Bryant University

HONORS THESIS

Cloning and Characterization of the Cell Wall Acting Enzyme CD1034 from the Pathogen Clostridium difficile

BY: Zachary Suter

ADVISORS • Christopher Reid

Submitted in partial fulfillment of the requirements for graduation
with honors in the Bryant University Honors Program

APRIL 2012

Table of Contents

Cloning and Characterization of the Cell Wall Acting Enzyme CD1034 from the Pathogen *Clostridium difficile*
Senior Capstone Project for Zachary Suter

ABSTRACT

The manifestation of multidrug resistance in bacteria over the past several decades has resulted in one of the foremost challenges in the management of infectious diseases. The question arises, "How do we address this growing problem?" One solution to stem the growing rise in antimicrobial resistance is to investigate new targets, while another approach is to re-examine classical antibacterial targets with a fresh perspective. The aim of this paper is to begin the process of antibacterial development for the pathogen *Clostridium difficile* by characterizing the cell wall acting glucosaminidase CD1034. It is inunderstanding how CD1034 functions biochemically that it can be targeted for antibacterial development. The gene CD1034 was successfully cloned and sequenced in this project. Results have implications in the further development of an antibiotic for *C. difficile* and other Gram-positive pathogens.

INTRODUCTION

Hospitals today are ridden with disease, whether it is from the patients entering to be treated or the dormant bacteria loitering within. Nosocomial infections are defined by the World Health Organization (WHO) as infections acquired by a patient who was admitted for reasons other than that infection (World Health Organization Department of Communicable Disease, Surveillance and Response, 2002). These infections are prevalent world-wide, from hospitals in developing nations to countries such as the United States. In this study by WHO, it was found that nosocomial infections were most common at the sites of surgical wounds, in the urinary tract, and in the lower respiratory tract. Nosocomial infections are one of the leading causes of death in hospitals, and can also lead to a reduction in the quality of life of patients due to excess stress and emotions from the infection. Many of these bacterial infections are becoming larger problems due to the bacteria's resistance to antibiotics.

Antibiotic resistance in a particular bacterium can happen in a few different ways. If the bacterium is constantly being exposed to a specific type of antibiotic treatment, there are ways in which it is able to adapt to not be affected by that treatment. This can happen in one of two ways. The first is through the mutation of chromosomes, which is extremely rare. The second method, which is far more common, is through a method called transmissible drug resistance, or TRD for short (Black's Veterinary Dictionary, 2005). This is a method where bacteria that carry what are known as R factors (resistance factors) in their cytoplasm are able to pass the R factors on to other bacteria via contact. The R factors are transferred through amedium called plasmids. This most commonly happens between bacteria that occupy the intestinal tracts of humans, and usually occurs when the person who is hosting the bacteria is receiving antibiotics for another infection. When that patient receives antibiotics, it disrupts the normal colonic flora and makes the conditions perfect for the R factor transfer. This can cause a patient who is already infected to become infected with a new bacterium, one which is antibiotic resistant.

One particular type of bacteria that is becoming particularly troublesome in hospitals is *Clostridium difficile* (*C. difficile*). *C. difficile*is a gram-positive organism, which means that the cell wall is made of a thick layer of peptidoglycans. This bacterium currently has no easy

and effective treatment, and has not responded to any type of antibiotics thus far. *C. difficile* is a fecal organism that is rampant in hospitals and the major cause of hospital acquired cases of diarrhea (Youngson, 2005). *C. difficile* targets those patients in a hospital that are at the highest risk for nosocomial infections. These are the elderly patients, those who are immunocompromised, and those who are on antibiotic regiments. Those who are on antibiotics are at risk because the antibiotics disrupt the normal bacteria of the bowel, allowing the bacterium to become well-established in the colon and intestines (Webster's New World Medical Dictionary). Those who are elderly and immunocompromised are at risk of non-normal flora, which, as previously stated, is the height of when *C. difficile*will establish itself in the body. It is also a tough bacterial infection to fight because some patients show symptoms of infection rather quickly, while others do not show them whatsoever and become what is known as a "carrier." These carriers are dangerous because *C. difficile* is a spore-producing bacterium and can quickly and easily spread from the carriers to the new victims. The ability of the bacterium to produce spores, create carriers within the hospital environment, and infect and re-infect those patients at the highest risk are the main reasons why the spread of *C. difficile*has significantly increased over the past decade (Wheeldon, Worthington, Hilton, Elliott, & Lambert, 2008). Figure 1 reveals infection rates for those patients aged 65 and older since only 1996, as shown in a study by the Centers for Disease Control and Prevention.

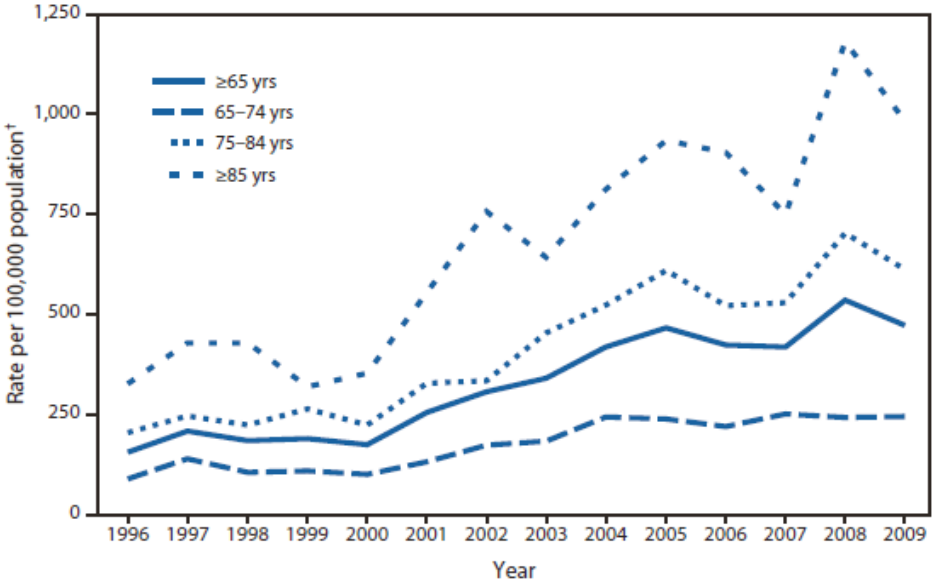


Figure 1 – Infection Rates of C. difficile *in US Hospitals from 1996 – 2009*

The figure shows increases in infection rates of 175% for those aged 65 – 74 years old, 198% for those aged 75 – 84, and 201% for those aged ≥85 years of age. A total infection rate increase of 200% for all patients aged ≥65 was found in this study as well. This is an enormous increase from 1996 alone, never mind from decades prior. This study by the CDC confirms prior studies that the elderly are at a higher risk of infection as well(Centers for Disease Control and Prevention, 2011).

C. difficile is not only a major problem for patients in the hospital, but also to the hospital itself.Hospitals are at risk of becoming subject to extremely high treatment costs when looking into infections caused by *C. difficile*. Average daily costs for treating infections causes by *C. difficile* ranges from 17.6 – 51.5 million dollars (Stabler, 2009). The range of costs for first-time infected patients is $2,871 - $4,846 per case and $13,655 - $18,067 per case for those patients who are re-infected (Ghantoji, Sail, Lairson, DuPont, & Garey, 2010). This leads to costs in excess of $1 billion in the US annually. These numbers are staggering, and are far too high for hospitals struggling to control costs. The need for an antimicrobial now is extremely high. According to Herbert Dupont, other researchers are in the field looking to develop a way to inhibit the infection process, much like this project intends to do. However, some of those projects are looking to develop a way to restore or preserve the colonic flora while taking antibiotics (Dupont, 2011). This approach is new and innovative,

much like the approach this project takes on to inhibit the cell wall adhesion process of *C. difficile* to the intestine wall.

This project will focus on the beginning stages of antimicrobial development for *C. difficile*, more specifically the cloning and characterization of the cell wall acting enzyme CD1034. CD1034 is a glucosaminidase specific to *C. difficile*, however has homologs in other Gram-positive pathogens, which will lead to outside applications of the results. A glucosaminidase is an enzyme which has the ability to hydrolyze peptidoglycans, which means that CD1034 cleaves the glycosidic bond via the addition of water. This is very important due to the fact that *C. difficile*is gram-positive with that thick layer of peptidoglycans in its cell wall. From this, it is hypothesized that CD1034 is annotated in the in the *C. difficile* genome to act on bacterial peptidoglycan as a β-glucosaminidase with the ability to cleave peptidoglycans. Overall, this project seeks to begin the discovery process of potential proteins that will be able to be developed further into methods of preventing or stopping the infection process of *C. difficile*. The lab work, data collected, and further analysis and conclusions that will be developed over the course of this Senior Capstone will help all further researchers in taking this project a step further into developing an antimicrobial against *C. difficile*.

METHODS AND MATERIALS

Getting to Know the Lab

The first week of work for this Capstone began with learning more about the equipment I would be using daily throughout the course of the experimentation. A sample of the equipment I learned about this beginning week is:

1. Pipets (used to measure out small amounts of chemicals for solutions and can be easily misued),
2. Autoclave (used to sterilize equipment such as flasks in a high pressure/high heat environment for use in experimentation),
3. Polymerase chain reaction machine, and
4. Gel electrophoresis (used to run the polymerase chain reaction solutions in to check for success in the reaction against a base).

This first week of learning about the equipment to be used was crucial for the success of the remainder of the project.

PCRof CD1034 with AccuTaq Polymerase

A 50x TAE buffer was first made for the gel electrophoresis stage of this step. This buffer was a solution of Trizma base, glacial acetic acid, EDTA, and deionized water. With this buffer made, the first of three polymerase chain reactions (PCR) was set up. The purpose of this reaction is to amplify the CD1034 gene, the cell wall acting enzyme that is strongly thought to be involved in *Clostridium difficile*growth and division. The reagents of the reaction included genomic DNA Cd630, which is the DNA of *C. difficile* that contains the gene CD1034. The Sigma AccuTaq PCR Kit was used for this reaction, which utilizes taq polymerase. Taq polymerase is one of the more cost-effective and commonly used polymerases on the market. The DNA primer AV-1 was used as the forward primer and has the sequence:

5’ – AAGACCCCATATGGCTAGAAAATTAATAAAAAATTTGGG – 3’

This forward primer is specifically engineered with an NdeI restriction site for cloning into the plasmid pET28. The reverse primer AV-3 was used with the sequence:

5’ – TCTCGAGTTATTTTGTCTCAACTTTATTATCTATC – 3’

This reverse primer was also engineered specifically with an XhoI restriction site for cloning into the plasmid pET28. The conditions of the first PCR can be found in Table 1.

Table 1 – Conditions of the PCR of CD1034 with AccuTaq Polymerase

Denaturing	Annealing			Extension	Sit Stage
98º C	94º C	55º C	68º C	68º C	4º C
48 sec.	20 sec.	20 sec.	1 min.	10 min.	Infinite

The reaction was analyzed in a 1% agarose gel electrophoresis, which is a scientific technique that separates a population of DNA by size. This step is crucial in determining if the PCR was successful in replicating the CD1034 portion of the genomic DNA. A GeneRuler 1 kb ladder was placed into the first lane of the gel and the reaction was placed into the second lane of the gel. The GeneRuler ladder is run against the reaction because the section of the DNA that is expected to show on the gel (it will be a thick stain if the DNA was replicated because the DNA is separated by size and amount) is approximately 1000 bases from the start of the strand.

Effect of $MgCl_2$on PCR of CD1034 withAccuTaq Polymerase

A second PCR was performed to amplify CD1034. The same genomic DNA, PCR kit, and forward and reverse primers were used as from the first PCR, however additional magnesium chloride ($MgCl_2$) was added in different levels to this reaction. The first reaction contained an additional 0.5 µL (microliters) of $MgCl_2$. The second reaction contained an additional 1 µL of $MgCl_2$. The third reaction contained an additional 1.5 µL of $MgCl_2$. Finally, the fourth reaction contained an additional 2 µL of $MgCl_2$. These four concentrations of $MgCl_2$ were added to try to find the ideal conditions in which the PCR would run. The conditions of the PCR can be seen in Table 2. The gel was run on these four PCR products, with the GeneRuler in the first lane, the four different reactions mentioned above in lanes 2-5 (respectively) and the genomic DNA in lane 6.

Table 2 –Conditions of the PCR of CD1034 with AccuTaq Polymerase & Addition of $MgCl_2$

Denaturing	Annealing			Extension	Sit Stage
98º C	94º C	50º C	68º C	68º C	4º C
48 sec.	20 sec.	20 sec.	1 min.	10 min.	Infinite

PCR of CD1034 with 5 Prime PCR Extender System

The third attempt at amplifying CD1034 was taken in a different direction. The 5 Prime PCR Extender System was used in place of the Sigma AccuTaq PCR Kit, in addition to the same genomic DNA and primers as stated in previous reactions. The 5 Prime PCR Extender System includes a polymerase that is a blend of thermostable DNA polymerases with a processivity-enhancing factor that provides a high extension rate and maximal proofreading capability and also two different types of buffer – tuning buffer and high fidelity buffer. Four reactions were developed for this PCR, two reactions with tuning buffer and two reactions with high fidelity buffer. The two reactions for each buffer included different levels of genomic DNA Cd630 as to find the optimal level. The conditions of the PCR can be seen in Table 3. The gel was run with the following lanes:

1. GeneRuler 1 kb ladder
2. Tuning Buffer with Mg with 1.25 µL of genomic DNA CD630
3. Tuning Buffer with Mg with 2.5 µL of genomic DNA CD630
4. High Fidelity Buffer with Mg with 1.25 µL of genomic DNA CD630
5. High Fidelity Buffer with Mg with 2.5 µL of genomic DNA CD630.

Table 3 – Conditions of the PCR using the 5 Prime PCR Extender System

Denaturing	Annealing			Extension	Sit Stage
93º C	93º C	55º C	68º C	68º C	12º C
2 min.	30 sec.	45 sec.	1 min.	10 min.	Infinite

Clean-up of PCR Reactions

The successful polymerase chain reaction solution must be cleaned up before any further experimentation can take place. A cleanup of this reaction was performed using a Sigma GenElute PCR Cleanup Kit. This cleanup was performed to rid the solution of any excess nucleotides that were not used in the reaction, excess primers, and other excess material from the PCR. The manufacturer's instructions were followed to clean up the successful PCR solution. First, DNA is bound on a silica membrane within the spin column. The DNA bound to the column is washed and the clean DNA is eluted in buffer. This purifies and recovers

approximately 95% of PCR product and more than 99% of primers and primer-dimers are removed, leaving product ready for further experimentation.

Isolation of Plasmids

pET28 is the plasmid that is to be used for the purposes of this project. This particular plasmid was propagated from Escherichia coli DH5α competent cells. The plasmids were prepared using a Sigma GenElute Plasmid MiniPrep Kit. The manufacturer's instructions were followed to prepare the plasmids. The cells were first harvested from recombinant *E. coli*. Next, the cultured cells were re-suspended in a re-suspension solution and from here they were lysed by the addition of a Lysis Solution. The solution was mixed by means of gentle inversion as to prevent the shearing of genomic DNA. The cells are then neutralized and gently inverted again, and this solution is then transferred to a binding column. The elution is performed, and thus the DNA is now present in the eluate and ready for experimentation.

RestrictionDigest of CD1034 & pET28

CD1034 and pET28 were digested after being cleaned and prepared. The purpose of the digestion process is to ensure that all DNA fragments that have been replicated are the same size. The reagents for the digestion included 10 µL of nuclease-free H_2O, 2 µL of 10x buffer, 6 µL of the successful PCR product, 6 µL of the vector (pET28 plasmid), and also 0.5 µL of both NdeI and XhoI. The product of the restriction digest was placed on the hot block for approximately one hour, and after that hour an addition 0.5 µL of both NdeI and XhoI were added to the solution.

Ligation of CD1034 into pET28

The first ligation was begun with a double restriction site cut of the vector. The reagents in the ligation included 2 µL of 10x buffer for T4 DNA ligase with 10 mM ATP, 10 µL of PCR insert, 5 µL of vector, 1 µL of ligase, and 2 µL of sterilized H_2O. The resulting solution was plated and left to grow overnight in the incubator.

The second ligation was begun with individual cuts of both the promoter and terminator ends of the vector, which would increase the accuracy of the cut to nearly 100%. Two trials with individual cuts were made, and the reagents for these ligations can be seen in Table 4. Both ligation products were plated and left to grow overnight in an incubator.

Table 4 – Reaction Conditions for Ligations with Individual Restriction Site Cuts

	10 ng Vector:60 ng Insert	10 ng Vector:120 ng Insert
Vector	0.8 µL	0.8 µL
Insert	1 µL	2 µL
10x Buffer	1 µL	1 µL
Ligase	0.5 µL	0.5 µL
H_2O	6.7 µL	5.7 µL
Total Volume	10 µL	10 µL

Sequencing of Successful Ligation

The colonies of the successful ligation were sent to the Rhode Island Genomics and Sequencing Center to be sequenced. The sequencing results were promptly sent back to Bryant Labs to be further analyzed and discussed.

RESULTS

Different attempts to amplify the gene CD1034 were made in the beginning stages of this project. After three attempts, the polymerase chain reaction to amplify CD1034 was best when using the 5 Prime PCR Extender Kit. The specific reaction using this kit that was successful had the addition of the Tuning Buffer with magnesium to 1.25 microliters of genomic DNA. With the addition of the magnesium, this adds a positively charged ion to the reaction, thus helping the negatively charged genomic DNA to react differently. The results of the first PCR with only AccuTaq polymerase can be seen in Appendix A. Appendix B shows the results from the second PCR with the AccuTaq polymerase and addition of $MgCl_2$. Finally, Appendix C shows the gel electrophoresis results from the third and final PCR with the 5 Prime PCR Extender System. These results show that lane 2 (lane assignments are from left to right, starting at lane 1) of the agarose gel electrophoresis (the successful reaction) has the stain at approximately the 1000 base pair line on the GeneRuler, thus confirming successful amplification of the CD1034 gene from the PCR. The successful PCR reaction was the one in which tuning buffer was used in combination with 1.25 25 µL of genomic DNA Cd630.

After a successful polymerase chain reaction was completed, it was then found that ligation was only successful when the cutting of the plasmid was performed in two separate steps – the forward cut and the reverse cut. The attempts at DNA ligation were unsuccessful when a more efficient and less accurate double cut were made, thus the extra step is necessary for 100% accuracy. A 12:1 ratioof PCR insert to vector was also found to be successful in ligation.

Finally, the sequencing results revealed that the gene CD1034 was successfully transformed into the plasmid, pET28. Appendix D shows the forward sequencing results, revealing a 99% match of the gene sequenced check against the known sequence of CD1034. The 1% mistake is a silent transformation and is irrelevant. Appendix E shows the reverse sequence of the gene sequenced check against the known CD1034 sequence. This 99% match is also only off by 1% again because of a silent transformation that is irrelevant and does not change the gene itself.

DISCUSSION

Understanding the steps of the polymerase chain reaction process are key to seeing why it is possible to amplify a particular gene over a billion replications in just over 2 hours' time. In the denaturing stage, the DNA is brought to a high temperature to separate it into single strands (in laymen's terms, unzipping the DNA strand to its forward and reverse sequences). After this stage, the annealing stage begins. The beginning of this stage cools the DNA to allow for the primers to attach to their specific sites on the individual strands. The final stage, extension, is when the polymerase used in the process attaches to the priming sites and extends, or synthesizes, the strands into exact replications of the original DNA strands. This cycle of three stages is repeated 25-30 times, and this will yield upwards of 1 billion replications of the original strand of DNA if successful. The "sit" stage is not timed and is a setting in the PCR machine to allow for a reaction to run without human supervision and be ready to go when necessary.

It is also necessary to understand why the magnesium was added to the reactions and why it is important in a successful polymerase chain reaction. Magnesium, or some form of it (commonly used $MgCl_2$, or magnesium chloride) is necessary as a cofactor to the DNA polymerase used in the reaction. It is not consumed in the reaction, but without it the reaction will be unsuccessful. The phosphate backbone of DNA has a negative charge to it, and thus the positive charge of $MgCl_2$ blocks this negative charge and allows for the DNA polymerase to bind to the primers. Without this charge blockage, the polymerase would be unable to bind and thus the replication process would fail.

The type of polymerase was also a major factor in the success of the PCR. It was found that the polymerase from the 5 Prime PCR Extender System was successful. This more-expensive DNA polymerase is marketed as having an additional component that increases accuracy of the reaction and the proofreading capability. The buffers, both tuning and high fidelity, also add to the success of the reaction by better maintaining pH balance at high temperatures, thus reducing the possibility of DNA degradation at high temperatures.

One other point of discussion that was discussed in the Methods and Materials section was the plasmid used for experimentation. pET28 is the vector used, and it was chosen for a few reasons. The first is because of the presence of the Kan resistance factor, which is the

antibiotic resistance factor that is necessary for growth when plated. The second reason is because of the NdeI forward restriction site and XhoI reverse restriction site. These were easily engineered into the forward and reverse primers to allow for easy ligation. Finally, the vector has six histidines that are specifically engineered for easier post-transformation clean-up of the vector.

Finally, a discussion of how a ligation was deemed unsuccessful is necessary. This was determined when no colonies were found on the media plate the next day, thus confirming that no vectors were able to survive the conditions (they would have survived and formed colonies had the PCR insert successfully ligated into the vector). The ligation process was unsuccessfully attempted four trials; however it could not be determined why the ligation was not working. Finally, it was determined that instead of performing a less-accurate double cut of the vector; both cuts on the vector would be performed separately, thus ensuring 100% accuracy. The final reactions where individual restriction site cuts were made finally yielded success.

ACKNOWLEDGEMENTS

Throughout the course of this project, my advisor, Christopher Reid, has been nothing short of incredible. He has been able to help me move from very limited knowledge in the field of microbiology to being my own expert on this particular topic. He has always been there throughout the Honors Program Senior Capstone process, from the very beginning when I approached him about working together, to the grueling process of presenting the project and editing the manuscript. I would like to personally thank him again for everything because without him, this project would not have been possible.

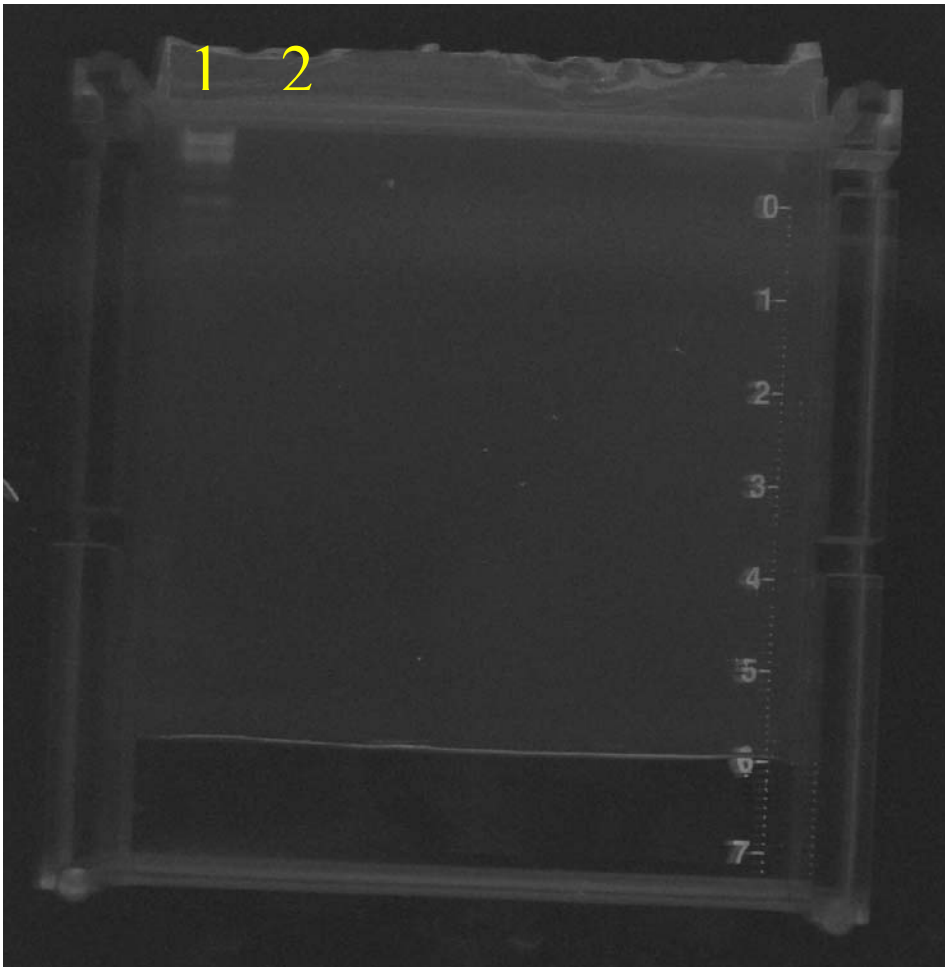
I would also like to thank Danielle Gutelius, an addition to my project as of the spring semester. She jumped right into helping me with some of my lab work, teaching me the new techniques I was getting into and, more importantly, helping me get through failed experiments. Again, this project wouldn't have gone as well without her help.

Kirsten Hokeness was also a major help in this process. She was able to direct me to Chris to get the project started, and has been a constant support when I needed it. She became my editorial reviewer and I could always count on her praising my work when I needed it most.

Finally, I would like to acknowledge the Rhode Island Genomics and Sequencing Center. "This research is based in part upon work conducted using the Rhode Island Genomics and Sequencing Center which is supported in part by the National Science Foundation (MRI Grant No. DBI-0215393 and EPSCoR Grant Nos. 0554548 & EPS-1004057), the US Department of Agriculture (Grant Nos. 2002-34438-12688 and 2003-34438-13111), and the University of Rhode Island." The RIGSC is funded by the same grant that helped fund this project. I would also like to acknowledge the Rhode Island INBRE Collaboration as well.

APPENDICES

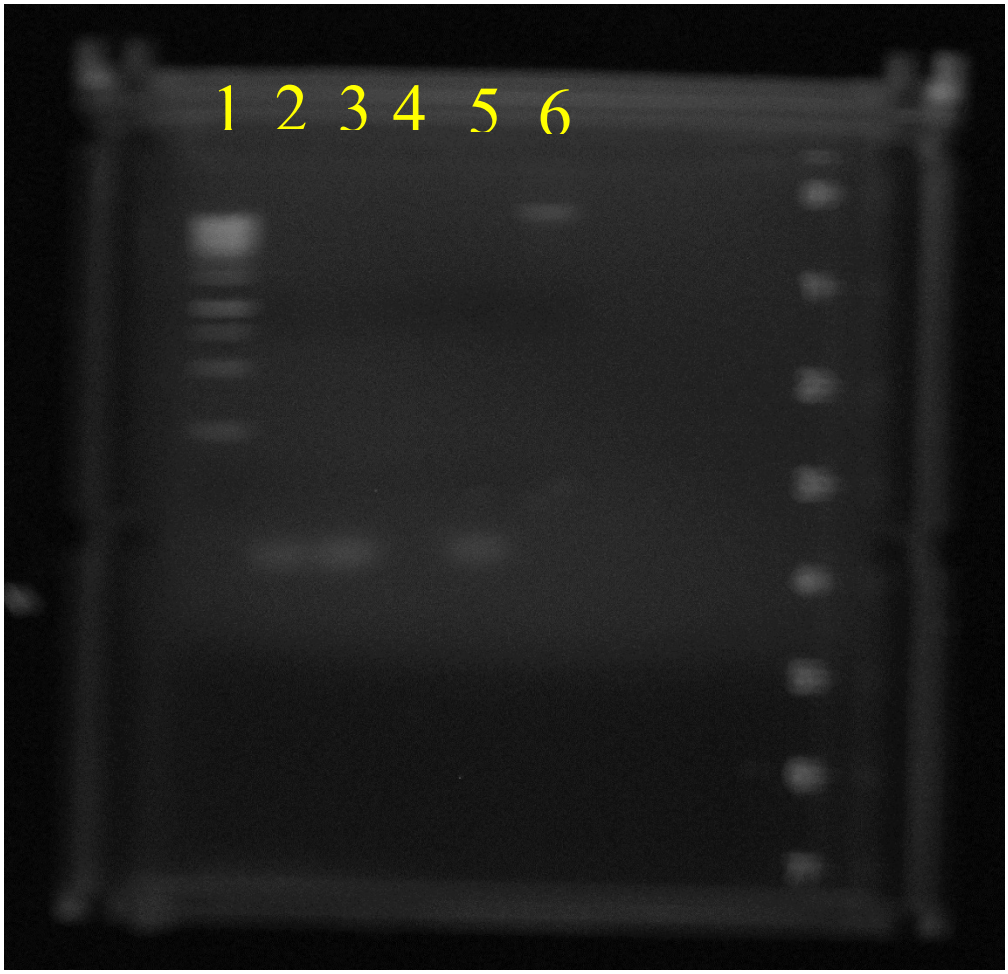
Appendix A – Gel Electrophoresis Output of PCR with AccuTaq Polymerase



First gel electrophoresis output with lane products as follows:

1. 1 kb GeneRuler
2. Product of PCR using AccuTaq Polymerase

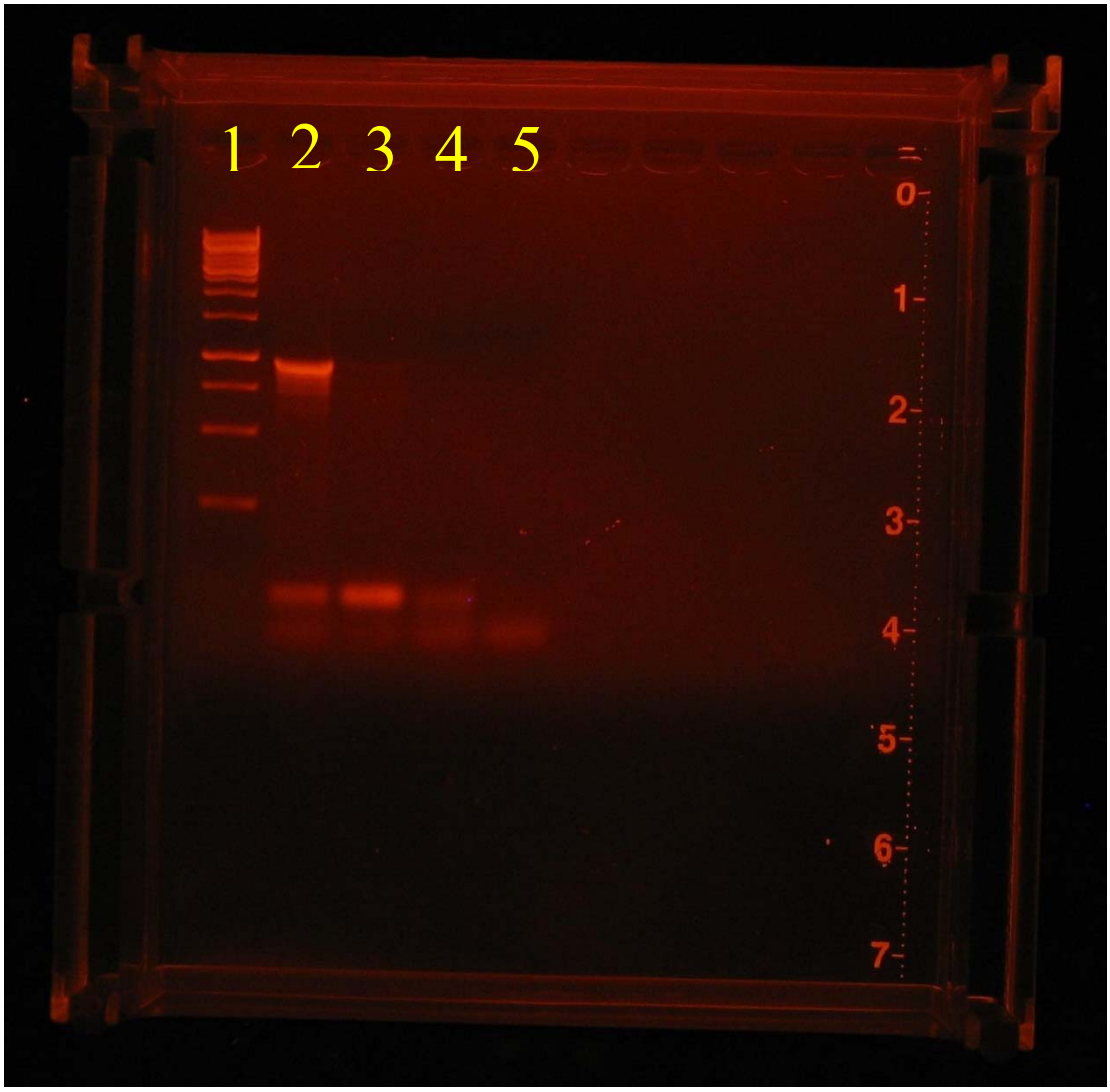
Appendix B – Gel Electrophoresis of PCR with AccuTaq Polymerase with Additional $MgCl_2$



The second gel electrophoresis output with lane products as follows:

1. GeneRuler 1 kb ladder
2. Product of PCR with additional 0.5 µL of $MgCl_2$
3. Product of PCR with additional 1 µL of $MgCl_2$
4. Product of PCR with additional 1.5 µL of $MgCl_2$
5. Product of PCR with additional 2 µL of $MgCl_2$
6. Genomic DNA Cd630

Appendix C – Gel Electrophoresis of PCR using 5 Prime PCR Extender System



The third gel electrophoresis output with lane products as follows:

1. GeneRuler 1 kb ladder
2. Tuning Buffer with Mg with 1.25 µL of genomic DNA CD630
3. Tuning Buffer with Mg with 2.5 µL of genomic DNA CD630
4. High Fidelity Buffer with Mg with 1.25 µL of genomic DNA CD630
5. High Fidelity Buffer with Mg with 2.5 µL of genomic DNA CD630

Appendix D – Forward Sequence of Successful Ligation Compared to Known CD1034 Sequence using Primer Complementary to the T7 Promotor on pET28

Forward Sequence – T7 Promoter Primer

```
>lcl|43923
Length=975

 Score = 1504 bits (814),  Expect = 0.0
 Identities = 816/817 (99%), Gaps = 0/817 (0%)
 Strand=Plus/Plus

Query  87   ATGGCTAGAAAATTAATAAAAAATTTGGGTAAAAGTAAAAGTGTAAAGAGAGTGAAACTT  146
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1    ATGGCTAGAAAATTAATAAAAAATTTGGGTAAAAGTAAAAGTGTAAAGAGAGTGAAACTT  60

Query  147  TTATTTAAGAAGATTTTTATTACTGTATTCATAGTAGCAAGTATAGTTGCTATTTTTAAT  206
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  61   TTATTTAAGAAGATTTTTATTACTGTATTCATAGTAGCAAGTATAGTTGCTATTTTTAAT  120

Query  207  ATAACGAAATATTTTGAGGAATTATACAAAGTAAGGGATTTAAAAAGTACTAAAATTGAA  266
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  121  ATAACGAAATATTTTGAGGAATTATACAAAGTAAGGGATTTAAAAAGTACTAAAATTGAA  180

Query  267  TACTATATGGATGTGGCTGATGAAGCAGGAGATGGAAAGGTCCAATTAAGTTGGAAGGCC  326
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  181  TACTATATGGATGTGGCTGATGAAGCAGGAGATGGAAAGGTCCAATTAAGTTGGAAGGCC  240

Query  327  TTACTTGCTATAGACATGGTAATACATGATGAAGATTTAAGTAATATAAAAAAGAAAGAC  386
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  241  TTACTTGCTATAGACATGGTAATACATGATGAAGATTTAAGTAATATAAAAAAGAAAGAC  300

Query  387  ACATTGGATATAGGGGAAAAGTTTATAGTAGAAGATAAAAATGATAAAGGCGAAAAGGTG  446
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  301  ACATTGGATATAGGGGAAAAGTTTATAGTAGAAGATAAAAATGATAAAGGCGAAAAGGTG  360

Query  447  TATAAGGTAAAAAAGTTTAATAAAGTATTAAGCGAATTGAAATTTGACTCTTCTCAAAAA  506
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  361  TATAAGGTAAAAAAGTTTAATAAAGTATTAAGCGAATTGAAATTTGACTCTTCTCAAAAA  420

Query  507  AGTAGAGCAAGAAAATACATGAAAGATTTAGAATACACATACCTTGGAAATAAACAATTA  566
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  421  AGTAGAGCAAGAAAATACATGAAAGATTTAGAATACACATACCTTGGAAATAAACAATTA  480

Query  567  GATAGTAGTGATGAAAAAATTAAATTTATAAAGAAGTTAGAAGACTCAGCTATAAGAGAA  626
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  481  GATAGTAGTGATGAAAAAATTAAATTTATAAAGAAGTTAGAAGACTCAGCTATAAGAGAA  540

Query  627  TATATTGATTATGGAATATTGCCCTCTATAACAATTGGACAAGCTATATTAGAATCTGGT  686
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  541  TATATTGATTATGGAATATTGCCCTCTATAACAATTGGACAAGCTATATTAGAATCTGGT  600

Query  687  TGGGGAAATTCTAAACTTACAAAACAGAGTAATAATTTATTTGGTATAAAAGCAGATAAA  746
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  601  TGGGGAAATTCTAAACTTACAAAACAGAGTAATAATTTATTTGGTATAAAAGCAGATAAA  660

Query  747  GCATGGAAAGGAAAAAGTGTAGAAATTTCAACTTCAGAGCATTATAATGAAAAAATTGTT  806
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  661  GCATGGAAAGGAAAAAGTGTAGAAATTTCAACTTCAGAGCATTATAATGAAAAAATTGTA  720

Query  807  GCTAGTTTTAGGTCGTATAATTCATTACAAGATTCTGTCAAAGACCACAGTTTATTTTTA  866
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  721  GCTAGTTTTAGGTCGTATAATTCATTACAAGATTCTGTCAAAGACCACAGTTTATTTTTA  780

Query  867  ATTAATAATAAAAGATATAGAAAACATGGGTTGTTTG  903
            |||||||||||||||||||||||||||||||||||||
Sbjct  781  ATTAATAATAAAAGATATAGAAAACATGGGTTGTTTG  817
```

Appendix E –Reverse Sequence of Successful Ligation Compared to Known CD1034 Sequence using Primer Complementary to the Polymerase Termination Sequence on pET28

Reverse Complement – T7 Terminator Primer

```
>lcl|56645
Length=975

 Score = 1676 bits (907),  Expect = 0.0
 Identities = 913/916 (99%), Gaps = 1/916 (0%)
 Strand=Plus/Minus

Query  47   TTATTTTGTCTCAACTTTATTATCTATCAATTGCAAATTATAACTCCTAATAACATCTAT  106
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  975  TTATTTTGTCTCAACTTTATTATCTATCAATTGCAAATTATAACTCCTAATAACATCTAT  916

Query  107  TAATAGCTCTGCATATATACGATTTCCTTTTTTATCTTCTGCTGTACTATATCCTGCATT  166
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  915  TAATAGCTCTGCATATATACGATTTCCTTTTTTATCTTCTGCTGTACTATATCCTGCATT  856

Query  167  TTCTAATGCTTGAGCTTGACTAATATAATCTTTTGCCTCAAACAACCCATGTTTTCTATA  226
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  855  TTCTAATGCTTGAGCTTGACTAATATAATCTTTTGCCTCAAACAACCCATGTTTTCTATA  796

Query  227  TCTTTTATTATTAATTAAAAATAAACTGTGGTCTTTGACAGAATCTTGTAATGAATTATA  286
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  795  TCTTTTATTATTAATTAAAAATAAACTGTGGTCTTTGACAGAATCTTGTAATGAATTATA  736

Query  287  CGACCTAAAACTAGCAACAATTTTTTCATTATAATGCTCTGAAGTTGAAATTTCTACACT  346
            |||||||||||||||| ||||||||||||||||||||||||||||||||||||||||||
Sbjct  735  CGACCTAAAACTAGCTACAATTTTTTCATTATAATGCTCTGAAGTTGAAATTTCTACACT  676

Query  347  TTTTCCTTTCCATGCTTTATCTGCTTTTATACCAAATAAATTATTACTCTGTTTTGTAAG  406
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  675  TTTTCCTTTCCATGCTTTATCTGCTTTTATACCAAATAAATTATTACTCTGTTTTGTAAG  616

Query  407  TTTAGAATTTCCCCAACCAGATTCTAATATAGCTTGTCCAATTGTTATAGAGGGCAATAT  466
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  615  TTTAGAATTTCCCCAACCAGATTCTAATATAGCTTGTCCAATTGTTATAGAGGGCAATAT  556

Query  467  TCCATAATCAATATATTCTCTTATAGCTGAGTCTTCTAACTTCTTTATAAATTTAATTTT  526
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  555  TCCATAATCAATATATTCTCTTATAGCTGAGTCTTCTAACTTCTTTATAAATTTAATTTT  496

Query  527  TTCATCACTACTATCTAATTGTTTATTTCCAAGGTATGTGTATTCTAAATCTTTCATGTA  586
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  495  TTCATCACTACTATCTAATTGTTTATTTCCAAGGTATGTGTATTCTAAATCTTTCATGTA  436

Query  587  TTTTCTTGCTCTACTTTTTTGAGAAGAGTCAAATTTCAATTCGCTTAATACTTTATTAAA  646
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  435  TTTTCTTGCTCTACTTTTTTGAGAAGAGTCAAATTTCAATTCGCTTAATACTTTATTAAA  376

Query  647  CTTTTTTACCTTATACACCTTTTCGCCTTTATCATTTTTATCTTCTACTATAAACTTTTC  706
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  375  CTTTTTTACCTTATACACCTTTTCGCCTTTATCATTTTTATCTTCTACTATAAACTTTTC  316

Query  707  CCCTATATCCAATGTGTCTTTCTTTTTTATATTACTTAAATCTTCATCATGTATTACCAT  766
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  315  CCCTATATCCAATGTGTCTTTCTTTTTTATATTACTTAAATCTTCATCATGTATTACCAT  256

Query  767  GTCTATAGCAAGTAANGCCTTCCAACTTAATTGGACCTTTCCATCTCCTGCTTCATCAGC  826
            |||||||||||||||| ||||||||||||||||||||||||||||||||||||||||||
Sbjct  255  GTCTATAGCAAGTAAGGCCTTCCAACTTAATTGGACCTTTCCATCTCCTGCTTCATCAGC  196

Query  827  CACATCCATATAGTATTCAATTTTAGTACTTTTTAAATCCCTTACTTTGTATAATTCCTC  886
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  195  CACATCCATATAGTATTCAATTTTAGTACTTTTTAAATCCCTTACTTTGTATAATTCCTC  136
```

REFERENCES

Antibiotic.(2000). In *Dictionary of Environmental Science and Technology*. Retrieved from http://www.credoreference.com/entry/wileyenvsci/antibiotic

Antibiotic Resistance.(2005). In *Black's Veterinary Dictionary*. Retrieved from http://www.credoreference.com/entry/acbvet/antibiotic_resistance

Centers for Disease Control and Prevention. (2011, September 2). Rates of Clostridium difficile Infection Among Hospitalized Patients Aged >65 Years. *Morbidity and Mortality Weekly Report*, 60.

Clostridium difficile.(2005). In *Collins Dictionary of Medicine*. Retrieved from http://www.credoreference.com/entry/collinsmed/clostridium_difficile

Clostridium difficile.(2003). In *Webster's New World™ Medical Dictionary*. Retrieved from http://www.credoreference.com/entry/webstermed/clostridium_difficile

Dupont, H. L. (2011). The search for effective treatment of Clostridium difficile infection. *The New England Journal of Medicine*, 473-475.

Ghantoji, S., Sail, K., Lairson, D., DuPont, H., & Garey, K. (2010). Economic healthcare costs of Clostridium difficile infection: a systematic review. *The Journal of Hospital Infection*, 309-318.

Polymerase Chain Reaction. (2008). In *The Columbia Encyclopedia*. Retrieved from http://www.credoreference.com/topic/polymerase_chain_reaction/

Reid, C. W., 2005. *Bacterial Lytic Transglycosylases: Biochemical Characterization and Rational Design of Inhibitors*. PhD, Thesis. University of Guelph, Canada.

Stabler, R. A. (2009). Comparative genome and phenotypic analysis of Clostridium difficile 027 strains provides insight into the evolution of hypervirulent bacterium. *Genome Biology*.

Wheeldon, L., Worthington, T., Hilton, A., Elliott, T., & Lambert, P. (2008). Physical and chemical factors influencing the germination of Clostridium difficile spores. *Journal of Applied Microbiology*, 2223-2229.

World Health Organization Department of Communicable Disease, Surveillance and Response. (2002). Prevention of hospital-acquired infections.

Youngson, R. M. (2005). *Clostridium difficile*. Retrieved from Collins Dictionary of Medicine.