Summer 7-2022

Molecular Mechanisms Underlying Mucosal Attachment and Colonization by *Clostridioides difficile*

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MOLECULAR MECHANISMS UNDERLYING MUCOSAL ATTACHMENT AND COLONIZATION BY *CLOSTRIDIOIDES DIFFICILE*

by

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A THESIS

Presented to the Faculty of

The Graduate College at the University of Nebraska

In Partial Fulfillment of Requirements

For the Degree of Master of Science

Major: Food Science and Technology

Under the Supervision of Professor Kurt H. Piepenbrink

Lincoln, NE

July 2022

MOLECULAR MECHANISMS UNDERLYING MUCOSAL ATTACHMENT AND COLONIZATION BY CLOSTRIDIOIDES DIFFICILE

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University of Nebraska, 2022

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Clostridioides difficile is a Gram-positive, spore-forming, obligate anaerobic bacterium which causes gastrointestinal disease and is a leading cause of nosocomial infections. Although infection typically occurs following antibiotic therapy, in recent years there has been an increase in infections which are not preceded by antibiotic use. Additionally, community-associated infections and rates of disease recurrence have increased. While it is understood that a healthy gastrointestinal microbiota provides protection against infection, the molecular mechanisms which underly *C. difficile'*s ability to colonize and persist in the gut are mostly unknown. Building on work from others that suggests *C. difficile* associates with the outer mucus layer during infection, we utilized an *in vitro* mucus layer model to probe attachment and colonization mechanisms. Using gene-interruption mutants of the major subunits of type IV pili and flagella, two extracellular appendages implicated in adhesion, we observed that the presence of flagella facilitates initial mucus attachment. Adhesion was variable across multiple strains of *C. difficile* and was also dependent on the source of mucin derivation. When mucin glycans were modified by mucin-degrading bacteria, *C. difficile* attachment decreased in our model. Collectively, our findings suggest that adherence to mucin likely influences *C. difficile'*s ability to colonize and may offer novel strategies for future therapeutics once the underlying mechanisms are better understood.

ACKNOWLEDGMENTS

I would like to express my appreciation for my advisor, Dr. Kurt Piepenbrink, for providing me with the opportunity to work and grow in his lab. Through his mentorship, my interest in research and scientific curiosity has grown immensely. I'm truly grateful for the skills and lessons I have learned as a member of his research group, and I'm confident they will serve me well in future pursuits.

I would also like to acknowledge the support and guidance provided by the members of my advisory committee, Dr. Jennifer Auchtung and Dr. Devin Rose. Their advice on this project, suggestions for navigating career decisions, and commitment to my academic success were integral to my professional development and the completion of this thesis. A special thanks to Dr. Jennifer Auchtung, Dr. Tommy Auchtung, and all the members of the Auchtung laboratory for sharing space and time in the anaerobic chamber.

Thank you to my laboratory mates, Leslie Ronish and Yafan Yu, for experimental advice, help with preparing reagents, and companionship through the stressful days of graduate work. Thank you to Hugh McCullough for mucus samples and training on mammalian cell culture maintenance.

Finally, a huge thank you to family and friends near and far for advice, open ears, and encouragement. I'm so appreciative of your support over the years, and feel I'll never be able to adequately express my gratitude enough.

This research was performed in part in the Nebraska Nanoscale Facility National Nanotechnology Coordinated Infrastructure and the Nebraska Center for Materials and

Nanoscience, which are supported by the National Science Foundation under Award ECCS: 2025298, and the Nebraska Research Initiative.

PREFACE

This thesis is comprised of three chapters. Chapter 1 includes a brief overview of *Clostridioides difficile* infection and the current state of scientific knowledge regarding mechanisms of colonization. Chapter 2 explores the possible role of two putative colonization factors, type IV pili and flagella, in mediating *C. difficile* adhesion to mucus. Additionally, this chapter includes investigations into the conservation of mucus adhesion across several *C. difficile* strains, and adhesion to mucins derived from different sources. Finally, Chapter 3 describes the possible role of inter-microbial competition between mucus-associated microbes in modulating the attachment of *C. difficile* to mucus.

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CHAPTER ONE: LITERATURE REVIEW

1.1 *CLOSTRIDIOIDES DIFFICILE*: A NOSOCOMIAL GUT PATHOGEN

Clostridioides difficile (recently reclassified from *Clostridium difficile* (Lawson *et al.*, 2016)) is an opportunistic, spore-forming pathogen capable of causing gastrointestinal illness. Disease severity ranges from mild, self-limiting diarrhea to life-threatening pseudomembranous colitis or toxic megacolon. Recent emergence of hypervirulent clinical isolates with increased antibiotic resistance and rates of disease recurrence has led the United States Centers for Disease Control and Prevention (CDC) to classify *C. difficile* under the 'urgent' public health threat level (Fatima & Aziz, 2019). Even so, rates of community transmission continue to rise (Durovic *et al.*, 2018). More research into *C. difficile* pathogenesis and disease mechanisms is needed to develop novel prevention strategies and precision therapeutics. In this review, I will briefly highlight the pathogenic strategy of *C. difficile*, discuss general mechanisms of gastrointestinal colonization and persistence, and provide direction for future investigation into *C. difficile*'s interaction with mucus.

1.2 MECHANISMS OF COLONIZATION AND DISEASE PROGRESSION

1.2.1 Spore transmission and germination

As an anaerobic bacterium, vegetative *C. difficile* cells are unable to survive the aerobic environment outside the host (Zhu *et al.*, 2018). Fecal-oral transmission of dormant spores between hosts is therefore a key aspect of *C. difficile* pathogenesis. The

protective coating of spores allows them to persist on surfaces and in the soil for months and sometimes years. Potential reservoirs of infectious spores include asymptomatic carriers, infected patients, livestock or animal intestinal tracts, and contamination in the environment (Czepiel *et al.*, 2019). In addition to oxygen, spores are resistant to ethanol, hydrogen peroxide, chloroform, and heat, greatly limiting the effectiveness of decontamination efforts (Edwards, Karim, *et al.*, 2016; Gil *et al.*, 2017).

Following ingestion by the host, *C. difficile* spores transit the gastrointestinal tract (GIT), experiencing no apparent loss in viability after exposure to digestive enzymes (Escobar-Cortés *et al.*, 2013). Although spores are considered metabolically dormant, they still respond to signaling in the intestinal environment for germination-inducing small molecules known as germinants (Shrestha *et al.*, 2019). These small molecules are species specific despite significant conservation of endospore structure across endospore-forming bacteria. In the case of CDI, a few important germinants include bile acids in combination with specific amino acids (Howerton *et al.*, 2011; Shrestha & Sorg, 2018). One mechanism by which the natural gut microbiota provides protection against *C. difficile* germination is through the conversion of primary conjugated bile acids to unconjugated primary and secondary bile acids which are less effective at promoting germination, and sometimes even inhibitory (Theriot *et al.*, 2016). Like other forms of colonization resistance, microbial bile acid conversion can be greatly diminished because of antibiotic therapy, aging, and immune deficiency.

1.2.2 Colonization resistance: protection by resident gut flora

Beyond regulating levels of primary and secondary bile acids, the resident gut flora provides additional forms of colonization resistance against *C. difficile*. Other

nutrient sources or metabolites can be converted to inhibitory molecules that impact vegetative cell proliferation or the ability of spores to germinate. There can also be direct competition for nutrients between *C. difficile* and other microbes. For example, the availability of different carbon sources and the way in which they are metabolized by *C. difficile* has been shown to greatly influence toxin production, likely playing a key role in mediating the progression of CDI (Neumann-Schaal *et al.*, 2015).

The availability of dietary and host-derived carbohydrates in the colon is known to greatly influence gut microbial diversity and therefore protection against CDI. It was recently shown that *C. difficile* is able to cross-feed and catabolize mucin-derived glycans following enzymatic liberation by other gut bacteria (Engevik *et al.*, 2020). By controlling access to host-derived nutrients, the abundance of primary mucin degrading species may modulate *C. difficile*'s ability to colonize and proliferate. Indirectly, microbes can also influence host immunity, production of antimicrobial peptides, and mucus production, which collectively provide a barrier to colonization by *C. difficile* (Britton & Young, 2014).

Developing a better understanding of the nutrient niche filled by *C. difficile* and its role in colonization resistance may offer promising alternative therapeutic strategies. For example, it was recently demonstrated that pre-colonization of hamsters with non-toxigenic strains of *C. difficile* was capable of protecting against disease following successive challenge with toxigenic *C. difficile* (Gerding *et al.*, 2018). Recent work from Leslie and colleagues investigating the possible mechanisms underlying this conferred protection found that a reduction in intestinal glycine levels and decreased ability for the second strain to germinate may be responsible (Leslie *et al.*, 2021). Future work is

needed to understand the direct role of specific antibiotics in driving the metabolic and ecological shifts which abrogate colonization resistance and promote disease.

1.2.3 Toxin production and sporulation

Following germination and subsequent stages of outgrowth, vegetative cells in the GIT eventually encounter cellular stress which leads to toxin production and spore formation. Although little is known about the regulation of sporulation, it's known to repress motility and toxin production, suggesting that regulation of these phenotypes may be related (Edwards, Tamayo, *et al.*, 2016). The three major toxins produced by *C. difficile* include TcdA, TcdB, and CDT, also known as binary toxin (Aktories *et al.*, 2017). Following secretion, TcdA and TcdB glucosylate small GTP-binding proteins and contribute to disease progression by disrupting tight junctions in the intestinal epithelium, inhibiting actin polymerization, and promoting apoptosis (Di Bella *et al.*, 2016; Voth & Ballard, 2005). Interestingly, CDT promotes the formation of microtubule protrusions at the surface of intestinal epithelial cells, increasing adherence of *C. difficile* (Schwan *et al.*, 2009). Anti-toxin and vaccine therapies have been developed over the last two decades, but they do not prevent colonization or destroy spores (Zhu *et al.*, 2018). Therapeutics which accomplish these goals and protect against the possibility of relapsing CDI should therefore be a primary focus of future investigations.

Although studies focused on *C. difficile* sporulation and toxin production have revealed their important contributions to CDI, little is known about the role of *C. difficile*'s adherence to the host. Furthermore, TcdA and TcdB are thought to be essential for disease development, but are not required for colonization in humans or animals

(Kuehne *et al.*, 2011). For many different enteric pathogens, adherence to the host mucosa is a known requirement for colonization of the GIT and disease progression. Developing an understanding of the genetic and molecular basis for mucosal adherence by *C. difficile* may therefore provide context for mechanisms underlying colonization and persistence during CDI.

1.2.4 Adherence to the gut mucosa

It has been widely demonstrated that adherence to host tissues is a prerequisite for colonization and expression of virulence factors for many enteric pathogens. For example, adherence to the host mucosa is considered to be essential for infection and disease caused by *Helicobacter pylori* (Testerman *et al.*, 2014) and *Campylobacter jejuni* (Konkel *et al.*, 2010). Vegetative *C. difficile* cells are known to colonize the colon during human CDI; colonization of other human bodily sites, including the small intestine, is considered rare (Heinlen & Ballard, 2010). While the colon has been identified as a primary site of colonization, mechanisms underlying host adherence by *C. difficile* are not completely understood. Collective evidence from several previous studies suggest that adherence may be relevant for CDI and should be further probed.

An early study investigating colonization of hamsters by *C. difficile* found that a virulent, toxigenic strain had greater adherence to the gut mucosa than both a less virulent toxigenic strain and a non-toxigenic strain (Borriello *et al.*, 1988). These findings provided preliminary evidence that adherence might determine virulence, but a lack of strain diversity limited formation of such broad conclusions. Later work by another group compared the adherence of 12 different isolates of *C. difficile* in a murine model of infection, finding that toxigenic strains had greater *in vivo* mucosal adherence than non-

toxigenic strains (Gomez-Trevino *et al.*, 1996). Several years later, a separate study observed no difference in *in vitro* adherence between toxigenic and non-toxigenic strains to Vero cells (Anne Judith Waligora *et al.*, 1999). One possible reason for these conflicting findings may stem from the use of Vero cells to model adherence to the intestinal epithelium. Vero cells are derived from the kidney of an African green monkey (Ammerman *et al.*, 2008) and therefore likely don't possess exposed receptors identical to those expressed by intestinal epithelial cells.

In addition to receptors on the surface of epithelial cells, the luminally exposed protective outer mucus layer and extracellular matrix (ECM) offer potential receptor binding sites for intestinal microbes (Figure 1.1). If these constituents do in fact contain binding sites that are important for *C. difficile* attachment, this would also help explain conflicting findings in the literature. In fact, evidence suggesting *C. difficile* adheres to mucus has existed in the literature for several decades. Recognizing that mucosal adherence may be a determinant for virulence, Karjalainen and colleagues were interested in identifying a possible bacterial adhesin which could mediate attachment (Karjalainen *et al.*, 1994). A surface protein found to be upregulated following heat-shock and hypothesized to mediate adherence was recombinantly expressed and purified. The 27-kDa hypothetical adhesin adhered to Caco-2 and Vero cells, but this interaction was diminished by co-incubation with axenic murine mucus or N-acetylgalactosamine, a mucus glycan. This was one of the earliest examples of indirect evidence suggesting that *C. difficile* possesses surface proteins which bind mucus or its glycan components. Despite the significance of these findings, the relevance of possible *C. difficile* adherence to mucus during CDI is largely unknown as many investigations have focused on

adherence to epithelial cells. Studies from the last decade have renewed interest in microbe-mucus interactions during CDI.

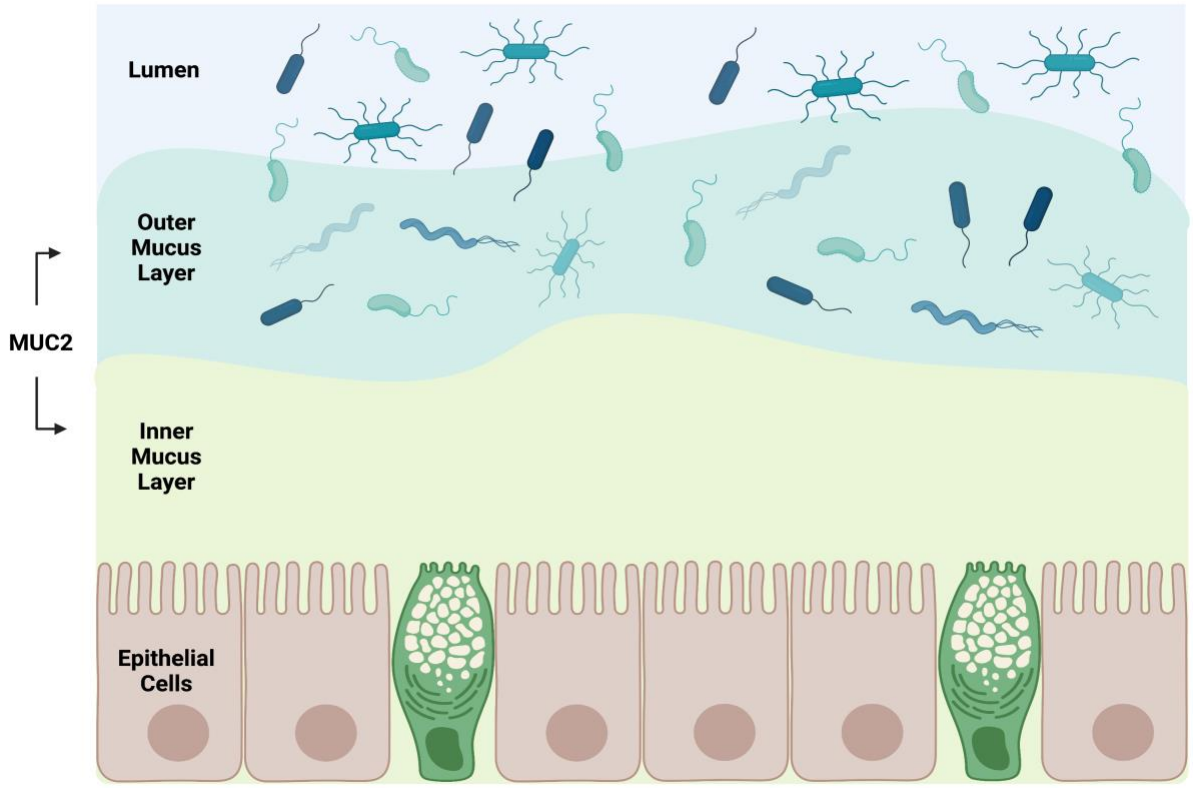

Figure 1.1 Structure of the colonic mucus layer. The colonic mucus layer consists of a firm, adherent inner mucus layer and a loose, outer mucus layer. The most abundant colonic mucin, MUC2, is secreted by goblet cells (green) in the epithelium. O-linked glycans on the surface of mucins provide attachment sites and nutrients for mucus-associated microbes in the outer mucus layer while microbes are typically excluded from the inner layer under healthy conditions. (This figure was created under a paid subscription to Biorender.com)

1.2.5 Association with mucus layer

The mucus layer has unique physical and chemical properties that cause it to compress rapidly under desiccating *ex vivo* conditions such as exposure of excised tissue sections to the open air. Additionally, fixation of tissue sections by conventional cross-linking agents causes complete mucus layer compression, limiting our understanding of its architecture and microbial composition (Johansson *et al.*, 2011). However, tissue fixation with Carnoy's fixative or paraformaldehyde has allowed for better preservation of mucus structure (Hasegawa *et al.*, 2017; Swidsinski *et al.*, 2005) and microscopic observation of bacterial species localized to this region of the mucosa. Recently, *C. difficile* was found to associate with the loose outer mucus layer of the cecum and colon in a murine model of infection (Semenyuk *et al.*, 2015). In contrast to work from other groups, Semenyuk and colleagues found no evidence of colonization or attachment to epithelial cells, suggesting preferential colonization of the murine mucus layer by *C. difficile*.

Although ethical conflicts prohibit researchers from directly evaluating *C. difficile*'s possible association with mucus in the human gut, recent studies have provided compelling evidence that microbe-mucus interactions, including attachment, are relevant to human CDI. For example, CDI patients were reported to have decreased MUC2 mucin expression relative to healthy subjects (Engevik *et al.*, 2015). MUC2 is the most abundant mucin glycoprotein in the human colon (Johansson *et al.*, 2011a) and contains many receptors important for colonization by mucus-associated bacterial species. Altered mucin expression in CDI patients could therefore plausibly impact availability of binding sites for *C. difficile*, greatly influencing virulence and disease severity.

In the same study by Engevik and colleagues, *C. difficile* alone was capable of decreasing MUC2 expression in an infection model using human intestinal organoids (Engevik *et al.*, 2015). Additionally, using mucus extracted from CDI and healthy patient stools, *C. difficile* was found to preferentially adhere to CDI patient mucus (Engevik *et al.*, 2015). A later study also reported preferential *in vitro* adherence to mucus from mucus-secreting human intestinal epithetical cell lines (Engevik *et al.*, 2020). Observed differences in mucin gene expression and relative *C. difficile* adhesion between healthy and infected populations suggest that further investigation of microbe-mucus interactions may elucidate novel targets for therapeutic development in the treatment of CDI.

1.3 TOOLS FOR STUDYING *C. DIFFICILE* ADHESION TO MUCUS *IN VITRO*

Because attachment to the host is considered essential for numerous enteric pathogens and commensals, many *in vitro* models of adhesion to the GIT mucus layer have been developed. The most commonly used method involves passive immobilization of mucus onto 96-well polystyrene plates by overnight incubation at 4°C (Cohen & Laux, 1995). The main advantage of this method is the simplicity of immobilization and that it's relatively inexpensive compared to other models. Additionally, direct bacterial interactions with mucus can be isolated. Hydrophobic interactions with polystyrene can be a problem, however, and background bacterial adherence to plastic wells has been shown to account for a significant amount of detected adhesion (Laparra & Sanz, 2009). This may limit the application of *in vitro* findings to host attachment during CDI *in vivo*.

Adherence to tissue culture cells is another common investigative approach. For enteric bacteria, Caco-2 and HT-29 human intestinal epithelial cell lines are most commonly used. Cell surface factors important for bacterial adherence are incorporated into this model making it a better representation of *in vivo* conditions. However, Caco-2 and HT-29 cell line models do not account for the intestinal mucus layer (van Tassell & Miller, 2011) and are derived from cancer cells, so they may not be representative of healthy tissue. HT-29 MTX cells secrete high levels of mucins and can be used to incorporate a mucus layer into adhesion studies, though they also are not representative of healthy tissue or mucin glycosylation. Furthermore, models utilizing a single cell type don't reflect the cell diversity found within the epithelium. Cell culture models which incorporate cell lines representative of both enterocytes and goblet cells into a single monolayer have been used to study drug absorption, and would likely provide utility in studying adhesion of gut pathogens and commensals (Béduneau *et al.*, 2014; Hilgendorf *et al.*, 2000; Pan *et al.*, 2015).

Finally, tissue sections from healthy digestive tracts can be used for *in vitro* adhesion assays. While human gastrointestinal tissue sections can be difficult to obtain, it's possible to use tissue from livestock and other animals. In the case of gut pathogens like *C. difficile* which are known to infect humans and animals (Weese, 2020), tissue sections from multiple hosts would be desirable for evaluation of possible strain or ribotype adaptation. Adhesion to tissue sections is likely to be most representative of *in vivo* conditions, particularly due to the incorporation of competitive exclusion by the naturally occurring GIT flora (van Tassell & Miller, 2011). However, as with the cell culture models, direct microbe-mucus interactions can be difficult to distinguish from

microbial adhesion to other cell-surface factors. Therefore, if investigation of specific adhesion to mucus is desirable, reducing the complexity of the *in vitro* model used may be desirable prior to use of tissue sections.

1.4 PUTATIVE ADHESINS MEDIATING HOST-MICROBE INTERACTIONS DURING CDI

Building on the early studies highlighting the importance of GIT adherence to CDI, more recent studies have focused on characterizing different spore and cell-surface proteins as putative adhesins (Table 1). While many studies have investigated *C. difficile*'s colonization factors, conflicting findings have limited conclusions regarding their relative importance. A major reason why conclusions cannot be drawn lies in the lack of standardization of *in vitro* and *in vivo* adhesion models across studies. For example, prior studies attempting to model adhesion *in vitro* have used many different cell lines, including Caco-2, Vero, Hep-2, HT-29, and MDCK cells. Some of these cell lines are not derived from the intestinal epithelium and are less than ideal for modeling GIT adherence. Furthermore, differences in media used during the adhesion stage of the assay, inoculum concentration/growth stage, length of incubation, washing steps, and other experimental parameters likely influence results. Despite these limitations, results from previous studies are still important and can be used to guide the development of future investigations. Due to available access to *C. difficile* R20291 type IV pili and flagellar mutants in the Piepenbrink lab, our investigation focuses on characterizing the putative role of these appendages in mediating adhesion to mucus. Other putative adhesins are beyond the scope of this work but have been included in Table 1 for reference and future investigations.

Table 1. Summary of putative *C. difficile* adhesins previously investigated.

Experimental Model	Putative Adhesin	*Cd* Genetic Background	Results	Reference
Caco-2 Cells	Spore Surface Proteins	630	Two 40-45 kDA spore-specific proteins mediate adherence to Caco-2 monolayers	(Paredes-Sabja & Sarker, 2012)
Vero Cells	Cwp66	79-685	Polyclonal antibodies to Cwp66-N and Cwp66-C and Cwp-66N/Cwp66-C protein recombinant proteins inhibited Vero cell adherence	(A. J. Waligora *et al.*, 2001)
Vero Cells	GroEL (Hsp60)	79-685	Polyclonal GroEL-specific antibodies and recombinant GroEL inhibited Vero cell adherence	(C. Hennequin *et al.*, 2001)
Dot Blotting & C3H Mice	FliC and FliD	79-685 ATCC 43593 EX560 ATCC 43598 6058	Recombinant FliC, FliD, and crude flagella adhered to axenic mouse cecal mucus in vitro; Flagellated strains were significantly more adherent to the mouse cecum than non-flagellated strains in vivo	(Tasteyre *et al.*, 2001b)
Hep-2 Cells & Mouse Colon Tissue Sections	Surface Layer Proteins (SLPs)	1 17 630	Native and recombinant High-MW SLPs bound to Hep-2 cells and mouse colon tissue; Low-MW SLPs did not bind as efficiently	(Calabi *et al.*, 2002)
Fibronectin & Vero Cells	Fbp68 (FbpA)	79-685	FbpA-specific antibodies inhibited adherence to fibronectin and Vero cells	(Claire Hennequin *et al.*, 2003)
Caco-2/HT-29 MTX Cells, Mono- and dixenic mice, HMA mice	FbpA	630Δerm	CdΔFbpA mutant had increased adherence to Caco-2/HT-29MTX cells; Colonization of the cecal wall greatly reduced for CdΔFbpA mutant in monoxenic mouse but not in dixenic or HMA model	(Barketi-Klai *et al.*, 2011)
Caco-2 Cells & Hamster	FliC and FliD	630Δerm	FliC and FliD mutants had increased adherence to Caco-2 cells; Flagellar mutants were more virulent in hamster model of infection	(Dingle *et al.*, 2011)

Caco-2 BBE Cells	SlpA	36 Clinical Isolates (epidemic and non-)	Crude and purified SlpA subunits and SlpA antisera inhibited adherence to Caco-2 BBE cells	(Merrigan *et al.*, 2013)
Caco-2 Cells, C3H and HeN Germ-free or HMA Mice	FliC and FliD	630Δerm R20291	R20291 flagellar mutants (FliC and FliD) had decreased adherence to Caco-2 cells while 630Δerm mutants increased; Intact flagellum important for R20291 adherence in vitro and in vivo	(Baban *et al.*, 2013)
Collagen V, IMR-90 Cells	CbpA	630Δerm	ΔCbpA mutant showed no defect in adherence to collagen V or IMR-90 cells; CbpA-expressing L. lactis had increased adherence to collagen V	(Tulli *et al.*, 2013)
Caco-2 Cells	Lipoprotein CD0873	630Δerm	CDΔCD0873 mutant showed deficiency in adherence to Caco-2 cells; Antibodies against CD0873 inhibited adherence	(Kovacs-Simon *et al.*, 2014)
HT-29/Caco-2/MDCK Cells C57BL/6 Mice	PilA1	630Δerm	TFP important for prolonged adherence to epithelial cells in vitro; TFP-null strains had reduced adherence to cecal mucosa in murine infection model	(Mckee *et al.*, 2018)
Caco-2 Cells	Cwp66	630	Cwp66 mutant had decreased adherence to Caco-2 cells; Restored WT adhesion through plasmid-complementation of Cwp66	(Zhou *et al.*, 2022)

1.4.1 Flagella and colonization

The most well-studied colonization factor of *C. difficile* is the flagellum. Flagellum-mediated motility enhances the ability of pathogenic bacteria to move towards an initial site of colonization, but flagella also function as direct adhesins in a number of enteric species (Friedlander *et al.*, 2013; Haiko & Westerlund-Wikström, 2013). One early study focused on the role of flagella in mediating *C. difficile*'s adherence found that recombinant FliC and FliD (flagellin or flagellar-cap, respectively) as well as crude flagella displayed binding to cecal mucus from axenic mice (Tasteyre *et al.*, 2001a). Even though *C. difficile* (including human clinical isolates) is known to cause disease in swine (Steele *et al.*, 2010), these proteins did not bind to porcine gastric mucin. Because tools for genetic manipulation of *C. difficile* were limited at the time, Tasteyre and colleagues attempted to validate their *in vitro* findings in an axenic mouse model utilizing flagellated and non-flagellated strains from the same serotype. While this indirect approach limits the strength of experimental conclusions, non-flagellated strains had significantly less cecal adherence than flagellated suggesting that flagella may be important for CDI *in vivo*.

Today, ClosTron mutagenesis (Heap *et al.*, 2007) and other genetic techniques enable researchers to directly evaluate the role of specific proteins in *C. difficile*'s pathogenic strategy, including attachment and colonization. Gene interruption mutants of *fliC* or *fliD* in the 630Δerm strain of *C. difficile* were non-flagellated and had increased adherence to intestinal Caco-2 tissue culture cells relative to the wild-type strain (Dingle *et al.*, 2011). Wild-type levels of adhesion were restored upon complementation of *fliC* and *fliD* encoded on a plasmid. In the same study, flagellar mutant and complement

strains had greater virulence relative to 630Δerm in a hamster model of infection. These results might lead to the conclusion that flagella are not important for colonization, but differences in toxin production between mutant and parental strains and the overall extreme sensitivity of hamsters to TcdA and TcdB make the *in vivo* results difficult to interpret (Libby *et al.*, 1982).

While results from Dingle and colleagues were duplicated by another group in a later study (Baban *et al.*, 2013), findings were not consistent across different strains of *C. difficile*. Baban and colleagues found that gene interruption mutants of *fliC* or *fliD* in the hypervirulent epidemic R20291 strain had reduced adherence to Caco-2 tissue culture cells relative to the R20291 wild-type strain. In the same study, the *fliC* and *fliD* mutants also had reduced adherence to Caco-2 cells relative to a *motB* mutant, which possessed a paralyzed flagella. Furthermore, the *motB* mutant had higher relative colonization and cecal adherence than the *fliC* mutant in murine co-challenge experiments. Based on these results, the authors concluded that flagellum-mediated motility is not required for colonization and that the flagellum itself may function as an adhesin. Recently it was suggested that the observed phenotypic differences between the epidemic R20291 strain and the non-epidemic 630Δerm strain may be explained by differences in the ability of each strain to phase vary flagellar production (Mckee *et al.*, 2018).

1.4.2 Type IV pili and colonization

Another extracellular proteinaceous fiber which may play a role in *C. difficile* colonization is type IV pili (T4P). T4P are polymers of a major pilin protein (PilA1 in *C. difficile*) and have functions in adhesive processes such as microcolony formation, DNA uptake, and adherence to eukaryotic cells (Melville & Craig, 2013; Piepenbrink *et al.*,

2015). T4P promote early biofilm formation in *C. difficile in vitro* (Maldarelli *et al.*, 2016) and were found to be contained in the core genome during microarray analysis of 74 isolates, suggesting they are likely indispensable for CDI in humans (Stabler *et al.*, 2006). Because T4P serve a wide array of functions in different bacterial systems, there are several possible mechanisms by which they could mediate adherence. For example, they could mediate direct adhesion to host cells, simply promote microcolony formation and resistance to peristaltic removal, or promote invasion of epithelial cells (McKee *et al.*, 2018).

Beyond their role in promoting early *in vitro* biofilm formation, little is known about these versatile appendages in *C. difficile*. A non-piliated *pilA1* mutant in *C. difficile* 630Δerm had no observable differences in adherence to HT-29 cells after one hour relative to the parental strain (McKee *et al.*, 2018). However, the same *pilA1* mutant was deficient in adherence to MDCK cells after 24 hours relative to the parental strain, suggesting that T4P may be unnecessary for initial attachment to host cells but necessary for prolonged adherence. It's therefore important to consider multiple time points when investigating the role of T4P in adherence and colonization. As previously mentioned with flagella, investigation of T4P in multiple strains of *C. difficile* is also necessary before any major conclusions can be drawn.

1.5 CONCLUSIONS

The emergence of hypervirulent *C. difficile* strains and increasing rates of community transmission necessitate development of novel therapeutics and precision treatment options for CDI. Combined evidence from early and recent studies provide support for mucus as a possible site of attachment and colonization by *C. difficile* during

in vivo infection. Preventing or diminishing adhesion to mucus or the metabolic substrates it provides to *C. difficile* may therefore offer new options for therapeutic targets. A greater understanding of the molecular mechanisms underlying initial mucosal colonization and persistence in the GIT are needed before such therapeutics can be developed. Many different surface proteins have been demonstrated to mediate adherence *in vitro* and *in vivo*, and it's likely that multiple adhesins play a role in this process during infection. Beyond the colonization factors found on the surface of vegetative cells and spores, it will be important to consider *C. difficile* colonization in the ecological context of a complex, adaptive microbial community and host immune system.

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CHAPTER TWO: INVESTIGATION OF *C. DIFFICILE* COLONIZATION FACTORS NECESSARY FOR *IN VITRO* ADHERENCE TO AN ARTIFICIAL MUCUS LAYER

2.1 ABSTRACT

Clostridioides difficile is a Gram-positive, spore-forming anaerobe and is the most common cause of antibiotic-associated diarrhea, representing a major public health threat. Although intestinal dysbiosis and immune deficiency are known to favor *Clostridioides difficile* infection, the underlying host-pathogen interactions that promote colonization and persistence in humans are unknown. Recent *in vitro* and animal studies have provided compelling evidence that *C. difficile* associates with the colonic mucus layer during infection creating interest in investigating mucus as a putative site of colonization. Using an improved quantitative model of *in vitro* adherence to mucus, we explored the molecular mechanisms underlying association with mucus. Specifically, we explored the potential for extracellular appendages, Type IV pili (T4P) and flagella, to mediate mucosal adherence. Using immobilized mucins and gene-interruption mutants of the primary T4P (*pilA1*) and flagellar *(fliC*) subunits, flagella were found to be important for adhesion while T4P were not necessary. Through chemical modification of an *in vitro* mucus layer, we also observed that *O*-linked glycans facilitate adhesion. Finally, by comparing multiple strains of *C. difficile* and mucins derived from human and animal origin, we observed the ability of mucin structural and chemical variation to impact *C. difficile* attachment with possible implications for insights into host tropism.

2.2 INTRODUCTION

Clostridioides difficile is a Gram-positive, spore-forming, obligate anaerobe and was the most common healthcare-associated infection prior to recently being surpassed by SARS-CoV-2 (Khanna & Kraft, 2021). Disease severity ranges from mild, self-limiting diarrhea to life-threatening pseudomembranous colitis and toxic megacolon. Recent emergence of hypervirulent clinical isolates with increased antibiotic resistance and rates of disease recurrence has led the United States Centers for Disease Control and Prevention (CDC) to classify *C. difficile* under the 'urgent' public health threat level (Fatima & Aziz, 2019). Data from the last decade also shows that roughly 40% of *C. difficile* infections in the United States are community-associated cases which often do not involve predisposing antibiotic use or hospitalization (Khanna *et al.*, 2012). To identify novel therapeutic targets for CDI, investigations of the molecular mechanisms which enable *C. difficile* to colonize and persist in the gut are necessary.

Logically, attachment to host mucosal surfaces allows both pathogens and commensals to increase time of intestinal residence and associate with regions of the digestive tract harboring a favorable ecological niche (Adlerberth *et al.*, 2000). It is therefore possible that host attachment and colonization of the gut mucosa may be important to the pathogenic strategy of *C. difficile*. Numerous early *in vitro* studies (See Table 1.1) have yielded conflicting results for the role of molecular colonization factors (CFs) in adherence to intestinal epithelial cell lines. However, recent studies have provided some evidence that *C. difficile* associates with the mucus layer during infection (Engevik, Danhof, *et al.*, 2020a; Engevik, Engevik, *et al.*, 2020; Semenyuk *et al.*, 2015a). Therefore, some of the previously explored CFs may be essential for adherence to the

gastrointestinal mucus layer and yet unessential for adherence to commonly used cell lines which do not produce a mucus layer.

Type IV pili (T4P) and flagella have been implicated as adhesive appendages in numerous commensal and pathogenic bacteria (Carbonnelle *et al.*, 2006; Melican *et al.*, 2013; Piepenbrink & Sundberg, 2016; Xicohtencatl-Cortes *et al.*, 2007) and have been specifically suggested to promote *C. difficile*'s attachment to epithelial cell monolayers *in vitro* (McKee *et al.*, 2018). Investigation of these appendages directly mediating adherence to the mucus layer are limited and often conflicting. A main objective of this work was therefore to compare the relative adherence of *C. difficile* R20291 T4P and flagellar mutants to immobilized mucus. Importantly, the adhesion assay model chosen has the advantages of directly investigating microbe-mucus interactions while preventing interference from non-specific adhesion caused by hydrophobic interactions.

In parallel with identification of the CFs which mediate adherence, investigation of the structural features of the mucus layer which serve as potential adhesin receptor sites is necessary. The mucus layer which lines the GIT is principally composed of mucin, a glycoprotein containing a protein core which is heavily *O*-glycosylated. Glycans account for approximately 80% of the mass of mucin molecules and influence the functional properties of mucus like viscoelasticity and gel-formation (Naughton *et al.*, 2014). There are at least 17 different mucin families in humans, composed of both membrane-bound and secreted mucins (Juge, 2012; Voynow & Fischer, 2006). MUC1, MUC5AC, and MUC6 predominate in the stomach while secreted MUC2 mucin is most abundant in the small intestine and colon (Juge, 2012).

Differences in mucin expression and glycosylation between bodily sites, different individuals, healthy and diseased tissues, and different hosts represent a wide diversity of bacterial receptors and nutrients which could explain some of the bacterial-host tropism observed in nature (Adlerberth *et al.*, 2000). *C. difficile* is known to infect a wide range of hosts including pigs, cattle, horses, dogs, and other animals (Gould & Limbago, 2010; Knight & Riley, 2019; Rabold *et al.*, 2018). By using human and animal-derived bacterial isolates in combination with gastrointestinal mucus from different hosts, an additional objective of this work was to investigate preferences in adherence to structurally-varied mucins, providing preliminary insights into possible specificity for mucus receptors and evolutionary adaption for mucus attachment by vegetative *C. difficile*.

2.3 MATERIALS AND METHODS

2.3.1 Bacterial strains and growth conditions

C. difficile 630, R20291, pilus, and flagellar mutants were a generous gift from Dr. Glen Armstrong's laboratory at the University of Calgary. CD2015, CD1015, and VPI-10463 isolates were kindly provided by Dr. Jennifer Auchtung's lab at the University of Nebraska-Lincoln.

Overnight cultures of *C. difficile* were grown from frozen glycerol stocks on BHIS(TA) agar plates (37 g/L RPI brain heart infusion broth, 0.5% yeast extract, 0.1% L-cysteine, 0.1% sodium taurocholate) at 37°C in an anaerobic chamber (Coy Lab Products) with an atmosphere of 5% H_2, 5% CO_2, and 90% N_2. Thiamphenicol (15 μg/mL) or lincomycin (20 μg/mL) were added for mutant strains as necessary. Cycloserine-cefoxitin-fructose agar supplemented with 0.1% sodium taurocholate

(TCCFA) selective media was used periodically, as previously described (Sorg & Dineen, 2009). For experiments, individual colonies from overnight plates were used to inoculate BHIS(TA) broth with appropriate antibiotics, unless otherwise specified.

2.3.2 Preparation of artificial mucosal surface

LS174T/HT-29 MTX human epithelial cell-derived mucins or partially purified porcine stomach mucins were covalently immobilized on glass coverslips (Engevik *et al.*, 2020; Landry *et al.* , 2006). Coverslips were washed in 1 M HCl overnight at 60°C. Clean coverslips were washed in sterile deionized water, then autoclaved. Once dry, coverslips were transferred to a 4% solution of (3-aminopropyl)triethoxysilane (Sigma #440140) in acetone for one hour, washed with acetone (Fisher), then heat treated at 110°C for one hour. Terminal aldehydes were formed on the surface by incubating the coverslips in 2.5% aqueous glutaraldehyde (v/v) (Fisher) for one hour and rinsing with water. Finally, coverslips were incubated in 1 mg/mL PBS-mucin overnight at 4°C. Coverslips were air-dried and UV-treated for sterility, then stored at 4°C.

2.3.3 Partial purification of commercial porcine stomach mucin

Commercial porcine stomach mucin (Sigma #M1778) was partially purified prior to use (Beighton *et al.*, 1988; Gargano *et al.* , 2014). Crude mucin was suspended at approximately 20 mg/mL in 0.1M NaCl. The pH was adjusted to 7.0 and the solution was stirred overnight at 4°C. The following day, insoluble residues were pelleted by centrifugation (10,000 x g for 10 min.). Mucin contained in the supernatant was precipitated in ice cold 60% ethanol (v/v), then recovered by centrifugation (10,000 x g

for 10 min.). Dissolving and precipitation steps were repeated twice more. The final pellet was resuspended in 0.1 M NaCl pH 7.0 and stored at -80°C.

2.3.4 Extraction and purification of porcine colonic mucin

Porcine colonic mucin was purified from pig digestive tracts obtained from the University of Nebraska-Lincoln Department of Animal Science. Colonic mucosal tissue samples were prepared by gentle scraping of the mucosa with glass coverslips. Samples were stored at -80°C until further processing.

Extraction and purification of mucins was performed similar to previously described methods (Asker *et al.*, 1995; Carlstedt *et al.*, 1983). Briefly, mucosal tissue samples were suspend in extraction buffer (6 M guanidium chloride, 5 mM EDTA, 0.01 M NaH_2PO_4, 100 mM PMSF, pH 6.5) and homogenized, then stirred overnight at 4°C. The insoluble fraction containing mucins was removed by centrifugation using a JA-20 rotor (18,000 rpm, 10°C, 30 min.). The resulting pellet was resuspended in extraction buffer and the process was repeated 3 times, limiting stir durations to 2-3 hours for remaining extractions.

Extracted insoluble mucins were solubilized in reduction buffer (6 M guanidium chloride, 0.1 M Tris, 5 mM EDTA, 25 mM dithiothreitol, pH 8.0) by stirring at 37°C for approximately 5 hours. Alkylation was performed by addition of 62.5 mM iodoacetamide (final concentration) and the solution was stirred overnight at room temperature in the dark. The next day, contaminant proteins were removed by centrifugation (10,000 rpm, 4°C, 30 min.) and the resulting supernatant containing soluble mucins was lyophilized.

For experiments, mucins were resuspended in appropriate buffer at a final concentration of 1 mg/mL unless otherwise specified.

2.3.5 Fluorescent labeling of *C. difficile*

For microscopy experiments, *C. difficile* cells were fluorescently labeled with CFDA-SE (5(6)-carboxyfluorescein diacetate succinimidyl ester; STEMCELL Technologies) (Engevik, Danhof, *et al.* , 2020). Briefly, mid-exponential phase cultures were washed three times with anaerobic PBS by gentle centrifugation (4,500 x 5 for 6 min.). After washing, cells were incubated in anaerobic PBS containing 10 mM CFDA-SE at 37°C for one hour. Following incubation, excess dye was removed by three consecutive washing and centrifugation steps as previously performed.

2.3.6 Mucosal adhesion assay

Prepared mucin-coated coverslips were used for evaluating the ability of *C. difficile* R20291 to interact with an artificial mucosal surface *in vitro*. Individual colonies were used to inoculate BHIS(TA) broth, and bacteria were grown to mid-log phase (4-6 hours) at 37°C. Cells were gently centrifuged (4,500 x g for 8 minutes), then resuspended in phosphate-buffered saline (PBS; 137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4, pH 7.4) at OD600 =2.0. Suspensions were added to sterile 6-well cell culture plates containing mucin-coated coverslips or clean glass controls and incubated at 37°C for one or 24 hours. Following incubation, unadhered bacteria were removed by washing 3 times with 1x PBS. Adhered bacteria were then removed by incubation with 0.25% trypsin-EDTA solution (Gibco) at 37°C for 8-10 minutes. Trypsin was neutralized

by addition of 2 volumes BHIS(TA), then recovered bacteria were plated on BHIS(TA) agar plates and enumerated after 24-48 hours of incubation at 37°C.

2.3.7 Swimming motility assay

To assess swimming motility, *C. difficile* R20291 and flagellar mutant colonies obtained on TCCFA plates were used to inoculate BHIS(TA) broth with antibiotics added where appropriate. Cultures were grown overnight at 37°C under anaerobic conditions. The following day, culture tubes containing pre-reduced 0.175%-0.5xBHIS (Dingle *et al.*, 2011; Purcell *et al.*, 2017) were stab inoculated with each strain. Tubes were placed in a seal-lock bag, the bag was partially closed, and the tubes were incubated at 37°C overnight. The next day, tubes were visually assessed for swimming motility. For strain comparison, overnight broth cultures were used to inoculate 0.3%-0.5xBHIS agar plates and measurements were recorded every 24 hours for 72 hours (Anjuwon-Foster *et al.*, 2018b).

2.3.8 6xHis-FliD expression and purification

A pET-28(a) plasmid construct containing *C. difficile* 630 FliD (amino acid residues 67-507) and an N-terminal 6x His-tag was kindly gifted by Dr. Eric Sundberg's lab at Emory University. Plasmid DNA was transformed into *E. coli* NiCO21(DE3) competent cells (New England BioLabs Cat No. C2529H) using the heat shock method (Froger & Hall, 2007). Isolated colonies obtained on Luria Broth (LB) agar plates containing kanamycin (50 mg/mL) for positive selection were cultured overnight in LB broth at 37°C with shaking. The following day, plasmid DNA from LB broth culture growth was extracted and purified using the Wizard *Plus* Minipreps DNA Purification

System (Promega). DNA samples were Sanger sequenced (Eurofins) to confirm accuracy.

For FliD protein expression, *E. coli* NiCO21(DE3) cells containing FliD(67-507)-pET-28(a) were grown overnight in 300 mL of LB broth containing kanamycin at 37°C shaking at 200 rpm. The following day, 35 mL of overnight growth was used to inoculate each of one 6 L flasks containing LB broth supplemented with kanamycin. Cells were grown at 37°C with shaking until reaching OD60 = 0.4-0.6, at which point induction with 0.5 mM isopropyl Beta-d-1-thiogalactopyranoside (IPTG) was performed. Following induction, cells were grown for 24-32 additional hours at 18°C and shaking before being harvested by centrifugation (4,700 x g; 30 min, 4°C). The resulting cell pellet was collected and stored at -80°C until purification.

For purification, frozen cell pellets were lysed in a hot water bath from frozen. Thawed cells were suspended in 30 mL of suspension buffer (20 mM Tris-HCl, 500 mM sucrose, 5 mM NaEDTA, 2 mM $MgCl_2$, 50 mM NaCl, 0.1% NaN_3). 1 mL DNase (2 mg/mL), 1 mL phenylmethylsulfonyl fluoride (PMSF; 0.1M), 1 mL lysozyme (25 mg/mL), and 1 mL $MgCl_2$ (2 M). Sodium deoxycholate and Triton-X 100 were added to give final concentrations of 1% and 0.5%, respectively. 80 mL of lysis buffer (50 mM Tris-HCl pH 8.3, 0.1M NaCl, 1% Triton-X, 20 mM sodium deoxycholate) was added, and the resulting lysate was centrifuged at 20,000xg for 30 minutes at 4°C. FliD was purified from the resulting supernatant using a nickel-NTA column on a GE Akta start. Elution fractions were further purified by size-exclusion chromatography over a GE S200 Superdex column using a Bio-Rad NGC FPLC.

2.3.9 Competitive exclusion assay

Adherence assays were performed following blocking of mucin-coated 96-well polystyrene plates with recombinant flagellar proteins. Porcine colonic mucus (PCM; 1 mg/mL) was passively immobilized in wells by overnight incubation at 4°C. The following day, wells were washed to remove unadhered mucus, and blocking with 0, 0.1, or 1 mg/mL protein was performed for 1 hour (Miyoshi *et al.*, 2006). Protein solutions were removed, wells were gently washed once, and adherence assays were performed with colony forming units (CFU) enumeration as previously described. Recombinant PilA1 protein (1 mg/mL) was used as a negative control.

2.3.10 Statistical analyses

All statistical analyses were performed using GraphPad Prism version 9.3.1 for macOS (GraphPad Software, San Diego, California USA). All significance differences were determined at p-value<0.05. Relevant statistical tests used for comparisons are discussed in figure captions.

2.4 RESULTS

2.4.1 Covalent immobilization of mucins as an *in vitro* mucus layer model

The most common method of evaluating microbe-mucus interactions *in vitro* involves passive immobilization of mucus onto 96-well polystyrene microtiter plates (Cohen & Laux, 1995). In preliminary studies, 1 mg/mL partially purified porcine gastric mucin (PGM) was incubated overnight at 4°C in a sterile high-binding 96-well plate (Corning Costar REF# 3361). Wells containing 1 mg/mL bovine serum albumin (BSA) or buffer alone were added as controls for non-specific binding. The next day, wells were

washed with buffer and an adherence assay involving incubation of bacteria in treated wells, washing to remove unadhered bacteria, and enumeration of adhered bacteria was performed (similar to steps described in Materials and Methods). The relative adherence of *C. difficile* R20291 to wells treated with BSA or buffer alone was greater than to wells treated with PGM (Figure 2.1A). Based on the observed amount of non-specific binding to plastic, it was determined that the commonly used 96-well plate model would not be suitable for future adhesion studies.

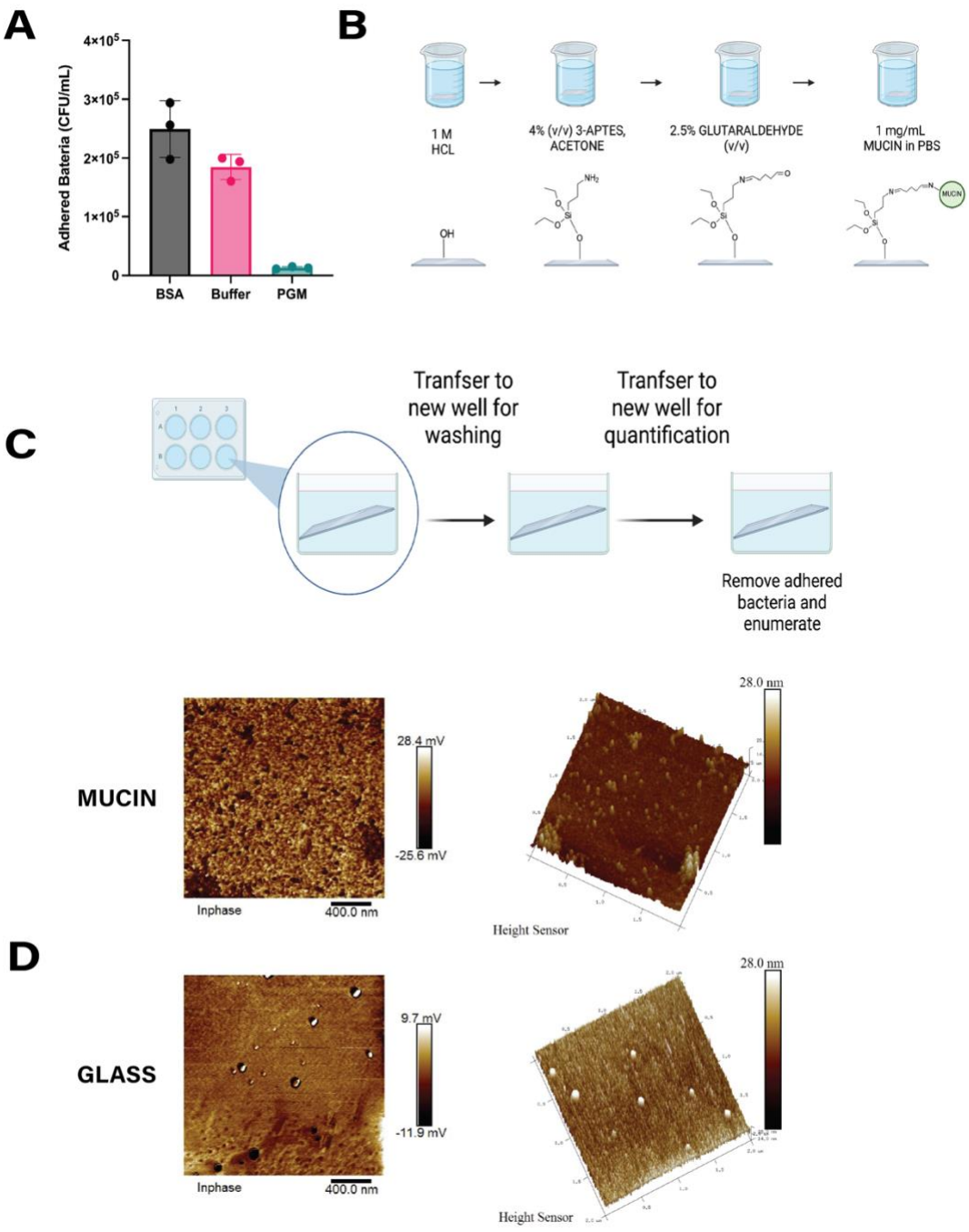

Figure 2.1 Establishment of an *in vitro* model of direct mucus adherence by *C. difficile*. (A) 1-hour *C. difficile* R20291 adhesion to immobilized porcine gastric mucin (PGM), BSA, or buffer alone in 96-well microtiter plate. **(B)** Schematic of covalent immobilization of mucin molecules to glass coverslips for *in vitro* modeling of the gastrointestinal mucus layer (see Materials and Methods). **(C)** Schematic of coverslip transfer during *in vitro* mucosal adhesion assay. **(D)** AFM topographical phase (left) and

3D images (right) of mucin-derivatized coverslips (top) or clean glass control (bottom). Images were obtained on a Dimension Icon AFM (Bruker).

As an alternative method of evaluating *C. difficile*-mucus interactions, mucins were covalently immobilized onto glass coverslips (Figure 2.1B). Because the model mucus layer is adhered to the coverslip, it is possible to transfer the surface of interest to sterile wells for direct enumeration of mucus-associated bacteria (Figure 2.1C). Mucin-derivatized coverslips were analyzed using atomic force microscopy (AFM) to confirm proper coating. As anticipated, the mucin-derivatized coverslips had a surface roughness and topography much different than a clean glass control (Figure 2.1D), demonstrating that mucus was surface-immobilized. Some dust particle artifacts were observed on the clean glass slide.

2.4.2 *C. difficile* preferentially adheres to mucus

Following AFM confirmation of mucus coverslips, an adherence assay was performed to determine the ability of *C. difficile* R20291 to associate with mucus. Fluorescently-labeled *C. difficile* cells were incubated with either mucus coverslips or clean glass coverslips. Unadhered bacteria were removed by washing, and adhered bacteria were visualized using fluorescence microscopy. As previously reported (Engevik, Engevik, *et al.*, 2020), *C. difficile* was found to preferentially adhere to mucus (Figure 2.2A). To quantify this adherence, a similar assay was performed with un-labelled *C. difficile*. Adhered bacteria were treated with trypsin for removal and quantified by CFU enumeration. In agreement with the microscopy data, adherence to mucus coverslips was significantly greater than adherence to glass (Figure 2.2B).

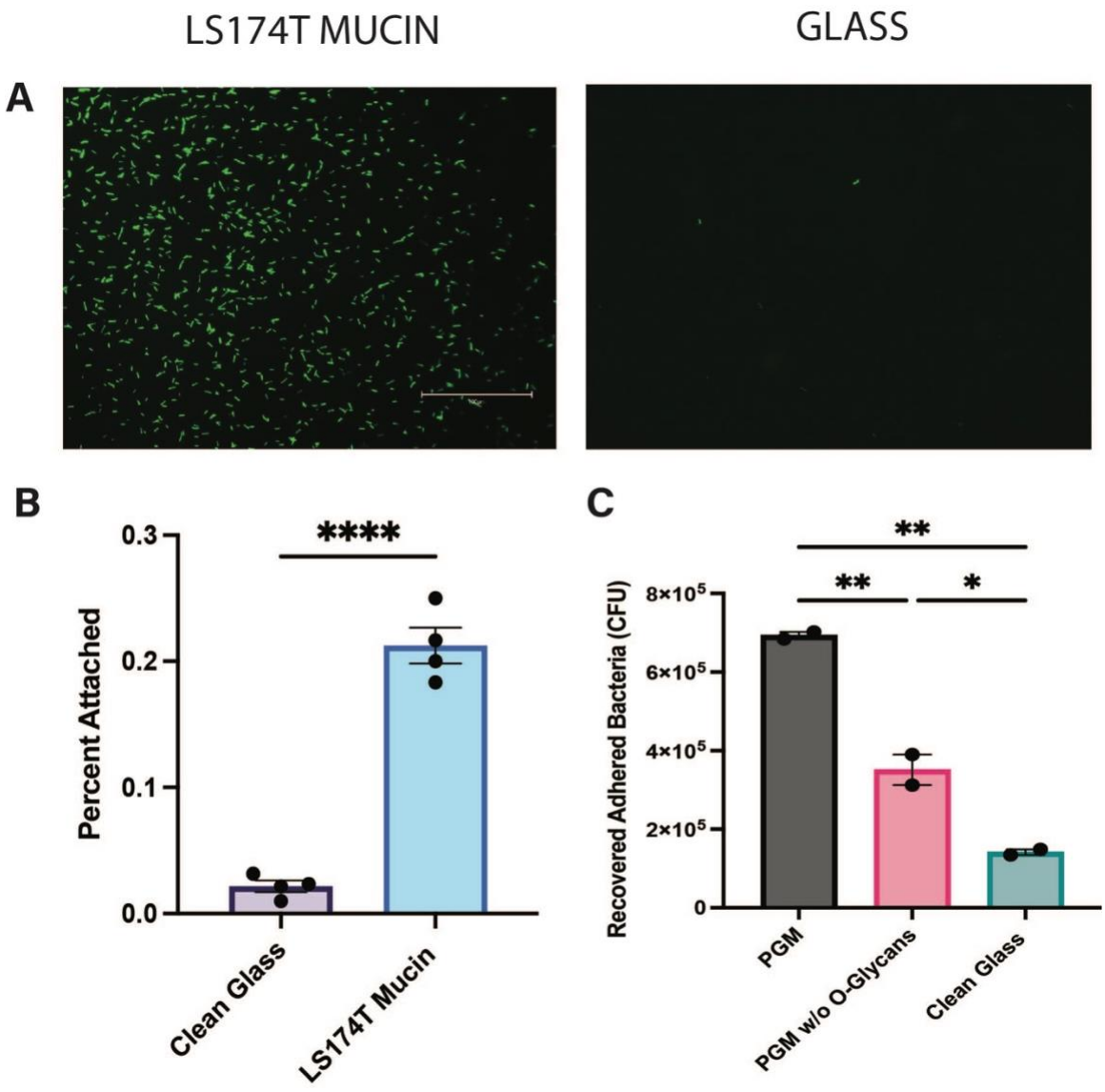

Figure 2.2 *C. difficile* preferentially adheres to mucin O-linked glycans *in vitro*. (A) Adherence of CFDA-SE fluorescently tagged *C. difficile* R20291 to LS174T mucin-derivatized coverslips or clean glass control (20x EVOS AMPER4682; Scale bar = 100 mm). **(B)** CFU enumeration of adherence to mucin-derivatized coverslips or clean glass control ($p < 0.0001$; Two-tailed unpaired t-test). **(C)** *C. difficile* R20291 adherence to porcine gastric mucin (PGM) coated coverslips with or without O-linked glycans, or clean glass control (n=2 coverslips; * indicates $p < 0.05$; Ordinary one-way ANOVA with Tukey's multiple comparisons test). Mean and SEM shown for B and C.

It has been previously reported that *C. difficile* chemotaxes towards and adheres to human epithelial cell-derived MUC2 O-linked glycans (Engevik, Engevik, *et al.*,

2020). To compare these findings in our quantitative model of adherence, PGM O-linked glycans were removed by reductive Beta-elimination (0.5 M $NaBH_4$, 50 mM KOH, 50°C overnight). Coverslips were prepared using the resulting mucin protein and adherence was measured by CFU enumeration. Indeed, adherence to intact PGM was significantly greater than adherence to PGM without O-linked glycans (Figure 2.2C). Adherence to the modified PGM was still greater than adherence to glass, implying either that the Beta-elimination reaction was incomplete or that adhesion to other moieties like the mucin polypeptide or N-linked glycans is also a factor.

2.4.3 A *C. difficile* R20291 *fliC* mutant is deficient in *in vitro* adherence to mucus

Due to access to *C. difficile* R20291 T4P and flagellar mutants in the Piepenbrink lab and the implication of these appendages in mediating colonization (see Chapter 1), we evaluated their relative ability to adhere to mucus. *C. difficile* R20291, *pilA1* mutant (major pilin), *fliC* mutant (flagellin), and mutant complements were incubated with PGM coverslips for 1 hour. An adhesion assay utilizing CFU enumeration was performed as before. The non-flagellated *fliC* mutant adhered significantly less to mucus than the R20291 parental strain (Figure 2.3A). This finding was consistent with the observation that an R20291 *fliC* mutant was deficient in adherence to Caco-2 cells *in vitro* (Baban *et al.*, 2013). Despite T4P having a demonstrated role in attachment in several bacterial species (Piepenbrink & Sundberg, 2016), the T4P-null *pilA1* mutant was not deficient in adherence, and in fact appeared to have increased attachment to mucus. Importantly, trends in relative adherence obtained with PGM coverslips were conserved for coverslips coated with HT-29 MTX human epithelial cell derived mucus (Figure 2.3B).

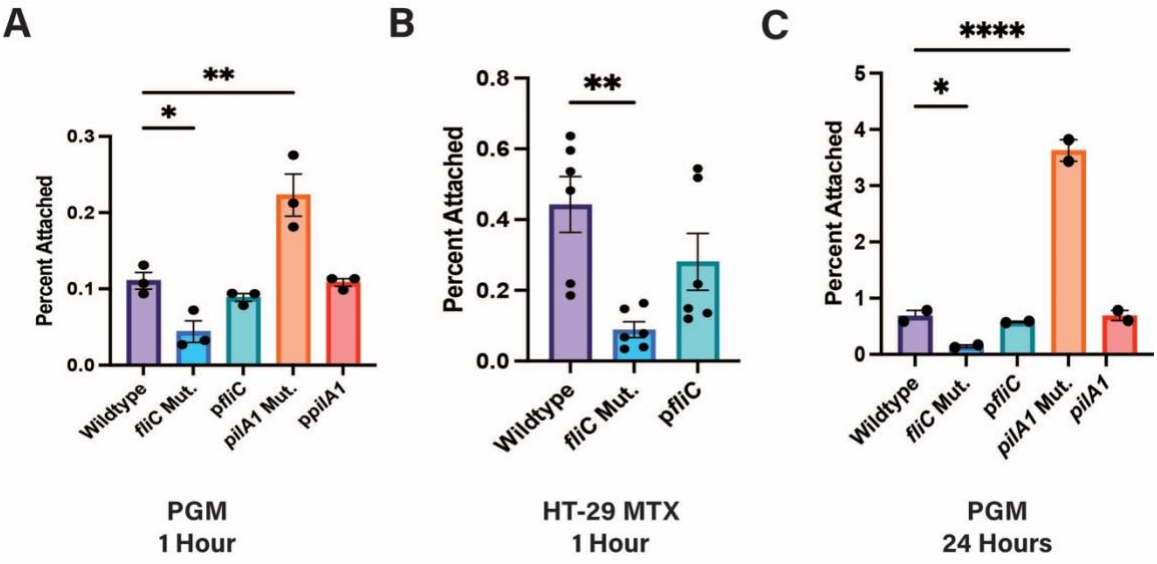

Figure 2.3 Role of T4P and flagella in mediating adhesion of *C. difficile* to mucus. **(A)** CFU enumeration of 1-hour attachment by *C. difficile* R20291, T4P, and flagellar mutants to PGM (n = 3 coverslips per strain) or HT-29 MTX **(B)** coated coverslips (n = 6 coverslips per strain). **(C)** 24-hour attachment to PGM coated coverslips. * indicates $p < 0.05$; Ordinary one-way ANOVA with Dunnett's multiple comparisons test; Mean and SEM shown.

In *C. difficile* 630Δerm, it was recently hypothesized that T4P may not be essential for initial host attachment, but could be important for prolonged adherence and colonization (McKee *et al.*, 2018b). Our 1 hour adhesion assay may have effectively measured initial attachment, but is insufficient for determining relative long-term adherence. To control for this, we performed the same adhesion assay previously described using a 24 hour incubation period. We hypothesized that an extended incubation period would decrease the relative contribution of flagella and increase the importance of T4P for promoting prolonged adherence. Surprisingly, relative adherence trends observed at 24 hours were similar to those obtained at 1 hour (Figure 2.3C). In

summary, these data demonstrate that a *C. difficile* R20291 *fliC* mutant is deficient in adherence to mucus *in vitro*.

2.4.4 *C. difficile* R20291 *fliC* mutant is deficient in swimming motility

Flagella are primarily known for their role in facilitating motility, often driven by a chemotactic response (Nakamura & Minamino, 2019). It is therefore not surprising that *C. difficile fliC* mutants, which do not produce a flagellum, are non-motile (Baban *et al.*, 2013; Dingle *et al.*, 2011). The phenotype of our *C. difficile* R20291 *fliC* mutant and complement were evaluated using a swim agar assay. As expected, *C. difficile* R20291 and *fliC* complemented strains were motile while the R20291 *fliC* mutant was deficient in swimming motility (Figure 2.4). Decreased motility in the intestinal tract during infection could impact colonization.

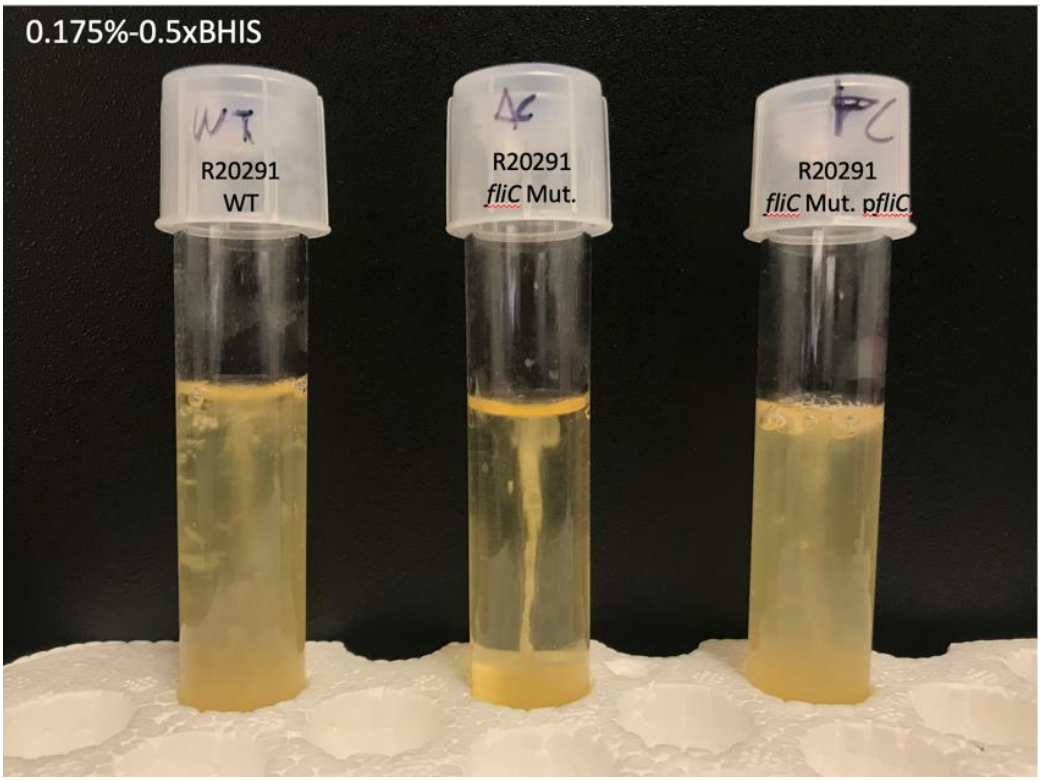

Figure 2.4 A *C. difficile fliC* mutant strain is deficient swimming motility. Swimming motility of *C. difficile* R20291 or flagellar mutant strains in swimming motility agar (0.175%-0.5xBHIS) following overnight incubation at 37°C.

2.4.5 Competitive exclusion of microbe-mucus interactions by protein blocking

Flagella have previously been shown to impact adhesion by *C. difficile* R20291 (Baban *et al.*, 2013), but it remains unclear if lack of motility explains decreased attachment or if the flagellum serves as a direct adhesin. The role of extracellular proteins in mediating adhesion can be assessed *in vitro* using a competitive adhesion assay (Arora *et al.*, 1998; Miyoshi *et al.*, 2006). Proteins assumed to be adhesins are incubated with the surface of interest to occupy binding sites, then an adhesion assay is performed. For evaluation of *C. difficile* flagella, we attempted to obtain a crude flagellar extract (Tasteyre *et al.*, 2001) for blocking. However, inconclusive results were obtained from the subsequent adhesion assay and production of large enough quantities of crude flagellar extract was not feasible for our adhesion model. As such, it was decided to express and purify recombinant FliC (flagellin) and FliD (flagellar cap) for blocking experiments.

Recombinant FliD purity was assessed by SDS-PAGE (Figure 2.5A) after purification by size-exclusion chromatography. Lower molecular weight proteins were not considered a concern for the purposes of blocking experiments. Following blocking of immobilized mucus by incubation with protein solutions, relative adhesion of *C. difficile* R20291 wild-type was evaluated. Because adhesion assays utilizing mucin-derivatized coverslips requires large volumes of protein solutions at high concentration, the necessary yield of protein was still difficult to achieve despite use of a recombinant

expression system. The commonly used 96-well plate adhesion assay was therefore deemed most appropriate for our investigation (Cohen & Laux, 1995). There were no significant differences observed between mucus-coated wells which were treated with recombinant proteins and those treated with buffer alone, suggesting that incubation with CD630 FliD(67-507) did not mask adhesion receptors (Figure 2.5B).

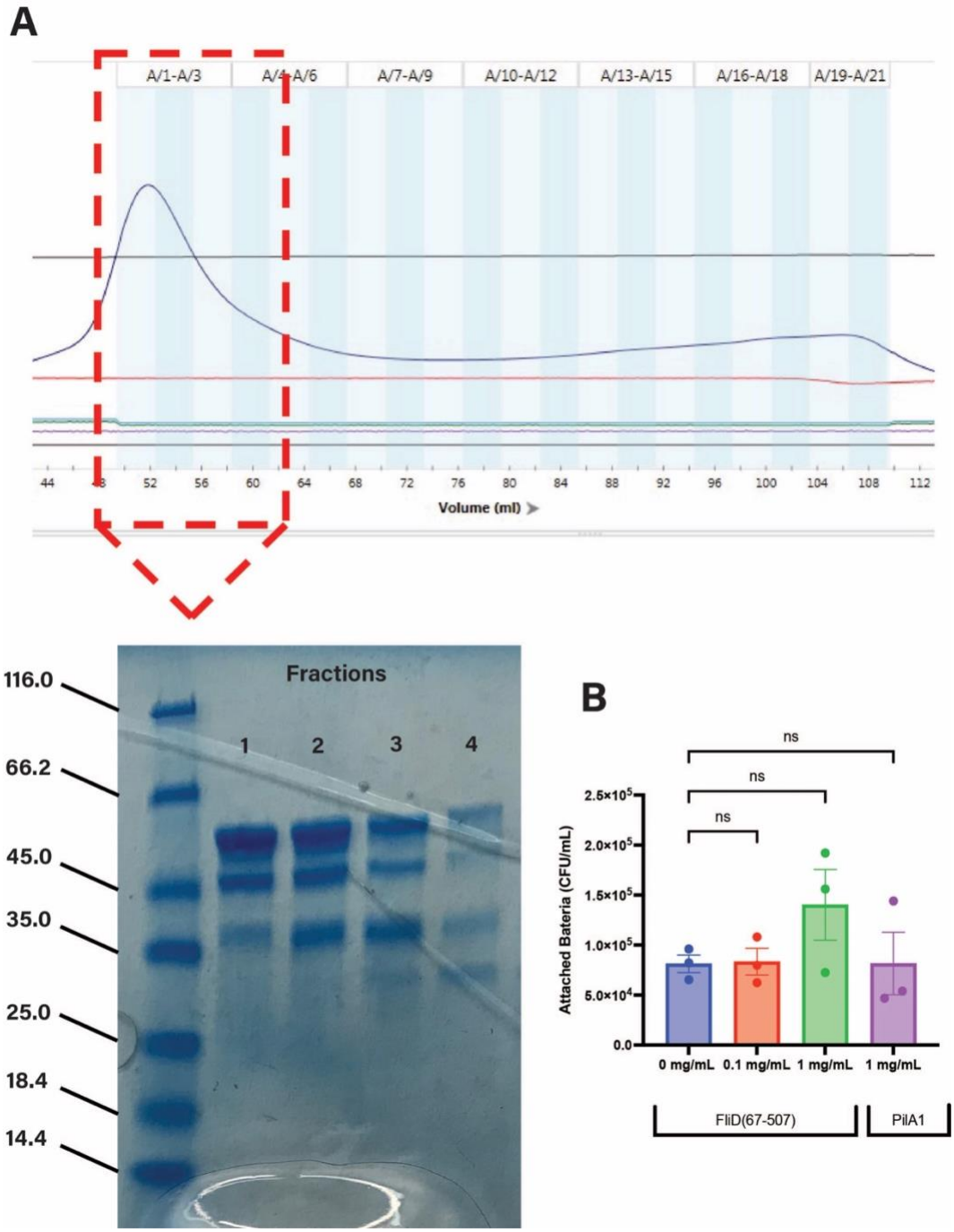

Figure 2.5 FliD(67-507) blocking does not impact *C. difficile* adherence to mucus. **(A)** Size-exclusion chromatogram and SDS-PAGE gel of FliD(67-507) purification. The predicted molecular weight of FliD(67-507) is approximately 49.2 kDa. **(B)** Porcine colonic mucin (PCM) coated wells of a 96-well polystyrene plate were incubated with FliD(67-507) or PilA1 (negative control) purified proteins to evaluate potential receptor blocking. Following incubation with purified proteins, 1-hour adhesion of *C. difficile*

R20291 to mucus was evaluated (n = 3 wells per treatment; ns = no significant difference; Ordinary one-way ANOVA with Dunnett's multiple comparisons test).

2.4.6 Swimming motility and mucus adherence vary by strain

C. difficile occupies many environmental niches and colonizes multiple different hosts, leading to a large degree of intraspecies genetic and phenotypic diversity (Knight *et al.*, 2015; Knight & Riley, 2019). Relative 1-hour adherence to PGM coated coverslips was determined for 5 *C. difficile* strains. BHIS(TA) broth cultures were prepared for each strain (CD630, R20291, CD2015, CD1015, and VPI-10463), and the mucosal adhesion assay was performed as described previously. CD1015 had significantly greater adherence to PGM coverslips than all other strains tested (Figure 2.6A). CD630 was the least adherent *C. difficile* strain in our model.

Because we observed that flagella may play an important role in adhesion to mucus, we also explored phenotypic differences in swimming motility across multiple strains. To quantitatively assess motility, 0.3xBHIS-0.3% agar was used (Anjuwon-Foster *et al.*, 2018), and motility was measured every 24 hours for 72 hours. R20291 and CD2015 (both ribotype (RT) 027 strains) were highly motile, while CD1015 (RT 078) and VPI-10463 (RT 003) had very little swimming motility after 72 hours (Figure 2.6B). Growth curves of each strain in BHIS broth over time were obtained for comparison using a Tecan Sunrise plate reader (Figure 2.6C).

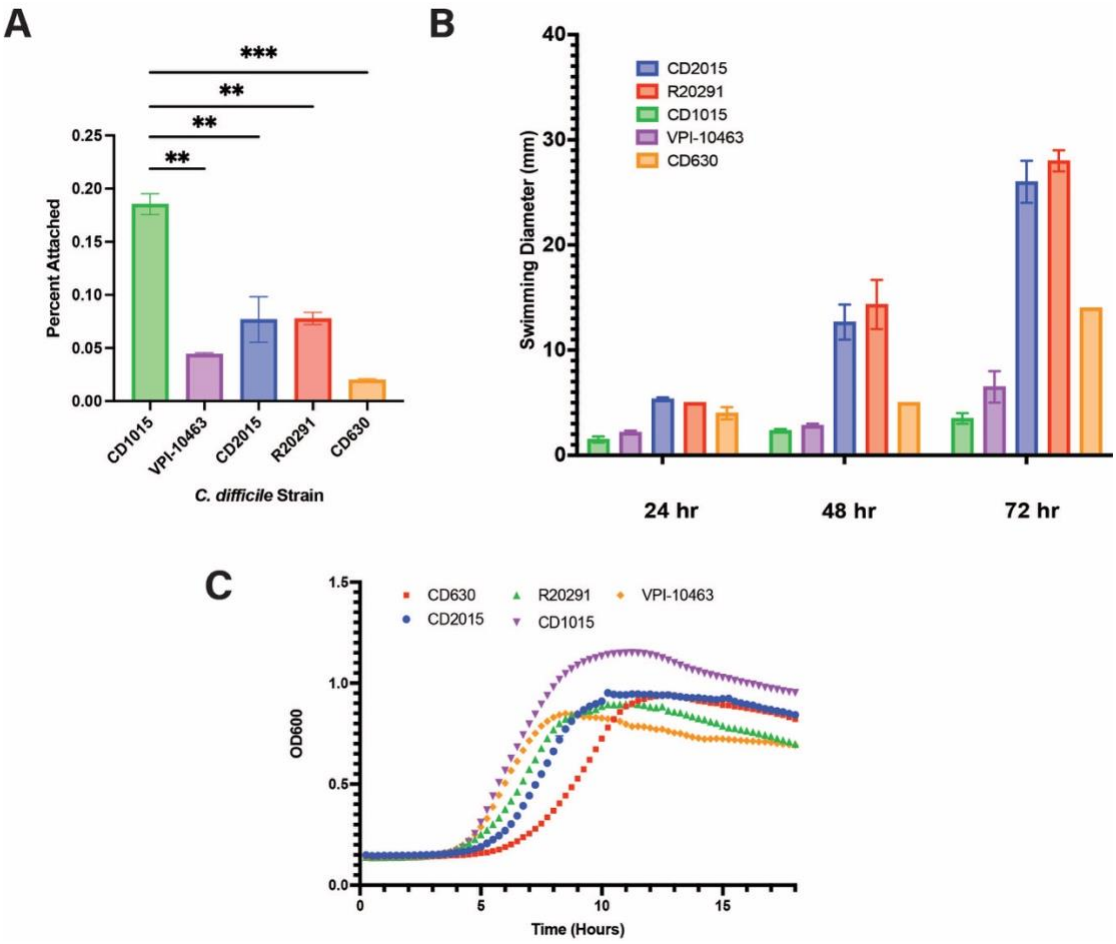

Figure 2.6 Comparison of swimming motility and mucus attachment across multiple *C. difficile* strains. (A) 1-hour attachment of multiple *C. difficile* strains to PGM coated coverslips (* indicates $p < 0.05$; Ordinary one-way ANOVA with Tukey's multiple comparisons test; n = 2 coverslips per strain with mean and SEM shown). **(B)** Swimming motility of *C. difficile* strains in swim agar (0.5xBHIS-0.3% agar) over time. Data shown are mean and SEM (n = 3 replicates per strain per timepoint). **(C)** Growth curve of *C. difficile* strains (ΔOD600 value over time) in BHIS broth. Data shown are mean of 12 replicates per strain with measurements obtained every 15 minutes.

2.4.7 Structural features of mucin impact *in vitro* adherence

Mucin proteins and their glycosylation varies by host and tissue, offering different receptors for possible *C. difficile* binding. To explore the impact of this variation in our model, coverslips were coated with porcine colonic mucin (PCM), porcine gastric mucin

(PGM), HT-29 MTX, or LS174T human intestinal epithelial cell-derived mucins. Relative adherence of *C. difficile* R20291 wild-type was then assessed following a one hour incubation as previously described (see section 2.3.6). Adherence to LS174T-derived mucins was significantly greater than to all others tested (Figure 2.7). PCM and HT-29 MTX-derived mucins had slightly greater adherence than PGM, although the difference was not statistically significant.

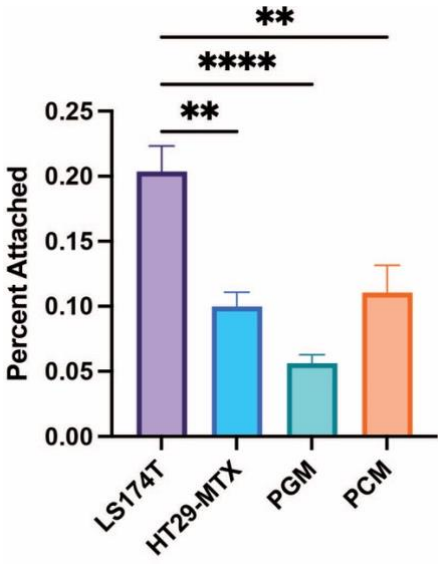

Figure 2.7 Structural and chemical features of mucin facilitate attachment. 1-hour adherence of *C. difficile* R20291 to coverslips coated with various mucins (* indicates $p < 0.05$; Ordinary one-way ANOVA with Tukey's multiple comparisons test; n = 5 coverslips per mucin type).

2.5 DISCUSSION

Adhesion to the mucus layer is an important step in colonization for many enteric bacteria, and recent studies have provided evidence that *C. difficile* associates with mucus during infection (Engevik *et al.*, 2015, 2021; Semenyuk *et al.*, 2015). Using a quantitative model of *in vitro* adhesion to mucus, we also observed that *C. difficile* preferentially

adheres to mucus. By covalently immobilizing mucin molecules onto glass coverslips (Engevik, Engevik, *et al.*, 2021; Landry *et al.*, 2006), the *in vitro* model used in this investigation allows for direct evaluation of microbe-mucus specific interactions without interference from hydrophobic interactions with polystyrene. As previously observed (Engevik, Engevik, *et al.*, 2021), when *O*-linked glycans were removed from mucin molecules, adhesion of *C. difficile* to the mucin backbone was reduced. These data demonstrate that *O*-linked glycans play a role in facilitating adhesion by *C. difficile*, potentially mediating its ability to colonize. Understanding exactly which glycans promote adhesion may offer novel therapeutic alternatives which decrease virulence without directly killing *C. difficile*, protecting against the development of treatment resistance.

While a number of different cell-surface proteins have been implicated in the attachment of *C. difficile* to the host, current findings are often conflicting and *in vitro* models fail to account for the presence of the mucus layer. In our *in vitro* model, flagella, but not T4P, appear to play a role in initial attachment of *C. difficile* to mucus. Interestingly, attachment to mucus appeared to be enhanced in a T4P-null *pilA1* mutant, though possible pleiotropic changes in gene regulation make these results difficult to interpret. Because T4P were found to promote prolonged attachment to MDCK cell monolayers by *C. difficile* 630Δerm (McKee *et al.*, 2018), future transcriptomic work investigating gene expression in the *pilA1* mutant used in our investigation is warranted. The importance of flagella is not surprising as many other groups have previously reported flagella to be an adhesive appendage used by *C. difficile* (Stevenson *et al.*, 2015; Tasteyre *et al.*, 2001).

Even though we observed that the loss of flagella resulted in deficient adherence to mucus, it's not clear if flagella simply facilitate chemotaxis-driven motility, or if they are a direct adhesin (Stevenson *et al.*, 2015). In *Psuedomonas aeruginosa*, FliD has been found to directly mediate attachment to mucus (Arora *et al.*, 1998). Attempted blocking of mucus receptors with recombinantly expressed FliD protein (flagellar cap) had no impact on *C. difficile* mucus adherence, suggesting that FliD is not a direct adhesin. However, part of the N-terminus of FliD was not included in our expression construct (to increase protein product solubility) and this portion of the native protein should still be explored as a potential adhesin. We also attempted to perform blocking experiments with a FliC construct found in the literature (Ghose *et al.*, 2016), but the recombinant product obtained was found to be in inclusion bodies. Future experiments should be performed to investigate recombinant FliC as well as crude intact flagellum as potential adhesins to mucins.

Surprisingly, comparison of relative adhesion to PGM across multiple strains of *C. difficile* found that CD1015, an RT 078 strain, was most adherent. RT 078 strains are highly prevalent in pigs and cattle (Goorhuis *et al.*, 2008), so this could potentially be a sign of host tropism. However, as with other RT 078 strains, we found that CD1015 was practically non-motile in a swimming motility assay. Therefore, if flagella do play a role in mediating attachment to mucus, it's possible that the intact flagellum is important for adherence and flagellar motility is dispensable. Many proteins other than flagella are likely involved in mediating adhesion and could instead be responsible for CD1015's enhanced attachment. Adhesion comparisons of a larger, more diverse library of strains

coupled with analysis of isolate genomic data could bring clarity to these phenotypic differences.

When comparing adhesion of *C. difficile* R20291 (human clinical isolate) to animal and human cell-derived mucins, we observed the greatest attachment to mucins from LS174T human epithelial cells. Adherence to PGM, mucins which are widely used in adhesion studies due to their commercial availability, was lowest of those tested. PGM has been shown to contain virtually no sialylated oligosaccharides while human intestinal mucins were found to be highly acidic (Ringot-Destrez *et al.*, 2018). Based on these results, this may suggest that acidic glycan epitopes like sialic acid are important for adherence by human isolates of *C. difficile*. Furthermore, LS174T cells are known to have greater expression of MUC2 mucin than HT-29 MTX cells (Bu *et al.*, 2011) and also had greater *C. difficile* attachment suggesting that MUC2 may contain important adhesion receptors, as was previously hypothesized (Engevik, Engevik, *et al.*, 2020).

In conclusion, *C. difficile* preferentially adheres to *O*-linked glycans found on mucin molecules. Flagella appear to play a role in this process, although the mechanism by which they facilitate attachment remains unclear and should be a focus of future investigations. Given that mucin expression and glycosylation vary widely between healthy and diseased tissues and also different hosts, more attention should be given to identifying differences in mucins between healthy and CDI individuals as this could provide insight into disease susceptibility. Because mucin glycans are known to inhibit virulence factor expression in mucus-associated pathogens like *P. aeruginosa* and *C. albicans* (Takagi *et al.*, 2022; Wheeler *et al.*, 2019), their ability to modulate *C. difficile* virulence should be explored for novel therapeutic development.

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CHAPTER THREE: MUCIN ENZYMATIC MODIFICATION BY PRIMARY DEGRADERS ALTERS *C. DIFFICILE* ADHERENCE

3.1 ABSTRACT

Several bacterial species that reside in the mucus layer of the gastrointestinal tract harbor the enzymes necessary for removing glycans from mucin molecules. The liberated monosaccharides can be used as nutrients by these primary degraders or benefit other microbes like *C. difficile* through nutrient cross-feeding. Enzymatic cleavage of mucin glycans can also mediate the availability of specific glycan epitopes necessary for attachment by bacterial adhesins or their toxins. Therefore, the ability of *Bacteroides thetaiotaomicron* or *Ruminococcus gnavus* or their cell-free supernatants to modulate *in vitro* attachment of *C. difficile* to mucus-coated coverslips was investigated. Enzymatic activity of both the live microbes and their respective supernatants was capable of reducing *C. difficile*'s attachment to mucus. Further characterization of the enzymatic repertoire of these bacteria may facilitate a greater understanding of the inter-microbial competition between *C. difficile* and other mucus-associated species.

3.2 INTRODUCTION

Metabolic and ecological perturbation of the healthy colonic microbiota following antibiotic therapy, or by other means, is known to favor *C. difficile* infection. As previously discussed in Chapter 1, protection from infection afforded by the natural gut flora, known as colonization resistance, is mediated through both direct and indirect mechanisms. Direct mechanisms include the production of antimicrobials (Mathur *et al.*, 2014), competition for nutrients (Aguirre *et al.*, 2021; Leslie *et al.*, 2021; Wilson &

Perini, 1988), and metabolism of bile acids (Allegretti *et al.*, 2016; Tam *et al.*, 2020). Indirect mechanisms which protect the host include microbial production of short-chain fatty acids (SCFAs) and polyamines, as well as amino acid metabolism (Pérez-Cobas *et al.*, 2015). The complex community of mucus-associated bacteria inhabits a distinct ecological niche in the digestive tract. Due to its uniqueness, there are likely unexplored mechanisms of colonization resistance that occur within the mucus layer to protect against infection by *C. difficile* and other enteric pathogens.

A major driver of microbial community interactions at the mucus layer involves the production of hydrolytic enzymes which cleave the oligosaccharide side chains of mucins. It's thought that only a few members of the gut microbial community are primary-degraders (Hoskins *et al.*, 1985), or microbes that harbor the hydrolytic enzymes necessary for direct cleavage of mucin glycans. However, bacteria that don't harbor enzymes for direct cleavage still benefit through cross-feeding mechanisms. For example, 25 *C. difficile* genomes surveyed were found to lack the genetic capacity for *O*-linked glycan removal and *C. difficile* R20291 was unable to grown on mucins as a sole carbon source *in vitro* (Engevik, Engevik, *et al.*, 2020). Despite this, *C. difficile* growth on mucin as a sole carbon source was possible following glycan enzymatic cleavage by co-incubation with the primary degraders *Akkermansia muciniphila, Bacteroides thetaiotaomicron*, or *Ruminococcus torques*.

The importance of cross-feeding by *C. difficile* has also been observed in a mouse experiments. *B. thetaiotaomicron* is a primary degrader capable of cleaving sialic acids from mucin, but lacks pathways required for their catabolism once liberated. *C. difficile* does possess catabolic pathways for sialic acid metabolism, and the genes responsible are

upregulated following monosaccharide liberation by *B. thetaiotaomicron in vivo* (Ng *et al.*, 2013). Therefore, liberated mucin-derived monosaccharides provide an important nutrient source for *C. difficile* during colonization and outgrowth. However, in addition to liberating sialic acid, *B. thetaiotaomicron* has also been shown to increase goblet cell differentiation and the ratio of sialylated to sulfated mucins in mono-associated rats (Wrzosek *et al.*, 2013). Therefore, beyond nutrient cross-feeding, primary mucin degrading species could also influence *C. difficile*'s ability to colonize by modulating host mucin production and glycosylation, particularly under conditions of a perturbed gut ecosystem. To fully understand conditions which promote colonization or dictate disease severity, pathogen, host, and microbiota interactions must all be taken into consideration.

In addition to liberating nutrients, the hydrolytic activity of primary degraders also has the potential to expose or destroy adhesin receptors necessary for attachment by microbes or their toxins. By influencing available receptor sites, mucus-associated bacterial species play a key role in determining which commensal and pathogenic microbes are able to colonize and persist in the gut mucosa. For example, in a recent preprint, desulfation of mucin glycans by *B. thetaiotaomicron* was shown to decrease binding of *Escherichia coli* to mucin (Al-Saedi *et al.*, 2017).

We speculate that modification of the structural and chemical features of the mucus layer by commensal microbes is a plausible additional mechanism of colonization resistance against *C. difficile*. Therefore, the aim of this investigation was to begin characterizing the bacterial species and enzymatic functions which impact *C. difficile*'s adhesion to the mucus layer through modification of surface-exposed receptors. Using our *in vitro* model of mucus adherence, we explored the capacity of *B. thetaiotaomicron*

and *Ruminococcus gnavus* to biologically modify surface-exposed glycans and examined the impact of these modifications on *C. difficile*'s ability to bind mucus. Determining the potential of other bacterial species to alter the prevalence of *C. difficile* ligand receptors may allow for the development of precision biotherapeutics for improved treatment of CDI.

3.3 MATERIALS AND METHODS

3.3.1 Bacterial strains and growth conditions

B. thetaiotaomicron VPI-5482 and *R. gnavus* were kindly provided by the labs of Drs. Robert Hutkins and Jennifer Auchtung, respectively, at the University of Nebraska-Lincoln. Before use in experiments, *B. thetaiotaomicron* was selectively isolated on Brain-Heart Infusion (BHI; Remel) media supplemented with 5 g/L yeast extract, 0.5 g/L L-cysteine, 0.5 g/L hemin, 4 mg/mL gentamicin, 100 mg/mL kanamycin, and 30 mg/mL novobiocin to obtain pure cultures (Ho *et al.*, 2017). To confirm the identity of the *B. thetaiotaomicron* VPI-5482 isolate, PCR was performed using the species-specific primers BT_FWD (5'-AACAGGTGGAAGCTGCGGA-3') and BT_RVS (5'-AGCCTCCAACCGCATCAA-3') as previously described (Teng *et al.*, 2004).

Overnight cultures of *C. difficile* R20291 were grown from frozen glycerol stocks on BHIS(TA) agar plates at 37°C in an anaerobic chamber (as described in Chapter 2). For experiments, individual colonies from overnight plates were used to inoculate BHIS(TA) broth.

3.3.2 Biological mucin modification by live primary degraders

The ability of *R. gnavus* and *B. thetaiotaomicron* to modulate *C. difficile*'s adherence to mucin was investigated using an *in vitro* coverslip adherence assay similar to the one described in Chapter 2 (2.3.5). Individual colonies of *R. gnavus* or *B. thetaiotaomicron* were used to inoculate BHIS broth, and cultures were grown to mid-exponential phase (approximately 4-5 hours). Bacterial cultures or BHIS alone were then added to sterile 6-well cell culture plates containing PCM-coated (porcine colonic mucin) coverslips, and plates were incubated at 37°C anaerobically for 3 hours.

Following incubation, PCM coverslips were washed 3 times with pre-reduced PBS to remove attached *R. gnavus* and *B. thetaiotaomicron* cells. To ensure full removal of cells, the coverslips were removed from the anaerobic chamber and exposed to environmental oxygen while simultaneously treated in a digital laboratory ultraviolet oven for 3 minutes (Stratalinker UV Crosslinker; Stratagene). Coverslips were then returned to the anaerobic chamber and quickly washed three times with 10 mL of anaerobic PBS for further removal of attached bacterial cells. *C. difficile* R20291 suspended in PBS (OD600=2.0) was added to the wells, and the remainder of the mucosal adhesion assay was performed as before (see section 2.3.5), with the exception of enumeration media. *C. difficile* selective taurocholate-cycloserine-cefoxitin-fructose agar (TCCFA) (Edwards *et al.*, 2013) was used to ensure accurate quantification of *C. difficile* colony forming units (CFU) alone.

3.3.3 Sialidase activity of cell-free supernatants

To evaluate the sialidase activity of cell-free supernatants (CFS), the commercially available fluorogenic substrate 4-Methylumbelliferyl N-acetyl-a-D-

neuraminic acid sodium salt (4MU-Neu5Ac; Carbosynth) was used as previously described (Crost *et al.*, 2013). Colonies of *B. thetaiotaomicron* and *R. gnavus* were used to inoculate BHIS broth supplemented with 0.3% (w/v) PGM. Cultures were grown at 37°C under anaerobic conditions. After 24 hours of growth, 1 mL aliquots (2 x each strain) were collected. Cells were removed from aliquots by centrifugation (17,000 x g, 5 min., 4°C). The resulting supernatant from one aliquot of each strain was then passed through a 0.45 µm filter (VWR), while activity of the second aliquot was evaluated without filtration. 50 mL of each supernatant was transferred to a black 96-well plate (Corning) and 150 mL of 650 mM 4MU-Neu5Ac (Carbosynth) in PBS (pH 7.40) was added to each well as substrate. Reactions were carried out at 37°C for 2.5 hours with continuous orbital shaking using a BioTek Synergy H1 plate reader. Fluorescence measurements (Excitation 360 nm., Emission 465 nm.) were obtained every 5 minutes. Relative fluorescence unit (RFU) values obtained at 20 and 40 minutes were used to determine relative rates of MU release per minute (see Figures 3.1C&D) after subtracting negative control measurements (BHIS + mucus; no enzyme).

3.3.4 Biological mucin modification by cell-free supernatant

Cell-free supernatant from *B. thetaiotaomicron* VPI-5482 was evaluated for its ability to modulate *C. difficile*'s mucin-binding capacity. Following overnight growth, colonies of *B. thetaiotaomicron* were used to inoculate BHI media supplemented with 5 g/L yeast extract, 0.5 g/L L-cysteine, 0.5 g/L hemin, and 0.3% type III porcine stomach mucin (HiMedia Laboratories). Following 48 hours of anaerobic growth at 37°C, the culture was centrifuged (3,000 x g, 10 min., 4°C) and the resulting supernatant was passed through a 0.2 mm bottle-top filter (ThermoFisher Cat No. FB12566512). Filtered

supernatant containing putative mucin-degrading enzymes, boiled supernatant, or media alone were then incubated with porcine colonic mucin coated coverslips overnight at 37°C. Following treatment, incubation solutions were removed and coverslips were gently washed with sterile PBS (pH 7.2). The mucosal adhesion assay was then performed with *C. difficile* R20291 as previously described (see Chapter 2 Materials and Methods).

3.4 RESULTS

3.4.1 *R. gnavus* and *B. thetaiotaomicron* decrease mucosal adherence by *C. difficile*

B. thetaiotaomicron and *R. gnavus*, two mucin-degrading bacterial species, were incubated with PCM-coated coverslips to allow for enzymatic modification of available surface glycans by the live microbes. Following enzymatic modification, relative attachment of *C. difficile* R20291 to the coverslips decreased for both bacterial species when compared to control coverslips incubated with BHIS alone (Figure 3.1A). Because mucin degrading species are known to secrete the hydrolytic enzymes necessary for glycan cleavage, the ability of cell-free supernatant of *B. thetaiotaomicron* grown in the presence of mucin was also used to treat PCM-coated coverslips. Adherence by *C. difficile* R20291 was similarly significantly decreased (Figure 3.1B) compared to a BHIS treated control coverslip or coverslip treated with boiled cell-free supernatant.

3.4.2 Sialidase activity of cell-free supernatants from primary degraders

To begin characterizing the enzymatic activity of cell-free supernatants from primary mucin-degrading species grown in the presence of mucin, the fluorogenic substrate 4MU-Neu5Ac was used (Crost *et al.*, 2013). As a substrate, 4MU-Neu5Ac is

hydrolyzed by sialidase activity to release 4-methylumbelliferone (4MU) (Minami *et al.*, 2021). The resulting molecule is fluorescent, allowing for detection of relative fluorescence units (RFU) for quantitative comparison of sialidase activity over time (Figure 3.1C). When compared to substrate hydrolysis of the negative control (BHIS supplemented with mucin; no enzyme), both filtered and un-filtered cell-free supernatants from *B. thetaiotaomicron* and *R. gnavus* cultures demonstrated sialidase activity (Figure 3.1D).

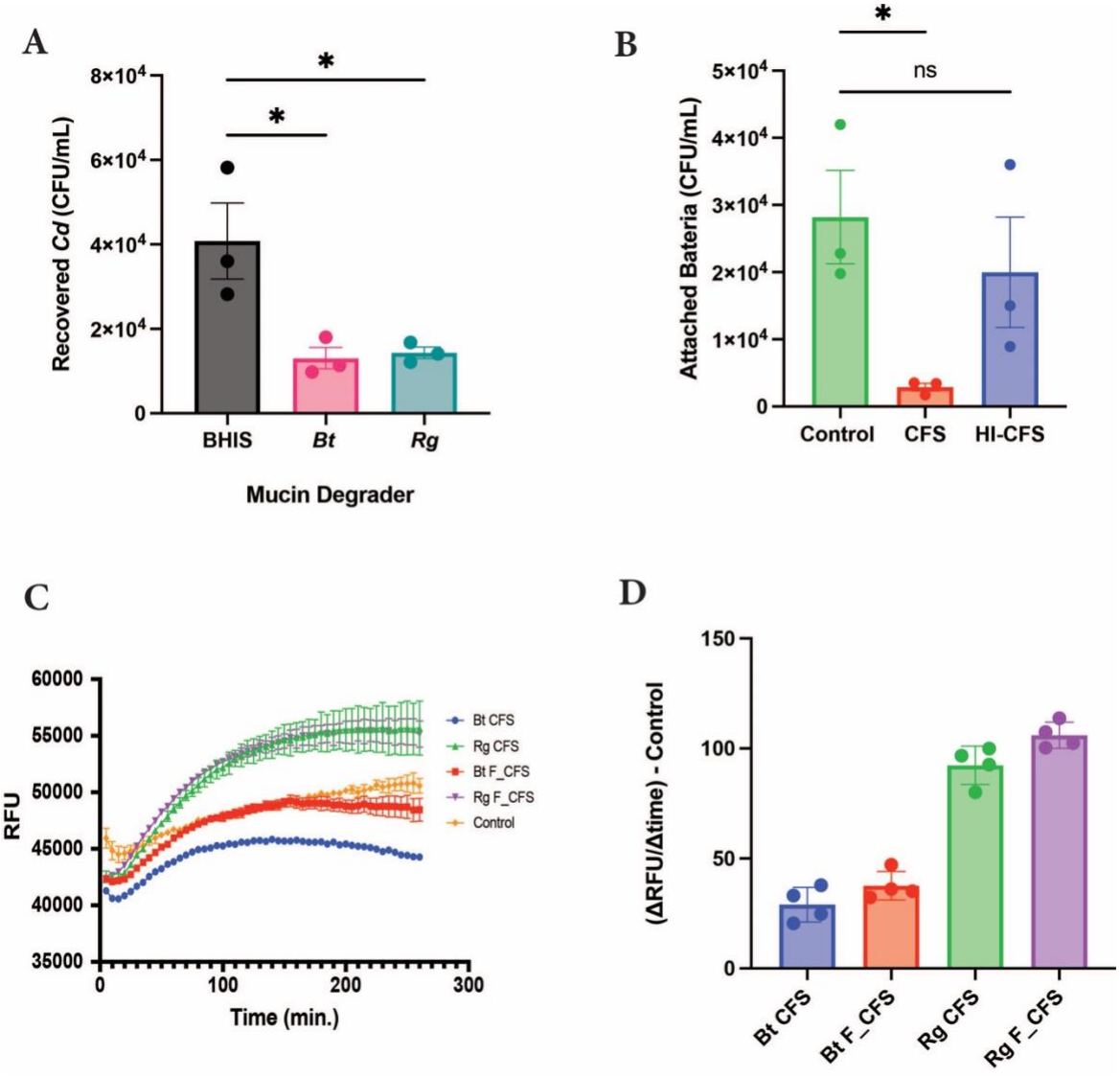

Figure 3.1 Primary mucin degrading bacteria influence *C. difficile* adherence to mucin. (A) Attachment of *C. difficile* R20291 to PCM coverslips following 3-hour coverslip incubation with *B. thetaiotaomicron* (*Bt*), *R. gnavus* (*Rg*), or BHIS (control) with n = 3 coverslips per treatment (Ordinary one-way ANOVA with Dunnett's multiple comparisons test). **(B)** R20291 attachment to PCM coverslips following overnight incubation of coverslips with *B. thetaiotaomicron* cell-free supernatant (CFS), heat-inactivated CFS (HI-CFS), or growth media alone (control) with n = 3 coverslips per treatment (Ordinary one-way ANOVA with Dunnett's multiple comparisons test). **(C)** Sialidase actitvty of *Bt* and *Rg* cell-free supernatants (CFS), filtered CFS (F_CFS), or BHIS supplemented with 0.3% porcine gastric mucin as determined by relative fluorescence units (RFU) (n = 4 reactions per condiition). **(D)** Relative sialidase activity of *Bt* and *Rg* CFS or F_CFS after subtraction of control reaction from the linear portion of Fig. 3.1C (t=20 min. to t=40 min.).

3.5 DISCUSSION

Ruminococcus gnavus and *Bacteroides thetaiotaomicron* are two mucin-degrading microbes which are thought to cross-feed *C. difficile* during infection through enzymatic cleavage of mucin glycans (Engevik, Engevik, *et al.*, 2020; Ng *et al.*, 2013). By modulating nutrient availability, these interactions may shape disease susceptibility and progression during CDI. Because *C. difficile* appears to colonize the mucus layer during infection (Semenyuk *et al.*, 2015), we speculated that mucin glycan cleavage would also modify the accessibility of bacterial adhesin receptors on the surface of mucin molecules, thereby impacting *C. difficile* attachment. When an artificial mucus layer consisting of immobilized porcine colonic mucins was briefly incubated with *R. gnavus* or *B. thetaiotaomicron*, we observed a subsequent decrease in relative mucin adhesion by *C. difficile*. Similarly, treatment with cell-free supernatant from *B. thetaiotaomicron* also decreased adhesion. These results suggest that primary mucin degrading species could influence *C. difficile* colonization by directly altering the epithelial surface glycan landscape and receptors available for attachment. Further characterization of this

enzymatic activity and confirmation of putative mucin glycan hydrolysis is necessary to better understand their relevance to *C. difficile* colonization. Targeted biological glycan modification in combination with rationally selected microbial consortiums known to deplete NeuAc and GlcNAc sugars (Pereira *et al*., 2020) could greatly reduce dependency on destructive courses of antibiotic therapies administered to treat resistant and recurrent CDI.

While *C. difficile* is thought to utilize sialic acid liberated by mucin-degrading microbiota following antibiotic disruption (Ng *et al.*, 2013), other bacterial species also likely benefit from an aberrant nutrient landscape. For example, similar to *C. difficile*, *Fusobacterium nucleatum* does not harbor the enzymes necessary for sialoglycan foraging but does encode the machinery required for their transport and catabolism (Agarwal & Lewis, 2021). Interestingly, it was recently reported that *F. nucleatum* and *C. difficile* co-aggregate in the context of an *in vitro* mixed microbial community, promoting biofilm formation and changes in the expression virulence-associated genes (Engevik, Danhof, *et al.*, 2020). It may therefore be possible that increased levels of mucin-derived monosaccharides (e.g., free sialic acid) promote synergistic interactions between cross-feeding benefactors like *F. nucleatum* and *C. difficile*. In the context of CDI, in addition to interactions with mucin-degraders, understanding polymicrobial relationships with other cross-feeding species may further elucidate mechanisms of inter-microbial competition which control or potentiate disease.

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