

**CHARACTERIZATION OF BCLA1, A PUTATIVE *C. difficile*  
EXOSPORIUM GLYCOPROTEIN**

A Senior Scholars Thesis

by

JAMES BREGG REEDY

Submitted to Honors and Undergraduate Research  
Texas A&M University  
in partial fulfillment of the requirements for the designation as

UNDERGRADUATE RESEARCH SCHOLAR

May 2012

Major: Biology

**CHARACTERIZATION OF BCLA1, A PUTATIVE *C. difficile*  
EXOSPORIUM GLYCOPROTEIN**

A Senior Scholars Thesis

by

JAMES BREGG REEDY

Submitted to Honors and Undergraduate Research  
Texas A&M University  
in partial fulfillment of the requirements for the designation as

UNDERGRADUATE RESEARCH SCHOLAR

Approved by:

Research Advisor:

Associate Director, Honors and Undergraduate Research:

Joseph Sorg

Duncan MacKenzie

May 2012

Major: Biology

## ABSTRACT

Characterization of BclA1, a Putative *C. difficile*  
Exosporium Glycoprotein. (May 2012)

James Bregg Reedy  
Department of Biology  
Texas A&M University

Research Advisor: Dr. Joseph Sorg  
Department of Biology

*Clostridium difficile* is a spore forming, anaerobic bacterium that can cause diarrhea, pseudomembranous colitis, and toxic mega colon in susceptible hosts. *C. difficile* spores are highly resistant to extreme chemical and physical environments, and are likely the infectious agent. Spores are formed and matured inside of a mother cell and released into the environment when the mother cell lyses. During spore formation, a loosely attached outer layer, the exosporium, is added before mother cell lysis. The function of the *C. difficile* exosporium is not known, but has been studied in several *Bacillus spp.* In *Bacillus cereus*, the exosporium is the primary barrier that interacts with the host environment, helps the spore adhere to surfaces, and escape phagocytosis by macrophages. In *C. difficile* 630, *bclA1* is an orthologue of *B. anthracis bclA* and *B. cereus bclA*, and is a possible exosporium glycoprotein. Mutant *C. difficile* strains lacking these proteins may have effects on spore adhesion, germination, and spore architecture. Here we attempt to mutate *C. difficile bclA1* through TargeTron technology. Though no mutations were observed, we did isolate erythromycin-resistance

colonies, suggesting a mutation had been generated, possibly at another locus.

Erythromycin-resistance is likely induced by a RAM erythromycin-resistance gene activated upon a TargeTron insertional event. Further studies will be done to determine the location of the TargeTron mutation. In addition, to determine if BclA1 is located in the *C. difficile* exosporium, spores with fluorescently tagged *bclA1* were generated and will be viewed under fluorescent microscopy.

## TABLE OF CONTENTS

|   | Page |
|---|------|
| ABSTRACT .....  | iii  |
| TABLE OF CONTENTS .....   | v    |
| LIST OF FIGURES.....  | vi   |
| LIST OF TABLES .....  | vii  |
| CHAPTER   |      |
| I INTRODUCTION.....   | 1    |
| II METHODS.....   | 6    |
| Strains and growth conditions .....                                     | 6    |
| Plasmid and strain generation .....                                     | 6    |
| Preparation of <i>C. difficile</i> spores .....                         | 8    |
| Imaging by phase contrast microscopy .....                              | 9    |
| III RESULTS.....  | 12   |
| Inducing a mutation into <i>C. difficile bclA1</i> .....                | 12   |
| Production of <i>C. difficile</i> spores with <i>bclA1-bsfbfp</i> ..... | 19   |
| IV CONCLUSION .....   | 21   |
| REFERENCES .....  | 26   |
| CONTACT INFORMATION .....   | 31   |

## LIST OF FIGURES

| FIGURE  | Page |
|---|------|
| 1 Amplification of <i>bclA1</i> from erythromycin-resistant <i>C. difficile</i> with <i>bclA1</i> 666a-targeted TargeTron .....                           | 14   |
| 2 Amplification of <i>bclA1</i> TargeTron insertion junctions from potential <i>C. difficile bclA1</i> mutants .....                                      | 15   |
| 3 Amplification of <i>bclA1</i> from erythromycin-resistant <i>C. difficile</i> with <i>bclA1</i> 666a-targeted TargeTron of a separate conjugation ..... | 16   |
| 4 Amplification of <i>bclA1</i> from erythromycin-resistant <i>C. difficile</i> with <i>bclA1</i> 81-targeted TargeTron .....                             | 17   |
| 5 Amplification of <i>bclA1</i> from erythromycin-resistant <i>C. difficile</i> with iron induced <i>bclA1</i> 81-targeted TargeTron .....                | 18   |
| 6 Amplification of ErmB RAM marker from erythromycin-resistant <i>C. difficile</i> strains with <i>bclA1</i> 666a-targeted TargeTron .....                | 19   |
| 7 Phase contrast microscopy of <i>C. difficile</i> spores .....   | 20   |

**LIST OF TABLES**

| TABLE                                  | Page |
|--|------|
| 1 Bacterial strains and plasmids ..... | 7    |
| 2 Oligonucleotide sequences .....      | 8    |

## CHAPTER I

### INTRODUCTION

*Clostridium difficile* is a Gram-positive, spore-forming, anaerobic bacterium (28, 35). Diseases caused by *C. difficile* include diarrhea, pseudomembranous colitis, and toxic megacolon (28). Epidemiological studies report that *C. difficile* causes up to one-third of antibiotic-associated diarrheal cases, over 50% of all cases of antibiotic-associated colitis, and over 90% of all cases of antibiotic-associated pseudomembranous colitis (1, 33). Outbreaks of *C. difficile* infections (CDI) are increasing worldwide and the exact reasons are unknown (33, 44). The increase in CDIs is likely multifactorial, including more virulent strains and increased antibiotic exposure (33). To limit the spread of *C. difficile* in humans, more research needs to be done to understand how: *C. difficile* spores are generated, how these spores germinate, and how germinated spores colonize a host.

*C. difficile* infections are often precipitated by antibiotic use (28). In healthy individuals, the normal intestinal flora is thought to inhibit *C. difficile* colonization by competing for nutrients and / or growth sites or by producing inhibitory molecules (43). However, when a patient receives antibiotic treatment for another medical condition, the intestinal flora is disrupted and *C. difficile* spores can germinate, colonize the host, and cause disease (43). *C. difficile* produces two enterotoxins, TcdA and TcdB (Toxin A and Toxin

---

This thesis follows the style of Journal of Bacteriology.

B respectively), which are responsible for symptoms of disease (22, 26). These toxins elicit symptoms through UDP-glucose-dependent glycosylation of Rho family of small GTPases in colonic epithelial cells (38). The actions of these toxins cause a loss of barrier function of colonic epithelial cells, and massive fluid secretions that cause the symptoms of disease (48). Treatment of *C. difficile* infections annually cost between \$750 million and \$3.2 billion in the United States alone (7, 13, 23, 30).

The production of *C. difficile* spores inside a host creates a major problem in the treatment of a CDI. Antibiotics are commonly prescribed to treat CDIs to kill the vegetative cells and stop the spread of disease. However, antibiotics do not affect spores, and when antibiotic treatment stops, the spores can germinate, and reinitiate infection. Since multiple antibiotic treatments are sometimes needed to treat CDIs, new treatment options are needed that either prevent germination of spores or block sporulation of vegetative cells.

*C. difficile*, an anaerobe, must be in the spore form to survive outside of the anaerobic intestine environment (19). Once the spores have been ingested, they germinate inside the gut to produce actively growing bacilli that produce the toxins necessary for disease (22, 26). Sporulation has been studied extensively in *Bacillus subtilis* and is used as a model organism to study spore formation and spore germination. The first stage of spore formation occurs when a chromatin filament aligns along the axis of the cell (17). The cell then asymmetrically divides into the larger mother cell and forespore (17). The

mother cell engulfs the forespore and allows the forespore to be a free-floating structure enclosed by two membranes (17). Next the spore is matured by the addition of a peptidoglycan cortex and layers of coat proteins (17). Once the spore has fully matured, the mother cell can lyse, releasing the spore into the environment (17). *C. difficile* spores cannot produce toxins without first germinating to a vegetative cell, thus it is generally accepted that to cause disease, *C. difficile* spores must germinate inside the host (19).

*C. difficile* spores are encased in a protein coat that helps protect the spores from harsh environments. Surrounding the coat is an additional structure termed the exosporium (16). The exosporium helps the spore resist extreme physical and chemical stresses (16). Even though the spore is metabolically dormant, the exosporium proteins must interact with the surrounding environment (16).

*Bacillus anthracis* and *Bacillus cereus* are both spore-producing bacteria whose spores are surrounded by an exosporium. These exosporiums are comprised of two layers: an outer, hair-like nap and an inner, basal layer (12). In these bacteria, the exosporium appears to be a film-like membrane that loosely envelops the spore (12). This exosporium is the primary physiological barrier that interacts with the environment and is often the first point of contact with the immune system of infected hosts (12, 42). In *B. anthracis* the exosporium is a semi-permeable barrier that protects the spore from potentially harmful molecules, such as antibodies and hydrolytic enzymes (10, 11).

In *B. cereus*, the exosporium first appears after the mother cell engulfs the forespore. (32). During this stage of sporulation, the exosporium appears in the cytoplasm, next to the outer forespore membrane (32). In later stages of spore development, the exosporium moves further away from its origin toward the polar end of the cell (32). By the late stage of spore formation the exosporium has completely engulfed the forespore (32). After the spore has matured, the spore is released from the mother cell with the exosporium attached (42).

The components of the *C. difficile* exosporium have yet to be identified. Based upon homology to exosporium proteins found in *B. anthracis* and *B. cereus*, *C. difficile* has three orthologues: *bclA1*, *bclA2*, and *bclA3*. In *B. cereus* and *B. anthracis*, BclA is the major glycoprotein that comprises the outer layer of the exosporium (16). BclA is comprised of three domains: (i) the N-terminal domain (NTD); (ii) the intermediate collagen-like region (CLR); (iii) and the C-terminal domain (CTD) (37, 45, 47).

In *Bacillus spp.*, BclA is suggested to form trimers in the hair-like nap (45). Interactions between the three CTD domains on BclA could facilitate trimerization and strong resistance to heat, photolytic activity, and detergents (2, 37). The BclA CLR and CTD allow *B. cereus* to adhere to surfaces, while the NTD anchors BclA into the hair-like nap of the exosporium (24, 46). In *B. anthracis*, ExsFA/BxpB incorporates BclA into the exosporium (46). No homologues of these proteins are found in *C. difficile*.

While little is known about the *C. difficile* spore compared to spores of other sporulating bacteria, even less is known about the *C. difficile* exosporium. In *C. difficile*, *bclA1* is a predicted exosporium glycoprotein. This gene will be mutated and once confirmed, *C. difficile* spores will be produced and the spore architecture of both the wild-type and mutant spores will be analyzed using electron microscopy. Further, we will analyze the effect of *C. difficile bclA1* on spore germination. Since electron microscopy cannot identify the location of BclA1, the gene will be fluorescently tagged to confirm its location in the exosporium.

## CHAPTER II

### METHODS

#### Strains and growth conditions

Table 1 contains the bacterial strains and plasmids used in this study. BHIS (Brain Heart Infusion [BHI] supplemented with 10% L-cysteine) medium was used to grow *C. difficile* (40). When needed, *C. difficile* growth was supplemented with 250 µg/mL D-cycloserine, 50 µg/mL kanamycin, 10 µg/mL thiamphenicol, or 5 µg/mL erythromycin. *C. difficile* strains were grown at 37°C in an anaerobic chamber (10% hydrogen, 5% carbon dioxide, 85% nitrogen). *Escherichia coli* and *Bacillus subtilis* strains were grown at 37°C in LB or BHIS medium supplemented with 20 µg/mL chloramphenicol, 50 µg/mL kanamycin, or 100 µg/mL ampicillin (25).

#### Plasmid and strain generation

Table 2 lists the oligonucleotides used in this study. *C. difficile* JIR8094 genomic DNA was used as a template for PCR amplification of *C. difficile bclA1* (31). Generation of a TargeTron (Sigma-Aldrich) construct to introduce a mutation into *C. difficile bclA1* was accomplished in several steps. The TargeTron system creates a mutation in the *C. difficile* genome by insertion of a mobilizable group II intron into the selected gene. The intron sequence was targeted to *C. difficile bclA1* through an algorithm that generates specific DNA primers for PCR. Splicing by Overhand Extension (SOE) PCR was done using templates pBL64 and pBL65 (gift from L. Bouillaut and A. L. Sonenshein, Tufts University) and primers EBS Universal, *bclA1* (666a) EBS1, *bclA1* (666a) EBS2, and

TABLE 1. Bacterial strains and plasmids

| Plasmid or Strain   | Key Features   | Reference  |
|---------------------|--|--|
| <b>Strains</b>      |  |  |
| <i>E. coli</i>      |  |  |
| HB101               | pRK24 that provides conjugation machinery and Amp <sup>R</sup>               | (18)   |
| <i>B. subtilis</i>  |  |  |
| Bs49                | <i>Tn916</i> that induces conjugation  | (29)   |
| BRS1                | <i>bclA1-bsfbfp</i>  | This Study   |
| <i>C. difficile</i> |  |  |
| 630                 | Clinical isolate   | (39)   |
| JIR8094             | Erm <sup>S</sup> -CD630 derivative   | (31)   |
| BRC1                | <i>bclA1-bsfbfp</i>  | This Study   |
| BRC2                | Erm <sup>R</sup> with possible TargeTron mutation                            | This Study   |
| <b>Plasmids</b>     |  |  |
| pCR2.1-TOPO         | Amp <sup>R</sup> , Kan <sup>R</sup>  | Invitrogen   |
| pJS107              | TargeTron construct derived from pBL100, contains <i>Tn916 oriT</i>          | (J. A. Sorg, Texas A&M University, unpublished data) |
| pBsfbfp             | Kan <sup>R</sup> , <i>B. subtilis</i> fluorescent protein derived from pET28 | (6)  |
| pBR1                | <i>bclA1</i> (666a) Intron fragment in pCR2.1                                | This Study   |
| pBR2                | <i>bclA1</i> (666a) Intron fragment in pBL100                                | This Study   |
| pBR3                | <i>bclA1</i> (666a) Intron fragment in pJS107                                | This Study   |
| pBR4                | <i>bclA1</i> in pCR2.1   | This Study   |
| pBR5                | <i>bclA1-bsfbfp</i> in pCR2.1  | This Study   |
| pBR6                | <i>bclA1</i> in pMTL84151  | This Study   |
| pBR7                | <i>bclA1-bsfbfp</i> in pMTL84151   | This Study   |
| pBR8                | <i>bclA1</i> (81) Intron fragment in pCR2.1                                  | This Study   |
| pBR9                | <i>bclA1</i> (81) Intron fragment in pJS107                                  | This Study   |
| pBR10               | pBR7 with <i>Tn916</i> homology  | This Study   |

TABLE 2. Oligonucleotide sequences

| Primer            | Sequence (5' → 3')   |
|-------------------|--|
| bclA1 (666a) EBS2 | TGAACGCAAGTTTCTAATTTTCGGTTGGAAATCGAT<br>AGAGGAAAGTGTCT           |
| bclA1 (666a) EBS2 | AAAAAAGCTTATAATTATCCTTATTTCCCGTTGGG<br>GTGCGCCAGATAGGGTG         |
| bclA1 (666a) EBS1 | CAGATTGTACAAATGTGGTGATAACAGATAAGTC<br>GTTGGGCCTAACTTACCTTCTTTGT  |
| EBSU              | CGAAATTAGAACTTGC GTTCAGTAAC                                      |
| 5' bclA1          | ATGAGAAATATTATACTTTATTTAAATGATG                                  |
| 3' bclA1          | TTAAGTCATAACAGTATCAGCTATTC                                       |
| 3' bclA1- no stop | AGTCATAACAGTATCAGCTATTCTA  |
| 5' bclA1-FP       | TAGAATAGCTGATACTGTTATGACTATGGCTAGTT<br>TTC AATCATT TGGGA         |
| 3' bsfbfp         | AATCTAGATTACATAATCGGAAGCACTTTAACG                                |
| 3' bclA1-stp      | AATCTAGATTAAGTCATAACAGTATCAGCTATTC                               |
| 5' bclA1 prom     | AAAGATCTGATTGTTTATATATTTATTTTAAATA<br>ATAATG                     |
| bclA1 (81) EBS1   | CAGATTGTACAAATGTGGTGATAACAGATAAGTC<br>AATGTTCACTTACCTTCTTTGT     |
| bclA1 (81) EBS2   | CGCAAGTTTCTAATTTTCGGTACTTCTCGATAGAG<br>GAAAGTGTCT                |
| bclA1 (81) IBS    | AAAAGCTTTTGCAACCCACGTCGATCGTGAAGAA<br>GTAAATGTTGTGCGCCAGATAGGGTG |
| 5' Erm(td)        | GTGTGCTTAAATGGTAAGGAATACTCC                                      |
| 3' Erm(td)        | CTTGTTACATTACTGTGACTGGTT   |

bclA1 (666a) IBS to generate a 350 bp retargeting intron fragment (Table 1, Table 2).

The intron fragment was cloned into pCR2.1-TOPO (Invitrogen) to create pBR1. pBR1 was digested with BsrGI/HindIII and the retargeted intron fragment was cloned into the *E. coli* – *C. difficile* shuttle vector, pJS107, yielding pBR3. A separate retargeting intron fragment was created using primers EBS Universal, bclA1 (81) EBS1, bclA1 (81) EBS2, bclA1 (81) IBS, and templates pBL64 and pBL65 (Table 1, Table 2). Using the same

techniques previously stated, the newly created intron fragment was inserted into pCR2.1-TOPO and pJS107 to make pBR8 and pBR9, respectively. pBR9 will be used if pBR3 is not successful in producing a mutation in *C. difficile bclA1*; pBR9 uses a different intron insertion site than pBR3. pBR3 was transformed into *E. coli* HB101 pRK24. *E. coli* HB101 pRK24 pBR3 was then conjugated with *C. difficile* strain JIR8094 creating *C. difficile* JIR8094 pBR3. Transconjugants were selected on BHIS agar medium containing kanamycin and thiamphenicol (BHIS-TK). Insertion of the intron into *C. difficile bclA1* was selected by screening transconjugants for erythromycin resistance on BHIS agar medium supplemented with erythromycin. Functional erythromycin resistance upon intron insertion is provided by the activation of a retrotransposable activated marker (RAM). Insertional disruption of *C. difficile bclA1* was determined by PCR of the disrupted gene, the 5' and 3' junctions of the intron insertion site, and active ErmB RAM (Table 2). The same protocol was used to introduce pBR9 into *E. coli* HB101 pRK24 and to conjugate with *C. difficile* JIR8094. Once a mutation of *C. difficile bclA1* had been confirmed, the mutated strain was frozen and renamed *C. difficile* BRC3.

The *C. difficile bclA1* gene complement was amplified using primers 5'bclA1 prom and 3'bclA1-stp, and cloned into pCR2.1-TOPO to create pBR4 (Table 1, Table 2). pBR4 was digested with XbaI/BglIII and the fragment was ligated into pMTL84151 to generate pBR6. pMTL84151 is an *E. coli* – *C. difficile* shuttle vector that will allow the *bclA1* complement to be transferred into *C. difficile* (15).

The fluorescent gene, *bsfbfp*, was PCR amplified from pBsfbfp and fused to *C. difficile* *bclA1* via SOE PCR using primers 5'bclA1prom, 3'bclA1-no stop, 5'bclA1-FP, and 3'bsfbfp (Table 1, Table 2) (6). *bclA1-bsfbfp* was cloned into pCR2.1-TOPO to create pBR5, which was digested with XbaI/BglIII. The fragment was ligated into pMTL84151 to generate pBR7. To allow the fusion gene to be recombine into the *C. difficile* genome, pBR7 was digested with FseI and AscI and ligated with a region of *Tn916* homology, to yield pBR10. pBR10 was transformed into *Bacillus subtilis* Bs49 (29), to generate *B. subtilis* BRS1, and was conjugated with *C. difficile* JIR8094. Transconjugants were selected on BHIS containing kanamycin and thiamphenicol (BHIS-TK). Once a *C. difficile* strain containing *bclA1-Bsfbfp* had been confirmed, the strain was stored as *C. difficile* BRC1.

### **Preparation of *C. difficile* spores**

Spores from *C. difficile* strains JIR8094 and BRC1 were prepared as described in (41) with the following modifications. Each strain was grown on SMC (90 g BactoPeptone, 5 g Protease peptone, 1 g NH<sub>4</sub>SO<sub>4</sub>, 1.5 g Tris Base) agar medium and allowed to grow for 4 days at 37°C. Cell growth from each plate was collected and washed five times with sterile water. Spores were purified from plate debris by centrifugation at 4,000 rpm, for 10 minutes, on a 50% sucrose gradient. Spores formed as a pellet during centrifugation, while other cells and debris were collected in the gradient interface. To remove the

sucrose, the spore pellet was washed five times with water and spores were resuspended in water.

### **Imaging by phase contrast microscopy**

A sample of purified spores from *C. difficile* JIR8094 and BRC1 were observed under phase contrast microscopy at 400x magnification. *C. difficile* BRC1 spores were visualized and compared to wild-type *C. difficile* JIR8094 spores.

## CHAPTER III

### RESULTS

#### **Introducing a mutation into *C. difficile bclA1***

The use of a TargeTron technology has previously been effective in generating mutations in *C. difficile* (21, 27). The TargeTron system uses mobile group II introns to introduce site specific mutations. The self-splicing activity of group II introns allows the intron to splice out of a mRNA transcript. Mobility of the group II intron (*ltrB*) is aided by LtrA, an intron-encoded protein (IEP) (14). Binding of the IEP to the spliced intron creates the ribonucleoprotein (RNP) complex (14, 20). The RNP scans DNA for the target site, and is recognized by intron-binding sites 1 and 2 (IBS1 and IBS2), and the complementary exon-binding sites 1 & 2 (EBS1 and EBS2) (20). Through reverse splicing, the intron RNA is inserted into one strand of the targeted DNA site by the RNP, while the IEP cleaves the complementary strand and uses the 3' end as a primer to begin reverse transcribing the intron (20). Once the transcribed intron is ligated into the DNA, a stable disruption is made in the coding sequence of the gene.

In *Bacillus spp.*, *bclA* has been shown to produce a major glycoprotein of the exosporium (5). In *C. difficile* there are three orthologues of *bclA* (*bclA1*, *bclA2*, *bclA3*) whose functions remain unknown. Mutation of *C. difficile bclA1* may affect spore adhesion, germination, and spore architecture based off studies of mutations in *bclA* of *Bacillus spp.* (4). To inactivate *C. difficile 630 bclA1*, a mobile group II intron was retargeted for insertion at nucleotide 666 of *C. difficile bclA1* and should insert in the

anti-sense orientation with respect to the *C. difficile bclA1* coding sequence. The *C. difficile bclA1* intron insertion site was chosen using an algorithm which generates DNA primers specific to that insertion site ([http://dna.med.monash.edu.au/~torsten/intron\\_site\\_finder/](http://dna.med.monash.edu.au/~torsten/intron_site_finder/)). These primers alter the IBS and EBS sites of the intron using PCR. Using pBL64 and pBL65 as templates, a 350 bp retargeting intron sequence was ligated into pBL100 to generate pBR2. The pBL100 backbone contains genes for LtrA, chloramphenicol resistance (*catP*), and a retrotransposition-activated selectable marker (RAM) that activates erythromycin resistance upon insertion of the intron into the target gene. In addition, a second TargeTron construct was made using the same 666 retargeted intron as pBR2, except it was ligated into pJS107, to generate pBR3; the pJS107 backbone is identical to pBL100 except that it contains origin of transfer (*oriT*) from the *Tn916* transposon. The *Tn916 oriT* allows for mobilization of the plasmid from *B. subtilis* to *C. difficile* through conjugation using *Tn916*. pBR3 was conjugated into *C. difficile* JIR8094 using *B. subtilis* as donor, and transconjugants were selected for thiamphenicol resistance ( $Tm^R$ ); *catP* provides both thiamphenicol and chloramphenicol resistance. Thiamphenicol-resistant strains were grown on BHIS-Erm to select for colonies with the activated ErmB RAM marker, suggesting possible *C. difficile bclA1* mutations. Erythromycin resistant colonies were isolated and DNA was extracted. Full-length *C. difficile bclA1* was amplified to screen for the presence of a mutation. If *C. difficile bclA1* was disrupted, we would observe a 1.8 kb insertion of the intron fragment into the 2,082 bp coding sequence of *C. difficile bclA1*, yielding a 3.8 kb product. Many of the PCR products of the erythromycin resistant strains had 3.8 kb fragments when

*bclA1* was amplified (Fig. 1). To ensure that the retargeted intron has been inserted into *C. difficile bclA1*, the 5' and 3' junctions of the 1.8 kb intron in *C. difficile bclA1* were amplified. The 5' junction of the mutant gene would produce a 2.5 kb fragment and the 3' junction would produce a 1.3 kb fragment. However, no PCR fragments for either the 5' or 3' junction were amplified (Fig. 2). These results suggested that no mutation of *C. difficile bclA1* was made by pBR3. The production of the 3.8 kb fragment from full length *C. difficile bclA1* was likely a false positive. An incorrect result for the positive control also suggests that this was a failed PCR. Another possibility is the presence of erythromycin-resistant colonies were produced by activation of a known, inactive *ermB* gene in *C. difficile* JIR8094 that provides erythromycin-resistance in the parent *C. difficile* 630, and not from RAM activation (9).

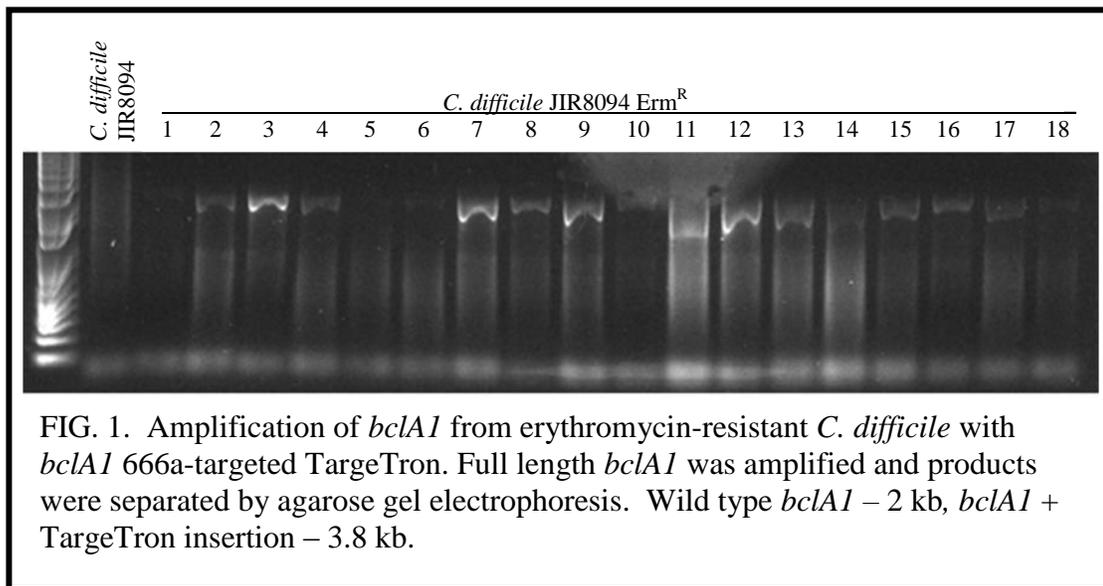
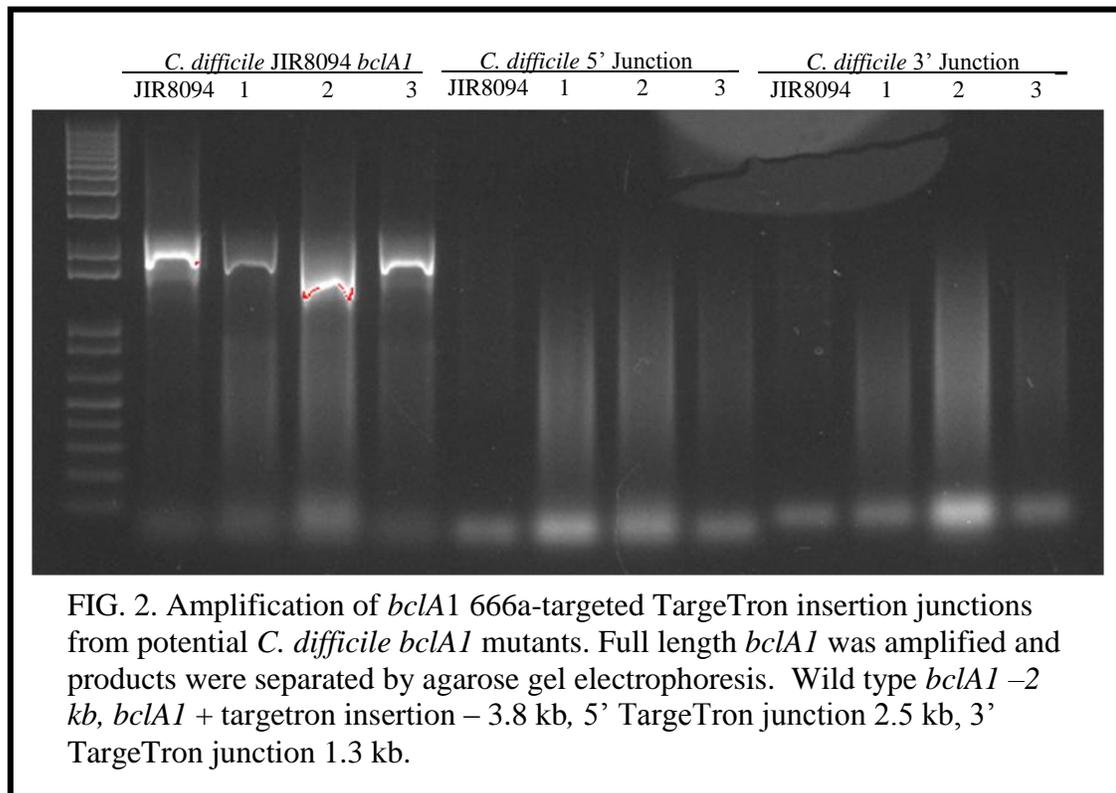


FIG. 1. Amplification of *bclA1* from erythromycin-resistant *C. difficile* with *bclA1* 666a-targeted TargeTron. Full length *bclA1* was amplified and products were separated by agarose gel electrophoresis. Wild type *bclA1* – 2 kb, *bclA1* + TargeTron insertion – 3.8 kb.

Since pBR3 did not produce a mutation in *C. difficile bclA1*, pBR2 was conjugated in to *C. difficile* JIR8094 using *E. coli* HB101 as a donor. Selection for mutant strains followed the same procedure as above, however only the wild-type size of *C. difficile bclA1* was amplified (Fig. 3). The intron sequence used in pBR2 and pBR3 may have targeted the intron into a DNA sequence other than *C. difficile bclA1*, creating erythromycin-resistant colonies. Even though *C. difficile bclA1* had not been mutated, insertion of the intron could have created a mutation in another gene besides *C. difficile bclA1*.



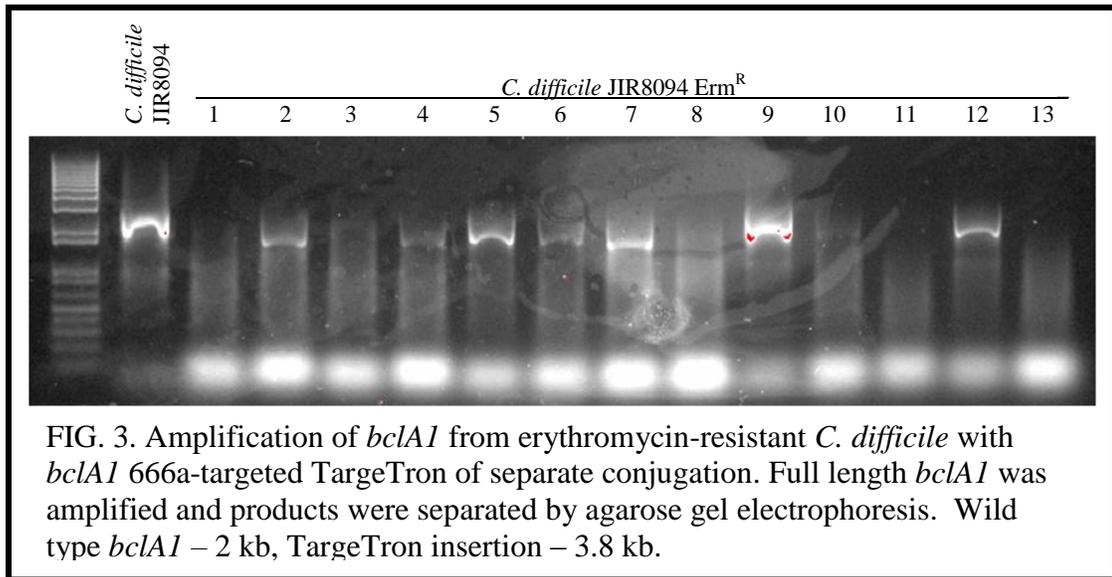
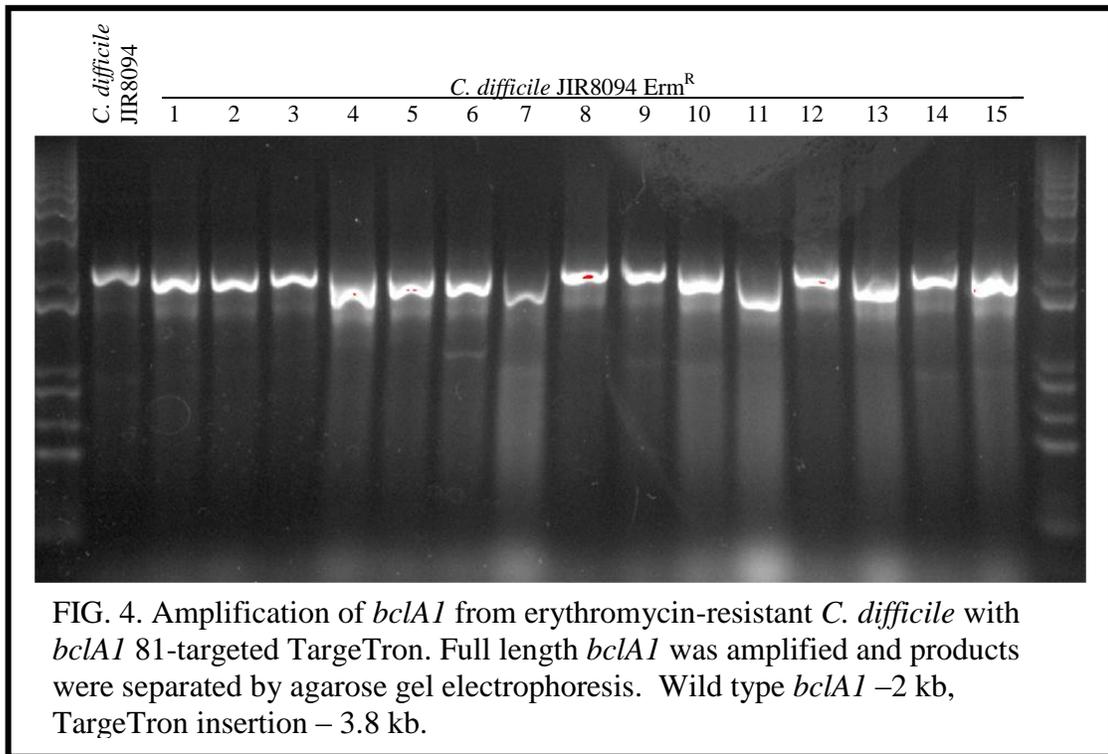


FIG. 3. Amplification of *bclA1* from erythromycin-resistant *C. difficile* with *bclA1* 666a-targeted TargetTron of separate conjugation. Full length *bclA1* was amplified and products were separated by agarose gel electrophoresis. Wild type *bclA1* – 2 kb, TargetTron insertion – 3.8 kb.

Since pBR2 and pBR3 were unsuccessful in producing a mutation in *bclA1*, another TargetTron, pBR9, was made from a new, retargeted intron sequence. The new intron sequence was re-targeted for nucleotide 81 of the *C. difficile bclA1* coding sequence. pBR9 was conjugated into *C. difficile* JIR8094 using *E. coli* HB101 as a donor, and erythromycin-resistance colonies were isolated as above. Amplification of *bclA1* from erythromycin-resistant strains indicated that all strains were wild-type for *bclA1* (Fig. 4). To increase TargetTron expression, *C. difficile* pBR9 strains on BHIS-TK were streaked on BHIS-Erm supplemented with 10 mM iron sulfate; TargetTron expression is driven from an iron inducible promoter. Significantly less erythromycin-resistant colonies formed on BHIS-Erm Fe medium. Amplification of *C. difficile bclA1* from erythromycin-resistant, iron-induced strains revealed that all strains were wild-type for

*bclA1* (Fig. 5). Thus, no insertion of the intron into *C. difficile bclA1* was detected in any *C. difficile* strains conjugated with pBR9.



Since pBR9 was not able to generate a mutation in *C. difficile bclA1*, pBR3 was used again to try to create a mutation in *C. difficile bclA1*. pBR3 was conjugated into *C. difficile* JIR8094 using *E. coli* HB101 as a donor, and erythromycin-resistance colonies were isolated as above. Amplification of *C. difficile bclA1* from erythromycin-resistant strains indicated that all strains were wild-type for *bclA1*. To determine if erythromycin-resistance was caused by a spontaneous mutation that activated an ErmB genes already present in the *C. difficile* genome, or from ErmB RAM activation by TargeTron

insertion, erythromycin-resistant strains were screened for an active ErmB RAM. All strains were able to amplify an active ErmB RAM, suggesting TargeTron insertion into the *C. difficile* genome creating a mutation somewhere other than *C. difficile bclA1* (Fig. 6). Erythromycin-resistant *C. difficile* strains were frozen and renamed *C. difficile* BRC2.

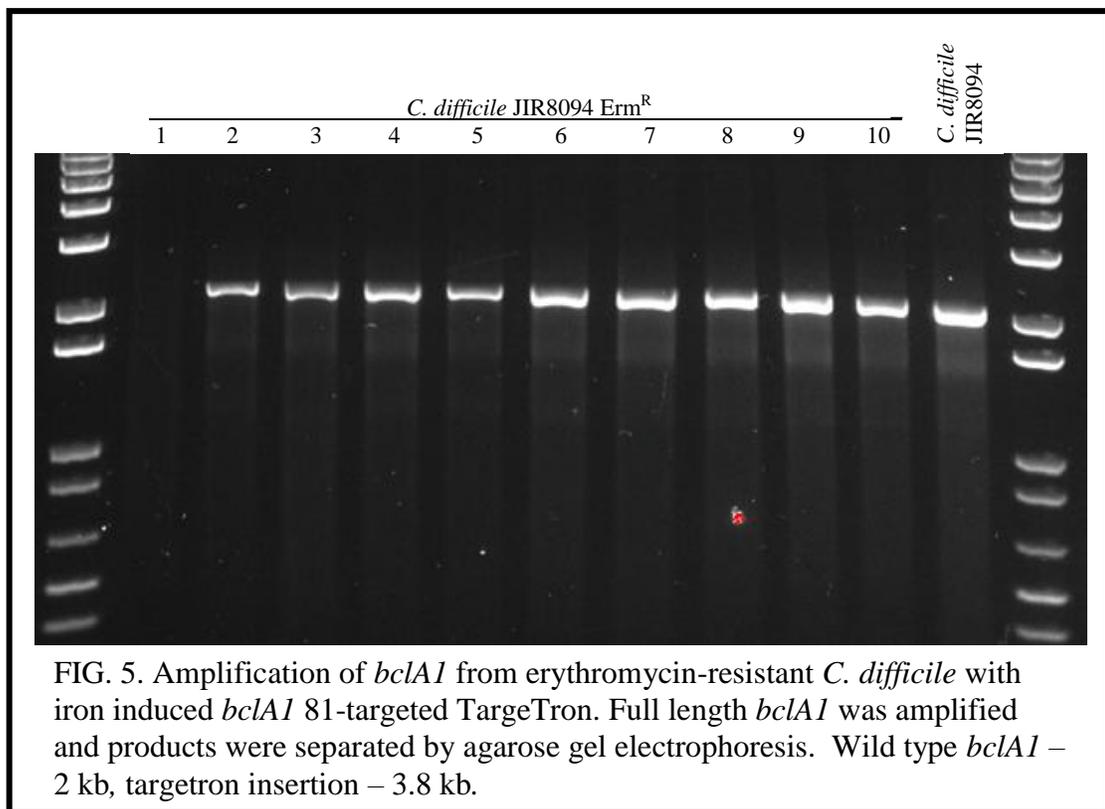
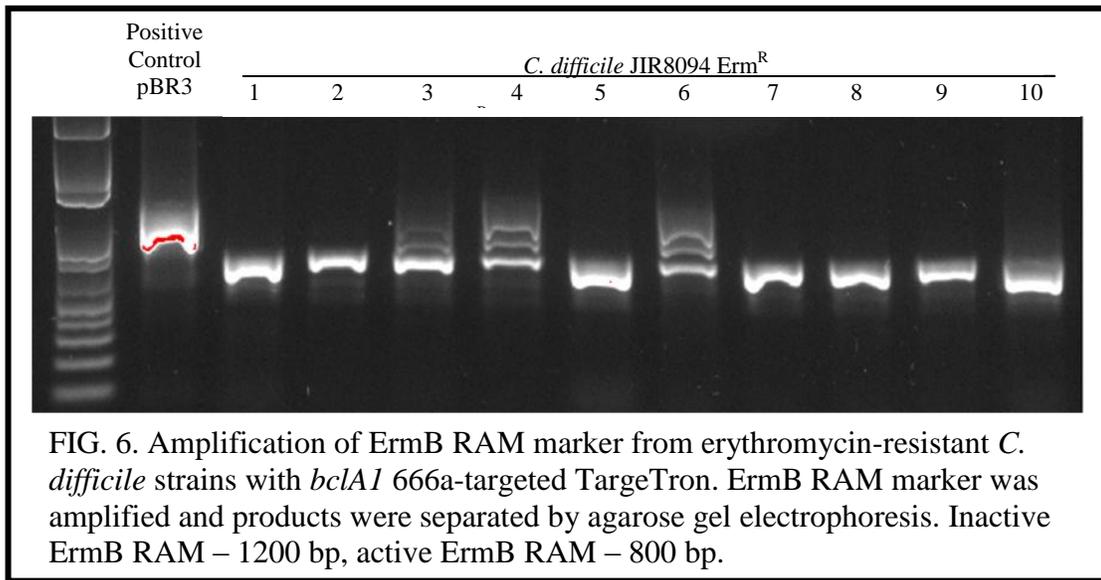


FIG. 5. Amplification of *bclA1* from erythromycin-resistant *C. difficile* with iron induced *bclA1* 81-targeted TargeTron. Full length *bclA1* was amplified and products were separated by agarose gel electrophoresis. Wild type *bclA1* – 2 kb, targetron insertion – 3.8 kb.



### Production of *C. difficile* spores with *bclA1-bsfbfp*

Traditionally, using fluorescent reporters to analyze the cellular location of target proteins in anaerobic bacteria has been met with frustration. Most fluorophores require molecular oxygen to fold the fluorophore properly. However, recently a novel, flavin-based fluorescent protein has been described and allows fluorescence even in the absence of oxygen (6). To identify the location of BclA1 in the *C. difficile* spore exosporium, *bclA1* was fused to a fluorescent gene, *bsfbfp* (6). The gene fusion, *bclA1-bsfbfp*, was integrated into a pBR7 containing *catP* and *Tn916* homology, to generate pBR10. The *Tn916* homology region allows recombination of pBR10 into *Tn916* of the *B. subtilis* genome to create *B. subtilis* BRS1. *B. subtilis* BRS1 was conjugated with *C. difficile* JIR8094 to allow the insertion of *Tn916* containing *bclA1-bsfbfp* into the *C. difficile* genome. Transconjugants were selected on thiamphenicol-containing medium.

DNA was extracted from thiamphenicol-resistant strains, and *bsfbfp* was amplified from the resulting DNA. The presence of the 786 bp product from amplification of *bsfbfp* confirmed the creation of *C. difficile* BRC1. To generate spores, *C. difficile* BRC1 was allowed to sporulate on SMC media. *C. difficile* BRC1 spores were purified and imaged under phase contrast microscopy (Fig. 7). Both strains appear to have produced many spores of similar size, however, *C. difficile* BRC1 spores appeared to form more large clumps than wild-type spores, suggesting different adhesion properties between the two strains. Wild-type *C. difficile* JIR8094 and *C. difficile* BRC1 spores will be visualized using fluorescent microscopy to identify if: (i) the fluorescent gene is capable of producing a fluorescent protein in *C. difficile*; and (ii) if BclA1 is expressed in and deposited on the *C. difficile* exosporium.

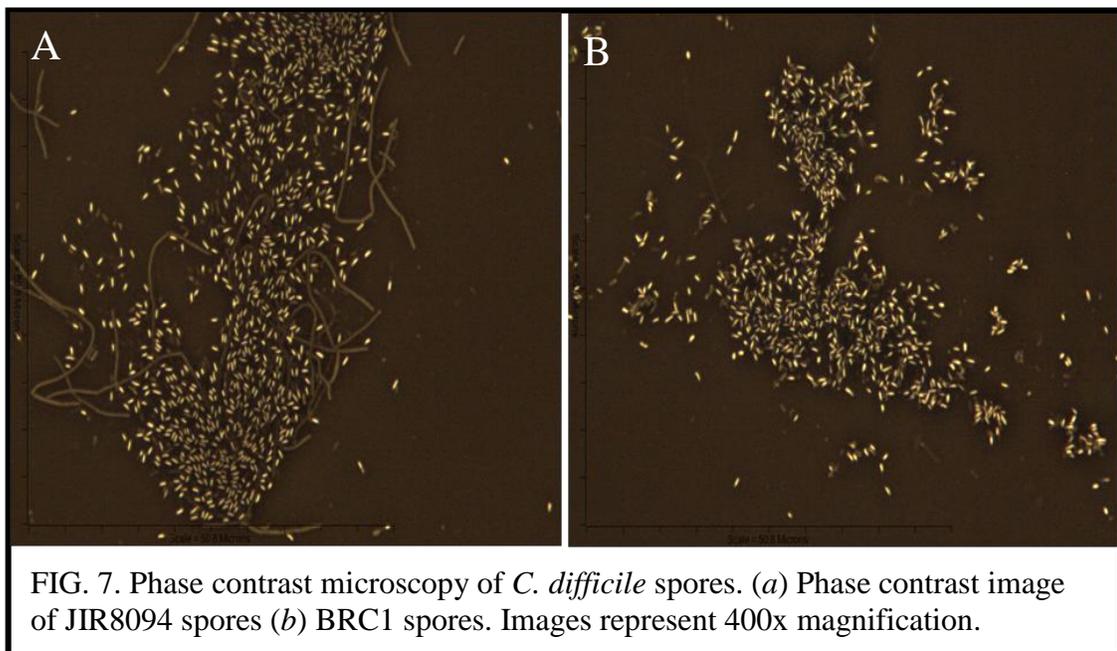


FIG. 7. Phase contrast microscopy of *C. difficile* spores. (a) Phase contrast image of JIR8094 spores (b) BRC1 spores. Images represent 400x magnification.

## CHAPTER IV

### CONCLUSION

Surrounding the outer surface of the *C. difficile* spore is the exosporium. No proteins have been identified in the *C. difficile* exosporium, and the role of the *C. difficile* exosporium is not known, but is thought to play a role in spore adhesion (34). Many spore-forming *Bacillus spp.* produce an exosporium during sporulation. In *B. cereus*, the exosporium is the first layer of the spore to interact with the environment and plays a role in spore adhesion, evasion from macrophages, and spore germination (3, 8, 36). In both *B. cereus* and *B. anthracis*, BclA is the major glycoprotein that comprises the outer layer of the exosporium, to form a hair-like nap that surrounds the spore (5, 24). *B. cereus* BclA is thought to be used for spore adhesion and for spore hydrophobicity, however the adhesion properties of *B. cereus* BclA are not fully understood (24). In *C. difficile* 630, *bclA1*, *bclA2*, and *bclA3* are orthologues of *B. cereus bclA* and *B. anthracis bclA*, a known exosporium glycoprotein. Characterization of *C. difficile* BclA1 could lead to an understanding of the function of the *C. difficile* exosporium and how it enables spore adhesion on epithelial cells lining the intestine.

Originally, the *C. difficile* exosporium was thought to play a minor role in spore germination, but now is thought to be a major factor in spore adhesion and cell colonization in pathogenesis (34). When *C. difficile* spores are grown *in vitro*, the exosporium is covered with projections termed “bumps” and “knobs” (34). Upon germination, the exosporium produces elongated projections that attach to the media,

and are later replaced by a thick, anchor-like structure (34). This large appendage appears to attach the spore to the medium, and prevents the spore from being removed during washing, agitation in water, solvent baths, or point drying (34). *C. difficile* BclA1 is not thought to be the elongated projections protruding from the exosporium, based off homology to *bclA* in *B. anthracis*, but it is possible that *C. difficile* BclA1 comprises the bumps and knobs seen on the outer-layer of the exosporium. These structures could play a role in initial spore adhesion until germination, which results in the production of separate protein appendages that bind to the host surface.

To determine the function of *C. difficile* BclA1, we sought to inactivate the *C. difficile* *bclA1* coding sequence. The method we used creates a site specific mutation in *bclA1* utilizing TargeTron technology. We were able to retarget a TargeTron construct to *C. difficile* *bclA1* and selected for erythromycin-resistant *C. difficile* colonies, suggesting a mutation had been generated. Originally, amplification of *C. difficile* *bclA1* produced a 3.8 kb fragment, indicating that the 1.8 kb TargeTron had been inserted into the 2 kb *C. difficile* *bclA1* coding sequence. However, upon amplification of the TargeTron junctions in *C. difficile* *bclA1*, no DNA was amplified, indicating no mutation of *C. difficile* *bclA1* was generated. Upon further analysis of the full-length *C. difficile* *bclA1* coding sequence, we observed wild-type 2 kb fragments, instead of the previous, false-positive, 3.8 kb fragments. Another attempt to disrupt *C. difficile* *bclA1* was made by creation of another TargeTron construct, pBR9, but yielded similar results. Similar difficulties arose in attempts to mutate *C. difficile* *bclA2* and *bclA3* (Nathan Oehring,

Kevin Slaughter and Joseph Sorg, Texas A&M University, personal communication). The generation of erythromycin-resistant colonies without having a TargeTron insertion in *C. difficile bclA1* begs the question: how can erythromycin-resistance arise without RAM activation of ErmB? One possibility is a spontaneous mutation activated either of the 2 inactive ErmB genes already present in the *C. difficile* genome (9, 39). Another possibility is that ErmB RAM is activated by random insertion of the TargeTron construct into the *C. difficile* genome. Since *C. difficile bclA1* shares homology to *bclA2* and *bclA3*, TargeTron insertion may have happened in either of these genes. To test this hypothesis, we amplified *C. difficile bclA2* and *bclA3* of the erythromycin-resistant colonies. This amplification produced wild-type fragments, concluding a mutation was not introduced into *C. difficile bclA1*, *bclA2*, or *bclA3*.

Erythromycin-resistant strains were screened for an active ErmB RAM caused by TargeTron insertion. All strains had an active ErmB RAM, suggesting that erythromycin-resistance is caused by RAM activation and not by a spontaneous mutation that activates the *C. difficile ermB* genes. More importantly, this result indicates RAM activation caused by an insertion of the TargeTron into the *C. difficile* genome, somewhere other than *C. difficile bclA1*, *bclA2*, or *bclA3*. This data concludes that a mutation has been made in *C. difficile*, but at an unknown location.

To determine if *C. difficile BclA1* is located in the *C. difficile* exosporium, we tagged *C. difficile bclA1* with the fluorescent gene *bsfbfp*. Most fluorescent proteins require

molecular oxygen to fluoresce, however, *C. difficile* is an anaerobe and cannot grow in the presence of oxygen. Hence, we used the flavin-based protein, Bsfbfp, which has been shown to fluoresce in the absence of oxygen (6). *C. difficile* spores containing the fluorescent gene *bclA1-bsfbfp* were collected, purified, and compared to wild-type *C. difficile* JIR8094 spores; *C. difficile* JIR8094 is an erythromycin-sensitive derivative of *C. difficile* 630. Previously, collection of *C. difficile* JIR8094 spores had been very difficult because this strain does not sporulate well on BHIS. Thus, we tested a different medium, SMC, to promote sporulation of *C. difficile* JIR8094. A sample was collected and phase-contrast microscopy determined that SMC was successful in inducing sporulation of *C. difficile* JIR8094. SMC was then used to generate spores of *C. difficile* BR1, containing fluorescently tagged *C. difficile* *bclA1*. When compared to wild-type spores, *C. difficile* BR1 spores appeared to form more groups of clumped spores, suggesting that these spores had different adhesion properties from wild-type. An alteration in adhesion properties may be facilitated by the presence of an additional *C. difficile* *bclA1* coding sequence found in the fluorescently tagged gene. These preliminary results indicated that over-expression of *C. difficile* BclA1 may influence adhesion properties of spores.

Understanding the role of *C. difficile* BclA1 is important since (i) it will determine part of the exosporium function and how the spore interacts with the host environment, and (ii) will determine how this protein influences spore germination, adhesion, and pathogenesis. In *B. anthracis*, mutation of BclA enables a decreased germination time.

(4). If this is also true for mutation of *C. difficile bclA1*, the role of the *C. difficile* exosporium can be correlated to *C. difficile* spore germination. Additionally, if *C. difficile* BclA1 does affect spore adhesion to surfaces, chemical treatments can be found that will lead to better sanitation that will decrease the amount of CDI relapse, and to decrease the adhesion of *C. difficile* spores in the intestine.

## REFERENCES

1. **Aslam, S., R. J. Hamill, and D. M. Musher** Sep 2005, posting date. Treatment of *Clostridium difficile*-associated disease: old therapies and new strategies 2005/08/27. [Online.]
2. **Boydston, J. A., P. Chen, C. T. Steichen, and C. L. Turnbough, Jr.** 2005. Orientation within the exosporium and structural stability of the collagen-like glycoprotein BclA of *Bacillus anthracis*. *J. Bacteriol.* **187**:5310-5317.
3. **Bozue, J., K. L. Moody, C. K. Cote, B. G. Stiles, A. M. Friedlander, S. L. Welkos, and M. L. Hale.** 2007. *Bacillus anthracis* spores of the *bclA* mutant exhibit increased adherence to epithelial cells, fibroblasts, and endothelial cells but not to macrophages. *Infect. Immun.* **75**:4498-4505.
4. **Brahmbhatt, T. N., B. K. Janes, E. S. Stibitz, S. C. Darnell, P. Sanz, S. B. Rasmussen, and A. D. O'Brien.** 2007. *Bacillus anthracis* exosporium protein BclA affects spore germination, interaction with extracellular matrix proteins, and hydrophobicity. *Infect. Immun.* **75**:5233-5239.
5. **Daubenspeck, J. M., H. Zeng, P. Chen, S. Dong, C. T. Steichen, N. R. Krishna, D. G. Pritchard, and C. L. Turnbough, Jr.** 2004. Novel oligosaccharide side chains of the collagen-like region of BclA, the major glycoprotein of the *Bacillus anthracis* exosporium. *J. Biol. Chem.* **279**:30945-30953.
6. **Drepper, T., T. Eggert, F. Circolone, A. Heck, U. Krauss, J. K. Guterl, M. Wendorff, A. Losi, W. Gartner, and K. E. Jaeger.** 2007. Reporter proteins for in vivo fluorescence without oxygen. *Nat. Biotechnol.* **25**:443-445.
7. **Dubberke, E. R., K. A. Reske, M. A. Olsen, L. C. McDonald, and V. J. Fraser.** 2008. Short- and long-term attributable costs of *Clostridium difficile*-associated disease in nonsurgical inpatients. *Clin. Infect. Dis.* **46**:497-504.
8. **Faille, C., G. Tauveron, C. Le Gentil-Lelievre, and C. Slomianny.** 2007. Occurrence of *Bacillus cereus* spores with a damaged exosporium: consequences on the spore adhesion on surfaces of food processing lines. *J. Food. Prot.* **70**:2346-2353.
9. **Farrow, K. A., D. Lyras, and J. I. Rood.** 2000. The macrolide-lincosamide-streptogramin B resistance determinant from *Clostridium difficile* 630 contains two *erm(B)* genes. *Antimicrob. Agents Chemother.* **44**:411-413.

10. **Gerhardt, P.** 1967. Cytology of *Bacillus anthracis*. Fed. Proc. **26**:1504-1517.
11. **Gerhardt, P., and S. H. Black.** 1961. Permeability of bacterial spores. II. Molecular variables affecting solute permeation. J. Bacteriol. **82**:750-760.
12. **Gerhardt, P., and E. Ribi.** 1964. Ultrastructure of the Exosporium Enveloping Spores of *Bacillus Cereus*. J. Bacteriol. **88**:1774-1789.
13. **Ghantaji, S. S., K. Sail, D. R. Lairson, H. L. DuPont, and K. W. Garey.** 2010. Economic healthcare costs of *Clostridium difficile* infection: a systematic review. J. Hosp. Infect. **74**:309-318.
14. **Heap, J. T., S. T. Cartman, S. A. Kuehne, C. Cooksley, and N. P. Minton.** 2010. ClosTron-targeted mutagenesis. Methods Mol. Biol. **646**:165-182.
15. **Heap, J. T., O. J. Pennington, S. T. Cartman, and N. P. Minton.** 2009. A modular system for *Clostridium* shuttle plasmids. J. Microbiol. Methods **78**:79-85.
16. **Henriques, A. O., and C. P. Moran, Jr.** 2007. Structure, assembly, and function of the spore surface layers. Annu. Rev. Microbiol. **61**:555-588.
17. **Hilbert, D. W., and P. J. Piggot.** 2004. Compartmentalization of gene expression during *Bacillus subtilis* spore formation. Microbiol. Mol. Biol. Rev. **68**:234-262.
18. **Hussain, H. A., A. P. Roberts, and P. Mullany.** 2005. Generation of an erythromycin-sensitive derivative of *Clostridium difficile* strain 630 (630Deltaerm) and demonstration that the conjugative transposon Tn916DeltaE enters the genome of this strain at multiple sites. J. Med. Microbiol. **54**:137-141.
19. **Jump, R. L., M. J. Pultz, and C. J. Donskey.** 2007. Vegetative *Clostridium difficile* survives in room air on moist surfaces and in gastric contents with reduced acidity: a potential mechanism to explain the association between proton pump inhibitors and *C. difficile*-associated diarrhea? Antimicrob. Agents Chemother. **51**:2883-2887.
20. **Karberg, M., H. Guo, J. Zhong, R. Coon, J. Perutka, and A. M. Lambowitz.** 2001. Group II introns as controllable gene targeting vectors for genetic manipulation of bacteria. Nat. Biotechnol. **19**:1162-1167.

21. **Kirby, J. M., H. Ahern, A. K. Roberts, V. Kumar, Z. Freeman, K. R. Acharya, and C. C. Shone.** 2009. Cwp84, a surface-associated cysteine protease, plays a role in the maturation of the surface layer of *Clostridium difficile*. *J. Biol. Chem.* **284**:34666-34673.
22. **Kuehne, S. A., S. T. Cartman, J. T. Heap, M. L. Kelly, A. Cockayne, and N. P. Minton.** 2010. The role of toxin A and toxin B in *Clostridium difficile* infection. *Nature* **467**:711-713.
23. **Kyne, L., M. B. Hamel, R. Polavaram, and C. P. Kelly.** 2002. Health care costs and mortality associated with nosocomial diarrhea due to *Clostridium difficile*. *Clin. Infect. Dis.* **34**:346-353.
24. **Lequette, Y., E. Garenaux, T. Combrouse, L. Dias Tdel, A. Ronse, C. Slomianny, X. Trivelli, Y. Guerardel, and C. Faille.** 2011. Domains of BclA, the major surface glycoprotein of the *B. cereus* exosporium: glycosylation patterns and role in spore surface properties. *Biofouling* **27**:751-761.
25. **Luria, S. E., and J. W. Burrous.** 1957. Hybridization between *Escherichia coli* and *Shigella*. *J. Bacteriol.* **74**:461-476.
26. **Lyras, D., J. R. O'Connor, P. M. Howarth, S. P. Sambol, G. P. Carter, T. Phumoonna, R. Poon, V. Adams, G. Vedantam, S. Johnson, D. N. Gerding, and J. I. Rood.** 2009. Toxin B is essential for virulence of *Clostridium difficile*. *Nature* **458**:1176-1179.
27. **McBride, S. M., and A. L. Sonenshein.** 2011. The *dlt* operon confers resistance to cationic antimicrobial peptides in *Clostridium difficile*. *Microbiology* **157**:1457-1465.
28. **McFarland, L. V.** 2008. Update on the changing epidemiology of *Clostridium difficile*-associated disease. *Nat. Clin. Pract. Gastroenterol. Hepatol.* **5**:40-48.
29. **Mullany, P., M. Wilks, L. Puckey, and S. Tabaqchali.** 1994. Gene cloning in *Clostridium difficile* using Tn916 as a shuttle conjugative transposon. *Plasmid* **31**:320-323.
30. **O'Brien, J. A., B. J. Lahue, J. J. Caro, and D. M. Davidson.** 2007. The emerging infectious challenge of *Clostridium difficile*-associated disease in Massachusetts hospitals: clinical and economic consequences. *Infect. Control Hosp. Epidemiol.* **28**:1219-1227.

31. **O'Connor, J. R., D. Lyras, K. A. Farrow, V. Adams, D. R. Powell, J. Hinds, J. K. Cheung, and J. I. Rood.** 2006. Construction and analysis of chromosomal *Clostridium difficile* mutants. *Mol. Microbiol.* **61**:1335-1351.
32. **Ohye, D. F., and W. G. Murrell.** 1973. Exosporium and spore coat formation in *Bacillus cereus* T. *J. Bacteriol.* **115**:1179-1190.
33. **Owens, R. C.** 2007. *Clostridium difficile*-associated disease: changing epidemiology and implications for management. *Drugs* **67**:487-502.
34. **Panessa-Warren, B. J., G. T. Tortora, and J. B. Warren.** 1997. Exosporial membrane plasticity of *Clostridium sporogenes* and *Clostridium difficile*. *Tissue & cell* **29**:449-461.
35. **Poutanen, S. M., and A. E. Simor.** 2004. *Clostridium difficile*-associated diarrhea in adults. *CMAJ : Canadian Medical Association journal = journal de l'Association medicale canadienne* **171**:51-58.
36. **Ramarao, N., and D. Lereclus.** 2005. The InhA1 metalloprotease allows spores of the *B. cereus* group to escape macrophages. *Cell. Microbiol.* **7**:1357-1364.
37. **Rety, S., S. Salamitou, I. Garcia-Verdugo, D. J. Hulmes, F. Le Hegarat, R. Chaby, and A. Lewit-Bentley.** 2005. The crystal structure of the *Bacillus anthracis* spore surface protein BclA shows remarkable similarity to mammalian proteins. *J. Biol. Chem.* **280**:43073-43078.
38. **Rupnik, M., B. Dupuy, N. F. Fairweather, D. N. Gerding, S. Johnson, I. Just, D. M. Lyrly, M. R. Popoff, J. I. Rood, A. L. Sonenshein, M. Thelestam, B. W. Wren, T. D. Wilkins, and C. von Eichel-Streiber.** 2005. Revised nomenclature of *Clostridium difficile* toxins and associated genes. *J. Med. Microbiol.* **54**:113-117.
39. **Sebahia, M., B. W. Wren, P. Mullany, N. F. Fairweather, N. Minton, R. Stabler, N. R. Thomson, A. P. Roberts, A. M. Cerdeno-Tarraga, H. Wang, M. T. Holden, A. Wright, C. Churcher, M. A. Quail, S. Baker, N. Bason, K. Brooks, T. Chillingworth, A. Cronin, P. Davis, L. Dowd, A. Fraser, T. Feltwell, Z. Hance, S. Holroyd, K. Jagels, S. Moule, K. Mungall, C. Price, E. Rabinowitsch, S. Sharp, M. Simmonds, K. Stevens, L. Unwin, S. Whithead, B. Dupuy, G. Dougan, B. Barrell, and J. Parkhill.** 2006. The multidrug-resistant human pathogen *Clostridium difficile* has a highly mobile, mosaic genome. *Nat. Genet.* **38**:779-786.

40. **Smith, C. J., S. M. Markowitz, and F. L. Macrina.** 1981. Transferable tetracycline resistance in *Clostridium difficile*. *Antimicrob. Agents Chemother.* **19**:997-1003.
41. **Sorg, J. A., and A. L. Sonenshein.** 2010. Inhibiting the initiation of *Clostridium difficile* spore germination using analogs of chenodeoxycholic acid, a bile acid. *J. Bacteriol.* **192**:4983-4990.
42. **Steichen, C. T., J. F. Kearney, and C. L. Turnbough, Jr.** 2007. Non-uniform assembly of the *Bacillus anthracis* exosporium and a bottle cap model for spore germination and outgrowth. *Mol. Microbiol.* **64**:359-367.
43. **Surawicz, C. M., and L. V. McFarland.** 1999. Pseudomembranous colitis: causes and cures. *Digestion* **60**:91-100.
44. **Surowiec, D., A. G. Kuyumjian, M. A. Wynd, and C. E. Cicogna.** 2006. Past, present, and future therapies for *Clostridium difficile*-associated disease. *Ann. Pharmacother.* **40**:2155-2163.
45. **Sylvestre, P., E. Couture-Tosi, and M. Mock.** 2002. A collagen-like surface glycoprotein is a structural component of the *Bacillus anthracis* exosporium. *Mol. Microbiol.* **45**:169-178.
46. **Thompson, B. M., H. Y. Hsieh, K. A. Spreng, and G. C. Stewart.** 2011. The co-dependence of BxpB/ExsFA and BclA for proper incorporation into the exosporium of *Bacillus anthracis*. *Mol. Microbiol.* **79**:799-813.
47. **Thompson, B. M., and G. C. Stewart.** 2008. Targeting of the BclA and BclB proteins to the *Bacillus anthracis* spore surface. *Mol. Microbiol.* **70**:421-434.
48. **Voth, D. E., and J. D. Ballard.** 2005. *Clostridium difficile* toxins: mechanism of action and role in disease. *Clin. Microbiol. Rev.* **18**:247-263.

**CONTACT INFORMATION**

Name: James Bregg Reedy

Professional Address: c/o Dr. Joseph Sorg  
Texas A&M University  
Department of Biology  
3258 TAMU  
College Station, TX 77843

Email Address: bregg1@msn.com

Education: B.S., Biology, Texas A&M University, May 2012  
Undergraduate Research Scholar