Cloning, Expression, and Sequence Analysis of Camelysin, a Zinc Metalloprotease from *Bacillus anthracis* and *B. cereus*

by

Andrew Ross Myers

A thesis submitted in partial fulfillment of the requirements for the degree of Master of Science
Department of Biology
College of Arts and Sciences
University of South Florida

Major Professor: My Lien Dao, Ph.D.
Steven Grossman, Ph.D.
Brian Livingston, Ph.D.

Date of Approval: July 18, 2005

Keywords: metalloenzyme, collagen, pathogenic factor, recombinant protein, nosocomial infections

© Copyright 2005, Andrew Ross Myers
AKNOWLEDGEMENTS

I would like to express my sincere gratitude to my major professor, Dr. My Lien Dao, for her encouragement, guidance, and her expertise during both my undergraduate and graduate careers. While in her lab I have gained valuable management, research, and teaching experience because of her strong dedication to her students. I would also like to thank my committee members, Dr. Steven Grossman and Dr. Brian Livingston for their valuable advice and patience.

Of course I cannot forget about the great friendships that were formed with my fellow lab occupants. I would like to thank Marios Ioannides, Valerie Carson, Thomas Han, and Justin Federico for their help, positive attitudes, wonderful personalities, and willingness to work together, which helped to make our lab a great place to work in. I would also like to thank Chi Zhang, Hong Yang, and Wenli Li for their advice, technical assistance, and friendliness.

I would like to thank my parents, grandparents, my brother, and my sister for their confidence, financial support, and for being there when I needed them the most. Over the past seven years I have maintained my close family ties, gained many friends, and sadly some have passed on. There are no words to describe how lucky I am to have such a great family and the best group of friends anyone could ever ask for, thank you for your love and support.
# TABLE OF CONTENTS

- LIST OF FIGURES iv
- LIST OF TABLES vi
- ABSTRACT vii
- INTRODUCTION 1
- MATERIALS AND METHODS 10
  - Chemicals and Reagents 10
  - Bioinformatics Analysis 10
  - Bacterial Strains, Eukaryotic Cells, and Growth Conditions 11
  - Genomic DNA Isolation 12
  - PCR Amplification 13
  - Ligation into Expression Vectors and Cloning 14
  - Plasmid Isolation and Analysis of Recombinant Clones 16
  - Transfection and Expression of $BCcalY$-pcDNA in HeLa Cells 17
  - Expression of $BAca/Y$-pBAD and $BCcal/Y$-pBAD in $E. coli$ TOP 10 Cells 18
  - Expression of $BCca/Y$-pET100D in $E. coli$ BL21 (DE3) pLysS Cells 19
  - SDS-PAGE 19
  - Western Blotting Analysis 20
  - Azocasein Assay 20
RESULTS

PCR Amplification of the Camelysin Gene from *B. cereus* and Cloning into pcDNA3.1/V5-His\(^\text{®}\) TOPO\(^\text{®}\) TA 23

Confirmation of the Construction of *BC*calY-pcDNA 26

Transfection into HeLa Cells and Expression of *BC*calY-pcDNA 28

Beta Galactosidase Assay after Expression of the pcDNA3.1/V5-His-TOPO/\(\text{lacz}^\text{®}\) Product 28

PCR Amplification of the Camelysin Gene from *B. anthracis* and *B. cereus* for Cloning into pBAD TOPO\(^\text{®}\) TA 29

Confirmation of the Construction of *BA*calY-pBAD and *BC*calY-pBAD 31

Expression of *BA*calY-pBAD and *BC*calY-pBAD in *E. coli* TOP10 Cells 33

Analysis of Gene Expression by SDS-PAGE 34

PCR Amplification of the Camelysin Gene and Cloning into pET100/D-TOPO\(^\text{®}\) 35

Confirmation of the Construction of *BC*calY-pET100D 37

Transformation and Expression of *BC*calY-pET100D in *E. coli* BL21 (DE3) pLysS cells 39

Analysis of Gene Expression by SDS-PAGE 40

Western Blotting Analysis of *BA*calY-pBAD, *BC*calY-pBAD, and *BC*calY-pET100D 40

Analysis of Enzymatic Activity by Azocasein Assay 41

Sequence Analysis of *calY* in *B. anthracis* and *B. cereus* ATCC 14579 42

Prediction of the Putative Signal Peptide of Camelysin from *B. anthracis* and *B. cereus* ATCC 14579 using Neural Network and Hidden Markov Models 45
LIST OF TABLES

Table 1  Conditions used for PCR Amplification  14
Table 2  Primers used for PCR Amplification of the Camelysin Gene and for Confirmation of Successful Ligations  24
LIST OF FIGURES

Figure 1  PCR amplification of the camelysin gene of *B. cereus* for cloning into pcDNA3.1/V5-His®-TOPO® TA 25

Figure 2  Map of the mammalian expression vector pcDNA3.1/V5-His®-TOPO® TA containing *calY* 25

Figure 3  Insertion of the camelysin gene into pcDNA3.1/V5-His®-TOPO® TA 27

Figure 4  Restriction enzyme analysis of *BCcalY*-pcDNA 27

Figure 5  IPTG assay of the expression product obtained from pcDNA3.1/V5-His-TOPO//lacZ® 29

Figure 6  PCR amplification of the camelysin gene of *B. anthracis* and *B. cereus* for cloning into pBAD TOPO® TA 30

Figure 7  Map of the prokaryotic expression vector pBAD TOPO® TA containing *calY* 31

Figure 8  Insertion of the camelysin gene into pBAD TOPO® TA 32

Figure 9  SDS-PAGE analysis of the expressed protein from *BAcalY*-pBAD 34

Figure 10  SDS-PAGE analysis of the expressed protein from *BAcalY*-pBAD 34

Figure 11  PCR amplification of the camelysin gene from *B. cereus* for cloning into pET100/D-TOPO® 36

Figure 12  Map of the prokaryotic expression vector pET100/D-TOPO® containing *calY* 36

Figure 13  Insertion of the camelysin gene into pET100/D-TOPO® 37

Figure 14  SDS-PAGE analysis of the expressed protein from *BCcalY*-pET100D 40
Figure 15 Western blotting analysis of $B{Acal}\text{Y}}$-pBAD, $B{Ccal}\text{Y}$-pBAD, and $B{Ccal}\text{Y}$-pET100D

Figure 16 Degradation of azocasein by camelysin

Figure 17 Multiple sequence alignment of the amino acid residues for camelysin from $B.\text{anthracis}$ and $B.\text{cereus}$

Figure 18 Multiple sequence alignment of the nucleotide residues for camelysin from $B.\text{anthracis}$ and $B.\text{cereus}$

Figure 19 SignalP 3.0’s neural network model prediction of the camelysin protein’s signal peptide

Figure 20 SignalP 3.0’s hidden Markov model prediction of the camelysin protein’s signal peptide

Figure 21 Secondary structure comparison between camelysin from $B.\text{cereus}$ and $B.\text{anthracis}$

Figure 22 Secondary structure prediction of camelysin from $B.\text{anthracis}$ using PSIPRED

Figure 23 Secondary structure prediction of camelysin from $B.\text{cereus}$ using PSIPRED
Cloning, Expression, and Sequence Analysis of Camelysin, a Zinc Metalloprotease from *Bacillus anthracis* and *B. cereus*

Andrew Ross Myers

**ABSTRACT**

*Bacillus anthracis* and *B. cereus* are well known etiological agents, which cause disease in healthy and immunocompromised individuals. Considering the abundance and lethality of these organisms it is imperative that research is performed to identify and analyze new factors that may contribute to their pathogenicity. Camelysin is a membrane bound, zinc metalloprotease isolated from *B. cereus*. Assays performed on purified camelysin demonstrate that the protease exhibits fibrinolytic, collagenolytic, and actin degradation activity, any of which can contribute to the organism’s ability to invade host tissues and cause damage. Considering the putative role of camelysin in pathogenicity, it would be beneficial to study the effects of camelysin in tissue cultures or animal models. The goal of this study focused on the cloning and expression of camelysin from *B. cereus* and its homolog in *B. anthracis*. Expression of a fusion tagged protein may assist in the purification of camelysin as well as overcoming the native protein’s extreme insolubility. Primers were designed to amplify the camelysin gene from *B. cereus* for cloning into the prokaryotic pBAD TOPO® TA, pET100/D-TOPO®, and the eukaryotic pcDNA3.1/V5-His® TOPO® TA expression
vectors. Primers were also designed to amplify the gene from *B. anthracis* for cloning into the pBAD TOPO® TA vector. The recombinant clones were induced and successful expression of the protein was confirmed by performing SDS-PAGE, Western blotting, and an azocasein protease assay. The recombinant proteins exhibited casein degradation activity which is observed with purified camelysin from *B. cereus*. This study successfully demonstrated the presence of the camelysin protein in *B. anthracis*. Furthermore, the recombinant clones obtained will be useful for purification and analysis of camelysin and delineation of its role in the pathogenicity of *B. cereus* and *B. anthracis*. 
INTRODUCTION

*Bacillus anthracis* and *Bacillus cereus* are known pathogens, which have various effects on the human body. Both healthy and immunocompromised individuals can become infected with these organisms and both *B. anthracis* and *B. cereus* contain many similar virulence factors (20). There have been numerous studies performed which have identified the main factors of pathogenicity in these organisms, and most have resulted in therapeutic treatments against these commonly known factors. Most of these treatments however are ineffective by the time physicians identify the presence of these pathogens, due to the fact that some are often considered clinical laboratory contaminants (10, 31). However, to this date there have been no recent studies aimed at identifying any new pathogenicity factors in these organisms that could be of therapeutic value. This study focused on the identification and expression of a zinc metalloprotease, camelysin, in *B. cereus* ATCC 14579 and *B. anthracis*, which share many common factors of pathogenicity (15).

*B. anthracis, B. cereus, and B. thuringiensis* are all Gram positive, rod shaped, spore forming, facultative aerobes, and most have plasmids that encode toxins. They are commonly found in the soil, and all are classified as group I bacilli, which contains most of the bacilli that cause infection, and all three organisms are indistinguishable from each other based on 16srRNA analysis (10,
20, 31, 36, 38). All three have plasmids, some encoding toxins like the pXO1
found in *B. anthracis*, and pBc10987 of *B. cereus* which is homologous to the
pXO1 plasmid, but lacks the genes producing the lethal and edema toxins (38).
The spore forms of these organisms are resistant to heat, gamma radiation, and
even hospital based antimicrobial handwashes and disinfectants (22, 10). *B.
anthracis* and *B. cereus* mainly affect humans, while *B. thuringiensis* affects
insects. Due to this fact, our study concentrated on the screening and
identification of the camelysin gene from *B. anthracis* and *B. cereus* ATCC
14579.

The ten main infections resulting from *B. cereus* include local infections of
burns or trauma, septicemia and bacteremia, meningitis, respiratory infections,
endocarditis, urinary tract infections, skin infection, eye infections, osteomyelitis,
and food poisoning (10, 22, 23). *B. cereus* mainly causes gastrointestinal
distress resulting from contaminated food, but is also know to cause non-
gastrointestinal disease in immunocompromised people such as individuals with
AIDS, newborns, people recovering from surgery, and IV drug users (10, 22).
The main drug users affected by *B. cereus* infections are those who are heroin
addicts, not just because the needles may become contaminated with
pathogens, but due to the fact that *B. cereus* often is found as a contaminate of
heroin (22).

The most prevalent infection associated with *B. cereus* is a result of
ingestion of contaminated foods such as rice, pasta, and dairy products (22).
There are two types of food poisoning that affect humans: the emetic food
poisoning, and the diarrheal food poisoning. The diarrheal food poisoning is caused by the HBL and NHE enterotoxin complexes, which induce the following symptoms: abdominal pain, cramps, rarely a fever, occasional vomiting, and diarrhea which last for 1 to 2 days (10, 22). Vomiting usually starts 10 hours after ingestion since the toxin can be preformed (10). This type of food poisoning is similar to that of *Clostridium perfringens*, which may lead to misdiagnosis of *B. cereus* infections.

The emetic food poisoning is caused by the heat resistant toxin, cereulide, which is a type of hemolysin (22). Symptoms of the emetic food poisoning appear similar to the food poisoning of *Staphylococcus aureus* and include nausea, vomiting, and occasional diarrhea (10, 22). Since the toxin is always preformed it can cause symptoms in as little as 15 minutes.

Since the gastrointestinal infections are the most common result of a *B. cereus* infection, physicians tend to overlook cases in which *B. cereus* is the source of the infection. This stems from the fact that *B. cereus* infections are not routinely screened for in some countries because they are not listed as reportable illnesses (22). This is a strange fact, especially since *B. cereus* is commonly found in the hospital environment, sometimes causing nosocomial infections (2, 5, 19, 27). One patient undergoing neurosurgery developed meningitis due to linens contaminated with *B. cereus* (22). Another example included the misdiagnosis of *B. cereus* infections in two people in Louisiana (31). The first patient exhibited symptoms of pneumonia 5 days before admission into the hospital, where he was diagnosed with a lung tumor and died that same day.
The second patient was admitted to the hospital after 3 days of symptoms, was diagnosed with tuberculosis and died 3 days later. Both were given antibiotics that should have treated the infection, however they received them too late, sepsis overtook their bodies and was later determined to be the cause of death in both individuals (31). This is why it is important that studies be continued on B. cereus that focus on identification of new virulence factors and faster methods to detect the pathogen.

*B. anthracis* mainly infects the respiratory tract of individuals although rare cases of gastrointestinal infections have been reported. The main factors contributing to the virulence of *B. anthracis* include a toxin complex, the ability of the organism to form spores that are easily aerosolized, as well as a capsule that is present in its virulent form which may help the organism evade the immune system (29, 34, 38). *B. anthracis* has two large plasmids (pXO1 and pXO2) that encodes for proteins that must be present for the organism to be fully virulent (1). pXO1 is the plasmid that contains the genes for the toxin complex. The toxin complex is made up of the edema factor, lethal factor, and protective antigen encoded by *cya*, *lef*, and *pagA* respectively (38). The protective antigen is a major component of the cell free vaccines on the market that are for human use (1). pXO1 is 182 kb and the pathogenicity island which contains *cya*, *lef*, and *pagA*, is only 44.8 kb in size, the island is surrounded by two IS1627 elements thus suggesting that it may be mobile (38). Plasmid pXO2 contains the gene that encodes for the Poly-d-glutamic acid capsule that aids in antiphagocytic activity (34). Although treatments and a vaccine are available for this organism, they are
not as effective as treatments for simple infections such as gastroenteritis. More work needs to be done to identify new virulence factors, and this study will encompass the use of *B. anthracis*, since sequence information has identified a putative camelysin gene in its genome.

Camelysin is a membrane bound neutral zinc metalloprotease with a molecular mass of approximately 19 kDa and is found in an uncharacterized strain of *B. cereus* while in the logarithmic phase of growth (15, 16, 24). The camelysin protein also known as casein cleaving metalloprotease, has been studied and characterized within the last ten years. Recent studies have outlined camelysin as a possible pathogenicity factor as it has fibrinolytic, collagenolytic, and actin degradation activity (15). These activities may contribute to the ability of *B. cereus* or any other organisms that contain the camelysin protein to invade host tissues and cause damage.

Proteases are divided into two major groups; those that cleave peptide chains at the ends, and those that cleave the peptide chains towards the middle, these are exopeptidases and endopeptidases respectively (37). Next the proteases are further classified by the residues that are at their active site into serine, aspartic, cysteine, and metallo-proteases. Metalloproteases usually require divalent metal ions for activity (37). Zinc peptidases consist of 30 families and are divided into 5 major clans (25, 37). The HEXXH motif is typically found in zinc metalloproteases at the zinc-binding region and is usually contained in an alpha helical region of the protein (26).
The first studies on camelysin were focused on establishing a number of general characteristics. The molecular weight was determined first by SDS-PAGE and then by mass spectrometry. SDS-PAGE performed (under reducing conditions, beta-mercaptoethanol) showed two bands, one at 58 kDa and the other at 64 kDa (13). Under non-reducing conditions the protease exhibits self-aggregation on the top part of the gel (13, 15). Even after boiling for several minutes with SDS, the protease was able to form aggregates. This self-aggregation is possibly caused by the oligomerization of the protease, which may explain the different molecular mass obtained by mass spectrometry (indicated MW= 19 kDa) as compared to SDS-PAGE.

Purified camelysin is shown to have the characteristics of an endopeptidase, which is a protease that cleaves towards the middle of the proteins, and may be involved in several pathogenicity pathways (37). Camelysin is able to cleave collagen type I, the most abundant collagen type that is found in skin, bones, and tendons (15). The protease cleaved the heterotrimer (collagen) in a similar manner to other bacterial collagenases (15). Other studies have found unidentified collagenases present during the log phase of growth on the outer membrane of *B. cereus* that may be responsible for binding to host cells. There is another hypothetical collagenase that may play a role in the degradation of collagen, which protects the lens capsule of the eye (2). *B. cereus* is known to cause ocular infections (endophthalmitis) by interacting with other pathogenicity factors which may play a role in these infections (15).
Camelysin has also been shown to activate plasminogen to plasmin and also destroys $\alpha_2$-antiplasmin. $\alpha_2$-antiplasmin keeps plasmin from degrading fibrin clots by forming a complex with it, and inactivating it (15). Cleavage of actin is also catalyzed by camelysin, and this could be a possible mechanism associated with the diarrheal effects of the organism (15).

Other zinc metalloproteases of different organisms have been shown to cause damage to cellular structures (7). One example is from *Vibrio vulnificus*, which has a protease that destroys collagen type IV, which results in the breakdown and collapse of capillary vessels (32). The destruction is due to the fact that the basal membranes surrounding the capillaries are made of collagen and laminin and once these are digested the capillaries have no structure holding them together, resulting in collapse and hemorrhaging. Venom from certain snakes may also contain zinc metalloproteases that act in the same fashion. In multiple sclerosis patients, zinc dependant matrix metalloproteases cause the breakdown of connective tissue; under normal circumstances the collagen is resistant to proteases because of its supercoiled triple helices and this is not the case in MS patients (4). In some people with cancer, the concentration of matrix metalloproteases is higher in patients who are sick as compared to those who are healthy. In certain cancers the concentration of matrix metalloproteases is increased during the beginning stages of metastasis and matrix metalloprotease inhibitors are used to prevent metastasis from occurring (11, 40). Zinc metalloproteases do not always cause damage: some actually are involved in the formation of bone tissue and a variety of other useful biological materials (18).
When it came to the purification of camelysin, solubilization of the active protein was difficult. Several non-ionic detergents were experimented with (Triton X-100, Brij 35, and Octylthioglucoside) and none were able to solubilize the protein in its active form (13). Later, Fricke et al. (13, 15) experimented with Sulfobetain SB-12 and it successfully solubilized the protein in its active form. This detergent was used to form a stable complex with the protein so it remained stable outside of the cell membrane and it also protected the hydrophobic ends from hydrophilic environments. Protease activity remained for several days without the addition of phospholipids when stored at 4° C. The mechanism of the protein removal involved the detergent replacing the phospholipids molecules in the cell membrane when the detergent was in high concentration. Decreasing the detergent concentration resulted in increased activity of the protease, which shows that the absence of the phospholipids did not have an inactivating effect.

Due to the high hydrophobicity of the protein, purification of the solubilizate is difficult. Mixed micelles form during purification between phospholipids and different membrane proteins as well as between different membrane proteins and the detergent (15). The detergent is also known to cause problems column matrix, thus it must be removed or diluted before addition to the columns (13). Conventional techniques for purification such as hydrophobic interaction chromatography (Butyl sepharose and Phenyl sepharose), chromatofocusing (PBE 94), and most forms of ion exchange chromatography cannot be used due to the hydrophobicity of the aggregated protease (15). However, in one study, ion exchange chromatography using EMD
TMAE Fractogel resulted in the partial purification of the protein (13). The only way to elute the protein from the column matrix involved the use of high concentrations of isopropanol. Subsequent studies showed that the polar solvents caused denaturation and inactivation of camelysin. In the latest study the purified protein was obtained using a Butyl Toyopearl column without any major loss in enzyme activity, and the usage of polar solvents was omitted (15).

In Grass et al., 2004, the molecular characteristics of the camelysin gene and protein were studied. Inverse PCR allowed the cloning of the gene and a gene knockout demonstrated that it was indeed camelysin that caused the protease activity in *B. cereus* (16). The studies also revealed that camelysin lacked the HEXXH motif commonly found in zinc metalloproteases and it had no conserved domains like most proteins, thus making it very unique. Our study will focus on the identification of the secondary structure of camelysin using the sequence information obtained from Dr. Grass and will be used to clone the gene into various expression vectors. Once a suitable vector system is identified, Dr. Grass will perform mutational studies to identify this rare zinc binding motif. This study will also identify whether or not the camelysin gene is present in *B. anthracis* and if so, further experiments will allow us to look at its possible role in the pathogenicity of these organisms.
MATERIALS AND METHODS

Chemicals and Reagents

The Wizard® Genomic DNA Purification Kit, restriction enzymes, and the PCR reagents were purchased from Promega Inc. (Madison, WI) and were used according to the manufacturer’s protocols. PCR primers were synthesized by Operon Biotechnologies (Huntsville, AL). PfuTurbo® DNA polymerase was purchased from Stratagene Inc. (La Jolla, CA). The expression vectors pBAD TOPO® TA, pcDNA3.1/V5-His® TOPO® TA, and pET100/D-TOPO® were obtained from Invitrogen Life Technologies (Carlsbad, CA). All other chemicals and reagents were purchased from Fisher Scientific (Pittsburg, PA), Sigma-Aldrich Co. (St. Louis, MO), or Bio-Rad Laboratories (Hercules, CA) unless otherwise noted.

Bioinformatics Analysis

The DNA sequence for the camelysin gene in B. anthracis Ames Strain and B. cereus ATCC 14579 were obtained from the National Center for Biotechnology Information (NCBI) Entrez server. Multiple sequence analysis was performed using the GAP alignment tool from the GCG Wisconsin Package® software (Accelrys Inc., San Diego, CA) using nucleotide, amino acid, and
secondary structure sequences. Predictions of the signal peptides were obtained from the SignalP 3.0 server (3). Secondary structure predictions were obtained using PSIPRED Protein Structure Prediction Server (21, 28).

**Bacterial Strains, Eukaryotic Cells, and Growth Conditions**

*B. cereus* ATCC 14579 was obtained from Dr. Daniel Lim (University of South Florida Biology Department, Tampa, FL) and cultured at 37°C in brain heart infusion broth (Difco, Detroit, MI). HeLa cells (CCL-2) were obtained from ATCC (Manassas, VA) for eukaryotic expression and were cultured in EMEM supplemented with 10% FBS at 37°C in 5% CO₂. Chemically competent *Escherichia coli* TOP10 cells were purchased from Invitrogen and used for the cloning of all constructs, and the expression of *BAcalY*-pBAD and *BCcalY*-pBAD. *E. coli* BL21 Star™ (DE3)pLysS cells were purchased and used for the cloning and expression of *BCcalY*-pET100D. Luria-Bertani (LB) broth (Difco, Detroit, MI) supplemented with 100μg/mL ampicillin (LB-AMP) was used for culturing and expression of camelysin in the TOP10 and BL21 transformants. Conditions for expressing the pBAD and pET constructs included growth in LB-AMP broth at 30°C, while conditions for mammalian cells remained the same except for the absence of 10% FBS for four and a half hours during the transfection.
**Genomic DNA Isolation**

Genomic DNA was isolated using the Promega Wizard® genomic DNA purification kit. A 10mL overnight culture was grown in LB broth in a shaking incubator at 37ºC. 1mL of the culture was removed and centrifuged at 16,000 X g for 2 minutes. The cell pellet was resuspended in 480µL of 50mM EDTA and 60µL of a 10mg/mL solution of lysozyme, which was incubated at 37ºC for 45 minutes. The sample was centrifuged at 16,000 X g for 2 minutes and 600µL of Nuclei Lysis Solution was added to resuspend the cells. To continue the cell lysis, the sample was incubated at 80ºC for 5 minutes. The samples were then incubated at 37ºC for 45 minutes with 3µL of RNase Solution to remove any RNA present in the samples. After cooling to room temperature, 200µL of Protein Precipitation Solution was added to the sample, which was then placed on ice for 5 minutes to aid in the precipitation. The sample was then centrifuged at 16,000 X g for 3 minutes and the supernatant containing the DNA was transferred to a fresh tube that contained 600µL of isopropanol to precipitate the DNA. The DNA was pelleted by centrifugation at 16,000 X g for 2 minutes. Next, the supernatant was removed and 600µL of 70% ethanol was added to rinse the pellet. The sample containing the purified DNA was then centrifuged for another 2 minutes at 16,000 x g. The supernatant was removed and the pellet was allowed to air dry, after which 100µL DNA Rehydration Solution was added. The resulting DNA
was analyzed on a 1% agarose gel for quality control, and the concentration and purity of the DNA was determined using the SmartSpec Plus Spectrophotometer (Bio-Rad).

**PCR Amplification**

PCR was performed on the genomic DNA for identification and cloning of the camelysin gene. The PCR products for the pBAD TOPO® TA vector were amplified using the Expand Long Template PCR System (Roche Applied Science, Indianapolis, IN), in which each reaction contained 3 units of *Taq* and *Tgo* polymerases, 200µM of dNTP’s, 250ng of genomic DNA, 50µL of Buffer 3, and 0.4µM of each primer were used (Table 2). PCR products for the pcDNA3.1 TOPO® TA vector were amplified using the PCR Mastermix (Promega) which contains a *Taq* polymerase with terminal transferase activity that adds adenosine residues onto the amplification product. For this amplification, 0.625 units of *Taq* polymerase, 200µM of dNTP’s, 137ng of genomic DNA, and 0.4µM of each primer were used. The PCR products for the pET100/D-TOPO® were amplified using the PfuTurbo® DNA polymerase (Stratagene), which resulted in blunt ended PCR products. For this amplification, 2.5 units of *PfuTurbo®* DNA polymerase, 200µM of dNTP’s, 100ng of genomic DNA, 0.4µM of each primer (Table 2), and 1x PCR reaction buffer (Stratagene) were used. The conditions of each PCR reaction are described in Table 1.
Table 1. Conditions used for PCR Amplification of camelysin from *B. anthracis* and *B. cereus*

<table>
<thead>
<tr>
<th>Primer Pair</th>
<th>PCR Conditions</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>B. anthracis</em> DNA: pBAD calY forward, pBAD calY reverse</td>
<td>95°C for 2 minutes (Initial Denaturation)</td>
</tr>
<tr>
<td></td>
<td>95°C for 30 seconds (Denaturation)</td>
</tr>
<tr>
<td></td>
<td>55°C for 30 seconds (Annealing)</td>
</tr>
<tr>
<td></td>
<td>68°C for 1 minute (Extension)</td>
</tr>
<tr>
<td></td>
<td>Repeated 25 times</td>
</tr>
<tr>
<td></td>
<td>68°C for 7 minutes (Final Extension)</td>
</tr>
<tr>
<td><em>B. cereus</em> DNA: pBAD calY forward, pBAD calY reverse</td>
<td>95°C for 2 minutes (Initial Denaturation)</td>
</tr>
<tr>
<td></td>
<td>95°C for 30 seconds (Denaturation)</td>
</tr>
<tr>
<td></td>
<td>55°C for 30 seconds (Annealing)</td>
</tr>
<tr>
<td></td>
<td>68°C for 1 minute (Extension)</td>
</tr>
<tr>
<td></td>
<td>Repeated 25 times</td>
</tr>
<tr>
<td></td>
<td>68°C for 7 minutes (Final Extension)</td>
</tr>
<tr>
<td><em>B. cereus</em> DNA: pcDNA calY forward, pcDNA calY reverse</td>
<td>96°C for 2 minutes (Initial Denaturation)</td>
</tr>
<tr>
<td></td>
<td>96°C for 30 seconds (Denaturation)</td>
</tr>
<tr>
<td></td>
<td>56°C for 30 seconds (Annealing)</td>
</tr>
<tr>
<td></td>
<td>72°C for 1 minute (Extension)</td>
</tr>
<tr>
<td></td>
<td>Repeated 26 times</td>
</tr>
<tr>
<td></td>
<td>72°C for 15 minutes (Final Extension)</td>
</tr>
<tr>
<td><em>B. cereus</em> DNA: pET calY forward, pET calY reverse</td>
<td>95°C for 2 minutes (Initial Denaturation)</td>
</tr>
<tr>
<td></td>
<td>95°C for 30 seconds (Denaturation)</td>
</tr>
<tr>
<td></td>
<td>53°C for 30 seconds (Annealing)</td>
</tr>
<tr>
<td></td>
<td>72°C for 1 minute (Extension)</td>
</tr>
<tr>
<td></td>
<td>Repeated 26 times</td>
</tr>
<tr>
<td></td>
<td>72°C for 10 minutes (Final Extension)</td>
</tr>
</tbody>
</table>

**Ligation into Expression Vectors and Cloning**

The PCR products amplified using the Promega Taq polymerase, and the Roche Tgo and Taq polymerases were ligated into the pcDNA3.1 TOPO® TA and pBAD TOPO® TA vectors respectively. Briefly, 2μL of the PCR products were added to 2μL of water, and 1μL of the appropriate linearized vector. The ligation reaction was incubated at room temperature for 5 minutes and placed on ice. Transformation was completed by taking 2μL of the ligation products and adding
them to *E. coli* TOP10 cells, which were incubated on ice for 15 minutes. Next, the cells were heat shocked for 30 seconds in a 42°C water bath and 250µL of S.O.C. medium (Invitrogen) was added, followed by incubation at 37°C for one hour in a shaking incubator. LB-AMP plates were inoculated with 20µL and 40µL volumes of each transformation reaction and incubated overnight at 37°C. Individual colonies were removed and screened for *BAcαlY*-pBAD, *BCcalY*-pBAD, or *BCcalY*-pcDNA using PCR amplification with primers (Table 2) specific for either the native plasmid or the inserted camelysin gene. Restriction enzyme digestion of *BCcalY*-pcDNA was also carried out using 25 units of *BstX I* per 1µg of plasmid DNA and results were observed on an agarose gel.

The PCR products amplified using the *Pfu Turbo*® polymerase were ligated into the pET100/D-TOPO® vector. The PCR product was diluted 1:25 in nuclease free water and 3µL of this product was added to 1µL of dilute salt solution, 1µL of water, and 1µL of linearized vector. Both the ligation reaction and transformation into *E. coli* TOP10 cells were carried out according to the protocols listed above. Two different sets of PCR primers (Table 2) were used to identify colonies containing *BCcalY*-pET100D. Sequencing analysis of each recombinant plasmid was performed at SeqWright DNA Technology Services (Houston, TX) using a forward primer (Table 2) specific for the vector, and a reverse primer specific for the inserted camelysin gene.
Plasmid Isolation and Analysis of Recombinant Clones

Plasmid isolation was performed using the FastPlasmid™ Mini kit from Eppendorf (Westbury, NY). *E. coli* TOP10 cells containing the recombinant plasmids were grown in LB-AMP for 15 hours in a shaking incubator at 37°C to an OD$_{600}$ of 2.4. 1.5μL of each culture was removed and were centrifuged at 15,000 x g for 1 minute. The supernatant was removed and 400μL of the lysis solution, containing RNase A and lysozyme was added to the pellet. The pellet was resuspended by vortexing on high speed for approximately 30 seconds, followed by a 3 minute incubation at room temperature. The lysate was then transferred to a spin column assembly which was then centrifuged for 1 minute at 15,000 x g. The remaining plasmid DNA contained in the spin column matrix was washed with 400μL of diluted wash buffer and centrifuged for another minute. Filtrate remaining in the bottom of tube was removed and the spin column assembly was centrifuged for 1 minute to remove any excess isopropanol that was introduced with the washing buffer. Next, the spin column was placed into a collection tube and the DNA was eluted by adding 50μL of buffer containing 10mM Tris-HCl / 0.1mM EDTA, pH 8.5 followed by centrifugation at 15,000 x g for 1 minute. Plasmid DNA used for transfections was prepared using the Qiagen Plasmid Midi kit according to the manufacturer’s protocol.
Transfection and Expression of BCcalY-pcDNA in HeLa cells

Transient transfections were performed using the following constructs; BCcalY-pcDNA (pcDNA3.1 with the camelysin gene), calY-pcDNA(-) (pcDNA3.1 without any insert), and pcDNA3.1/V5-His-TOPO/lacZ® (pcDNA3.1 that contained the ß-galactosidase control). A negative control designated HeLa1 (HeLa cells with no vector) underwent the same treatments as transfected cells to make sure the lipid had no toxic effects on the cells. Prior to transfection the HeLa cells were plated in a 12-well plate at a cell density of approximately 7.8 x 10⁴ cells per well and were incubated overnight at 37°C in 5% CO₂ until mostly confluent (~90-100%). The following day, plasmid DNA obtained from the Midi preps was diluted at a ratio of 1.6μg of DNA per 100μL of Opti-MEM (Invitrogen) and the Lipofectamine™ 2000 (Invitrogen) reagent was diluted at a ratio of 4μL lipid per 100μL of Opti-MEM according to the manufacturer’s protocol. The diluted DNA/Lipid mixtures were combined and incubated at room temperature for 20 minutes. The growth medium was then removed and the entire plate was washed with PBS, then 1mL of EMEM without 10% FBS was added to each well. Next, 200μL DNA/Lipid mixture was added to each well and the plates were incubated for 4.5 hours at 37°C in 5% CO₂. The growth media then was removed and replaced with fresh EMEM containing 10% FBS and the transfection reaction was allowed to continue for 24 hours. The next day, the
cells were detached from their respective plates using sterile cell scrapers and transferred to microcentrifuge tubes. After spinning down the cells, both the pellet and supernatant were frozen at -20°C for western blotting analysis, SDS-PAGE, and an azocasein enzyme assay.

Expression of \textit{BAcalY}-pBAD and \textit{BCcalY}-pBAD and in \textit{E. coli} TOP10 Cells

The pBAD vector in TOP10 cells, containing either the camelysin gene from \textit{B. anthracis} (\textit{BAcalY}-pBAD) or from \textit{B. cereus} (\textit{BCcalY}-pBAD) were used to express the camelysin protease (35). One other construct designated \textit{calY}-pBAD(\text{-}) (pBAD with no insert) was also induced during the expression experiment, along with TOP10 cells containing no vector. All cultures were grown in 10mL of LB-AMP overnight at 37°C in a shaking incubator. 7mL of the cultures containing \textit{BAcalY}-pBAD and \textit{BCcalY}-pBAD were added to 70mL of LB-AMP, and 2mL of cultures containing \textit{calY}-pBAD(\text{-}) and TOP10 cells were added to 20mL of LB-AMP. All cultures were grown for approximately 2 hours to an \textit{OD}_{600} of ~0.75 and then induced with various concentrations of arabinose before a 4 hour incubation at 30°C. At the end of the induction, 5μL of each sample was removed to obtain their respective \textit{OD}_{600}. Finally, the samples were centrifuged and the supernatant was transferred to a fresh tube, then both the supernatant and pellet from each culture were frozen at -20°C for future analysis.
Expression of BCcalY-pET100D in *E. coli* BL21 Star™ (DE3)pLysS Cells

The pET vector containing the camelysin gene from *B. cereus* (BCcalY-pET100D) was obtained from the TOP10 cells using the plasmid miniprep kit as mentioned above. BL21 Star™ (DE3)pLysS cells were transformed with 10ng of BCcalY-pET100D in a volume of 3μL using the heat shock method described previously and the transformed bacteria, along with untransformed bacteria (as a negative control) were incubated in 10mL of LB-AMP overnight at 37ºC in a shaking incubator. Next, 20mL of LB-AMP was inoculated with 2mL of the overnight culture and was incubated under the same conditions as above for 1 hour and 20 minutes to an OD$_{600}$ of 0.7. The cultures were then split into two tubes (10mL in each), one containing 1mM isopropyl β-D thiogalactoside (IPTG), for induction of expression, and one without IPTG. At each hour, 500μL of each culture was removed. Once removed, the samples were centrifuged and the supernatant was transferred to a fresh tube, then both the supernatant and pellet from each culture was frozen at -20ºC for future analysis.

SDS-PAGE

The cell pellets were resuspended in 1X SDS-Page Sample Buffer (Invitrogen) and the mixture was boiled for 5 minutes. Next, 20μL of each sample was loaded into a 12% SDS-PAGE gel which underwent electrophoresis.
at 200V for 45 minutes using the Mini-Protean II Electrophoresis Cell (Bio-Rad). After the electrophoresis was complete, the gel was stained with 0.1% coomassie blue R-250 (in 40% MeOH / 10% HOAc) for 30 minutes. The gel was destained with several changes of 40% MeOH / 10% HOAc with a total incubation time of 2 hours.

**Western Blotting Analysis**

Following separation by SDS-PAGE, the proteins were transferred to a nitrocellulose membrane using a Mini Trans-Blot® electrophoretic transfer cell (Bio-Rad) overnight at 30V/100mA. Next, the membrane was placed in a blocking solution (TBST with 5% dry milk) for 25 minutes. The primary antibody (rabbit-anticamelysin) was diluted 1:5,000 in 5mL of blocking solution and the membrane was incubated for 45 minutes at room temperature (14). The membrane was washed three times with 20mL of TBST (TBS with 0.05% Tween 20), 8 minute incubations were carried out for each wash. The secondary antibody, anti-rabbit IgG-Alkaline phosphatase (Sigma) was diluted 1:20,000 in blocking buffer and the washed membrane was incubated for 45 minutes, followed by three more washes in TBST. Next, the membrane was placed into 10mL of TBS (0.1M NaCl, 0.5M Tris, pH 7.4) for rinsing.

**Azocasein Assay**

An azocasein assay was used to measure the proteolytic activity of the recombinant camelysin proteins using standard methods (30, 41). Equal
volumes of the cell pellets were resuspended in 1mL of 50mM Tris-HCl, pH 7.4 using a pipet. After resuspension the cells were subjected to freezing and thawing in order to lyse the cells, alternating between a dry ice/ethanol bath and a 50ºC water bath. The lysate was then subjected to sonication at 100W, 4 times at 20 second intervals in an ice bath. Next, the lysate was centrifuged for 10 minutes at 12,000 x g and 4ºC for 10 minutes. The supernatant was removed and the pellets from each sample were resuspended in 400μL of 50mM Tris-HCl, pH 7.4. Then, 200μL of each sample were separately added to tubes containing 200μL of the azocasein substrate solution (5mg/mL azocasein resuspended in 50mM Tris-HCl, pH 7.4) and incubated at 37ºC for 16 hours. The proteolytic activity was stopped with the addition of 800μL of 10% trichloroacetic acid (TCA), which also precipitates any undigested azocasein. The samples were centrifuged at 12,000 x g for 4 minutes and 1mL of the supernatant was transferred to a cuvette. The samples were placed in a SmartSpec Plus spectrophotometer (Bio-Rad) and the OD₃₆₅ for each sample was recorded.

**Beta Galactosidase Control Assay**

The HeLa cell pellets were washed twice with 1X PBS, pH 7.4 (Mg²⁺/Ca²⁺ free) and resuspended in TEN buffer (40mM Tris-HCl, pH 7.5, 1mM EDTA, pH 8.0, and 150mM NaCl). The cell suspension was centrifuged at 18,000 x g for 1 minute at 4ºC, resuspended in 0.25M Tris-HCl (pH 8.0), and subjected to 3 freeze thaw cycles by alternating between dry ice/ethanol and a 37ºC water bath. The lysate was then centrifuged at 18,000 x g for 2 minutes at 4ºC and the
supernatant was assayed. A 96 well plate was loaded with 50μL samples from each transfection reaction, either diluted (1:10) or undiluted. Next, 50μL of assay buffer (200mM sodium phosphate buffer, pH 7.3, 2mM MgCl₂, 100mM β-mercaptoethanol, 1.33mg/mL ONPG) was added to each well, mixed by pipetting, and incubated at 37°C for 50 minutes. At the end of the incubation, the plate was visually observed for color change and 150μL of 1M sodium carbonate was added to each well to stop the reaction.
RESULTS

Amplification of calY by PCR and Cloning into the pcDNA3.1/V5-His®

TOPO® TA Expression Vector

Due to the fact the proteases mode of toxicity is not known, this study used the eukaryotic expression vector pcDNA3.1 first to examine if mammalian expression was possible, and secondly to observe any intracellular protease activity, like the lethal factor of B. anthracis. After isolation of the genomic DNA from B. cereus, camelysin gene (calY) was amplified by PCR. Primers were designed to amplify the 594 bp gene using the genomic DNA sequence of B. cereus ATCC 14579 obtained at the NCBI website, gene ID # 1203630 (Table 2). Due to the fact that pcDNA3.1/V5-His® TOPO® TA is a eukaryotic expression vector, the start codon (ATG) was incorporated into the forward primer. This allowed correct translational recognition by the mammalian cells ribosome’s (17). This vector also has a cytomegalovirus (CMV) promoter that allows for overexpression of proteins once transfected into mammalian cells. Agarose gel electrophoresis was performed and confirmed the successful amplification of calY (Figure 1). Next, calY was ligated into the pcDNA3.1/V5-His® TOPO® TA expression vector and was transformed into E. coli TOP10 cells. Ligation into TOPO TA vectors employs the use of deoxyadenosines left on the 3’ ends of PCR products by Taq polymerase which allowed the amplified DNA to bind to the
3’ deoxythymidines located on the linearized vector. The vector is kept in a linearized form before the insertion of amplified DNA by topoisomerase I that cleaves one strand of the vectors DNA after the sequence 5’-CCCTT, which is located at the cloning site. The energy of this broken bond was conserved by a covalent bond between the 3’ phosphate of the vector DNA and a tyrosyl residue of the topoisomerase I. The energy was released and the bond was broken when the newly inserted DNA was introduced at the cloning site, which in turn released the topoisomerase and allowed the formation of the double stranded recombinant vector (Figure 2).

Table 2. PCR primers used for amplification of genomic DNA and confirmation of gene insertion.

<table>
<thead>
<tr>
<th>Primer name</th>
<th>Primer sequence</th>
<th>DNA amplified</th>
<th>Vector used</th>
</tr>
</thead>
<tbody>
<tr>
<td>pBAD calY forward</td>
<td>AGTCTGAAAAAGAAATTAGGTATGGGGAGT</td>
<td>Genomic DNA from <em>B. anthracis</em> and <em>B. cereus</em> without the native GTG start codon.</td>
<td>pBAD</td>
</tr>
<tr>
<td>pcDNA calY forward</td>
<td>ATGGGTAGTGTCAGAAAAGAAATTAGGTATGG</td>
<td>Genomic DNA from <em>B. cereus</em> with ATG added for mammalian expression system.</td>
<td>pcDNA3.1</td>
</tr>
<tr>
<td>pET calY forward</td>
<td>CACCGTGAGTCTGAAAAAGAAATTAGGGAGT</td>
<td>Genomic DNA from <em>B. cereus</em> containing CACC needed for insertion into vector.</td>
<td>pET100/D</td>
</tr>
<tr>
<td>pBAD forward</td>
<td>ATGCCATAGCATTTTTATCC</td>
<td>Vector DNA, used to confirm the insertion of calY.</td>
<td>pBAD</td>
</tr>
<tr>
<td>pBAD reverse</td>
<td>ATGCCATAGCATTTTTATAT</td>
<td>Vector DNA, used to confirm the insertion of calY.</td>
<td>pBAD</td>
</tr>
<tr>
<td>T7 forward</td>
<td>TAATACGACTCACTATAGGG</td>
<td>Vector DNA, used to confirm the insertion of calY.</td>
<td>pcDNA3.1 &amp; pET100/D</td>
</tr>
<tr>
<td>Native calY reverse</td>
<td>TAGTTATGCTGACGCGGTTG</td>
<td>Genomic DNA from <em>B. cereus</em> containing the native stop codon.</td>
<td>pET100/D &amp; pBAD</td>
</tr>
<tr>
<td>pcDNA calY reverse</td>
<td>TAGTTATGCTGACGCGGTTG</td>
<td>Genomic DNA from <em>B. cereus</em> containing the native stop codon.</td>
<td>pcDNA3.1</td>
</tr>
<tr>
<td>pBAD BA-calY reverse</td>
<td>TAGTTATGCTGACGCGGTTG</td>
<td>Genomic DNA from <em>B. anthracis</em> containing the native stop codon.</td>
<td>pBAD</td>
</tr>
<tr>
<td>pcDNA reverse</td>
<td>TAGTTATGCTGACGCGGTTG</td>
<td>Genomic DNA from <em>B. cereus</em> containing the native stop codon.</td>
<td>pET100/D</td>
</tr>
<tr>
<td>T7 reverse</td>
<td>TAGTTATGCTGACGCGGTTG</td>
<td>Vector DNA, used to confirm the insertion of calY.</td>
<td>pET100/D</td>
</tr>
</tbody>
</table>
Figure 1. PCR amplification of the 594 bp camelysin gene (3 additional nucleotides for the mammalian start codon ATG) from *B. cereus* ATCC 14579 using primers pcDNA *calY* forward and pcDNA *calY* reverse. The 1% agarose gel confirms the presence of the 594 bp DNA fragment. Lane 1: 100 bp ladder and Lane 2: amplified *calY* from genomic DNA.

Figure 2. Map of the mammalian expression vector pcDNA3.1/V5-His\(^\circ\) TOPO\(^\circ\) TA containing *calY* with the mammalian start codon ATG. The stop codon, TAA was used to end translation and allowed the overexpression of the native protein without any fusion tags. Ampicillin was used to screen for positive clones.
Confirmation of the Successful Cloning into pcDNA3.1/V5-His\textsuperscript{®} TOPO\textsuperscript{®} TA

Following the amplification and ligation of \textit{calY} into the expression vector, the vector was used to transform \textit{E. coli} TOP10 cells and was propagated. Plasmid DNA was then isolated from eight colonies and screened for correct insertion of \textit{calY} into the vector by PCR analysis (Figure 3), restriction enzyme digestion with \textit{Bst}X I (Figure 4), and then DNA sequencing. PCR amplification was carried out using 100ng of the \textit{calY}-pcDNA as a template and the primers T7 forward / pcDNA \textit{calY} reverse. The products were subjected to agarose gel electrophoresis and the 668 bp fragment confirmed that both the ligation into the vector and the insertion of \textit{calY} in the correct orientation was successful. Restriction digestion was carried out on the recombinant plasmid and yielded two bands, the first representing the plasmid (6120 bp) and the second representing the insert with some excess vector nucleotides (638 bp), confirming the cloning was successful. DNA sequencing was performed at the H. Lee Moffitt Cancer Center’s Molecular Biology Core Facility. Once the sequence information was obtained a BLAST2seq (39) was performed against the original gene sequence from \textit{B. cereus} ATCC 14579’s \textit{calY}, confirming no mutations were introduced during the cloning process.
Figure 3. PCR amplification products on a 1% agarose gel used for identifying correct insertion of calY. Lane 1: 100 bp ladder, Lane 2: 323 bp PCR control, and Lanes 3-10 are recombinant plasmid amplified using the primers T7 forward / pcDNA calY reverse to give the 668 bp product. This represented a 63% cloning efficiency.

Figure 4. Restriction enzyme analysis on a 1% agarose gel using BstXI after incubation at 50°C. Lane 1: 100 bp ladder, Lane 2: λ HindIII ladder, Lane 3: 1hr incubation with BstXI, Lane 4: 1hr incubation without BstXI, Lane 5: 2hr incubation with BstXI, Lane 6: 2hr incubation without BstXI, Lane 7: 3hr incubation with BstXI, Lane 8: 3hr incubation without BstXI, Lane 9: 4hr incubation with BstXI, Lane 10: 4hr incubation without BstXI, Lane 11: 5hr incubation with BstXI, Lane 12: 5 hr incubation without BstXI. Uncut plasmid is 6120 bp in size, cut plasmid has two distinct bands, one 5482 bp and the other 638 bp.
Transfection into HeLa cells and Expression of \textit{BCcalY}-pcDNA

After obtaining large amounts of the recombinant plasmid using a Midi-Prep Kit, \textit{BCcalY}-pcDNA (pcDNA3.1 with the camelysin gene), \textit{calY}-pcDNA (-) (pcDNA3.1 without any insert), and pcDNA3.1/V5-His-TOPO/lacZ\textsuperscript{©} (pcDNA3.1 that contained the ß-galactosidase control) were transiently transfected into HeLa cells. The HeLa cells appeared to be confluent both before and after transfection in all cultures, as did a culture containing only HeLa cells that underwent the same lipid and medium treatments as transfected cells. SDS-PAGE was performed on each of the cell cultures and no differences in banding patterns were observed except for the 122 kDa band present for the ß-galactosidase control. Western blotting, an azocasein assay, and immunoblotting were also performed on each culture and all results were negative. To test the positive control for ß-galactosidase activity, a ß-galactosidase assay was performed and demonstrated that not only did the transfection work, but it also produced the active ß-galactosidase protein (Figure 5).

Amplification of calY by PCR and Cloning into the pBAD TOPO® TA Expression Vector

Genomic DNA containing the camelysin gene from both B. anthracis and B. cereus was amplified by PCR using the Expand Long Template PCR system for insertion into the pBAD TOPO® TA vector (Figure 6) to examine if prokaryotic expression was possible without the generation of a fusion tagged protein. This system contains a mixture of two polymerases (Taq and Tgo) that gave a high yield of PCR product that was proofread via the Tgo polymerases 3’-5’ exonuclease activity. The pBAD vector is advantageous for use in expressing suspected toxic proteins as it contains the tightly regulated araBAD promoter, which controls the level of protein expression. Another advantage to using this vector system is the fact that it contains a short N-terminal leader peptide, which improved translation of the protein and also increased its solubility (42). The vector also allows a V-5 epitope and a polyhistidine tag to be placed at the C-
terminal end of a recombinant protein, which could prove useful for detecting a fusion protein. However, in this experiment the tags were left out since we already had the antibody for purified camelysin and the tags cannot be cleaved off once placed onto the native protein. The genomic DNA sequence for the *B. anthracis* camelysin gene was obtained from the NCBI website (39), protein ID # AAP25246.1. The start codon was removed from the forward primer since the vector contains its own initiation codon, and the stop codon was left to prevent formation of a fusion tagged protein. The PCR product was obtained and then ligated into the TA vector as previously described (Figure 7). Following ligation, *E. coli* TOP10 cells were transformed with the recombinant vector and grown in LB-AMP to propagate the plasmid.

Figure 6. PCR amplification of *calY* from *B. anthracis* using primers pBAD *calY* forward / pBAD BA-*calY* reverse and from *B. cereus* using primers pBAD *calY* forward / Native *calY* reverse for cloning into pBAD TOPO® TA. The 1% gel confirms the presence of the 591 bp camelysin gene (no start codon on primer). Lane 1: 1kb ladder, Lanes 2-3: *B. anthracis calY*, and Lanes 4-5: *B. cereus calY*. 

30
Figure 7. Map of the prokaryotic expression vector pBAD TOPO® TA containing calY without its own start codon, GTG. The stop codon, TAA was used to end translation to allow expression of the native protein without any fusion tags. Ampicillin was used to screen for positive clones once transformed into E. coli TOP10 cells.

Confirmation of the Successful Cloning into pBAD TOPO® TA

The products obtained by PCR amplification of calY from B. anthracis and B. cereus were ligated into the pBAD vector and used to transform TOP10 cells, recombinant plasmid was then isolated from five colonies using Mini-Preps. The recombinant plasmid was then screened for correct insertion of calY into the vector by PCR analysis (Figures 8A and 8B), and DNA sequencing. PCR amplification was carried out using approximately 100ng of BAcalY-pBAD and BCcalY-pBAD as templates and the primers for both the camelysin gene and the vector. The products were subjected to agarose gel electrophoresis and the distinct bands at 594 bp and 773 bp confirmed that both the ligation into the vector and the insertion of calY in the correct orientation were successful.
Figure 8A. PCR amplification products on a 1% agarose gel used for identifying the correct insertion of BAcalY and BCcalY into the pBAD vector. Lane 1: 1kb ladder, Lanes 2-6: PCR of BAcalY using pBAD calY forward / pBAD BA-calY reverse, Lane 7: Empty well, and Lanes 8-12: PCR of BAcalY using pBAD forward / pBAD BA-calY reverse. This represented a 60% cloning efficiency.

Figure 8B. PCR amplification products on a 1% agarose gel used for identifying the correct insertion of BAcalY and BCcalY into the pBAD vector. Lane 1: 1kb ladder, Lanes 2-6: PCR of BCcalY using pBAD calY forward / Native calY reverse, Lane 7: 1kb ladder, Lane 8: Empty well, and Lanes 9-13: PCR of BCcalY using pBAD forward / Native calY reverse. This represented a 40% cloning efficiency.
DNA sequencing was performed at SeqWright DNA Technologies (Houston, TX). Once the sequence information was obtained a BLAST2seq (39) was performed against the original gene sequences from both *B. anthracis* and *B. cereus* ATCC 14579’s *calY*, confirming no mutations were introduced during the cloning process.

**Expression of BAcalY-pBAD and BCcalY-pBAD**

Expression of *BAcalY*-pBAD and *BCcalY*-pBAD was induced in *E. coli* TOP10 cells by using different concentrations of arabinose to identify the amount needed for successful expression of the protease. The induced culture was incubated at 30ºC in a shaking incubator for 4 hours. At the end of the 4 hours, the OD$_{600}$ was recorded for each culture and the averages were 2.4 for *BAcalY*-pBAD, 2.2 for *BCcalY*-pBAD, and 3.1 for *E. coli* TOP10 with no vector. The lower values for *BAcalY*-pBAD and *BCcalY*-pBAD indicate the possible presence of toxicity in the cells expressing camelysin. SDS-PAGE was performed on each of the samples and the 19 kDa mature camelysin protein was observed for both the *B. anthracis* and *B. cereus* constructs (Figures 9 and 10 respectively). The greatest amount of protein seemed to be produced when each induced sample had arabinose at a final concentration of 0.002%.
Figure 9. Expression of calY from BAcalY-pBAD construct. Lane 1: MW marker, Lane 2: uninduced BAcalY-pBAD, Lanes 3-7: BAcalY-pBAD induced with 0.2%, 0.02%, 0.002%, 0.0002%, and 0.00002% arabinose respectively, Lane 8: pBAD vector with no insert induced with 0.2% arabinose, and Lane 9: E. coli TOP10 cells with no vector induced with 0.2% arabinose. The camelysin protein (~19 kDa) can be seen in lanes 3-7, however the strongest bands are in lanes 5 and 6.

Figure 10. Expression of calY from BCcalY-pBAD construct. Expression of calY from BCcalY-pBAD construct. Lane 1: MW marker, Lane 2: empty well, Lane 3: uninduced BCcalY-pBAD, Lanes 4-8: BCcalY-pBAD induced with 0.2%, 0.02%, 0.002%, 0.0002%, and 0.00002% arabinose respectively, Lane 9: pBAD vector with no insert induced with 0.2% arabinose, and Lane 10: E. coli TOP10 cells with no vector induced with 0.2% arabinose. The camelysin protein (~19 kDa) can be seen in lanes 4-8, however the strongest bands are in lanes 6 and 7.
Amplification of \textit{calY} by PCR and Cloning into the pET100/D-TOPO\textsuperscript{®}

Expression Vector

DNA from \textit{B. cereus} ATCC 14579 was amplified by PCR using the \textit{PfuTurbo}\textsuperscript{®} DNA polymerase for integration into pET100/D-TOPO\textsuperscript{®} (Figure 11) to examine if the creation of a fusion tagged protein would effect expression levels of camelysin. The \textit{Pfu} polymerase contains 3'-5' exonuclease activity much like the \textit{Tgo} polymerase used for cloning into the pBAD system. However, the \textit{Pfu} polymerase does not add any deoxyadenosines onto the PCR product because it lacks this activity, which leaves the PCR products with blunt ends. Ligation of the PCR product into this TOPO vector required the sequence CACC to be added onto the forward primer of camelysin. The linearized vector contains a region complimentary to this sequence, thus allowing directional binding of the PCR product to the vector that is also in frame with the other genes on the vector. Primers were designed with the CACC located in the forward primer (pcDNA \textit{calY} forward) without a stop codon following the sequence that allowed expression of a fusion protein containing the N-terminal polyhistidine tag and X\textsuperscript{TM}press epitope, while the reverse primer (Native \textit{calY} reverse) contained camelysin’s native stop codon (Figure 12). Once the PCR product was obtained, it was ligated into the vector and transformed into \textit{E. coli} TOP10 cells for propagation.
Figure 11. PCR amplification of the 598 bp camelysin gene (4 additional nucleotides for the addition of CACC) from *B. cereus* ATCC 14579. The 1% agarose gel confirms the presence of the 598 bp DNA fragment. Lane 1: 1kb plus ladder and Lane 2: amplified calY from genomic DNA.

Figure 12. Map of the prokaryotic expression vector pET100/D-TOPO® containing the native calY with the addition of CACC at the 5’ end. The stop codon, TAA was used to end translation and ampicillin was used to screen for positive clones once transformed into *E. coli* TOP10 cells.
Confirmation of the Successful Cloning into pET100/D-TOPO®

Plasmid DNA from five *E. coli* clones were obtained and screened for the presence of the camelysin gene using PCR (Figures 13A and 13B) and DNA sequencing. The plasmid DNA containing the camelysin gene was PCR amplified using the primers sets T7 forward / Native calY reverse and pET calY forward / T7 reverse yielding fragments of 794 bp and 679 bp respectively, confirming that camelysin was inserted into the vector.

![PCR gel](image)

Figure 13A. PCR amplification products on a 1% agarose gel used for identifying the correct insertion of *BCcalY* into the pET vector. Lane 1: 1kb plus ladder and Lanes 2-6: PCR of *BCcalY*-pET100D using T7 forward / Native calY reverse. This represented a 20% cloning efficiency.
Figure 13B. PCR amplification products on a 1% agarose gel used for identifying the correct insertion of BCca/Y into the pET vector. Lane 1: 1kb, plus ladder and Lanes 2-6: PCR of BCca/Y-pET100D using pET cal/Y forward / T7 reverse.

DNA sequencing on BCca/Y-pET100D was performed at SeqWright DNA Technologies (Houston, TX). Once the sequence information was obtained a BLAST2seq (39) was performed against the original gene sequences from B. cereus ATCC 14579’s cal/Y, confirming no mutations were introduced during the cloning process and that the gene was inserted into the TOPO vector. The vector was then transformed into E. coli BL21 Star™ (DE3)pLysS for expression.
Expression of *BCcalY*-pET100D

The vector contains a T7lac promoter that was induced by production of T7 RNA polymerase in the host cells and a lac operator that the lacI repressor protein (expressed in the BL21 cells) binds, thus decreasing the chances of basal expression. *E. coli* BL21 Star™ (DE3)pLysS cells have the lambda DE3 lysogen which has the gene for the T7 RNA polymerase and is tightly regulated by the lacUV5 promoter in the cells chromosome. IPTG was used to turn on the lacUV5 promoter, which resulted in overexpression of the protein encoded by the PCR product that was inserted into the pET vectors cloning site. Since overexpression can be a problem with toxic proteins, the BL21 Star™ (DE3)pLysS strain were used to express camelysin since they also produce a T7 lysozyme that slowed expression by binding to T7 RNA polymerase and inactivating it. The BL21 cells transformed with *BCcalY*-pET100D were induced with IPTG for 4 hours. After each hour, samples were taken and stored for further analysis. SDS-PAGE was performed on the all of the samples (Figure 14), and no noticeable differences were apparent upon staining with Coomassie blue.
Figure 14. Expression of BCca/Y-pET100D in BL21 Star™ (DE3)pLysS cells. No banding pattern differences were discernable. Lanes 1 & 11: MW marker, Lane 2: Induced BL21 Star™ (DE3)pLysS cells, Lanes 3, 5, 7, & 9: Uninduced BL21 cells at 1, 2, 3, and 4 hours respectively, Lanes 4, 6, 8, & 10: Induced BL21 cells at 1, 2, 3, and 4 hours respectively.

Western Blotting Analysis

After performing SDS-PAGE on samples from each vector system, a Western blot was performed and confirmed that the protein bands that were apparent on some of the gels were indeed that of camelysin (Figure 15). Despite not seeing any noticeable bands on the SDS-PAGE performed on the BCca/Y-pET100D expression, two bands were apparent in the samples that were induced with IPTG for 3 hr and 4 hr. These bands were located around 65 kDa and the purple color is indicative of a positive reaction, while the brown colored bands are negative. The 19 kDa bands seen on the SDS-PAGE from the BAca/Y-pBAD and BCca/Y-pBAD expression were apparent on the Western blot at the same location, thus indicating successful expression in both the pET and pBAD vector systems.
Azocasein Assay

To test the proteolytic activity present in all three vector system expression experiments, an enzyme assay using azocasein as a substrate was performed. Azocasein is made up of orange sulfanilamide groups that are covalently bound to the peptide bonds of casein. Once a protease attacks these bonds, small peptides and sometimes amino acids are released giving the solution a dark orange color. TCA was added after an overnight incubation at 37°C, which precipitated the protease and any undigested azocasein. Following centrifugation, the small digested particles (giving the orange color) are contained in the supernatant and the OD$_{365}$ is read (Figure 16), thus the higher the OD, the greater the amount of proteolytic activity present. The supernatants
of \( BAcalY\)-pBAD, \( BCcalY\)-pBAD, and \( BCcalY\)-pET100D appear to have the highest proteolytic activity indicating the presence of camelysin. The pellets also contain some activity, but less than the supernatants. Negative controls included HeLa cells transfected with the pcDNA vector with no insert (\( calY\)-pcDNA(-)) and \( E. coli\) TOP10 cells transformed with the pBAD vector with no insert.

![Proteolytic Activity of Cells](image)

Figure 16. Degradation of azocasein by camelysin. An increase in the OD\(_{365}\) indicated high proteolytic activity in the supernatants of the cells that were transformed and induced with camelysin using either the pBAD or pET vectors. Top10 cells transformed with the empty pBAD vector appear to have some proteolytic activity, but is insignificant when compared to the Top10 cells that were transformed with either \( BAcalY\)-pBAD, \( BCcalY\)-pBAD, or \( BCcalY\)-pET100D. HeLa cells transfected with the empty pcDNA vector exhibited minute levels of proteolytic activity. Error bars indicate the standard deviation from the mean for each cell type used.

**Sequence Analysis of \( calY\) in \( B. anthracis\) and \( B. cereus\) ATCC 14579**

A multiple sequence alignment was performed on both the nucleotide (Figure 18) and amino acid (Figure 17) sequences of \( calY\) from \( B. anthracis\) and \( B. cereus\) using the GAP alignment tool from the GCG Wisconsin Package\(^\circ\).
software to check for the degree of homology between the two proteins and their genes. The results of the alignment concluded there is a 94.78% similarity between the two organism’s nucleotide sequences. Out of the 594 nucleotides in both camelysin genes, there were 30 differences between the two sequences (Figure 18). There are 197 amino acids in both camelysin genes; the alignment identified 9 amino acid differences between the two sequences and concluded there was a 95.94% similarity between the two amino acid sequences (Figure 17).

Figure 17. Multiple sequence alignment of the amino acid residues for camelysin from B. anthracis and B. cereus. The first amino acid sequence is that of B. anthracis and the second is that of B. cereus. The amino acids that are highlighted in gray indicate a difference between the two proteins. Two dots between each residue that differs indicates a threshold of 2 for the alignment score conveying a strong similarity between the two amino acids, and one dot between each residue indicates a threshold of 1 for the alignment, conveying a similarity between the two amino acids, but not as high. A blank space between the residues indicates no similarity between the different amino acid residues.
Figure 18. Multiple sequence alignment of the nucleotides for camelysin from *B. anthracis* and *B. cereus*. The first nucleotide sequence is that of *B. anthracis* and the second is that of *B. cereus*. The nucleotides that are highlighted in gray indicate a difference between the two genes.
Prediction of the Putative Signal Peptide of Camelysin from \textit{B. anthracis} and \textit{B. cereus} ATCC 14579 using Neural Network and Hidden Markov Models.

Both neural network and hidden Markov models were used to predict where the putative signal peptide of the camelysin protein is located using the SignalP 3.0 server (3). Due to the fact this protease exhibits unusual behavior and has no conserved domains it was imperative to perform several forms of sequence analysis. The signal peptide analysis allows us to view differences between the two proteins signal peptides as well as differences in their cleavage sites. The neural network model calculates where both the predicted signal peptide and the position of the signal peptidase I cleavage site are located using two separate networks. The signal peptide was predicted to contain amino acids 1-29 with a cleavage site between residues 29 and 30 for the camelysin protein of \textit{B. anthracis} and \textit{B. cereus} ATCC 14579 (Figures 19A and 19B).

The hidden Markov model calculates whether or not a given amino acid sequence contains a signal peptide. If the sequence of interest is predicted to contain a signal peptide the location of the cleavage site, N-terminus, C-terminus, and alpha-helical segments of the signal peptide are included in the output. The Markov model predicted the signal peptide to consist of amino acid residues 1-27 with a cleavage site between residues 27 and 28 for the camelysin protein of \textit{B.}
*anthracis* and *B. cereus* ATCC 14579 (Figures 20A and 20B). The N-terminal region of the signal peptide was predicted to be comprised of residues 1-6, the alpha helical region 7-20, and the C-terminal region to be from 21-27 in the protease from both organisms.

Figures 19A and 19B. SignalP 3.0’s neural network model prediction of the camelysin protein’s signal peptide. The C score corresponds to the probability that a particular residue is located at the signal peptidase I site, the S score reflects the probability that residues are part of the signal peptide, and the Y score helps to predict the cleavage site at points where the steep slopes of the C score line are located. Figure A is the sequence from *B. anthracis* and Figure B is the sequence from *B. cereus*. Camelysin from both organisms are predicted to contain the same signal peptide and are predicted to be secreted.
Figures 20A and 20B. SignalP 3.0’s hidden Markov model prediction of the camelysin protein’s signal peptide. The cleavage probability is the location where cleavage of the signal peptide is most likely to occur; the N-region, H-region, and C-region probabilities correspond to the predicted N-terminal, alpha helical, and C-terminal regions of the signal peptide. Figure A is the sequence from *B. anthracis* and Figure B is the sequence from *B. cereus*. Camelysin from both organisms are predicted to contain the same signal peptide and are predicted to be secreted.
Secondary Structure Prediction of Camelysin from *B. anthracis* and *B. cereus* ATCC 14570

The predicted secondary structure of camelysin was obtained from the PSIPRED Protein Structure Prediction Server (21, 28). A multiple sequence alignment was performed using the GAP alignment tool from the GCG Wisconsin Package® software to identify similar structural characteristics between the two peptides from *B. anthracis* and *B. cereus* ATCC 14579 (Figure 21). The alignment revealed that the secondary structure between the two proteins is 95.94% similar. The alpha helical region of the predicted signal peptide is also apparent in this secondary structure analysis, however there are also two other helical regions, possibly involved with anchoring the protein to the membrane of the cell (Figures 22 and 23).

![Secondary structure comparison between camelysin from *B. cereus* and *B. anthracis*](image)

Figure 21. Secondary structure comparison between camelysin from *B. cereus* and *B. anthracis*. Out of the 197 peptides, only 4 peptides exhibited different secondary structure characteristics between the two peptide strands (gray residues). C, E, and H correspond to coil, strand, and helix respectively.
Figure 22. Secondary structure prediction of camelysin from *B. anthracis* using PSIPRED. The protein is predicted to contain 4 alpha helices and 12 strands.
Figure 23. Secondary structure prediction of camelysin from *B. cereus* using PSIPRED. The protein is predicted to contain 4 alpha helices and 12 strands, identical to the prediction for the proteins secondary structural characteristics for *B. anthracis*. 
DISCUSSION

The focus of this study was aimed at identifying, cloning, and expressing the active camelysin protease from *B. cereus* ATCC 14579 and *B. anthracis*. Previous studies demonstrated that camelysin isolated from an uncharacterized, unsequenced strain of *B. cereus* may play a role in the pathogenicity of this organism, which is why *B. cereus* ATCC 14579 was chosen. Blastn and blastp searches revealed a putative camelysin gene may also be located in *B. anthracis*. These findings along with the fact that *B. anthracis* is such a potent pathogen were the reasons why it was chosen for this study. Identifying a suitable method for obtaining large amounts of this highly hydrophobic membrane protease was another goal of this project due to the difficulty in obtaining even minute amounts of the protease from the native organism and also due to the dangers of culturing live *B. anthracis*. Obtaining large amounts of the camelysin protease, with or without a fusion tag will allow future studies to be performed that are focused on the delineation of camelysin’s possible role in the pathogenicity of these organisms.

The camelysin gene from *B. cereus* ATCC 14579 was first cloned into the mammalian expression vector pcDNA3.1/V5-His<sup>®</sup> TOPO<sup>®</sup> TA. Several proteins are linked to pathogenicity by causing damage intracellularly, like the lethal factor of *B. anthracis*. A mammalian expression vector was used to see if it would be
possible to express the protease intracellularly in a mammalian cell line to observe any possible pathogenic effects of the enzyme. This would allow intracellular studies of camelysin’s involvement in pathogenicity without purification of the protease, reconstitution into liposome’s, and then adding the protein to a cell culture. PCR, restriction digestion analysis, and DNA sequencing confirmed that BCcalY was successfully inserted into the vector. Plasmid was obtained by performing large scale plasmid isolation from the transformed TOP10 cells and the DNA electrophoresis confirmed the purity of the recombinant plasmid. HeLa cells were grown to confluency and the recombinant vector was transiently transfected into the cells. Unfortunately there was no expression of the camelysin protein as indicated by SDS-PAGE, western blotting, immunoblotting analysis, and an azocasein assay. To make sure there were no errors with the transfection, the positive control pcDNA3.1/V5-His-TOPO/lacZ® (pcDNA3.1 that contained the ß-galactosidase control) was transfected into the same HeLa cell cultures during the expression experiment. The IPTG assay (Figure 5) demonstrated that there was ß-galactosidase activity, indicating there were no problems with the transfection methodology.

There are several possible explanations for the mammalian expression failure. Mammalian systems often do not process or fold heterologous proteins properly because of different translational and post-translational machinery. The mature camelysin protein exhibits self aggregation and is extremely hydrophobic most probably due to oligomerization (13). These characteristics are only exhibited in the properly folded protein; if camelysin is not properly folded it could
be recognized by intracellular proteases which would destroy any camelysin protein produced immediately after translation. In addition, bacterial membrane proteases often need specific bacterial transport systems, like the Sec pathway for crossing and integrating into the membrane of the cells; in HeLa cells these pathways are not present. HeLa cell membranes also contain a much different phospholipid environment than the ones found in prokaryotic cell membranes. Direct membrane insertion of membrane bound proteases is often based on phospholipid composition (15).

Following the expression trial in the mammalian system, the camelysin gene was cloned into the pET100/D-TOPO®, vector. The pET vector has several advantages over pcDNA3.1/V5-His® TOPO® TA. There are two N-terminal fusion tags, a histidine tag and an X™press epitope which can be used for purification or detection of a fusion protein and both can be removed using enterokinase, due to the presence of an enterokinase cleave site located C-terminal to the tags. After successful PCR amplification the gene was inserted into the vector and transformed into the TOP10 cells. PCR and DNA sequencing confirmed that BCcalY was successfully inserted into the vector.

Mini-preps were performed on the TOP10 cells and the vector was transformed into E. coli BL21 Star™ (DE3)pLysS cells. Once induced with IPTG, the cells expressed the recombinant protease in a controlled manner. SDS-PAGE was unable to demonstrate the successful expression of camelysin once stained with coomassie blue. This is not unusual since previous studies have shown (13) that camelysin in its mature state forms hydrophobic aggregates that
do not stain with coomassie blue. However, western blot analysis detected a positive result with band at 65 kDa, similar to the molecular weight obtained of aggregated camelysin on SDS-PAGE gels that were stained with colloidal gold in previous studies (15). In addition to the positive results obtained for the western blot, the protein also exhibited proteolytic activity using an azocasein assay (Figure 16), indicative of the presence of camelysin in its active form.

Next, the camelysin gene from \textit{B. anthracis} and \textit{B. cereus} was cloned into the pBAD TOPO® TA expression vector. The camelysin gene from \textit{B. anthracis} was only cloned into this vector due to the difficulty in obtaining the genomic DNA. This allowed us to examine the expression of camelysin from both organisms using an expression vector designed especially for producing toxic proteins. Additionally, this vector contains an N-terminal leader peptide that is shown to increase the solubility of recombinant proteins. The C-terminal histidine tag and V-5 epitope were purposely left out of the recombinant protein since they can not be removed from the protein after purification, and their effects on the enzymatic activity were not known at the time. Once inserted into the vector, it was transformed into TOP10 cells and confirmation of the genes correct orientation and sequence was accomplished by PCR and DNA sequencing.

The recombinant clones expressed camelysin after induction using various concentrations of arabinose. SDS-PAGE confirmed the expression of the mature, non-aggregated 19 kDa protease after staining with coomassie blue. There are many possible explanations for the difference in molecular weight between expressions, the first being the presence of fusion tags on the protease
of the pET vectors product. By adding the tags, the protease may have taken on a more native and aggregated form as seen in previous studies, which does not stain well when coomassie blue is used. The hydrophobic aggregation of this protease makes it difficult for it to travel through SDS-PAGE gels even after using reducing conditions (13). The proteins expressed in the pBAD system, lacked these fusion tags and also contained a leader peptide which increases the solubility of most the proteins that are expressed, which would explain the different results on SDS-PAGE between the pBAD and pET systems. A western blot performed on both the BAcalY-pBAD and BCcalY-pBAD recombinant proteins demonstrated a positive result which included a band at 19 kDa, backing up the SDS-PAGE analysis. An azocasein assay was performed on the two recombinant proteins following the western blot, and both demonstrated proteolytic activity, consistent with the active native protease obtained from B. cereus and the pET vectors recombinant protease.

Various methods of sequence analysis were performed on the camelysin gene and protein from both B. cereus ATCC 14579 and B. anthracis Ames strain. The first analysis included a multiple sequence alignment which demonstrated the high similarity between the two sequences at both the nucleotide and amino acid level. Since camelysin from B. cereus is shown to have properties suggesting it may be involved in pathogenesis, it gave reason to clone the gene from both organisms. Signal peptide analysis using SignalP 3.0 allowed prediction of the position of the signal peptide, its cleavage site, and whether or not the peptide is soluble or insoluble. The mature protein was show to have 2
different predicted sites of cleavage, probably due to the different models used, the first site was between amino acid residues 29 and 30 and the other was between amino acids 27 and 28, the latter was previously reported as the putative site in another paper (16).

Secondary structure analysis was performed using PSIPRED. Results of the structure analysis further strengthened the possibility that the signal peptide of camelysin was located within the first 30 amino acids. Previous studies on camelysin suggest this location due to the fact that signal peptides usually have basic amino acids in the N-terminus, followed by a hydrophobic section of amino acids (usually represented by helical secondary structures), and Gly residues at the C-terminus (16, 25, 30, 33). The PSIPRED prediction found two more possible alpha helices not previously described that could be involved in with the hydrophobic interactions that keep camelysin associated to the cell membrane. Grass, et al, have already demonstrated that camelysin is neither bound to the cell membrane by electrostatic interactions (camelysin was not removed from cell membrane after washing under high salt conditions and after using enzymes that cause lysis), transmembrane stretches of hydrophobic amino acids (which is only found in the N-terminal signal peptide), the modifications of lipoproteins (the recognition domain for the lipoprotein signal peptidase is not present in camelysin), or covalent linkages (molecular mass of protein matches well with predicted molecular mass from sequence) (16). The two new alpha helices seem to exhibit amphipathic properties (data not shown) from a hydrophobicity prediction performed in the Clone Manager software (Science and Educational
Software, Inc. Cary, NC.). These amphipathic alpha helices are one possible way that the protease may bind to the cell membrane and they fall within one of the six hydrophobic domains found using the DAS transmembrane server (8) identified in previous studies (Grass 2004).

This study indeed proves that the camelysin gene is found both in *B. cereus* and *B. anthracis*, and that the active protease, although only weakly proteolytic, can be expressed in gram negative prokaryotic expression systems. This will prove extremely useful in the future as this system, or newer gram positive vector systems can be used to create knock-out clones to identify the active region of the camelysin protein (9). Due to the fact that camelysin was found in *B. anthracis* and *B. cereus* and since previous studies demonstrate it contains pathogenic activity, some of the recombinant clones used in this study can be useful for large scale preparation of camelysin for cell culture and possibly animal models to examine the role it may have in the pathogenicity of these organisms.
REFERENCES


