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# Redesigning gfp Reporter System for Utilization in Clostridium Difficile

Laura E. Fitzgerald

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# **Redesigning *gfp* Reporter System for Utilization in *Clostridium Difficile***

An Honors Thesis submitted in partial fulfillment  
Of the requirements for Honors Studies in  
Biological Sciences

By

Laura E. Fitzgerald

Spring 2016  
Biological Sciences  
J. William Fulbright College of Arts and Sciences  
**The University of Arkansas**

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## Abstract

*Clostridium difficile* (*C. difficile*) is a gram-positive bacterium that comprises part of the healthy human gut microbiome. When it gains sufficient access to peptides, *C. difficile* flourishes and releases tissue-damaging toxins, which cause inflammation of the colon that can develop into a *Clostridium difficile* Infection (CDI).<sup>10</sup> The Ivey Laboratory believes that the best tactic in preventing CDIs is stopping peptide ingestion, which theoretically could be accomplished by manipulating the oligopeptide permease (App) system.<sup>7</sup> In order to verify that altering the App system would successfully impede peptide uptake, first the expression of the app Promoter Region (*appProR*) of *C. difficile*'s DNA needs to be better understood. This characterization can be accomplished by fusing *appProR* to the *gfp*-reporter gene, which codes for Green Fluorescent Protein (GFP). GFP emits green fluorescent light when exposed to blue or ultraviolet light, and the degree of fluorescence can be used to quantify the gene expression of whatever DNA sequence to which the *gfp*-reporter gene is fused.<sup>9</sup>

The specific aim of this project was to incorporate the *appProR-gfp*-reporter gene complex first into *Escherichia coli* (*E. coli*), and then into *Bacillus subtilis* (*B. subtilis*). Those two bacterial species were chosen as hosts for the transformations, for *E. coli* and *B. subtilis* are known for being more receptive to recombinant DNA techniques than *C. difficile*.<sup>22</sup> By ligating the *appProR-gfp*-reporter gene sequence of pUA321 to pG<sup>+</sup>host4, the resulting plasmid, pUA625, contained a broad enough host range to transform both gram-negative *E. coli* and gram-positive *B. subtilis*. Those successful transformations indicate that pUA625 could be integrated into *C. difficile* in the future, an achievement which would lead to a better understanding of the expression of *C. difficile*'s App system.

## Introduction

*Clostridium difficile* is a gram-positive, pathogenic obligate anaerobe that resides in the human gastrointestinal tract. As is typical of many strictly anaerobic bacteria that inhabit the human gut, *C. difficile* produces endospores as a means of pathogenesis and, ultimately, survival.<sup>10</sup> While vegetative *C. difficile* cells are anaerobic, its spores do not require oxygen-free conditions and can persist in a variety of environments for extended periods of time.<sup>18</sup> Once a potential host consumes *C. difficile* spores, the spores can develop into vegetative, toxin-releasing cells. These cytotoxins damage epithelial colon tissue and cause what has appropriately been termed a *Clostridium difficile* Infection.<sup>10</sup> CDIs often involve pseudomembranous colitis, or inflammation of the large intestine. Symptoms of CDI-associated pseudomembranous colitis can include abdominal pain and fever, but its most characteristic presentations are diarrhea (which can range from mild to extreme) and the accompanying unpleasant odor. The more severe cases can be lethal, earning CDIs the reputation of being the main cause of gastroenteritis fatalities in the United States.<sup>3</sup> From 2005 to 2009 the CDI prevalence rate doubled to 140 cases per 10,000 hospital discharges, resulting in upwards of 500,000 new instances of CDI in the U.S. each year.<sup>3,4</sup> These alarming statistics demonstrate how critical it is that better CDI treatment options are developed.

While *C. difficile* is a standard part of the human gut microbiome, CDIs do not become an issue until the normal intestinal flora has become disrupted. This disruption most often occurs through the use of antibiotics, thus explaining why CDIs are almost exclusively restricted to patients in hospitals who are receiving antibiotic therapy.<sup>3</sup> Antimicrobial treatments specifically involving penicillin analogues, fluoroquinolones,

clindamycin, or cephalosporins are implicated in most CDI incidences.<sup>11</sup> These antibiotics kill a large portion of the bacteria in the gastrointestinal tract, but the spores of *C. difficile* are resistant to these antimicrobials.<sup>10</sup> When other intestinal bacterial species

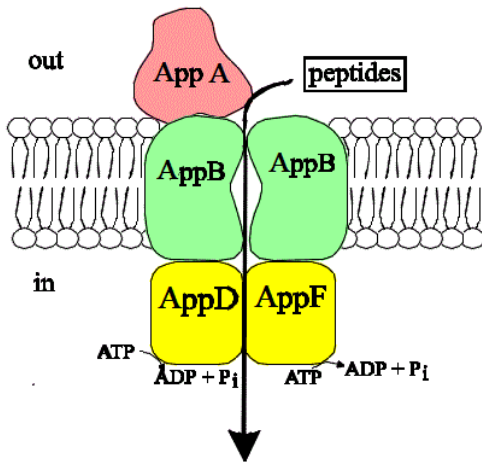


Figure 1: Subunits of the App System; image generated using the Microsoft Paint Application.

have been suppressed, *C. difficile* no longer has to compete for its nutrient of choice, peptides.

The uptake of these peptides is facilitated by the oligopeptide permease (App) system, particularly by subunits App A, B, D, and F (figure 1).<sup>7</sup> The App system also plays a role in the colonization abilities of *C. difficile*, for App

A gives *C. difficile* the ability to bind to intestinal epithelial cells.<sup>7</sup>

Once well-nourished *C. difficile* has attached to the luminal side of the epithelial cells of the colon, it begins releasing cytotoxins A and B. It is hypothesized that the release of these toxins is prompted by the peptides' dual function as pheromones. Gram-positive bacteria, such as *Bacillus subtilis* and *Staphylococcus aureus*, have been known to utilize peptide pheromone systems as a means of chemical signaling to stimulate synchronized cellular responses among bacterial communities.<sup>6</sup> Though this phenomenon has not yet been confirmed in *C. difficile*, the notion that the uptake of peptides communicates to *C. difficile* that the weakened gut microbiome is now vulnerable to its toxins seems a likely possibility.

Following their release from the vegetative *C. difficile* cells, the cytotoxins bind to the epithelial intestinal cells' receptors and enter the cytosol of their new host cells. Next,

Toxins A and B target the host Rho GTPases, which are proteins that regulate numerous cellular processes, including transcription, phagocytosis, actin cytoskeleton maintenance, and other vital activities.<sup>5</sup> The cytotoxins alter the Rho GTPases via mono-O-glucosylation, a modification that disrupts the functionality of the Rho GTPases and eventually leads to cell death by apoptosis (figure 2). This widespread intestinal epithelial cell death causes inflammation of the colon, which then progresses into a CDI.<sup>5</sup>

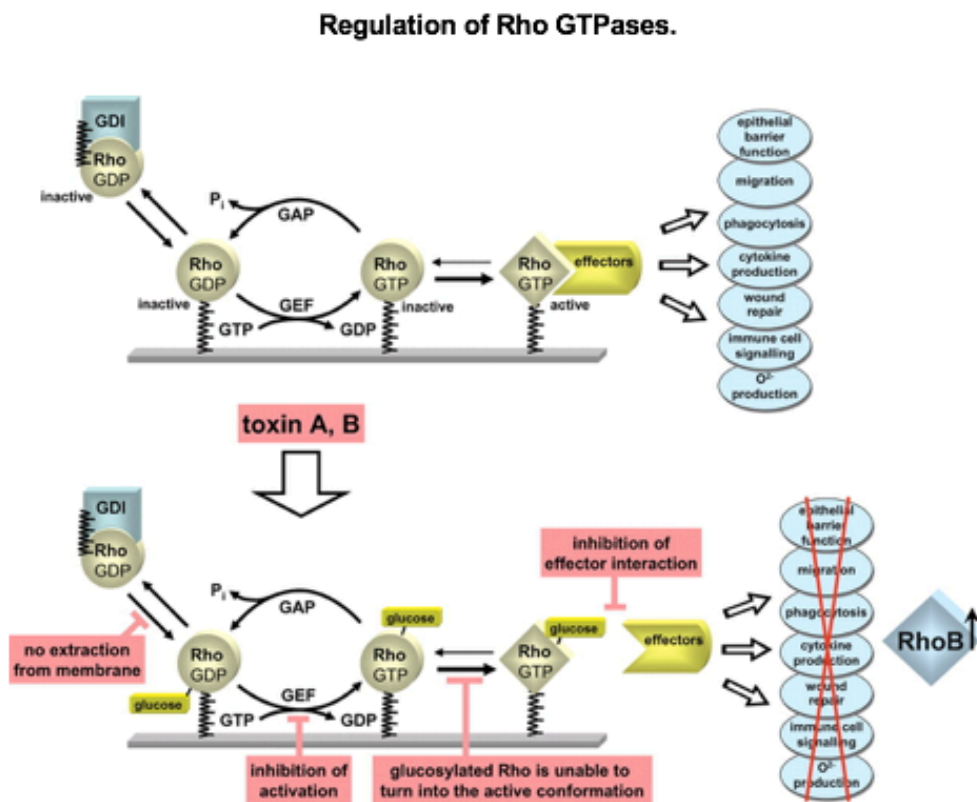


Figure 2: Effects of Glucosylation by Toxin A and B on RhoGTPases.<sup>14</sup>

Once a patient has been diagnosed with a CDI, the first step of treatment involves stopping whatever antibiotics the patient is currently consuming. Next, the patient is typically prescribed a different antibiotic that specifically targets the endospore-forming capacities of *C. difficile*, a skill that is crucial for the survival and consequent pathogenesis of the bacterium. Without its highly resistant spores, *C. difficile* can no



longer withstand penicillin or other common antibiotic treatments associated with CDIs; therefore, *C. difficile* cannot persist long enough to gain access to peptides and produce its infection-causing toxins. Historically vancomycin has been thought to be the most effective antibiotic in battling CDIs, but recent studies have offered a better option: fidaxomicin. While both drugs rapidly reduce the amount of vegetative cells in CDI patients, patients receiving vancomycin have a much higher recurrence rate. Fidaxomicin's dominance is likely attributed to two factors: it causes a larger decrease in *C. difficile* spore counts, and it maintains a greater portion of the healthy gastrointestinal microbiome. By preserving other bacterial species, *C. difficile* remains starved for peptides and is unable to cause an infection. While fidaxomicin may produce better results than vancomycin, the 20% relapse rate of patients using fidaxomicin suggest that there is still plenty of room for CDI treatment improvement.<sup>12</sup>

Studies suggest that *C. difficile* is evolving at a rapid rate, become more virulent and even less responsive to traditional antibiotic treatments. This worrisome observation highlights the need for a new type of CDI therapy. A promising alternative to drugs is the fecal microbiota transplant (FMT). As its name suggests, FMT is the transfer of fecal matter from a healthy individual into the gastrointestinal tract of a CDI patient. The healthy stool sample contains normal gut flora, and the re-introduction of those bacteria into the colon of the CDI patient reestablishes bacterial homeostasis and disturbs *C. difficile*'s unregulated proliferation. While the gruesome nature of an FMT may not seem like a desirable option for CDI patients, its impressive cure rate of roughly 94% will likely increase the frequency of FMTs in the future.<sup>18</sup>

Ideally, a technique should be developed that prevents CDIs from occurring and, thus, renders CDI antibiotic treatments and FMTs unnecessary. The work performed in the Ivey Laboratory operates under the notion that the best method of stopping CDIs is to inhibit *C. difficile*'s peptide intake, which could have a two-fold affect. First, vegetative *C. difficile* cell growth should be hindered, for the lack of nutrient consumption should limit *C. difficile*'s proliferation capacities. Ceasing peptide ingestion could also obstruct cytotoxin release, assuming that peptides do indeed act as a communication method in *C. difficile*. In theory, this goal of peptide restriction could be accomplished by manipulating the *app* promoter region (*appProR*), which is the sequence of DNA that controls gene expression of the peptide-consuming App system.<sup>7</sup> Before this manipulation can occur, however, a better understanding of *appProR* is required. To further characterize this segment of DNA, the Ivey Laboratory utilizes the green fluorescent protein (GFP). GFP is a protein comprised of 238 amino acids and is found in the jellyfish species *Aequorea victoria*.<sup>9,17</sup> When exposed to U.V. or blue light, GFP emits green fluorescence.<sup>9</sup> Since its discovery, this fluorescent aspect of GFP has been used to analyze various cellular activities, including gene expression.<sup>17</sup> By fusing the *gfp*-reporter gene to the DNA sequence of choice and cultivating cells containing this gene construct, the degree of fluorescence in the resulting colonies serves as an indicator of that specific gene's expression. Since the Ivey Laboratory aims to characterize the App system of *C. difficile*, the *gfp*-reporter gene has been attached to the *appProR* sequence in a plasmid called pUA321. By inserting the DNA sequence coding for the *appProR*-*gfp*-reporter gene complex into *C. difficile*, the amount of fluorescence in the transformed *C. difficile* cells can be quantified as a means of measuring the degree of *appProR* expression. Further

characterizing *appProR* expression should clarify whether or not manipulating the App system is a viable option for impeding *C. difficile* growth and pathogenesis.

Unfortunately, transforming *C. difficile* is no easy task. Before attempting to insert pUA321 into *C. difficile*, one must start with a more cooperative bacterial species. For this experiment, *E. coli* was chosen as the primary transformation candidate, for it is celebrated as being one of the most compliant bacterial species when subjected to recombinant DNA techniques.<sup>22</sup> Once the desired DNA sequence has successfully been inserted into the *E. coli* DNA, one can then attempt to transform a species that is more similar to *C. difficile*, such as *B. subtilis*. This transition is necessary because gram-negative *E. coli* (while it can be fairly easily transformed) is not a good model organism for how a gram-positive species such as *C. difficile* would react during a transformation. Therefore, the *appProR-gfp*-reporter gene complex from *E. coli* must be transferred to the more cooperative gram-positive *B. subtilis* before a *C. difficile* transformation would be a feasible endeavor.

The specific goal of this project is to transform *B. subtilis*, the realization of which would be indicated by the production of green fluorescent *B. subtilis* colonies. The successful recombination of the *appProR-gfp*-reporter gene construct into *B. subtilis* would pave the way for the future incorporation of the DNA sequence into *C. difficile*, which would help the Ivey Laboratory further analyze *C. difficile*'s App system and bring CDIs one step closer to eradication.

## Materials and Methods

### Materials and Definitions:

Material	Definitions	Source
pUA321	Plasmid containing <i>appProR</i> and <i>gfp</i>	Ivey Lab
pG <sup>+</sup> host4	Broad host range plasmid containing Ori T <sub>s</sub>	Ivey Lab
pUA625	Plasmid created via the ligation of pUA321 and pG <sup>+</sup> host4	
LB Miller Broth	Base for maintenance and proliferation of <i>E. coli</i>	IBI Scientific
Brain Heart Infusion (BHI) Broth	Base for maintenance and proliferation of <i>B. subtilis</i>	EMD
Bacto agar	Solidifying agent for LB, BHI broth media	SIGMA Chemical Company
Erythromycin (Em)	Antibiotic used to select for transformed cells containing pUA625 and to prevent unwanted bacterial growth	Ivey Lab
UP101	Universal primer	Bio-Rad
AppProR	Primer for <i>appProR</i>	IDT
Master mix, 2x	Mixture containing Taq DNA polymerase, dNTPs, MgCl <sub>2</sub> , and reaction buffer; used for PCR amplification of DNA templates ranging from 0.2-2 kb	Promega
Vent	DNA polymerase; proofreads and prevents mutations in DNA of PCR product	New England BioLabs, Inc.
dH <sub>2</sub> O	Deionized water	Ivey Lab
TAE, 1x	Buffer utilized in gel electrophoresis	Ivey Lab
NEBuffer 2.1	Digest buffer	New England BioLabs, Inc.
HindIII	Restriction enzyme	Life Technologies
EcoRI	Restriction enzyme	Promega

XbaI	Restriction enzyme	Life Technologies
T4 Buffer, 10x	Buffer for ligation involving T4 ligase	New England BioLabs, Inc.
T4 Ligase	Ligation enzyme	New England BioLabs, Inc.
MicroElute Cycle Pure Kit	Kit containing materials and protocol for a purification	Omega Bio-Tek
MicroElute DNA Cleanup Kit	Kit containing materials and protocol for a DNA purification	Omega Bio-Tek
DH5 $\alpha$	Electrocompetent <i>Escherichia coli</i> cells	Invitrogen
BD366	Electrocompetent <i>Bacillus subtilis</i> cells	Ivey Lab
Glycerol	Base for the stock solution containing pUA625	Ivey Lab
Plasmid DNA Maxi Kit	Kit containing materials and protocol for a large-scale plasmid preparation	Omega Bio-Tek
NaCl, 5M	Component of ethanol precipitation	Ivey Lab
Ethanol, 95%, 70%	Components of ethanol precipitation	Ivey Lab
SeaKem GTG Agarose	Agarose specifically designed for gel electrophoresis	FMC BioProducts
SYBR Safe	DNA stain used in gel electrophoresis; gets excited by blue light	Life Technologies
1 kb marker	Creates a ladder from which the size of the sample is assessed via gel electrophoresis	Ivey Lab
Loading buffer, 2x	Dyes the sample DNA so that it can be assessed via gel electrophoresis	Ivey Lab

## Methods:

### **Media and Growth Conditions:**

#### LB Miller Broth, Erythromycin Plates:

The medium for the LBEm<sup>500</sup> plates was prepared using 200mL H<sub>2</sub>O, 5g LB Miller Broth, 3g bacto agar, and 1mL Em<sup>100</sup>. The transformed *E. coli* (DH5 $\alpha$ ) cells were spread on these plates, which were then placed in a 30°C incubation chamber for 7 days.

#### LB Miller, Erythromycin Broth Culture:

The broth culture utilized the same medium as the LBEm<sup>500</sup> plates described above. A fluorescent colony from the LBEm<sup>500</sup> plates was picked and placed in a test tube containing LBEm<sup>500</sup> broth. The test tube was placed in a 37°C shaking incubator overnight.

This broth culture was also used in an inoculation involving the glycerol stock solution of the transformed *E. coli* cells. The inoculated flask was placed in the 37°C shaking incubator overnight.

#### Brain Hearth Infusion (BHI) broth, Erythromycin Plates:

The medium for the BHIEm<sup>1</sup> plates was prepared using 200mL H<sub>2</sub>O, 7.4g BHI broth, 3g bacto agar, and 20 $\mu$ L Em<sup>10</sup>. The transformed *B. subtilis* (BD366) cells were spread on these plates, which were then placed in a 30°C incubation chamber for 3 days.

#### Electroporation Recovery Media:

The recovery medium used for each electroporation product corresponded to the plates onto which the transformed cells were spread; the recovery medium for the transformed DH5 $\alpha$  cells was LBEm<sup>500</sup>, while the recovery medium for the transformed BD366 cells was BHIEm<sup>1</sup> broth.

## **Recombinant DNA Techniques:**

### Polymerase Chain Reaction (PCR):

PCR is a standard method for rapidly producing numerous copies of a DNA segment.<sup>20</sup> The first step of PCR is combining the plasmid possessing the DNA sequence of interest, the master mix, deionized water, and two primers that mimic the beginning and ending nucleotide sequences that encompass the desired DNA segment. Next, that mixture is placed in some sort of heating device, such as a thermocycler. The thermocycler heats the sample to three different temperatures, the first of which being the temperature required to denature the double-stranded DNA of the plasmid. Once the two DNA strands have been separated, the sequences are ready to act as templates for the construction of new strands. Thus begins the annealing stage, during which the temperature is reduced to allow the primers to adhere to their complementary sequences on the now single-stranded DNA segments. Finally, the thermocycler raises the temperature once more to the optimum temperature at which the specific DNA polymerase functions. During this last step, the DNA polymerase prompts the deoxynucleotide triphosphates (dNTPs) from the master mix to bind to their complimentary nucleotides on the DNA segment, starting with the primer. By the end of this final stage, the single copy of double-stranded DNA coding for the desired DNA segment has now become two identical copies. This three-stage cycle can be repeated as many times as necessary until the desired amount of DNA copies have been created.<sup>20</sup>

The components of the PCR typically include master mix, vent, the target plasmid, two primers associated with that plasmid, and dH<sub>2</sub>O. While the master mix contains Taq, the additional DNA polymerase vent is often utilized because it is capable

proofreading the new DNA strands and eliminating any potential mutations that occur during the elongation process. For this experiment, the thermocycler was set to 94°C for the denaturing stage, which lasted 30 seconds. The second step ran for 45 seconds at 45°C, which was deemed the appropriate annealing temperature for the AppProR and UP101 primers. The final stage occurred at 72°C, the optimum temperature for the DNA polymerases Taq and vent; this step lasted for 60 seconds. This three-step cycle was set to repeat 30 times.

#### Restriction Enzyme Digests:

The purpose of a restriction enzyme digest is typically to produce compatible ends of DNA sequences that can be ligated together, or “sticky ends.” DNA digests typically involve restriction enzymes that recognize 6-8 consecutive bases.<sup>21</sup> This project included two digests in preparation for a ligation: a pUA321 digest and a pG<sup>+</sup>host4 digest. The pUA321 digest consisted of 11µL purified pUA321 PCR product, 32µL dH<sub>2</sub>O, 5µL NEBuffer 2.1, 1µL HindIII, and 1µL EcoR1. The pG<sup>+</sup>host4 digest consisted of 1µL pG<sup>+</sup>host4, 42µL dH<sub>2</sub>O, and equivalent amounts of NEBuffer 2.1, HindIII, and EcoR1 as outlined in the pUA321 digest. Both digests utilized the same restriction enzymes, HindIII and EcoR1, in the hopes of producing sticky ends for a future ligation. Each digest was placed in the 37°C shaking incubator for 1 hour.

Restriction enzyme digests can also be used as a means of preparing a plasmid for transformation. Studies have shown that transformations of naturally competent *B. subtilis* cells produce better results when transformed with linear DNA rather than circular DNA.<sup>8</sup> While this linear DNA preference has not been shown conclusively for transformation via electroporation, a digest was performed on the ethanol precipitation



product (EPP) in the hopes of increasing the transformation efficiency of BD366 with the EPP. The components of this restriction enzyme digest were 2 $\mu$ L EPP, 5 $\mu$ L NEBuffer 2.1, 1 $\mu$ L XbaI, and 42 $\mu$ L dH<sub>2</sub>O. This digest was also placed in the 37°C shaking incubator for 1 hour.

#### Ligation:

Ligations are performed to unify two linear DNA fragments into one circular piece of DNA. The components of a ligation include a ligation enzyme, the ligation buffer associated with that enzyme, and the two digests that are being joined. In this experiment, the ligation mixture was incubated at room temperature overnight.

#### **Purification Techniques:**

##### MicroElute Cycle Pure Kit:

This purification kit is typically used to remove impurities from PCR products. The protocol in this kit was followed for two purifications during this experiment: the purification of the pUA321 PCR product and the purification of the pUA321 and pG<sup>+</sup>host4 digests prior to their ligation.

##### MicroElute DNA Cleanup Kit:

This purification kit is typically used to remove impurities from DNA segments that have been subjected to enzymatic reactions of some sort. The protocol in this kit was followed to purify the EPP digest prior to the transformation of the BD366 cells via electroporation.

#### **Electroporation of DH5 $\alpha$ and BD366:**

Electroporation is the most common physical technique utilized to transform bacterial cells. Electroporation uses a high-strength electrical pulse to generate temporary

pores in the membranes of the electrocompetent cells. The plasmid containing the DNA sequence to be incorporated into the bacterial DNA may enter the cells through those pores and be recombined into the electrocompetent cells.<sup>1</sup> First, the plasmid of interest is pipetted into a solution of electrocompetent cells. This mixture is pipetted into a chilled cuvette, which is then placed into a chilled cuvette holder. The cuvette holder is positioned into the electroporator, which is set according to the needs of the electrocompetent cells being utilized. After the electrical charge is applied, the time constant is measured. Finally, recovery medium is added to the cuvette before the contents of the cuvette are transferred to a Falcon tube for incubation. In this experiment, the Falcon tube was placed in the 37°C shaking incubator for 2 hours prior to plating the transformed cells.

#### **Glycerol Stock Solution:**

A glycerol stock solution (created from the fluorescent *E. coli* colony) was produced so that the transformed *E. coli* cells containing the pUA625 could be stored in the -80°C deep freezer and retrieved during future related experiments.

#### **Inoculation:**

A flask containing LBEm<sup>500</sup> broth was inoculated with 100µL glycerol stock solution in order to grow cells to be used for the maxi plasmid preparation.

#### **Plasmid Preparation:**

##### Plasmid DNA Maxi Kit:

This plasmid preparation kit produces high copy number plasmid DNA, which is desirable when executing a bacterial transformation.

**Ethanol Precipitation:**

The purpose of an ethanol precipitation is to concentrate DNA through the centrifugation, washing, and consequent drying of the DNA of interest. The first step in an ethanol precipitation is to add NaCl and ethanol to the plasmid DNA. The mixture is then centrifuged to produce a pellet. The pellet is then washed with ethanol, dried by vacuum centrifugation, and then suspended in a solution.

**Horizontal Agarose Gel Electrophoresis:**

Agarose gel electrophoresis is a means of separating DNA fragments based on size. The DNA being evaluated is placed in wells at one end of the gel, and an electric current is then applied to the gel. The negatively-charged phosphate backbone of the DNA causes the fragments to move towards the positively charged anode. As the fragments are migrating through the gel, the network of agarose particles acts as a filter and catches the DNA fragments in a size-dependent manner; the smaller the DNA fragment, the more easily it can move through the agarose pores and, thus, the farther it will travel in the gel.<sup>15</sup>

This project utilized the Embi Tec “RunOne Electrophoresis Unit” when performing horizontal agarose gel electrophoresis. The gel itself was composed of TAE, SeaKem GTG agarose, and SYBR Safe. A 1 kb marker, which produces multiple bands of varying sizes, was run during every gel that was executed. By simultaneously running the 1 kb marker with the DNA samples, the ladder created by the marker allowed the relative sizes of the DNA samples to be estimated. These size estimations served as a means of verifying various experimental steps, for the expected size of the samples was

known. The gels, therefore, were extremely helpful in validating the success of the other experimental methods (figures 3, 4, 8, and 9).

**Fluorescence Microscopy:**

Fluorescence microscopy is a technique that separates fluorescence excitation and detection into two distinct light paths. By juxtaposing the axis of illumination and the axis of detection in a perpendicular manner, the amount of excitation light that is captured in the image is reduced. This means that the final image is a clearer, better representation of the degree of fluorescence being emitted by the cells.<sup>19</sup>

## Results

**PCR amplification of Papp:gf<sub>p</sub>.** A fragment suitable for cloning to the app promoter was produced by PCR, using pUA321 as target DNA, and the primers AppProR and UP101. The purpose of the PCR was to create numerous linear copies of the DNA

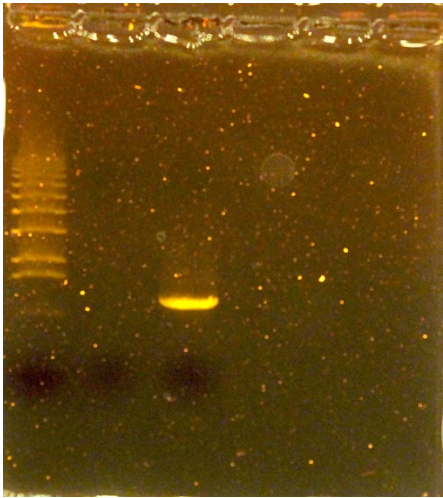


Figure 3: Gel electrophoresis of pUA321 PCR Product.

segment that codes for the desired *appProR-gfp*-reporter gene sequence, with flanking DNA sequences containing restriction endonuclease sequences for the subsequent cloning experiment. The PCR product was subjected to horizontal gel electrophoresis, which revealed a band of 1.2 kb (figure 3), corresponding to the Papp:gf<sub>p</sub> gene fragment.

The PCR product was then subjected to the MicroElute Cycle Pure Kit in preparation for the subsequent ligation. Horizontal gel electrophoresis was then utilized to verify the success of this purification (figure 4); the 1.2 kb band produced was consistent with the expected size of the purified PCR product.

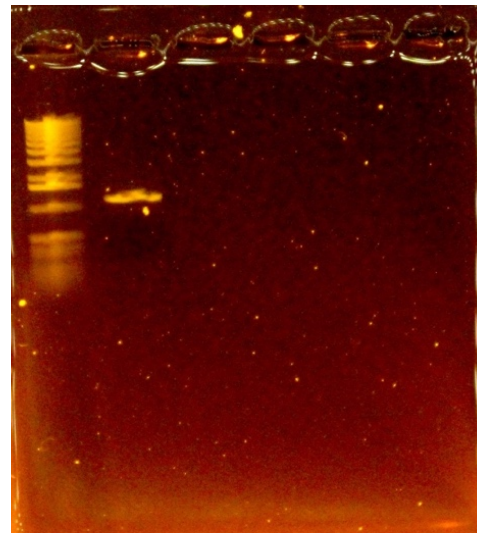


Figure 4: Gel electrophoresis of purified pUA321 PCR Product.

**Ligation of pUA321 and pG<sup>+</sup>host4.** The ligation was executed utilizing T4 ligase and T4 ligation buffer to unite the purified pUA321 digest with the purified pG<sup>+</sup>host4 digest. This fusion of pUA321 with pG<sup>+</sup>host4 was a vital step in the experiment, for pUA321 would be unable to replicate in both *E. coli* and *B. subtilis* if left to its own devices. The plasmid pG<sup>+</sup>host4, however, contains a broad host range that permits it to survive in a variety of gram-negative and gram-positive bacterial species.<sup>13</sup> Therefore, the ligation was carried out to insert the *appProR-gfp*-reporter sequence from pUA321 into pG<sup>+</sup>host4 to create a new plasmid, pUA625 (figure 5). This new plasmid was utilized in all subsequent transformations and steps in the project.

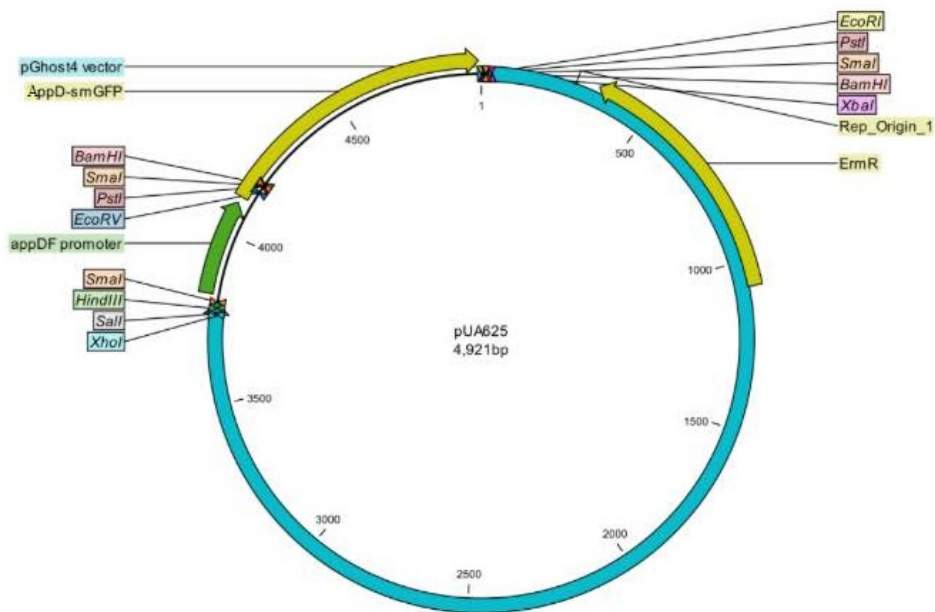


Figure 5: Plasmid Map of pUA625; image generated from the nucleotide sequence using CLC-Bio DNA Workstation.

**Electroporation of *E. coli* cells.** This electroporation was conducted to incorporate pUA625 into electrocompetent *E. coli* cells, or DH5 $\alpha$  cells. This experiment used the Bio-Rad Electroporator, which was set to 25 $\mu$ F, 200 Ohms, and 1.7kV/cm pulse rate, as is dictated by the DH5 $\alpha$  cells. The time constant was recorded as 4.1 ms, which fell into the range of satisfactory time constants for DH5 $\alpha$  cells (3.5 to 4.5 ms).

The time constant of the transformed *E. coli* cells suggested that the electroporation was successful, so the cells were spread on an LBEm<sup>500</sup> plate. The cells produced a green fluorescent colony when subjected to epilluminescence with blue light (figure 6).

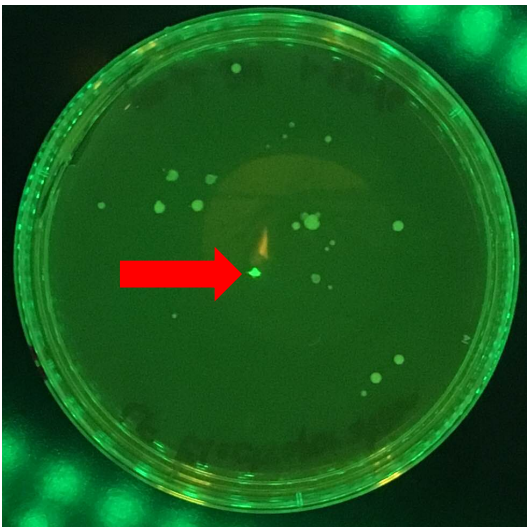


Figure 6: A fluorescent *E. coli* colony on an LBEm<sup>500</sup> plate. This image was captured with a standard Canon camera that was covered with an orange lens.

**Fluorescence microscopy of transformed *E. coli* cells.** Cells from the fluorescent *E. coli* colony (figure 6) were subjected to fluorescence microscopy using a Zeiss Fluorescent AxioImager M1 Upright Motorized Microscope in conjunction with AxioVision Image Analysis Software, yielding an image of bright green cells (figure 7).

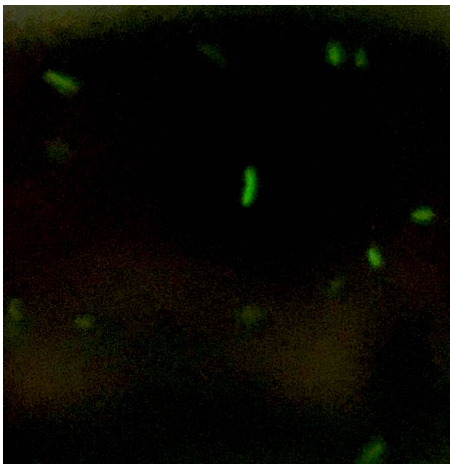


Figure 7: Fluorescent *E. coli* cells.

**Plasmid preparation of transformed *E. coli* cells.** A plasmid preparation was performed according to the protocol outlined in the Plasmid DNA Maxi Kit. This step was executed in order to produce a large amount of the transformed *E. coli* DNA containing the *appProR-gfp*-reporter sequence, increasing the likelihood that the upcoming *B. subtilis* transformation would be successful. The product of this plasmid preparation was subjected to horizontal agarose gel electrophoresis (figure 8), which produced a blurry band of roughly 5 kb that was consistent with the expected band size of the plasmid. The smallest bands (less than 500 bp) indicated the presence of residual RNA.

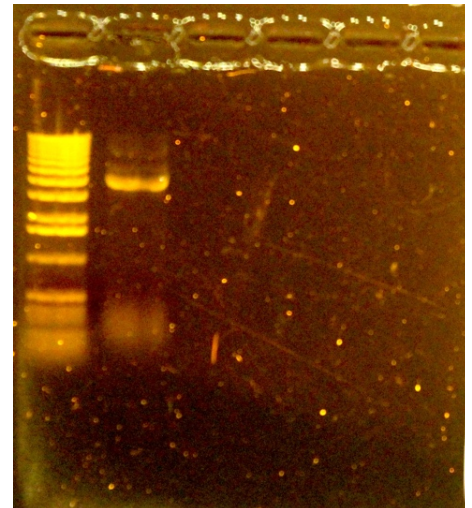


Figure 8: Gel electrophoresis of large-scale plasmid preparation product.



**Ethanol precipitation of the large-scale plasmid preparation product.** The purpose of this precipitation was to concentrate the DNA from the preceding plasmid preparation product in order to increase the chances for a successful subsequent *B. subtilis* transformation. This ethanol precipitation was executed as described by Lever et al (2015), which started by adding 0.2 volumes 5 M NaCl and 2.5 volumes ethanol (95 %) to the plasmid preparation product.<sup>16</sup> After centrifuging at 20,000 x g for 10 minutes, the pellet was washed with 70% ethanol and dried by vacuum centrifugation before being suspended in 10 mM Tris-C (pH 8.0). The resulting ethanol precipitation product (EPP) was then subjected to horizontal agarose gel electrophoresis (figure 9). Electrophoresis produced a band of approximately 5 kb, as was expected of the EPP. Additional large bands arose due to DNA supercoiling, as well small bands (less than 500 bp) indicative of residual RNA.

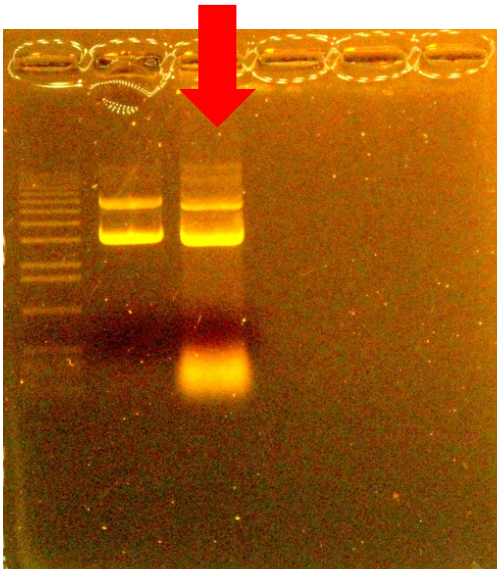


Figure 9: Gel electrophoresis of EPP (third well).

**Electroporation of *B. subtilis* cells.** Another electroporation was executed, this time involving the integration of the purified, digested EPP into electrocompetent *B. subtilis* (BD366) cells. For this electroporation, the Bio-Rad Electroporator was set to 25 $\mu$ F, 400 Ohms, and 1.4kV/cm pulse rate. The time constant was recorded as 5.3ms, which fell under the range of suitable time constants for BD366 cells (4.8 to 5.8 ms).

The time constant of the transformed BD366 cells, suggested that the electroporation was successful, so the cells were spread on a BHIEm<sup>1</sup> plate. The cells produced multiple green fluorescent colonies when subjected to transillumination with blue light (figure 10). The fluorescent colonies were labeled pUA630, a designation which will be used when referring to this strain of transformed *B. subtilis* cells containing the *appProR-gfp*-reporter gene sequence in future Ivey Laboratory experiments.

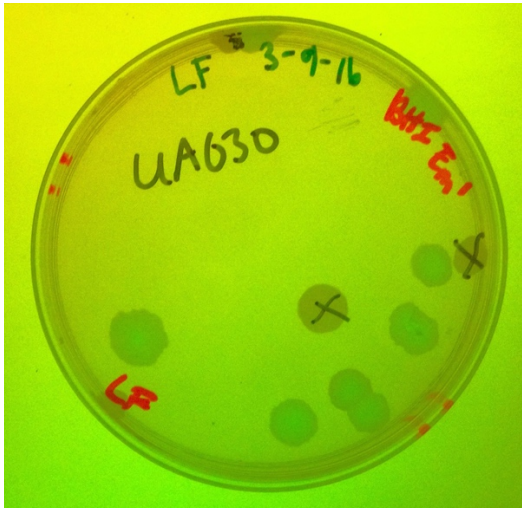


Figure 10: Fluorescent *B. subtilis* colonies on a BHIEm<sup>1</sup> plate. The colonies marked through with an "X" are non-fluorescent. This image was captured with a standard Canon camera that was covered with an orange lens.

**Fluorescence Microscopy of transformed *B. subtilis* cells.** Cells from the fluorescent pUA630 colonies (figure 10) were subjected to fluorescence microscopy using a Zeiss Fluorescent AxioImager M1 Upright Motorized Microscope in conjunction with AxioVision Image Analysis Software microscope, producing an image of vibrant green cells (figure 11).

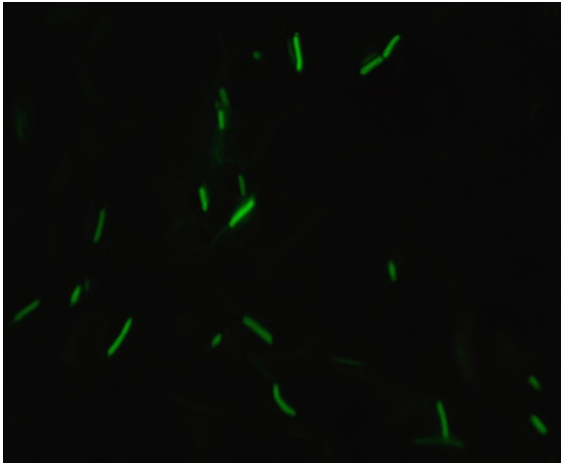


Figure 11: Fluorescent *B. subtilis* cells.

## Discussion

The first achievement of this experiment was the successful cloning of plasmid pUA625 through the ligation of the purified pUA321 and pG<sup>+</sup>host4 digests. This step was essential for the future success of this project, for the *appProR-gfp*-reporter gene sequence from pUA321 would not have the host range potential to be incorporated into *C. difficile* without pG<sup>+</sup>host4. The creation of pUA625 was verified when the transformation of the *E. coli* cells with the ligation product produced fluorescent colonies, since *E. coli* colonies are not naturally fluorescent and must have been expressing the green fluorescent protein from pUA625. Once the *appProR-gfp*-reporter construct was integrated into *E. coli*, the next phase of the experiment was to incorporate pUA625 into *B. subtilis*. This step would be more indicative of the likelihood that a future transformation of the plasmid into *C. difficile* would work, since *B. subtilis* behaves more similarly than *E. coli* to *C. difficile* as a host organism. *B. subtilis* is not as easily transformed as *E. coli*, so the outcome of the second electroporation was less certain. Thankfully, the transformed *B. subtilis* strain (pUA630) produced fluorescent colonies, signifying that this electroporation was also executed effectively.

The incorporation of the *appProR-gfp*-reporter gene into *B. subtilis* was a triumph for the Ivey Laboratory, for it created a model organism in which various aspects of *C. difficile* can be further characterized. *B. subtilis*'s sporulation developmental program, for example, is one promising target for future research. Since endospore formation is crucial for *C. difficile* survival, one could attempt to modify the spore-forming capacities of pUA630 and then apply those findings to *C. difficile*. Additionally, pUA630 could be used in conjunction with the results from previous projects conducted in the Ivey

Laboratory. CodY, a protein found in gram-positive bacterial species, has been found to bind and repress the expression of the App promoter in response to *C. difficile*'s nutritional environment.<sup>2</sup> This repressive function could be instrumental in preventing CDIs, for stopping the expression of the App system would render the bacterium incapable of ingesting peptides and perhaps hinder cytotoxin release. By conducting experiments to further analyze CodY's suppressive effects on pUA630's *appProR* sequence, the protein's mechanisms could be better understood and, hopefully, exploited in *C. difficile*. By taking advantage of the similarities shared between *B. subtilis* and *C. difficile*, the Ivey Laboratory can determine which methods would be most effective in preventing *C. difficile* proliferation by performing tests on a much safer experimental host, pUA630.

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