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Antibodies to Inhibit Clostridium Difficile Adhesion to Human Gut Epithelial Cell Line

Wonbeom Paik

Loyola University Chicago, wpaik89@aol.com

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LOYOLA UNIVERSITY CHICAGO

ANTIBODIES TO INHIBIT
CLOSTRIDIUM DIFFICILE ADHESION TO
HUMAN GUT EPITHELIAL CELL LINE

A THESIS SUBMITTED TO
THE FACULTY OF THE GRADUATE SCHOOL
IN CANDIDACY FOR THE DEGREE OF
MASTER OF SCIENCE

PROGRAM IN INFECTIOUS DISEASE AND IMMUNOLOGY

BY
WONBEOM PAIK
CHICAGO, IL
AUGUST 2013
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<tr>
<td>--------------</td>
<td>-------------</td>
<td></td>
</tr>
<tr>
<td>°C</td>
<td>Degrees Celsius</td>
<td></td>
</tr>
<tr>
<td>6x His</td>
<td>Hexahistidine tag</td>
<td></td>
</tr>
<tr>
<td>AAD</td>
<td>Antibiotic-associated diarrhea</td>
<td></td>
</tr>
<tr>
<td>ABTS</td>
<td>2, 2’-Azino-bis-3-ethylbenzothiazoline-6-sulfonic acid</td>
<td></td>
</tr>
<tr>
<td>ABX</td>
<td>Antibiotics</td>
<td></td>
</tr>
<tr>
<td>ADP</td>
<td>Adenine diphosphate</td>
<td></td>
</tr>
<tr>
<td>APC</td>
<td>Antigen presenting cell</td>
<td></td>
</tr>
<tr>
<td>AT</td>
<td>Adenine and thymine</td>
<td></td>
</tr>
<tr>
<td>BBE</td>
<td>Brush border expressing clone</td>
<td></td>
</tr>
<tr>
<td>Cbp</td>
<td>Collagen binding protein</td>
<td></td>
</tr>
<tr>
<td>CDI</td>
<td><em>Clostridium difficile</em> infection</td>
<td></td>
</tr>
<tr>
<td>CDT</td>
<td><em>C. difficile</em> binary toxin</td>
<td></td>
</tr>
<tr>
<td>CFU</td>
<td>Colony forming unit</td>
<td></td>
</tr>
<tr>
<td>Cwp</td>
<td>Cell wall protein</td>
<td></td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco’s modified Eagle’s medium</td>
<td></td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribose nucleic acid</td>
<td></td>
</tr>
<tr>
<td>ECM</td>
<td>Extracellular matrix</td>
<td></td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylene diamine tetraacetic acid</td>
<td></td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
<td></td>
</tr>
<tr>
<td>--------------</td>
<td>-----------</td>
<td></td>
</tr>
<tr>
<td>EGTA</td>
<td>Ethylene glycol tetraacetic acid</td>
<td></td>
</tr>
<tr>
<td>ELISA</td>
<td>Enzyme-linked immunosorbance assay</td>
<td></td>
</tr>
<tr>
<td>Fbp</td>
<td>Fibronectin binding protein</td>
<td></td>
</tr>
<tr>
<td>FDA</td>
<td>Food and Drug Administration</td>
<td></td>
</tr>
<tr>
<td>GI</td>
<td>Gastrointestinal</td>
<td></td>
</tr>
<tr>
<td>HEPES</td>
<td>4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid</td>
<td></td>
</tr>
<tr>
<td>HMW</td>
<td>High molecular weight</td>
<td></td>
</tr>
<tr>
<td>HRP</td>
<td>Horseradish peroxidase</td>
<td></td>
</tr>
<tr>
<td>Hsp</td>
<td>Heat shock protein</td>
<td></td>
</tr>
<tr>
<td>Ig</td>
<td>Immunoglobulin</td>
<td></td>
</tr>
<tr>
<td>IPTG</td>
<td>Isopropyl β-D-1-thiogalactopyranoside</td>
<td></td>
</tr>
<tr>
<td>kb</td>
<td>Thousand base pairs</td>
<td></td>
</tr>
<tr>
<td>kD</td>
<td>Kilodaltons</td>
<td></td>
</tr>
<tr>
<td>LB</td>
<td>Luria-Bertani</td>
<td></td>
</tr>
<tr>
<td>LMW</td>
<td>Low molecular weight</td>
<td></td>
</tr>
<tr>
<td>LSR</td>
<td>Lipolysis-stimulated lipoprotein receptor</td>
<td></td>
</tr>
<tr>
<td>mAb</td>
<td>Monoclonal antibody</td>
<td></td>
</tr>
<tr>
<td>MALT</td>
<td>Mucus associated lymphoid tissue</td>
<td></td>
</tr>
<tr>
<td>mg</td>
<td>Milligram</td>
<td></td>
</tr>
<tr>
<td>mL</td>
<td>Milliliter</td>
<td></td>
</tr>
<tr>
<td>mM</td>
<td>Millimolar</td>
<td></td>
</tr>
<tr>
<td>MOI</td>
<td>Multiplicity of infection</td>
<td></td>
</tr>
<tr>
<td>MRSA</td>
<td>Methicillin-resistant <em>Staphylococcus aureus</em></td>
<td></td>
</tr>
</tbody>
</table>
NAP  North American pulse-field type
ng   Nanogram
Ni-NTA Nickel (II) nitrilotriacetic acid

*Number x G*  Centrifugal force relative to gravitational force
PBS  Phosphate buffered saline
PCR  Polymerase chain reaction
PCV7  Heptavalent pneumococcal conjugate vaccine
Pfam 04122  Putative cell wall binding repeat 2
pIgR  Polymeric immunoglobulin receptor
REA  Restriction endonuclease analysis
RFLP  Restriction fragment length polymorphism
SAP  Shrimp alkaline phosphatase
SAV  Streptavidin
SDS  Sodium dodecyl sulfate
SDS-PAGE  SDS-polyacrylamide gel electrophoresis
SEM  Scanning electron microscopy
sIgA  Secretory immunoglobulin A
SLP  S-layer protein
TcdA  Toxin A
TcdB  Toxin B
TE  Tris-EDTA
TSB  Tryptic soy broth
U  Enzyme unit
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
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<tbody>
<tr>
<td>w/v</td>
<td>Mass concentration</td>
</tr>
<tr>
<td>Δgene</td>
<td>Functional disruption of gene</td>
</tr>
<tr>
<td>μg</td>
<td>Microgram</td>
</tr>
<tr>
<td>μL</td>
<td>Microliter</td>
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ABSTRACT

*Clostridium difficile* infection (CDI) is a major hospital-acquired diarrhea that can cause life-threatening complications such as pseudomembranous colitis. CDI is caused by colonization of host with *C. difficile*, a Gram positive, anaerobic bacterium known to produce toxins that cause disease. Normally, the gut microbiota protects the host from CDI, but disruption of the microbial composition through antibiotic treatment can leave one vulnerable for CDI. To date, no vaccines for preventing CDI are available.

In this study, the potential of antibodies directed against specific surface molecules of *C. difficile* to block bacterial adherence to host gut epithelial cells was studied, in hopes for developing a vaccine that could prevent colonization and disease. Antibodies against recombinant FliD (flagellar cap protein), HMW SLP (surface-associated protein), and Cwp84 (cysteine protease) were generated in rabbits and tested for their ability to reduce *C. difficile* adherence to Caco-2 BBE cells, a human colon-derived epithelial cell line. In this model, the antibodies against target molecules did not significantly decrease *C. difficile* adherence, although the target molecules had been found to be adhesins in the literature using other models. Thus, these data suggest that blocking of *C. difficile* adherence to epithelial cells using antibodies against adherence-associated surface molecules may be assay-dependent, highlighting a need to develop other models that can effectively simulate the *in vivo* conditions of CDI pathogenesis.
CHAPTER ONE
REVIEW OF LITERATURE

Introduction

*Clostridium difficile* (*C. difficile*) is a Gram positive, anaerobic bacterium that causes diarrhea and in severe cases, pseudomembranous colitis\(^1\). *C. difficile* is able to form spores that are extremely durable and can contaminate various surfaces including those within healthcare environments\(^2\). The spores can be ingested by people coming in contact with the spore-contaminating surfaces, and the bacteria can then colonize the gut if the intestinal microbiota is already disrupted\(^3\). Since antibiotics, the treatment of choice for a variety of bacterial infectious diseases in modern medicine, cause disruptions in the composition of the host microbiota, people undergoing antibiotic therapy are often at increased risk for *C. difficile* infection (CDI)\(^4\). Since the use of antibiotics is common in various healthcare settings today, many of the CDI cases are known to be healthcare-associated\(^5\). Although the major risk factor for contracting CDI is the use of antibiotics, the latest treatment recommendation for CDI is still antibiotic therapy using metronidazole or oral vancomycin in severe cases\(^6\). Consequently, a major problem with CDI is that about 12-25% of patients undergoing CDI treatment suffer from recurrent disease when treatment is stopped\(^3\). Thus, novel approaches to treat CDI are needed.
Several new approaches for treating CDI have been described, and this includes three broad approaches: antibiotic agents with higher specificity to *C. difficile* spare the normal microbiota[^7], biotherapeutic agents[^8][^9][^10], and vaccines[^11][^12][^13]. In the case of vaccine development, the primary goal in the field has been to induce an anti-toxin immune response, as CDI is known to be a toxin-mediated disease. Some of these vaccines that target the toxins are currently undergoing clinical trials[^12][^13]. However, an anti-toxin immune response is not likely to prevent colonization of patients with *C. difficile*, as many protected individuals that develop anti-toxin antibody responses can still shed spores into the environment and contaminate their surroundings. From an epidemiological standpoint, a vaccine that prevents colonization of patients and thus prevents the transmission of infective spores would be more ideal for containing the disease’s spread. Still, developing such a vaccine is a challenging process due to the complex nature of *C. difficile* colonization of the human host that is yet to be fully understood. Studies need to be conducted to identify target antigens and their ideal formulations in order to develop vaccines that prevent colonization.

This study aims to test the possibility of blocking *C. difficile* adherence to human gut epithelial cells, in order to identify candidate antigens and formulations for novel vaccine development that can prevent colonization. Bacterial adherence is thought to be an important part of colonization process. Thus, if a vaccine formulation could block adherence in an immunized host, it could prevent colonization. Antibodies against such antigen should block adherence of the bacteria to gut epithelial cells *in vitro*. If not, a different formulation may be needed for effective vaccines. Three candidate molecules of *C. difficile*, HMW SLP, Cwp84, and FliD, are tested in this study to determine if
antibodies against these molecules can inhibit *C. difficile* adherence to Caco-2 BBE cells, a human colonic epithelial cell line.

**Clostridium difficile Infections**

*C. difficile*, first identified in stool samples from infants and formerly known as *Bacillus difficilis*, is a Gram positive anaerobe that is known to be one etiological agent of antibiotic-associated diarrhea (AAD). *C. difficile* produces two major toxins, toxin A (TcdA) and toxin B (TcdB), both of which are known to damage the gut mucosal barrier function through the disruption of the intracellular cytoskeletal network. The disruption of the gut mucosal barrier function leads to the recruitment of inflammatory cells to the site of damage and the patients subsequently suffer from diarrheal disease. The traditional treatment for CDI, and the current one for mild cases, has been simply the stoppage of current antibiotic therapy until diarrhea resolves. Unfortunately, the cases of CDI, based on hospital discharge diagnoses, increased in prevalence and also in disease severity between 1993 and 2005. Now, the rate of nosocomial (hospital-acquired) CDI has increased to the point that it rivals methicillin-resistant *Staphylococcus aureus* (MRSA) infections, and currently accounts for 20-30% of all cases of healthcare-associated diarrhea.

The explanation for the recent increase in the rate of CDI appears to be multifactorial. One major factor is the widespread use of antibiotics. It is known that use of antibiotics is a major risk factor for CDI, supported by the fact that about 95% of the cases of CDI are healthcare-associated; patients who develop CDI typically have exposure to healthcare facility or service. Another factor to be considered is the
emergence of hypervirulent strains of *C. difficile*. One prominent hypervirulent strain is known as BI, NAP1, or 027, depending on the method in which they were typed; there are other strains that are also considered hypervirulent but the BI group is the most common.

Compared to strain 630, the most well-characterized strain of *C. difficile* to date, the hypervirulent BI strains are noted for their resistance to fluoroquinolones and a deletion in *tcdC* gene\(^2\); TcdC is thought to regulate the expression of *tcdA* and *tcdB* which code for the two major toxins of *C. difficile*\(^3\). The mechanism by which the change in TcdC expression in this strain contributes to hypervirulence is unclear\(^4\) as association of the mutation to actual disease severity has not been established\(^5\). Rather, factors contributing to the regulation of toxin production are very complex, as it has been demonstrated that toxin production is also linked to flagellar regulation\(^6\),\(^7\) and response to environmental stress such as antibiotic exposure\(^8\). It is, however, clear that the severity and mortality rates of CDI have increased following the emergence of the hypervirulent strains, and many more cases today require measures beyond traditional methods to deal with CDI such as vancomycin\(^6\); in very severe cases, surgical removal of inflamed colon is performed.

A challenging issue that clinicians face today is recurrent CDI following treatment. Most CDI patients resolve their diarrhea and disease symptoms upon treatment. However, after treatment is stopped, about 12-25\% of patients experience disease relapse\(^3\); these recurring patients can experience multiple episodes of CDI, each after a course of CDI treatment. Repeated treatments and the added problem of serious complications requiring surgery have put a heavy financial burden on the healthcare
system, estimated to be $500 million annually in the United States\textsuperscript{29}, another study estimates the cost to be over $3 billion\textsuperscript{113}. Antibiotics are the treatment of choice when it comes to managing CDI. Currently, physicians try to manage disease recurrence with tapering antibiotic regimen, where the frequency of each antibiotic dosage is decreased over time\textsuperscript{3}. However, antibiotics used for CDI treatment are also risk factors for CDI\textsuperscript{4}, as they also disrupt the normal microbiota which prevents host colonization with \textit{C. difficile}.

Animal models of CDI, traditionally using hamsters\textsuperscript{30} and more recently mice\textsuperscript{31}, utilize antibiotic treatment followed by administration of infectious dose of \textit{C. difficile} spores to induce disease. In these models, antibiotic administration is thought to disrupt the microbiota colonizing the intestine, leaving the animals permissive for \textit{C. difficile} colonization. It has been demonstrated that a single dose of clindamycin, an antibiotic historically linked to CDI, causes significant alterations to the gut microbiota, leaving mice susceptible to CDI\textsuperscript{32}. Likewise, another mouse model for CDI administers metronidazole and vancomycin to mice prior to \textit{C. difficile} spore challenge\textsuperscript{31}; these are the same drugs currently recommended for treatment of CDI patients. Thus, treatment of CDI puts some patients at increased risk for CDI, and when more contaminating \textit{C. difficile} are ingested while at risk\textsuperscript{33} or persisting \textit{C. difficile} grow, the patients can contract CDI again. This cycle of CDI recurrence is illustrated in Figure 1; patients undergo antibiotic therapy for CDI which can lead to subsequent CDI recurrence, and the disease cycle continues until successful therapy leads to a cure. To break such a destructive cycle, a novel approach to treating or preventing CDI is urgently needed.
Figure 1. Schematic diagram of CDI recurrence cycle. Antibiotic treatment leads to CDI which can be cured once antibiotic treatment is stopped. In some cases, CDI is treated using antibiotics. While this can cure CDI, it also puts one at risk for CDI. Thus, a destructive cycle of recurrent CDI can occur. ABX: antibiotics. Black Arrow: non-recurrence disease pathway. Red Arrow: recurrent disease pathway.
**Novel CDI Therapeutics**

Looking at the rate of recurrent disease, and the financial burden it puts on the healthcare industry from repeated treatment regimen and severe complications, standard antibiotic therapy for CDI is becoming less effective. In response, there are many novel approaches to CDI treatment in development today. These new approaches can be classified into three groups: antimicrobial therapy, biotherapeutics, and immunologic therapy.

Currently, there are some novel antibiotic compounds tested to improve CDI treatment effects compared to vancomycin\(^{34}\). One of the novel antimicrobial therapeutic agents is called fidaxomicin, an antibiotic agent recently approved by the Food and Drug Administration (FDA) for the treatment of CDI. During clinical trials, this drug was shown to be equivalent to vancomycin, but a lower rate of recurrence was achieved compared to vancomycin in clinical trials\(^{35}\). A possible advantage of fidaxomicin is that, compared to standard antibiotics for CDI, the drug is more specific for *C. difficile*; the microbiota isolated from stool samples from patients undergoing fidaxomicin therapy was altered to a lesser degree compared to samples from patients undergoing vancomycin therapy\(^{36}\). Fidaxomicin may lessen the risk for CDI recurrence by having a lesser impact on the microbiota compared to standard treatment options. This suggests that the increase in antibiotic specificity can lead to a better treatment for CDI that decreases the risk of recurrence.

Knowing about the importance of normal microbiota in CDI, treatment approaches using probiotics have been studied extensively. An alternative therapy that has shown success in treating CDI is fecal transplantation. This therapy seeks to restore
the balance in the microbiota of CDI patients, whose microbiota has been disrupted by
antibiotic use and subsequent infection with \textit{C. difficile}. The human gastrointestinal tract
houses a large population of microbes consisting of fungi, viruses, archaea, small
eukaryotes, and bacteria. These intestinal symbionts play a crucial role in the
maintenance of human health, as the microbiota are involved in development and
modulation of the host immune system\textsuperscript{37,38,39,40}, host metabolic processes\textsuperscript{41}, and
protection from pathogens\textsuperscript{42,43}. In the case of CDI, the normal microbiota seems to confer
colonization resistance against \textit{C. difficile}, as antibiotic administration alters the
microbiota to confer susceptibility to CDI\textsuperscript{32}. Fecal transplantation transfers microbiota
from a healthy donor to a CDI patient whose microbiota is altered, and thus colonization
resistance is established in the CDI patient. This therapy has shown success, including a
recent clinical trial showed greater success with fecal transplants when compared to
standard vancomycin therapy in curing CDI and in limiting recurrence\textsuperscript{10}. While the
therapy is effective, the specific effector organisms in fecal transplantation involved in
protection from CDI have not been identified, not to mention that many have never been
successfully cultured for study before. As studies continue, the microbes needed to
induce colonization resistance against \textit{C. difficile} will be identified and lead to specific
probiotic therapeutics in the near future.

Immunologic therapy for CDI has been under study for more than twenty years\textsuperscript{44};
a vaccine for CDI was studied in hamsters using formalin-inactivated \textit{C. difficile} cells and
culture filtrates in 1995\textsuperscript{45}. Since CDI is mediated by the two major toxins, vaccines
targeting these toxins as antigens remain the best studied so far. The development of anti-
toxin antibody response is suggested to be protective against disease symptoms, since
asymptomatic carriers have increased levels of anti-TcdA IgG serum antibodies compared to symptomatic patients\textsuperscript{46}. Also, human monoclonal antibodies (mAb) against toxins have been shown to protect hamsters from lethal \textit{C. difficile} challenge\textsuperscript{47}, and to reduce the rate CDI recurrence among patients given toxin-specific mAb\textsuperscript{48}. The most promising vaccine target currently seems to be a fusion protein of TcdA and TcdB receptor binding domains that was shown to be effective in a mouse model\textsuperscript{49}; targeting both toxins is preferred because the relative importance of TcdA versus TcdB in disease is not entirely clear (strains expressing either both toxins or TcdB only can all cause disease). Meanwhile, it has been shown that a toxin B targeting vaccine does not prevent \textit{C. difficile} colonization in hamsters while it does protect the animals from disease when challenged with a strain only producing TcdB\textsuperscript{11}. Because toxins are not assumed to be involved in the initial colonization process, vaccines targeting the toxins would not likely prevent colonization in patients whose intestinal microbiota is disrupted. Thus, a successful toxin-based CDI vaccine may result in asymptomatic carriage and subsequent spread of infectious spores to the environment including hospital surfaces. In order to design a vaccine that prevents colonization and subsequent CDI, this study aims to test candidate adhesins of \textit{C. difficile} to determine if antibodies against chosen molecules could block adherence. Such a vaccine, by preventing adherence and subsequently colonization, could prevent disease and its spread.
<table>
<thead>
<tr>
<th>Therapy</th>
<th>Examples</th>
<th>Potential Cost</th>
</tr>
</thead>
<tbody>
<tr>
<td>Microbiota-sparing ABX</td>
<td>Fidaxomicin</td>
<td>High</td>
</tr>
<tr>
<td>Biotherapeutics</td>
<td>B. coagulans, Lactobacilli, Fecal transplantation, Non-toxigenic C. difficile</td>
<td>Low</td>
</tr>
<tr>
<td>Immunologics</td>
<td>mAb</td>
<td>High</td>
</tr>
<tr>
<td></td>
<td>Vaccines</td>
<td>Likely low</td>
</tr>
</tbody>
</table>

Table 1. Summary of developing therapeutics for CDI and potential cost estimate of each category.
Candidate Molecules for Vaccine Development

Bacterial flagella are recognized to be virulence factors in many different pathogens, serving a variety of functions including role in motility\(^50\). In particular, FliD, the capping protein for the flagellar apparatus in many bacteria, has been demonstrated to play a role in colonization of mammalian hosts in different bacteria\(^51,52\). In the case of \textit{C. difficile}, FliC is the flagellin subunit that makes up the fibrous portion of the flagella, while FliD serves as the capping protein at the outer tip of the flagellar structure, as illustrated in Figure 2. Both FliC and FliD have been shown to bind to mucin, a protein component of intestinal mucus, and FliD has also been found to bind to epithelial cells \textit{in vitro}\(^53\). The \textit{in vivo} significance of FliD in colonization and disease is not clear, as studies with a mutant unable to produce FliD found the mutant to have enhanced virulence\(^26\); this is inconsistent with the previous finding that flagellated strains tend to have increased adherence compared to non-flagellated strains within the same serogroup\(^53\). However, this increase in virulence may be a byproduct of mutagenesis itself, as toxin production was found to be increased in \textit{fliD} mutants\(^26\); the regulation of toxin production was found to be partly related to flagellar regulation\(^27\). While both FliC and FliD seem to be involved in the colonization process, it was shown that CDI patients tend to generate more antibodies against FliD than FliC\(^54\). FliC has been shown to have variability in DNA restriction fragment length polymorphism (RFLP) patterns and in protein sequence amongst different serogroups of \textit{C. difficile}\(^55\), meaning there is limited antibody cross-reactivity for FliC among \textit{C. difficile} strains. In contrast, antibodies produced against FliD of one serotype cross-reacts with FliD from other serotypes\(^56\). This can make FliD a
good vaccination target as the immune response generated by this molecule likely will be
directed against multiple strains and thus confer protection against multiple strains.
Figure 2. Illustration of *C. difficile* cell with a flagellar structure. FliC is the flagellin subunit while FliD is the capping protein. The flagellar fiber is attached to a flagellar base apparatus on the cell. *C. difficile* is known to express multiple flagellar fibers, but only one is illustrated in this figure.
C. difficile, like many other bacteria, has a protein array that covers the surface of the cell in close association with its cell surface\textsuperscript{57}. The proteins, termed surface layer proteins (SLPs), serve a variety of functions including protective coating, adhesion, ion trapping, cell shaping, and cell division in a variety of different organisms of both bacteria and archaea\textsuperscript{58}. Although the sequence and structure of SLPs are very diverse amongst different species, SLPs have co-evolved to share some properties such as self-assembly, regularity of pores, and association with the underlying cell surface structure\textsuperscript{58}. In C. difficile, the SLP monomers are made of two subunits of different size, termed low-molecular weight (LMW) and high-molecular weight (HMW) SLP\textsuperscript{57}. These proteins are encoded by slpA gene which translates into a precursor protein that is cleaved by proteolysis into the two subunits\textsuperscript{59}. The subunits then form a stable complex that serves as the monomer to subsequent S-layer self-assembly as illustrated in Figure 3\textsuperscript{60}. One suggested function of the SLPs in C. difficile is adhesion to host intestinal mucosa, as the proteins were found to be associated with colonic sections of both humans and mice upon co-incubation with tissue samples; SLPs were also shown to interfere with C. difficile adhesion to HEp-2 cells\textsuperscript{61}, an epithelial cell line derived from HeLa cell contaminants\textsuperscript{62}. Thus, SLPs seem to be a good target for vaccine development since they are associated with adhesion process and covers the entire cell surface. Also, the proteins form an array of identical subunits, which likely makes them good immunogens. The structural studies indicate that LMW SLP is more surface exposed\textsuperscript{60} and likely more immunodominant. However, LMW SLP shows high antigenic diversity among the different strains of C. difficile and such, a vaccine may confer protection against only a few strains\textsuperscript{59}. 
Figure 3. Illustration of *C. difficile* cell wall structure with overlaying S-layer. **A.** The plasma membrane is covered with a thick peptidoglycan layer characteristic of a Gram positive organism. The SLP paracrystalline layer is closely associated with the peptidoglycan layer through conserved domains in HMW SLP. Other cell wall proteins are incorporated into the S-layer. **B.** Illustration of the SlpA, the precursor molecule for the SLPs. The triangles denote proteolytic cleavage sites in the maturation process. SlpA does contain a signal peptide (left-most portion of SlpA in B) believed to be involved in the secretion pathway for the molecule. Figure from Fagan *et al.* 2009. *Molecular Microbiology* 60.
In contrast, HMW SLP is less exposed to the host immune system to recognize and is thought to be localized more towards the interior part of the S-layer due to its interactions with the underlying cell wall. Despite the localization, HMW SLP was found to be an immunoreactive cell wall protein in sera of CDI patients, indicating that the antigen can be exposed to the host immune system. The HMW SLP is more conserved amongst different strains of *C. difficile*, since it must have domains that interact with the Gram positive peptidoglycan layer. Indeed, antibodies raised against HMW SLP of strain 630 cross-reacts with HMW SLP of other strains including toxigenic and non-toxigenic strains. Thus, in contrast to LMW SLP, a vaccine targeting HMW SLP could confer protection from many different strains of *C. difficile*.

As illustrated in Figure 3, the S-layer of *C. difficile* does contain various other molecules. Many of these proteins are paralogs of SlpA called cell wall proteins (Cwp), as they share three putative cell wall binding repeat 2 (Pfam 04122) domains to interact with the underlying cell wall. Each paralog has its own unique domain that may provide a variety of different functions. One particular Cwp of interest is Cwp84, a cysteine protease known to be responsible for the proteolytic cleavage of SlpA into the LMW and HMW SLP subunits. This processing of SlpA is critical for the generation of a proper S-layer, since insertional disruption of *cwp84* gene expression results in cell wall proteins and SlpA being found in the culture supernatant, indicating poor incorporation of the proteins into the S-layer. Multiple forms of Cwp84 are known to exist during its maturation process, but which form or forms, under *in vivo* conditions, cleaves SlpA is not yet clear. Other than the cleavage of SlpA, Cwp84 protease function extends to many other targets including host extracellular matrix proteins. Precisely what the *in*
**Other Virulence Factors**

Studies of *C. difficile* adhesion interference in the literature have always reported partial decreases in adherence$^{61,73}$, and vaccination studies of animals, while they have suggested protection, have not demonstrated complete clearance of pathogen from the animal$^{71,74}$. This suggests that *C. difficile* utilizes many different molecules and pathways in its colonization process of the host. Studies of *C. difficile* proteins have been difficult due to the lack of genetic tools to generate mutants. However, a new system utilizing homologous insertion with bacterial conjugation, named ClosTron$^{75}$, has allowed for the generation of functional knockouts in *C. difficile* and study of specific molecules became more available. Many different factors for colonization in addition to the ones listed in the previous section have been previously described, and many hold therapeutic potential for CDI.

One of the earliest discovered adhesin for *C. difficile* was Cwp66, a SlpA paralog like Cwp84. The study of Cwp66 revealed that antibodies against Cwp66 inhibited *C. difficile* adherence *in vitro*. 

*vivo* role of degrading extracellular matrix proteins serves for *C. difficile* pathogenesis is unclear. While the question of whether an antibody response directed against Cwp84 is protective is unknown for humans, some patients do make antibodies against Cwp84$^{63}$, and Cwp84 has been tested in active immunization of mice for protection along with FliD$^{71,72}$. Perhaps an antibody response could interfere with the incorporation of Cwp84 into the S-layer during its assembly or affect the protein in a way the function can be blocked. This study is designed to test whether antibodies against Cwp84 could alter *C. difficile* adherence *in vitro*. 
difficile adhesion to Vero cells, a monkey kidney epithelial cell line\textsuperscript{76}. An interesting aspect of Cwp66 is that it is not present at the cell surface under normal culture conditions, but upon heat-shock at 60°C for twenty minutes, Cwp66 localizes to the cell surface, perhaps to the S-layer since the protein is a SlpA paralog. Also, the antibody-mediated adhesion interference with Cwp66 was limited to the heat-shocked \textit{C. difficile} with antibodies against Cwp66 from heat-shocked bacteria\textsuperscript{76}. It was subsequently found that Cwp66 has distinct antigenic properties after heat-shock, as antibodies against normal Cwp66 did not recognize the heat-shock induced Cwp66 and vice versa\textsuperscript{76}. Thus, it is not clear yet how Cwp66 actually may mediate adhesion \textit{in vivo}, and the question of Cwp66 as a potential vaccine antigen remains open.

Another heat-shock protein identified to have a role in \textit{C. difficile} adhesion is GroEL (or heat-shock protein 60, Hsp60). In heat-shocked \textit{C. difficile}, GroEL was found to be localized to the cell surface and recombinant GroEL or antibodies against GroEL both inhibited adherence of heat-shocked \textit{C. difficile}\textsuperscript{77}. However, GroEL lacks the signal peptide normally present in bacterial molecules that localize to the cell surface, so how GroEL may be exposed to antibodies in this case is unclear. Seeing that many environmental stress factors such as high acidity, high osmolarity, iron deficiency, and heat stress modulate adherence in \textit{C. difficile}\textsuperscript{78}, that transcription of genes from the pathogenicity locus that codes for TcdA and TcdB increases during later phases of growth in culture when nutrients are relatively scarce\textsuperscript{79}, and that many other virulence genes are upregulated under stress conditions like high salt concentration and subinhibitory antibiotic dose in culture\textsuperscript{80}, there may be an association between \textit{C. difficile} virulence and environmental stress the bacteria face. Although it is unknown if an
immune response against stress-induced molecules including GroEL would be able to protect from CDI since the expression profile of these molecules is unknown in *in vivo* conditions, it may still be a feasible approach to target stress-related molecules for vaccine development to prevent *C. difficile* colonization.

It has been thought that *C. difficile* adheres to the brush border components of the intestine since the bacteria were observed to be associated with the apical tip of the brush borders of Caco-2 cells, a human intestinal cell line derived from a colon carcinoma. Also, it was also shown that the increase in brush borders, through spontaneous differentiation of Caco-2 cells in culture, seemed to increase the level of adherence. However, it was demonstrated that treatment of Caco-2 cells with calcium ion (Ca$^{2+}$) chelators such as ethylene glycol tetraacetic acid (EGTA) to expose the basolateral side of the Caco-2 through the disruption of the intercellular junctions increases adherence of *C. difficile*, and that *C. difficile* seems to associate with projections that look like extracellular matrix (ECM) components when observed by scanning electron microscopy (SEM); it was confirmed that the bacteria can bind to immobilized ECM proteins, suggesting that *C. difficile* can bind to ECM proteins for adherence to Caco-2 cells.

Fibronectin is a ubiquitous protein of about 440kD that is a major part of the ECM that supports various tissues. It is known that *Streptococcus pneumoniae* (*S. pneumoniae*) and *Streptococcus pyogenes* (*S. pyogenes*) bind to fibronectin, and that this process seems to be mediated by fibronectin-binding proteins (Fbp). *C. difficile* also possesses FbpA (noted FbpA in strain 630 and Fbp68 in strain 79-685) that mediates *C. difficile* binding to immobilized fibronectin in manganese (Mn$^{2+}$) dependent manner. Although anti-Fbp68 antibodies showed interference of *C. difficile* adherence to Vero
cells\textsuperscript{87}, suggesting that Fbp68 may mediate adherence to epithelial cells, disruption mutants of \textit{fbpA} (\textit{ΔfbpA}) showed increase in adherence to Caco-2 and HT29-MTX (mucus producing human intestinal epithelial cell line)\textsuperscript{89}, suggesting that the role FbpA plays in overall \textit{C. difficile} adherence remains to be understood. Still, the same study demonstrated that the \textit{ΔfbpA} mutant poorly colonized mice in two of three models of differing intestinal microbiota compared to wild-type \textit{C. difficile}\textsuperscript{89}, and the bacteria possesses Cwp84 which can exert proteolytic functions against ECM proteins\textsuperscript{70}, suggesting that \textit{C. difficile} interaction with ECM proteins like fibronectin may still be important in the overall colonization process.

The emergence of hypervirulent strains of \textit{C. difficile} has compounded the challenge for clinicians to treat CDI. One feature of the BI group (by restriction endonuclease analysis (REA) typing and 027 by polymerase chain reaction (PCR) ribotyping), one of the hypervirulent strains, is the production of binary toxin (CDT) in addition to TcdA and TcdB. CDT is an actin-specific adenine diphosphate (ADP)-ribosyltransferase that has cytotoxic effects in Vero cells that can be neutralized by antibodies against \textit{Clostridium perfringens} (\textit{C. perfringens}) iota toxin\textsuperscript{90}. CDT is a member of iota-family binary toxins present among many different species of \textit{Clostridia}\textsuperscript{91}. The iota family binary toxin functions are known to be mediated in part by CD44 on host cell surface that serve a variety of functions in different cells\textsuperscript{92}, as CD44\textsuperscript{−/−} cells are known to be resistant to the cytotoxic effects of these toxins\textsuperscript{93}. Also, lipolysis-stimulated lipoprotein receptors (LSR) seem to be important for CDT uptake since CDT can induce clustering of LSRs into lipid rafts\textsuperscript{94}, a possible mechanism for subsequent signaling. Other than cytotoxic effects in some cell lines, an interesting function that
CDT has the effect on microtubular network in Caco-2 cells, where the treatment of Caco-2 cells with CDT induced microtubule-based thin projections from the host cell surface that formed a net that seemed to trap *C. difficile*, and this indeed correlated with increased adhesion; this process was demonstrated to also be neutralized by treatment with anti-iota toxin antibodies\(^95\). While it can be assumed that the production of binary toxin by the hypervirulent strains could help the *C. difficile* to adhere better to intestinal epithelium via this microtubule extension mechanism, further studies are needed to see if the production of binary toxin indeed contributes to colonization and subsequent disease.

**Biofilms and Spores**

Many bacteria form a biofilm, a complex structure consisting of specialized microbial communities, polysaccharides, and other compounds\(^96\). These community structures can form on many different surfaces including biological surfaces like the gastrointestinal epithelium. Biofilms provide the microbial community many characteristics such as resistance to antimicrobial compounds and resistance to sheer forces\(^96\). It is thought that a freely moving, planktonic bacterium comes in contact with a surface to which the bacterium first associates with then adheres to. At certain point, the bacteria start to produce extracellular material in which the colony becomes encased. Subsequently, signals are produced to differentiate cells within the biofilm structure and more matrix components are produced. Fragments of biofilm can become detached from the biofilm structure and cells are dispersed. The general process of bacterial biofilm development is illustrated in Figure 4.
Figure 4. Illustration of bacterial biofilm formation. A. Free-swimming, planktonic bacterium comes in contact with intestinal epithelial surface. Adherence to surface usually induces downregulation of mobility-associated gene expression. B. Adherence bacteria grow and form a colony. Colony starts to produce extracellular components. C. Community of bacteria is now encased in thick extracellular matrix which serves a variety of functions including resistance to antimicrobial compounds. D. Certain factors, such as strong sheer forces can induce dispersal of new planktonic bacteria or even a part of biofilm matrix containing matrix components and bacterial cells to be released to spread elsewhere. Based on Hall-Stoodley *et al.* 2004. *Nature Reviews Microbiology.*
*C. difficile* has also been demonstrated to form biofilms, especially when co-cultured with certain bacteria\(^97\). It was also determined that biofilm formation by *C. difficile* *in vitro* requires *cwp84*, *spo0A*, and *luxS* gene expression since the insertional disruption mutants of each gene did not form biofilms\(^98\). This suggests that proper S-layer processing, sporulation, and quorum sensing are required for biofilm formation, respectively. It is interesting to note that *spo0A*, whose protein product Spo0A is known to be the master regulator of sporulation that determines cell fate to become a spore in *Bacillus subtilis* (*B. subtilis*)\(^99\) and *C. difficile*\(^100\), is required for biofilm formation. The authors of the *C. difficile* Spo0A study also found that defect in sporulation increases TcdA and TcdB, increases virulence in the hypervirulent strain in mice, and decreases transmission of CDI to other mice as fecal shedding was eliminated. This is interesting because one speculated mechanism in which recurrence can occur is through persistence of *C. difficile* in the patient gastrointestinal (GI) tract which may be mediated in part by resistant biofilm formation. The persisting *C. difficile* could then grow again while the intestinal microbiota is still depleted following antibiotic treatment for CDI. If sporulation is indeed a critical process for biofilm formation *in vivo* as suggested by *in vitro* experiments, perhaps sporulation could be a target for novel therapeutics that could alleviate disease recurrence. However, the degree to which biofilm formation actually contributes to *in vivo* colonization is unknown, and further studies need to be done to confirm that biofilm can be a target for preventive therapeutics for CDI.

Bacterial spores are a dormant form of the organism designed for prolonged survival and resistance to a variety of environmental stresses. Thus, *C. difficile* is not metabolically active and does not produce toxins while in the spore form; for CDI to
occur, the spores must germinate and form vegetative cells. *C. difficile* spores are known to adhere to surfaces such as semi-solid agar surface and the intestinal cell line Caco-2. While it may be that the spores of *C. difficile* likely can adhere to GI epithelium, the extent to which such process contributes to overall colonization of the host is unknown. If spores contribute in part to adherence and persistence of the organism in the host, vaccines targeting spores or spore components may be a possible way to prevent CDI and its recurrence.

There is an interesting surface protein on *C. difficile* S-layer known as CwpV. CwpV is also a paralog of SlpA that contains a repeat domain unique to CwpV; this domain seems to have antigenic variability amongst different strains. Like many of the SlpA paralogs, CwpV also undergoes maturation process through proteolytic cleavage, in this case by autoproteolysis. While variable amongst different strains, this protein does seem to have a conserved function in promoting bacterial self-aggregation. While the *in vivo* significance of this function is still unknown, it can be speculated that this function may be important in biofilm formation; it has been demonstrated in other bacteria that autoaggregation seems to contribute to initial stages of biofilm formation. Interestingly, CwpV is known to be regulated at the transcriptional level by DNA inversion mediated by specific recombinases, allowing for phase-variable expression. Although the expression of CwpV under *in vivo* settings is unknown, CwpV was demonstrated to be the major Cwp within the S-layer when its recombinational transcription switch is turned on. Perhaps when the conditions are set for biofilm formation, CwpV may be expressed within the S-layer to promote self-aggregation which then may contribute towards biofilm formation. An antibody response that blocks this
autoaggregation function may affect biofilm formation. However, more studies are needed to evaluate this possibility.

There are many molecules of *C. difficile* that have been identified to potentially play a role in colonization of the host along with a variety of mechanisms of colonization. Many of these molecules possess the potential for being targeted by vaccines to induce protection in human hosts from CDI. The molecules described in this document are listed in Table 2. In addition, there are certainly other molecules and mechanisms contributing to host colonization and disease yet to be identified. Thus, the potential for developing and improving a vaccine that could prevent CDI is vast.
<table>
<thead>
<tr>
<th>Virulence Factor</th>
<th>Description</th>
<th>Possible contribution to CDI</th>
</tr>
</thead>
<tbody>
<tr>
<td>TcdA/B</td>
<td>Major toxins</td>
<td>Disruption of host epithelium</td>
</tr>
<tr>
<td>FliC</td>
<td>Flagellin subunit</td>
<td>Mucin binding</td>
</tr>
<tr>
<td>FliD</td>
<td>Flagellar cap</td>
<td>Mucin/Epithelial binding</td>
</tr>
<tr>
<td>SlpA</td>
<td>S-layer protein</td>
<td>Molecular exclusion, host epithelial binding</td>
</tr>
<tr>
<td>Cwp84</td>
<td>Cysteine protease</td>
<td>SlpA processing</td>
</tr>
<tr>
<td>Cwp13</td>
<td>Cysteine protease</td>
<td>SlpA processing, Cwp84 processing</td>
</tr>
<tr>
<td>Cwp66</td>
<td>Heat-shock adhesin</td>
<td>Adhesion to epithelium under heat-shock</td>
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<tr>
<td>GroEL</td>
<td>Heat-shock protein</td>
<td>Protein refolding, adhesion under heat-shock</td>
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<td>Collagen binding protein</td>
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<td>CdtA/B</td>
<td>Binary toxin</td>
<td>Cytotoxicity, microtubular trapping of bacteria for enhanced adhesion</td>
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<td>Spo0A</td>
<td>Master regulator of sporulation</td>
<td>Sporulation, biofilm formation</td>
</tr>
<tr>
<td>CwpV</td>
<td>Autoaggregation protein</td>
<td>Biofilm formation?</td>
</tr>
</tbody>
</table>

Table 2. List of *C. difficile* virulence factors, description of their function, and potential contributions to disease.
Mucosal Vaccines Targeting Colonization

The mucosal layer serves as the interface in which host and microbes interact, and thus the host immune system is highly active at mucosal surfaces. A major component of the mucosal immune system is secretory immunoglobulin A (sIgA). In the mucosa, sIgA serves a variety of functions including prevention of bacteria adhesion\textsuperscript{107}. As many infectious agents initiate pathogenesis at this mucosal interface, developing a mucosal immune response such as sIgA can prevent pathogenic processes from initiating and thus protect the host from disease. However, the mechanism by which the sIgA (dimeric form of IgA) response is generated is separate from the systemic antibody response that is primarily IgG and monomeric IgA. As the transcytosis of immunoglobulins into the mucosal layer is mediated by polymeric Ig receptor (pIgR), systemic antibodies generated through systemic vaccine administration are thought not to cross into the mucosal surface. In contrast, a mucosal vaccine would induce specific responses targeting to the mucosa that would cross into the mucosal layer and function against mucosal pathogens. In targeting microbial factors for colonization, such a mucosal response may prevent host colonization with the target pathogen.

There are many vaccines that are designed to induce a mucosal immune response, including vaccines for cholera, typhoid fever, rotavirus, influenza, and polio\textsuperscript{108}. One interesting vaccine in current use is the heptavalent pneumococcal conjugate vaccine (PCV7). This vaccine consists of surface carbohydrates from different serotypes of \textit{Streptococcus pneumoniae} (\textit{S. pneumoniae}) conjugated to diphtheria toxin. While this vaccine is delivered systemically and not by a mucosal route, it was demonstrated that in children vaccinated with PCV7, the \textit{S. pneumoniae} colonizing the nasopharyngeal
mucosa becomes seroconverted, where the strains of bacteria colonizing the vaccinated children converts to non-vaccine strains\textsuperscript{109}. This suggests that vaccination prevents children from being colonized with vaccine-targeted strains \textit{S. pneumoniae}. In the case of \textit{C. difficile}, toxin-targeting vaccines have been the focus of research, whereas studies to develop vaccines that could prevent colonization have been few. One study measured the level of \textit{C. difficile} recovery from feces after challenging mice with or without immunization with FliD, Cwp84, cell wall extract, flagellar preparation, or a combination through various mucosal routes\textsuperscript{71}. While the study demonstrated a slight decrease in \textit{C. difficile} recovery in immunized mice, the vaccine failed to prevent colonization. This may have to do with the efficacy of the vaccination itself, since only the rectal administration resulted in the production of antigen-specific IgA in the intestinal lavages of mice while other routes had resulted in almost undetectable IgA levels. With the uncertainty about the mechanisms behind \textit{C. difficile} adhesion and colonization process, further studies are still needed to develop a vaccine that can prevent colonization. However, other mucosal vaccines already licensed for clinical use, and the success that some vaccines have had in preventing colonization, suggest that prevention of CDI by targeting colonization is possible.

\textbf{Caco-2 BBE Colon Adenocarcinoma Cell Line}

In order to test antibodies for their ability to interfere with \textit{C. difficile} adherence, a system modeling the gut epithelial surface has to be established. The Caco-2 cell line is derived from human colon adenocarcinoma and is able to spontaneously differentiate and polarize to have enterocyte-like qualities such as brush borders. Caco-2 brush border
expressing clone (BBE) cells, derived from Caco-2 cells\textsuperscript{110}, have been chosen as the model for gut epithelial surface in this study. In the past, Caco-2 cell line has been used in bacterial adherence assays under anaerobic conditions, which is crucial for the growth and survival of vegetative cells of \textit{C. difficile}. The Caco-2 survival was reported to be 98\% after three hours of incubation under anaerobic conditions\textsuperscript{111}. Studies involving adhesion interference in \textit{C. difficile} have utilized mainly Vero cells\textsuperscript{53} and Hep-2 cells\textsuperscript{61}, which can bind enteric pathogens but are not of colonic origin. Thus, the Caco-2 BBE cell line has been chosen since the cells are of colonic origin and express brush borders which \textit{C. difficile} has been suggested to associate with\textsuperscript{81}.

**Experimental Design**

In order to test antibodies targeting certain molecules of \textit{C. difficile} in their ability to interfere with adherence, target molecules, FliD, HMW SLP, and Cwp84 will be produced as recombinant proteins from \textit{Escherichia coli} (\textit{E. coli}). The recombinant proteins will be used as immunogens for antibody production in rabbits. Adhesion levels to Caco-2 BBE cells will be compared between \textit{C. difficile} pre-treated with pre-immune serum and serum specific for the target molecules. If the molecules are indeed involved in \textit{C. difficile} adhesion to Caco-2 BBE cells, the antibodies should reduce the number of adherent bacteria compared to naïve serum.
CHAPTER TWO
MATERIALS AND METHODS

C. difficile Culture

C. difficile strain 630 spores were streaked onto 10% sheep blood agar plates (BAP) (BD Bioscience, Franklin Lakes, New Jersey) and incubated for 48 hours at 36°C under anaerobic conditions (80% N₂, 15% CO₂, and 5% H₂) in a Bactron IV anaerobic chamber (Shel Lab, Cornelius, Oregon). A single colony of C. difficile was inoculated into 20mL anaerobic tryptic soy broth (TSB) (BD Bioscience) and grown overnight (16-18 hours) at 36°C anaerobically. Alternatively, a C. difficile colony was inoculated into brain-heart infusion broth (BHI) (BD Bioscience) and grown overnight.

Isolation of C. difficile Genomic DNA

An overnight culture of C. difficile 630 was prepared in TSB, and the cells were pelleted by centrifugation at 3,000xG for 10 minutes at 4°C using Allegra™ 6R centrifuge (Beckman-Coulter, Fullerton, California). Cells were resuspended in 3mL 50mM EDTA solution. Lysozyme, mutanolysin, and lysostaphin were added to 15mg/mL, 1U/mL, and 500μg/mL respectively into the resuspension. RNase A was added to active concentration of 200μg/mL and the mixture was incubated at 37°C for 1 hour. Proteinase K and SDS was added to 2.25mg/mL and 1.8% (w/v) respectively, and the mixture was
incubated at 50°C for another 1 hour. Phenol:chloroform:isoamyl alcohol (3mL) was added to the mixture, and the mixture was centrifuged at 1,500xG for 5 minutes. The upper phase was taken and the DNA was precipitated by adding 8mL 100% ethanol and incubating overnight at -20°C. DNA was pelleted by centrifugation at 3,000xG for 30 minutes at 4°C and the pellet was resuspended in 100μL TE buffer after washing and air-drying. Genomic DNA was stored at 4°C until use.

**PCR Amplification of Target Genes**

Target coding regions of genes *fliD*, *slpA*, and *cwp84* were amplified by PCR with Phusion high-fidelity DNA polymerase (Thermo Scientific, Pittsburg, Pennsylvania) using specific primers as listed in Table 3. Primers were designed to incorporate BamHI and XhoI restriction sites to the PCR products. PCR products were separated by 1% agarose gel electrophoresis and bands corresponding to target size were cut out and eluted from gel using Wizard® PCR Preps DNA Purification System (Promega, Madison, Wisconsin). PCR product was then ligated into pGEM®-T Easy vector (Promega) and subsequently transformed into *E. coli* DH10B by electroporation using Gibco-BRL Cell-Porator (Life Technologies, Grand Island, New York). Transformed cells were selected for resistance against 100μg/mL ampicillin and by blue-white colony screening on Luria-Bertani (LB) (BD Bioscience) agar plates overnight at 37°C. Selected clones were cultured overnight at 37°C in LB broth then stored at -80°C as 20% glycerol stocks.
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Table 3. List of primers used in the study. The incorporated restriction site sequences are noted in bold. The entire fliD coding region was amplified, compared to slpA and cwp84 where only a part of the coding region of interest was amplified. All primers were ordered from Integrated DNA Technologies (Coralville, Iowa). Bold text indicates the BamHI and XhoI restriction sites.
**Construction of Expression Plasmids**

*E. coli* clones harboring pGEM®-T Easy constructs with target gene inserts were streaked onto LB agar plates containing 100μg/mL ampicillin and incubated overnight at 37°C. Single colonies were inoculated into LB broth with 100μg/mL ampicillin for overnight culture at 37°C. Plasmid DNA from the overnight culture was isolated using the Wizard® Plus SV Minipreps DNA Purification System (Promega). DNA was stored at -20°C. pGEM®-T Easy constructs were digested with BamHI and XhoI for 1 hour at 37°C, and pET-21a(+) vector (Merck KGaA, Darmstadt, Germany) was also digested with BamHI and XhoI for 1 hour. The pET-21a(+) vector was treated with shrimp alkaline phosphatase (SAP) for 30 minutes at 37°C. Resulting DNA fragments were separated by 1% agarose gel electrophoresis and bands corresponding to target genes and pET-21a(+) were cut out, eluted from gel, then subsequently ligated to generate the expression plasmid constructs. The expression plasmid constructs were transformed into *E. coli* DH10B cells by electroporation and colonies were selected by 100μg/mL ampicillin on LB agar plates after overnight culture at 37°C. Plasmids were extracted from overnight LB broth culture and digested for 1 hour with BamHI and XhoI at 37°C. The products were analyzed by 1% agarose gel electrophoresis to confirm the presence of correct plasmid and insert. Clones harboring correct plasmid construct were cultured overnight in LB broth and stored at -80°C as 20% glycerol stocks. A list of plasmid constructs and the parent plasmids can be found in Table 4. All plasmid constructs, including pGEM®-T Easy and pET-21a(+) constructs were sequenced using ABI Prism 3130 Genetic Analyzer (Life Technologies) to confirm that the cloning was successfully done in frame. For sequencing, lowered extension temperature (50°C for 4 minutes) was
used due to the high AT content of target genes, as it has been demonstrated that lowered extension temperatures in PCR enhances amplification in AT-rich templates\textsuperscript{112}. For pGEM\textsuperscript{®}-T Easy constructs, T7 promoter-specific and SP6 promoter-specific primers were used while for pET-21a(+) constructs, the T7 promoter-specific and T7 terminator-specific primers were used, all ordered from Integrated DNA Technologies.
<table>
<thead>
<tr>
<th>Plasmid</th>
<th>Description</th>
<th>Marker</th>
</tr>
</thead>
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<tr>
<td>pGEM®-T Easy</td>
<td>Plasmid for cloning PCR products, contains hanging T edges</td>
<td>Ampicillin, Blue-white</td>
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<td></td>
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<tr>
<td>pGEM®-T Easy-\textit{cwp84}</td>
<td>\textit{cwp84} cloning plasmid construct</td>
<td></td>
</tr>
<tr>
<td>pET-21a(+)</td>
<td>Parent plasmid for expression</td>
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<td>pET-21a(+)-\textit{fliD}</td>
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<td>pET-21a(+)-\textit{cwp84}</td>
<td>\textit{cwp84} expression plasmid construct</td>
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<tr>
<td>pRARE2</td>
<td>Encodes tRNA genes rare in \textit{E. coli} to address codon bias</td>
<td>Chloramphenicol</td>
</tr>
</tbody>
</table>

Table 4. List of plasmids used in this study. The pRARE2 plasmid is already contained by the \textit{E. coli} Rosetta 2 (DE3) cells by design (Merck KGaA).
Expression of Target Proteins

The pET-21a(+) expression plasmid constructs were transformed into *E. coli* Rosetta™ 2 (DE3) cells (Merck KGaA) by electroporation. The Rosetta™ 2 strain of *E. coli* contains pRARE2, which is a plasmid encoding tRNA genes rare in abundance in *E. coli*. This addresses codon bias in *E. coli* that limits the proteins bacteria can express, and allows for genes from *C. difficile*, whose proteins utilize some of these rare codons, to be expressed more proficiently. The transformed cells were selected with 34μg/mL chloramphenicol and 200μg/mL ampicillin on LB agar plates. Clones harboring the plasmid constructs were screened by colony PCR using primers specific for each target insert (listed in Table 4). Briefly, the PCR template was prepared by picking a single colony into 100μL dH2O and heating at 100°C for 90 seconds. This sample (1μL) was used as the template for colony PCR using the Phusion DNA polymerase. PCR products were analyzed by 1% agarose gel electrophoresis. Clones confirmed to harbor correct plasmid construct were stored at -80°C as 8% glycerol stocks.

Expression clones were cultured overnight in LB broth containing 34μg/mL chloramphenicol and 200μg/mL ampicillin. The overnight culture was diluted into fresh LB broth 1:50 and cultured until OD_{600nm} reached 0.5. At this point, IPTG was added to 0.5mM to induce protein expression for 3 additional hours. Cells were harvested by centrifugation at 3,000xG for 5 minutes at 4°C, and resuspended in 1mL of 50mM NaH₂PO₄, 300mM NaCl solution. Lysozyme was added to 100μg/mL and the mixture was stored at -80°C to facilitate lysis by freeze-thaw cycles. Thawed lysate was subsequently cleared by sonication and the soluble and insoluble molecules were separated by centrifugation at 14,000xG for 15 minutes at 4°C using Sorvall Legend
Micro 21R centrifuge (Thermo Scientific). The supernatant was labeled “soluble fraction” and stored at -20°C. The pellet was resuspended in 1mL NaH₂PO₄, 300mM NaCl solution containing 6M urea to solubilize the pellet, and this mixture was labeled “insoluble fraction”. Both fractions were analyzed for target protein expression by 10% SDS-PAGE followed by Coomassie brilliant blue staining or western blot. For western blot, the presence of target protein’s N-terminal T7·tag® was detected with biotinylated anti-T7·tag® mAb (Merck KGaA) at 20ng/mL followed by streptavidin (SAV) conjugated to HRP at 100ng/mL. Films were developed using Pierce ECL western blotting substrate (Thermo Scientific).

**Purification of Target Proteins**

Target proteins expressed in the “soluble fraction” were purified by Ni-NTA column chromatography using Ni-NTA His·Bind® Resin (Merck KGaA) under native conditions. Proteins were eluted using increasing concentration of imidazole in buffer (50mM NaH₂PO₄ and 300mM NaCl, 20-250mM imidazole, pH 8.0). For proteins expressed in the “insoluble fraction”, purification was performed in the same way as those in the “soluble fraction” under denaturing conditions (6M urea). 1mL fractions collected from column chromatography were analyzed for the presence of target protein by dot blotting. The dot blot was probed in the same way as the western blot for the T7·tag®. Select fractions were then analyzed by 10% SDS-PAGE to determine the purity of the sample. Fractions from initial “soluble fraction” that contained the most purified target protein were dialyzed into PBS (pH 7.6) and stored at -20°C. For fractions from initial “insoluble fraction” that contained the most purified target protein, a stepwise
dialysis was performed to remove urea from solution in order to allow refolding of the protein. Once dialyzed into buffer without urea (50mM NaH$_2$PO$_4$ and 300mM NaCl), the fraction was dialyzed in PBS and stored at -20°C.

**Immunization of Rabbits**

New Zealand White rabbits of at least 3 months of age bred in house were immunized with target antigens in TiterMax® Gold adjuvant (Sigma-Aldrich, St. Louis, Missouri) by intramuscular and subcutaneous injections. Rabbits were boosted three times every two weeks with the same antigen in Freund’s incomplete adjuvant. Blood was collected every two weeks throughout the immunization procedure to collect serum. Sera were stored at -20°C. All animal protocols were used as per protocols approved by the Institutional Animal Care and Use Committee of Loyola University Chicago.

**Determination of Antigen-specific Serum Titer**

The target antigen-specific titer of each serum collected from rabbits was determined by enzyme-linked immunosorbance assay (ELISA). Briefly, Costar® 96-well ELISA plates (Corning, Tewksbury, Massachusetts) were coated with 2μg/mL target antigen overnight. Coated plates were blocked with PBS containing 2.5% Difco™ skim milk (BD Bioscience) for 2 hours at room temperature, then probed with serial dilutions of antigen-specific serum. Antibodies reactive to specific antigens were detected with donkey anti-rabbit IgG$_{H\%L}$ (heavy and light chains) conjugated to HRP (Jackson ImmunoResearch, West Grove, Pennsylvania) at 80ng/mL. The plate was then stained with ABTS staining (0.1M sodium citrate buffer pH 4.5, 1.37mg/mL ABTS, 0.015%
H$_2$O$_2$) and the absorbance at 405nm using ELx800 Absorbance Microplate Reader (BioTek®, Winooski, Vermont).

**C. difficile S-layer Extraction**

Overnight culture of *C. difficile* 630 or BI-17 was prepared in BHI broth. Cells were washed in PBS and resuspended in 1mL 200mM glycine buffer, pH 2.2. This mixture was incubated for 30 minutes at room temperature and subsequently pelleted by centrifugation at 14,000x$G$ for 15 minutes at 4°C. The resulting supernatant containing the extracted proteins were dialyzed in PBS and stored at -20°C. The extracted proteins were analyzed by 8% SDS-PAGE followed by Coomassie brilliant blue staining.

**C. difficile Adhesion Interference Assay**

Caco-2 BBE cells were grown in Costar® 24-well tissue culture plates (Corning) until the monolayer reached confluence with high-glucose DMEM (Life Technologies) with following additives: 10mM HEPES, 1mM pyruvate, 50U/mL penicillin, 50μg/mL streptomycin, 30μg/mL gentamicin, 550μM L-glutamine, 0.0004% 2-mercaptoethanol, and 500ng/mL amphotericin B. Once confluent, the monolayer was maintained for three additional days. One day before the assay, the medium was switched to DMEM supplemented with only 1.5mM CaCl$_2$. Overnight culture of *C. difficile* was washed and resuspended in PBS. 1mL aliquots of *C. difficile* (diluted to about 6 million colony forming units (CFU)/mL) were made and each aliquot was incubated with either naïve or serum specific for target antigens for 15 minutes at 36°C at 1:100 dilution. The antibody-*C. difficile* mixture was washed once with PBS then resuspended in DMEM with CaCl$_2$. 
Caco-2 BBE monolayer was introduced into the anaerobic chamber and medium was replaced to anaerobic DMEM with 1.5mM CaCl$_2$, 250μL per well. The pre-incubated C. difficile mixture was then added to wells 250μL each, achieving about $1.5 \times 10^6$ CFU per well in 500μL total volume (MOI 1, 1:1 ratio of bacteria:host cells). Bacteria were allowed to adhere for 1 hour at 36°C, then the wells were washed twice with 1mL PBS to remove non-adherent bacteria. The monolayer was then treated with 1mL PBS containing 1mM EDTA to disrupt tight junctions, and the entire monolayer was dislodged from the well by vigorous pipetting. A relatively homogenous mixture was obtained for each well by subsequent vortexing and CFU was determined for each well by plating dilutions onto BHI agar plates overnight at 36°C. As a control, adherence interference of C. difficile by pre-incubation of cell monolayer with extracted SLPs, as previously reported in the literature$^{61}$, was used. Briefly, the medium for the Caco-2 BBE monolayer in a 24-well plate was replaced with DMEM containing 1.5mM CaCl$_2$ and 200μg/mL SLP extract, 250μL per well. The cells were incubated under anaerobic conditions at 36°C for 1 hour before C. difficile in DMEM was added to Caco-2 BBE cells at a MOI of 1. Adherence was then carried out as outlined above for the antibody assay.
CHAPTER THREE

RESULTS

Cloning of Target Genes into Expression Vector Constructs

From genomic DNA of *C. difficile* 630, whose genome has been fully sequenced, *slpA* (region coding for HMW SLP), *fliD* (full length), and *cwp84* (region coding for mature form) were amplified by PCR to generate vector constructs for expressing target proteins. The amplified products were cloned into pET-21a(+) which utilizes a IPTG inducible system. After obtaining *E. coli* clones that gained resistance to ampicillin by the transformation of the pET-21a(+) vector constructs, plasmid DNA was analyzed by restriction endonuclease digestion with BamHI and XhoI (the sites were introduced into target genes by PCR) followed by agarose gel electrophoresis. As shown in Figure 5, digestion of plasmid constructs released DNA bands corresponding to expected sizes of each target insert, suggesting successful cloning of target genes into the pET-21a(+) vector. In order to confirm insert as the intended targets and to check for in-frame cloning, the pET-21a(+) constructs were sequenced at the insertion site. Initial attempts at sequencing with BigDye® Terminator system (Life Technologies) under standard conditions repetitively failed to yield product, and so the lowered extension temperature protocol, as suggested in the literature\textsuperscript{112} for AT-rich templates, was used to successfully sequence plasmid DNA at the insertion site. The plasmid constructs were confirmed by sequencing to be the correct target gene sequences and to be in-frame.
Figure 5. Restriction digestion analysis of pET-21a(+) expression vector constructs. Each vector constructs were digested with BamHI and XhoI for 1 hour then analyzed by 1% agarose gel electrophoresis. In lane 2, a band below the 2.0kb mark can be observed which corresponds to cwp84 PCR product (about 1.5kb). Also in lane 3, fliD (about 1.5kb) can be observed below 2.0kb mark. In lane 4, slpA-HMW SLP (about 1.1kb) can be observed below 2.0kb mark and below the 1.5kb bands shown in lane 2 and 3. In lanes 2, 3, and 4, the pET-21a(+) vector without the insert (about 5.4kb) can be observed between the 6.5kb and 4.4kb marks. Some other minor bands are present but likely represent undigested plasmid DNA.
The correct plasmid constructs were subsequently transformed into *E. coli* Rosetta 2 (DE3) cells. Because this strain of *E. coli* does not have *endA* mutations, plasmid extraction using standard protocols does not yield quality DNA needed for restriction digestion analysis (the DH10B strain we used for cloning contains a single point mutation in the *endA* gene that inactivates the enzymatic activity of the translated protein). In order to confirm that the plasmid construct was successfully transformed, colony PCR was performed instead. As shown in Figure 6, each expression clone harbors their corresponding target gene insert. Along with selection with ampicillin for the presence of the pET-21a(+) vector itself, this confirms that correct plasmid constructs were transformed successfully into their respective expression host.
Figure 6. Colony PCR of each *E. coli* clone transformed with pET-21a(+) expression constructs. Each transformed clone of *E. coli* was probed with three different specific primer sets (listed in Table 3) for the presence of target insert. Lanes 5-7 show that the Cwp84 clone harbors only *cwp84*; no bands were found using primers for *fliD* or *slpA* in this clone. Likewise, lanes 8-10 show *fliD* specificity in FliD clone and lanes 11-13 show the same for HMW SLP clone. No bands are found in lanes 2-4 in which no template was added to reaction mixture.
Expression of Target Proteins

*E. coli* clones harboring the expression plasmid constructs were tested for their ability to express target proteins. Initially, the expression plasmid constructs were transformed into *E. coli* BL21 (DE3) cells (Merck KGaA), a standard strain for recombinant protein expression. However, only Cwp84 was successfully expressed using this system, while the induction experiments under various conditions repetitively failed to produce FlID and HMW SLP. *C. difficile* genomic DNA is known to be rich in AT content, and thus quite different from the genomic DNA content of *E. coli*. The problem, in the context of recombinant protein production, is that *C. difficile* proteins utilize codons that are relatively rare in *E. coli* since *E. coli* does not utilize those codons much for producing its own proteins. This codon bias results in truncated proteins or even early translational termination which leads to poor or no protein yield. To address the issue of codon bias, the expression host strain was switched to *E. coli* Rosetta 2 (DE3) cells, which possess a pRARE2 plasmid which encodes tRNA genes rare in *E. coli*, essentially supplementing the host with rare codons. As shown in Figure 7A, induced lysates of each of the clones have T7 tagged target proteins which indicate successful expression. The Cwp84 previously expressed from the BL21 strain was used as a positive control here, and it can be observed that Cwp84 expression level is much greater in the Rosetta 2 strain. Thus, subsequent production of Cwp84 was performed using the Rosetta 2 clone instead.
Figure 7. Analysis of induced *E. coli* Rosetta 2 (DE3) expression hosts for expressed proteins. **A.** Western blot analysis for expressed proteins. Uninduced and induced *E. coli* clone lysates were analyzed for the presence of T7·Tag® which is on the N-terminus of expressed protein construct. HMW SLP (about 45kD), FliD (about 53kD) and Cwp84 (about 50kD) clones show bands at correct respective sizes. The Cwp84 lane is difficult to visualize due to overpowering signal, but the major banding seems to be ~50kD. SDS-PAGE analysis as shown in Figure 7B supports Cwp84 band ~50kD. Uninduced lysates do not show any bands. (+): control T7 tagged protein. **B.** SDS-PAGE analysis of expressed proteins. Induced lysates were separated into insoluble and soluble fractions and analyzed in separate lanes. For Cwp84, only the insoluble fraction was analyzed. (-): uninduced sample. (+): induced sample. Box indicates the expected band sizes for each target proteins.
Because the purification process for the expressed proteins differ based on the solubility state of the molecule, a SDS-PAGE analysis was performed for the lysates that were separated into soluble and insoluble fractions. If the protein is expressed as a soluble protein, purification is performed under native conditions while if the protein is insoluble, the expressed protein need to be denatured during purification and subsequently be refolded into native conditions. As observed from previous work in the BL21 strain (data not shown), Cwp84 was produced only in the insoluble fraction when expressed in the Rosetta 2 strain. As for FliD and HMW SLP, the majority of the expressed proteins are in the soluble fraction while a lesser amount are produced as insoluble inclusion bodies, indicated in Figure 7B by the darker band staining in the soluble fraction sample than the insoluble fraction. Therefore, FliD and HMW SLP was determined to be purified from the soluble fraction using native conditions while Cwp84 was determined to be purified from the insoluble fraction using denaturing conditions.

**Purification of Target Proteins**

The pET-21a(+) vector construct encodes a C-terminal hexahistidine tag (6x His), which has affinity to certain metal ions such as Ni^{2+}. Thus, the expressed proteins, possessing the C-terminal 6x His tag, were purified from *E. coli* lysates by Ni-NTA column chromatography. Column fractions (1mL) were collected and each fraction was tested for the presence of the T7·Tag® by dot blot to determine which fractions contain the target proteins. One representative dot blot (for FliD) is shown in Figure 8. As shown in Figure 8, the presence of the fusion tagged proteins in the flow-through fractions indicate that some expressed target proteins did not bind to column. Only minimal
amounts of target protein eluted during the wash fractions. Strong signals for the T7·Tag®
can be observed in the first few fractions of the elution fractions, indicating that the
majority of target proteins eluted in those first few fractions.

The dot blot analysis reveals the presence of the target proteins, but it does not
measure the relative purity of the proteins. To determine the degree of protein
purification, SDS-PAGE analysis of select fractions was performed. As shown in Figure
9A, there is a decrease in the number of bands present in the fraction beginning with the
initial, unpurified sample to purified elutions, indicating that the target protein was
selectively purified from the lysate. The same purification procedure was followed for the
other two target proteins HMW SLP and Cwp84, although with Cwp84 purification
followed denaturing conditions containing 6M urea since it was expressed as insoluble
proteins. Figure 9B shows the purified proteins analyzed by SDS-PAGE. Interestingly,
HMW SLP and Cwp84 both show major co-purifying bands. If contaminating E. coli
proteins were present in the purified fractions, the co-purifying bands would be of similar
size across samples since ones that have affinity to Ni²⁺ would have bound to column.
However, the co-purifying bands are different in HMW SLP and Cwp84 purification,
indicating that degradation products of target proteins during expression are causing these
extra bands. This was later supported by western blot analysis where antibodies raised
against each purified sample did not cross-react against each other, indicating that the co-
purified bands are not shared between the samples (data not shown).
Figure 8. Representative dot blot analysis of Ni-NTA fractions (FliD). Flow-through samples represent fractions collected without imidazole. Wash samples were collected in the presence of 20mM imidazole (20 Wash), or with 50mM imidazole (50 Wash). Elution samples were collected in the presence of 250mM imidazole, and the clean samples were collected with 1M imidazole to elute any remaining proteins. Starting material is the sample loaded onto the column. The blot was probed for the presence of T7-Tag®. SW: first fraction collected immediately after switching buffer.
Figure 9. Purification of target proteins by Ni-NTA column chromatography. A:
Representative SDS-PAGE analysis of select Ni-NTA fractions (FliD). B: SDS-PAGE
analysis of target protein elution fractions containing the most protein. HMW SLP
(~45kD), Cwp84 (~50kD), FliD (~53kD). Gels were all stained with Coomassie Brilliant
Blue.
Generation of Target Antigen-specific Serum

Purified target proteins were used as immunogens in rabbits to produce antigen-specific antisera. To determine if the rabbits immunized with the antigen indeed generated antigen-specific antibodies, sera were collected at various points throughout the immunization protocol and their reactivity to respective immunogens was tested by ELISA. As shown in Figure 10, there is a significant increase in absorbance in the serum collected from immunized rabbits compared to naïve serum; in Figure 10, one representative pre-immune serum (from the FliD-immunized rabbit) is shown, though it was confirmed that all immunized sera resulted in increased absorbance compared to pre-immune serum from other rabbits (data not shown). Also, in other ELISA and western blot analyses where sera were tested against antigens other than the respective immunogens, such as anti-FliD antiserum against recombinant HMW SLP, no cross-reactivity was observed, indicating that the produced antisera were indeed target antigen-specific (data not shown).
Figure 10. Production of target antigen-specific antisera from rabbits. Sera from rabbits were collected after the final booster injection and analyzed for reactivity against respective antigens by ELISA. Sera were serially diluted 1:2 from 1:1,000 to 1:1,024,000.
**Adherence Interference**

To test if antibodies against FliD, HMW SLP, and Cwp84 could block *C. difficile* adherence *in vitro*, an adherence interference assay with Caco-2 BBE cells was performed. Caco-2 BBE cells are brush-border expressing epithelial cell line of colonic origin, and serve as an *in vitro* model for the GI tract epithelial surface to where *C. difficile* may initially bind during CDI pathogenesis. Thus, if the target antibodies can interfere with *C. difficile* adherence, the adherence of *C. difficile* pre-incubated with target-molecule specific antiserum should be decreased compared to those treated with naïve serum. The adherence interference assay was performed, and no significant decrease in adherence in *C. difficile* treated with immune serum compared to naïve serum was observed as shown in Figure 11. Only one group reached statistically significant decrease in group pre-treated with all three antisera, but as none of the individual components achieved significant decrease in *C. difficile* adherence, it seems likely that the antibodies targeting FliD, HMW SLP, and Cwp84 do not affect *C. difficile* adherence to Caco-2 BBE monolayers.

Interestingly, when Caco-2 BBE monolayers were pre-treated with S-layer extracts for 1 hour, the subsequent adherence of *C. difficile* significantly increased as shown in Figure 11. In the literature, *C. difficile* SLPs were described as adhesins as the S-layer extract was shown to decrease *C. difficile* adherence to Hep-2 cells and also found to be in proximity of human GI tract sections\(^6^1\). As such, the SLP pre-treatment of Caco-2 BBE cells were tested essentially as a positive control for this system. However, in Caco-2 BBE cells which express brush borders, a characteristic of GI tract epithelium, the SLPs seem to have the opposite effect. This suggests that while SLPs are still
potential adhesins, they may not serve a major function in *C. difficile* adherence to Caco-2 BBE cells. Similarly, antibodies directed against FliD and Cwp84 also do not affect *C. difficile* adherence significantly, indicating that these molecules also may not play a role in adherence to Caco-2 BBE cells.

It was demonstrated that environmental factors such as divalent cations have dramatic effects in *C. difficile* adherence. Divalent cations are a crucial component of cellular physiology, thus *in vitro* culture media are often supplemented with a calcium source, typically serum. In serum-free media, as used in this *C. difficile* adherence interference assay, calcium chloride is added as a source of divalent cation supplementation. Taking into account the report in which high levels of CaCl$_2$ (up to 25mM) in solution increased *C. difficile* adherence to Vero cells by 13 to 17-fold, the effect of Ca$^{2+}$ on *C. difficile* adherence interference in Caco-2 BBE cells was investigated. When no exogenous calcium was added to the medium, the adherence interference of *C. difficile* to Caco-2 cells by target antibodies did not differ significantly from the data shown in Figure 11 (performed with 1.5mM CaCl$_2$), suggesting that soluble levels of Ca$^{2+}$ did not affect adherence interference by target antibodies. Typically, Ca$^{2+}$ is supplied to the medium at concentrations below 1.7mM CaCl$_2$ due to its poor solubility in media containing abundant phosphate and carbonate ions. Nevertheless, 25mM CaCl$_2$ supplementation in DMEM for the *C. difficile* adherence interference assay was attempted. While this resulted in significant increase in overall adherence levels (data not shown), precipitates forming in the medium may have caused this increase in adherence. Thus, the assay was subsequently performed with only soluble levels of CaCl$_2$ also because the absence of calcium may affect the monolayer detrimentally.
Figure 11. *C. difficile* adherence interference assay with rabbit antisera against target proteins. % adherence was calculated by dividing the number of CFU *C. difficile* recovered by the number of CFU initially added to wells and multiplying by 100. All % adherence was normalized to *C. difficile*-only samples. Data pooled from seven different experiments, normalized to percent adherence of *C. difficile* alone group in each experiment. Total sample size (N): *C. difficile* alone – 17, SLP Pre-treatment – 11, Naïve serum – 3, α-HMW SLP – 3, α-FliD – 3, α-Cwp84 – 3, Pooled antisera – 11. Statistical analysis was performed using two-tailed student’s t-test at α=0.05.
CHAPTER FOUR
DISCUSSION

Vaccines and Colonization Resistance

CDI is the major cause of hospital-acquired infectious diarrhea, and its treatment remains a heavy burden to healthcare systems and a difficult challenge for physicians. As shown in animal and epidemiological studies, exposure to antibiotics leads to severe alterations of the gut microbiota, and it is the major risk factor for contracting CDI. Even though antibiotics cause CDI, they are also used to treat CDI. Consequently, the microbiota is still left disrupted for *C. difficile* to take foothold again and cause recurrent CDI.

Several new therapies in development seek to address the issue of recurrent disease. As described in Table 1, new antibiotics have narrower spectrum and cause a relatively mild alteration of the gut microbiota, and fecal transplantation seeks to restore the microbiota altered by CDI or antibiotic therapy. Both therapies focus on the importance of normal gut microbiota, as it provides colonization resistance against *C. difficile*. If the microbial balance can be maintained during antibiotic therapy, or be re-established, recurrent CDI can be prevented through colonization resistance.

Vaccines for CDI have been in development for a while, but most success has come from targeting TcdA and TcdB, aiming to induce an anti-toxin antibody response in the host. While antibodies against toxins have been shown in both animal studies and in
preliminary human trials to have protective effects in CDI, it is suggested that such
response does not prevent colonization with \textit{C. difficile}\textsuperscript{11}. As such, an individual
successfully vaccinated to generate a neutralizing anti-TcdA/TcdB antibody response
may be asymptotically colonized, and may still shed infectious spores into the
environment where another individual may contact. If an immune response were
generated that confers colonization resistance against \textit{C. difficile} in individuals, the
spread of infectious spores could be prevented. Potential for vaccines that could induce
an immune response that confers colonization resistance have thus been investigated in
this study.

\textit{C. difficile} Adherence to Host GI Tract Mucosa

Bacterial adherence to host gut mucosal surface is a complex process involving
many different factors such as physical mechanics, protein-protein interactions, and
others. For example, both the bacterial cell membrane and the mammalian epithelial cell
membrane is made of a lipid bilayer. The lipid bilayer is kept electrically polarized by the
cells and thus the bacteria must overcome charge-mediated repulsive to get in proximity
with the host cell. Once this barrier is overcome, specific interactions between the host
and the bacteria including adhesin-receptor complexes can mediate tight adhesion
processes. However, the dynamic process that is bacterial adherence to host mucosal
surface is more complex. This is partly due to the nature of the host gut mucosa,
consisting of a heterogeneous population of cells that form the epithelial barrier such as
specialized M cells and goblet cells, immune components that constantly interact with the
mucosa, and even minor damage that occurs in the epithelium that can cause potentially significant changes to the mucosa.

In the case of *C. difficile*, literature suggests that the nature of *C. difficile* adherence to host gut mucosa is also complex. While the initial studies suggested that *C. difficile* associates with the apical tip of the brush borders on differentiated Caco-2 cells\(^8\), another study suggested that *C. difficile* binding to Caco-2 cells decrease significantly upon differentiation of the Caco-2 cells when the brush border expression would increase\(^2\). The latter study suggested that the extracellular matrix is also involved in *C. difficile* adherence. In addition, *C. difficile* flagella can bind to mucus\(^5\), which is yet another surface in the gut mucosa that the bacteria can interact with. Because *in vitro* cell line models cannot provide all of the components of the gut mucosal surface, the *in vivo* significance of the data on adherence studies are still difficult to interpret in the context of *in vivo* pathogenesis.

**Models and Adherence Assays**

The adherence interference assay with the Caco-2 BBE monolayer seems to suggest that antibodies against HMW SLP, FliD, and Cwp84 fail to interfere with *C. difficile* adherence. This is inconsistent from the published data which suggests that HMW SLP and FliD work as adhesins *in vitro*. In the case of Cwp84, the molecule is not an adhesin but is involved in SlpA processing\(^6\), and thus antibody binding may lead to disrupted S-layer function. While there may be multiple factors that contribute to this discrepancy, one deviation of this experiment from the literature is the use of a different cell line. Studies of *C. difficile* adherence have been initially performed with Caco-2
cells\textsuperscript{81}, as it is a cell line derived from human colonic origin and expresses qualities resembling enterocytes that line the intestine\textsuperscript{110}. However, published studies of \textit{C. difficile} adhesins were not done using Caco-2 cells. FliD was originally identified as an adhesin using radiolabeled Vero cells, an epithelial cell line of kidney origin that bound to immobilized FliD\textsuperscript{53}. SLPs were identified as adhesins in studies showing that S-layer extract mediated adherence interference of \textit{C. difficile} using Hep-2 cells\textsuperscript{61}, an epithelial cell line derivative of HeLa cells. At the same time, IgY(major antibody class found in chicken egg yolk) specific for FliD and Cwp84 was shown to decrease \textit{C. difficile} adherence in T84 cells that are metastatic colon cancer cells from the lung\textsuperscript{72}. As for SLPs, acid-extracted SLPs were also found to be associated with the epithelium of human GI tract tissue sections\textsuperscript{61}, though no adherence studies with \textit{C. difficile} were performed with these tissue sections. Additionally, studies of \textit{ΔfilD} mutant and \textit{ΔfbpA} mutant showed that adherence is increased in the absence of these potential adhesion molecules\textsuperscript{26, 89} which also makes the role these molecules play in adherence unclear. Along with the data from Caco-2 BBE epithelial model, the data seem to suggest that antibodies against FliD, HMW SLP, and Cwp84 can block adherence in some models but not in others. Adding to this issue, FliD can bind mucus\textsuperscript{53} while HMW SLP can bind to certain ECM proteins\textsuperscript{61}, which complicates the matter deciding which binding activity is actually more important for \textit{C. difficile} adherence to mucosa. \textit{In vitro} models of bacterial adherence do not provide all possible components to which \textit{C. difficile} may bind, nor can such an approach easily address the issue of determining which mechanism contributes more than another. Overall, the failure of the antibodies against FliD, HMW SLP, and Cwp84 to block adherence in Caco-2 BBE cells highlight the need to recognize that results from one \textit{in
vitro model may not be reproduced in other models. Thus, a different model that better simulates the in vivo conditions is needed to confirm the adhesion activity of target molecules and to test if antibodies can block adherence and subsequent colonization. Some possible models are ex vivo gut mucosal tissue sections to determine if antibodies block adherence of C. difficile to gastrointestinal mucosa directly and using in vivo animal model to determine if antibodies can reduce colonization of animal host with C. difficile.

Alternative Approaches to CDI Vaccines

While the interference of adherence can be a mechanism on which antibodies against C. difficile surface molecules could protect one from CDI, there are other possible mechanisms by which the antibodies may protect. Thus, antibodies that do not block C. difficile adherence may still protect individuals from CDI. One example demonstrating potential protection employs passive immunization of hamsters using antibodies against SLPs\textsuperscript{74}. In this study, all hamsters immunized with C. difficile became colonized but survival was prolonged in immunized hamsters compared to unimmunized hamsters. This suggested that the antibodies conferred slight resistance to disease in hamsters. While the level of colonization was not determined in this study, C. difficile pre-incubated with anti-SLPs antiserum showed enhanced uptake by THP-1 cells, a human phagocytic monocyte cell line, in vitro. This indicated that the antibodies opsonized C. difficile for enhanced uptake by THP-1 cells. While protection was modest at best, and it was unclear if opsonization did play a role in vivo to provide that protection, the study showed that effective opsonization of C. difficile with antibodies can contribute to protection.
against CDI. Additionally, enhanced uptake of \textit{C. difficile} can contribute to enhanced antigen presentation to the adaptive immune system which in turn could enhance protection. To date, only few opsonization studies with \textit{C. difficile} have been reported. Further studies are required to identify which antigens, when targeted by antibodies, induce opsonization that could enhance host protection.

Many potential targets for vaccine development remain to be tested, including molecules involved in pathogenesis and those yet to be discovered. As illustrated in Figure 12, there are multiple mechanisms by which \textit{C. difficile} interact with the host gut mucosa that can potentially be targeted by vaccines for protection. The adherence of \textit{C. difficile} to gut mucosa, if blocked efficiently by antibodies, could protect one from colonization. If \textit{C. difficile} uptake by phagocytic cells can be enhanced by antibodies, it may also lead to enhanced protection from CDI. It is also known that epithelial cells harbor receptors that mediate bidirectional transport of antibodies and its antigens across the epithelium. These mechanisms are capable of transporting in opsonized bacteria for enhanced immune recognition or transporting invading bacteria out from the lamina propria to the gut lumen, potentially contributing to protection. There may be additional mechanisms in which a vaccine could protect such as mechanisms involving complement activation or other novel mechanisms, but further studies are required to test this possibility.
Figure 12. Illustration of known *C. difficile*-host interaction pathways that have potential for vaccine development. Blocking of *C. difficile* interaction with mucus, epithelial surface, or ECM proteins may interfere with colonization. Alteration of *C. difficile* uptake by antigen presenting cells (APC) or transcytosis through the epithelial barrier could expose bacteria to immune system.
Concluding Remarks

Prevention and treatment of CDI is a major challenge for healthcare systems. Because it is initiated by the disruption of the microbiota through antibiotics, CDI represents a call to develop novel therapeutics that take into consideration a previously underappreciated aspect of mammalian biology, the microbiota. Vaccines have the potential to prevent the spread of CDI by preventing colonization that can last an individual’s lifetime given an effective immunization strategy. Many candidate molecules have been identified for vaccine development to prevent CDI, but the complexity of \textit{C. difficile} interaction with the host gut mucosal environment presents a challenge to extrapolate the current \textit{in vitro} findings for \textit{in vivo} significance. In this work, antibodies against FliD, HMW SLP, and Cwp84, molecules suggested in the literature to be involved in \textit{C. difficile} adherence, were tested for their ability to reduce \textit{C. difficile} adherence to Caco-2 BBE cell line, a model for gut intestinal epithelium not described previously in studies of \textit{C. difficile} adherence interference. The antibodies failed to significantly reduce \textit{C. difficile} adherence in this model which suggest that antibody-mediated adherence inhibition may be dependent upon different assay conditions. There are many more vaccine candidates to be tested and discovered including those that may confer protection through mechanisms not involving adherence. The data here highlight a need to study different, alternative models of \textit{C. difficile} interactions with host mucosa in order to identify and test vaccine potential of molecules and mechanisms yet to be discovered.
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VITA

Wonbeom “Won” Paik was born in 1989 and raised in Seoul, Republic of Korea. In 2000, he came to United States where he attended middle and high school in Chicago and in Aurora, Illinois. He went on to DePaul University to earn his Bachelor of Science degree *cum laude* in biotechnology, minoring in sociology. During his undergraduate studies, he was introduced to research by studying chromosomal congression in female *Drosophila melanogaster* during meiosis I in the lab of Dr. William Gilliland. Also, he studied the increased risk of osteoporosis in HIV patients at an internship through the Department of Sociology at DePaul. He also participated in the university honor’s program throughout his undergraduate career.

After graduation in 2007, he joined the Master’s program in Infectious Disease and Immunology at Loyola University Chicago. He then joined the laboratory of Dr. Katherine Knight and studied *Clostridium difficile* infection and vaccine development with the guidance of Dr. Knight and Dr. Dale Gerding. After the completion of his Master’s degree, Won will be joining medical school at Stritch School of Medicine in Maywood, Illinois.