THE PENNSYLVANIA STATE UNIVERSITY
SCHREYER HONORS COLLEGE

DEPARTMENT OF BIOCHEMISTRY AND MOLECULAR BIOLOGY

CLONING OF INSECTICIDAL BT GENES FOR APPLICATION TO PROTECTION OF MUSHROOMS FROM FUNGUS GNATS

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ABSTRACT

This thesis describes the initiation of a project to generate mushrooms that will be protected from pests using the insecticidal proteins of the bacterial *Bacillus thuringiensis* (BT). The concept builds on the discovery of a previous student that found that proteins were transported from the lower mycelial layer up into the mushroom cap in such a way that the resulting edible mushroom is only contains the insecticidal proteins and does not contain the bacterial genes. This is accomplished by generating a BT-transgenic mycelium to be used as the lower under-layer, with a non-transgenic over-layer that generates the edible mushroom cap. The thesis work started with learning the basics of molecular biology for gene cloning and mushroom transformation, while conducting the background research on the BT insecticidal proteins as well as the target insect pests. The pests are 'fungus gnats' where their larval 'maggot' stage feeds on the mycelium within the compost. It is this larval stage that the BT can kill very selectively due to the behavior of the BT proteins that are in a crystal form until the dissolve in the alkaline gut of the larva to be active. This same behavior is why these proteins are non-toxic to humans and used in many GMO crops. This discovery phase of the research was conducted largely independently, since this project was not a part of an existing research project. The effort revealed the wide array of BT proteins where there is not only many BT genes in a given strain of *B. thuringiensis*, but there are many different strains - each of which has different specificity for killing larva of different insects. The literature review revealed that *Bacillus thuringiensis* serovar Israelensis was a good candidate for BT genes. The experimental component of the thesis involved obtaining these strain and the subsequent cloning of eight genes from its native megaplasmid. The genes were each PCR amplified with individually designed primers that included restriction fragment ends to facilitate subsequent DNA manipulation. The PCR product was digested and ligated into the PET28C cloning vector which was placed into two *E.coli*

strains. The high-copy number *E.coli* DH5α was used for sequence verification; the *E.coli* BL21 is an expression vector that will be used to test the expressed proteins on fungus gnat larva to determine the relative toxicity of the various BT gene candidates. As part of training for this project, transformation methods for *Agaricus bisporus* (common supermarket button mushroom) were learned to transform reporter genes into this mushroom using hypertranslation sequences. As a result of this aggregate of general methods and specific molecular biology, the stage has been set to conduct the BT bioassay and generate the transgenic mushrooms, where the final stage of the thesis has been 'passing the torch' to a freshmen honors student who will continue towards the goal of generating BT protected mushrooms which do not contain the transgene and therefore provide for gene containment as well as the very interesting question of whether the mushrooms are a GMO food or not - since they do not contain the transgene (the current paradigm of GMO crops).

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I would like to give my thanks to my advisor, Dr. Wayne Curtis, for his continual guidance and an outlook on life that inspires intensity and focus in every aspect of the lives of those who work with him. The members of the Curtis lab, both past and present, have had an immeasurable impact on my own growth both as a student and a person. Both April Hile and Elicia Yoffee are specifically acknowledged for their immeasurable aid in the development of fly cultures. I would like to give special thanks to Benjamin Woolston, whose mentorship and expertise in all things molecular has made this work possible.

CHAPTER I

INTRODUCTION AND BACKGROUND

The Target Mushroom, *Agaricus bisporus*

In attending Penn State I was not aware of the importance of the mushroom industry to Pennsylvania. PA is the largest producer of edible mushroom in the world. In fact, the 'Plant Pathology' department has a long history of mushroom research, including ongoing collaborations with Penn State's "Mushroom Research Center." A predominant focus is on the common supermarket 'button mushroom,' a crop granted its appealing white coloration by research efforts in Pennsylvania (personal communication). The basidiomycete *Agaricus bisporus* is cultivated around the world for its edible fruiting body. Responsible for commercial mushroom varieties ranging from the button mushroom to the portabella depending on degree of maturity, industrial processes are highly adept at rapidly producing large volumes of mushroom, exceeding 12 million tons annual production worldwide[1].

Mushrooms can be grown in a relatively wide variety of settings. Where the industrial scale growth facilities built in the limestone mines of Pennsylvania can produce as many as 23 million pounds of the mushrooms per year[2], mushroom growing is also common in small scale 'home-brewed' operations in growth suitable for producing a household mushroom supply under the kitchen sink[3]. In all cases, the mushrooms are grown in a staged process. Cultures are initiated in extremely nutrient rich substrate that contains grain. This "grain spawn" allows mycelia to rapidly grow to high densities, and is used to initiate cultures on less expensive substrates, such as manure or hay. Mycelia will continue to grow in an amorphous web throughout the material so long as nutrients are present. The edible mushroom cap, however, will not be produced until the

fungus senses the need to reproduce by making spores. By adding an upper 'casing layer' of nutrient-depleted peat and mycelial inoculum over the more nutrient dense 'compost layer', the fungal culture can be induced to produce the reproductive cap to be collected as the mushroom crop. While the initial production of caps by this fashion represents the majority of the biomass yield of a cycle, additional fruiting events (flushes) will occur at one-week intervals, producing as many as five batches from a single inoculated tray.

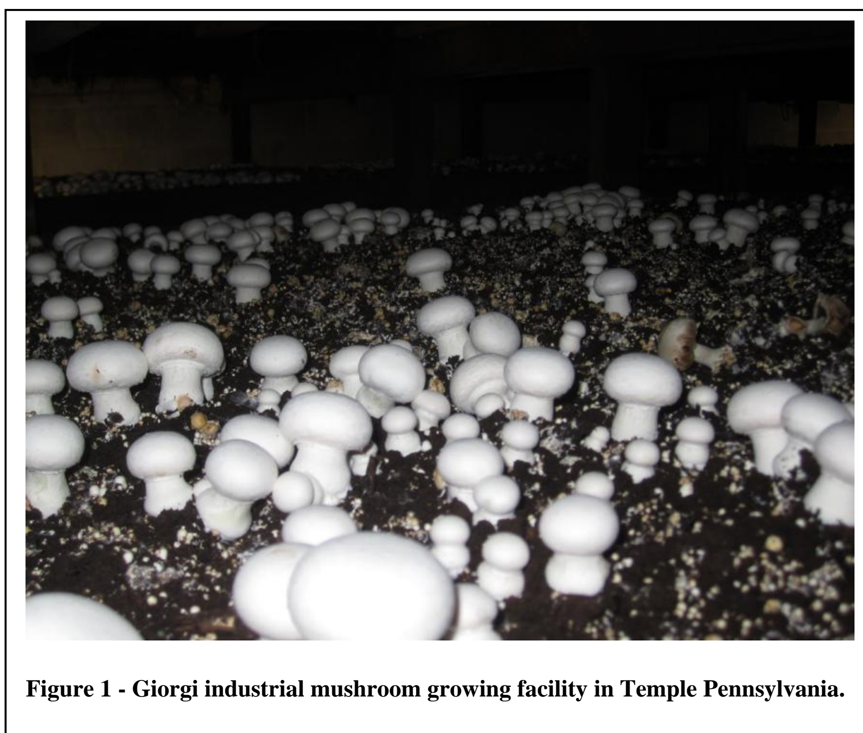

Figure 1 - Giorgi industrial mushroom growing facility in Temple Pennsylvania.

Recent efforts in the Plant Pathology department have been focused on the development of genetic tools for the manipulation of *Agaricus bisporus*. This has led to the development of a robust *Agrobacterium*-mediated transformation protocol for the fungus[4, 5]. In addition, it was recently discovered in a collaboration between the Romaine and Curtis lab, that the introduction of different genotypes in the compost and casing layers of a mushroom growing inoculation, it is possible to produce mushroom caps containing a genetically engineered protein product, while lacking the associated genes[6]. Together, these two recent findings put into place the framework

necessary to facilitate research toward large scale production of eukaryotic proteins such as vaccines to replace current time-consuming and expensive methods. To date, these mechanisms have only been reported publically for marker genes. A major goal of this project is to demonstrate the proper production, transport, and activity of useful, non-marker transgenic proteins in the mushroom host.

Mushroom Pests Hurt the Industry

One of the most significant barriers to the growth of mushrooms on any scale is the presence of a variety of mushroom pests and diseases. *Agaricus bisporus* is susceptible to attack by insects, bacterium, viruses and even other fungi. The most widespread and well known of these pests, however, are the mushroom flies. These pests include flies of both the *Phoridae* and *Sciaridae* families. Phorid grubs such as *Megaselia halterata* are known to inhabit the compost of a growing mushroom bed, and cause damage primarily by consuming the mycelia in this nutrient rich substrate. Sciarid flies, however, are known as the single most destructive of mushroom pest. While these grubs of this family also inhabit the compost layer of a mushroom bed, they are also known to burrow through the stem of the mushrooms and infest the mushroom fruiting body itself. Studies have shown that the mushroom sciarid *Lycoriella auripila* is capable of reducing the mushroom yields by as much as 80%[7]. In addition to the direct damage that these pests are capable of causing to a mushroom crop, flies have a tendency to act as vectors for the spread of less mobile mushroom bacterial and viral diseases within a facility, making the control of these pests critical to mushroom growing on all scales[3].

A variety of methods are already in place for the control of mushroom pests in growth facilities. These range from spraying down the premises with toxic pesticides on a regular basis, to the introduction of very selective insect parasitic nematodes in the compost prior spawning[3].

These methods, however, incur significant additional costs, food safety ramifications, and labor required to prevent infestations from harming mushroom crops. Research efforts in the field have begun to turn toward the use of highly selective biological agents as alternatives to chemical pesticides due to growing health concerns in the food industry, however, these alternatives still utilize a labor- or capital-intensive soil drench method to apply the compounds to growing mushroom beds[8]. By utilizing the previously mentioned genetic engineering techniques it should be possible to provide an inherent resistance in a mushroom spawn to such pests without incurring additional costs or labor requirements.

CHAPTER II

SELECTION OF A SUITABLE RESISTANCE GENE

Pest management has always been an issue of primary concern in the biotechnology industry. A growing trend in plant biotechnology is the use of the bacterium *Bacillus thuringiensis* (BT) as a biological alternative to toxic chemical pesticides. Different serovars of BT have been shown to produce crystalline proteins with very specific activity against different orders, families, and even genera of insects[9]. Literature review has shown that while most of the Cry proteins are specific for insects in the order Lepidoptera, a particular strain called *Bacillus thuringiensis* serovar *israelensis* (BTi) has been shown to provide specific activity against dipteran insects, most testing having occurred to define the toxin's specificity against mosquitoes[10].

BTi has been formulated by a variety of companies, and put into common use as a standalone insecticide in recent years. Because of its ability to prevent infestations from a variety of pests ranging from gnats to mosquitoes, it is often used in sports fields and low lying areas to prevent the growth of these species[11]. BT produces the crystallized form of its toxins under stressed and nutrient deprived conditions. As the organism begins to sporulate, the crystalline proteins begin to accumulate in inclusion bodies[12]. As a result, BT formulations for commercial use can include just the purified crystalline proteins, or the entire spores themselves. In both cases, the toxic component is most often dispersed in liquid water and sprayed over crops in a manner similar to standard chemical pesticides[13].

BT toxins are widely considered a safe insecticide because of their ability to selectively target insects without harming humans or other mammals. The crystalline toxin proteins of BT require the unique alkaline environment of the insect midgut to solubilize, and require proteolytic

activation by enzymes of the insect midgut. Furthermore, the active form of the Cry proteins are required to bind with a very high degree of specificity to the protein targets on the intestinal walls of the insect's midgut. Once bound, the toxin is able to insert itself into the membrane, and associate as a tetramer to generate a potassium selective pore, leading to the osmotic lysis of sheath cells on the intestinal wall. Insects ingesting this toxin are killed by septicemia caused by the loss of the integrity of the midgut membranes, as well as starvation caused by the inability to absorb nutrients[12,14,15]. Because the mammalian stomach is a highly acidic environment, these proteins are destroyed before they can ever achieve an active and toxic form. In addition, the mammalian digestive tract lacks the target receptors required for Cry proteins to bind and associate to form lytic pores in the cell membrane. As a result, these toxins are completely inert in the human physiology, and have been demonstrated as safe to consume[16].

Although the use of BT formulations has allowed a reduction in the use of potentially dangerous chemical pesticides, the use of these as topically applied pesticides suffers from many of the same disadvantages regarding the need for increased labor and/or capital costs to be used. To ease the burden on crop growers, a recent movement in the biotechnology industry has been the genetic engineering of selected BT proteins for constitutive production in plants[17]. These efforts have seen the greatest degree of success in cotton and corn, although many additional crops are being evaluated for the efficacy of the use of these genes. While there is still some degree of debate in the public sphere regarding the safety and ethics of using these toxins, EPA and FDA approval has been granted for many varieties of food crops that express these toxin proteins directly, making the constitutive expression of BT toxins generally accepted as a safe and effective method of pest control[16].

Specific proof of action of BTi against the target mushroom dipteran *Lycoriella auripila* has not been shown; however, the broad spectrum of insects against which *Bacillus thuringiensis* sp. *israelensis* has been commercially formulated would suggest that it is the most likely

candidate for the control of mushroom pests. Studies have shown that whole organism formulations of BTi are an effective pesticide for killing very early populations of the mushroom sciarid *Lycoriella ingenua*; however, the conclusion was reached that this formulation is ineffective for the control of insect pests in mushroom growing due to its relatively low half-life. Because the effective action of BT occurs in early sciarid pests, it is necessary for the bioactive BT toxins to be present in the environment during early colonization of the pest. This makes BTi highly ineffective for the standard paradigm of rescuing crops once infestation has been observed[18]. The production of BTi toxins within the growing fungus itself, however, would serve a constant protective function against the establishment of pest colonies throughout the lifetime of a mushroom bed, allowing these disadvantages to be overcome.

Because of the difficulty of protein purification and the ease of whole organism BT spore formulation, the precise interactions and specificity of the various toxic proteins of BT remain largely unstudied. In addition, the precise protein content of the crystals can vary depending on the specific conditions under which BT begins to sporulate[15]. As a result, while it has been shown that the full complement of proteins produced in BTi crystals have activity against *Lycoriella* fungus gnats, the effectiveness of individual proteins are unknown, and thus must be tested for efficacy through the utilization of an insect bioassay[19]. The toxins of BTi are all found on a single plasmid called pBtoxis. Four genes producing toxins from the crystalline protein family were selected, named CryIVA, CryIVB, CryIVC, and CryIVD. Each of these genes has significant similarities, with as high as 40% sequence homology at the C-terminus, but they have been shown to give slightly different biological specificities. In addition to these Cry proteins, two genes for toxins in the cytolytic domain have been selected for testing, as it has been shown that the inclusion of these proteins in toxin crystals can significantly increase their toxicity. Finally, a 15 kDa protein was identified as a "helper protein" that may posttranslationally modifies the Cry toxins to increase their potency[20].

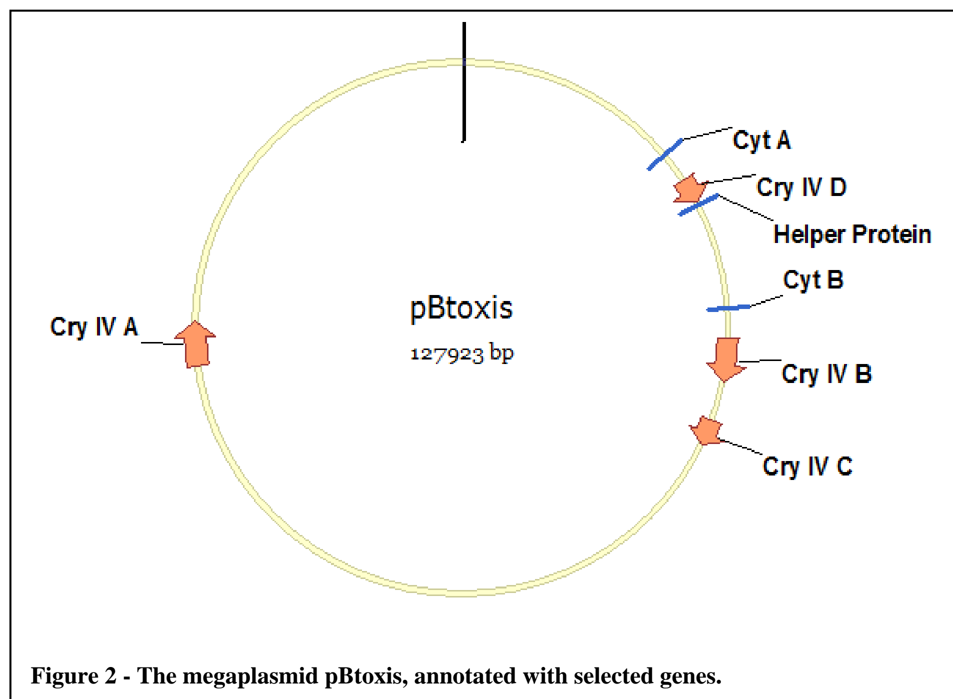

Figure 2 - The megaplasmid pBtoxis, annotated with selected genes.

BT samples were obtained from the Bacillus Genetic Stock Center, a repository for the various discovered and modified BT strains. Strains of BT typically have multiple plasmids, including the *israelensis* serovar. To aid the process of gene amplification from the pBtoxis plasmid, a plasmid cured strain modified to bear only the pBtoxis megaplasmid was selected, with a catalog number of 4Q5[21].

CHAPTER III

MOLECULAR CLONING OF BT GENES

Cloning Strategy Overview

The cloning strategy for transferring the crystalline proteins CryIVA-D, cytotoxins A and B, and the 15 kDa helper protein identified previously all follow the same basic principles. The plasmid, pBtoxis, was isolated to provide a stable, reliable source of toxin genes. To aid in the downstream characterization process, a set of reverse primers were prepared for each gene target both with and without the inclusion of a histidine tag. PCR was used to amplify each gene individually, and was followed by sticky end ligations with the pET-28c vector to generate the expression plasmid for each gene. Heat shock was used to insert the plasmid first into the cloning strain DH5α for plasmid amplification and insert verification, then into the expression host BL21.

Megaplasmid Isolation

The very large size of the pBtoxis plasmid (nearly 128 kbp) meant that traditional plasmid preparation protocols utilizing column filtration to separate plasmid from genomic DNA could not be used. In addition, such large plasmids generally have very low copy numbers within the bacterium, thus reducing the potential yield from the culture. A procedure was adapted from methods described in the Bacillus Genetic Stock Center catalog and methods developed for the isolation and study of Bacterial Artificial Chromosomes[21,22]. Procedure was performed as described in Appendix B, yielding 740 μg in plasmid for storage and further analysis.

To confirm the identity of the isolated plasmid, a restriction digest with the enzyme BglII was performed. Using Vector NTI, it was determined that the predicted pattern should be expected: two bands near 11 kbp before the 10 kbp ladder fragment, two bands between the 10 and 8 kbp ladder fragments, two bands between the 8 and 6 kbp fragments, followed by many smaller bands. Figure 3 shows that the bands above 6000 bp align correctly with the predicted values; however, due to the concentration of DNA present in the smaller bands, they cannot be resolved from the background noise.

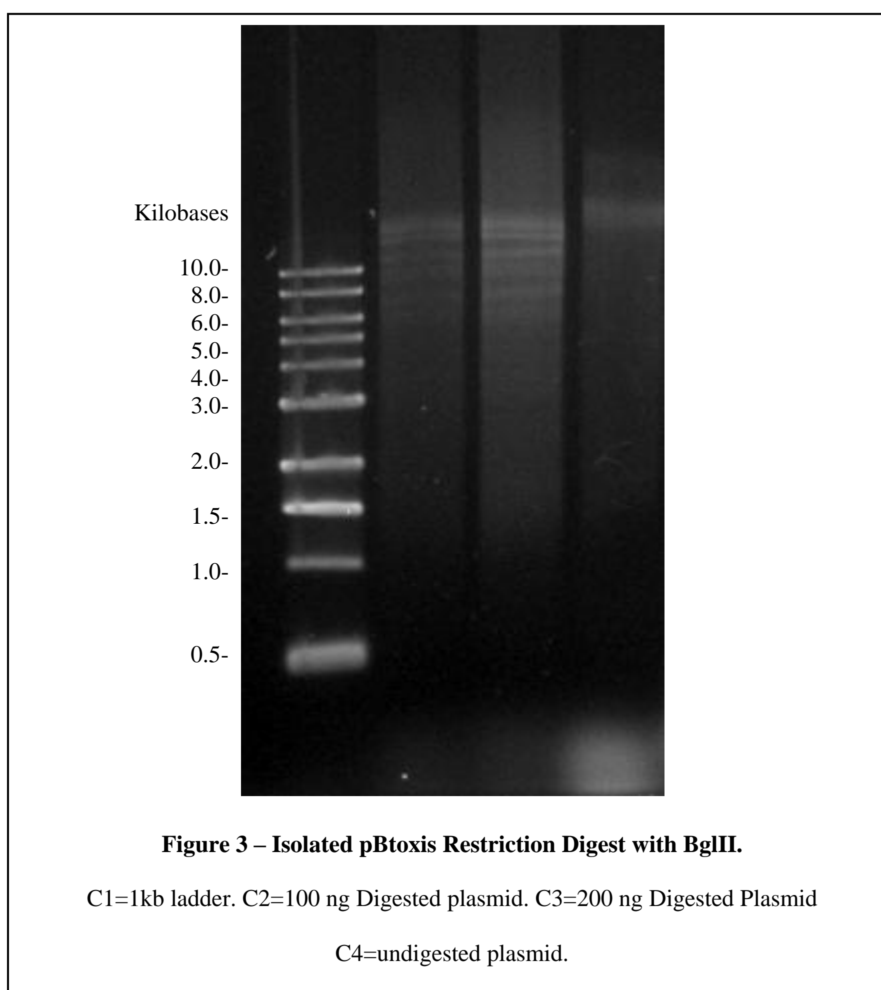

Figure 3 – Isolated pBtoxis Restriction Digest with BglII.

C1=1kb ladder. C2=100 ng Digested plasmid. C3=200 ng Digested Plasmid

C4=undigested plasmid.

Vector Design

The vector selected for this work was the pET-28c expression vector, detailed in Figure 4. This expression vector was selected for its general ease of use. It provides the constitutive expression of kanamycin resistance for cloning selection, and allows control of expression of the selected insert through a lac inducible promotor and associated LacI repressor to provide improved expression control. Multiple cloning sites are available both before and after a histidine tag, allowing the design of primers that include and exclude this tag from the protein product. The restriction site NcoI was selected at the 5' end of the insertion site for its inclusion of the methionine start codon. At the 3' end of the insertion site, the restriction site SalI preceding the histidine tag was selected to allow modification of the primer to produce both a native or his-tagged protein product upon induction. By using only these cloning sites on the vector, preparation of the vector for ligations is significantly simplified.

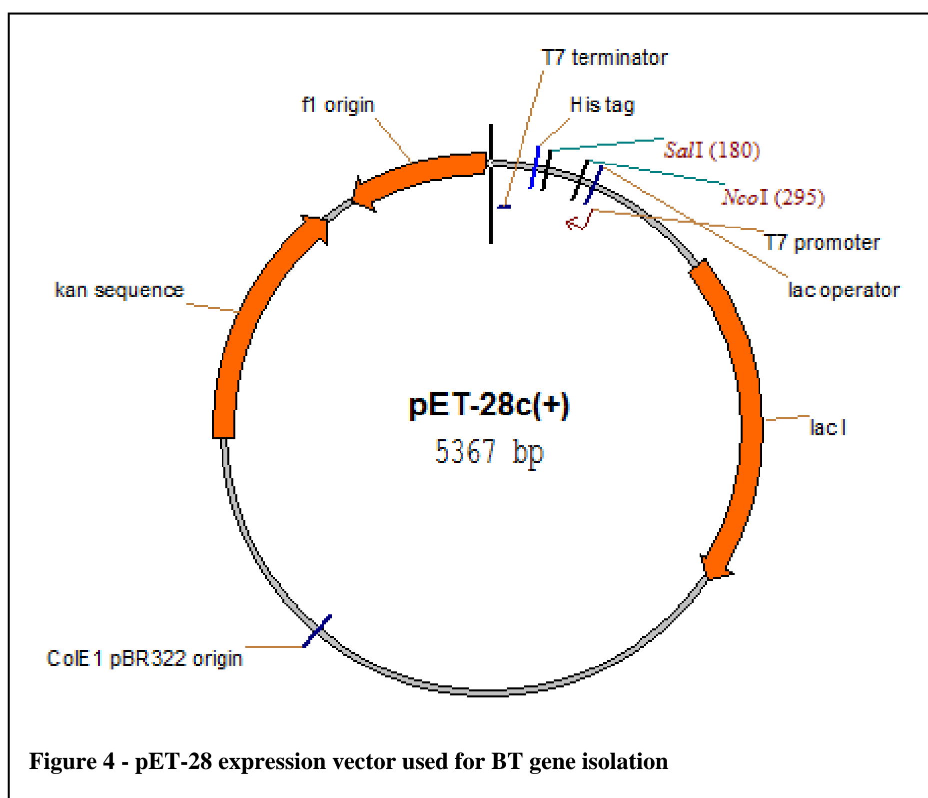

Figure 4 - pET-28 expression vector used for BT gene isolation

To confirm to identity and purity of the pET28c samples received, a restriction digest with SalI was performed. The results shown in Figure 5 show that while there is a significant degree of RNA contamination associated with the plasmid prep performed, the plasmid linearizes to approximately the correct size (5300 bp), and is cut in only one location by this restriction enzyme. The non-digested lanes show the presence of closed ring DNA, confirming that the full plasmid has been isolated.

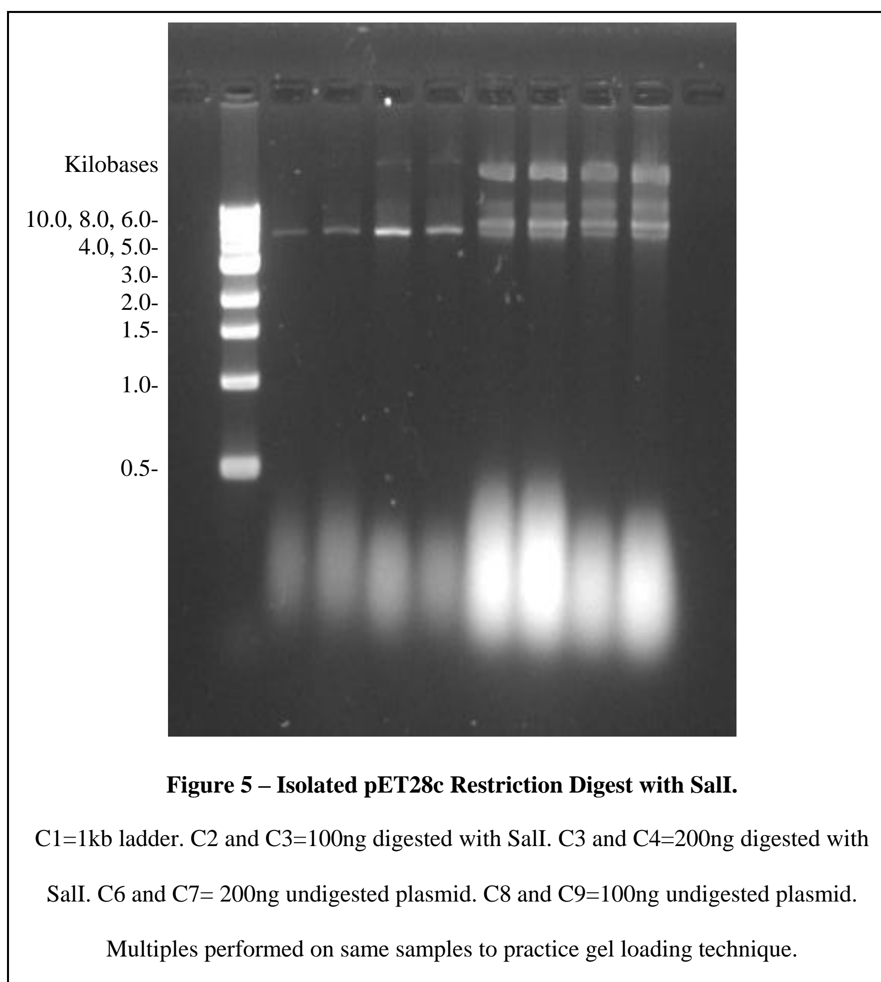

Figure 5 – Isolated pET28c Restriction Digest with SalI.

C1=1kb ladder. C2 and C3=100ng digested with SalI. C3 and C4=200ng digested with SalI. C6 and C7= 200ng undigested plasmid. C8 and C9=100ng undigested plasmid. Multiples performed on same samples to practice gel loading technique.

Primer Design

All primers were designed using the same basic set of guidelines. To facilitate the ease of running PCR reactions, it is desirable that the primers all have similar melting temperatures. For this experiment, a melting temperature of approximately 65°C was selected as a target. In addition, it is typical that primer GC contents be approximately 40-60% to facilitate annealing. Utilizing the software PerlPrimer[23], primer secondary structures and dimerization energies were minimized through the avoidance of repeats and primer runs.

The forward restriction site selected on the vector was NcoI; however, due to the presence of this cut site within the sequences for CryIVA and CryIVB, the restriction enzyme BspHI producing compatible sticky ends with NcoI was also utilized as a forward restriction site for primer design. In both cases, as the methionine start codon is included in the restriction site, the binding region of the primer was selected exclusively from the coding region of the gene. For the targets Cyt A, Cyt B, CryIVA, and CryIVB, the cut sites could not be matched exactly to the coding sequence of the protein. In the case of Cyt A, a single isoleucine was added to the N-terminus of the protein. For Cyt B, CryIVA, and CryIVB, a single glycine was added to the N-terminus. According to the N-end rule, glycine and isoleucine are stabilizing residues in *E. coli* that protects against proteolytic degradation of these gene products[24]. In addition, the small size, nonpolarity, and location of the amino acids inserted, it is considered extremely unlikely that they will have an effect on the folding and activity of the protein products.

The reverse restriction site selected on the vector was SalI. To produce the native protein from BT, a reverse primer for each gene was designed with a stop codon placed just before the SalI restriction site. By excluding the native stop codon in the primer, it is possible to include the following amino acid sequence of the vector to the end of the protein: V D K L A A A L E H H H

H H H. The inclusion of this six-X poly-histidine tag can significantly improve the ease of purification and verification of the expressed protein. Because of its location at the end of the C-terminus of the protein, it is again unlikely that the addition of this sequence will significantly affect the folding or activity of the protein; however, the length of this addition makes interference a possibility. Primers were ordered for both the tagged and non-tagged versions of the protein. Exact primer sequences can be found in Appendix A.

PCR Amplification

PCR amplification of all genes was performed using Phusion® polymerase. Each gene was successfully amplified from pBtoxis. Figure 6 shows the amplification product for the six toxin genes, while Figure 7 shows the amplification of the additional soluble helper protein.

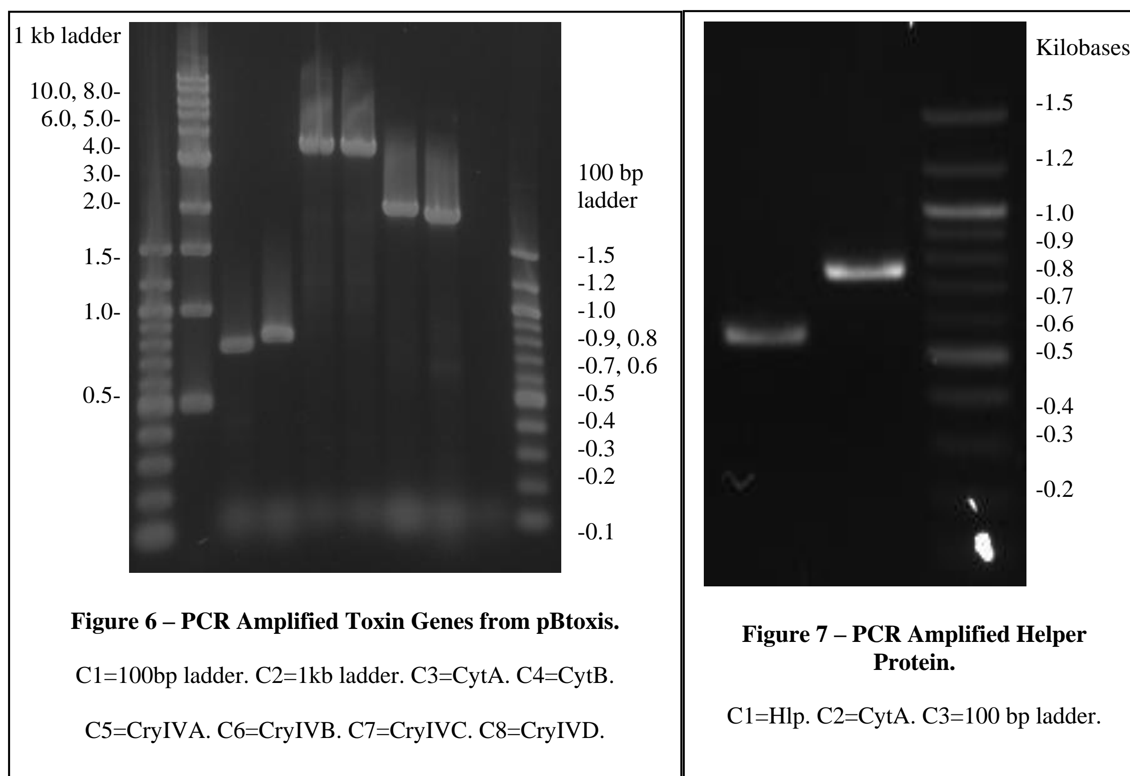

Figure 6 – PCR Amplified Toxin Genes from pBtoxis.

C1=100bp ladder. C2=1kb ladder. C3=CytA. C4=CytB. C5=CryIVA. C6=CryIVB. C7=CryIVC. C8=CryIVD.

Figure 7 – PCR Amplified Helper Protein.

C1=Hlp. C2=CytA. C3=100 bp ladder.

Digestion, Ligation, and Transformation

Restriction digests of the PCR products were performed using the appropriate restriction enzyme as noted in Appendix A. Digestion, Ligation, and heat shock procedures are described in Appendix B. The constructs were first ligated into the cloning host, *E.coli* DH5α. After allowing colonies to grow on kanamycin selection plates overnight, six resistant colonies were selected and evaluated for the size of the plasmid insert using colony PCR. To minimize the risk of false positives based on the PCR products used in ligation, T7 primers bordering the vector insert site were utilized for colony PCR. Two colonies for each construct were selected and grown up for cryopreservation. Figure 8 shows the results of colony PCR for the genes CytA (top) in which only one colony contained an insert at 750 bp, and CytB (bottom) where all colonies contained the successfully ligated plasmid as indicated by the PCR product at 800 bp.

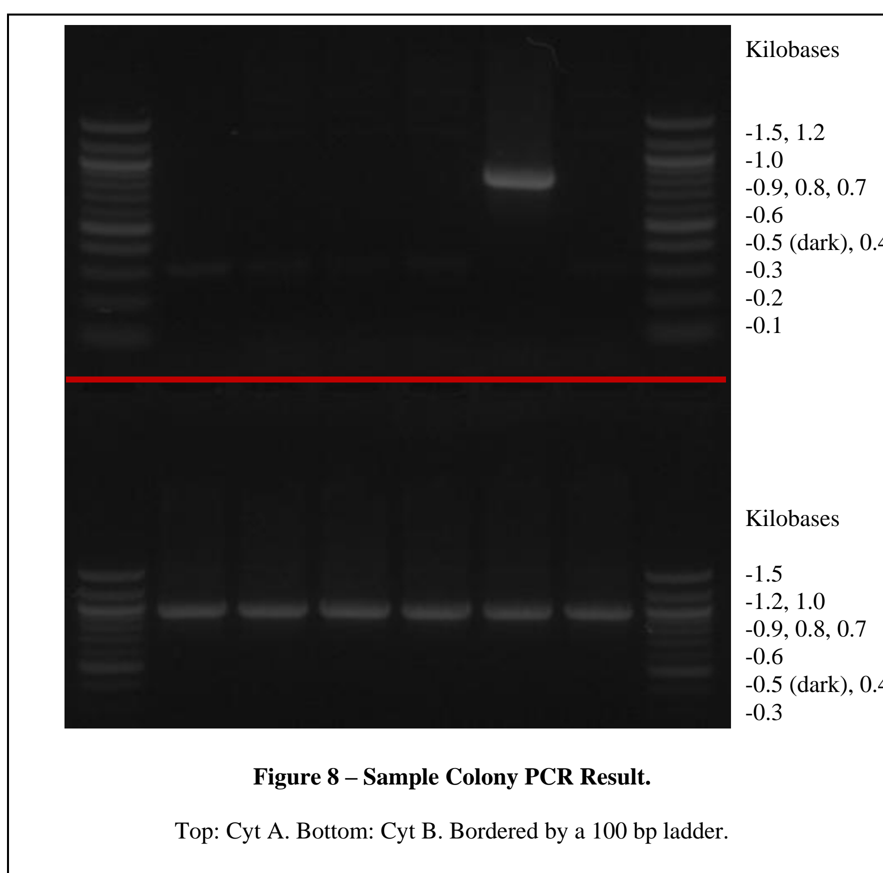

Figure 8 – Sample Colony PCR Result.

Top: Cyt A. Bottom: Cyt B. Bordered by a 100 bp ladder.

Sequence Verification

Plasmid preparations of each construct were performed from the DH5α cloning host utilizing Omega Bio-tek E.Z.N.A Plasmid Mini Kit I[25]. To obtain suitable yields of the non-cloning vector, cultures of 10 mL were grown and spun down for use in the columns, rather than the recommended 5 mL. The plasmid from each gene insertion was sent to the Huck Institute Genomics core facility for sequencing analysis using T7 primers. In all cases, sequencing results confirmed the proper integration of the targeted genes. Sequencing results are presented in Appendix A. Once the sequence for each gene was confirmed, the chemically competent cells of the expression host BL21 were prepared and transformed with the isolated plasmid using heat shock procedures, verified by colony PCR, and cryopreserved for future use.

CHAPTER IV

ONGOING AND FUTURE WORK

Mushroom Transformation

A major ongoing aspect of this work is the final development and genetic engineering of *Agaricus bisporus* to produce the BT toxins isolated in this work. To learn the techniques necessary for the transformation of *Agaricus bisporus*, three gene constructs originally developed by the alumni honors student Benjamin Woolston (plyGusNos, plyCPMVGus, and plyCPMVGus19) were inserted randomly into the *A. bisporus* chromosome utilizing the Agrobacterium-mediated vacuum filtration method using the bacterial strain AGL-1 and 30 μg/mL hygromycin B for selection[4,5]. These constructs were then evaluated using the histochemical GUS stain to evaluate differences in expression between these three constructs. While no major differences were seen between the three constructs used, significant differences in expression level and pattern were observed between insertional events of the same gene, as shown in Figure 9. This can most likely be attributed to differences genetic context of the insertional event. This is due to the nature of random insertion that is the basis of *Agrobacterium* genetic transformation in the genes in the transfer (T-DNA) are delivered into the fungal cell using the virulence mechanism of this plant pathogen[26]. It was noted that in some cases, the gene was expressed differentially in the newly growing regions of the mycelia, while others saw accumulation only in the more mature regions of the growth. These insertional differences in expression could have extremely important effects on the protection afforded by the expression of BT toxins in a mushroom, as proteins expressed only in old growth may leave the growing mycelia susceptible to pests.

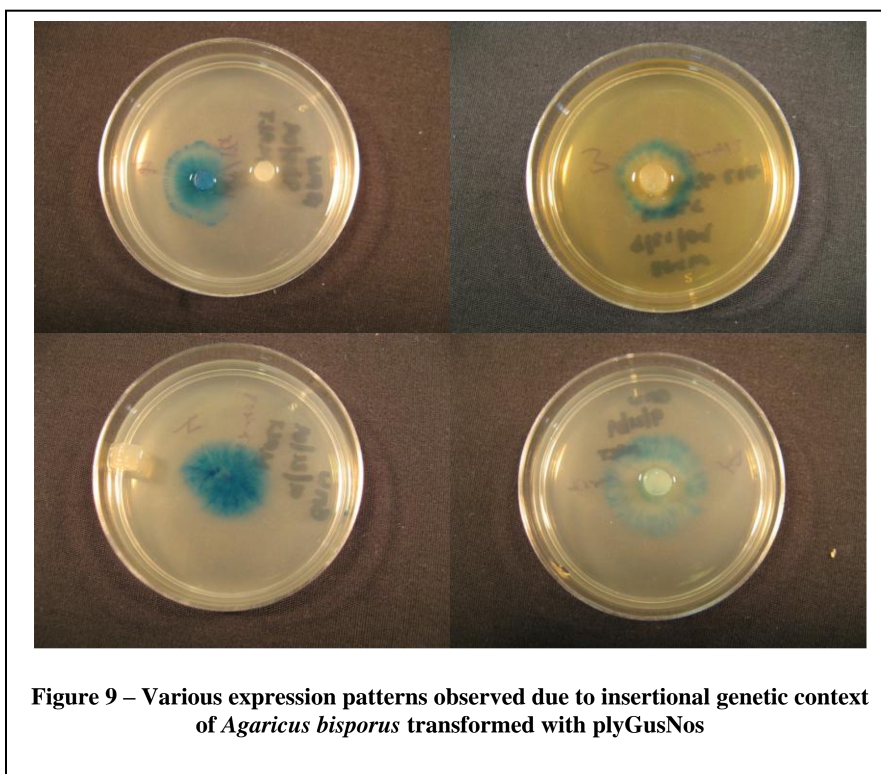

Figure 9 – Various expression patterns observed due to insertional genetic context of *Agaricus bisporus* transformed with plyGusNos

To carry out genetic transformations of *A. bisporus* using *Agrobacterium*, the BT genes will need to be moved into an *Agrobacterium* binary vector. This binary vector system has been created to retain the functions of the DNA transfer functions of the tumor-inducing (Ti) plasmid, while separating a smaller cloning plasmid that is compatible for *E. coli* replication and inter-species transformation[26]. While cloning within these binary vectors is straight-forward, expression within the *A. bisporus* fungal host will require selection of appropriate promoters. The prior work by Ben Woolston utilized the laccase promoter, since this is highly expressed in the compost layer since it would normally drive the expression of hydrolytic enzymes in this growth phase. Since the Curtis Laboratory has extensive experience in plant genetic engineering, further work will be facilitated by combining the prior experience of the Romaine laboratory with ongoing efforts to develop improved *Agrobacterium* transformation methodologies for fungi involved in the breakdown of lignocellulosic materials.

Preparation for Insect Bioassay

Simultaneous efforts were made by a separate student in the lab to develop the capability to grow sterile *Lycoriella auripila* cultures for use in insect bioassays. Unfortunately, due to extenuating personal circumstances of that student, this project was never completed. However, she had managed to make contacts through the Penn State Agricultural extension agents with a Giorgi mushroom growth facility manger (Phillip Coles, who is conveniently an enthusiastic Penn State Alumni). These efforts have been reinitiated as a mentorship project in which the Chemical Engineering freshman Elicia Yoffee. She has been directing efforts to obtain and maintain mushroom pests in a lab setting. The primary goal of this work is to develop an aseptic fly culture system that can be reproducibly cultured with quantifiable growth rates and viability of growth through multiple generations from the larval stage to adults. This baseline is critical for any attempts to measure the effectiveness of the BT genes against mushroom pests. As part of that effort, the concept of utilizing *Agrobacterium rhizogenes* transformed 'hairy root' cultures as a food source for these pests is being tested due published observations of related fly larva being problematic plant pests (and leveraging this additional expertise of the Curtis laboratory).

The second major component of this future work is a literature review and development of techniques to quantify the insecticidal capacity of a protein. While the techniques used to transform *Agaricus bisporus* are relatively simple to use, attempting to clone all of the constructs and combinations of protein products into mushrooms is an inefficient use of resources. As such, it is desirable to utilize a technique in which sterile fly cultures can be exposed to the proteins produced by an *E. coli* expression host. Utilizing such an insect bioassay will allow the rapid testing of the proteins selected for this project both alone, and in combinations that may increase the potency of the toxin product. Once the combinations with the highest degree of insecticidal

activity are selected, transformation of the mushrooms themselves can be used to develop and test the efficacy of insect resistant mushroom crops.

Public Acceptance of Genetic Engineering

One of the largest barriers for the actual utilization of a mushroom crop protected by genetically engineered biological pesticides is the public's perception and acceptance of the effects and utility of genetic engineering. Many people throughout the world hold the opinion that any genetically modified crop is inherently more dangerous, and should be avoided in the market. While genetically engineered agricultural products have been accepted and commercialized in the United States, there are still major differences in the regulatory procedures and outlook of nations throughout the world that could interfere with the marketing of mushrooms developed through this method.

In addition, BT has its own set of current issues in the public sphere. There has been some research that shows BT has the capability to interfere with the natural pollinating insects necessary to the ecology of agricultural production. A study performed in Cornell has shown that BT-engineered corn pollen causes significant mortality in the monarch butterfly simply when dusted on milkweed leaves[27]. This has significantly raised public suspicion of BT products despite follow-up studies that showed that if presented with treated and untreated leaves, the monarch larva would avoid the BT – and the counter arguments of the effects of less specific pesticides. While it would be difficult to argue that the presence of BT in mushroom crops could harm the biosphere, avoidance of the BT conflict may harm attempts to market a pest-resistant mushroom. In addition, the recent discovery unauthorized genetically engineered BT wheat in Oregon fields has led to significant international backlash as countries with bans on the use of genetically engineered products have postponed their imports of US wheat. Again, the issue of

bio-containment in mushroom houses is significantly reduced as cultures must be replaced entirely on a regular basis. In addition the utilization of the proposed protein transportation mechanism can exclude BT genes from the reproductive fruiting body and thus spores of the mushroom to further mitigate the possibility of contamination of non-GMO mushroom crops.

CHAPTER V

CONCLUSIONS

Conclusions from this work fall into both general conclusions about scientific research as well as specific technical conclusions associated with the objective of generating BT-protected mushrooms.

Specific Conclusions:

S1) **The crystalline genes of *Bacillus thuringiensis* serovar isrealensis can be successfully cloned into *E. coli* expression vectors.**

S2) Transformed *E.coli* with the pET-28c vector is viable after 2 years of cryopreservation at -80°C in a glycerol solution which provides for rapid re-initiation of research after an extended period (in addition to storing plasmids).

S3) The plant pathogen *Agrobacterium tumafaciens* can be used to efficiently transform heterologous genes into the genome of *Agaricus bisporus*.

S4) Hygromycin antibiotic provides a selectable marker that allows for isolating transformants of linked genes into *A. bisporus*, and placement of the selectable marker adjacent to the 'left border' provides higher probability of desired transgene integration.

S5) Due to insertion into the chromosome, *Agrobacterium* transformation results in considerably different expression levels with the same promoter and reporter gene construct.

General Conclusions:

G1) Research is not simply at the bench. In conducting a research project that was not a simple extension of an existing laboratory project, I was exposed to the very early stage of literature review and the developing of methods. Where I had previously understood BT was used as a protein to protect plants, I was completely unaware that this involved so many different genes, strains and helper proteins.

G2) Research methods are often guidelines that must be modified. Where many procedures that I learned - such as PCR - are common methods, even small variants such as 'colony PCR' have nuances that can require 'tweaking' to make them work. In addition, the specifics of a project introduce unforeseen problems. For example, the Mega-plasmid on which the BT genes resided was too large to be captured by typical plasmid prep kits. As a result, older bulk plasmid isolation methods had to be adapted for this purpose.

G3) Projects which initially seem rather simple and straightforward require an extended effort to see them through to completion. As a result, the mentoring that I received to initiate the project has to be efficiently transferred to the next student in order to see the project through to a reasonable state of completion (publication).

APPENDIX A

GENE SEQUENCES, PRIMER DETAILS AND SEQUENCING

Genbank Sequences:

CytA:

ATGGAAAATTTAAATCATTGTCCATTAGAAGATATAAAGGTAAATCCATGGAAAACCCCTCAATCAACAGCAAGGGTTATTACATTACGT
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CytB:

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CryIVA

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CryIVB

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CryIVC

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CryIVD

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Hlp

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ACTTAA

Primer Details

Table 1: Primer Selection

Gene	Restriction Sites		Primer	Tm	GC%
CytA	BspHI, SalI	Forward	AACAGCTCATGATCGAAAATTTAAATCATTGTCCA	66.5	31
		Reverse His	GATACATGTCGACTTAGAGGGTTCCATTAATAGC	67.1	45
		Reverse	GATACATGTCGACGAGGGTTCCATTAATAGC	66.6	41
CytB	NcoI, SalI	Forward	ATCTTGCCATGGGCCACCTTAATAATTTGAATAATTT	67.8	32
		Reverse His	GCTTAGAGTCGACCGATTTTATTGGATTAACATTCAG	67.8	37
		Reverse	GCTCAGAGTCGACTTACGATTTTATTGGATTAACA	67	37
CryIVA	NcoI, SalI	Forward	ACATAGCCATGGGCAATCCTTATCAAAATAAAAATGA	67.6	32
		Reverse His	AGTATTCGTCGACCTCGTTCATGCAAATTAATTC	67.7	38
		Reverse	AATATTCGTCGACTCACTCGTTCATGCAAATTAATTC	67.9	35
CryIVB	NcoI, SalI	Forward	TAAATTCCATGGGCAATTCAGGCTATCCGTTAGCG	71.6	45
		Reverse His	AGTATTCGTCGACCTCGTTCATGCAAATTAATTC	67.7	38
		Reverse	AATATTCGTCGACTCACTCGTTCATGCAAATTAATTC	67.9	35
CryIVC	BspHI, SalI	Forward	GGTACGTCATGAATCCATATCAAAATAAGAATG	63.4	33
		Reverse His	TACTAACGTCGACATTAACAAATAAATCATTCACTA	64	27
		Reverse	TACTAACGTCGACTTAATTAACAAATAAATCATTCAC	63.5	27
CryIVD	NcoI, SalI	Forward	CTAACGCCATGGAAGATAGTTCTTTAGATACTT	64.9	36
		Reverse His	GCAATCTGTCGACCTTTAGTAACGGATTAAT	65.6	38
		Reverse	TCTCAATGTCGACCTACTTTAGTAACGGATTAAT	65.8	34
Hlp	BspHI, SalI	Forward	TCGAGATCATGACAGAAAATGGAGTGTTTTAT	65.3	34
		Reverse His	ATCGTAAGTCGACAGTTAAATAAGTCATTGTTACC	65.6	34
		Reverse	ATCTTAAGTCGACCCGATTAAGTTAAATAAGTCATTG	65.6	37

Sequencing Results:

Sequencing results were prepared by aligning both forward and back sequencing reactions utilizing the alignment software of Vector NTI. Alignment diagrams show graphically the overall sequence homology between the consensus sequence received from overlapping sequencing results and the target gene sequences. Mismatches appear in the alignment diagram as values less than one. Sequencing chromatographs provided tended to break down near 1000 base pairs, thus inserts approaching 2000 bp in length could not be fully sequenced. This was deemed an acceptable degree of uncertainty, as the enzyme used in PCR was a Phusion high-fidelity polymerase from New England Biolabs.

CytA

ATGATCGAAAATTTAAATCATTGTCCATTAGAAGATATAAAGGTAAATCCATGGAAAACCCCTC
AATCAACAGCAAGGGTTATTACATTACGTGTTGAGGATCCAAATGAAATCAATAATCTTCTTTCTATTAA
CGAAATTGATAATCCGAATTATATATTGCAAGCAATTATGTTAGCAAATGCATTTCAAAATGCATTAGTT
CCCACTTCTACAGATTTTGGTGATGCCCTACGCTTTAGTATGCCAAAAGGTTTAGAAATCGCAAACACAA
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CAATTAACTGCAGCAGTTACAAATACGTTTACAAATTTAAATACTCAAAAAAATGAAGCATGGATTTTCT
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CTGGTGGCGTTATGTATTGTGTACCAGTTGGTTTTGAAATTAAAGTATCAGCAGTAAAGGAACAAGTTTT
ATTTTTCACAATTCAAGATTCTGCGAGCTACAATGTTAACATCCAATCTTTGAAATTTGCACAACCATTAG
TTAGCTCAAGTCAGTATCCAATTGCAGATCTTACTAGCGCTATTAATGGAACCCTCGTCGACAAGCTTGC
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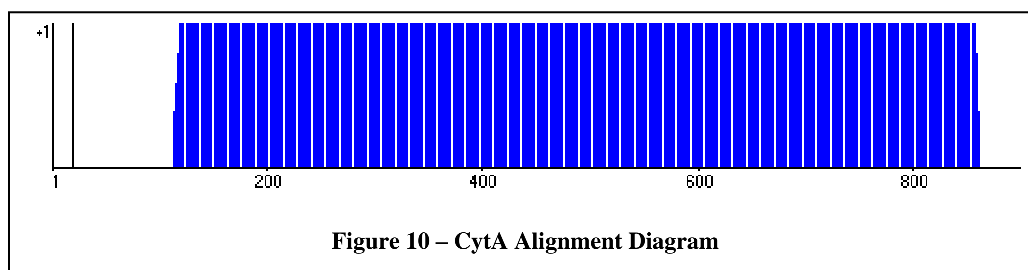

Figure 10 – CytA Alignment Diagram

CytB

ATGGGCCACCTTAATAATTTGAATAATTTTAATAATTTAGAAAACAATGGTGAATATCACTGTTC
AGGACCTATTATTAAAAAACCGTTTAGGCATATTGCATTAACAGTTCCATCCAGTGATATAACTAATTTTA
ATGAAATTTTTTATGTAGAGCCACAATACATTGCTCAAGCAATTCGTTTAACAAATACATTTCAAGGAGC
TATAGATCCACTTACCTTAAATTTCAATTTTGAAAAAGCTTTACAAATTGCAAATGGTCTTCCTAATGCAG
GAGTTACAGGAACTATTAATCAAAGTGTAATTCATCAAACAATTGAAGTTTCAGTTATGATTAGTCAAAT
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GCTATTACAAATACATTTACAAATTTAGAACCTCAAGTAGATGAAAATTGGATTGTTTGGCGTAATTTATC
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CAATATTGCCTATAGCATTTGAAATTACTGTGGATGTTCAAAAACAACAATTATTATTCATTACAATAAA
AGATAGTGCACGATATGAAGTTAAAATGAAAGCTCTTACTGTAGTTCAAGCTTTAGATTCTTATAATGCA
CCCATTATAGATGTATTTAATGTTCGTAACTATAGCTTACATCGTCCAAATCATAATATTCTTCAAAATCT
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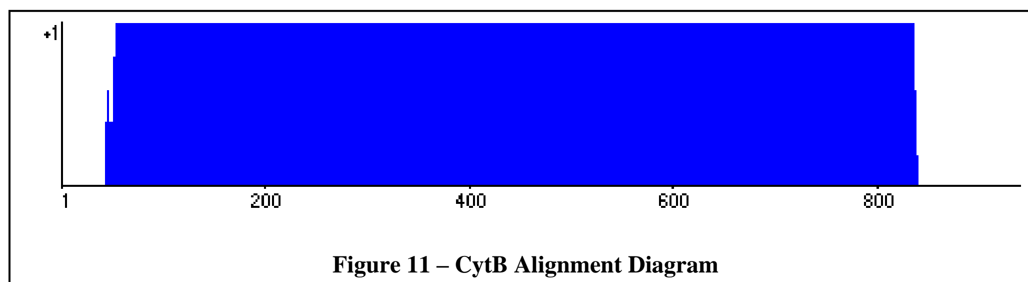

Figure 11 – CytB Alignment Diagram

CryIVA

AATTTTGTTTAACTTTAAGAAGGAGATATACCATGGGCAATCCTTATCAAAATAAAAATGAATA
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GATTGCGATTACTATAACATACTAGTATTATCTAGTTATGCACAAGCAGCAAACTTACATCTGACTGTATT
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AAGCCCCTATNAANNTNTGANTTTCAATATNANANGANTCACTTNNNCGTAGACCGC

GCTGNTTTAAANTATCTGTATNCTTCTANNNTTGATNGTGAAGGGTCTAATCGTTGTGAGACGTN
CGCTGTGCCGGCTAACATTGGGANCACTTNTGATATGTTGTATTCATGCCAATATGATACAGGGAAAAAG
CATGTCGTATGTCAGGATTCCCATCAATTTAGTTTCACTATTGATACAGGGGCATTAGATACAAATGAAA
ATATAGGGGTTTGGGTCATGTTTAAAATATCTTCTCCAGATGGATACGCATCATTAGATAATTTAGAAGT
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GATACAGATCGTGTACGAATTGAGATAGGCGAAACCGAAGGTTCGTTTTATATCGAAAGCATTGAATTAA
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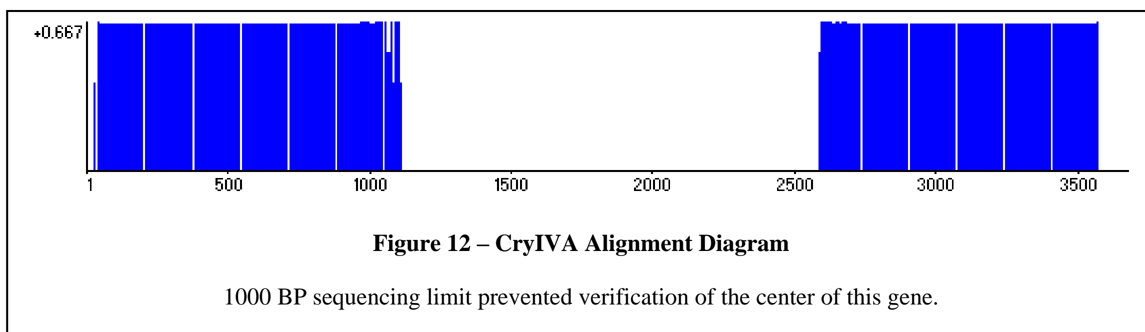

Figure 12 – CryIVA Alignment Diagram

1000 BP sequencing limit prevented verification of the center of this gene.

CryIVB

AATTTTGTTTAACTTTAAGAAGGAGATATACCATGGGCAATTCAGGCTATCCGTTAGCGAATGA
CTTACAAGGGTCAATGAAAAACACGAACTATAAAGATTGGCTAGCCATGTGTGAAAATAACCAACAGTA
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ATCCACGTCGATACCCTGCGGACAAAATAGATAATACGAAACTATCAAAAACAGAATTTACAAGAGAGA
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TGTTCATTTATTCACTTGGNTAAAGAGAGTAGATTTCNGGACCANN-CTANNTATCANN-
TTTANNANTTTNATCTGC

GCTAACATTGGGAACACTTCTGATATGTTGTATTCATGCCNATATGATACAGGGAAAAAGCATG
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AGGGGTTTGGGTCATGTTTAAAATATCTTCTCCAGATGGATACGCATCATTAGATAATTTAGAAGTAATT
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CAAGAAAAATTGACGTTTACGTCTTGTGAAGAAGGATATATTACGAAGACAGTAGATGTATTCCCAGATA
CAGATCGTGTACGAATTGAGATAGGCGAAACCGAAGGTTCGTTTTATATCGAAAGCATTGAATTAATTTG
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TAACAAAGCCCGAA

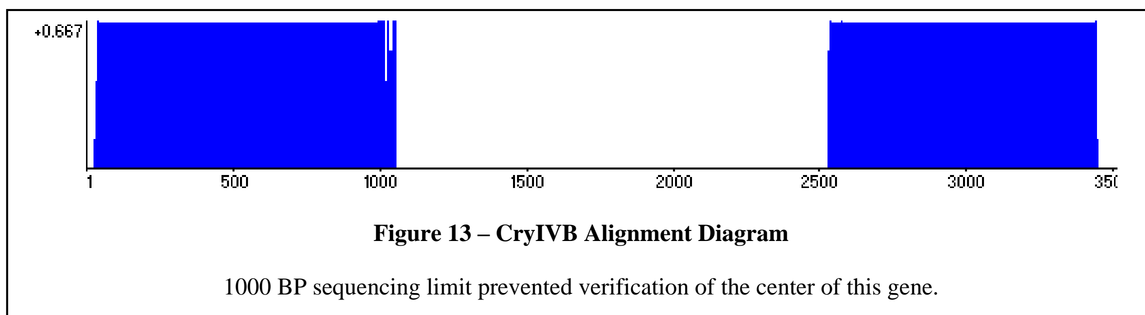

Figure 13 – CryIVB Alignment Diagram

1000 BP sequencing limit prevented verification of the center of this gene.

CryIVC

ATGAATCCATATCAAAATAAGAATGAATATGAAATATTCAATGCTCCATCCAATGGTTTTAGCA
AGTCTAATAACTATTCTAGATATCCATTAGCAAATAAGCCAAATCAACCACTGAAAAACACGAATTACAA
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AGCTGCAGGTATAATATCTTTTGGGACTTTGTTGCCGATCTTTTGGCAAGGATCTGACCCTGCAAATGTTT
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GTATACAAACGTACAATGCANGACTAACTATGATTAGAACTAATACTAACGCAACATGGAATATGTATA
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TTTTACNCNANAANTTCTCGAGACATTCTTGATNCTTATGATANTTTTTTCTTTTACAGGTANCCAGATGN
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CACATCCAAAGTATTTCCTTTTTATAGAAACAAACCTATTGATAAGGTCGAAATTGTCAGACATAGAGAG
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ATGGTACACCAAATCATTCATATAATATATATATTGACAAAATCGAATTTATTCCAATCACTCAATCTGTA
TTAGATTATACAGAGAAGCAAAATATAGAAAAAACACAGAAAATAGTGAATGATTTATTTGTTAATGTC
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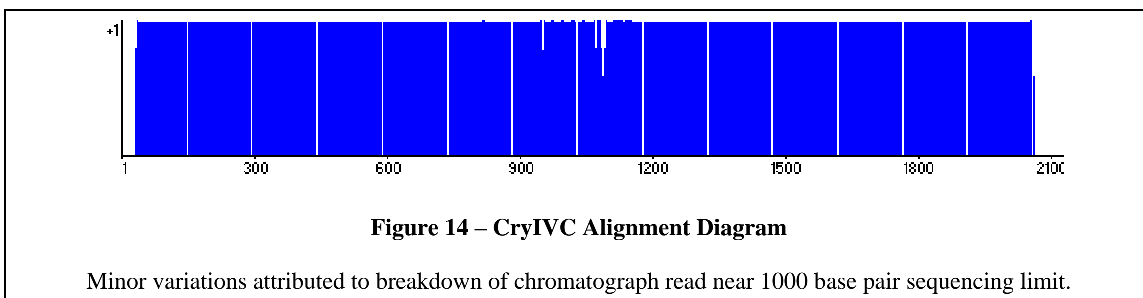

Figure 14 – CryIVC Alignment Diagram

Minor variations attributed to breakdown of chromatograph read near 1000 base pair sequencing limit.

CryIVD

ATGGAAGATAGTTCTTTAGATACTTTAAGTATAGTTAATGAAACAGACTTTCCATTATATAATAA
TTATACCGAACCTACTATTGCGCCAGCATTAATAGCAGTAGCTCCCATCGCACAATATCTTGCAACAGCT
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CTACTATGGAAAAAGTTCGTACAGAAGTGGAAACACTTATAAATCAAAAATTAAGCCAAGATCGAGTCA
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GATTGTGTAAAGTCAGTCTTAAAGATGGATTGACGTTCCGGAATATGTGTAATTTATATGTGTTTCCATTT
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CAGCAGACCTATTTTTTAAAAATGCAGATATAAATGTAAAGTTCACACAGTGGTTTCAGTCTACTCTATAT
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CCGGGTTTAATACGGCAACTAGATATAAATTAATTATCAGGGTAAGAGTACCTTATCGCTTACCTGCTGG
AATACGGGTACAATCTCAGAATTCGGGAAATAATAGAATGCTAGGCAGTTTTACTGCAAATGCTAATCCA
GAATGGGTGGATTTTGTCACAGATGCATTTACATTTAACGATTTAGGGATTACAACTTCAAGTACAAATG
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CCACCACCACCACCACTGAGATCCGGCTGCTA

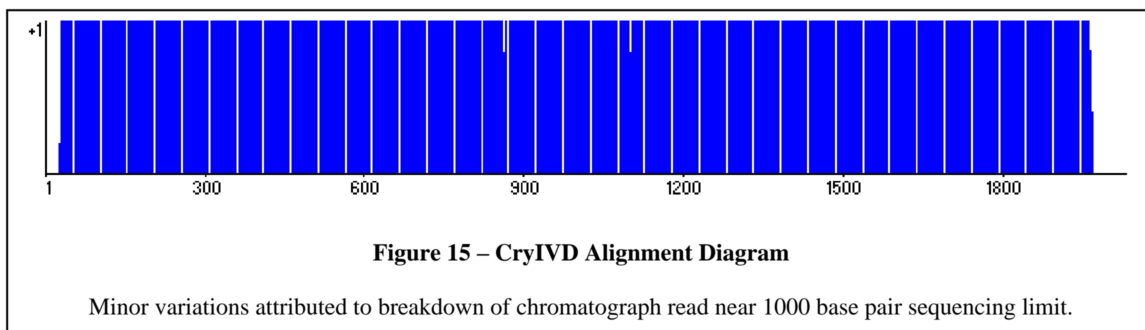

Figure 15 – CryIVD Alignment Diagram

Minor variations attributed to breakdown of chromatograph read near 1000 base pair sequencing limit.

Hlp

AATTTTGTTTAACTTTAAGAAGGAGATATACCATGACAGAAAATGGAGTGTTTTATAAAATATT
CACAACAGAAAATAATAATTTTTGTATAAATCCTACTTTGTTAGAAAGGGTTTTTAAAAATAATTTAGAT
GAATTTGATTTTTCGCTAGTAAAAAAAAACTTAGAACATGAGAAGAATTGTGTGATTACTTCTACAATGA
ATCAAACAATTTCTTTCGAGAATATGAATAGTACAGAAATGGGGCATAAGACATATTCTTTTTTAAATCA
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TTGTAGTTTGCTCTTTATAGTGCCCTATAAGAATAACATAACATCAATTATTCCAGTAAATTTACAACTAA
CATTATTATCGAAAAATGTAAAACAATCCTCTTCTACAAATATATTTTCAGGAGATATACATTTTAATATG
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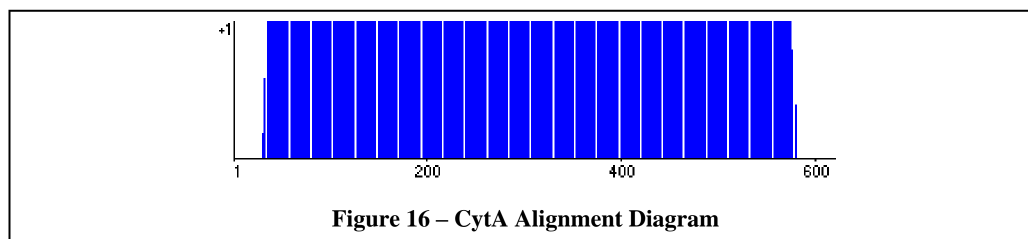

Figure 16 – CytA Alignment Diagram

APPENDIX B

PROTOCOLS

Plasmid Preparation Procedure:

1) Grow strains in broth overnight
2) Pellet 1.5 - 2 ml of cells by centrifugation.
    a) Remove supernatant.
3) Resuspend by vortexing in 100 µL resuspension buffer (50 mM glucose/10 mM EDTA/10 mM TrisCl, pH 8.0, 100 µg/mL RNAse A).
4) Add 200 µL lysis solution (0.2 M NaOH/1% sodium dodecyl sulfate [SDS]).
    a) Mix by inversion.
    b) Incubate 5 minutes at room temperature.
5) Add 150 µL 7.5 M ammonium acetate and 150 µl chloroform.
    a) Mix by inversion.
    b) Chill on ice 10 minutes.
    c) Centrifuge 10 minutes.
6) Transfer supernatant to 200 µL precipitation solution (30%polyethylene glycol 8000/1.5 M NaCl).
    a) Mix by inversion.
    b) Chill on ice 15 minutes.
7) Centrifuge to pellet DNA. Remove supernatant.
    a) Resuspend in TE or water.

Restriction Digest Protocol:

1) Reaction Volume: for cloning, use a 50 uL reaction(<10 ug DNA); for verification, use 20 uL
2) Add Buffer 1X
3) Add BSA 1X
4) Add enough units to digest your DNA in 1 hour. (<10% Volume)
    a) No less than 1.25 uL enzyme into 50 uL reaction.
5) Add water to volume.
6) Dephosphorylate as needed.

Incubation temperature is enzyme dependant.
Incubation time can be variable if you have “Time saver” qualified enzymes.

Ligation Protocol:

1) ReactionVolume: 20 uL reaction
2) Add 50 ng dephosphorylated vector
3) Add 3:1 molar ratio insert:vector
4) Add 1X T4 Ligase Buffer
    a) Thaw ligase buffer slowly (not at 37C, due to degradation of ATP)
    b) Smell for wet dog odor.
5) Add Water to balance volume
6) Add 1 uL T4 Ligase
    a) Keep in ice caddy while using
7) Refrigerate at 16C overnight.
8) 4 uL of the product are immediately used for the heat shock protocol.

Heat Shock Protocol:

1) Thaw chemically competent cells on ice.
    a) Requires about 5 minutes
    b) Turn on water bath 42 C.
2) Add DNA mixture
    a) 4 uL from ligation.
3) Chill on ice for 30 minutes
4) Dip for 45 seconds in a 42 C water bath
5) Move back to ice (about 2 minutes), add 1 m C medium.
6) Incubate @ 37 C for 1 hour.
7) Plate onto pre-warmed selection plates
    a) Use about 100 uL
8) Grow overnight at 37 C
9) Screen for colonies.
    a) Count Colonies/DNA used to calculate transformation efficiency.

APPENDIX C

NOTABLE LESSONS LEARNED

Ordered Sequences Are Not Always Correct

As PCR amplification of the selected genes from the pBtoxis plasmid began, there were several issues that lead to a lack of amplification, ranging from miss-programming the order of steps to the incorrect calculation of appropriate annealing temperatures. While troubleshooting allowed the correct amplification of each of the toxin genes, running the PCR with annealing temperatures as low as 20 degrees below recommended continued to yield no results when utilizing the Hlp reverse his-tagged primer. An experiment was run utilizing the non-his tagged protein along with a control gene that had worked before (CytB). Figure 17 shows that while the control and non his-tagged amplification worked appropriately, the his-tagged reverse primer failed to yield any results. Upon reordering this primer, the amplification worked appropriately, showing that the initial primer was incorrect, and paid services are not above making mistakes.

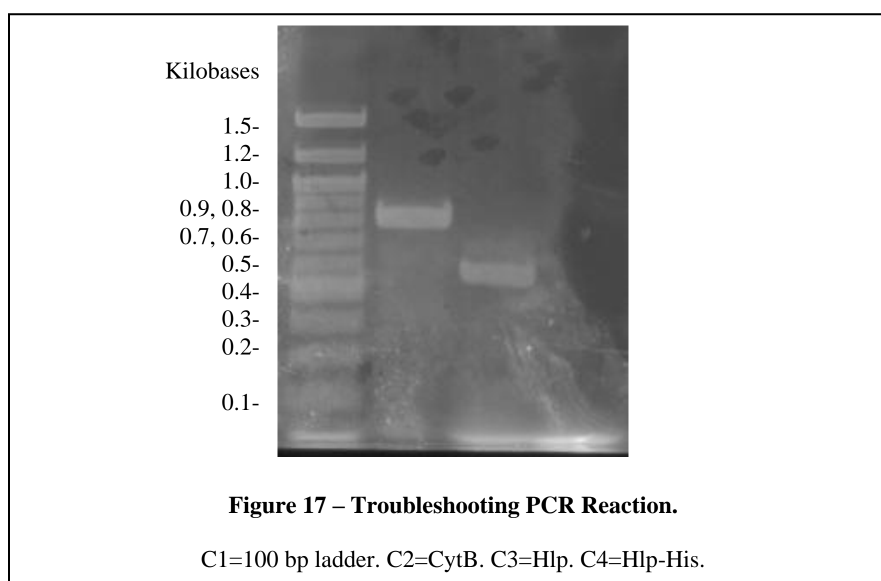

Figure 17 – Troubleshooting PCR Reaction.

C1=100 bp ladder. C2=CytB. C3=Hlp. C4=Hlp-His.

Selection of Proper Restriction Sites is Critical

In the first attempt to perform a restriction digest and ligate the genes for CryIVA and CryIVB, it was observed that the restriction enzyme chosen, BspHI, cut both of these genes into smaller fragments, as shown in Figure 18. It was found that this was caused by an error in which a BamHI restriction map was inappropriately used instead of that for BspHI. While this meant the products were unusable, it served as the first major confirmation that PCR correctly amplified the desired genes because the restriction fragment pattern matched that predicted by the gene sequences. As a result of this error, the primers were redesigned to utilize the NcoI restriction site and reordered.

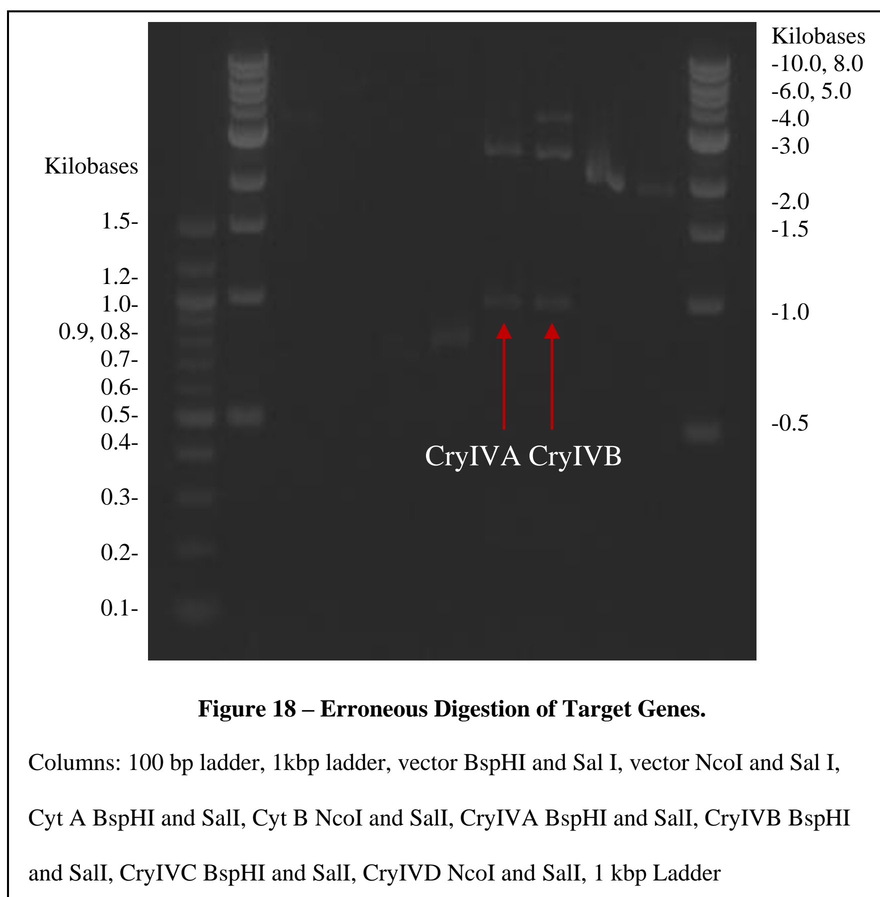

Figure 18 – Erroneous Digestion of Target Genes.

Columns: 100 bp ladder, 1kbp ladder, vector BspHI and Sal I, vector NcoI and Sal I, Cyt A BspHI and SalI, Cyt B NcoI and SalI, CryIVA BspHI and SalI, CryIVB BspHI and SalI, CryIVC BspHI and SalI, CryIVD NcoI and SalI, 1 kbp Ladder

Genetic Events Can Be Unpredictable

The first transformation event to produce colonies with a gene of approximately the correct size to be the anticipated CytA toxin gave a sequencing read with no homology to the desired gene. Utilizing the NCBI nucleotide BLAST algorithm, it was found that the sequence had perfect homology with an *E.coli* gene fragment encoding the protein D-mannonate oxidorectase. It is interesting to note that the laboratory in which this work was performed was previously occupied by a group performing research on pentose metabolism which could have been a possible source of this seemingly random gene. Looking at how this could have possibly been introduced into the cloning vector I've used, it was noted that the restriction sites at the boarders of this gene were NcoI and SalI, the restriction sites used for the vector, but not the restriction sites to be introduced on the PCR product. The most likely way for this gene to have made it into the construct was to have been cut with vector due to minor genomic contamination of the plasmid prep, failure to dephosphorylate during treatment with Antarctic Phosphatase, and re-ligation into the vector, adding to the sheer coincidence that this gene is the same length as the insert that I was looking for. This stresses the need for sequence or restriction digest verification of the constructs prior to induction. A second attempt to clone this gene yielded the proper result without incident.

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ACADEMIC VITA

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Education

The Pennsylvania State University (University Park, PA)
Schreyer Honors College – Graduation in August 2013
B.S. Chemical Engineering (Bioprocess Option)
B.S. Biochemistry and Molecular Biology
Dean's List, all semesters

Honors and Awards

2010: President's Freshman award
2011: President's Sparks Award
2011: Biomolecular Engineering Research Fellowship
2011: John and Jeanette McWhirter Chemical Engineering Scholarship
2012: Larry K. Duda Award for Undergraduate Research
2012-2013: Evan Pugh Scholar Award
2012-2013: George Gleason Memorial Scholarship
2009-2013: Schreyer Honors College - Academic Excellence Scholarship

Association Memberships/Activities

2011-2013: Omega Chi Epsilon, Chemical Engineering Honors Society – Webmaster
2009-2013: The Pennsylvania State University Glee Club –Webmaster
2009-2013: American Institute of Chemical Engineers (Penn State) – Member

Professional Experience

Undergraduate Research at Penn State University (University Park, PA)
Supervisor: Wayne R. Curtis
August 2009 – August 2013

Dow AgroSciences R&D Summer Internship (Indianapolis, IN)
Supervisors: Jeff Larson, Paul Samuel, Jagdish Tewari
May 2012 – August 2012

Professional Presentations

M. Curtis. "*An Improved Temporary Immersion Bioreactor for Plant Tissue Culture Propagation*" AICHE National Conference, Lawrence Convention Center, Pittsburgh, PA, Oct. 29, 2012. (Oral Presentation)

M. Curtis. "*Plant Propagation Bioreactors"* AICHE Regional Conference, Stevens Institute of Technology, Hoboken, NJ, April 13-15, 2012. (Oral Presentation, 1st Place Winner)

M.CURTIS, S.Florez, and W. Curtis. *"Engineering the Tissue Culture Environments"* SIVB National Conference, Rhode Island Conference Center, Providence, RI, June 17, 2013. (Poster)