INSERTING A MUTATION INTO THE PUTATIVE *CLOSTRIDIUM DIFFICILE* BILE SALT HYDROLASE-ENCODING GENE

An Undergraduate Research Scholars Thesis

by

NATALIE SATTERFIELD

Submitted to the Undergraduate Research Scholars program
Texas A&M University
in partial fulfillment of the requirements for the designation as an

UNDERGRADUATE RESEARCH SCHOLAR

Approved by
Research Advisor: Dr. Joseph Sorg

May 2016

Major: Microbiology

TABLE OF CONTENTS

ABSTRACT

Inserting a Mutation into the Putative *Clostridium difficile* Bile Salt Hydrolase-Encoding Gene

Natalie Satterfield
Department of Biology
Texas A&M University

Research Advisor: Dr. Joseph Sorg
Department of Biology

Clostridium difficile is a Gram-positive, anaerobic, spore-forming bacterium that causes infections in the gastrointestinal tract. The mechanisms by which *C. difficile* is able to germinate in such a toxic environment are not fully understood. However, our lab and others have shown that certain bile components have the capacity to induce *C. difficile* spore germination and affect growth of *C. difficile* cells. Interestingly, *C. difficile* encodes a bile salt hydrolase (cholylglycine hydrolase), but it is unknown how this affects the bacterium's ability to grow in the presence of bile acids. This research project is centered around the goal of inserting a mutation into the *C. difficile* bile salt hydrolase. This will be completed using the TargeTron Gene Knockout System, which inserts group II introns into a specific DNA location, and allelic-coupled exchange. Successful completion of this project would allow further investigation into the relationship between *C. difficile*'s bile salt hydrolase activity and its ability to grow in the presence of bile acids. Ultimately, fully understanding the bacterium's method of growth could help with the development of more effective treatments for *C. difficile* infections in the future.

CHAPTER I

INTRODUCTION

Clostridium difficile is a spore-forming bacterium that has proven to be a large problem in the healthcare environment [1]. *C. difficile* infects patients who have received antibiotic treatment and results in symptoms such as diarrhea, fever, and abdominal pain [1]. Unfortunately, the treatment for a *C. difficile* infection is more antibiotics (e.g. vancomycin or metronidazole) [1]. Because these antibiotics further disrupt the colonic microflora, patients frequently relapse with *C. difficile* infections [1].

When antibiotics are administered to patients, the colonization resistance afforded by the colonic microflora is compromised [1]. The loss of colonization resistance provides to *C. difficile* the opportunity to invade and colonize the colonic environment [1]. Upon colonization, *C. difficile* produces two toxins (toxins A and B) that bind to receptors on the cell surfaces of epithelial cells [2]. These toxins are then endocytosed and eventually enter the cytosol of these cells [2]. By enzymatically glycosylating GTPases, the toxins affect the assembly of cellular fibers and the polymerization of actin filaments in epithelial cells [2]. This causes tight junctions to deteriorate, and it prevents epithelial cells from attaching to the colonic basement membrane, or the lamina propria [2]. Eventually, this leads to apoptosis of the damaged cell [2]. Ultimately, this chain reaction leads to inflammation of the intestines, damage to the colonic tissues, and diarrhea which spreads *C. difficile* into the surrounding environment [2].

Due to the strict anaerobic nature of *C. difficile* vegetative cells, *C. difficile* survives in the healthcare environment in the form of a metabolically dominant spore [3]. Spores are dormant forms of bacteria that are formed in hostile conditions and are highly resistant to desiccation and extreme temperatures [3]. They provide a mechanism of self-protection for bacteria to use in conditions that are not ideal for growth [3]. Once environmental conditions improve, bacteria are able to respond to the presence of germination-inducing molecules (germinants) to germinate into vegetative cells [3]. Structurally, spores contain a core, a dehydrated environment containing the genomic DNA, RNA, and essential proteins [3]. Protecting this core are various layers composed of phospholipids, proteins, and peptidoglycan, which work to provide a defensive barrier between the spore core and the environment [3]. Upon certain environmental stimuli that indicate ideal growth conditions, the outer layers of the spore are degraded, and the spore core is re-hydrated and a vegetative bacterium grows out from the spore [3].

Because of the relative toxicity of the gastrointestinal environment, it is interesting that *C. difficile* has developed a method to survive in such harsh conditions. Our lab has found that the germination of *C. difficile* spores is either activated or inhibited by the presence of certain bile acids [4]. For example, glycine and cholate derivatives work to initiate germination of *C. difficile* spores while chenodeoxycholate inhibits cholate-mediated germination [5]. During synthesis in the liver, bile acids are conjugated with either glycine or taurine [6]. During gastrointestinal transit, bile acids help emulsify fats and cholesterol. Most of the bile acids are absorbed by the intestine and recycled back to the liver to be used in other rounds of digestion [6]. Here, about 10% of the total bile acids enter the colonic environment where they are acted

upon by members of the microbiota [6]. However, these conjugated bile acids are hydrolyzed back into their base bile acid molecules by bile salt hydrolases (e.g. taurocholate is deconjugated to cholate), which are expressed on the cell surfaces of many members of the colonic microbiota [4].

Selective pressure from the toxicity of bile forces only bacteria best suited to the toxic environment to survive [7]. Bile salt hydrolases provide multiple benefits to the bacteria that express them and are likely a substantial factor that allows some bacteria to survive in the colon [7]. For example, bile salt hydrolases often help bacteria obtain nutrition [7]. Amino acids are freed when bile salts are deconjugated, and these can be broken down by bacteria to be used as sources of energy, carbon, and nitrogen [7]. Therefore, bacteria encoding bile salt hydrolases may be more fit than others to survive in nutritionally depleted environments [7]. In addition, bile salt hydrolases allow bacteria to alter their cell membranes in order to protect themselves from host defense mechanisms [7]. For example, some bacteria utilize their bile salt hydrolase activity to add bile acids to their membranes in order to increase the strength of the membrane or alter the net charge and fluidity of the membrane [7]. Because these factors affect the susceptibility of certain bacteria to host defense mechanisms, membrane modifications may provide a means for bacteria to protect themselves from defense systems in the colon [7]. Lastly, it has been found that conjugated bile salts, especially glycoconjugated bile salts, are very toxic to bacteria due to their ability to acidify intracellular components of microorganisms [7]. Therefore, bile salt hydrolases may provide a mechanism of self-defense that protects bacteria from the toxicity of bile salts [7].

C. difficile encodes a homologue of a bile salt hydrolase (CD2311). Because bile acids impact *C. difficile* germination and growth, I hypothesize that the bile salt hydrolase activity affects *C. difficile* growth in the presence of certain bile acids [4]. In order to test for potential bile salt hydrolase activity, it is necessary to first inactivate the gene coding for the bile salt hydrolase in *C. difficile* [8]. While there are multiple methods of gene inactivation that are used on *C. difficile*, this project will use the TargeTron gene knockout system to inactivate CD2311. With this system, a group II intron is re-targeted to the CD2311 DNA sequence using a computer algorithm [8]. This region is subcloned into our targeting vector and then delivered to *C. difficile* through conjugation [9]. Once expressed in *C. difficile*, an RNA-protein complex is expressed from the cloned plasmid, which retro-homes the TargeTron RNA to the insertion site in the genomic DNA, and inserts the RNA sequence using reverse transcription to create double stranded DNA [9]. Ideally, this will disrupt the coding sequence of CD2311 [9]. The use of group II introns is widely used to inactivate genes in *C. difficile* today [9]. However, another method of gene inactivation in *C. difficile* is single crossover plasmid integration [9]. With this technique, a plasmid is integrated into the genome through a single crossover event during homologous recombination [9]. While this method has been successful at producing the desired mutants in the past, the insertion is often unstable . Therefore, the use of group II introns is often preferred [9].

If *C. difficile* expresses a bile salt hydrolase, successful insertion of a group II intron into the gene encoding the bile salt hydrolase will enable an investigation of the relationship between any bile salt hydrolase activity of *C. difficile* cells and the effects of this on its growth. Importantly, if CD2311 is associated with bile acid tolerance in the presence of certain bile

acids, it may serve as an effective target for future chemotherapeutic treatment of *C. difficile* infections.

CHAPTER II

METHODS

Bacterial strains and growth conditions

C. difficile UK1 was grown on brain heart infusion medium containing 5 g/L of yeast extract and 0.1% L-cysteine (BHIS) at 37ºC in an anaerobic chamber containing 85% N_2, 10% H_2, and 5% CO_2 [5]. *E. coli* DH5α and *E. coli* MB3436 were grown on Luria-Bertani (LB) medium at 37ºC aerobically [10]. *B. subtilis* BS49 was grown on LB medium at 30ºC aerobically. The antibiotics chloramphenicol (20 µg/mL for *E. coli* and 2.5 µg/mL for *B. subtilis*), thiamphenicol (10 µg/mL), tetracycline (5 µg/mL), lincomycin (20 µg/mL), and kanamycin (50 µg/mL) were added where necessary [10].

Generation of group II intron and creation of plasmids

A computer algorithm (provided by Targetronics, LLC.) determined potential insertion sites for a group II intron in *C. difficile* CD2311, and the insertion fragment was generated as a gBlock from Integrated DNA Technologies (IDT) [11]. This 350 base pair fragment was cloned into pCR-Blunt II-TOPO and then sub-cloned into the BsrGI and HindIII insertion sites of pJS107, a TargeTron vector used to insert mutations into *C. difficle* UK1, producing pNS02 [11]. pNS02 was then introduced into *B. subtilis* BS49 using standard techniques [10].

Conjugation and screening for mutants

B. subtilis BS49 was used as the donor for conjugation to introduce pNS02 into *C. difficile* UK1 using previously described techniques [10]. Colonies that showed thiamphenicol resistance,

indicating the presence of the pNS02 plasmid, and tetracycline sensitivity, indicating the absence of the Tn916 transposon, were then isolated and re-streaked onto BHIS medium containing lincomycin [10]. Lincomycin-resistant colonies indicated potential strains with a successful TargeTron insertion into CD2311 [10]. DNA was then isolated from the lincomycin-resistant colonies by first suspending a single colony in 95 µL of 1X PCR buffer. 4 µL of lysozyme (50 mg/mL) were then added to the suspension and incubated at room temperature for 15 minutes. Next, 1 µL of proteinase K (20 mg/mL) was added, and this incubated at 58ºC for 60 minutes. Proteinase K was then inactivated at 90ºC for 15 minutes, and the mixture was centrifuged at 5000 rpm for 5 minutes. The supernatant was removed and used to screen for the insertion into CD2311 using polymerase chain reaction and primers generated specifically for CD2311 [10].

CHAPTER III

RESULTS

Generating a site-directed mutation in *C. difficile* CD2311

The germination of *C. difficile* spores within the gastrointestinal tract has been found to be both activated and inhibited by the presence of different bile acids, and thus the resulting vegetative cells must survive their toxic actions [4,12]. Because CD2311 codes for a protein with potential bile salt hydrolase activity, it may play a role in surviving in the host colonic environment. To generate a site-directed mutation in *C. difficile* CD2311, two different TargeTron insertion sites were chosen (nt 532 and 583 of 1,170, respectively). If the retargeting algorithm correctly predicted the insertion sites, the ~1,800 nt group II intron would be inserted and reverse transcribed into the genome, thus disrupting the CD2311 gene sequence (Figure 1) [9].

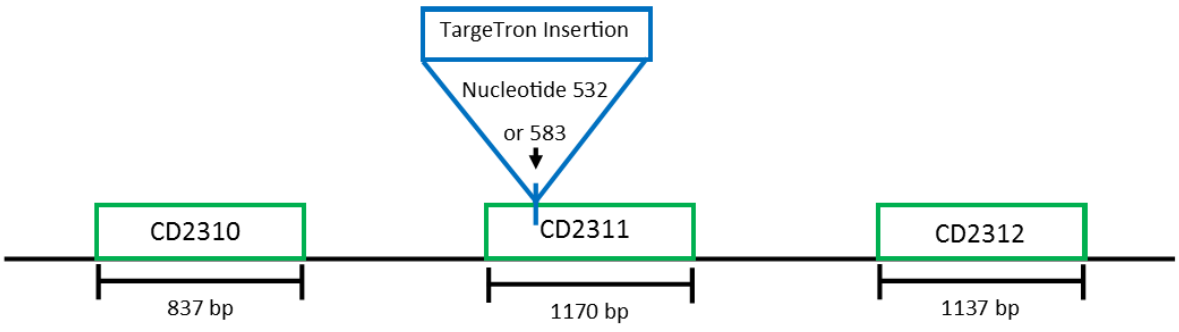

Figure 1. Diagram of TargeTron insertion site. Two different TargeTron insertion sites were used to retarget the TargeTron to nucleotide 532 or 583 of *C. difficile* CD2311.

The TargeTron re-targeting sequences were cloned into the *C. difficile* TargeTron shuttle vector, pJS107. The successful introduction of the 532 and 583 nt TargeTron re-targeting fragments

into pJS107 was verified using restriction analysis and gel electrophoresis (Figure 2). As a control, pJS107 was included in the digest (Lane 2, Figure 2 and Lane 2, Figure 3). In lanes 3 to 8 of Figures 2 and 3, the digested potential positive clones show a large fragment (12,500 bp) corresponding to the vector backbone. Lanes 3 to 8 in Figures 2 and 3 also show a fragment at ~350 bp, in addition to the vector backbone, which is indicative of the presence of the desired insert. Therefore, the gels confirm the successful ligations of pJS107 with the inserts.

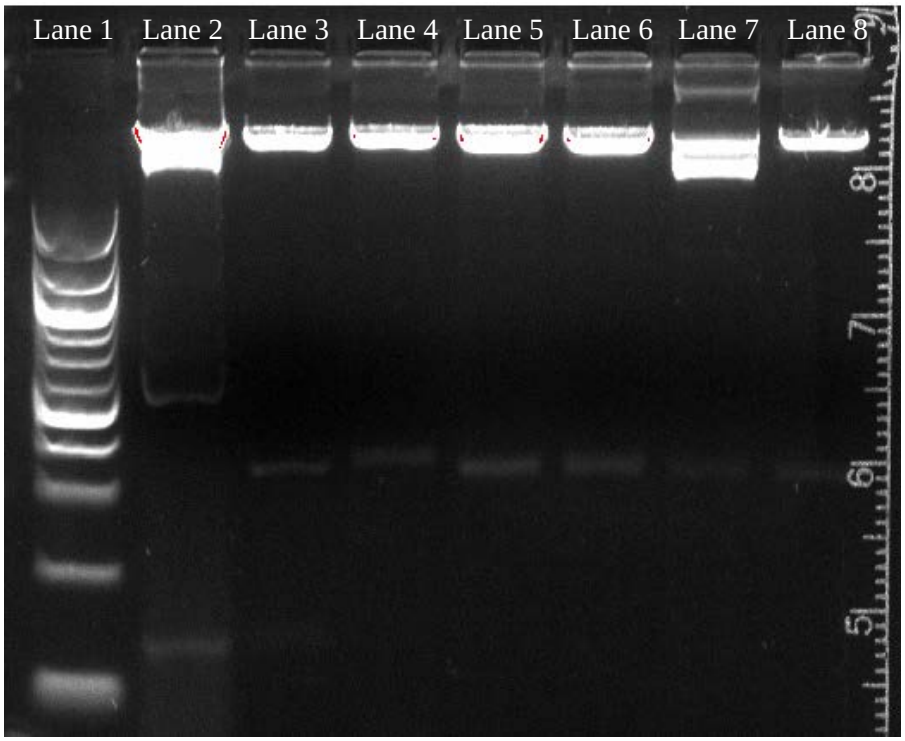

Figure 2. Verification of successful ligation of pJS107 (vector) and nucleotide 532 re-targeting fragment. Lane 1: 100 bp ladder, lane 2: digested pJS107 with *Hind*III and *Bsr*GI, and lanes 3-8: potential re-targeted isolates digested with *Hind*III and *Bsr*GI.

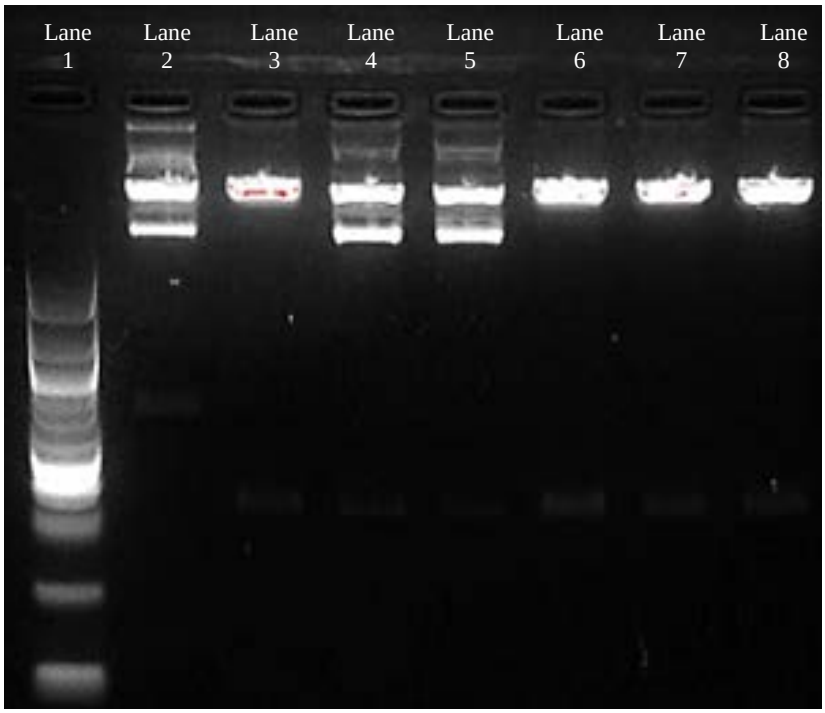

Figure 3. Verification of successful ligation of pJS107 (vector) and nucleotide 583 re-targeting fragment. Lane 1: 100 bp ladder, lane 2: pJS107 digested with *Hind*III and *Bsr*GI, and lanes 3-8: potential re-targeted isolates digested with *Hind*III and *Bsr*GI.

The re-targeted vectors were introduced into *B. subtilis* BS49 and then into *C. difficile* UK1 using conjugation. After conjugation, plasmid-positive *C. difficile* UK1 isolates (containing pNS02 and pNS04) were streaked onto medium supplemented with lincomycin to select for those cells that had the intron insert. DNA from the lincomycin-resistant colonies was isolated and screened for the presence of a TargeTron insertion into CD2311 using PCR (Figure 4 and Figure 5). Wildtype *C. difficile* UK1 CD2311 is approximately 1,500 bp in length (Figure 4 and Figure 5) and a ~3,300 bp fragment is expected if the intron inserts into the CD2311 gene. Amplification of the DNA extracted from each isolate, except for those in lanes 6 and 7 of Figure 4 and lane 10 of Figure 5 which show a failed PCR reaction, yield an approximately 1,500 bp fragment similar to that of the wildtype control. These results suggest that all tested

colonies were negative for the CD2311 mutation and that the intron likely inserted elsewhere in the genome (a possibility given the nature of the retargeting algorithm).

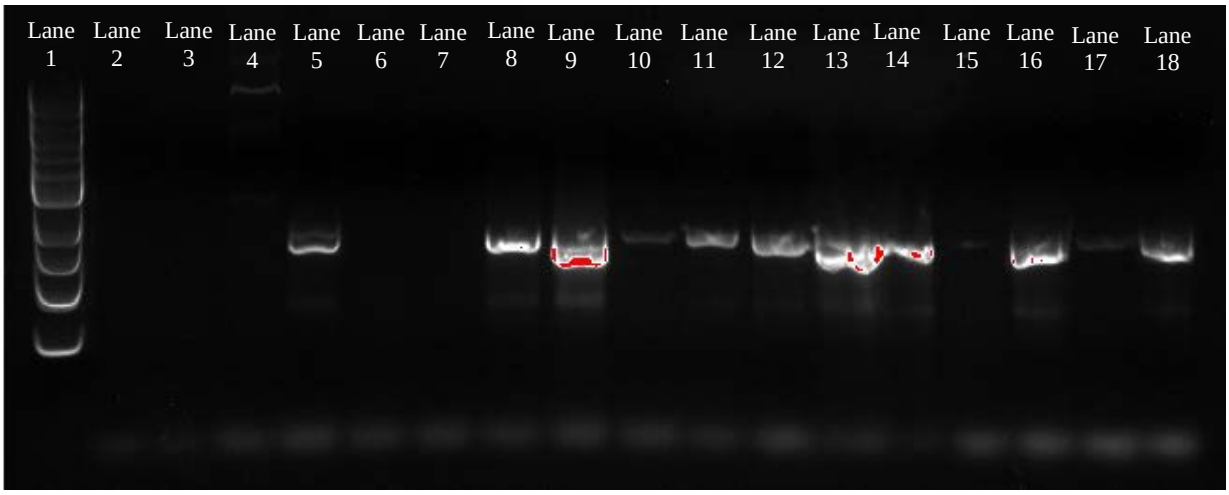

Figure 4. Screening for mutations in CD2311 at nucleotide 532. Lane 1: 1 kb ladder, lane 2: distilled water, lane 3: *E. coli* DH5α pNS02, lane 4: *E. coli* MB3436 pNS02, lane 5: wildtype *C. difficile* UK1, and lanes 6-18: lincomycin-resistant colonies that were screened for the CD2311 mutant.

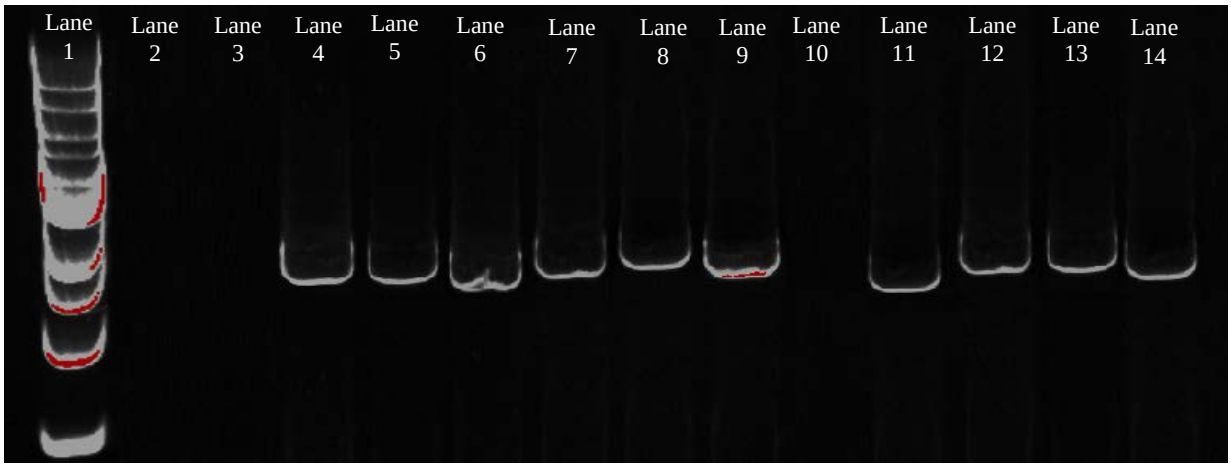

Figure 5. Screening for mutations in CD2311 at nucleotide 583. Lane 1: 1 kb ladder, lane 2: distilled water, lane 3: *E. coli* DH5α pNS04, lane 4: wildtype *C. difficile* UK1, and lanes 5-14: lincomycin-resistant colonies that were screened for the CD2311 mutant.

Inactivating CD2311 using Allelic-Coupled Exchange

Given the difficulties in genetically manipulating *C. difficile*, we also chose to inactivate CD2311 using allelic-coupled exchange (ACE) technology [13]. This method involves the use of a vector that is segregationally unstable in *C. difficile*. By amplifying 1 kb upstream and downstream of the gene of interest and piecing the products together, we create a deletion in the CD2311 coding region (Figure 6) [13].

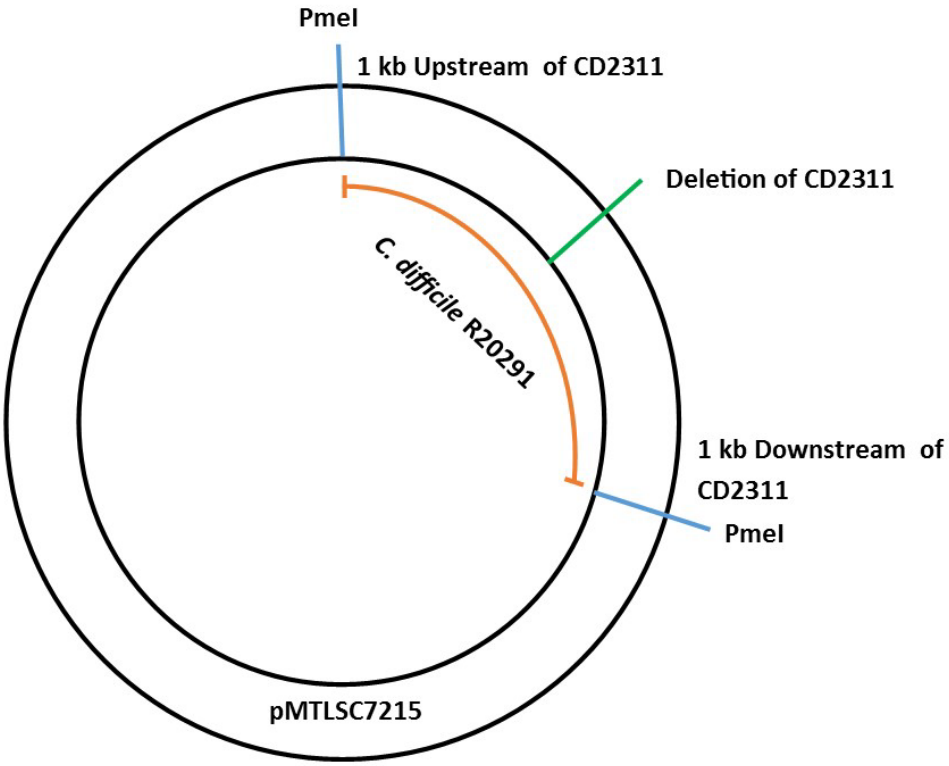

Figure 6. Diagram of CD2311 deletion using allelic exchange. 1 kb upstream of CD2311 and 1 kb downstream of CD2311 cloned into the allelic exchange vector pMTLSC7215 will yield a complete deletion of CD2311 coding sequence upon homologous recombination in the *C. difficile* genome.

The 1 kb upstream and downstream fragments of CD2311 were inserted into the *Pme*I sites of the pMTLSC7215 ACE vector using Gibson assembly, yielding pNS05 [13]. Positive clones

were verified by restriction digest and by PCR (Figure 7). As a control, pMTLSC7215 was included in the digest (Lane 2, Figure 7). In lanes 3 to 6 of Figure 7, the digested potential positive plasmids show a large fragment corresponding to the pMTLSC7215 vector. Lanes 3 to 6 of Figure 7 also show fragments at ~2000-2500 bp, in addition to the vector backbone. This confirms the presence of the desired plasmid. In lanes 9 to 16 of Figure 7, the PCR reaction amplifying the 2 kb *C. difficile* fragment of the allelic-coupled exchange plasmid show a band at ~2000-2500 bp. This corresponds to the ~2000-2500 bp fragment shown in lanes 3 to 6 of the same figure and further confirms the presence of the desired plasmid.

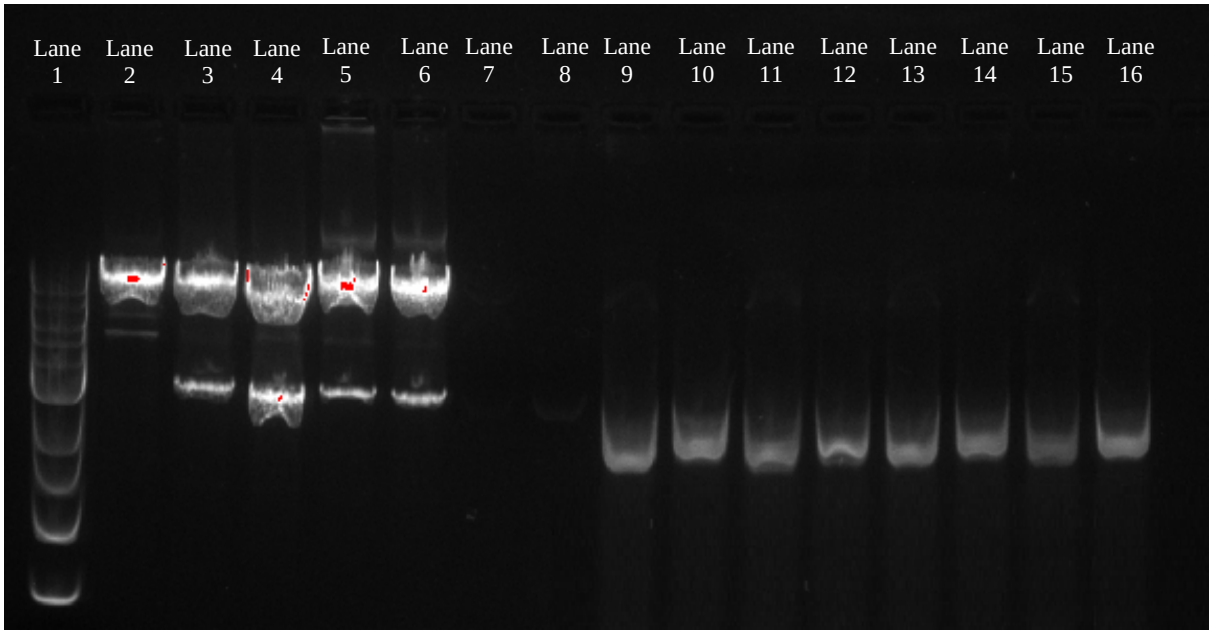

Figure 7. Verification of allelic-coupled exchange plasmid. Lane 1: 1 kb ladder, lane 2: *E. coli* DH5α pMTLSC7215 digested with *Pme*I, lanes 3-6: *E. coli* DH5α pNS05 digested with *Pme*I, lanes 7-8: *E. coli* DH5α pMTLSC7215 PCR, lanes 9-16: *E. coli* DH5α pNS05 PCR.

The resulting plasmid was introduced into *E. coli* HB101 and then into *C. difficile* via conjugation. Plasmid-positive *C. difficile* isolates were identified and passaged to identify potential single-integrants of the pNS05 plasmid. Due to the plasmid being segregationally

unstable in *C. difficile*, single-integrants appear as a ‘large colony’ phenotype among a small colony background. Excision of the plasmid from the *C. difficile* genome will be induced by inoculating onto medium supplemented with 5-fluorocytosine (FC). DNA will be extracted from the FC-resistant strains, and the CD2311 coding regions amplified by PCR. Because this method results in a deletion in the CD2311 coding region, the resulting potential mutants will be amplified using outside primers to detect a smaller fragment compared to a wild-type control.

Following multiple attempts of conjugation, results showed either no colonies or colonies that appeared explicitly as ‘small colony’ phenotypes. Therefore, this suggests that no single-integrants were present and that the allelic-coupled exchange method was also unsuccessful at inactivating CD2311.

CHAPTER IV

CONCLUSION

Hospital-acquired *C. difficile* colitis has become a growing problem to health care providers. In the environment, *C. difficile* exists in its dormant yet highly protective spore form, making the bacteria challenging to completely remove from patient facilities and effective at colonizing susceptible hosts [14,15]. However, in order to cause symptoms and disease, *C. difficile* spores must first germinate within the colon [11]. Previous research has found that, when tested in vitro, bile acids act as activators and inhibitors of *C. difficile* germination [4,12]. For example, glycine and cholate derivatives were found to work as activators of germination [4]. Conversely, chenodeoxycholate was found to act as a competitive inhibitor of germination mediated by taurocholic acid [12]. Ultimately, the composition of bile acids within the gastrointestinal environment plays a critical role in *C. difficile* spore degradation and vegetative cell colonization [4,11,12].

C. difficile encodes a bile salt hydrolase, yet it is currently unknown whether this gene is expressed [4]. Given that the bile acid composition of its environment works to optimize growth in *C. difficile* cells, an expressed bile salt hydrolase could play a vital role in maintaining infection [4]. If a bile salt hydrolase is expressed, its catalytic activity could provide a mechanism that frees nutrients, such as glycine, to aid in vegetative cell growth [16]. Ultimately, if expressed, a bile salt hydrolase could influence the makeup of bile acids within the human colonic environment and impact a host's susceptibility to infection.

Studying the potential *C. difficile* bile salt hydrolase is important as it could unveil a possible pathway to target that effectively combats the disease. As the bile salt hydrolase would most likely affect *C. difficile* growth, targeting the supposed bile salt hydrolase could provide a mechanism to slow or stop vegetative cell growth in patients completely. Ultimately, for future directions of this research, efforts should be made to generate a CD2311 mutant, and the relationship between any bile salt hydrolase activity and growth should be investigated.

REFERENCES

1. McFarland LV (2008) Update on the changing epidemiology of Clostridium difficile-associated disease. Nat Clin Pract Gastroenterol Hepatol 5: 40-48.

2. Voth DE, Ballard JD (2005) Clostridium difficile toxins: mechanism of action and role in disease. Clin Microbiol Rev 18: 247-263.

3. Paredes-Sabja D, Shen A, Sorg JA (2014) Clostridium difficile spore biology: sporulation, germination, and spore structural proteins. Trends Microbiol 22: 406-416.

4. Sorg JA, Sonenshein AL (2008) Bile salts and glycine as cogerminants for Clostridium difficile spores. J Bacteriol 190: 2505-2512.

5. Sorg JA, Sonenshein AL (2010) Inhibiting the initiation of Clostridium difficile spore germination using analogs of chenodeoxycholic acid, a bile acid. J Bacteriol 192: 4983-4990.

6. Ridlon JM, Kang DJ, Hylemon PB (2006) Bile salt biotransformations by human intestinal bacteria. J Lipid Res 47: 241-259.

7. Begley M, Hill C, Gahan CG (2006) Bile salt hydrolase activity in probiotics. Appl Environ Microbiol 72: 1729-1738.

8. Wang Y, Li X, Milne CB, Janssen H, Lin W, et al. (2013) Development of a gene knockout system using mobile group II introns (Targetron) and genetic disruption of acid production pathways in Clostridium beijerinckii. Appl Environ Microbiol 79: 5853-5863.

9. Heap JT, Pennington OJ, Cartman ST, Carter GP, Minton NP (2007) The ClosTron: a universal gene knock-out system for the genus Clostridium. J Microbiol Methods 70: 452-464.

10. Francis MB, Allen CA, Sorg JA (2015) Spore Cortex Hydrolysis Precedes Dipicolinic Acid Release during Clostridium difficile Spore Germination. J Bacteriol 197: 2276-2283.

11. Francis MB, Allen CA, Shrestha R, Sorg JA (2013) Bile acid recognition by the Clostridium difficile germinant receptor, CspC, is important for establishing infection. PLoS Pathog 9: e1003356.

12. Sorg JA, Sonenshein AL (2009) Chenodeoxycholate is an inhibitor of Clostridium difficile spore germination. J Bacteriol 191: 1115-1117.

13. Heap JT, Ehsaan M, Cooksley CM, Ng YK, Cartman ST, et al. (2012) Integration of DNA into bacterial chromosomes from plasmids without a counter-selection marker. Nucleic Acids Res 40: e59.

14. Milligan M, George L, Rolfe RD, Finegold SM (1979) Contamination of a Hospital Environment by Clostridium difficile. Curr Microbiol 3: 173-175

15. Kim KH, Waters D, Fekety R, Batts DH, Brown D, et al. (1981) Isolation of Clostridium difficile from the Environment and Contacts of Patients with Antibiotic-Associated Colitis. J Infect Dis 143: 42-40

16. Bouillaut L, Dubois T, Dupuy B, Sonenshein AL (2015) Integration of metabolism and virulence in Clostridium difficile. Res Microbiol 166: 375-383