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Characterization of the Clostridium Difficile Biofilm

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CHARACTERIZATION OF THE *CLOSTRIDIUM DIFFICILE* BIOFILM

A THESIS SUBMITTED TO
THE FACULTY OF THE GRADUATE SCHOOL
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MASTER OF SCIENCE

PROGRAM IN INFECTIOUS DISEASE AND IMMUNOLOGY

BY

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The views expressed in this article are those of the author and do not reflect the official policy or position of the United States Air Force, Department of Defense, or the U.S. Government.
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For Dad, Christine, Garrett, Nick, and Mom
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CHAPTER ONE
LITERATURE REVIEW

Introduction

*Clostridium difficile* infection is one of the most common nosocomial infections [1], and *C. difficile* is one of the most commonly isolated causes of hospital acquired diarrhea [2]. Currently, between 15-23% of nosocomial diarrhea cases are caused by *C. difficile* [3]. *Clostridium difficile* is a Gram-positive, spore forming, rod shaped, obligately anaerobic bacterium. *C. difficile* belongs to the family Clostridiaceae, in the class Clostridia, and of the phylum Firmicutes. *C. difficile* is an opportunistic gastrointestinal pathogen. While it is not part of the normal flora in adults, patients can be colonized with *C. difficile* upon antibiotic treatment. Antimicrobial treatment results in the reduction of the commensal microbiota, allowing for the efficient colonization of pathogenic *C. difficile*. As a result of the widespread usage of antibiotics, *C. difficile* is prevalent in hospitals worldwide. *C. difficile* is acquired by the ingestion of spores. Spores are a highly resistant cell type that allows the bacteria to remain dormant and persist on several hospital surfaces, including hospital equipment and the hands of health care workers. Spores are resistant to most decontamination methods, with the exception of diluted bleach used to clean hospital surfaces. This makes decontamination efforts challenging for health care systems. Once the spore is ingested, it passes through the stomach and germinates in the small intestine. The resulting vegetative bacteria then
efficiently colonize the colon. After colonization, vegetative cells produce toxins that accumulate in the colon and cause epithelial damage, with disease symptoms ranging from mild diarrhea, to pseudomembranous colitis, and even death from multi organ failure [4, 5]. Patients can be treated successfully with antibiotics for Clostridium difficile infection (CDI). However, an estimated 20% of patients develop recurrent infection [6, 7].

The interaction between host epithelium and the bacteria during C. difficile colonization is a critical step in creating infection, yet the mechanism of interaction is not understood. It is also not known how C. difficile evades host immune responses. These are important questions for clinicians and scientists to answer in advancing treatment and prevention of CDI.

Bacterial biofilms have been identified in several human diseases. Biofilms can facilitate attachment to host mucosa, and can protect bacteria from critical host defense molecules like antibodies and anti-microbial peptides. Additionally, biofilms can help bacteria avoid host immune recognition and persist in the host [8]. A C. difficile biofilm could promote persistence and relapse (a recurrence with the same strain) if it serves as a reservoir for spores.

I have sought to characterize a previously unidentified C. difficile biofilm. C. difficile biofilms could contribute to CDI by facilitating attachment of C. difficile to host epithelium; by resisting host defenses and antimicrobial drugs; by accumulating toxin and directing it to host tissues; and by harboring dormant spores that could facilitate recurrent
disease. Understanding how *C. difficile* interacts with host tissues, and evades host defenses and therapeutics, would greatly facilitate development of novel treatments.

**Epidemiology of CDI**

**Disease Characteristics and Epidemiology**

The earliest indication of CDI includes symptoms of diarrhea, fever, and bloating. The toxins damage the gut mucosa, which creates an inflammatory response. If the infection continues, CDI can become more severe with the formation of a pseudomembrane. A pseudomembrane is made up of dead epithelia, sloughed-off mucous, leukocytes, and fibrin which forms over the inner surface of the colon [8]. Finally, toxic megacolon can occur in which the colon becomes distended and bloated with gas and the colon becomes paralyzed with the possibility of rupture. Kidney failure can occur, as well as heart damage, from the systemic spread of toxin. Large sections of colon may need surgical removal, and if left untreated, any of the severe symptoms can result in death.

The rates of CDI are increasing worldwide, in both the number of infected patients and in mortality. In the USA in 1996, the number of reported CDI cases was 82,000. By 2003, the rate had doubled [9]. The mortality rate also increased. One study indicates that between 1999-2004 the mortality rate has increased 35% each year [10]. These increases are primarily attributed to the emergence of hypervirulent strains that have greater antibiotic resistance than previous epidemic strains.
Treatment and Prevention

The widespread use of antibiotics has caused *C. difficile* to be endemic in hospitals worldwide. Use of nearly any antibiotic will compromise the gastrointestinal microbiota, and can make a patient susceptible. Several antibiotics can place a patient at greater risk for CDI than others, including cephalosporins and ampicillin [11, 12]. *C. difficile* has developed resistance to some antibiotics, most notably the third generation cephalosporins, clindamycin, and recently the floroquinolone class [11-13]. One study indicates that fluoroquinolones were responsible for 55% of infections during the hypervirulent *C. difficile* outbreak in Quebec in the mid-2000’s [14]. As these antibiotics are widely used, the most prevalent epidemic *C. difficile* strains are often resistant to these common antibiotics. Vancomycin and metronidazole are effective antibiotics currently in use to treat CDI, and presently there has not emerged any resistant *C. difficile* strains to either antimicrobial [8]. However, treatment with metronidazole has required an increase in the dosages for effective treatment of symptoms [15].

The costs associated with CDI are great. CDI prolongs a patient’s hospital stay, treatments are extended, and a recent estimate placed the cost of CDI for each case can add up to $4000 to a U.S. patient’s hospital bill. This estimate can increase up to $18,000 for recurrent CDI [5, 16]. With the onset of recent epidemics, the current estimate for U.S. healthcare systems places the annual cost of CDI treatment to be $3.2 billion [17].

To acquire CDI, a susceptible patient must ingest *C. difficile* spores, which are widespread in hospital environments. Spores can be found on hospital surfaces, medical devices, and the hands of healthcare workers [5]. *C. difficile* spores can remain on
hospital surfaces for extended periods of time, in one estimate as much as 7 months [18]. Therefore, it is understandable that aggressive decontamination methods for \textit{C. difficile} spores are a main priority for hospitals. \textit{C. difficile} spores are highly resistant to many decontamination methods. Most importantly, antibacterial alcohol-based hand gels are not effective in killing \textit{C. difficile} spores [19]. With the widespread use of hand gels that can often be used in lieu of traditional soap and water hand washing methods, there is evidence that the recent rise in CDI can be partially attributed to hand gel use [20]. Proper hand washing methods have been encouraged, along with the use of gloves to reduce the incidence of transmission from health care workers to patients [21, 22]. Additionally, the use of bleach to clean surfaces and using disposable medical equipment has been shown to reduce rates of transmission [21].

Finally, limiting the use of antibiotics can be effective in preventing CDI acquisition. A recent study with hypervirulent \textit{C. difficile} using a comprehensive approach to reduce epidemics showed that increased hygiene and cleaning measures, along with controlled and limited antibiotic usage can reduce the CDI incidence rate by as much as 78% [23]. Limited usage of cephalosporins, clindamycin, macrolides and ciprofloxacin with a hypervirulent \textit{C. difficile} outbreak in Quebec reduced the total number of CDI cases by 60% [24].

\textbf{Recurrence of Disease}

Most patients are successfully treated with antibiotics, which clears the infection and eradicates disease symptoms. However, the antibiotic therapies used do not always eradicate the organism. In 20-35\% of CDI cases, the disease returns even though the
initial symptoms have been treated [7]. Disease recurrence usually occurs within 3-30 days after the completion of antibiotic treatment [6]. Disease recurrence can be due to either relapse with the same strain of *C. difficile*, or reinfection with a new strain. The time for relapse with the same strain in general is much shorter than reinfection with a new strain [6]. One study indicates the mean time for relapse is 14.5 days, with 42.5 days being the mean time to reinfection [25]. There are several potential explanations pertaining to the mechanism of recurrent infections, including inadequate immune responses, and persistent disruption of the microbiota [26-29]. One study suggests that the diversity of the microbiota in recurrent CDI patients is significantly reduced compared to healthy individuals, indicating that the microbiota has not adequately regenerated to exclude future colonization with toxigenic *C. difficile* [26]. While these are likely contributing factors to recurrence, there are quite possibly other unknown mechanisms that contribute to the frustrating issue of recurrent CDI.

**Sporulation**

Spores are a highly resistant, metabolically inactive dormant cell type. *C. difficile* spores are resistant to heat, chemical and pH extremes, mechanical forces, and aerobic environments. Spores persist on hospital surfaces and, once ingested, spores are resistant to destruction by stomach acid, and travel to the small intestine for germination. The ability of spores to persist in the environment is a key factor in CDI acquisition and transmission [52].

The process of sporulation creates a resistant spore from a vegetative cell. This complex process is highly regulated by a sophisticated gene expression network. A
signaling cascade of sigma factors creates a stepwise response to several signals that can induce sporulation to begin. The most common signal that can initiate sporulation is starvation. In the case of *C. difficile*, an obligate anaerobe, oxygen can initiate sporulation as well. Little is known about the signaling network in *C. difficile*. However, the molecular mechanisms are better understood in *B. subtilis*.

Spo0A is a master regulator, and is phosphorylated by a phospho-relay cascade, leading to the activation of Spo0A. Spo0A~P can then activate downstream regulators which initiate steps to sporulation, and repress vegetative cell functions [30]. A sigma factor cascade is the major controller of sporulation, which occurs in both the mother cell and the spore. Spo0A~P and sigma H are activated in the pre-divisional cell, which leads to an asymmetric division creating the prespore and the mother cell divided by a septum [30]. Sigma F is then activated in the prespore, while sigma E is activated in the mother cell. Sigma E leads to the production of coat proteins and, by communication with the forespore, activates Sigma G in the forespore. Sigma G initiates a signaling cascade that results in the activation of sigma K, leading to the assembly of the outer spore coat [30].

In the initial stages of sporulation, asymmetric division occurs two z rings at either pole of the sporangium. One z ring forms the septum, while the other z ring is disassembled. During the septum formation, the chromosomes replicate and form an axial filament. The remaining section of the chromosome is actively transported into the forespore and segregated from the mother cell with the completion of the septum. This results in two distinct cells, both with a complete chromosome. Then, the mother cell migrates around the forespore and pinches the membrane off to completely engulf the
forespore. At this point, the chromosome in the forespore is remodeled into a circular structure, the cortex forms a thick layer of cell wall material and, finally, the protective protein spore coat forms around the outside, completing the synthesis of the spore. Then, the mother cell lyses by the action of lytic enzymes and liberates the mature spore [31].

Pathogenesis

Toxins and Other Virulence Factors

_Clostridium difficile_ produces two large toxins, A and B, encoded by _tcdA_ and _tcdB_, respectively. Initially, toxin A was believed to be the most important virulence factor, when the work of Lyerly and Wilkins determined that Toxin A given intragastrically produced similar disease symptoms to a total supernatant, while toxin B did not produce significant disease. Toxin B was only effective as a cytotoxin when given to animals with bruised ceca, or given with small amounts of toxin A [32]. The view that toxin A was the important virulence factor was challenged when clinical reports provided evidence of disease causing strains that did not produce toxin A. Strain 8864 is a naturally occurring toxin A-, toxin B+ strain capable of producing infection [33]. Additional A-/B+ strains were later reported that were responsible for fatal human cases and epidemics, as well as producing disease in hamsters [34-36]. Genetic manipulation of _C. difficile_ has allowed for molecular analysis of the importance of toxins A and B as virulence factors. By constructing isogenic mutants of _tcdA_ and _tcdB_ in strain 630, followed by introduction of the mutant strain to a hamster model, it was shown that toxin B is the essential virulence factor [37]. Hamsters infected with the toxin B mutant displayed significantly less mortality than either the wild type or toxin A mutant. The
fatal cases of hamsters infected with toxin B mutants were found to have revertant strains in which the recombination vector was excised, providing additional support that toxin B production is favored for pathogenesis [37]. A more recent study determined that both toxins contribute to virulence in a hamster model. Null mutations in tcdA and tcdB were followed with infection in the master model. Hamsters infected with C. difficile without toxin B (A+, B-) survived several days longer than those infected with C. difficile lacking toxin A (A-, B+) [38]. It appears that both toxins contribute to pathogenesis, though toxin B makes a larger contribution to virulence. It is likely though that the contribution of toxins to virulence is not yet fully understood.

The Pathogenicity Locus or PaLoc encodes the genes for toxin A and B, tcdA and tcdB, as well as three other genes tcdR, tcdE, and tcdC [39]. Non-toxigenic strains lack the PaLoc entirely [40]. Toxin A and B are large toxins that belong to the family of Large Clostridial toxins, which includes other toxins from C. sordelli and C. novyi, all of which share similar 3-domain structure and mechanism of action [41]. In C. difficile strain VPI 10463, tcdA is an 8133 base pair gene, encoding a 2710 amino acid, 308 kDa protein. tcdB is 7098 nucleotides, encoding a 3266 amino acid, 279 kDa TcdB protein [42, 43].

The three other genes encoded in the locus include tcdC, tcdE, and tcdR [39]. tcdC encodes a 26 kDa protein that is thought to act as a negative regulator for toxin production by acting as an anti-sigma factor [44]. tcdE encodes a protein with unknown function, but shares homology with phage holins. TcdE protein has been shown to play a role in toxin release during stationary phase [45]. tcdR codes for a sigma factor and is
required for \textit{tcdA} and \textit{tcdB} expression, acting as a positive regulator \cite{46, 47}. TcdR is a 22 kDa protein sigma factor, which binds to RNA polymerase core enzyme and allows transcription at specific promoters.

Separate from the PaLoc, \textit{C. difficile} encodes an additional toxin called binary toxin. Binary toxin is produced in two individual fragments, CdtA and CdtB, which are secreted separately and join extracellularly to form a functional toxin. CdtA is the enzymatic fragment, while CdtB is responsible for binding and translocation \cite{48}. Host cells take up binary toxin through receptor-mediated endocytosis. Once inside the cell, binary toxin ADP-ribosylates toxin, which leads to the degradation of the cytoskeleton \cite{49}. Binary toxin is not present in all \textit{C. difficile} strains, and it has been found in strains that do not contain the PaLoc. Strains that contain the binary toxin without toxins A and B are able to colonize animals, but do not create any disease symptoms. In the ligated ileal loop assay, however, fluid accumulation can be caused by purified binary toxin \cite{50, 51}.

In addition to the toxins produced by \textit{C. difficile}, there are several other virulence factors including the bacterial capsule, fimbriae, and hydrolytic enzymes. These virulence factors have been shown to be present more frequently in highly toxigenic strains \cite{52, 53}.

\textbf{Mechanisms of Host Response and Pathophysiology}

Following secretion from the bacteria, toxins A and B bind to the host cell surface. Once bound, the toxins are endocytosed via clathrin-mediated endocytosis. During cellular uptake, the toxins are proteolytically processed. The toxins contain both
enzymatic and translocation domains. The C terminus contains the translocation domain and remains in the endosome after endocytosis, while the enzymatic domain is delivered into the cytoplasm [54]. Autocatalytic cleavage of toxin B is carried out by a cysteine protease, which activates toxin B in the cytoplasm [55]. Once inside the cytoplasm, toxin A and B can glucosylate GTPases. The GTPases are signaling proteins that cycle between an active and inactive state. When bound with GTP, the GTPase is active and can interact with numerous effector molecules. GTP hydrolysis to GDP results in the inactivation of the GTPase [56]. When the toxins glucosylate the GTPase, the protein is permanently inactivated. This blocks the action of GEF and GAP proteins preventing any new GTP exchange, and ultimately blocking the interaction with effectors [57]. The inactivated GTPases affect the host cell cytoskeleton causing cell rounding and inducing apoptosis [7, 58]. In addition to GTPase activity, toxin A and B can damage the mitochondria, which can lead to apoptosis [59], and disrupt the tight junctions in the gut epithelia [60].

**Spore Germination, Colonization and Adherence**

CDI occurs when antimicrobial-treated patients ingest *C. difficile* spores, spores germinate in the small intestine, and the resulting vegetative cells colonize the colon. The vegetative cells produce toxin, which damages the mucosal and epithelial cell surfaces and creates symptoms of infection. *C. difficile* spores do not create toxins on their own, and are metabolically inactive. Therefore, *C. difficile* spores are incapable of producing infection without germination. Spore germination occurs when spores undergo a complex process to resume metabolic activity and growth, resulting in vegetative cell
growth. The process of spore germination in *C. difficile* is not well characterized. In general, spores begin to germinate in response to environmental conditions that indicate a favorable environment for vegetative cell growth. In *B. subtilis*, germination begins in response to several germinants, including the nutrient glucose. When glucose binds receptors in the inner membranes of the spore, this interaction triggers the release of cations and dipicolinic acids, leading to an influx of water to rehydrate and expand the spore core. This is followed by the hydrolysis of the cortex peptidoglycan and germination is complete when the spore resumes full metabolic activity and outgrowth [61].

While *C. difficile* spore germination is not well understood, it has been noted that a common bile acid, taurocholate, activates germination in *C. difficile* spores [62]. Taurocholate is hydrolyzed by the action of other bacteria that are normally present in the microbiota, which breaks taurocholate into secondary bile salts cholate and chenodeoxycholate [63, 64]. These secondary bile salts have been shown to inhibit *C. difficile* germination, which may explain the contribution of the microbiota in preventing *C. difficile* colonization [63].

Colon colonization is a key event in the course of CDI. After a patient ingests spores, *C. difficile* must colonize the gut to produce high quantities of toxins, which create symptoms and colonic tissue damage during CDI. Colonization can occur with both toxigenic and non-toxigenic strains. Interestingly, early work by KH Wilson indicated that colonization of animals with non-toxigenic strains of *C. difficile* can prevent colonization with toxigenic strains and development of disease [62].
indicates that competition for colonization is an important factor in CDI. As referenced earlier, the normal gastrointestinal microbiota, when present in a healthy individual, excludes *C. difficile* from the gastrointestinal tract and results in protection from CDI. Further studies from Dr. Dale Gerding’s laboratory indicate that colonization with non-toxigenic *C. difficile* is effective as a preventative treatment for CDI with a toxigenic strain in both humans and hamsters [65].

For colonization to occur, the bacteria must associate with the mucosal epithelium. Unfortunately, relatively little is known about the interaction of *C. difficile* with the gut. During infection, the colonic mucosa is shed from the epithelial surface. Additionally, the flow of luminal contents in the colon creates an environment with considerable mechanic force. Yet, despite these physical stresses *C. difficile* is able to persist and remain in the gut. Clearly the adhesion of *C. difficile* to the gut is a strong and resistant interaction.

Several adhesins have been identified in *C. difficile*. Surface layer protein SipA is from a protein class of S-layer proteins that are secreted in many bacterial species [66]. After secretion, S-layer proteins self assemble into a lattice on the cell surface and provide structural integrity. In addition to its role in cell structure, S-layer proteins have been implicated in immune evasion and adhesion [67]. S-layer proteins are glycoproteins, and glycosylation on the bacterial surface has been shown to block complement-mediated lysis enabling immune system evasion [68].

Cwp66 is a surface-associated adhesin, and is described as a heat shock protein located on the bacterial surface [69]. Additional adhesins that have been identified in *C.
*Clostridium difficile* include the flagellin FliC and the flagellar cap FliD [70], the heat-shock protein GroEL [71], and fibronectin-binding protein Fbp68 [72].

**Biofilm Structure and Components**

Biofilms are organized bacterial 3-dimensional communities surrounded and embedded in an extracellular matrix [73]. Biofilms are ubiquitous in nature, and are prevalent in natural, industrial, and hospital settings [74]. Traditionally, it has been understood that biofilms must attach to a surface, either living or non-living, though it has now been determined that biofilms may not always be attached to a surface in the human host, as has been demonstrated in *P. aeruginosa* and *S. aureus* [75-77]. The cells in a biofilm are distinct from planktonic cells of the same organism. Bacteria in biofilms exhibit community behavior and specialized functionality [78], creating a functionally heterogeneous community [74]. Large gene loci have been found to regulate biofilm formation and regulation [79]. When a cell switches to a biofilm mode of growth, changes in gene expression lead to varying phenotypes. Cellular specialization and heterogeneous cell populations will be discussed further with regard to *Bacillus subtilis* biofilms.

The first step in biofilm formation is the attachment of planktonic cells to a surface, creating a small microcolony. Cell-cell communication in this small microcolony is accomplished through quorum sensing (QS), which allows bacteria to survey and sense population density through a series of secreted signals and receptors. With QS, bacterial cells are able to coordinate behavior and respond to environmental factors as a community [74,81]. The initial microcolony then expands through
recruitment of additional planktonic cells and cellular division, while an extracellular matrix develops to encase the bacteria. The biofilm is composed of cells and a surrounding self-produced extracellular matrix that covers and protects the bacteria. The extracellular matrix is typically composed of three primary components, including protein, nucleic acid, and polysaccharides, and is often referred to as the extracellular polymeric substance (EPS). The extracellular matrix can facilitate extracellular signaling and cell-cell communication. Biofilms are porous, and some biofilms contain channels that aid in nutrient and signaling distribution [73, 74, 80, 81]. The final step in biofilm development is dispersion. Motile cells disperse from the mature biofilm which allows bacteria to colonize new surfaces. Erosion of the biofilm refers to continuous release of single cells or small clusters over an extended period. Sloughing dispersal liberates large portions of the biofilm late in biofilm development. Finally, seeding dispersal is a rapid method of dispersal to release single cells or small clusters in a shortened time period [82]. Dispersal can be accomplished through the production of several matrix-degrading enzymes, including proteases, deoxyribonucleases, and glycosidases [82]. Both nitric oxide and D-amino acids have been demonstrated to facilitate dispersal from the B. subtilis biofilm [83, 84].

Biofilms have been implicated in numerous human infections, and biofilms contribute to survival in a variety of ways. Biofilms help bacteria avoid phagocytosis, exclude host defense molecules like antibodies and anti-microbial peptides, adhere to epithelial tissues, and resist antibiotics [85, 86]. Antibiotic resistance can be increased in some cases up to a thousandfold-greater than planktonic cells [87].
**Biofilms in Human Disease**

Biofilms are found in many niches in the mammalian host, including the surface of the gut mucosa [88-90]. Biofilms have now been recognized as the cause or as an exacerbation of numerous chronic infections, including periodontitis, device-related infections, CF pneumonia, chronic urinary tract infections, recurrent tonsillitis, chronic rhinosinusitis, chronic otitis media, and chronic wound infections [85]. Hall-Stoodley and Stoodley suggest that biofilm development is a “persistence factor” rather than a virulence factor. Biofilms are found during infection, but also can be found in control specimens without infection, as seen in the case of chronic rhinosinusitis [85]. Additionally, patients with biofilm infections will often exhibit signs and symptoms of infection, yet diagnostic attempts at culture are negative. Culture tests and detection of *P. aeruginosa* by FISH in chronic wounds showed no correlation with infection, despite demonstrated biofilms present in the wound [91]. Due to the difficulty associated with diagnosing biofilm infections, efforts have been made to outline criteria for chronic biofilm infections. Parsek and Singh in 2003 proposed 4 diagnostic criteria; 1) surface associated, 2) cell clusters encased in matrix, 3) localized infection, 4) antibiotic resistant [86]. These criteria were followed in 2009 by Hall-Stoodley and Stoodley with 2 additional diagnostic postulates to include 5) culture-negative result, and 6) ineffective host clearance [85].

Due to enhanced antibiotic resistance and evasion of host defenses, biofilm infections can be difficult to treat. Several studies describe biofilm matrix and dead cells as a mechanism for delayed antibiotic delivery and “dilution” of antibiotics to live cells in
the biofilm [92]. Jefferson et al. described a delay in antibiotic penetration of the biofilm matrix. While the extracellular matrix does not inhibit the penetration of antibiotics into the biofilm, the rate of penetration may be slowed enough to allow biofilm cells to express particular resistance genes [93]. Biofilms often exhibit resistance to host defense mechanisms in addition to antibiotics. Lam and Costerton demonstrated biofilm growth in post-mortem lung tissue from *P. aeruginosa*-infected cystic fibrosis patients. The lung tissue showed that biofilm was surrounded, but not penetrated, by antibodies and inflammatory cells [94]. A 1997 study also demonstrated that IgG was bound to the surface of biofilms, yet the antibody failed to penetrate the EPS [95]. Resistance to phagocytosis by the action of the extracellular matrix has been documented in *P. aeruginosa* and *S. aureus* [96, 97].

**Bacillus subtilis Biofilm**

*Bacillus subtilis* is a Gram-positive, rod-shaped, aerobic, spore-forming bacterium that is commonly found in the soil. *B. subtilis* is not typically classified as a pathogenic bacterium, though it can cause disease in immunocompromised patients [98]. It is frequently used as a probiotic supplement. *B. subtilis* is a model organism for Gram-positive sporulation and biofilm growth, and is commonly used in laboratories for genetic research as it is highly responsive to genetic manipulation. The *Bacillus subtilis* biofilm has been well characterized and is the best studied Gram-positive biofilm. For this reason, we base several of our experimental questions from characteristics seen in *B. subtilis* biofilm development, conscious of the reality that *B. subtilis* and *C. difficile* are quite different.
Wild type (WT) strains of *Bacillus subtilis* form a biofilm, or pellicle, in standing liquid culture at the air-liquid interface. The WT biofilm displays a structurally complex morphology, with aerial projections serving as the preferential site of sporulation [99]. *B. subtilis* biofilms exhibit cellular differentiation through the formation of a heterogeneous cell population. Individual cells in the biofilm exhibit specialized behavior. Initially, biofilm development begins with the aggregation of motile, swimming cells to the air-liquid interface. From there, cells adhere to one another and cellular division results in long, ordered chains of cells within 12-24 hours of culture incubation. Biofilm formation is controlled by the master regulator Spo0A. Spo0A is activated by phosphorylation in response to nutrient limitation and regulates both matrix production and sporulation [100, 101]. Once cells have reached stationary phase, cellular signaling begins, which initiates matrix production. At about 48 hours of growth, the level of Spo0A−P is at a low concentration to permit cells to differentiate from motile cells to matrix producers/cannibal cells [78, 102]. Spo0A−P regulates several genes responsible for matrix production, notably *eps* and *tasA*. TasA is a major structural protein component of the BS biofilm, and *eps* is responsible for the polysaccharide component of the extracellular matrix [103]. Two other regulators are also involved in cellular differentiation during this stage of biofilm development. DegU is a protein regulator responsible for the production of miner cells. These cells produce exoproteases that degrade extracellular proteins, resulting in amino acid availability for the cells. This delays nutrient limitation and thus sporulation. Another regulator, ComA, allows for the specialization of surfactin producers and competent cells [104]. Matrix production is
thought to be a metabolically taxing event during biofilm formation. This metabolic strain creates distinct regions of the biofilm with severe nutrient limitation. Nutrient limitation initiates additional signaling cascades resulting in high levels of Spo0A~P, leading to sporulation [78, 104]. By 72 hours of growth, the majority of cells in the biofilm have specialized from matrix-producing cells to sporulating cells [78]. For a detailed review of sporulation in *B. subtilis* please refer to page 7-8. The study of cell specialization and cellular fate in the *B. subtilis* biofilm has led to a model of natural sporulation not previously identified. Previous studies had not outlined biofilm growth as a step in sporulation. Laboratory studies detailed the steps of sporulation from liquid growth, which may not be representative of what is occurring in nature. We are particularly interested in the cellular fate of the *B. subtilis* biofilm in comparison with the *C. difficile* biofilm, in which I will propose a unique model for cell fate in Gram-positive biofilms.
CHAPTER TWO
MATERIALS AND METHODS

*C. difficile* strains

Seven human clinical isolates of *C. difficile* were chosen for study. All strains were obtained from the culture collection of Dr. Dale Gerding. Two toxigenic strains, BI6 and BI17, are part of the BI genetic group and have been classified as hypervirulent [13]. Two additional toxigenic strains were chosen including J9 and K14, known to cause disease but not classified as hypervirulent. Strains M3, BY1, and CH1 are non-toxigenic.

**Biofilm Culture Conditions**

All *C. difficile* strains were grown under anaerobic conditions (85% N₂, 5% H₂, and 10% CO₂) in a Bactron IV chamber (Sheldon Manufacturing, Cornelius, OR) at 36°C. Overnight cultures were grown in Tryptic Soy (Tsoy) broth (BD Biosciences, Boston, MA) until an O.D. of 0.8. 1ml of culture was then centrifuged and resuspended in 2ml of Tsoy broth. Black polycarbonate membranes (Catalog # HTBP02500 Millipore, Billerica, MA) were inoculated with 10 μl resuspended culture. The polycarbonate membranes were placed on Tsoy agar nutrient medium and incubated for 1-14 days at 37°C.
Visualization of Biofilm Architecture

Biofilms were cultured as described above. The polycarbonate filter is transferred to a glass slide. To preserve colony architecture, 1% gluteraldehyde was carefully pipetted onto the biofilm and incubated for 30 minutes. Biofilms were then stained with 0.05% calcofluor white in 1M Tris, pH 9 for 15 minutes, followed by rinsing with deionized water. Biofilms were then imaged using a confocal laser scanning microscopy, LSM 510 (Carl Zeiss Inc, NY, USA) using Diode laser (405-430nm). Confocal images were analyzed using Zeiss LSM Image Browser software.

Matrix protein Isolation

Biofilms were cultured as described above. The biofilm was scraped from the polycarbonate membrane and suspended in 0.5 ml 0.9% NaCl. Bacteria were separated from the matrix fraction by centrifugation (14,000 rpm for 20 minutes at 4°C) and filtration of the supernatant through 0.22um PVDF membrane filters (Millipore, Billerica, MA). For SDS-PAGE, the filtered supernatant was incubated with 1/100 volume 2% deoxycholate for 30 min at room temperature, then 1/10 volume of 100% trichloroacetic acid was added and the mixture incubated for 2 hours on ice. The pellet was centrifuged, washed with 500 μl cold 100% acetone, and sat for 10 minutes to dry, then resuspended in 50 μl MQ-H2O. Then, 12.5 μl 4x SDS-PAGE protein loading buffer was added for SDS-PAGE electrophoresis. Proteins were visualized using Coomassie Blue stain (Biorad, Hercules, CA).
**Total soluble protein Isolation**

Biofilms were cultured as explained previously. The biofilm is scraped from the polycarbonate membrane and suspended in 0.5 ml 0.9% NaCl. Bacteria were separated from the matrix fraction by centrifugation (5,000 rpm for 20 minutes at 4°C). The matrix supernatant was removed, and the cell pellet was resuspended in 0.5 ml 0.9% NaCl. The cells were again centrifuged (5,000 rpm for 20 minutes at 4°C) and the supernatant removed to wash the cells for a total of two washes. The biofilm cells were then resuspended for a third time in 0.9% NaCl. Cells were sonicated (Branson 450 Sonifier, Danbury, CT) on ice for 5 minutes total with 1 minute bursts (output control 5). After sonication, the cells were centrifuged at 14000 rpm for 30 minutes at 4°C. The supernatant was removed and 100 μl 4x SDS-PAGE running buffer added and boiled for 5 minutes. The lysed cells were electrophoresed through a 12% SDS-PAGE and proteins were visualized using Coomassie Brilliant Blue stain.

**Proteomic Identification of C. difficile Matrix Proteins**

Matrix protein extracts were electrophoresed using denaturing 12% SDS-PAGE and stained with Coomassie Brilliant Blue. We identified 4 abundant matrix protein bands which were distinct from a comparative SDS-PAGE gel of *C. difficile* surface protein bands. The 4 bands were excised, and the proteins were identified using MALDI-TOF-TOF mass spectrometry analysis. All mass spectrometry analyses were performed by Alphalyse Inc, Palto Alto, CA.
Phenol Sulfuric Acid Assay

Three standard concentrations of galactose were made by diluting galactose in MQ-H₂O, 50 μg/ml, 250 μg/ml, and 500 μg/ml. Matrix extracts were made as previously described using biofilms of three differing ages (1, 3, and 6 days). 0.5 ml of non-precipitated matrix suspended in 0.5 ml 0.9% NaCl, or 0.5 ml galactose standard, or 0.5 ml MQ-H₂O, was combined in a glass tube with 0.5 ml 5% phenol in 0.1M HCl and 2.5 ml H₂SO₄, and allowed to incubate for 5 minutes for color development. After incubation, the color of each matrix sample was then visually compared with the standards. A darker shade (range from clear to scarlet red) indicates a higher polysaccharide concentration.

Polysaccharide staining and Microscopy

Concanavalin A (ConA) lectin conjugated with Texas Red (Molecular Probes, Eugene, OR) was used to label extracellular polysaccharides. Stock solutions were prepared according manufacturer’s instructions and stored at -20°C in 100 μl aliquots. Con A was diluted in PBS before use to a lectin concentration 100 μg/ml. Wheat Germ Agglutinin (WGA) (Molecular Probes, Eugene, OR) was made to stock solutions according to manufacturer’s instructions and stored at -20°C in 100 μl aliquots. WGA was diluted in PBS before use to 3 lectin concentrations of 10 μg/ml, 50 μg/ml, and 100 μg/ml. 100 μl of WGA+PBS solution was carefully placed on the top of biofilms grown on polycarbonate membranes. After incubation for 1 hr at room temperature in darkness, excess lectin solution was removed by washing four times with PBS. To stain cells, 100 μL Syto 9 (Molecular Probes, Eugene, OR) was carefully pipetted onto biofilms and
incubated in darkness for 5 minutes. Samples were examined under a LSM 510 laser scanning microscope using an excitation wavelength of 595 nm and emission wavelength of 615 nm.

**Nucleic Acid Staining and Microscopy**

Biofilms are cultured as previously described. Syto 9 (live) and propidium iodide (dead) stain from LiveDead Bacterial Viability Kit (Molecular Probes Eugene, OR) were used to stain biofilms. Samples were examined under a LSM 510 laser scanning microscope using an excitation wavelength of 405 nm and emission wavelength of 525 nm.

**Nucleic Acid Degradation and Disruption of Biofilm Architecture**

Biofilm inoculums were prepared as described above. Before the inoculation of cells onto polycarbonate membrane, DNase I was added to resuspended bacteria to concentrations 0, 10, 50, 100 μg/ml. Bacteria + DNase were inoculated onto the membranes, and membranes were incubated for 24 hours, followed by staining with LiveDead Bacterial Viability Kit (Molecular Probes, Eugene, OR). Samples were examined under a LSM 510 laser scanning microscope (Carl Zeiss Inc, NY, USA) using an excitation wavelength of 405 nm and emission wavelength of 525 nm.

**Immunodetection**

Western immunoblotting experiments were performed using matrix protein extracts, harvested as explained previously. Total protein concentration was quantified using a BCA Protein Assay Kit (Pierce, Rockford, IL). Equal protein loads (3μg) were electrophoresed on denaturing 6% SDS-PAGE and transferred for 1.5 hours to 0.22 μM
nitrocellulose membrane at 330 mA, using Transfer Buffer (0.025M Tris, 0.192M Glycine, 20% Methanol) in a Mini Trans-Blot Electrophoretic Transfer Cell (Bio-Rad, Hercules, CA). Membranes were blocked overnight at 4°C, using 5% nonfat condensed milk, 0.1% Tween 20 in 1X PBS. The membrane was probed with 1:1000 dilution mouse anti-toxin antibody harvested from *C. difficile* infected mice [105] for 1 hour in 5% milk in PBS, 0.1% Tween 20 in PBS at room temperature with shaking. Membranes were washed 3 times with washing buffer (PBS, 0.1% Tween 20) for 10 minutes. The secondary antibody was goat anti-mouse IgG (H+L) HRP conjugate (Jackson Immuno-Research, West Grove, PA). Secondary antibodies were incubated for 1 hour at RT, then washed 3 times with washing buffer and three times with PBS. Proteins were visualized using the SuperSignal West Pico Chemiluminescent Substrate (Pierce, Rockford, IL) system, according to the manufacturer’s directions. ImageJ software (NIH, Bethesda, MD) was used to quantify Western signal.

**Immunofluorescence Microscopy**

Biofilms were cultured as described above to 3 days old. Polycarbonate membranes were transferred to a Silane coated glass slide (Fisher Scientific, Pittsburgh, PA). Biofilms were blocked with a 5% BSA blocking solution in PBS and was carefully added to biofilm and incubated for 1 hour. Primary antibody incubation was done with anti-toxin antibody from *C. difficile* infected mouse serum [105] in a 1:100 dilution overnight at 4°C. Biofilms were washed 3 times with PBS by carefully pipetting PBS onto the membrane, waiting 5 minutes, and then removing the PBS with a pipette and filter paper. The secondary antibody, rat anti-mouse IgG1 conjugated to FITC (Catalog #
406605, BioLegend, San Diego, CA), was added and the biofilm incubated for 1 hour at room temperature. Then biofilms were washed 3 times with PBS. Cells were stained with 0.05% calcofluor-white for 5 minutes. Samples were examined under a LSM 510 laser scanning microscope (Carl Zeiss Inc, NY, USA) using Diode laser (405-430 nm) and Argonne laser (488 nm).
CHAPTER THREE

EXPERIMENTAL RESULTS

Structure and Contents of the _C. difficile_ Biofilm

Colony Architecture

For an initial characterization, we needed to visualize the actual structure of the _C. difficile_ biofilm, including the orientation of cells and the extracellular matrix. We used calcofluor white staining and confocal imaging to visualize a laboratory cultured biofilm, and began to identify the contents of the biofilm, identify stages of growth, and determine the structure of the biofilm through the organization of cells, spores, and matrix. Figure 1 shows biofilms that we analyzed of three differing stages of 24 hours, 3 days, and 6 days. At 24 hours, we saw several small clusters of cells on the outer edges of the macrocolony. These colonies contained rod shaped staining particles, consistent with the shape and appearance of growing vegetative cells. At 3 days, the small cell clusters on the edge of the macrocolony we saw at 24 hours were no longer present, but we did see cell clusters on the interior of the macrocolony. Instead, 3 day old biofilms contained rod shaped vegetative cells, as well as smaller staining particles. To identify the smaller staining particles, we used phase contrast microscopy and saw ovoid shaped spores (Fig 1B). A 6 day old biofilm contained very few rod shaped vegetative cells, and contained abundant ovoid shaped spores on the inside of the colony. Small cell clusters containing vegetative cells were visible on the outer edges.
Figure 1. A) CLSM imaging, with calcofluor, of laboratory-cultured biofilms over 6 days. From left to right: Calcofluor stained 1 day (strain BI17), 3 day (BI6) and 6 day (BY1) biofilms. X-Y (center), X-Z (top), and Y-Z (right side) are shown. A cell “cluster” is evident in the upper right of the left panel. Rod shaped vegetative cells are evident at 3 days (middle) and spores (ovoid shaped) are predominant at 6 days (right). Data is representative of all strains analyzed.
Figure 1. B) Phase contrast and fluorescent images of a 6 day biofilm (strain BI6). The same field is imaged with calcofluor staining (left) and phase contrast (right). Circled areas emphasize clusters of spores.
Extracellular Nucleic Acid

In well studied biofilm species, extracellular nucleic acid (eDNA) has been demonstrated as a matrix component [106-108]. eDNA has been implicated as a necessary component for initial biofilm formation and may be important for the structural integrity of the biofilm [106-108]. As a preliminary experiment, we wanted to determine whether extracellular DNA was present in the C. difficile biofilm matrix. We used live-dead cell staining to identify and locate eDNA in the matrix. Figure 2 demonstrates both live and dead cell material in the biofilm. Syto-9 staining (shown in green) shows rod shaped particles, which are presumably live cells. Propidium iodide staining (shown in red) reveals diffuse staining throughout the entire field, as well as some red cells, which is evidence of dead or dying cells. When shown as individual fields, it is evident that propidium iodide is space filling in the field (circled areas in Fig 2). I interpret this as evidence for free nucleic acid content present in the matrix.
Figure 2. CLSM imaging of a laboratory cultured biofilm using the nucleic acid stains Syto-9 and propidium iodide. A. Syto-9 (green) and propidium iodide (red) double labeling of a 3-day biofilm (strain BI6). Syto-9 stains live cells and propidium iodide stains dead cell material. B and C shows the same field, without merging the two channels, so that the propidium iodide (right) and Syto-9 (left) staining can be seen individually. Circled areas indicate space filling extracellular DNA where cells are absent.
Extracellular Polysaccharides

Polysaccharide content is a major component of most biofilms and comprises a large part of the EPS. Biofilm sugars form a thick covering over cells, protecting the biofilm cells from physical stress, providing structural strength to the biofilm and evasion from host defense and antimicrobial degradation [74, 85]. As a preliminary experiment, we first wanted to identify whether polysaccharides were a component of the *C. difficile* biofilm matrix. We used a phenol sulfuric acid assay to test this possibility [114]. The phenol sulfuric acid assay of biofilm matrix extracts showed a slightly yellow color, which was darker than the control MQ-H2O and 50 μg/ml galactose standard, but lighter in color than the 250 μg/ml galactose sample (Figure 3). The coloration of the biofilm matrix extracts did not visually differ between the three biofilm ages (1, 3, and 6 days) (Fig 3). I used this visual comparison to interpret that indeed carbohydrate was present in the biofilm matrix.
Figure 3. Phenol sulfuric acid assay comparing the polysaccharide concentration of laboratory cultured biofilms to galactose concentrations. Polysaccharide concentration of *C. difficile* strain BI17 (right) biofilm matrix compared to galactose and water shows the polysaccharide concentration does not vary greatly by biofilm maturity, and appears to contain between 50 μg/ml and 250 μg/ml polysaccharide. Lanes: 1) MQ-H₂O, 2) 50 μg/ml galactose, 3) 250 μg/ml galactose, 4) 500 μg/ml galactose, 5) 1 day biofilm, 6) 3 day biofilm, 7) 6 day biofilm.
We then sought to identify whether the matrix polysaccharides exhibited any localization to particular areas of the biofilm. We hypothesized that the polysaccharide content would potentially vary with sugars being localized to cells and spores, or to the outer edges of the biofilm matrix. To address this hypothesis, we used specific sugar binding lectins and the polysaccharide stain calcofluor white along with confocal microscopy to determine polysaccharide binding in the biofilm.

CLSM imaging with calcofluor white showed rod shaped and ovoid structures, shown previously in Figure 1. I interpret these to be cells and spores, which I confirmed with phase contrast microscopy. The calcofluor staining revealed that cells and spores are surrounded by polysaccharide.

The fluorescently bound lectins, which bind particular subsets of sugars, interestingly revealed variable staining patterns that differ from calcofluor white staining. We first tested polysaccharide binding with Concanavalin A (ConA) lectin. For cellular identification, we also used Syto-9 staining. Syto-9 is a nucleic acid stain that binds DNA in live cells, and fluoresces green. When staining with ConA and Syto-9, cells were shown in green (Syto-9) and there was strong red fluorescence (ConA) in between cells (Figure 4). No cells were stained red, though there was a small amount of overlap with some cells stained yellow. On the basis of diffuse ConA binding present throughout the biofilm, we conclude that the lectin ConA binds the biofilm matrix.
Figure 4. CLSM imaging of a laboratory cultured biofilm using the nucleic acid stain Syto-9 and the lectin ConA. A. Syto-9 (green) and ConA (red) double labeling of a 3-day biofilm (strain BI6). ConA stains the apparent cellular debris but, for the most part, not the cells. B and C shows the same field, without merging the two channels, so that the ConA (left) and Syto-9 (right) staining can be seen individually.
When we tested a second lectin, *Triticum vulgaris* lectin (WGA), we identified yet a different pattern of polysaccharide binding. Small areas of high fluorescence showed that WGA binding polysaccharide is present in the biofilm (Fig 5), in small rod shaped particles, which are presumably cells. WGA appeared to bind only a small subset of cells, in an apparently random orientation in the biofilm. When we compared WGA staining using three different concentrations of lectin staining, the red WGA staining appeared to increase with increased lectin concentration (Fig 6). The WGA positively stained cells clustered in small areas, or “islands” of the biofilm. A potential explanation for the binding areas might be that WGA stains dead or dying cells, though this speculation is unconfirmed by experimental evidence. It is also possible that WGA stains somewhat differentiated cells, in which some cells may express WGA-binding matrix while other cells do not.
Figure 5. CLSM imaging of a laboratory cultured biofilm using the nucleic acid stain Syto-9 and the lectin WGA. A. Syto-9 (green) and WGA (red) double labeling of a 3-day biofilm (strain BY1). WGA stained small areas and some cells. B and C shows the same field, without merging the two channels, so that the WGA (right) and Syto-9 (left) staining can be seen individually.
Figure 6. CLSM imaging of a laboratory cultures biofilm using the nucleic acid stain Syto-9 and the lectin WGA. Syto-9 (green) and WGA (red) double labeling of a 3-day biofilm (strain J9). WGA stains small bright aggregates which are likely cells, possibly dead cells. From left to right, biofilms cultured with 0 μg/ml WGA (negative control), 10 μg/ml WGA, 50 μg/ml WGA, 100 μg/ml WGA.
Biofilm Matrix Proteins

Biofilm matrix proteins are of interest as they may be involved in biofilm structure and formation [74]. To visualize the protein composition of the matrix, we isolated extracellular matrix from cells and spores using centrifugation and filtration, followed by gel electrophoresis of the matrix extract. Our goal was to identify differences in the electrophoretic protein patterns amongst strains. Perhaps the toxigenic strains contained proteins that were distinct from non-toxigenic strains. Figure 7 shows a comparison of the electrophoretic profiles of all seven clinical isolate strains used in this study. We tested 3 non-toxigenic strains, 2 toxigenic but not hypervirulent strains, and two hypervirulent BI strains. We could not determine any noticeable differences in the protein electrophoresis between any of the strains, indicating there was not an obvious difference in protein electrophoresis between non-toxigenic, toxigenic, or hypervirulent strains.
Figure 7. SDS-PAGE analysis of the matrix. 5 day biofilms were salt-extracted and filtered to remove cells prior to solubilization and electrophoresis. SDS-PAGE (12%) of biofilm matrix extracts (coomassie stained) reveals similar electrophoretic patterns between all strains analyzed. Strains, from left to right: BY1, BI6, J9, BI17, CH1, M3, K14.
Once we knew the electrophoretic patterns of matrix proteins were not variable amongst individual strains, we next sought to determine if there were particular proteins that were distinct in the biofilm matrix when compared to planktonic cell surface proteins. To address this possibility, we visually compared the electrophoretic patterns of biofilm matrix with liberated biofilm cell surface proteins. Figure 8 provides evidence that matrix proteins are not merely liberated biofilm cell surface proteins present in the matrix due to our matrix extraction process. It also indicates multiple protein bands that were present in the matrix extract and not the cell surface proteins. We analyzed seven strains, and these data are representative of all strains, indicating the analysis shown is not strain specific. We chose 4 abundant matrix protein bands for mass spectrometry that were not visible in lanes containing cell surface proteins. We were able to identify eight metabolic enzymes present in the biofilm matrix. Shown in Figure 8, we identified the enzymes acetaldehyde/alcohol dehydrogenase, uppermost band; formate-tetrahydrofolate ligase, second band from top; acetyl-CoA acetyltransferase, hydroxyisocaproate CoA-transferase, glutamate dehydrogenase and amino acid aminotransferase, third band from top; hydroxybutyryl-CoA dehydrogenase, fructose-bisphosphate aldolase and formate-tetrahydrofolate ligase, bottom band. All of these proteins are common metabolic enzymes, normally found in the cytoplasm of cells.
Figure 8. SDS-PAGE analysis of the matrix. 5 day biofilms were salt-extracted and filtered to remove cells prior to solubilization and electrophoresis. SDS-PAGE (12%) of extracts from toxigenic strain J9 (Coomassie stained). Lanes: 1) planktonic cell surface extraction, 2) matrix extract. Arrowheads on the right indicate bands which were excised for MS.
**Cellular lysis and dead cell material as a matrix component**

Knowing then that matrix proteins are not simply cell surface proteins, and that cytoplasmic proteins are present in the matrix, we asked how such proteins are released into the matrix. It is well documented that a main protein component of the *B. subtilis* biofilm, TasA, is secreted into the matrix [103, 109]. Secretion may be involved in matrix protein delivery, as well as the possibility for cell lysis liberating cytoplasmic proteins. We used SDS-PAGE to compare the electrophoretic patterns of biofilm matrix proteins with whole biofilm cell lysates. Figure 9 reveals that the electrophoretic profiles of matrix proteins and whole biofilm cell lysates are very similar, with no noticeable differences between the protein patterns. We analyzed seven strains: BI6, BI17, BY1, CH1, M3, K14, and J9, with consistent results indicating the evidence is general for all strains. I interpret this to mean that a major component of biofilm matrix proteins come from lysed cell material, and is released cytoplasmic protein that has been liberated into the matrix.
Figure 9. SDS-PAGE analysis of the matrix. 5 day biofilms were salt-extracted and filtered to remove cells prior to solubilization and electrophoresis. A. SDS-PAGE (12%) of extracts from toxigenic stain BI17 (Coomassie stained). Lanes: 1) matrix extract, 2) biofilm cell lysate.
Structural Role of Extracellular DNA and Biofilm Formation

Extracellular DNA is a necessary component in initial biofilm formation

Extracellular DNA has been shown to be a crucial component in initial biofilm formation of multiple bacterial species [106, 107]. eDNA is involved in colony architecture and adhesion of cells to each other, as well as attachment to surfaces during colony formation. Previously, we identified eDNA in the laboratory cultured C. difficile biofilm, and next wanted to identify the role of eDNA in biofilm structure. The enzyme DNase degrades extracellular DNA and causes disruption in the biofilm architecture of several species [107]. By removing extracellular DNA, I was able to determine if eDNA is important to the structural integrity of the biofilm, and if the absence of eDNA disrupts the normal architecture of a biofilm.

We cultured biofilms with DNase, adding the enzyme at the time of inoculation. We then allowed the biofilms to grow for 24 hours and followed with LiveDead cell staining and confocal microscopy. Figure 10 shows biofilms grown with DNase at 24 hours old. The biofilms exhibited varying degrees of disruption when compared to a normal laboratory cultured biofilm. Disruption of the biofilm was characterized as decreased live cell density, and the presence of “holes” that were free of both propidium iodide and Syto 9 staining. We defined cellular density as the degree of how closely cells were packed together. The propidium iodide staining (red) was decreased, and the Syto-9 staining (green), which stains live cells, appeared less dense than the control biofilm (Fig 10A). I noticed “holes” present in the DNase treated biofilm, where no staining was present at all. The three concentrations of DNase also showed varying degrees of
disruption. When I inoculated biofilms with 10 μg/ml DNase, live cell density was decreased, and some small “holes” free of staining were present (Fig 10B). For 50 μg/ml biofilms, the live cell density was decreased even further, and I saw even larger holes in the biofilm (Fig 10C). At 100 μg/ml, I observed even greater disruption in the biofilm, with large holes present and the least densely packed cells (Fig 10D) compared to a standard biofilm (Fig 10A). However, over 6 repetitions, the amount of disruption seen was inconsistent. I was unable to produce consistent disruption in the biofilm. The three concentrations of DNase also showed varying degrees of disruption. Using three concentrations of DNase (10 μg/ml, 50 μg/ml, and 100 μg/ml), I saw concentration-dependent disruption in 2 trials. In 4 additional trials, disruption was either not seen at all (Fig 10E-H), or one biofilm would exhibit disruption while the other biofilms tested appeared to be unaffected by DNase treatment. Disruption also varied at differing locations in the biofilm. The amount of disruption at the outer edges of the biofilm was greatest, with many more holes visible on the outer edge of the biofilm than in the center of the biofilm.
Figure 10. CLSM imaging of DNase treated laboratory cultured biofilms using the nucleic acid stains Syto-9 and propidium iodide. Syto-9 (green) and propidium iodide (red) double labeling of 24 hour biofilms (strain J9). Syto-9 stains live cells and propidium iodide stains dead cell material. From left to right: A,E) 0 μg/ml DNase, B,F) 10 μg/ml, C,G) 50 μg/ml, D,H) 100 μg/ml. Disruption is evident in a concentration dependent manner compared to negative controls, but the effect of DNase is inconsistent. Images E-H represents unsuccessful trials where DNase did not produce biofilm disruption.
The absence of extracellular DNA alters cellular orientation in biofilm architecture

The effect of DNase on initial biofilm architecture appears to create a disruptive effect, albeit inconsistently. But we wanted to determine the effects of DNase in older biofilms. We again cultured biofilms with DNase, but allowed the biofilms to grow for 48 hours before staining and imaging. When I imaged 48 hour biofilms, I saw no “holes” present and no visible decrease in live cell density when compared to the control biofilm grown without DNase (Fig 11). However, there was an interesting effect in which the DNase treated biofilms appeared to show less cellular organization than the control. As shown in the control (Fig 11A), normally cultured biofilms exhibited some measure of organization, where cells were stacked in a linear fashion. We consistently saw this stacking in all regularly cultured biofilms. Compare this to the DNase treated biofilm, the biofilm appeared to be disorganized, with cells facing in all directions and no consistent pattern was visible (Fig 11B). These results are representative of only one experimental attempt.
Figure 11. CLSM imaging of 48 hour DNase treated laboratory cultured biofilms using the nucleic acid stains Syto-9 and propidium iodide. Syto-9 (green) and propidium iodide (red) double labeling of 48 hour biofilms (strain BI6). Syto-9 stains live cells and propidium iodide stains dead cell material. Left: biofilm cultured with 0 μg/ml DNase (negative control). Right: biofilm cultured with 100 μg/ml DNase.
Biofilm Toxins

Toxin in the Biofilm Matrix

Toxigenic Clostridium difficile produces two large toxins, A and B, which are primarily responsible for CDI. The immune system of the host recognizes and creates an immune response to both toxins, and this antibody response is a strong indicator of current or previous infection [36, 110, 111]. We wanted to identify toxin in the laboratory cultured biofilm as a preliminary experiment to verify that toxin was present in the biofilm. We used a Western immunoblot analysis with anti-toxin antiserum C. difficile infected mice [105] and biofilm matrix proteins which were extracted as described above, shown in Figure 12. From the Western blot, I saw reactive bands of high molecular weight (around 300 kDa), present in only toxigenic strains. No reactivity was seen in non-toxigenic strains. Also, as this serum has previously been demonstrated to contain anti-toxin antibodies by Pehga Johnston in the Knight Lab, I interpret these observations as evidence that toxin is present in the biofilm.
Figure 12. Western blot analysis of the matrix. SDS-PAGE (6%) of matrix extract and western blot analysis using a mouse anti-toxin antiserum. Lanes 1) non-toxigenic strain CH1, 2) non-toxigenic strain BY1, 3) toxigenic strain K14, 4) toxigenic strain BI6. There is an empty lane between 2 and 3.
Survey of multiple CD strains reveal variation in toxin levels

The amount of toxin present in a given biofilm matrix may identify particular *C. difficile* strains with potential to cause more severe disease with higher toxin production. There is evidence of differences in toxin production in toxigenic strains seen in liquid culture [112], but it is possible that a biofilm may be more representative of *in vivo* colonization and disease. Therefore, we wanted to compare the levels of toxin in the biofilm amongst multiple strains. We cultured biofilms for 5 days, using four toxigenic strains (BI17, BI6, K14, J9) and non-toxigenic BY1 as a negative control. The Western blot identified signals at around 300 kDa, indicating toxin was present in all toxigenic strains (Figure 13A). No signal was evident in non-toxigenic strain BY1 or using pre-immune serum. Amongst toxigenic strains, the intensity of signal is approximately equal. We used ImageJ technology (NIH, Bethesda, MD) to measure the density of the Western signal so that we could quantitatively compare each blot. This led us to interpret that the toxin levels in 5 day biofilms amongst these four toxigenic strains were at approximately equal concentrations.

Seeing little difference in toxin levels at 5 days old, we next compared toxin levels in younger biofilms. A 5 day biofilm consists of mostly spores, so we chose a 3 day biofilm where the cellular population consists of both vegetative cells and spores. In Figure 13B we see that the two hypervirulent strains BI6 and BI17 have a two fold increase in toxin when compared to the toxigenic, but not hypervirulent strains J9 and K14. These data are representative of one repetition.
Figure 13. Western blot analysis of toxin levels in the matrix. SDS-PAGE (6%) of matrix extract and western blot analysis using a mouse anti-toxin antiserum. A) 5 day biofilms, B) 3 day biofilms.
Variation in biofilm toxin over time

We next investigated the toxin content in biofilms of differing maturity. As a biofilm ages, the mass increases as cells divide and extracellular matrix increases with an increase in cell lysis. It is reasonable that the amount of toxin may increase in the biofilm with the increase in protein. Alternatively, the amount of toxin may correlate with the population of vegetative cells compared to spores in the biofilm. We may expect to see higher levels of toxin in younger biofilms, which consist primarily of vegetative cells. As a *C. difficile* biofilm ages, the population of vegetative cells declines, and the concentration of spores increases. Vegetative cells produce toxin, while spores do not. Therefore, the amount of toxin in a biofilm may correlate with the amount of vegetative cells, and sporulation may decrease toxin levels in the biofilm matrix.

The Western blot comparing biofilms from strain BI6 at 1, 3, 5, and 6 days old indicated that the level of toxin increases in biofilms over time (Fig 14). Each lane was loaded with the same amount of protein (1.16 μg). Therefore, the increase in toxin we saw in biofilms over time was independent of an increase in the total protein concentration we expected from an increase in biofilm size. The proportion of toxin to overall protein concentration was increasing with time. Also, we saw toxin production well into the time of sporulation, which begins at 3 days, and at 6 days the biofilm was almost entirely composed of spores. A potential explanation for this may be due to the increase of total cell mass as a biofilm ages. Older biofilms have undergone extensive cell division, possibly leading to more toxin producers in an older biofilm, which would potentially
account for the increased toxin levels despite high levels of sporulation. These data are representative of one repetition.
Figure 14. Timecourse western blot analysis of toxin levels in the matrix. SDS-PAGE (6%) of toxigenic strain BI6 matrix extract and western blot analysis using a mouse anti-toxin antiserum.
Toxin localization in the biofilm matrix

The accumulation of toxin in the biofilm, and possible localization to specific areas of the biofilm, may facilitate CDI. Different locations for toxin accumulation in the biofilm may indicate the method of toxin delivery to the host. The potential funneling of toxin to the biofilm-host epithelial interface could enable toxin to create the most epithelial damage. Or, if toxin is located near the edges of the biofilm matrix, pieces of biofilm matrix may break off to deliver toxin to the host. Finally, toxin may be cell associated, and cells may actually need to migrate to the host mucosal surface to introduce toxin and create epithelial damage. We used immunofluorescence to try to visualize the location of toxin in the biofilm matrix. We imaged 3 day old biofilms of three different strains, toxigenic BI17 and J9, and non-toxigenic BY1 as a negative control. As shown in Figure 15, in both toxigenic strains, we saw small bright aggregates of fluorescence that were absent in all negative controls. Cells and spores were stained in blue from the polysaccharide specific stain calcofluor white. The small punctate green aggregates were not cell or spore associated, and no overlap in fluorescence was seen between the green aggregates and blue cells or spores in the merged image. Using an anti-toxin antibody, which complexes with a goat anti-mouse FITC conjugated secondary antibody, we are confident that the bright green aggregates were toxin present in the biofilm matrix. It appeared that toxin molecules associated to form the aggregates seen in the matrix. Using a naïve mouse pre-immune serum, we saw that there was some cell associated background binding. This is also present in non-toxigenic strain BY1 (Figure
16). The toxin aggregates were absent from all negative controls. These data are representative of one repetition.
Figure 15. CLSM immunofluorescence images of toxigenic biofilm labeled with anti-toxin antibody. Anti-toxin antibody (FITC) and calcofluor white double labeling of a 3 day biofilm, toxigenic strain J9. The bright green punctate staining with antibody labeling shows aggregates of toxin in the extracellular matrix.
Figure 16. CLSM immunofluorescence images of nontoxigenic biofilm labeled with anti-toxin antibody. Anti-toxin antibody (FITC) and calcofluor white double labeling of a 3 day biofilm, non-toxigenic strain BY1.
CHAPTER FOUR

DISCUSSION

Cellular fate in biofilm matrix production

It has been well documented that biofilms are composed of cells and matrix, with the matrix frequently consisting of polysaccharide, protein, and nucleic acid [74]. In our preliminary experiments, we have shown that the C. difficile biofilm consists of all three traditional matrix components. Interestingly, we showed that the protein electrophoresis of matrix proteins was very similar in appearance to a protein electrophoresis from a biofilm cell lysate. When we excised 4 abundant protein bands for mass spectrometry, we identified 8 metabolic enzymes present, which are normally in the cytoplasm of cells. Therefore, it appears that released cytoplasmic contents are a major part of the C. difficile biofilm matrix. Cellular lysis has been indicated as an important contributor to biofilm development. The 2007 review by Kenneth Bayles describes the role and regulation of cell death in S. aureus biofilms. In this review, Bayles indicates that biofilm development is facilitated by a highly regulated gene network that selectively targets biofilm cells for apoptosis. The resulting cellular debris, including nucleic acid, serves as the “glue” to hold biofilm cells together during initial biofilm development [113]. The role of cell death has also been surveyed in Gram-positive B. subtilis. Cannibalism in the biofilm is a well documented trait of selectively differentiated cells. Differentiated cells act as cannibals, but they also play a second role as matrix producers. Cannibal cells
commit fratricide to neighboring cells, releasing nutrients to be used by the cannibals/matrix producers. Matrix production is thought to be a metabolically taxing event, therefore the cannibal cells utilize nutrients from neighbors to fuel matrix production and delay sporulation due to nutrient depletion [102].

The role of lysed cellular contents in *B. subtilis* biofilms has been indicated as a nutrient provider, not as an actual matrix component. The *B. subtilis* biofilm consists of two major secreted matrix components; the structural protein TasA and the extracellular polysaccharides EPS [103]. While *B. subtilis* is phylogenetically very distinct from *C. difficile*, *B. subtilis* is the best studied Gram-positive biofilm and a model organism upon which we base several experimental questions. In our studies, we did not find any major protein which would serve as a structural base for the *C. difficile* biofilm; instead we found lysed cell material to be a major component. This leads us to speculate about the cell fate and differentiation in *C. difficile* biofilms. Cellular differentiation has been extensively studied in *B. subtilis* biofilms and it implicates several different populations. Initially the biofilm consists of mostly motile cells. At about 24 hours of growth, many cells lose their motility and differentiate into matrix producers/cannibals, miners, surfactin producers, and later in biofilm growth, sporulating cells [78, 104]. While cellular differentiation in *C. difficile* biofilms is not known, we can speculate about two potential fates of *C. difficile* cells in biofilm growth. Assuming that *C. difficile* biofilms do not contain a major structural protein like TasA, and cell lysis is the major contributor to extracellular matrix production, we can hypothesize that *C. difficile* biofilm cells are initially undifferentiated and later differentiate into spore producers, or undergo apoptosis.
and lyse to provide matrix material. This is further supported by evidence that the structural protein TasA is not encoded in the *C. difficile* genome. We do not currently have evidence of matrix production in cells. Rather, we have identified cellular lysis as a major event that contributes to the *C. difficile* biofilm matrix. It is very plausible that vegetative cells produce matrix prior to lysis and sporulation. We can propose a model for cellular differentiation in a *C. difficile* biofilm that is distinct from *B. subtilis* and serves as an alternative model for Gram-positive biofilms (Fig 17). It is an intriguing idea to determine the cell fate of *C. difficile* cells during biofilm growth, and with greater study those biofilm developmental events will likely become clearer.
Figure 17. Model for cellular differentiation in *C. difficile* biofilms. Motile cells aggregate and are initially undifferentiated. Cells differentiate into matrix producers and secrete toxin, then eventually sporulate or lyse to contribute to the matrix. Sporulation produces a spore, and the lysed mother cell becomes part of the extracellular matrix.
Contribution of Extracellular DNA to Biofilm Formation and Architecture

The biofilm disruption I observed indicates that eDNA is potentially an important component to biofilm structure and is necessary for proper biofilm formation. The importance of eDNA to biofilm formation has been well documented in other biofilm species, showing results that are consistent with my findings. DNase treatment seems to have the greatest effect early in biofilm development [106]. 24 hour biofilms showed considerably greater disruption than 48 hour biofilms, indicating that eDNA may not be as crucial to the structure of more mature biofilms. This observation is consistent with Whitchurch et al which suggests that as biofilms mature, the extracellular DNA becomes less important to biofilm integrity, as polysaccharides and proteins in the matrix support the biofilm structure [106]. The dense packing of cells was decreased with increasing DNase concentration, leading to the interpretation that extracellular nucleic acid is important for adhesion of cells to one another. Also, the “holes” that I observed in DNase treated biofilms may show that eDNA is also important for biofilm adhesion to surfaces. We were unable to create consistent disruptive effects in the biofilm through the removal of extracellular DNA with DNase. Through 6 experimental attempts, disruption only occurred in 4 trials. Additionally, the concentration dependent disruption we observed in the first experimental attempt could not be recreated through multiple repetitions. I did see disruption in additional attempts, but could not repeat concentration dependent disruption. The severity of disruption was not consistent with each trial; therefore, we are unable to make any conclusive remarks about the effects of increased concentrations of DNase on biofilm development or organization. There were several
small variables in the experiment that make this experiment difficult to reproduce. The first potential variable is the time of inoculation. When our lab inoculates culture for biofilm growth, we attempt to achieve a consistent optical density of overnight culture, though this is not always the case. Due to small changes in the culture timing, the time of inoculation in regards to the density of culture may vary. For most experiments, this is not an issue. But we do expect the differences in eDNA to affect initial attachment of cells to the surface. As attachment is the initial step in biofilm formation, precise timing may very well determine the effect of DNase on initial attachment. The second variable to consider is the action of DNase. While the enzyme we use is taken from the same stock for each experiment, the action of the enzyme may fluctuate too greatly to produce consistent results. What we have determined from multiple repetitions is that extracellular DNA is important for initial biofilm formation, and the absence of eDNA disrupts the biofilm architecture. The extent of that disruption is unclear. Our lab will continue to explore the role matrix eDNA in biofilm formation with additional evaluation of the experimental design.

**Toxin Production in Biofilms of Hypervirulent Strains of C. difficile**

Despite great advances in the molecular study of *C. difficile*, the molecular mechanism underlying the emergence of hypervirulent strains is still far from understood. Specifically, the amount of toxin and onset of toxin production have been studied previously with several conflicting results. Most recently, the laboratory of Dr. Dale Gerding determined differences in toxin production for several hypervirulent strains and toxigenic, but not hypervirulent, strains. Through this work it was determined that the
hypervirulent strains tested did not produce greater amount of toxin than non-
hypervirulent strains tested in liquid culture [112]. Through my studies, we have only
experimented with two HV strains, BI17 and BI6. These two strains in no way represent
the entirety of the hypervirulent strains, making strain selection in future studies with
regard to hypervirulent C. difficile biofilms of critical importance. The difference in
growth and toxin production in biofilms rather than liquid culture could potentially be
more representative of toxin production during infection in the host. Our data indicates
toxin production in BI strains is higher in younger biofilms than non-hypervirulent strains
by a two-fold increase. This suggests that the hypervirulent strains we tested produce
toxin at a higher rate earlier in biofilm growth. Experiments to demonstrate and validate
this observation are ongoing to determine when toxin is being produced at the earliest
time during biofilm development. It is likely though that hypervirulence cannot be
attributed simply to differences in toxin production amongst strains. For example, strain
VPI10463, which is the highest known toxin producer, is not hypervirulent and is not
commonly found in clinical patient isolates. VPI10463 has not become an epidemic
strain, providing evidence that toxin production cannot be the sole factor in determining
hypervirulence. However, experiments including toxin quantification are critical in
defining the C. difficile biofilm as a virulence factor for CDI.

**Toxin Localization in CD Biofilms**

A potential explanation for the appearance of toxin aggregates in the matrix may
be due to cellular debris, also present in the matrix. Toxin may accumulate around a
piece of cell membrane, or around nucleic acid in the matrix. As we have seen that
cellular lysis is a common event in *C. difficile* biofilm growth (shown previously), that contributes to the formation of the matrix, it is plausible that toxin may aggregate around a piece of debris in the matrix. Toxin was diffuse throughout the matrix, with high concentrations located around small aggregates in the matrix, which are potentially cellular debris. It is possible therefore to speculate about the mechanism of toxin delivery to the host, as well as some experimental limitations from our immunofluorescence microscopy. The growth of laboratory cultured biofilms is accomplished on a solid polycarbonate membrane placed on top of agar medium. While it is an efficient method for *in vitro* biofilm growth, there are certainly events *in vivo* that are not represented by our model. The location of toxin would be best studied in an *in vivo* model where the surface association of biofilm to host mucosal surfaces would be more representative of biofilm growth during CDI.

The dispersal of motile bacteria in biofilm growth has been well documented in several species. Sloughing dispersal liberates large pieces of biofilm late in biofilm growth, while erosion of the biofilm involves biofilms breaking off in small pieces and being released over the development of the biofilm [81, 82]. The *B. subtilis* biofilm likely undergoes both methods of dispersal, and has been shown to completely disassemble with the addition of D-amino acids. While these observations have only been discussed in the context of motile cell release from the biofilm, it is intriguing to speculate about the role of dispersion in toxin delivery. It is possible that in the *C. difficile* biofilm, toxin is delivered to new parts of the gut when dispersal occurs. Pieces of the biofilm could break off, liberating cells and toxin to new areas of the gut. Then, as
the released bacteria colonize new tissue, toxin is already available and able to initiate epithelial damage before an immune response could be activated, and prior to the production of toxin from the new colony.

**Clinical Relevance of Biofilm Characterization**

There are several mechanisms by which *C. difficile* biofilms could contribute to CDI. The biofilm may contribute by resisting antimicrobial drugs and host defense mechanisms; by facilitating attachment of *C. difficile* to appropriate locations in the colon; by accumulating toxin and directing it to host tissues; and by harboring a reservoir of dormant spores that could facilitate relapsing disease. My characterization of the biofilm will provide a foundation for developing novel therapeutics, including vaccines and probiotics, which rely on knowledge of the host-pathogen interaction. Biofilms play a critical role in pathogenesis. Biofilms allow persistence in the host, and can facilitate relapsing infection. We will use what we have learned about the biofilm, and will analyze those properties that could influence CDI and treatment of the disease. Our preliminary *in vitro* characterization provides a foundation for *in vivo* studies, which could significantly accelerate development of novel treatments. In future work, we will use these data to further clarify our understanding of CDI pathophysiology. We will also more deeply analyze the molecular composition of the biofilm. We will focus especially on identifying targets for novel therapeutics, and the design of probiotic treatments.
Future Directions

Disruption of biofilm architecture with the addition of DNase

We have documented several stages in biofilm development, yet we do not know the molecular mechanisms of biofilm growth. Attachment of cells to a surface is an early step in biofilm growth and the creation of a microcolony. Certainly, in the course of CDI, attachment of bacteria to host mucosa is a critical step in pathogenesis. We need to determine the contribution of extracellular DNA in biofilm formation. As discussed previously, we will need to eliminate small variances in the inoculation of polycarbonate membranes to initiate biofilm development, as that could affect the initial formation of the biofilm. There are several additional experiments we would like to perform to determine the contribution of eDNA to biofilm formation. This includes the addition of DNase to degrade eDNA at varying timepoints. Our previous experiments involved the addition of DNase once during inoculation and imaging the biofilms at 24 hours and 48 hours of growth. 24 hours may be too late to visualize the important changes in biofilm attachment that could be affected by the absence of eDNA. We also will continually add DNase to visualize the disruptive effects of DNase over time. In our previous experiments, we likely lost the effect of DNase long before confocal microscopy. Also, cellular lysis continues with biofilm growth, and cellular lysis supplies the matrix with additional eDNA. If we can continually add DNase several times during biofilm development, we would likely see greater disruption and learn more about the role of eDNA in biofilm development.
Toxin Production and Localization in CD Biofilms

We have completed some preliminary experiments to determine the concentrations and location of toxin in the biofilm with a survey of 4 strains. Previous work by Michelle Merrigan demonstrated the absence of significant differences in toxin levels in liquid culture, indicating that differences in toxin levels cannot determine hypervirulence [112]. I agree that hypervirulence is likely due to many complex factors that have not yet been determined. Our preliminary results indicate that toxin levels in the hypervirulent BI strains are two fold higher than non-HV strains. Repetition and toxin quantification in younger biofilms will determine if this is a consistent result. Additionally, quantifying toxin in more strains will determine if the differences in the toxin levels that we see are due to strain variation or a true difference between HV and non-HV strains. Finally, we would like to identify the location of toxin in vivo rather than through a laboratory cultured biofilm. The delivery of toxin to the gut mucosal surfaces is an important step in the pathogenesis of CDI. We would like to determine how the biofilm contributes to this critical step, and subsequently may learn about dispersal in the C. difficile biofilm.

Immune Response to Biofilm Proteins

Through Western blot analysis we were able to identify toxin in the biofilm. We also did not observe any antibody binding to other biofilm matrix proteins other than toxin. This indicates that there was not a systemic immune response to matrix proteins other than toxin, as the IgG antibodies from C. difficile mice indicate only systemic immunity. However, there should not necessarily be a systemic response to biofilm
proteins. We would like to use mucosal antibodies from several mammalian sources to characterize the immune response to *C. difficile* biofilms, including hamsters and human patients. The effect of *C. difficile* infection has been well studied in hamsters, and the hamster model is used by our collaborators in Dr. Dale Gerding’s lab. The best mucosal antibody to test would be from human patients who have experienced CDI. Determining antigenic proteins in *C. difficile* biofilms could eventually lead to novel therapeutics and improved treatment of CDI.
REFERENCE LIST


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