The Influence of Mucins on Bacterial Communities

by

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B.S. Microbiology, Immunology, and Molecular Genetics
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Submitted to the Microbiology Graduate Program in partial fulfillment of the requirements for the degree of Doctor of Philosophy at the Massachusetts Institute of Technology

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ABSTRACT

Mucus is the hydrogel layer that coats all wet epithelia in the body. By supporting commensal microbes and preventing pathogenic invasion, mucus maintains host-microbe homeostasis. Mucin polymers, the primary gel-forming component of mucus, are an important mediator of mucus-microbe interactions. In this thesis, I demonstrate that mucins impact bacterial communities in their physical structure as well as microbe-microbe and microbe-host dynamics.

In Chapter 2, I study the ability of mucin surface coatings to suppress bacterial surface attachment, the first step in biofilm formation, for Streptococcus pneumoniae and Staphylococcus aureus. Mucin-bound glycans were identified as a critical structural component of mucin coatings that are necessary for bacterial repulsion.

In Chapter 3, I investigate how mucins impact established Pseudomonas aeruginosa biofilms. The data reveal that mucins cause disassembly and structural rearrangement in P. aeruginosa biofilms in a mucin concentration and flow rate dependent manner.

In Appendix A, I show evidence for the involvement of the bacterial flagella in mucin-mediated biofilm disruption. Deletion of flagellar cap fliD or flagellar stators motABCD results in biofilms that are resistant to mucin-mediated dissociation.

In Appendix B, I examine how mucins affect dual-species bacterial communities. I show that mucins promote S. aureus survival during co-culture with P. aeruginosa and also suppress the anti-staphylococcal effects of P. aeruginosa pyocyanin.

In Appendix C, I explore the impacts of mucins on P. aeruginosa quorum sensing, an important pathogenic determinant in P. aeruginosa infections. I found that mucins suppress the expression of P. aeruginosa Las and Rhl quorum sensing genes as well as downstream virulence factors.

In Appendix D, I assess how mucins modulate P. aeruginosa-epithelium interactions. The data show that mucins hinder the ability of P. aeruginosa to attach to epithelial cells in vitro. Additionally, mucins suppressed P. aeruginosa-associated epithelial cytotoxicity in a mucin concentration dependent manner.

Together, this thesis demonstrates that mucins modulate microbial communities in their behavior and interactions. Understanding how mucus and mucins impact microbes provides insight to host-microbe relationships, as well as for the development of novel bacteria-regulating strategies.
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Chapter 1

Introduction

Mucus is a biological hydrogel that lines all non-keratinized epithelia in the human body, including the lungs, intestines, and urogenital tracts. The mucus layer maintains homeostasis between the host and the environment, acting as a selective barrier that permits passage of desirable material such as nutrients and oxygen (1), while preventing access of potentially harmful agents such as toxins or pathogens (2, 3). In recent years, evidence has emerged for a role of the gel-forming polymers of mucus, the mucins, in regulating interactions of commensal and pathogenic microbes with the host (2-5). Specifically, mucins have been demonstrated to suppress virulence traits of opportunistic pathogens, including Pseudomonas aeruginosa (6), Streptococcus mutans (7), and Candida albicans (8), distinguishing them as important candidates for the regulation of microbial infections on mucosal epithelia. Despite the crucial role of mucus and mucins in maintaining health (9, 10), their effects on microbial behavior are understudied. This thesis focuses on the influence of mucins on structured bacterial communities called biofilms, which are responsible for many human infections. Furthermore, this work examines the impacts of mucins on both intraspecies and interspecies microbial interactions. Understanding the interplay between mucus and bacteria contributes to our biological understanding of both commensal and pathogenic mucosal microbes, and may provide insights for the development of new strategies to control microbial behavior.

Biofilms

Introduction to Biofilms

Biofilms are structured communities that secrete and encase themselves within a protective matrix (11-14). They are a common mode of bacterial existence found on both biotic and abiotic surfaces. Biofilms are often associated with mucosal infections such as dental cavity formation, cystic fibrosis, and ulcers, where their resistance to host immune defenses and
antibiotic treatments lead to their persistence (13). Furthermore, biofilms are highly relevant in the context of the colonization of medical devices. For example, biofilms are found on as many as 50% of all medical devices (12) and cause 25.6% of all hospital acquired infections (15). Despite their prevalence, effective strategies which prevent or remove biofilms are lacking.

Extensive studies of biofilms of several model organisms, including *P. aeruginosa*, *Escherichia coli*, and *Staphylococcus aureus*, have led to the emergence of a generalized model for bacterial biofilm development (Figure 1) (14). In brief, biofilm formation begins with the initial attachment of bacteria to a surface. Bacteria-substrate interactions are dependent on general physico-chemical properties including charge and hydrophobicity of both the bacteria and substrate surfaces (16–20), specific bacterial adhesins, and secreted matrix components. For example, initial surface attachment of *Staphylococcus* spp. is mediated by the surface protein Bap (21), extracellular DNA (22), and polysaccharide intercellular adhesin (PIA) (23). For some bacteria, surface attachment relies on motility, which improves surface accessibility, and motility appendages, which can act as adhesins (24–27).

Once attached to a surface, the bacteria proliferate and produce a structural and protective matrix to form a mature biofilm that is resistant to environmental challenges. The biofilm matrix consists of extracellular polymeric substances (EPS), including polysaccharides, nucleic acids, lipids, and proteins; the exact composition is specific to each microbial species. Differences in the composition of the EPS matrix can impact biofilm development and architecture. For
example, the thickness and smoothness of *P. aeruginosa* biofilms differ considerably depending on the amount of specific EPS polysaccharides Pel, Psl, and alginate that are produced (28-31). Biofilm and matrix development are also dependent on environmental conditions (32, 33) including nutrient availability (34, 35), shear stress (36, 37), and quorum sensing (38, 39). Moreover, the EPS matrix may regulate molecular transport, thereby creating nutrient and oxygen gradients throughout the biofilm.

Microbes within biofilms are not permanently anchored to the associated community, but instead can detach or disperse out of the biofilms upon certain perturbations to colonize a new or more favorable environment (40-43). Detachment occurs when individual cells or groups of cells are released from the outermost biofilm surfaces, and often results from mechanical forces or disruption of the biofilm matrix (37). Dispersal is when cells from the biofilm actively migrate out of the biofilm (40). This process is regulated by physiological pathways such as quorum sensing (44, 45), prophage activation (46), and response to nutrient availability (47, 48), and often involves regaining motility (40) or the secretion of rhamnolipids or matrix-hydrolyzing enzymes (22, 42, 49-51). Upon escape from biofilms by active or passive mechanisms, bacteria can attach to another surface to form a new biofilm (14, 40, 42).

**Challenges in treating biofilm infections**

Biofilm infections are problematic due to their array of resistance mechanisms that provide resilience against the immune system and antibiotics. First, the protective barrier property of the EPS matrix can restrict transport of host antimicrobial peptides (52) and other antibiotics, including colistin (53), tobramycin (54), and vancomycin (55), into the biofilm. Moreover, the sheer size of the biofilms can prevent clearance by immune cells, which are more effective in attacking planktonic microbes. Additionally, physiological and metabolic differentiation within the biofilm may lead to the emergence of subpopulations of protected phenotypes. For example, cells that are in a dormant state would be resistant to antibiotics that target metabolism or growth. Last, the high cell density within the biofilms is conducive to high frequencies of conjugation, transformation, and transduction, which can result in the horizontal transfer of antibiotic resistance genes (56).

The poor efficacy of antibiotic treatments for biofilm infections has led researchers to focus on two main strategies to prevent and combat biofilms. The first is focused on developing
surfaces, primarily coatings, that are not readily colonized by bacteria (57). Antibiotics have been bonded to surfaces to produce bacteriostatic or bactericidal coatings (58). However, the use of antibiotics can lead to the selection of resistant bacteria and can even stimulate biofilm formation for some microbes (59). Metals, toxic chemicals, and synthetic materials applied to coatings are effective in preventing bacterial attachment and viability, but these surfaces can become coated with biotic material or inactivated in clinical settings (57, 60, 61). Bio-inspired superhydrophobic surfaces with micron- and nano-scale roughness can also inhibit microbial attachment, though these effects are transient (62). For established biofilms, researchers have targeted biofilm detachment and dispersal mechanisms as alternative treatment strategies. Matrix-hydrolyzing enzymes (63, 64), phage treatments (65), and exposure to mechanical stress (66, 67) have successfully been used to disrupt biofilms in vitro. Unfortunately, these agents are often toxic or challenging to apply in a human host, and thus are not ideal for clinical applications.

The general problems with current anti-biofilms strategies are that they are short-lived, toxic, or technically difficult to implement. In this thesis, I explore the potential to harness the intrinsic protective properties of mucus, the material that shields the epithelial lining against damaging agents and microbes, to prevent biofilm formation and disrupt established biofilms. Because mucus is naturally present in the body, it is likely to be biocompatible. In the first chapter of my thesis, I focus on biofilm prevention and explore if mucins can interfere with surface attachment, the first step in biofilm formation. In the second chapter, I study already matured biofilms and investigate if mucins can disrupt their integrity and structure.

**Mucus and Mucins**

**Physiology of mucus**

The mucus layer covers all wet epithelia in the body, including the ocular, respiratory, digestive, and reproductive systems. Mucus maintains lubrication and hydration, and forms a selectively permeable barrier that allows passage of gasses, nutrients and other beneficial material, while blocking passage of foreign antigens and harmful agents (1–3). In all mucosal regions, mucus manages the interactions between the epithelia and microorganisms (68, 69). This regulatory function can in part be attributed to the mucin polymers, which can directly influence microbial behavior and virulence (discussed later in more detail) (6–8, 70). In addition,
mucus harbors immune components like immunoglobulin and antimicrobial peptides that control bacterial proliferation (3).

Mucus also has specific functions on different anatomical locations and accordingly, can differ in its composition, properties, and specific functions. For example, in the lungs, the mucus layer is up to 10 µm thick (71) and traps particles and microbes that enter the airways during inhalation. In healthy lungs, this unwanted matter is removed by mucociliary clearance (10). Dysfunctional respiratory mucus transport leads to chronic infections, which is common in cystic fibrosis (CF) patients that have dehydrated mucus and impaired mucociliary clearance (72). The mucus layer in the stomach is over 500 µm thick (73), and protects the epithelia from digestive gastric acids (74). Irregularities in the gastric mucus lining are associated with development of peptic ulcers (75). In the intestines, where the mucus layer ranges from 20 µm to 800 µm in thickness (76), mucus regulates nutrient absorption (76, 77) and houses the gut commensal microorganisms. In the female reproductive tract, mucus regulates sperm passage into the cervix (78) and prevents microbial access to the uterus (68).

**Structure and regulation of mucins**

The mucus hydrogel is composed of over 90% water, and the remaining content is a heterogeneous mix of macromolecules, including proteins, lipids, and nucleic acids that are either secreted or contributed from cellular debris of sloughed epithelial lining. The primary gel-forming component of mucus is the mucin glycoprotein, which contributes to both the physical and functional properties of mucus (2, 3, 10). Mucins are large biopolymers (up to tens of MDa in size) composed of a protein backbone with protruding oligosaccharides (3, 10). The high density of glycans causes the peptide backbone to exhibit an extended structural conformation (Figure 2) (79). Mucins are characterized by the hallmark proline, threonine, and serine (PTS) rich tandem repeat regions, which are the sites of the extensive O-glycosylations that contribute up to 90% of mucins' molecular weight (10, 80). During mucin synthesis, PTS domains are glycosylated in the Golgi apparatus, where an N-acetylgalactosamine (GalNAc) residue is linked to a serine or threonine (81). Elongation of the glycan chains results in both branched and linear oligosaccharides up to 20 residues in length (2). These oligosaccharides are composed of GalNac, N-acetylglucosamine (GlcNAc), galactose, mannose and fucose residues, and often terminate in anionic sialic acid or sulfate residues. The composition and arrangement of the oligosaccharide
Mucin Distribution

Secreted mucins

<table>
<thead>
<tr>
<th>Mucin</th>
<th>Distribution</th>
</tr>
</thead>
<tbody>
<tr>
<td>MUC2</td>
<td>Small intestine, colon, respiratory tract, eye, middle ear epithelium</td>
</tr>
<tr>
<td>MUC5AC</td>
<td>Respiratory tract, stomach, cervix, eye, middle ear epithelium</td>
</tr>
<tr>
<td>MUC5B</td>
<td>Respiratory tract, salivary glands, cervix, gallbladder, seminal fluid, middle ear epithelium</td>
</tr>
<tr>
<td>MUC6</td>
<td>Stomach, duodenum, gallbladder, pancreas, seminal fluid, cervix, middle ear epithelium</td>
</tr>
<tr>
<td>MUC7</td>
<td>Salivary glands, respiratory tract, middle ear epithelium</td>
</tr>
<tr>
<td>MUC19</td>
<td>Sublingual gland, submandibular gland, respiratory tract, eye, middle ear epithelium</td>
</tr>
</tbody>
</table>

Cell-surface mucins

<table>
<thead>
<tr>
<th>Mucin</th>
<th>Distribution</th>
</tr>
</thead>
<tbody>
<tr>
<td>MUC1</td>
<td>Stomach, breast, gallbladder, cervix, pancreas, respiratory tract, duodenum, colon, kidney, eye, B cells, T cells, dendritic cells, middle ear epithelium</td>
</tr>
<tr>
<td>MUC3A/B</td>
<td>Small intestine, colon, gall bladder, duodenum, middle ear epithelium</td>
</tr>
<tr>
<td>MUC4</td>
<td>Respiratory tract, colon, stomach, cervix, eye, middle ear epithelium</td>
</tr>
<tr>
<td>MUC12</td>
<td>Colon, small intestine, stomach, pancreas, lung, kidney, prostate, uterus</td>
</tr>
<tr>
<td>MUC13</td>
<td>Colon, small intestine, trachea, kidney, appendix, stomach, middle ear epithelium</td>
</tr>
<tr>
<td>MUC15</td>
<td>Spleen, thymus, prostate, testis, ovary, small intestine, colon, peripheral blood leukocyte, bone marrow, lymph node, tonsil, breast, fetal liver, lungs, middle ear epithelium</td>
</tr>
<tr>
<td>MUC16</td>
<td>Peritoneal mesothelium, reproductive tract, respiratory tract, eye, middle ear epithelium</td>
</tr>
<tr>
<td>MUC17</td>
<td>Small intestine, colon, duodenum, stomach, middle ear epithelium</td>
</tr>
<tr>
<td>MUC20</td>
<td>Kidney, placenta, colon, lung, prostate, liver, middle ear epithelium</td>
</tr>
</tbody>
</table>

Table 1: Mucin genes and their tissue distributions. Adapted from (2) with permission from Nature Publishing Group.

chains are dictated by the availability of glycosyltransferases as the mucins move through the Golgi, resulting in glycan heterogeneity (82, 83).

Mucins are encoded by MUC genes (Table 1), which differ in presence and abundance by anatomical location. Mucins are categorized into two main classes: cell-surface mucins and secreted mucins. Cell-surface mucins are tethered to epithelial cell membranes and contribute to the glycocalyx (84). These mucins are classified by the presence of a transmembrane domain and a short cytoplasmic domain. Most cell-surface mucins also have epidermal-growth-factor (EGF) family domains that presumably play a role in signaling with EGF receptors (ERBB) (84). There is evidence that the cell-surface mucins, MUC1 and MUC4, participate in cell signaling events (84). Additionally, enhanced expression cell-surface mucins have been associated with cancer (84). Secreted mucins are produced by goblet cells, and most can polymerize to form hydrogels that provide mucus with its characteristic viscoelastic properties (3, 10). The structure of secreted
mucins includes cysteine rich domains and D domains (homologous to von-Willebrand factor dimerization domains) that form disulfide bonds necessary for mucin oligomerization (Figure 2) (2, 85). Secreted mucins MUC5AC and MUC5B are dominant in the lungs, MUC5AC and MUC6 are the most prevalent in the stomach, and MUC2 is the primary mucin in the intestines (Table 1). These mucins are named based on differences in their gene structure, however their functional differences have not yet been discovered.

Mucin secretion is regulated by both constitutive and regulated pathways. Constitutive mucin production replenishes and maintains the constantly moving mucus layer. Regulated mucin production is an innate immune response that is stimulated by neutrophils, inflammatory cytokines, and other immunogenic material (86). Both routes of mucin regulation are important for maintaining the health of the mucosal epithelia.
Mucin-Microbe Interactions

Types of relationships between mucins and microbes

Mucins contribute to the ability of mucus to protect against unwanted microbial colonization. Although the physical and biochemical properties of mucins are appreciated as important for conferring their protective effects, the exact mechanisms of this protection are not well understood. One hypothesis is that mucins form a physical barrier to microbes. For example, mucins have been shown to restrict the motility of the gastric bacterium *Helicobacter pylori* (69), a well-known mucosal pathogen. Additionally, Johansson et al. demonstrated that the murine secreted mucin Muc2 was necessary to maintain a region of separation between the commensal microbes and the intestinal epithelia, and to prevent inflammation that likely stemmed from microbial contact with the epithelia (Figure 3A) (87). However, mucin gels do not exclusively hinder bacterial motility, as is seen in the cases of *P. aeruginosa* and *E. coli* (6), and therefore are selective filters rather than impenetrable barriers. Lastly, cell-surface mucin MUC16 protects human epithelial cells against *S. aureus* attachment (88–90), and Muc1 reduces *Helicobacter pylori* attachment to murine epithelial cells (Figure 3B) (91, 92).

In addition to modulating the transport of bacteria through the mucus, the mucin biopolymer network provides a selectively permeable barrier that can regulate passage of certain proteins, small molecules, and other matter which can impact the microbes inhabiting the mucus. The small pore size (100 nm-400 nm) of the mucin hydrogel mesh network may restrict transport of certain particles based on size exclusion (93–95). Additionally, hydrophobic domains and the negatively charged regions of mucins allow mucins to selectively modulate transport through the gel. Mucins therefore are likely to provide a framework for the presentation of immune factors like immunoglobulins and antimicrobial peptides in high local concentrations. For example, airway mucins can bind to cathelicidin LL-37 (96), and colonic mucins bind to a broad array of antimicrobial peptides including defensins HBD-1 and -3 and LL-37 without affecting their efficacy (97). Furthermore, mucins may sequester and alter the availability of nutrients and extracellular resources utilized by microbes, causing certain regions within the mucus to be more hospitable than others.

Mucins can also directly modulate the persistence and location of bacteria that reside within a mucus gel. Certain microbes express cell surface adhesins and mucus-binding proteins that can interact with mucin-bound glycans (98, 99) and mucin-peptide moieties (100). This
Figure 3. Mucins regulate bacterial attachment to the epithelia. (A) Histological sections of colon mucosa in WT and Muc2-/- mice show that mucin is necessary to maintain separation between the epithelium (stained blue) and resident gut bacteria (stained red). Scale bar: 100 µm. Reproduced from (87) Copyright (2008) National Academy of Sciences, USA. (B) H. pylori attachment to Muc1-/- epithelial cells is greater than that to WT epithelial cells (Muc1+/+). (C) Stomachs of Muc1-/- mice are hypercolonized by H. pylori relative to WT mice. (B, C) Reproduced from (91) with permission from Elsevier.

binding has been suggested to serve several functions: 1) mucin-bacteria binding could facilitate mucociliary removal of unwanted bacteria, 2) mucins may serve as competitive ligands for bacterial adhesins that would otherwise bind to epithelial cells, or 3) bacterial binding to mucin may promote mucus colonization (2, 3). The purposes of mucin-microbe interactions likely vary depending on the microorganism, anatomical location, and the nature and strength of mucin-microbe binding.

Certain species of mucosal bacteria are capable of metabolizing or modifying mucin molecules and networks. A subset of mucosal microbes can degrade (101–103) and utilize mucins as a nutrient source (104–107). Some bacteria synergize to metabolize mucins, for example the oral bacteria Streptococcus oralis and Streptococcus sanguis (108). For pathogenic
mucins retain cells in the planktonic state. Adapted from (6) with permission from Elsevier.

microbes, disruption of mucin hydrogels permits penetration of the mucus barrier. *H. pylori* secretes bicarbonate, which increases local pH and thus reduces mucin polymerization, to enhance its motility through gastric mucus (69). *P. aeruginosa* produces mucolytic enzymes to facilitate penetration through mucin solutions (109).

Mucins can also impact the expression of microbial virulence genes and traits. For example, the biopolymers can induce mucin adhesin and virulence gene expression in *H. pylori* (70), while they suppress virulent hyphae formation in *C. albicans* (8). Furthermore, mucins can interfere with microbial surface attachment and early biofilm formation (Figure 4), for example in *C. albicans* (8), *S. mutans* (7), and *P. aeruginosa* (6) (Figure 4). Moreover, mucin degradation can result in the introduction of metabolites that affect microbial pathogenicity. For instance, mucin utilization by the commensal microbe *Bacteroidetes thetaiotaomicron* leads to the release of fucose, which in turn suppresses enterohaemorrhagic *E. coli* (EHEC) virulence gene expression (110). In contrast, cleavage of mucin-associated sialic acids by influenza virus can exacerbate polymicrobial pneumonia by promoting *Streptococcus pneumoniae* proliferation (111). Thus, mucins are an important environmental factor that impact host-associated microbial behavior and colonization.

**Systems for investigating mucin-microbe interactions**

Microbial interactions with mucins have been explored using a variety of models. *In vivo* animal models in which mucin genes and glycosidases have been altered are useful to study systemic responses to changes in mucin availability and composition (87, 91, 111–113).
Explanted mucosal tissue and mucus-producing cell lines are valuable systems that facilitate controlled perturbations or the addition of microbes (8, 91). A caveat to these models is that disruption of mucin and therefore mucus homeostasis may stimulate systemic responses to compensate for mucin dysregulation. It is difficult to ascertain whether changes in the host and microbes are a direct result of changes in the mucins, or from downstream effects of altered mucins. Another important system for studying mucus-microbe interactions is the use of whole mucus or sputum isolated from human subjects (114-116). However, sample isolation can be difficult and highly invasive for many mucosal regions. Moreover, due to the complexity of whole mucus and sputum, it is difficult to pinpoint the specific effects of mucins.

In this work, I use native purified mucins to examine the influence of the biopolymers on biofilms and microbial interactions. Specifically, I use native mucins purified from fresh pig gastric mucus which have high homology to human MUC5AC (117), a primary mucin in the airways and stomach. Native purified mucins, unlike commercially purified mucins, recapitulate the structural and rheological properties of physiological mucus (118, 119), and have been previously used to study the behavior of H. pylori (69), P. aeruginosa (6), C. albicans (8), and S. mutans (7) in mucus environments. This thesis studies mucins in two different configurations (Figure 5): 1) adsorbed to synthetic surfaces as “2D” thin film coatings, and 2) solubilized in a ”3D” configuration, in which the mucins more closely represent the biochemical and rheological properties found in native mucus.

Although native purified mucins are an informative and experimentally advantageous model for mucus, there are limitations of this simplified system. While mucins are a primary gel-forming constituent of mucus, other mucus components can likely contribute to its physicochemical and microbial-modulating properties. The large and heterogeneous mucin glycoproteins are difficult to purify, and the possibility that associated factors may be present in the purified mucin preparations cannot be eliminated. Furthermore, mucus and mucins in physiological conditions are constantly replenished, a phenomenon which is not accounted for in some experiments in this thesis. Despite these caveats, native mucins remain a useful tool in which experimental conditions can be carefully controlled and perturbed, and are used in this thesis to study microbial behavior.
Figure 5. Schematic of mucin systems studied in this thesis. In Chapter 2, mucins are adsorbed to synthetic surfaces as "2D" thin film coatings. These mucin coatings are useful for bioengineering applications; however they do not represent physiological mucus environments. The remainder of this thesis examines mucins in their solubilized and native "3D" configuration, in which they more closely resemble the biochemical and rheological properties found in native mucus.

Thesis summary

Mucins have emerged as an important regulator of microbial behavior. Mucins in vivo can prevent microbial attachment to the epithelia, and mucin gels in vitro suppress microbial attachment and early biofilm formation on synthetic surfaces. This thesis aims to extend the current knowledge of how mucins influence microbial communities. Specifically, I investigate the possibility of exploiting the intrinsic bacteria-modulating properties of mucins to control biofilms (Chapter 2, Chapter 3 and Appendix A), and the impacts of mucins on bacterial interactions within planktonic cultures (Appendix B-C) and with the host (Appendix D).

In Chapter 2, I explore the application of mucins as surface coatings to reduce bacterial attachment. The development of anti-adhesive surfaces is desirable to reduce the incidence of unwanted bacterial colonization, for example on implanted medical devices. Together with colleagues, I show that thin film coatings of surface adsorbed mucins (Figure 5) suppress attachment of Streptococcus pneumoniae and S. aureus to polystyrene surfaces. Using deglycosylated mucins, we illustrate that mucin-bound glycans provide critical structural properties, and are imperative for the bacteria-repelling capability, of the mucin coatings.

In Chapter 3, I study the ability of mucins when presented in their native 3D configuration to disrupt established P. aeruginosa biofilms. Using reconstituted mucin gels (Figure 5), I demonstrate that the biopolymers can cause the disassembly and structural rearrangement of P. aeruginosa biofilms. Appendix A explores the role of the bacteria flagella in mucin-mediated biofilm disassembly and shows that loss of either the flagellar cap or flagellar motors renders the biofilms resistant to the dissociative effects of mucins.
In Appendix B, I describe the impact of mucins on bacterial interspecies dynamics, specifically between *P. aeruginosa* and *S. aureus*. *P. aeruginosa* kills *S. aureus* in most laboratory conditions, however, on native mucosal epithelia these organisms are frequently found together. I show that mucins support the co-existence of *S. aureus* with *P. aeruginosa*, and that the biopolymers protect *S. aureus* against the antagonistic effects of *P. aeruginosa* secreted pyocyanin.

In Appendix C, I present the finding that mucins can interfere with *P. aeruginosa* quorum sensing. Complex mucus environments can affect *P. aeruginosa* gene expression (114, 115, 120), however the specific effects of mucins were previously unknown. Using transcriptional reporters, I show preliminary data to illustrate that mucins can downregulate the expression of *lasI* and *rhlI* quorum sensing genes, two key regulators of *P. aeruginosa* quorum sensing, as well as several downstream virulence genes.

In Appendix D, I address the ability of mucins to shield intact epithelial cells from infection by *P. aeruginosa*. Mucins have been described to prevent surface attachment of several pathogens (89–92). My preliminary experiments show that mucins can also suppress the attachment of *P. aeruginosa* to epithelial cultured cells and thereby, render the bacteria less cytotoxic.

Together, this thesis provides evidence that mucins play an important role in regulating bacterial biofilms, as well as intraspecies and interspecies dynamics. These findings highlight the importance of mucins and mucus as a consideration when studying mucus-associated microbes. Furthermore, this work demonstrates the potential for the development of mucin-mediated or mucin-inspired strategies to regulate microbial behavior.

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Chapter 2

Mucin-bound glycans contribute to bacterial repulsion by mucin coatings

Abstract

Biomaterials that prevent bacterial colonization are needed to reduce the incidence of microbial colonization of implanted medical devices. This study investigates the use of mucin biopolymers to prevent attachment of selected respiratory pathogens to polystyrene surfaces. Our data show that coatings of porcine gastric mucins or bovine submaxillary mucins reduce surface attachment by *Streptococcus pneumoniae* and *Staphylococcus aureus*, but not *Pseudomonas aeruginosa*, highlighting the limitations of such coatings as universal antimicrobial surfaces. To elucidate how mucin coatings repel *S. pneumoniae* and *S. aureus*, the molecular components of mucins are examined. The data presented here suggest that mucin-bound glycans are key structural contributors of mucin coatings, and are responsible for the repulsive effects toward *S. pneumoniae* and *S. aureus*. 
Introduction

Microbial colonization is a leading cause of medical device failure. About 50% of indwelling devices become colonized by microbes (1), causing a significant fraction of hospital acquired infections (2). Bacterial colonization begins with cell attachment to the device surface. Attached cells proliferate and mature to form resilient matrix-encased communities called biofilms. Once established, biofilms are difficult to eradicate due to their resistance to antimicrobial treatments. Thus, there is a strong focus on developing new surfaces to prevent bacterial attachment.

This study explores the natural mucus barrier, and specifically its gel-forming mucin polymers, for strategies to prevent surface attachment. Mucus is the hydrated polymer network that lines all wet epithelia in the human body, including the respiratory, digestive, and reproductive tracts. The primary gel-forming components within mucus, the mucins, are highly glycosylated polymers which exist in secreted and cell-surface forms (3–6). Both mucin types can protect the underlying mucosal epithelia from microbial infection. For example, gastric mucin MUC5AC can maintain the bacteria *Pseudomonas aeruginosa* (7) and the yeast *Candida albicans* (8) in a planktonic state and impair subsequent biofilm development. A similar effect has been observed for human salivary mucin MUC5B toward the bacteria *Streptococcus mutans* (9). Moreover, cell-surface mucins can reduce *Staphylococcus aureus* attachment to corneal epithelial cells (10) and can limit *Helicobacter pylori* attachment to gastric epithelial cells (11).

Given their ability to suppress microbial surface attachment in native conditions, mucins have also been studied in the context of microbe-repelling surface coatings (12, 13). Although these thin film coatings do not represent physiological mucin gels in structure or thickness, the mucin coatings are able to reduce attachment of some microbes to underlying surfaces. Studies of mucin coatings on polyacrylic acid, poly(methyl methacrylate), silicone, polyurethane, and polystyrene surfaces show that the function of mucin coatings varies by mucin-type, the surfaces to which they are adsorbed, and the deposition conditions. For example, bovine submaxillary mucin coatings reduce *Candida albicans* attachment to silicon surfaces, but increase its attachment to poly(acrylic acid-b-methyl methacrylate) (PAA-b-PMMA) surfaces. This variability reflects a gap in our understanding of which functional domains and biochemical properties of mucin coatings are important in mucin-microbe interactions.
This study investigates which molecular components of mucin coatings contribute to bacterial repulsion, specifically in the context of polystyrene surfaces. The respiratory pathogens *Streptococcus pneumoniae*, *Staphylococcus aureus*, and *Pseudomonas aeruginosa* often colonize medical devices to cause infection (2), and thus were examined in this study. The data show that coatings made of gastric or submaxillary mucins efficiently prevented surface attachment of *S. pneumoniae* and *S. aureus*, but not of *P. aeruginosa*. To dissect the role of mucin-bound glycans in bacterial repulsion, properties of native mucin coatings were compared to those of deglycosylated mucin coatings. Upon deglycosylation, mucin coatings lost their ability to repel bacteria and exhibited a thinner and more rigid coating structure. Our findings indicate that mucin-bound glycans are structural components capable of regulating surface attachment by *S. pneumoniae* and *S. aureus*.

**Materials and Methods**

**Mucins**

Bovine submaxillary mucin (Sigma-Aldrich) was dialyzed for 4 days against ultrapure water using a Spectra/Por Float-A-Lyzer G2 100 kDa MW cutoff dialysis membrane (Spectrum Labs), then lyophilized for storage. Native porcine gastric mucin was purified as previously reported (14). Briefly, mucus was scraped from fresh pig stomachs and solubilized in saline buffer with protease inhibitors and sodium azide. Insoluble material was pelleted by ultracentrifugation and mucins were purified using size exclusion chromatography on a Sepharose CL-2B column. Mucin fractions were desalted, concentrated, and lyophilized for storage. To produce fluorescent mucins, gastric and submaxillary mucins were labeled with Alexa488 (Invitrogen) following the manufacturer's instructions. Briefly, 10 μl of 10 mg ml⁻¹ Alexa488 succinimidyl ester in DMSO was added to 1 ml of 3 mg ml⁻¹ mucins in 0.2 M bicarbonate buffer (pH 8). After incubation at room temperature for 1 hour, free dye was separated from the labeled mucin using a Macrosep 100 kDa MW cutoff centrifugal filter (Pall).

*Preparation of apo-mucins*

Mucin was deglycosylated by treatment with trifluoromethanesulfonic acid (TFMS), followed by oxidation and beta-elimination of the residual sugars as previously described (15). 5 mg lyophilized mucin was cooled on ice and mixed with 375 μl of ice-cold TFMS containing 10 %
(v/v) anisole. The solution was gently stirred on ice for 2 hours then neutralized by the addition of a solution containing 3 parts pyridine, 1 part methanol and 1 part water. Precipitates were dissolved by adding water. The solution was dialyzed for 2 days against ultrapure water using a 20 kDa MW cutoff dialysis membrane (Spectrum Labs). NaCl and acetic acid were then added to the solution to a final concentration of 0.33 M and 0.1 M, respectively. The solution was adjusted to pH 4.5 with NaOH. For the oxidation step, ice-cold 200 mM NaIO4 was added to the mucin solution to a final concentration of 100 mM NaIO4, and incubated at 4 °C for 5 hours in the dark. The unreacted periodate was destroyed by adding ½ volume of neutralizing solution containing 400 mM Na2S2O3, 100 mM NaI, and 100 mM NaHCO3. For elimination, the mucin solution was adjusted to pH 10.5 using 1 M NaOH and incubated at 4 °C for 1 hour. The solution was then dialyzed overnight at 4 °C against a 5 mM NaHCO3 buffer (pH 10.5) and further dialyzed for 2 days against ultrapure water. The resulting apo-mucin solution was then concentrated and dissolved in the appropriate buffer.

Apo-mucins were evaluated for glycan removal using the periodic acid-Schiff assay as previously described (16). Briefly, in a 96 well plate, 120 μL of a mixture of 7% acetic acid and 0.06% periodic acid was added to 20 μL sample. The solution was incubated for 1.5 hours at 37°C. 100 μL of Schiff reagent was added to the wells, and allowed to react at room temperature for 10 minutes before measuring absorbance at 550 nm using a SpectraMax M2 Microplate Reader (Molecular Devices). The ratio of absorbance to mucin mass contained in each sample was reported.

**Mucin coatings**

Mucin coatings were generated by incubating 200 μg ml⁻¹ gastric mucins or submaxillary mucins in 20 mM HEPES (pH 7.4) on the surface of interest. For mucin coatings in 96-well polystyrene microtiter plates, 200 μg ml⁻¹ native mucins were incubated in microtiter wells at room temperature for 1 hour, followed by 3 washes with dH2O. For apo-mucin coatings, 200 μg ml⁻¹ deglycosylated mucins were incubated in wells at room temperature for 1 hour, followed by 3 washes with dH2O.
Microscopy of mucin coatings

To verify mucin coating homogeneity, mucin coatings were visualized in the polystyrene microtiter wells. The wells were coated with gastric mucin-Alexa488, submaxillary mucin-Alexa488, or their Alexa488-labeled apo-mucin counterparts. A scratch was made in the film with a pipette tip for reference. The fluorescent coatings were imaged using an Observer Z1 inverted epifluorescence microscope (Zeiss) and a 40×/0.75 NA objective (Zeiss).

Measuring bacterial attachment to mucin and apo-mucin coatings

Bacterial attachment to mucin coatings and modified mucin coatings was evaluated for *Streptococcus pneumoniae*, *Staphylococcus aureus*, and *Pseudomonas aeruginosa*. *S. pneumoniae* TIGR4 serotype 19F was cultured in Todd Hewitt Broth (Becton Dickinson) supplemented with 0.5% Yeast Extract (Becton Dickinson) in static conditions at 37°C with 5% CO₂. *S. aureus* UAMS-1 was cultured in Brain Heart Infusion (Becton Dickinson). *P. aeruginosa* PAO1 was cultured in Luria Broth (Becton Dickinson). *S. aureus* and *P. aeruginosa* were grown shaking at 37°C. Bacteria were grown to logarithmic phase, centrifuged and resuspended in PBS to OD₆₀₀ 0.4. For the microtiter plate attachment assay, wells were coated with mucins as described above, or left untreated as a control. Bacteria were prepared as described for microscopic visualization, and then incubated in wells at 37°C for 1.5 hours for *S. aureus* and *P. aeruginosa* or 3 hours for *S. pneumoniae*. Unattached bacteria were aspirated, and wells were washed 3 times with dH₂O. Attached bacteria were fluorescently stained using the CyQuant Direct Cell Proliferation Assay (Life Technologies) following manufacturer's protocol, and quantified with a SpectraMax M2 Microplate Reader (Molecular Devices). Fluorescence of bacteria attached to mucin coated wells were normalized to fluorescence of bacteria attached uncoated control wells. Experiments were performed in triplicate.

QCM-D

Quartz crystal microbalance with dissipation monitoring (QCM-D, E4 system, Q-Sense) was used to measure the hydrated mass of mucins adsorbed to a polystyrene-coated quartz crystal (QSX305, Q-sense). Solutions of 200 µg ml⁻¹ native mucins or apo-mucins were adsorbed to the crystal. The crystal vibration was followed at its fundamental frequency (~5 MHz) and 5 overtones (15, 25, 35, 45, 55 and 65 MHz). Once the excitation was stopped, changes in the
resonance frequencies and dissipation of the vibration were followed at the 6 frequencies. When the adsorbed layers are highly hydrated they usually possess viscoelastic properties requiring the measurement data to be modeled. Hydrated thickness was calculated using the Q-Tools 3.0.12.518 software that includes the Voigt model (i.e., a spring and dashpot in parallel under no slip conditions) (17) assuming a density of 1050 kg.m$^{-3}$ (as validated for a related system (18)) and that the coating is homogeneous in thickness and over the crystal’s surface. Measurements were performed at least in duplicate.

Results

Mucin coatings reduce surface attachment of *S. pneumoniae* and *S. aureus*, but not *P. aeruginosa*

Surface attachment of three common respiratory pathogens (*S. pneumoniae*, *S. aureus*, and *P. aeruginosa*) to mucin coatings was evaluated using in-house purified porcine gastric mucins and commercial bovine submaxillary mucins. Mucins were adsorbed to polystyrene, then verified using fluorescence microscopy and QCM-D analysis, which confirmed that the mucins

![Figure 1](image)

**Figure 1.** Mucin coatings are homogeneous. (A, B) Coatings were produced on polystyrene slides of fluorescently labeled (A) gastric mucins or (B) submaxillary mucins. A scratch was made in the coatings with a pipette tip for visual comparison with uncoated polystyrene regions. Scale bars: 50 μm. (C, D) Representative QCM-D data shows saturation of mucins on a polystyrene coated crystal for (C) gastric mucins or (D) submaxillary mucins.
formed relatively homogeneous coatings (Figure 1). To assess bacterial repulsion by mucin coatings, bacteria bound to mucin-coated or uncoated polystyrene microtiter wells were fluorescently labelled and measured on a microplate reader. Figure 2B reveals that gastric mucin coatings reduced attachment of *S. pneumoniae* by 76.3% and attachment of *S. aureus* by 81.3% to underlying polystyrene. Submaxillary mucin coatings were comparably effective, reducing attachment of *S. pneumoniae* by 71.5% and of *S. aureus* by 81.0% to underlying polystyrene. In contrast, neither surface coating significantly altered *P. aeruginosa* attachment.

**Mucin-bound glycans contribute to repulsion of *S. pneumoniae* and *S. aureus***

Previous work demonstrated that mucin-bound glycans within mucin coatings contribute to the repulsion of mammalian cells (19). To examine if mucin-bound glycans also play a role in bacterial repulsion, bacterial attachment was tested on coatings made from deglycosylated mucins, which are henceforth referred to as apo-mucins. Apo-mucins were generated from native mucins by chemical removal of mucin-associated glycans. Mucin deglycosylation was verified using the periodic acid-Schiff assay (Figure 3A). Apo-mucins were adsorbed to polystyrene surfaces to produce coatings, and fluorescence microscopy of Alexa488-labeled apo-mucin

![](image)

**Figure 2.** Mucin coatings reduce bacterial attachment. Bacterial attachment to gastric mucin or submaxillary coatings generated in polystyrene microtiter wells was quantified using the CyQuant Assay (Life Technologies). Values represent relative attachment to mucin coatings (bacterial attachment to mucin coatings normalized to bacteria attached to uncoated polystyrene). Error bars represent standard deviation of 3 biological replicates. Reduction in attachment of *S. pneumoniae* and *S. aureus* to gastric mucin and submaxillary mucin coatings compared to polystyrene is statistically significant as determined by unpaired t-test with *p*≤0.05.
coatings confirmed relatively homogeneous surface coverage (Figure 3B, 3C).

The removal of glycans from mucins reduced the ability of the coatings to repel bacteria. Specifically, apo-gastric mucin coatings exhibited a 4.2 fold increase in *S. pneumoniae* attachment and a 10.8 fold increase in *S. aureus* attachment relative to native gastric mucin coatings (Figure 4A). Similarly, apo-submaxillary mucin coatings had 3.1 fold more *S. pneumoniae* and 8.3 fold more *S. aureus* attached compared to their glycosylated counterparts (Figure 4B). These results indicate that mucin-bound glycans are critical for preventing attachment by *S. pneumoniae* and *S. aureus*. In contrast, *P. aeruginosa* attachment was comparably efficient on native mucin and apo-mucin coatings, indicating that mucin-bound

![Figure 3](image)

**Figure 3.** Apo-mucin coatings are homogeneous. (A) Periodic acid-Schiff (PAS) assay of mucins and apo-mucins verified deglycosylation. Error bars represent standard deviation of 3 replicates. Reduction in absorbance/µg protein in apo-mucins relative to their native counterparts is statistically significant as determined by unpaired t-test with *p*<0.05. (A, B) Coatings were produced on polystyrene slides of fluorescently labeled apo-gastric mucins (A) or apo-submaxillary mucins (B). A scratch was made in the coatings with a pipette tip for visual comparison with uncoated polystyrene regions. Scale bars: 50 µm.

![Figure 4](image)

**Figure 4.** Quantification of bacterial attachment to (A) apo-gastric mucin coatings or (B) apo-submaxillary coatings generated in polystyrene microtiter wells. Values represent relative attachment to mucin coatings (bacterial attachment to mucin coatings normalized to bacteria attached to uncoated polystyrene). Error bars represent standard deviation of 3 biological replicates. Increase in attachment of *S. pneumoniae* and *S. aureus* to apo-mucin coatings relative to native mucin coatings is statistically significant as determined by unpaired t-test with *p*<0.05.
glycans, at least in the context of surface coatings, do not measurably affect *P. aeruginosa* binding. The glycan compositions of gastric and submaxillary mucins differ considerably (20, 21), suggesting that the repulsive effect of the coatings observed here is dictated not by the specific glycan components, but instead by general physico-chemical properties conserved among the different mucin types.

**Structural analysis of mucin coatings**

The deglycosylation of mucins alters the biochemistry and structure of mucin coatings, which in turn may affect bacterial attachment (22–24). To better understand the how mucin-associated glycans contribute to bacterial repulsion, QCM-D was used to examine the hydrated thickness and softness of native mucin and apo-mucin coatings. Figure 5A shows that gastric mucin coatings were 35.0 ± 9.9 nm thick, while submaxillary mucin coatings were 60.3 ± 3.2 nm thick. In contrast, apo-gastric mucins and apo-submaxillary mucins both formed thinner coatings that were less than 4 nm in thickness. QCM-D analysis also provided information about the softness of the coatings as a measure of energy dissipation of the acoustic waves applied to analyze the coatings. Figure 5B shows that the energy dissipation was greater for coatings of native mucins than for their apo-mucin counterparts. Upon deglycosylation, the energy

![Figure 5. Mucin glycans contribute structural properties to mucin coatings.](image-url)

(A, B) Coatings produced from native mucin and apo-mucin coatings were analyzed with QCM-D. Native mucin coatings exhibited (A) thicker coatings and (B) greater variation in energy dissipation, which is indicative of a softer coating. Error bars represent standard deviation of at least two replicates. Values for apo-mucins are statistically different from their native mucin counterparts as determined by unpaired t-test with p<0.05, except for variation in dissipation for sub-maxillary mucins and apo-submaxillary mucins. However, the trend of decreased variation in dissipation for apo-submaxillary mucins was conserved. (C) Model of glycan contributions to the structure of mucin coatings.
dissipation measurements decreased 21 fold for gastric mucin coatings and 2.5 fold for bovine submaxillary mucin coatings, indicating that apo-mucin coatings are stiffer than the native mucin coatings. These data suggest that mucin-bound glycans influence the thickness and softness of mucin coatings.

**Discussion**

Mucins have evolved to help protect the wet epithelia from microbial colonization and hence, have shown promise as building blocks for anti-microbial surface coatings. Previous work showed that coatings from submaxillary mucins can prevent *S. aureus* attachment to underlying surfaces (12). The current study extends the understanding of mucin coatings by demonstrating that coatings of submaxillary mucins can also repel *S. pneumoniae*. In addition the data here demonstrate that another species of mucins, namely from pig stomachs, is also effective at repelling both *S. aureus* and *S. pneumoniae*, providing a potential additional candidate biopolymer for the engineering of coatings.

This study also highlights the limitations of mucins as universal bacterial-repelling surfaces when displayed as 2D coatings. Specifically, neither type of mucin coating significantly altered *P. aeruginosa* attachment. When adsorbed to a surface, mucins may lose part of the biofilm-suppressing functionality, which is exhibited in a three dimensional hydrogel network. Moreover, mucin-digesting enzymes secreted by *P. aeruginosa* (25) may damage coating integrity and its repulsive properties. Last, adhesins on the bacterial surfaces (26, 27) which bind mucin-bound glycans (28, 29) and mucin-peptide moieties (30) may mediate interactions with the coatings. Of note is that the mucin coatings generated in this study only repelled Gram positive *S. pneumoniae* and *S. aureus*, but not Gram negative *P. aeruginosa*. However, previous work demonstrated that another Gram-negative bacterium, *Escherichia coli*, can be repelled by mucin coatings (13), hence, we conclude that the repulsive effect of mucin coatings may not be restricted to Gram-positive bacteria.

This study indicates that mucin-bound glycans provide mucin coatings with important structural properties, specifically thickness and softness. These properties have previously been shown to increase efficacy of bacterial repulsion by certain classes of polymer coatings, such as PEG (31–33). One possibility is that the glycans provide coating thickness and softness via their contributions to the mucin polymers’ bottle brush conformation. In solution, deglycosylation
causes the mucins’ bottle-brush architecture to collapse into a globular protein-like conformation (Figure 5C) (34). The structural conformation of polymers applied to coatings can determine antifouling properties, for example glycerol-based brush coatings with bulkier branched side chains form better antifouling surfaces than those with linear and shorter side chains (35). Thus, changes in mucin conformation upon deglycosylation may in part explain the apo-mucins coatings’ loss of repulsive ability. The identification of mucin-bound glycans as important bacteria-repelling, structural components of mucin coatings may inform the development of new antifouling materials and surfaces that reduce unwanted microbial colonization and prevent medical device associated infections.

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References


Chapter 3

Mucins trigger disassembly of *P. aeruginosa* biofilms

Abstract

The opportunistic pathogen *Pseudomonas aeruginosa* forms problematic biofilms that are resistant to immune clearance and antimicrobial agents. Mucins, the primary gel-forming biopolymers of the protective mucus layer, have previously been observed to suppress the generation of biofilms by a range of different microbes. In this study, I build on this work to examine if mucins can also disrupt already established biofilms, using *P. aeruginosa* as a model organism. Using a hydrodynamic flow cell biofilm system, I show that mucins can cause the disassembly and structural rearrangement of established *P. aeruginosa* biofilms. This biofilm response is dependent on mucin concentration and treatment flow rate. Other viscous polymer solutions cannot match the biofilm disruption by mucins, suggesting that there is specificity to mucin-mediated biofilm disruption. The discovery that mucins can disassemble biofilms may offer insight for the development of novel *P. aeruginosa* biofilm eradication strategies, both inside and outside of the body.
Introduction

The Gram-negative opportunistic pathogen *Pseudomonas aeruginosa* forms problematic biofilms—surface-attached bacterial communities within protective matrices—that cause morbidity in infections (1, 2) and medical device failures (3, 4). *P. aeruginosa* biofilms are challenging to treat because they are highly resistant to antibiotics (5) and harsh chemical treatments (6). Researchers have targeted biofilm disassembly mechanisms like matrix disruption (7), phage treatments (8), and exposure to mechanical stress (9–11) as new biofilm eradication strategies. However, these agents are often toxic or difficult to implement in a human host, and thus are not ideal for clinical applications. Thus, there is a pressing need for novel biocompatible biofilm removal methods.

Here, I explore the potential of the protective mucus layer, specifically its gel-forming mucin biopolymers, to disrupt biofilms. Mucins can promote the clearance of microbes, and have been demonstrated to impair surface attachment and early biofilm formation in *Streptococcus mutans* (12), *Candida albicans* (13), and *P. aeruginosa* (14). Mucins are thus interesting candidates for regulating established biofilms. Using a continuous flow cell biofilm model, I demonstrate that solutions of native purified mucins cause disassembly and structural rearrangement in *P. aeruginosa* biofilms. For comparison, other synthetic polymers were also able to partially disrupt the biofilms, but not to the same extent as the mucins, suggesting that features specific to the mucins are required to mediate the observed effect on biofilms.

Materials and Methods

Bacterial strains and culture conditions

The *Pseudomonas aeruginosa* wild type PAO1 was used in this study. For constitutive GFP expression, PAO1 was transformed with the plasmid pBBR1(MCS5)-Plac-gfp by standard electroporation methods to produce PAO1-GFP. *P. aeruginosa* strains were grown in Luria Broth (LB, Difco) with 30 µg/ml gentamicin to maintain the plasmid. For determination of planktonic cell viability, bacterial cultures were serially diluted in PBS and plated on LB agar for CFU counts. OD_{600} of 0.0025 represents a culture density of ~5.0 x 10^5 CFU/ml.
Mucin purification

This study used native porcine gastric mucin, which differ from industrially purified properties in their rheological properties (15). Native mucins were purified as previously reported (16). Briefly, mucus was scraped from fresh pig stomachs and solubilized in sodium chloride buffer with protease inhibitors and sodium azide. Insoluble material was pelleted by ultracentrifugation and mucins were purified using size exclusion chromatography on a Sepharose CL-2B column. Mucin fractions were desalted, concentrated, and lyophilized for storage. Peptide tandem mass spectrometry was used to determine preparation composition. Microcapillary reverse-phase HPLC nano-electrospray tandem mass spectrometry on a Thermo LTQ-Orbitrap mass spectrometer (Harvard Mass Spectrometry and Proteomics Resource Laboratory) confirmed that these mucin preparations were dominated by MUC5AC. Notably, MUC2, MUC5B, MUC6 as well as other proteins including actin, histones, and albumin were also present. To control for effects by contaminants, the mucin preparations were compared to mucins further purified by CsCl gradient centrifugation (17, 18).

Static adhesion and biofilm assays

PAO1-GFP was inoculated in a 96-well microtiter plate containing 70 μl of LB with or without 0.5% mucins at an OD₆₀₀ of 0.0025. Glass chips cut from a microscope slide were placed into each well. Cultures were grown for 8, 12, or 16 hours at room temperature. Glass chips were carefully removed, rinsed 3 times with PBS, and placed on a coverslip. Adherent bacteria were visualized with epifluorescence microscopy using an Observer Z1 inverted epifluorescence microscope (Zeiss) with a 20×/0.2 NA dry objective. Adherent bacteria were quantified by measuring the amount of surface coverage per frame, determined using ImageJ. Depicted images were taken using a 40×/0.75 NA dry objective (Zeiss).

Flow cell biofilm reactor

A PDMS (Polydimethylsiloxane; Sylgard 184; Dow Corning, MI, USA) microfluidic device was molded from capillaries anchored to a petri dish yielding a negative imprint of 4 straight microchannels (4 × 2 × 35 mm) which was then bonded to a glass slide. A suspension of PAO1-GFP at OD₆₀₀ 0.0025 was introduced into the microchannels under continuous flow (0.5 μl/min unless indicated otherwise) driven by a syringe pump (PHD Ultra, Harvard Apparatus,
MA, USA) for 48 hours at room temperature. Fresh media was introduced for 5 minutes at 25 
µl/min before analysis or treatment. Mucins, methylcellulose (15 cP Sigma), PEG 100 kDa 
(Sigma), or PEG 600 kDa (Sigma) in LB were introduced into channels at 0.5%, unless 
otherwise stated, for the indicated treatment times. Biofilms were imaged before (referred to as 
‘0 hours’) and after treatments at the middle of the channel using a Zeiss 510 confocal laser-
scanning microscope (CLSM) with 20×/0.5 NA dry objective with 2× or 4× zoom or, 100×/1.4 
NA oil immersion objective for high magnification images. Z-stacks were analyzed using 
COMSTAT (19) to determine biofilm biomass, average thickness, and surface roughness 
coefficient, and to produce thickness heat maps.

**Rheology**

Rheological tests were performed on an MCR 302 rheometer (Anton-Paar) in a cone-
plate geometry. The diameter of the cone was 25 mm, the cone angle was 1°, and the cone 
truncation was 51 μm. 90 µl of 0.5% polymer solutions solubilized in LB were applied to the 
rheometer. Shear stress was measured at shear rates between γ = 10/s to γ = 100/s. The viscosity 
was calculated assuming a Newtonian relationship between the stress and shear rate, τ (γ) = ηγ.

**Results**

**Mucins cause *P. aeruginosa* biofilm disassembly and structural rearrangement**

The main goal of this chapter was to test if mucins can disrupt existing biofilms. To 
address this question, biofilms were grown in hydrodynamic flow conditions, which are often 
found in biomedical and clinical settings. Continuous flow cell biofilm reactors are a useful 
system because 1) they better represent biofilms in non-static conditions, and 2) they are 
experimentally advantageous because they allow continuous replenishment of mucins, thereby 
preventing changes in mucin availability due to degradation or adsorption to biofilm or flow cell 
surfaces.

First, PAO1-GFP biofilms were grown under continuous flow at 0.5 µl/min for 48 hours. 
Confocal microscopy revealed that these biofilms exhibited a relatively smooth, flat architecture 
(Figure 1A). The mature biofilms were then exposed to LB containing 0.5% mucins, and after 1 
hour analyzed by confocal microscopy. Figure 1A shows that the initially smooth biofilms began 
to fragment after mucin exposure. In contrast, the biofilms remained unchanged in the mucin-
free LB control treatment (Figure 1A). A quantitative analysis of the biofilm structure and thickness using COMSTAT (19) revealed that exposure to mucins caused a reduction in biomass by 63.2% and a reduction of thickness by 56.0%, while the biofilms treated with the mucin-free LB control remained unchanged (Figure 1B-C). More detailed characterization of the biofilm structure after mucin exposure revealed a drastic structural rearrangement. In Figure 1D, the biofilm thickness map generated by COMSTAT shows differences in height and topography. The roughness coefficient, which quantifies the variability in biofilm height within an image stack, was 9.7 fold higher in mucin-exposed biofilms compared to the control LB-treated biofilms (Figure 1E). In addition, I observed that the bacteria within the mucin-exposed biofilms appeared to assemble in chains. Higher magnification images revealed that the cells were aligned along their long edges, like cars in a parking lot (Figure 2). This contrasted with the
LB treated control biofilms, in which bacteria did not appear to be directly associated with one another. Together, these data indicate that mucin treatment causes the biofilm surface to acquire a more uneven topography and structural rearrangements within the biofilm.

**Mucins are not toxic to *P. aeruginosa***

To determine if mucins disintegrate biofilms by killing the bacteria, I first tested the toxicity of mucins toward planktonic *P. aeruginosa* cells. The viability of planktonic PAO1-GFP in 0.5% mucins was evaluated by counting colony forming units (CFU) over the course of 20 hours of exposure. The number of viable PAO1-GFP cells is not reduced in the presence of mucins, nor are there effects on the growth rate of the planktonic bacteria over this time period (Figure 3). Hence, the absence of bactericidal or bacteriostatic effects in planktonic cells by mucins suggests that mucins are generally nontoxic to *P. aeruginosa*.
Figure 3. Mucins are neither bacteriostatic nor bactericidal toward PAO1-GFP. Error bars represent standard deviation of biological replicates (n=3).

It is possible that biofilm bacteria may respond differently than planktonic bacteria to mucins, thus I next asked if mucin exposure was toxic to the biofilm bacteria. To test this, I examined if biofilms could continue development after transient mucin exposure. PAO1-GFP biofilms were treated with 0.5% mucins for 1 hour, after which the mucin-containing medium was removed and replaced by LB without mucins. Figure 4 shows that after removal of the mucins, the biofilms resumed growth, increasing their biomass 3-fold and average thickness 2-

Figure 4. Biofilms can continue development after mucin treatments. (A) Confocal images of PAO1-GFP biofilms treated with 0.5% mucins for 1 hour, followed by a chase treatment for 19 hours with LB only or 0.5% mucins. Scale bars = 20 μm. (B) Biofilm biomass and (C) average thickness of biofilms treated for 1 hour with 0.5% mucins, followed by a chase treatment with LB only or 0.5% mucins (equivalent to 20 hour continuous mucin treatment). Error bars represent standard deviation of biological replicates (n≥3). *p≤0.05 by unpaired t-test.
fold. This result suggests that viable biofilm cells were still present even after exposure to mucins.

**Biofilm disassembly and structural rearrangement are mucin concentration dependent**

To further characterize the influence of mucins on *P. aeruginosa* biofilms, I asked if the quantity of mucins introduced to the biofilms would affect the extent of disassembly. 48 hour biofilms were exposed to 0.05%, 0.1%, 0.5%, or 1.0% mucins under continuous flow at 0.5 µl/min for 1 hour, and then analyzed. Confocal images revealed that treatment with 0.05% mucins caused no visible structural rearrangement in the biofilms (Figure 5A). Biofilms treated with 0.1% mucins did not appear fragmented, however small clusters of aligned cells were embedded throughout a flat biofilm architecture that resembled that of the LB control. COMSTAT analysis of the biofilms showed that the 0.1% and 0.05% mucin treatments did not significantly disassemble biofilms, as measured by biofilm thickness or biomass (Figure 5C-D), indicating that there is a threshold mucin concentration that is required for biofilm disruption. 0.5% and 1% mucin treatments resulted in fragmentation of the biofilm structures (Figure 5A). The 1%

![Figure 5](image.png)

**Figure 5.** Mucins impact *P. aeruginosa* biofilms in a concentration-dependent manner. (A) Confocal images of 48 hour PAO1-GFP biofilms treated with mucins for 1 hour 0.5 µl/min show mucin concentration-dependent biofilm fragmentation. Scale bars = 20 µm. (B) Quantification of biofilm biomass, (C) average thickness, and (D) roughness coefficient after 1 hour mucin treatment. Dotted lines represent mean of 48 hour biofilms prior to mucin treatments. Error bars represent standard deviation of biological replicates (n≥3). *p*<0.05 by ANOVA, however the trend of stronger biofilm disruption by higher mucin concentration treatments was reproducible.
mucin treatment resulted in stronger disassembly than the 0.5% mucin treatment (Figure 5B-C), suggesting that mucin-mediated biofilm disruption is dependent on mucin concentration.

**Mucin treatment flow rate affects the degree of biofilm disassembly**

Because these experiments are performed in a flow cell system, the fluid mechanics of the bulk liquid experienced by the biofilm surface may influence the biofilm phenotype. Shear stress, which is dictated by solution viscosity and flow rate, can impact biofilm growth and disassembly (9–11). I next asked if the mucin-mediated biofilm disruption was affected by the treatment flow rate. 48-hour PAO1-GFP biofilms were treated with 0.5% mucins for 1 hour at slow flow (0.5 μl/min) or fast flow (10 μl/min) (Figure 6A). The fast flow mucin treatment caused enhanced biofilm disassembly, resulting in a biofilm with 31.6% less biomass (Figure 6B) and a 41.6% decrease in average thickness (Figure 6C) relative to that of the slow flow treatment. Furthermore, the roughness coefficient of biofilms after the fast flow treatment increased 2-fold compared to that of the slow flow treatment (Figure 6D). Thus, a faster treatment flow rate enhances mucin-mediated disassembly and structural changes in *P. aeruginosa* biofilms.

![Figure 6](image)

**Figure 6.** Mucin treatment flow rate impacts *P. aeruginosa* biofilm disassembly and structural rearrangement. (A) Confocal images of PAO1-GFP biofilms treated with 0.5% mucins at slow flow (0.5 μl/min) or fast flow (10 μl/min) for 1 hour. Scale bars = 20 μm. (B,C) Quantification of biofilm (B) biomass and (C) average thickness and (D) structural rearrangement as measured by roughness coefficient after 1 hour of treatment with LB or 0.5% mucins. Error bars represent standard deviation of biological replicates (n≥3). *p≤0.05 by unpaired t-test.
Mucin specificity of biofilm disassembly

To determine if the biofilm disassembly was specific to mucins, I examined several commonly studied synthetic polymer solutions for their abilities to disrupt biofilms. Specifically, I tested methylcellulose, which has been studied as a mucin mimetic due to similarities in its viscoelastic properties (20, 21), and polyethylene glycol (PEG), which is commonly used in antifouling coatings (6). Since mucins are heavily glycosylated polymers (22, 23), it is interesting to compare them to glycan-based polymers like methylcellulose and non-glycan polymers like PEG to identify if there are general effects of glycans or viscous polymer solutions.

PAO1- GFP biofilms treated with 0.5% methylcellulose solutions or 0.5% PEG solutions (either 600 kDa or 100 kDa) for 1 hour were observed to exhibit some disassembly and structural rearrangement, however to a lesser extent than the mucin treated biofilms (Figure 7A). Methylcellulose treatment caused a 29.4% reduction in biomass (Figure 7B) and 33.9% reduction in average thickness (Figure 7C), and both 600 kDa and 100 kDa PEG treatments resulted in similar outcomes (Figure 7B-C). These data indicate that biofilm disruption is not restricted to glycan-based polymers. However, since neither the glycan-based nor non-glycan synthetic polymers tested here could disassemble the biofilms to the same degree as mucins, it suggests that there is some specificity to the effects of mucins on the biofilms.

Because the viscosity of the treatment polymers can contribute to biofilm-disrupting shear stress (9–11), I worked together with a colleague to characterize the rheological properties of the 0.5% polymer solutions. Data acquired using a rheometer shows that the mucin and 600 kDa PEG solutions had the highest viscosities, followed by methylcellulose, then 100 kDa PEG (Figure 7D). Although the mucins and the 600kDa PEG solutions had similar viscosities, mucins caused a greater degree of biofilm disassembly, suggesting that the viscosity of the polymer solutions is not directly correlated to the extent of biofilm disruption and that there may be specific biochemical or molecular properties of mucins responsible for the disruption of P. aeruginosa biofilms.
Figure 7. *P. aeruginosa* biofilm disassembly and structural rearrangement by synthetic polymer solutions. (A) Confocal images of PAO1-GFP biofilms after treatment with 0.5% polymer solutions in LB at 0.5 μl/min for 1 hour. Scale bars = 20 μm. (B, C) Quantification of biofilm disassembly measured by biofilm (B) biomass and (C) thickness. Reported values for biomass and thickness of biofilms after 1 hour treatment normalized to biofilms pre-treatment. (D) Viscosity of 0.5% polymer solutions measured by a rheometer. Error bars represent standard deviation of biological replicates (n=3). *Differs from LB control treatment determined by ANOVA, p≤0.05.

Discussion

Here, I report the discovery that mucin biopolymers can disrupt *P. aeruginosa* biofilms, which are highly resistant to antibiotics and other biofilm removal strategies. There are several possibilities by which mucins may be acting upon the biofilms. First, the viscoelastic properties of mucin solutions may contribute to shear stress, a property of solution viscosity and flow rate, which is known to impact biofilm detachment (9–11). Increasing the flow rate, and thus shear stress, of mucin solutions resulted in enhanced biofilm disassembly. Notably, a synthetic PEG solution with similar viscosity to the mucins exhibited a lesser degree of disassembly, suggesting that there are additional mucin-specific mechanisms of *P. aeruginosa* biofilm disruption.

In addition to presenting specific rheological properties, mucins may also trigger a physiological dispersal response in the biofilm bacteria, potentially via direct interactions between mucins and bacterial signaling systems. For example, *P. aeruginosa* is known to respond to N-
Acetylglucosamine (GlcNAc), a glycan found on mucins, via a two-component regulator (24). Mucins may also trigger signaling events by altering nutrient availability and environmental conditions, which are known regulators of *P. aeruginosa* biofilm dispersal (25, 26). Furthermore, mucins have charged and hydrophobic moieties that can potentially interact with matrix EPS or specific bacterial adhesins, and may compete for binding sites that maintain the biofilm structure. Preliminary experiments investigating how mucins disrupt *P. aeruginosa* biofilms, reported in Appendix A, indicate that flagella are involved in the biofilm response to mucins. The specific role of the flagella, whether via mucin binding, a dispersal signaling response, or bacterial motility, remains an open question. Mucins likely disrupt *P. aeruginosa* biofilms by a combination of these proposed mechanisms.

Mucin exposure gave rise to several interesting new biofilm phenotypes. First, microcolonies were observed that remained after mucin exposure under high flow. Why do these portions of the biofilm persist? Physiological differentiation of bacteria in mature biofilms (28) may lead to the emergence of subpopulations that vary in their expression of mucin interaction partners. Another notable phenotype within mucin-treated biofilms was a novel bacterial alignment, in which the rod-shaped cells aligned lengthwise in chains up to tens of cells long, similar to cars aligned horizontally in a parking lot. The functions and mechanisms of this phenotype require further investigation.

Together, this work identifies mucins as biofilm-disrupting agents that can disassemble and prevent subsequent growth of established biofilms. Further characterization of mucin-associated biofilm phenotypes may lead to the development of mucin-based strategies to regulate these microbial communities. For example, it remains unknown if mucin exposure can impact biofilms formed by other microorganisms, or can sensitize the biofilms to certain antibiotics. Better understanding of the extent and mechanisms of mucin-biofilm interactions may provide foundation for the innovation of novel mucin-inspired materials that can improve the efficacy of biofilm eradication treatments.
Acknowledgements

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References


Appendix A

Mucin-mediated *P. aeruginosa* biofilm disassembly is flagella dependent

Introduction

Chapter 3 demonstrated that mucins have the capacity to disrupt established *Pseudomonas aeruginosa* biofilms. In this appendix, I explore the role of bacterial flagella in mucin-mediated biofilm disassembly. Previous studies have demonstrated that flagella play an important role in *P. aeruginosa* responses to mucins (1–3). In addition, the flagella are involved in bacterial dispersal out of biofilms (11, 12). Here, I study how mucins influence biofilms of bacteria lacking the flagellar cap *fliD* or flagellar stators *motABCD*, both of which are impaired in swimming motility. I found that mucins could not dissociate Δ*fliD* biofilms. Moreover, biofilms of Δ*motABCD* bacteria, which that have intact flagella but are deficient in flagellar motility, were also resistant to mucin-mediated disassembly. Together these data suggest that *P. aeruginosa* flagellar function is important for mucin disruption of biofilms.

Materials and Methods

Bacterial strains and culture conditions

*Pseudomonas aeruginosa* strain PAO1 and its derivatives PAO1Δ*fliD* and PAO1Δ*motABCD* from (4) were used in this study. For constitutive GFP expression, *P. aeruginosa* strains were transformed with the plasmid pBBR1(MCS5)-Plac-gfp by standard electroporation methods to produce PAO1-GFP, PAO1Δ*fliD*-GFP, and PAO1Δ*motABCD*-GFP. *P. aeruginosa* strains were grown in Luria Broth (LB, Difco) with 30 μg/ml gentamicin to maintain the plasmid.

Mucin purification

This study used native porcine gastric mucins, which differ from industrially purified properties in their rheological properties (5). Native mucins were purified as previously reported.
Briefly, mucus was scraped from fresh pig stomachs and solubilized in sodium chloride buffer with protease inhibitors and sodium azide. Insoluble material was pelleted by ultracentrifugation and mucins were purified using size exclusion chromatography on a Sepharose CL-2B column. Mucin fractions were desalted, concentrated, and lyophilized for storage. Peptide tandem mass spectrometry was used to determine preparation composition. Microcapillary reverse-phase HPLC nano-electrospray tandem mass spectrometry on a Thermo LTQ-Orbitrap mass spectrometer (Harvard Mass Spectrometry and Proteomics Resource Laboratory) confirmed that these mucin preparations were dominated by MUC5AC. Notably, MUC2, MUC5B, MUC6 as well as other proteins including actin, histones, and albumin were also present. To control for effects by contaminants, the mucin preparations were compared to mucins further purified by CsCl gradient centrifugation (7, 8).

Flow cell biofilm reactor
A PDMS (Polydimethylsiloxane; Sylgard 184; Dow Corning, MI, USA) microfluidic device was molded from capillaries anchored to a petri dish yielding a negative imprint of 4 straight microchannels (4 × 2 × 35 mm), which was then bonded to a glass slide. A suspension of PAO1-GFP at OD₆₀₀ 0.0025 was introduced into the microchannels under continuous flow (0.5 μl/min) driven by a syringe pump (PHD Ultra, Harvard Apparatus, MA, USA) for 48 hours at room temperature. Fresh media was introduced for 5 minutes at 25 μl/min before analysis or treatment. Biofilms were treated with 0.5% mucins solubilized in LB for 1 hour. Biofilms were imaged before (referred to as ‘0 hours’) and after treatments at the middle of the channel using a Zeiss 510 confocal laser-scanning microscope (CLSM) with 20×/0.5 NA dry objective with 2× or 4× zoom or, 100×/1.4 NA oil immersion objective for high magnification images. Z-stacks were analyzed using COMSTAT (9) to determine biofilm biomass, average thickness, and surface roughness coefficient.

Results
Mucin-mediated biofilm disruption is dependent on the P. aeruginosa flagellar cap fliD
To elucidate the role of FliD in mucin-mediated biofilm disassembly, the effects of mucin exposure on P. aeruginosa fliD mutant biofilms were examined. PAO1ΔfliD-GFP biofilms were grown for 48 hours, at which point they closely resembled 48 hour PAO1 wildtype (WT)
biofilms (Figure 1A). Before and after a 1 hour treatment with 0.5% mucins, ΔfliD biofilms were analyzed by confocal microscopy. In contrast to WT biofilms, the mucin treatment did not significantly change the biofilm biomass, average thickness, or roughness coefficient in ΔfliD biofilms (Figure 1B-D). The resistance of fliD mutant biofilms to mucin-mediated disruption suggests involvement of FliD in the phenomenon. I hypothesized that FliD played a role in the biofilm response to mucins by its contribution to a flagellar motility-based dispersal response.

Figure 1. P.aeruginosa swimming fliD is required for mucin-associated biofilm disassembly and structural rearrangement. (A) Confocal images of 48-hour PAO1-GFP or PAO1ΔfliD-GFP biofilms before (0h) or after 1 hour treatment with LB control or 0.5% mucins at 0.5 μl/min. Scale bars = 20 μm. (B-C) Quantification of (B) relative biomass, (C) relative thickness, or (D) fold change in roughness coefficient. Reported values are biomass, thickness, or roughness coefficient of biofilms treated for 1 hour normalized to that of biofilms before treatment. Error bars represent standard deviation of biological replicates (n=3). *p<0.05 by unpaired t-test.

Role of flagellar motility in mucin-mediated biofilm disassembly

To examine whether mucin-mediated biofilm disassembly was dependent on general flagellar functioning or the presence of FliD specifically, I studied a mutant that lacked the motABCD flagellar motor genes. The motABCD mutant bacteria produce flagella (10), but like fliD mutants, are impaired in swimming motility (Figure 2). PAO1ΔmotABCD-GFP biofilms were grown in flow cell chambers for 48 hours at room temperature. Confocal microscopy
revealed that the ΔmotABCD biofilms resembled WT biofilms (Figure 1A). Similar to the ΔfliD biofilms, treatment of PA01ΔmotABCD-GFP biofilms with 0.5% mucins for 1 hour did not trigger biofilm disassembly (Figure 1A-C) or structural rearrangement (Figure 1D). This data indicates that the presence of the flagella is not sufficient for mucin-mediated biofilm disassembly, and suggests that functional flagella are involved in the biofilm response to mucins.

Figure 2. *P. aeruginosa* swimming motility. (A) PAO1 strains were inoculated into swim agar (M9 medium with 0.3% agar) using a toothpick. Plates were imaged after an 18 hour incubation at 30°C. (B) Swim zone diameters were measured for each strain. Error bars represent standard deviation of biological replicates (n=3). Swimming diameters of ΔfliD and ΔmotABCD bacteria were significantly different than WT using the unpaired t-test, p≤0.05.

**Discussion**

Biofilms of *P. aeruginosa* bacteria lacking flagellar cap *fliD* or flagella motor stators *motABCD* were resistant to disassembly by mucins, suggesting that mucin-mediated biofilm disruption of *P. aeruginosa* wildtype biofilms is flagella dependent. Based on these results, I propose that mucins disrupt biofilms by stimulating active dispersal, which in *P. aeruginosa* involves swimming motility (11, 12). To disperse out of the biofilm, the sessile bacteria must regain swimming motility, and thus must have functioning flagella. In this scenario, swimming-impaired ΔfliD and ΔmotABCD bacteria would not be able to disperse in response to mucins, potentially explaining the resistance of the mutant biofilms to mucin-mediated biofilm disassembly. This hypothesis is consistent with previous reports of mucin-associated changes in flagellar-based surface motility in *P. aeruginosa* (13) and enhancement of swimming motility in *Escherichia coli* (4).

The data presented here point to a role for flagella in mucin disruption of *P. aeruginosa* biofilms, however there is a caveat for studies using mutant bacteria. Although the ΔfliD and
ΔmotABCD strains formed biofilms that visually resembled wildtype biofilms, the possibility that mutant biofilms differ in matrix composition or bacterial physiology cannot be eliminated. Further investigation is required to elucidate the mechanisms of mucin-mediated *P. aeruginosa* biofilm disassembly.

References


Appendix B

Mucins protect Staphylococcus aureus against Pseudomonas aeruginosa pyocyanin

Introduction

Mucosal surfaces are colonized by both commensal and pathogenic microorganisms, whose interactions can influence the emergence of pathogenesis (1–3). *Pseudomonas aeruginosa* and *Staphylococcus aureus* are common nosocomial pathogens, frequently found together in wounds (4–6) and in mucosal environments (7–9), such as the thick, stagnant lung mucus of cystic fibrosis (CF) patients (7, 9). Co-colonization by these two bacteria can result in enhanced pathogenicity (10–13) and increased antibiotic resistance (14, 15), and cause delayed wound healing in the host (16). The dynamics of *P. aeruginosa* and *S. aureus* interactions inside mucosal environments are poorly understood, largely because in most laboratory conditions, *P. aeruginosa* inhibits and kills *S. aureus* (17–19), making them difficult to study together.

In contrast to common laboratory culture conditions, more complex in vitro and in vivo wound environments appear to stabilize the co-existence of *P. aeruginosa* and *S. aureus* (11, 14). For example, complex CF sputum alters the timing and degree of *P. aeruginosa* anti-staphylococcal activity (18). These findings indicate that the interactions between *P. aeruginosa* and *S. aureus* depend on the nature and composition of their environment. These observations point to mucin glycoproteins, the primary gel-forming polymers in the mucus gel, as prime candidates to influence the interaction dynamics between *S. aureus* and *P. aeruginosa*. The rationale is that mucins can modulate virulent behavioral traits of single species populations, such as *Helicobacter pylori* motility (20), *Candida albicans* hyphae formation (21), and *P. aeruginosa* (22) and *Streptococcus mutans* (23) biofilm formation. Hence, it is likely that mucins can also influence behavior of bacteria in polymicrobial communities.

To test this hypothesis, I studied whether *P. aeruginosa* and *S. aureus* population dynamics are altered by the presence of mucins. My data show that mucins protect *S. aureus* against antagonism by *P. aeruginosa*. I further demonstrate that mucins can suppress the
antagonistic effects of pyocyanin, the secreted phenazine which is largely responsible for \textit{P. aeruginosa} anti-staphylococcal activity (24). Specifically, mucins reduce the ability of pyocyanin to inhibit respiration, stimulate reactive oxygen species (ROS) production, and arrest growth of \textit{S. aureus}. Preliminary experiments show that mucins can limit pyocyanin diffusion, hinting that mucin sequestration of pyocyanin may be responsible for the protective effect. Better understanding of how mucins impact \textit{S. aureus} and \textit{P. aeruginosa} interactions can contribute to the development of better \textit{in vitro} infection models, and will provide valuable insight to how polymicrobial communities function in mucosal environments.

**Materials and Methods**

*Bacterial strains and culture conditions*

\textit{S. aureus} UAMS-1 and \textit{P. aeruginosa} PA14 were grown in Luria Broth (LB) at 37°C with shaking. \textit{S. aureus} and \textit{P. aeruginosa} were inoculated from overnight cultures at \~10^7 CFU/ml in monoculture or co-culture (1 \textit{S. aureus}: 1 \textit{P. aeruginosa} ratio) at a final volume of 100 µl in polypropylene 1 ml 96-well plates. Cultures were grown shaking at 37°C for 9 hours unless indicated otherwise. Native purified mucins (purification described below) were added to media before inoculation at a final concentration of 0.5%. To evaluate bacterial viability, samples were vortexed with bead bashing for 10 x 2 seconds to break up aggregates, serially diluted, and plated on LB agar plates for monocultures. For co-cultures, serial dilutions were plated on LB agar with 10 µg/ml chloramphenicol agar for \textit{P. aeruginosa} selection and Mannitol Salt Phenol Red Agar (Sigma) for \textit{S. aureus} selection.

For pyocyanin challenge experiments, pyocyanin (Cayman Chemicals) was added to culture media before inoculation with \textit{S. aureus}. Pyocyanin from a 5 mg/ml DMSO stock was diluted in culture media to a final concentration of 100 µM. To evaluate bacterial viability, samples were vortexed with bead bashing for 10 x 2 seconds to break up aggregates, serially diluted, and plated on LB agar plates.

*Mucin purification*

This study used native porcine gastric mucins, which differ from industrially purified properties in their rheological properties (25). Native mucins were purified as previously reported (26). Briefly, mucus was scraped from fresh pig stomachs and solubilized in sodium
chloride buffer with protease inhibitors and sodium azide. Insoluble material was pelleted by ultracentrifugation and mucins were purified using size exclusion chromatography on a Sepharose CL-2B column. Mucin fractions were desalted, concentrated, and lyophilized for storage.

**Pyocyanin extraction**

*P. aeruginosa* was inoculated in co-culture with *S. aureus* in LB with mucins as described above. Cultures were incubated with shaking in 1 ml polypropylene 96-well plates at 37°C for 9 hours. The cells were then pelleted at 12,000 × g for 5 minutes and the supernatants retained. One volume of chloroform was added to the supernatants, which was then vortexed for 3 minutes. The upper phase was removed and the chloroform extraction repeated. The chloroform phases were pooled and 110 µl 0.2 N HCl was added to further extract the pyocyanin. The pyocyanin extract (the aqueous phase) was transferred to a microplate and quantified by measuring OD 520 on a SpectraMax M3 plate reader (Molecular Devices).

**Respiration assay**

*S. aureus* cells were inoculated into LB and incubated with shaking at 37°C until in exponential phase. The cultures were pelleted, washed, and resuspended in PBS. Cells were diluted to achieve a final concentration of OD 600 0.1 in PBS containing pyocyanin (final concentration 100 µM) with or without mucins (final concentration 0.5%). Cells were incubated in a total 25 µl in a 96 well half area plate. 2.5 µl Alamar Blue respiratory indicator dye (Life Technologies) was added to samples and incubated at room temperature for 1 hour. Alamar Blue fluorescence (Ex 540 nm/Em 590 nm) was measured using a SpectraMax M3 plate reader (Molecular Devices). Cell-free controls of mucins and pyocyanin were incubated with Alamar Blue as controls.

**ROS**

*S. aureus* cells were grown in LB with shaking at 37°C until exponential phase. *S. aureus* was inoculated to reach an OD 600 of 1.0 into LB in the presence or absence of pyocyanin (final concentration 100 µM) and with or without mucins (final concentration 0.5%). Cultures were incubated for 2 hours with shaking at 37°C. Cells were pelleted and resuspended in PBS
containing 25 µM H2DCF ROS dye (Life Technologies) and incubated for 90 minutes at room temperature in the dark. Relative ROS production was determined by measuring fluorescence (Ex 488 nm/Em 515 nm) and normalizing to the OD_{600} of the culture. Measurements were collected using a SpectraMax M3 plate reader (Molecular Devices).

*Mucin binding assay*

Pyocyanin binding to mucins was investigated using a modified version of the differential radial capillary action of ligand assay (DRaCALA) as previously described (27). Mucins solubilized in PBS were mixed with pyocyanin (final concentration 200 µM) and 2 µl were dropped on a dry nitrocellulose membrane. Pyocyanin was allowed to diffuse until membranes were dry. Membranes were photographed and analyzed for intensity of the center spot using ImageJ.

**Results**

*Mucins protect S. aureus from P. aeruginosa antagonism*

*S. aureus* UAMS-1 and *P. aeruginosa* PA14 were grown as monocultures or co-cultures in Luria Broth (LB), in the presence or absence of 0.5% solubilized mucins. In monoculture conditions, the presence of mucins did not measurably impact the growth of either organism (Figure 1). When *S. aureus* and *P. aeruginosa* were grown together in co-culture without mucins, *S. aureus* viability decreased after 6 hours (Figure 1A). However, when mucins were present in the medium, there were nearly 2 orders of magnitude more viable *S. aureus* cell than in the mucin-free co-cultures. This result suggests that in the presence of mucins, *S. aureus* is protected from the antagonistic effects of *P. aeruginosa.*
Mucins suppress the antagonistic effects of pyocyanin toward S. aureus

The anti-staphylococcal activity of *P. aeruginosa* has been largely attributed to its production of the pigmented phenazine pyocyanin (24). Indeed, *P. aeruginosa* produced pyocyanin during co-cultures with *S. aureus* in both mucin-containing and mucin-free conditions, as determined by colorimetric measurement of chloroform extracts of supernatants (Figure 2C).

Figure 1. *P. aeruginosa*-S. aureus co-culture growth. (A) Growth of *S. aureus* monocultures and co-cultures with *P. aeruginosa*. Mucins significantly increased *S. aureus* viability in co-culture at 6, 9, and 12 hours using an unpaired t-test, *p*<0.05. (B) Growth of *P. aeruginosa* monocultures and co-cultures with *S. aureus*. Viability was determined by CFU counts. Error bars represent standard deviation of biological replicates (n=4).

Figure 2. Mucins suppress the inhibitory effects of pyocyanin. (A) Growth curve of *S. aureus* UAMS-1 in 100 μM pyocyanin. Mucins significantly increased *S. aureus* viability in pyocyanin cultures at 3, 6, and 9 hours using an unpaired t-test, *p*<0.05. (B) *S. aureus* respiration, as measured with Alamar Blue. Reported values are the relative Alamar Blue signal of bacteria treated with 100 μM pyocyanin normalized to control bacteria. (C) *S. aureus* ROS production, measured with H2DCF ROS dye. Reported values are fold change in H2DCF signal of bacteria treated with 100 μM pyocyanin normalized to control bacteria. (D) *P. aeruginosa* PA14 pyocyanin production. Pyocyanin was extracted from *P. aeruginosa* monocultures or co-cultures with *S. aureus*, and quantified colorimetrically. Error bars represent standard deviation of biological replicates (n≥3). *p*<0.05 by unpaired t-test.
To determine if mucins protect *S. aureus* against pyocyanin, I examined the antagonistic effects of the phenazine in the presence of mucins. First, I evaluated the viability of *S. aureus* cultures challenged with 100 µM purified pyocyanin. Without mucins, pyocyanin efficiently suppressed *S. aureus* growth (Fig. 2A). However, in the presence of mucins, *S. aureus* could partially overcome the pyocyanin-associated growth inhibition, suggesting that mucins can mitigate the antimicrobial activity of pyocyanin toward *S. aureus* (Figure 2A).

I next tested if mucins influence the ability of pyocyanin to inhibit aerobic respiration and stimulate reactive oxygen species (ROS). First, *S. aureus* respiration was measured in the presence and absence of mucins using the metabolic indicator Alamar Blue, which fluoresces upon reduction by the electron transport chain. In the absence of mucins, pyocyanin suppressed respiration by 65% (Figure 2B). In contrast, in the presence of mucins, pyocyanin reduced respiration only by 32% (Figure 2B), indicating that mucins can partially prevent pyocyanin-mediated inhibition of *S. aureus* respiration. Similarly, I measured the ROS levels in *S. aureus* after pyocyanin exposure using the fluorescent ROS indicator H2DCF. Figure 2C reveals that pyocyanin exposure stimulated a 5.0 fold increase in *S. aureus* ROS levels relative to that of the pyocyanin-free control. However, in the presence of mucins, the same concentration of pyocyanin only resulted in a 2.3 fold increase in ROS levels. Together these experiments show that mucins suppress the antagonistic effects of pyocyanin against *S. aureus*.

**Mucins restrict pyocyanin diffusion**

How do mucins suppress pyocyanin activity? Mucins may sequester pyocyanin to lower the effective concentration that can access *S. aureus* cells. To investigate this hypothesis, I used the differential radial capillary action of ligand assay (DRaCALA) (27), which exploits the ability of nitrocellulose membranes to immobilize proteins but not small molecules. 200 µM pyocyanin in buffer with or without mucins was applied to the membrane. Capillary action caused unbound pyocyanin to radially migrate from the initial spot of application, whereas in the presence of mucins, pyocyanin remained associated with the immobile initial spot, indicating an interaction between mucins and the pyocyanin (Figure 3A). Quantification of pyocyanin pigment intensity at the center revealed that the degree of pyocyanin retention at the initial spot of
Figure 3. Mucins restrict pyocyanin diffusion. (A) Schematic of DRaCALA. A mixture of mucins and pyocyanin are spotted onto a nitrocellulose membrane. Radial capillary action causes unbound pyocyanin to diffuse outward, while pyocyanin bound to immobilized mucins remain associated with the inner spot. (B) Quantification of pyocyanin bound to mucins in the inner spot of the membrane, measured by pigment intensity using ImageJ. Error bars represent standard deviation of biological replicates (n=3). *p<0.05 and **p<0.01 by ANOVA.

application correlated with the mucin concentration present (Figure 3B). This experiment suggests that pyocyanin can be bound by mucins. Future work is needed to determine if pyocyanin-mucin binding indeed leads to the reduced antagonism toward S. aureus.

Discussion

The goal of this study was to test if mucus components, specifically its gel-forming mucin polymers, influence the interactions between P. aeruginosa and S. aureus. This is an important question because microbial physiology and polymicrobial communities are often studied in laboratory conditions that lack essential elements of the native environments where these microbes are found. The data here show that the presence of mucins can alter P. aeruginosa and S. aureus dynamics. Specifically, mucins promote co-existence of these organisms, which is interesting because P. aeruginosa outcompetes S. aureus in most laboratory culture conditions (17, 18). This finding may explain why P. aeruginosa and S. aureus are found together on mucosal epithelia, but not in less complex laboratory broth cultures.
While the mechanisms by which mucins affect *P. aeruginosa* and *S. aureus* interactions remain unknown, the data here indicate that neutralization of pyocyanin by mucins may contribute to the protective effect. Mucins, which are known to interact with a range of particles, proteins, and small molecules like antibiotics (29, 30), may reduce the antagonistic effects of pyocyanin by direct binding with the phenazine. Mucin-pyocyanin interactions may lead to sequestration, effectively lowering the amount of pyocyanin that can access *S. aureus* cells. Supporting this hypothesis, the data presented here indicate that mucins can reduce the diffusion of pyocyanin. Because the pore size of mucin networks are two orders of magnitude larger than small molecules like pyocyanin (31–33), mucins probably inhibit diffusion via pyocyanin-mucin binding rather than by size exclusion. Specifically, hydrophobic moieties of pyocyanin may interact with hydrophobic regions on the mucin peptide backbone. The ability of mucins to reduce pyocyanin diffusion is somewhat specific, as preliminary experiments show that albumin cannot achieve this effect.

Another mechanism by which mucins could reduce antagonism of pyocyanin or other *P. aeruginosa* anti-staphylococcal systems is by altering the physiology of *S. aureus* to render it more resistant. It is possible that a metabolic switch from aerobic respiration to fermentation could confer resistance to pyocyanin and other respiratory inhibitors, which are only antagonistic under aerobic conditions (24). This possibility should be further examined, for example by analysis of oxygen levels and fermentation genes and products in mucin-containing and mucin-free cultures. Other physiological responses of *S. aureus* to mucins may also provide protection against *P. aeruginosa*, for example mucins could stimulate production of factors that degrade or inactivate pyocyanin or other *P. aeruginosa* toxins. Further investigation is required to elucidate the mechanisms of mucin protection of *S. aureus* against pyocyanin and broader *P. aeruginosa* antagonism.

The discovery that mucins impact *S. aureus* and *P. aeruginosa* interactions may have important implications for other mucosal microbes. The ability of mucins to dictate interspecies dynamics likely extends to other microbe-microbe, as well as microbe-host interactions. Additionally, since mucins can support the growth of *S. aureus* in otherwise non-permissive conditions, mucins may be a helpful tool for isolating other mucosal inhabitants that are difficult to culture, for example members of the gut microbiota. This work emphasizes the importance of recreating physiologically relevant conditions to study microbial behavior.
References


Appendix C

Mucins suppress *P. aeruginosa* quorum sensing genes

Introduction

Virulence of the opportunistic mucosal pathogen *Pseudomonas aeruginosa* is largely controlled by the quorum sensing cell communication systems. In these systems, bacteria secrete signaling molecules called autoinducers, which accumulate in the surrounding environment and are sensed by neighboring cells. Quorum sensing was first described as a bacterial response to cellular density (7), however recent work revealed that environmental conditions independent of cell density also regulate *P. aeruginosa* quorum sensing (8, 9). To better understand *P. aeruginosa* mucosal infections, it is necessary to study its quorum sensing systems in physiological environments because 1) autoinducers must diffuse through the extracellular environment—in this case mucus—to reach neighboring cells, and 2) environmental factors can impact quorum sensing signaling. Furthermore, recent studies point to mucin glycoproteins, the primary gel-forming mucus component, as regulators of microbial virulence (13–15). Using native purified mucins, I demonstrate that mucins suppress *P. aeruginosa* quorum sensing gene expression.

Materials and Methods

**Bacterial strains and culture conditions**

*P. aeruginosa* PA14 strains were maintained in M9 media with 1% glucose (w/v) and 1% casamino acids with 500 µg/ml kanamycin when required. To monitor gene expression, PA14 was transformed with lux reporter plasmids in the pMS402 background (8).

**Mucin purification**

This study used native porcine gastric mucin, which differ from industrially purified properties in their rheological properties (17). Native mucins were purified as previously
reported (19). Briefly, mucus was scraped from fresh pig stomachs and solubilized in sodium chloride buffer with protease inhibitors and sodium azide. Insoluble material was pelleted by ultracentrifugation and mucins were purified using size exclusion chromatography on a Sepharose CL-2B column. Mucin fractions were desalted, concentrated, and lyophilized for storage. Lyophilized mucins were reconstituted shaking gently at 4°C overnight in the M9 media.

**Transcriptional reporter assay**

*P. aeruginosa* gene expression was monitored in PA14 harboring pMS402 lux-based reporter plasmids (8). Reporter bacteria were inoculated from exponential growth to OD<sub>600</sub> 0.05 in M9 media a black, clear bottom 96 well plate. Cultures were grown shaking at 37°C in a Tecan Infinite 200 PRO microplate reader, and reporter activity (luminescence, 1000ms integration time) and bacterial growth (OD<sub>600</sub>) were measured every 30 minutes for 15 hours.

**Results**

**Mucins suppress *P. aeruginosa* Las and Rhl quorum sensing genes**

To determine the effect mucins on *P. aeruginosa* quorum sensing, gene expression of autoinducer synthases (*lasI, rhlI*) were measured using transcriptional reporters. PAO1 with plasmids containing lux promoter fusions were inoculated into M9 media and grown at 37°C with shaking. Mucins, which did not affect bacterial growth (Figure 1A), suppressed both *lasI* and *rhlI* quorum sensing genes. The maximum expression of *lasI* occurred around 5 hours of growth, and was suppressed by ~30% (Figure 1B) in the presence of mucins. For *rhlI*, maximum

![Figure 1](image-url)

*Figure 1.* Mucins suppress *P. aeruginosa* quorum sensing genes. (A) Growth curve of *P. aeruginosa* with M9 media with or without 0.5% mucins. (B, C) *P. aeruginosa* expression of (B) *lasI* or (C) *rhlI* determined using luminescent transcriptional reporters. Reported values are luminescence values normalized to the highest value within each experiment. Error bars represent standard deviation of biological replicates (n=3).
expression was delayed from early stationary phase (9 hours of growth) in the mucin-free control, to late stationary phase (15 hours of growth) in the presence of mucins (Figure 1C).

**Mucins suppress quorum sensing-regulated virulence genes**

Given that *P. aeruginosa* quorum sensing controls virulence genes, I next used transcriptional reporters to examine whether mucins could also suppress quorum sensing-regulated virulence genes phenazine *phzA1*, protease *aprA*, and rhamolipid *rhlA*. The expression of all three virulence genes was suppressed in mucins (Figure 2A-C). Of note, the virulence gene expression in mucins in late stationary phase is similar to transcriptional levels found in mucin-free media. This may be due to a growth-phase dependent mucin response or degradation of mucins such that the biopolymers can no longer impact quorum sensing or other genes. These results indicate that mucins suppress *P. aeruginosa* virulence genes at the level of transcription.

![Figure 2. Mucins suppress *P. aeruginosa* virulence genes. *P. aeruginosa* expression of (A) *aprA*, (B) *phzA1*, and (C) *rhlA* determined using luminescent transcriptional reporters. Reported values are luminescence values normalized to the highest value within each experiment. Error bars represent standard deviation of biological replicates (n=3).](image)

**Discussion**

Mucins have long been known to contribute to mucus' barrier properties (10–12). Recent studies describing mucins as microbial virulence modulators (14) led me to investigate the effect of mucins on *P. aeruginosa* virulence, specifically quorum sensing genes. The findings here indicate that mucins can suppress *P. aeruginosa* quorum sensing and virulence traits, and corroborate the broader notion that mucins regulate microbial virulence. One possible mechanism is that mucins sequester the autoinducer signals, thereby interrupting the positive feedback quorum sensing systems. Another possibility is that the mucin environment triggers a physiological change that results in intracellular regulation of quorum sensing signaling and virulence. Mucin-mediated virulence suppression may be a host defense strategy to keep these
opportunists in check. Further work is necessary to understand the mechanisms by which mucins interfere with *P. aeruginosa* quorum sensing.

**References**


Appendix D

Mucins reduce *P. aeruginosa* epithelial attachment and cytotoxicity

**Introduction**

*Pseudomonas aeruginosa* causes infection in mucosal environments, including the respiratory (1–3) and intestinal tracts (4, 5), where it can cause epithelial injury (6). *In vitro* studies of epithelial cytotoxicity by *P. aeruginosa* primarily use cell culture models that lack the protective mucus barrier. However, mucus and specifically its gel-forming polymers, mucins, play an important role in mediating host-microbe interactions *in vivo* (7, 8). Previous work examining the impact of mucins on *P. aeruginosa* behavior showed that the biopolymers could reduce bacterial surface attachment to abiotic surfaces (9). In this study, I use A549 lung epithelial cells as an *in vitro* model to study the effects of mucins on *P. aeruginosa* attachment. The data here demonstrate that mucins reduce *P. aeruginosa* attachment to epithelial cells. Furthermore, I investigated how mucins impacted epithelial viability in response to *P. aeruginosa* exposure because direct pathogen-host cell contact can lead to epithelial injury (10–12). I found that mucins can protect epithelial cells against *P. aeruginosa* cytotoxicity. The findings here support the notion that mucins act as a barrier to protect against microbial invasion (11–13).

**Materials and Methods**

*Bacterial strains and culture conditions*

*P. aeruginosa* PA14 strains were maintained in Luria Broth (LB) medium. For microscopic visualization, PA14-GFP was made by transforming PA14 with pBBR1(MCS5)-Plac-gfp, and grown in 30 μg/ml gentamicin to maintain the plasmid.
**Mucin purification**

This study used native porcine gastric mucins, which differ from industrially purified properties in their rheological properties (16). Native mucins were purified as previously reported (17). Briefly, mucus was scraped from fresh pig stomachs and solubilized in sodium chloride buffer with protease inhibitors and sodium azide. Insoluble material was pelleted by ultracentrifugation and mucins were purified using size exclusion chromatography on a Sepharose CL-2B column. Mucin fractions were desalted, concentrated, and lyophilized for storage. Lyophilized mucins were reconstituted shaking gently at 4°C overnight in the Ham’s F-12 (Kaighn’s) medium (Invitrogen).

**Epithelial cell culture**

A549 airway epithelial cells were maintained in T25 flasks in Ham’s F-12 (Kaighn’s) medium (Invitrogen) with 10% fetal bovine serum (Invitrogen) and 1% antibiotics (25 U/mL penicillin, 25 μg/mL streptomycin (Invitrogen)). The cells were detached using trypsin-EDTA (Invitrogen) and passaged before reaching confluence, approximately every 2-3 days.

**P. aeruginosa attachment assay**

A549 cells were seeded in 96 well plates at a density of 2 x 10⁴ cells/well and incubated overnight at 37°C with 5% CO₂. 1 hour prior to infection, wells were washed with PBS, and incubated in unmodified in Ham’s F-12 (Kaighn’s) medium (Invitrogen). A549 cells were exposed to PA14-GFP in unmodified Ham’s F-12 (Kaighn’s) medium at multiplicity of infection (MOI) 10 for 15 minutes at 37°C with 5% CO₂. Non-adherent bacteria were removed by aspiration and adherent bacteria were visualized with phase contrast and epifluorescence microscopy using an Axio Observer Z1 inverted epifluorescence microscope (Zeiss) with a 40x/0.75 NA dry objective (Zeiss). The number of fluorescent bacteria and epithelial cells in each frame were manually counted.

**Epithelial cytotoxicity assay**

A549 cells were seeded in 96-well plates at a density of 2 x 10⁴ cells/well and incubated overnight at 37°C with 5% CO₂. 1 hour prior to infection, wells were washed with PBS, and incubated in unmodified in Ham’s F-12 (Kaighn’s) medium (Invitrogen). PA14-GFP grown to
exponential phase in LB was pelleted, and resuspended in serum-free Ham’s F-12 (Kaighn’s) medium. PA14 was inoculated in serum-free Ham’s F-12 (Kaighn’s) medium containing mucin as indicated. For cytotoxicity experiments, A549 was infected with PA14 at multiplicity of infection (MOI) 50 for 1 h at 37°C with 5% CO₂. Bacteria were removed and replaced with serum-free Ham’s F-12 (Kaighn’s) medium with 200 μg/ml gentamicin for 30 min at 37°C with 5% CO₂ to kill adherent bacteria. The metabolic indicator dye Alamar Blue was then added to measure epithelial cell survival as a measure of metabolic activity. After 3 hours of incubation at 37°C with 5% CO₂, viability was determined by measuring Alamar blue fluorescence (Ex. 530 nm/Em. 590 nm).

Results

Mucins reduce *P. aeruginosa* epithelial attachment

Mucins can prevent *P. aeruginosa* attachment to synthetic surfaces (9), however the ability of native mucins to protect epithelial surfaces against *P. aeruginosa* has not yet been studied. Using A549 human lung carcinoma cells to model the respiratory epithelium, I examined epithelial attachment of the GFP-expressing *P. aeruginosa* strain PA14-GFP.

![Figure 1](image)

**Figure 1.** Mucins suppress *P. aeruginosa* attachment to A549 epithelial cells. (A) Top: Phase contrast images of A549 epithelial cells. Bottom: Fluorescent images of PA14-GFP attached to A549 epithelial cells. Scale bar=10 μm. (B) Quantification of *P. aeruginosa* attachment to A549 epithelial cells. Reported values are number of attached *P. aeruginosa* cells normalized to total number of epithelial cells. Error bars represent standard deviation of biological replicates (n=3). *p≤0.05 by unpaired t-test.
Epithelial cells were incubated with PA14-GFP for 15 minutes in the presence or absence of 0.5% mucins, and then non-adherent bacteria were removed. Microscopy of attached bacteria revealed that *P. aeruginosa* epithelial attachment was reduced from an average 0.81 bacteria per epithelial cell in the mucin-free control to 0.35 bacteria per epithelial cell in the presence of mucins (Figure 1).

**Mucins protect epithelial cells against *P. aeruginosa* cytotoxicity**

The ability of mucins to reduce *P. aeruginosa* attachment to epithelial cells suggests that the polymers may also affect the epithelial viability in response to bacterial exposure. Direct contact of *P. aeruginosa* with host cells is known to contribute to epithelial death via the type III secretion (T3SS) (10). To test if mucins affect *P. aeruginosa* epithelial cytotoxicity, I used the fluorescent dye Alamar Blue to measure A549 epithelial cell viability after exposure to PA14 for 1 hour in the presence or absence of the polymers. Figure 2 shows that mucins suppressed *P. aeruginosa* epithelial cytotoxicity in a concentration dependent manner: 0.25%, 0.5%, and 1% mucins reduced cytotoxicity from 80% (mucin-free control) to 43.8%, 37.8%, and 17.4%, respectively (Figure 2). These experiments suggest that mucins protect epithelial cells from cytotoxicity by *P. aeruginosa*.

![Figure 2](image)

*Figure 2. Mucins protect epithelial cells against *P. aeruginosa* cytotoxicity. A549 epithelial cells were evaluated for viability using the fluorescent Alamar Blue viability dye after exposure to *P. aeruginosa* PA14. Reported values are fluorescence of epithelial cells exposed to *P. aeruginosa* normalized to untreated control epithelial cells. Error bars represent standard deviation of biological replicates (n=3). *p≤0.05 by ANOVA.*
Discussion

Mucins have been demonstrated to protect epithelia from attachment by *Helicobacter pylori* (18), *Staphylococcus aureus* (19, 20), *Candida albicans* (21), and gut commensals (8). Here, I show that mucins can suppress *P. aeruginosa* epithelial attachment, as well as cytotoxicity. There are several mechanisms by which mucins could confer these protective effects. Mucins may block bacterial access to the epithelial cells by providing a physical or biochemical barrier. Furthermore, the biopolymers may prevent epithelial interactions by occupying bacterial receptors necessary for epithelial binding. Since the *P. aeruginosa* type 3 secretion system (T3SS), a primary mechanism of epithelial cytotoxicity, requires direct host cell contact, then suppression of *P. aeruginosa* epithelial attachment by mucins could contribute to the reduced levels of cytotoxicity. In addition, mucins might reduce bacteria-induced epithelial injury by triggering physiological changes in *P. aeruginosa* that downregulate virulence factors. This phenomenon has previously been demonstrated for *C. albicans*, which suppresses pathogenic traits like hyphal formation in the presence of mucins (21). The results presented here support the notion that mucins are a key contributor of the ability of mucus to regulate microbial invasion and toxicity.

References


Chapter 4

Discussion

Mucus and mucins in vivo protect the host epithelia against mucosal microbes by acting as a physico-chemical barrier and displaying antimicrobial factors (1, 2). Beyond its barrier function, mucins are emerging as modulators of microbial physiology. This thesis demonstrates several bacteria-regulating functions of mucins. Specifically, mucins can impact the development and disassembly of bacterial biofilms and regulate intraspecies and interspecies microbe-microbe single species and multi-species communities. Whereas most research prior to this thesis focused on how microbes affect mucins, this work focuses on the opposite question: how do mucins influence bacterial behavior? Based on my results, I suggest that mucins are potent regulators of bacterial behavior inside the body, and have strong potential to be used in antimicrobial strategies in clinical contexts.

In Chapter 2, we showed that purified mucins can be used to generate surface coatings that suppress the attachment of Streptococcus pneumoniae and Staphylococcus aureus. We demonstrated through the removal of mucin-associated glycans that these sugars provide structure to the coatings, and are necessary for bacterial repulsion. These findings implicate both mucin polymer brush structure and the use of glycans as considerations for the development of better anti-adhesive coatings.

Mucin-associated glycans also play a role in reducing bacterial attachment in vivo (3), however, it is unclear whether the mechanisms of repulsion are similar in vivo and for mucin coatings. While mucins in vivo are anchored to epithelial membranes or polymerized in a hydrogel network, mucins adsorbed to polystyrene as coatings may differ in their biochemistry and structure. For coatings, interactions between the mucins and the underlying polystyrene likely occupy or occlude bacterial binding sites. Additionally, surface adsorption may cause mucin denaturation, thereby altering mucin conformation and potentially introducing new binding sites. Thus, our insights to bacterial repulsion by mucin surface coatings cannot be directly applied to explain microbe-mucin interactions in vivo. Despite this, the success of mucin
coatings in our in vitro system suggests that they are valuable tools for hindering bacterial surface attachment.

In addition to hindering bacterial attachment, the first stage of biofilm formation, my results in Chapter 3 show that mucins mediate the dissociation of mature P. aeruginosa biofilms. This finding demonstrates the potential of mucins as anti-biofilm strategies, which is exciting because new and more efficacious biocompatible strategies are urgently needed. The data presented here suggests that this effect is specific to mucins, as other synthetic polymers tested in this study could not achieve the same outcome. Although the mechanism behind mucin-mediated biofilm disassembly is not fully understood, I demonstrate in Appendix A the involvement of the flagella in this phenomenon. Biofilms of swimming-impaired fliD or motABCD mutants were resistant to disruption by mucins, suggesting that flagellar motility plays an important role in the response of P. aeruginosa biofilms to mucins. Mucins may stimulate a chemotactic or biofilm dispersal response, which both engage swimming motility (4–6). To identify the physiological pathways that are triggered by mucins, transcriptomic or proteomic analyses of mucin-treated biofilms may be informative. Elucidating the specific mechanisms of mucin-mediated disassembly could reveal new pathways that can be targeted to control biofilms.

The work presented in Chapter 3 and Appendix A focus on how P. aeruginosa biofilms respond to mucins, but it is currently unknown whether mucins can disrupt biofilms formed by other microorganisms. Therefore, more exploration is necessary to understand the extent of the biofilm-disrupting capability of mucins. Moreover, the specific effects of mucins on biofilms require further characterization. For example, do mucins sensitize biofilms to antibiotic treatments? Broader understanding of the impacts of mucins on biofilms may lead to the use of mucins or mucin-inspired strategies to eradicate biofilms.

After establishing that mucins affect the physiology of monospecies cultures, I next explored the impacts of mucins on dual species interactions. In Appendix B, I found that mucins promoted S. aureus survival in the presence of P. aeruginosa. This finding provides an explanation for why these organisms are often co-isolated from mucosal infections in vivo (7–9), despite their inability to co-exist in most laboratory conditions (10–12). Furthermore, I demonstrate that mucins suppress the anti-staphylococcal effects of P. aeruginosa pyocyanin. Binding experiments suggest that mucins may protect S. aureus by sequestering pyocyanin, however this does not rule out the possibility that a physiological response of S. aureus to
mucins contributes to a protected state. Although the specific mechanisms by which mucins protect *S. aureus* against *P. aeruginosa* remain unclear, this phenomenon highlights the importance of mucins and mucus as a consideration when studying mucosal microbes.

Since biofilm formation and interspecies interactions both rely on bacterial communication, I hypothesized that quorum sensing may be regulated by mucins. Appendix C reveals that mucins regulate *P. aeruginosa* intra-species interactions by suppressing Las and Rhl quorum sensing and related virulence genes. The mechanisms of mucin quorum sensing regulation have not yet been elucidated, however this finding illustrates a specific microbe-microbe interaction that is modulated by mucins. Since quorum sensing is also used in interspecies communication (13, 14), it is possible that mucin modulation of quorum sensing systems can influence the behavior of multispecies communities. Notably, the quorum sensing regulated phenazine biosynthesis *phzA1* gene was downregulated by mucins, which contrasts with the finding in Appendix B that similar levels of pyocyanin are produced in the presence and absence of mucins. This discrepancy might be explained by compensation for *phzA1* suppression by expression of the redundant phenazine biosynthesis gene *phzA2* (15). Also, expression levels of downstream genes in the phenazine biosynthesis pathway, for example *phzM* or *phzS*, may be more important in determining pyocyanin production levels. Another explanation is that pyocyanin production is upregulated by alternate regulatory pathways. For example the mucin glycan N-Acetylglucosamine (GlcNAc) is known to stimulate pyocyanin via the two-component regulator PA0601 (16). While the specific pyocyanin biosynthesis genes involved in the PA0601-GlcNAc response have not yet been identified, it is possible that activation of this pathway could compensate for suppression of *phzA1* gene expression.

Although the exact mechanisms of mucin-mediated effects on bacterial behavior are currently unknown, the biopolymers remain an important consideration when examining mucosal microbes. Mucins may modulate microbial interactions by direct mucin-microbe interaction or indirectly by affecting the availability of nutrients or signaling molecules. These interactions are relevant for the study of both commensal and pathogenic microbes associated with mucus. Commensal microbes are likely well adapted for survival within the mucus layer, which may explain why many members of the gut microbiome have been difficult to culture in mucin-free laboratory conditions. The addition of mucins to cultures may better mimic the physiological environment and improve the ability to isolate otherwise unculturable mucosal
microbes. Furthermore, mucins are useful as an in vitro model for complex mucus during investigations of mucosal microorganisms individually and within polymicrobial communities.

Similarly, mucins are important for investigating interactions between mucosal microbes and the host epithelia. Although mucins have been shown to prevent epithelial attachment against pathogens Helicobacter pylori (17, 18) and S. aureus (19, 20), their role in epithelial protection against P. aeruginosa had not previously been demonstrated. Appendix D confirms the protective role for mucins against P. aeruginosa epithelial attachment and cytotoxicity. Mucins also likely impact the behavior of commensal microbes, whose interactions with each other and the epithelia regulate a multitude of host processes (21). Due to their impacts on microbial behavior, as well as their influence on the transport of microbial products and resources, mucins are an important factor to include when studying mucosal microbes.

Overall, this thesis shows that mucins are important regulators of biofilms and other microbial communities. Mucus is difficult to study due to its complexity and heterogeneity, however mucins are a useful tool to model physiological mucus conditions. Beyond its barrier functions, the roles of mucins in regulating microbial physiology are beginning to emerge (22–24). As we better understand the interactions of mucins with microbes, there is potential to translate this knowledge to apply mucins to modulate microbial behavior.

References


