THE EFFECT OF CD2118 ON *Clostridium difficile* BILE ACID RESISTANCE

An Undergraduate Research Scholars Thesis

by

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ABSTRACT

The Effect of CD2118 on *Clostridium difficile* Bile Acid Resistance. (May 2013)

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Clostridium difficile is a Gram-positive, anaerobic, spore-forming bacteria that is the leading cause of antibiotic-associated diarrhea. *C. difficile* infections (CDI) are thought to be caused by ingestion of spores by a host with an altered intestinal flora, often caused by antibiotic treatments for unrelated conditions. *C. difficile* spores germinate *in vivo* when exposed to bile acids, which are commonly toxic to bacterial pathogens, and glycine. The proliferation of the resulting vegetative cells suggests the presence of mechanism(s) to resist bile acid-mediated toxicity. Previous studies identified genes with altered mRNA abundance following bile acid exposure. The expression of *C. difficile* CD2118 increased 20-fold upon exposure to cholic acid or deoxycholic acid. *C. difficile* CD2118 encodes a putative ABC (ATP-binding cassette) transporter, a class of transporters known to participate in bile acid resistance mechanisms of other Gram-positive bacteria. Herein, we characterize the role of CD2118 in protecting *C. difficile* against bile acid-mediated toxicity by constructing a site-directed mutation in the coding region of *C. difficile* CD2118 using the TargeTron system.

CHAPTER I

INTRODUCTION

Clostridium difficile is a Gram-positive, spore-forming, bacteria. *C. difficile* is a strict anaerobe when in the vegetative state, but spores are able to tolerate oxygen and dry conditions (1). Exposure to *C. difficile* in hospital settings can occur when the spores are spread from infected patients to susceptible individuals by spore carriage on the gloves and clothing of healthcare providers, contaminated surfaces, and aerosols. Community-acquired CDI (CA-CDI) has also been reported, usually affecting the very young and those with chronic gastrointestinal conditions. Broad-spectrum antibiotic use is normally not associated with these cases, but a few have been reported following fluoroquinolone use (2). CA-CDI is much less common than hospital-acquired CDI, however, it is becoming more prevalent. Hospital-acquired CDI is usually precipitated by use of broad-spectrum antibiotics, which disrupt the balance of the natural intestinal microbiota of the host (1). *C. difficile* spores ingested by the host germinate and then proliferate, possibly due to increased nutrient availability and decreased levels of antimicrobial compounds normally produced by the natural flora. Vegetative *C. difficile* also demonstrates resistance against host-synthesized antimicrobial compounds such as bile acids and CAMPs (cationic antimicrobial peptides) (3). The symptoms normally associated with CDI are caused by two toxins, TcdA and TcdB, which disrupt the actin cytoskeleton of colonic epithelial cells (2). These toxins enter colonic epithelial cells through receptor-mediated endocytosis and the catalytic domain then enters into the host cell cytosol through a pore formed in the endosome and catalyzes the UDP-dependent glucosylation of Rho family of GTPases (4). The subsequent inhibition of the host Rho GTPases results in a catastrophic signaling cascade that leads to

morphological changes in the cell and apoptosis (5). TcdA and TcdB are produced only upon germination of the spore into a vegetative cell. Germination of *C. difficile* spores in the host is location-specific. Glycine and bile acids serve as signaling molecules and trigger the transition from spore to vegetative state (6).

Bile acids are biosynthesized in the liver from cholesterol and aid in absorption of fat and cholesterol during digestion (7). Because *C. difficile* spores use bile acids as signals to germinate, vegetative cells must be able to survive and multiply in their presence, an unusual ability given the toxicity of bile acids to many other bacterial pathogens (6). Bile acids cause lysis by increasing permeability of the cell membrane. If bile acids enter the cell without causing immediate lysis, cytoplasmic bile acids can lead to secondary structure formation in RNA, DNA damage, misfolding or denaturation of proteins, or damage from generation of free oxygen radicals. Cytoplasmic bile acids will also acidify the cytosol upon dissociation and cause further cellular stress and damage (8). Bacteria that colonize the small intestine have a variety of mechanisms to circumvent the antimicrobial actions of bile acids. One example is *Listeria monocytogenes*, the causative agent of listeriosis (a form of food poisoning). Like *C. difficile*, it is Gram-positive, but it is able to survive in a much wider range of environmental conditions. In order to resist the effects of bile acids, *L. monocytogenes* utilizes a bile salt hydrolase, a membrane-located bile exclusion system, and the regulatory factor sigma B, which increases expression of certain genes in response to environmental stressors like bile acids. The specifics of the *C. difficile* bile acid resistance mechanisms are unknown.

In preliminary studies, log-phase *C. difficile* was exposed to sub-MIC (minimal inhibitory concentration) levels of cholic acid and deoxycholic acid (bile acids). Total RNA was isolated and analyzed by RNAseq. This led to the identification of genes with altered mRNA abundance upon bile acid exposure. *C. difficile* CD2118 showed twenty-fold increase in mRNA abundance upon bile acid exposure, suggesting it may contribute to bile acid resistance (Allen and Sorg, unpublished). The gene CD2118 codes for an ABC (ATP-binding cassette) transporter, a transporter class that participates in several cellular processes, including CAMP and clindamycin resistance in *C. difficile* (3, 9). The role of CD2118 in bile acid resistance will be evaluated by rendering it nonfunctional through mutation and comparing the bile acid MIC (minimal inhibitory concentration) of mutants to that of wild-type *C. difficile*. The mutation of CD2118 will be introduced using the TargeTron system, which is currently the most reliable method for *Clostridium* mutagenesis. This system utilizes retargeted Group II introns, which insert directly into the gene of interest, and a retro-transposition-activated marker (RAM), which activates a lincomycin resistance gene upon successful insertion at the target site. Target sites are determined using a computer algorithm, which predicts the probability of successful insertion into specific areas of the gene. This method requires assembly of DNA fragments through PCR, usage of various recombinant vectors in *Escherichia coli*, and conjugation between *Bacillus subtilis* and *C. difficile*. Antibiotic screening is used throughout to eliminate contaminants and to verify the success of each step in the process (10). If complementation to CD2118 via plasmid genes restores mutants to wild type MIC for bile acids, it can be assumed that CD2118 plays a role in the *C. difficile* bile acid resistance mechanism.

CHAPTER II

METHODS

Strains and growth conditions

Table 1 lists the plasmids and bacterial strains used in this project. All strains were grown at 37°C. *Escherichia coli* DH5α and MB3436 were grown in Luria Broth (LB) medium supplemented with 50 μg/mL kanamycin or 20 μg/mL chloramphenicol as needed (11). *Bacillus subtilis* Bs49 was grown in Brain Heart Infusion supplemented with 5 g/L yeast extract and 0.1% L-cysteine (BHIS) or LB medium, supplemented with 2.5 μg/mL chloramphenicol and/or 5 μg/mL tetracycline (12). *Clostridium difficile* UK1 was grown in an anaerobic environment (10% hydrogen, 5% carbon dioxide, 85% nitrogen) in BHIS medium and antibiotics (10 μg/mL thiamphenicol, 50 μg/mL kanamycin, 5 μg/mL tetracycline, and 20 μg/mL lincomycin) were added where indicated (13).

Plasmid and Strain Generation

Table 2 lists the oligonucleotides used in this study. The TargeTron system was used to introduce a mutation into *C. difficile* CD2118 through insertion of a mobilizable group II intron into the CD2118 coding sequence (10, 14). The CD2118 DNA sequence was entered into the free TargeTron web-based algorithm that is maintained by Monash University. This algorithm predicts possible insertion sites of the group II intron into the gene. The primers CD2118 (6a) IBS, CD2118 (6a) EBS2, CD2118 (6a) EBS1d, and EBS Universal were used to assemble the retargeting intron fragment using PCR. The fragment was then ligated into the plasmid pCR2.1-TOPO (Invitrogen) to yield pSS1. The plasmid pSS1 was transformed into *E. coli* DH5α. The sequence of the insert was confirmed (Eurofins, Operon) and the fragment was liberated from the

vector by digesting purified DNA with HinDIII and BsrGI. The fragment was then ligated into the vector pJS107, also digested with HinDIII and BsrGI, to produce pSS2. Another method was used to determine possible insertion sites, the Targetronics LLC. web-based algorithm, which predicts likely insertion sites of the group II intron into the gene based on insertion efficiency and then filters the chosen sites based on the probability of insertion at undesired locations in the genome. The sequence of the 350-bp fragment that was used to retarget the group II intron to the CD2118 gene was compiled by the algorithm and synthesized by Integrated DNA Technologies (IDT). The synthesized DNA fragment was ligated into TOPO Zero Blunt (Invitrogen) and transformed into *E. coli* DH5α, yielding pSS3. The intron fragment in pSS3 was introduced into pJS107, as described above for pSS1, yielding pSS4. The plasmids pSS2 and pSS4 were transformed into *E. coli* MB3436, plasmid DNA extracted and used to transform *B. subtilis* Bs49 using standard techniques (12). *B. subtilis* Bs49 pSS2 and pSS4 were conjugated into *C. difficile* strain UK1. Transconjugants were identified by growth on BHIS medium containing thiamphenicol (to select for plasmid-containing *C. difficile*) and kanamycin (to counter-select *B. subtilis*). Colonies were then screened for loss of the conjugative transposon Tn*916* by tetracycline sensitivity (12). Tetracycline-sensitive *C. difficile* UK1 pSS2 or *C. difficile* UK1 pSS4 was plated on BHIS medium supplemented with lincomycin to select for the retrotransposition of the group II intron. DNA was then extracted from lincomycin-resistant colonies and tested for an intron insertion into CD2118 by amplifying the full-length CD2118 coding sequence using primers 5' *CD2118* and 3' *CD2118* (15). The 5' and 3' junctions of the intron insertion site were also amplified using primers CD2118 (6a) IBS and CD2118 (6a) EBS1d.

TABLE 1. Bacterial strains and plasmids

Plasmid or strain	Key features	Reference
Strains		
E. coli		
DH5α	Φ80d*lacZ*ΔM15	(Hanahan, 1983)
MB3436	*recA+*	(Wang & Benedik, 2012)
B. subtilis		
Bs49	CU2189*::*Tn*916*	(Haraldsen, 2003)
C. difficile		
UK1	Clinical isolate	(Sorg, 2010)
Plasmids		
pCR2.1-TOPO	Amp^R and Kan^R	Invitrogen
TOPO Zero Blunt	Amp^R and Kan^R	Invitrogen
pJS107	TargeTron construct derived from pBL100, contains *Tn*916 oriT	(Sorg, unpublished)
pSS1	CD2118 intron fragment in pCR2.1-TOPO	This Study
pSS2	CD2118 intron fragment in pJS107	This Study
pSS3	IDT-synthesized CD2118 intron fragment in TOPO Zero Blunt	This Study
pSS4	IDT-synthesized CD2118 intron fragment in pJS107	This Study

TABLE 2. Oligonucleotide sequences

Primer	Sequence (5'→ 3')
5' CD2118	AATATTACAGAAGGGAAGAAATGTATG
3' CD2118	CTACTCATTAGCTAGCTCCTCCTTTGA
CD2118 (6a) IBS	AAAAAAGCTTATAATTATCCTTAGATTTCTTTAAAGTGCGCCCAGAT AGGGTG
CD2118 (6a) EBS1d	CAGATTGTACAAATGTGGTGATAACAGATAAGTCTTTAAATATAACT TACCTTTCTTTGT
CD2118 (6a) EBS2	TGAACGCAAGTTTCTAATTTCGATTAAATCTCGATAGAGGAAAGTGT CT
EBS Universal	CGAAATTAGAAACTTGCGTTCAGTAAAC
CD2118 (671a) gBlock	TTCCCCTCTAGAAAAAAGCTTATAATTATCCTTAAGCATCTTAGCAG TGCGCCCAGATAGGGTGTTAAGTCAAGTAGTTTAAGGTACTACTCTG TAAGATAACACAGAAAACAGCCAACCTAACCGAAAAGCGAAAGCTG ATACGGGAACAGAGCACGGTTGGAAAGCGATGAGTTACCTAAAGAC AATCGGGTACGACTGAGTCGCAATGTTAATCAGATATAAGGTATAA GTTGTGTTTACTGAACGCAAGTTTCTAATTTCGATTATGCTTCGATAG AGGAAAGTGTCTGAAACCTCTAGTACAAAGAAAGGTAAGTTAGGTG CTAAGACTTATCTGTTATCACCACATTTGTACAATCTG

CHAPTER III

RESULTS

The TargeTron system was used to introduce a mutation into *C. difficile* CD2118. With this method, genes are rendered nonfunctional through site-specific mutation by mobile group II intron insertion (14). The sites of insertion into *C. difficile* CD2118 and intron fragment sequences were determined using web-based algorithms.

Initially, the web-based algorithm maintained by Monash University was used to generate possible TargeTron insertion sites into CD2118. After the targeting plasmid pSS2 was constructed, the DNA was mobilized from *B. subtilis* to *C. difficile* UK1. Insertion of the intron into the *C. difficile* chromosome was selected for by incubating *C. difficile* UK1 pSS2 on agar medium supplemented with lincomycin; the plasmid-encoded, retro-transposition-activated marker (RAM) provides lincomycin resistance (17). We screened 14 colonies for the insertion of the RAM into *C. difficile* CD2118 by amplifying the full-length CD2118 coding sequence. However, upon amplification of CD2118, we did not observe an amplified fragment at the size expected for an intron insertion into CD2118 (3.8 kb), suggesting that the intron had inserted elsewhere in the *C. difficile* UK1 genome (Fig. 1).

Because we observed aberrant targeting of the intron into the *C. difficile* genome, we generated a different targeting plasmid based upon a different TargeTron algorithm. The Targetronics web-based algorithm operates in much the same manner as the algorithm maintained by Monash University, except that possible insertion sites are also evaluated by the probability of insertion at

undesired sites in the genome. A new targeting plasmid (pSS4) was constructed and introduced into *C. difficile* UK1 and the resulting strain screened for lincomycin resistance. Lincomycin-resistant clones have been observed and purified colonies are currently being isolated. The final success of this method has yet to be determined, as research is still ongoing.

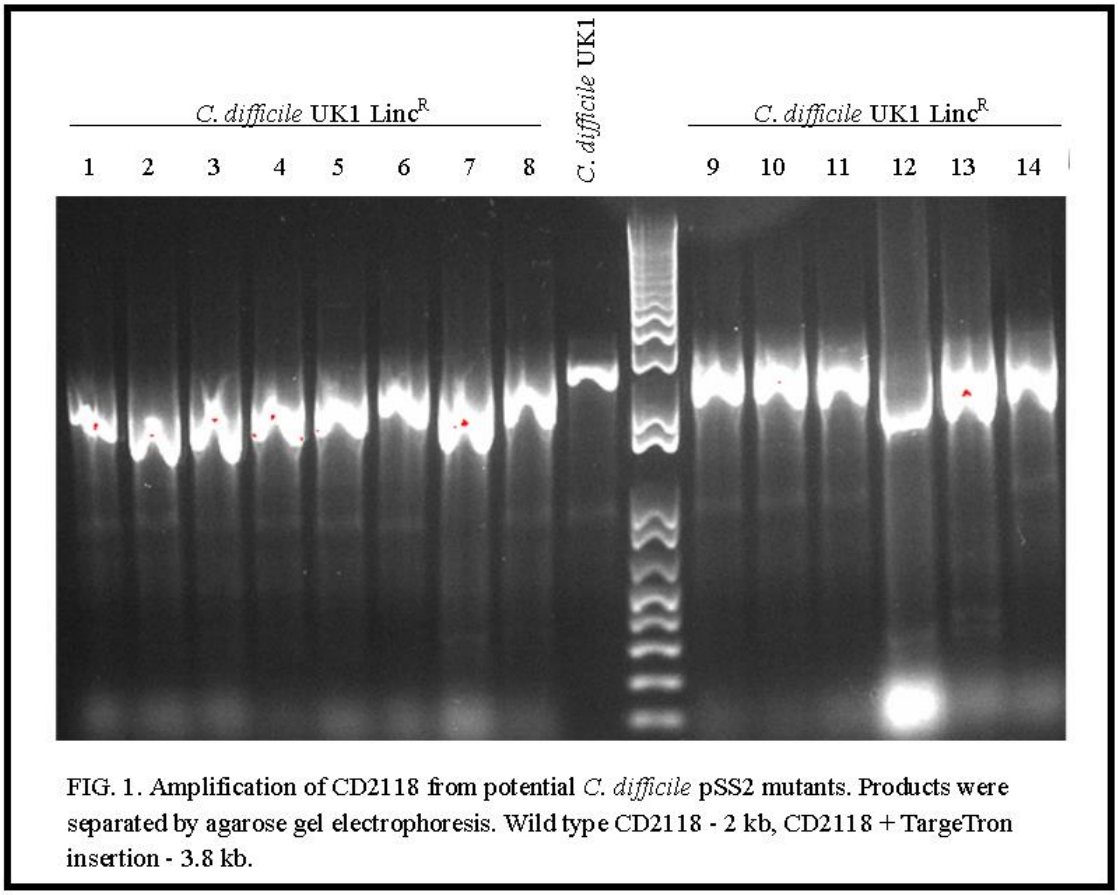

FIG. 1. Amplification of CD2118 from potential *C. difficile* pSS2 mutants. Products were separated by agarose gel electrophoresis. Wild type CD2118 - 2 kb, CD2118 + TargeTron insertion - 3.8 kb.

CHAPTER IV

CONCLUSIONS

Because *C. difficile* germinates in response to bile acids and subsequently survives in the presence of these normally toxic molecules, a resistance mechanism may exist (6, 8). In preliminary experiments, the effects of cholic acid and deoxycholic acid on gene expression in vegetative cells were analyzed using RNAseq. Altered transcript levels were observed for several genes, including CD2118 which exhibited a twenty-fold increase in expression compared to the negative control condition (Allen and Sorg, unpublished). The results of a BLAST search led to its identification as a putative ABC-transporter. In other bacterial species, ABC-transporters have been implicated in the resistance to antibiotics and environmental toxins (18). *Listeria monocytogenes*, another Gram-positive intestinal pathogen, utilizes ABC transporters to resist the toxic effects of bile (8). This prompted the choice of CD2118 as the gene of interest in this project.

Two web-based algorithms, one hosted by Monash University and the other by Targetronics, LLC., generated the insertion sites into CD2118. This information was used to determine the sequence of the targeted intron fragment, which was introduced into *C. difficile* through conjugation. A mutation to CD2118 could not be obtained using the algorithm hosted by Monash University, although lincomycin-resistant clones were observed. When these clones were analyzed for the presence of the inserted intron DNA, and there were no observable differences when compared to CD2118 amplified from wild type *C. difficile* (Fig. 1). A weakness of this algorithm is its inability to evaluate the entire genome for possible aberrant insertions. It is entirely possible that the fragment successfully inserted elsewhere in the genome, activating the

RAM and conferring lincomycin resistance without affecting CD2118. Because of the lack of success using this method, we chose a different algorithm to generate a list of potential insertion sites. The second algorithm, hosted by Targetronics, LLC., evaluates both the gene of interest and the entire genome, maximizing insertion efficiency while minimizing aberrant insertion. Lincomycin-resistant *C. difficile* growth was observed using this method, but the presence of the TargeTron insertion into CD2118 has not yet been verified. A final conclusion about the success of this method has not yet been reached, and is dependent on the outcome of forthcoming research.

In future experiments, the Targetronics, LLC.-based method will be fully assessed for its effectiveness in introducing a mutation into CD2118. If CD2118 mutants are obtained, they will be tested for altered bile acid resistance by comparing the bile acid MIC to that of wild type. If the mutants have diminished resistance, the function of CD2118 will be restored via complementation. A complete restoration of the mutant bile acid MIC to wild-type levels will confirm that CD2118 plays a major role in *C. difficile* bile acid resistance.

One potential explanation for our observations is that CD2118 is essential for survival of *C. difficile* and that an intron insertion into the CD2118 coding sequence would have detrimental effects for the bacterium. Alternatively, the lincomycin-resistant growth observed could be the result of chance insertions elsewhere in the genome, as we observed in our first attempt. These hypotheses could be tested by using testing several different potential insertion sites in CD2118, or a newly developed, allelic-exchange strategy for *C. difficile* (19).

If novel bile acid resistance mechanisms are elucidated for *C. difficile*, there could be major implications for the development of new treatments for CDI. A narrow spectrum antibiotic targeting these mechanisms could break the cycle of antibiotic treatment and subsequent CDI relapse by preventing the resistance of *C. difficile* to bile acids; a strategy that would spare the gut microbiota.

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