To better understand how MUC5B influences *S. mutans*, I determine if the reduction in surface colonization is mucin-specific or generalizable to other polymers that increase viscosity. To answer this question, I repeated surface colonization experiments with methylcellulose instead of MUC5B. Methylcellulose has been used in previous studies to mimic the viscosity of mucus, but does not have the same complex branched sugar chains as mucin (7, 8). I found that methylcellulose did not significantly reduce *S. mutans* attachment or biofilm formation, therefore viscosity is likely not causing the observed reduction in attachment and biofilm formation. Further experiments could be performed, however, using other polymers such as dextran and polyethylene glycol. If another polymer produced the same effect, this could indicate specific features of MUC5B that may be playing a role in the reduction of *S. mutans* surface colonization. Another key question is whether the observed reduction in bacterial surface colonization in the presence of MUC5B can be generalized to other mucins and microbes. The answer is most likely yes, since similar results have been shown for Muc5ac porcine gastric mucin, which reduces *Pseudomonas aeruginosa* and *Candida albicans* biofilm formation (9, 10).

To better understand how MUC5B would affect other aspects of bacterial physiology in a more complex environment, I developed a dual-species competition model with *S. mutans* and *S. sanguinis* in Chapter 3. In this model, I study bacterial viability to assess how MUC5B influences interspecies competition. Results show that **MUC5B significantly increases *S. mutans* and *S. sanguinis* coexistence.** This model represents a first step towards understanding how MUC5B influences the oral microbiota, but more complex models are needed to better recapitulate the environment of the oral cavity. Follow up studies could use a similar model with other oral bacteria and fungi or plaque from dental patients, which has been harvested and used

in several studies to gain an understanding of the oral microbiota in different disease states (11, 12). *In vivo* studies using hamster models have also been used to study methods for cavity prevention and could be used in this context to better understand how MUC5B modulates the oral microbiota (13, 14). For example, hamsters could be fed a high sucrose diet with or without mucin supplements then the composition of their oral microbiota could be evaluated using 16S RNA sequencing.

After determining that MUC5B increases bacterial coexistence, I evaluated several aspects of *S. mutans* and *S. sanguinis* growth in the presence of MUC5B to better understand how MUC5B could be influencing competition. Results indicate that **MUC5B could reduce interspecies competition by altering the cells' mode of growth.** Specifically, in the dual-species culture, MUC5B reduces the number of cells in the mixed-species biofilm, thereby increasing the relative number of cells in the less competitive planktonic state (15, 16). Although this dual-species competition model does not fully capture the complex environment of the oral cavity, it provides an initial understanding of how MUC5B could protect the oral cavity by preventing opportunistic or pathogenic microbes from becoming dominant species.

The work presented in this thesis provides a foundation for other studies that further characterize how MUC5B is able to reduce bacterial surface colonization and interspecies competition. Besides acting as a physical barrier, there are two primary mechanisms through which salivary mucins can interact with microbes to provide protection: 1) salivary mucins can agglutinate microbes, which would facilitate bulk removal during swallowing, and 2) salivary mucins can cause the dispersal of microbes. Because there are few studies indicating that *S. mutans* directly binds MUC5B, the second mechanism likely plays a larger role. The increase in *S. mutans* dispersal illustrated in this thesis may be a generalizable mechanism that mucins use to

reduce microbial virulence since other studies in the Ribbeck lab have also shown that Muc5ac porcine gastric mucin promotes *P. aeruginosa* and *C. albicans* dispersal (9, 10). Mucins' ability to reduce microbial virulence by keeping cells in an individualized state without killing the organism is a unique mechanism of protecting the body; it allows opportunistic pathogens to survive in the microbiota but limits their pathogenic potential. In contrast, many antibacterial proteins in the oral cavity, such as lysozyme, protect the body by killing harmful organisms (17).

One main question that warrants further investigation is whether the observed microbial phenotypes in the presence of MUC5B are caused by a physical effect or a change in bacterial gene regulation. An example of a physical effect that is not related to increased viscosity would be that the mucin polymer networks surrounding bacterial cells reduce diffusion of secreted factors, such as quorum sensing molecules, which are necessary for biofilm formation. For example, competence-stimulating peptides are secreted molecules that are known to play a role in *S. mutans* biofilm formation (18). These peptides have a prominent hydrophobic patch, which could facilitate mucin-peptide interactions (19). Another way MUC5B could influence bacteria is through specific glycan interactions, which could lead to downstream changes in genetic regulation that reduce microbial virulence. The ability of mucins to regulate gene expression has been demonstrated by several studies showing that intestinal and gastric mucins alter bacterial genetic regulation and by Dr. Kavanaugh in the Ribbeck who demonstrated that Muc5ac down-regulates *C. albicans* virulence traits (10, 20, 21).

There are several experimental strategies that could be used to further dissect the mechanism by which MUC5B causes *S. mutans* and *S. sanguinis* dispersal. First, a global gene analysis study, such as RNA-sequencing, could be performed to provide insight into changes in gene regulation that may take place when oral bacteria are exposed to salivary mucins. *S. mutans*

biofilms could be grown in medium with and without MUC5B, then cells from the supernatant and biofilm could be harvested separately. Comparing RNA expression in these four cell populations would indicate whether cells exposed to MUC5B have up- or down-regulated specific genes or pathways that lead to the observed reduction in surface colonization. If the genetic profiles of MUC5B and non-MUC5B exposed cells have similar genetic profiles, this would indicate that MUC5B likely alters bacterial surface colonization through a physical effect.

Another way to understand how MUC5B could be affecting *S. mutans* physiology is by studying how secreted molecules may change in the presence of MUC5B. Two important secreted molecules produced by *S. mutans* are competence-stimulating peptide, which plays a role in biofilm formation as mentioned above, and mutacin, which is important for interspecies competition (18, 22, 23). These molecules could be purified from *S. mutans* cultures grown in the presence and absence of MUC5B; a change in the amount of these molecules in the presence of MUC5B could indicate that MUC5B is influencing their production or directly interacting with them, which would decrease their effective concentration.

To better understand how oligosaccharides on MUC5B could be affecting *S. mutans*, specific saccharides could be grafted onto a polymer backbone then added to surface colonization experiments instead of MUC5B. Grafted oligosaccharides could be composed of a single type of saccharide or several different types in a configuration that mimics portions of oligosaccharide chains naturally found on MUC5B. For example, fucose has been identified as a common terminal sugar residue, and Lewis antigens are common terminal epitopes; both of these examples would be interesting to study and could provide insight into the effect of MUC5B sugars on oral bacteria (24, 25). If specific sugars or combinations of sugars reduce *S. mutans* surface colonization and interspecies competition, this would indicate that oral bacteria may

directly or indirectly interact with MUC5B sugar residues.

Because the oral cavity is easily accessible, determining if MUC5B levels change during disease states would be interesting and could provide insight into how MUC5B could be used as a diagnostic marker of disease. The oral cavity can be a mirror for changes that are happening in other parts of the body, therefore MUC5B could be an indicator of diseases that are not specific to the oral cavity. For example, one study shows that once an individual is infected with HIV-1, the concentration of MUC5B in whole saliva is significantly decreased compared with non-infected individuals, which could make MUC5B a diagnostic marker of HIV-1 infection (26).

Further research is needed to better understand how MUC5B is able to create a gel that houses millions of oral bacteria while coaxing potentially harmful microbes into passive existence. Understanding how mucins protect the body could open the doors to an entirely new set of therapeutic tools that aim to prevent microbes from transitioning into a pathogenic state as opposed to antibiotics, which treat the microbe once it is already virulent and can lead to antibiotic resistance. Based on the findings presented in this thesis, MUC5B could potentially be used as a useful therapeutic or preventative treatment for oral diseases like periodontal disease and Candidiasis that are caused by an overgrowth of opportunistic pathogens (27–29). In these cases, MUC5B could ideally be used to shift the oral microbiota back to a state of health. Although the use of mucins as therapeutics is promising, manufacturing full-length mucin mimetics in high yields is a challenge that has only begun to be investigated. One potentially more tangible goal is to identify specific sugars or sugar motifs on mucins that induce the same phenotypic effects described in this research. Once the influence of mucins on microbes is fully characterized, salivary mucins or engineered mimetics could potentially be used as therapeutic tools to prevent or treat diseases in novel ways.

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