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Is Cutinase The Smallest Possible Alpha/Beta Hydrolase? Insights Into Structure And Function Using Deletion Mutants

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IS CUTINASE THE SMALLEST POSSIBLE ALPHA/BETA HYDROLASE? INSIGHTS INTO STRUCTURE AND FUNCTION USING DELETION MUTANTS

by

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A thesis submitted to the faculty of The University of Mississippi in partial fulfillment of the requirements of the Sally McDonnell Barksdale Honors College.

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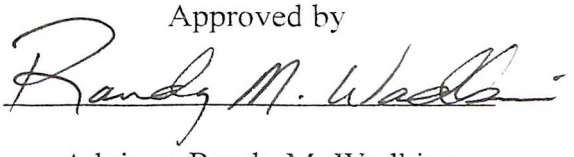
Approved by



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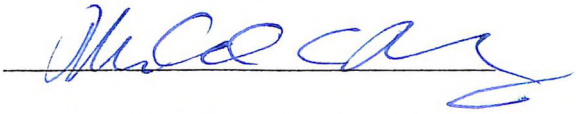

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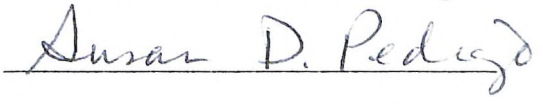

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ABSTRACT

Alpha/beta hydrolases are a sub-family of enzymes with a variety of different structures and functions. Though there is great diversity in size, structure, and function, there is a common fold within this alpha/beta hydrolase family called the alpha/beta fold. It consists of 8 mostly parallel beta sheets enclosed between two sets of alpha helices. Cutinase, the smallest of the alpha/beta hydrolases, is missing 2 alpha helices and 3 beta sheets. In this experiment we aimed to develop a set of plasmids with a gene for mutated cutinase DNA. Studying deletion mutations of cutinase can lead to understanding the structure of cutinase. Cutinase is missing so much of the characteristic fold; the question this lab would like to begin to answer is what is there about the cutinase structure keeps it active while missing so much of the alpha/beta hydrolase fold. There has not yet been a successful transformation of mutated plasmid DNA into the XL-10 gold bacteria but we have reason to believe that the mutated plasmid gene has been successfully made.

ACKNOWLEDGEMENTS

This thesis would not have been possible without the help of the other members of this research lab. I owe them much gratitude. I am grateful to Dr. Wadkins for the opportunity to work in his lab and learn from his team. I am in debt to the members of the team, especially Shana Stoddard, for spending many hours working with me on my writing. It has been an honor for me to work with this team.

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LIST OF ABBREVITIONS

ASP	Aspartate
BME	β-mercaptoethanol
DNA	Deoxyribonucleic Acid
dNTPs	Deoxynucleotide Triphosphates
H_2O	Water
HIS	Histidine
KB	Kilo-bases
NaCl	Sodium Chloride
NaOH	Sodium Hydroxide
NEB	New England Bio Lab
PAGE	Polyacrylamide Gel Electrophoresis
PCR	Polymerase Chain Reaction
RE	Restriction Enzymes
SER	Serine
TAE	Tris-acetate-EDTA
EDTA	Ethylenediaminetetraacetic acid
μL	Microliter

mL	Milliliter
L	Liter
G	Gram
Mg	Milligram
V	Volts
°C	Degrees Celsius

CHAPTER 1: INTRODUCTION

1.1 ALPHA/BETA HYDROLASES

The alpha/beta hydrolase family consists of several hydrolases, all of which have the alpha/beta hydrolase fold. The fold of each alpha/beta hydrolase enzyme is similar such that each contains an alpha/beta sheet consisting of eight mostly parallel beta-sheets surrounded by alpha helices [10]. In Figure 1.1 the subfamily of alpha/beta hydrolases is depicted in a phylogenetic tree. Starting with the smallest of the alpha/beta hydrolases, the family is mapped and compared based on similar protein structure. All of these alpha/beta hydrolases share the same core structure; yet there is huge sequence diversity. All of these groups within the subfamily of alpha/beta hydrolases have different size and shape, and hydrolyze different substrates. For example, acetylcholinesterase hydrolyzes acetylcholine in our neural synapses; the carboxylesterase hydrolyze carboxyl esters, and thioesterases hydrolyze thioesters. Between cutinase, one of the smallest alpha/beta hydrolases, and acetylcholinesterase, one of the largest, we have a huge range of sizes and substrates. Yet, all the members of this alpha/beta hydrolase family share the same canonical fold.

Though the subfamily of alpha/beta hydrolases is diverse in structure and catalytic function, they all share this canonical fold (Figure 1.2). They also share a similar catalytic triad, which consist of a nucleophile, a Histidine, and an acid residue. In the characteristic canonical fold of the alpha/beta hydrolases, there are 8 mostly parallel beta sheets and 6 alpha helices. All the alpha/beta hydrolases begin with the n-terminus followed by beta sheets 1 and beta sheet 2. The characteristic fold then tucks beta sheet 3 into the protein before alpha helix A. Alpha helix A leads into beta sheet 4, followed by alpha helix B. Alpha helix B leads into beta sheet 5, which is always associated with the nucleophile of the catalytic triad. The nucleophile is followed by alpha helix C, which leads into beta sheet 6 followed by alpha helix D. Alpha helix D leads into beta sheet 7, which is always associated with the acid of the catalytic triad. Then, following the acid, alpha helix E leads into beta sheet 8, which leads into the Histidine, alpha helix F, then the COOH terminus.

Alpha/beta hydrolases come in a variety of sizes. One of the largest enzymes in this subfamily is acetylcholinesterase with 534 amino acids. The smallest known alpha/beta hydrolase, cutinase, is a mere 197 amino acids (Figure 1.3).

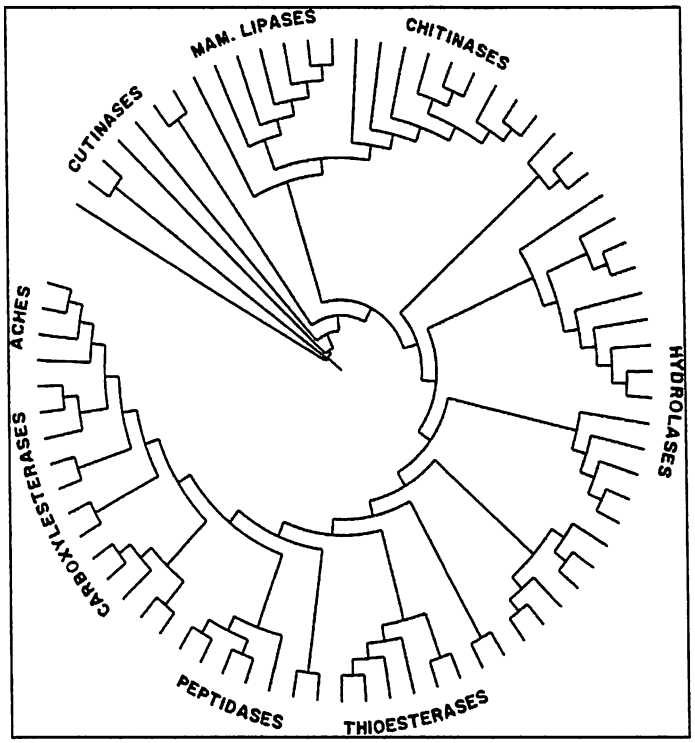

Figure 1.1 Phylogenetic Tree of Subfamily Alpha/Beta Hydrolase

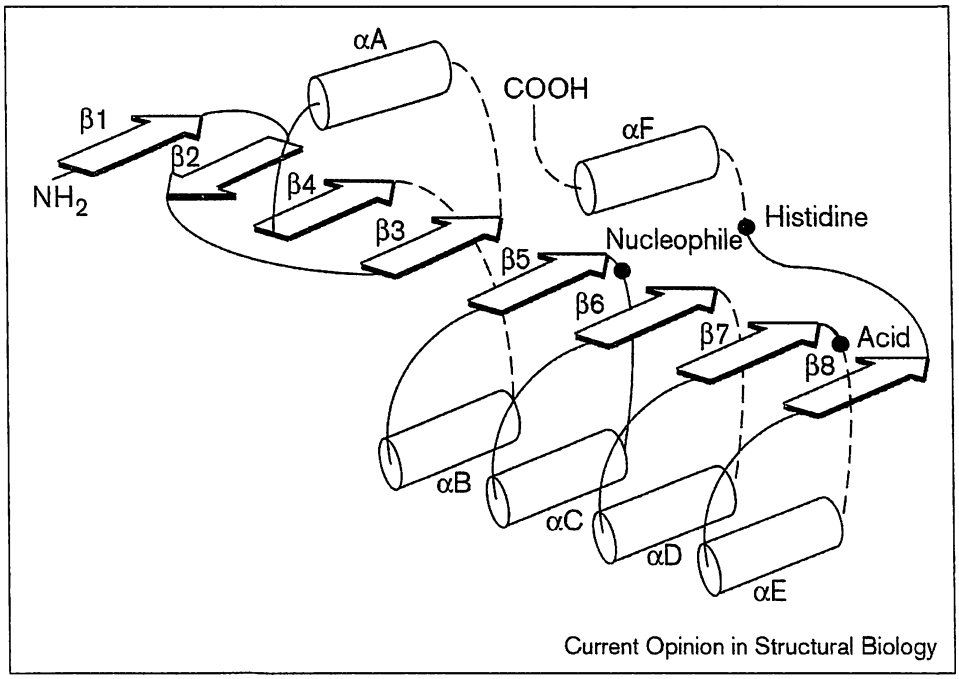

Figure 1.2 The Canonical Fold of Alpha/Beta Hydrolases*

*Canonical fold figure from: Nardini et al., *Curr Opin Struct Biol*, 1999, 9, pp 732-7

1.2 CUTINASE

Cutinase is named for the ability to degrade cutin polymers [1], which protect plants from infection by pathogens. The degradation of cutin is the first step in the process of fungi infecting the plant. The secretion of extracellular cutinase from pathogenic fungi degrades the cutin on the roots of plants [2]. Cutin is composed of ester linkages, which are cleaved by cutinase.

The structure of cutinase is much smaller compared to the larger alpha/beta hydrolases with several alpha helices and beta sheets removed from the characteristic canonical fold. Figure 1.3 is a comparison of cutinase, the smallest known alpha/beta hydrolase, and acetylcholinesterase, one of the largest alpha/beta hydrolases. Acetylcholinesterase has a massive 534 residues in comparison to cutinase with only 197 residues. In addition, acetylcholinesterase has all of the components of the canonical fold that is characteristic to all members in the alpha/beta hydrolase subfamily. In cutinase, however, alpha helices D, E and beta sheets 1, 2 and 8 are missing from the core structure.

The catalytic triad of cutinase is similar to the catalytic triad of all alpha/beta hydrolases in that it consists of a reaction between a nucleophile, a Histidine, and an acid. In cutinase, the nucleophile is Serine and the acid is Aspartate. As shown in figure 1.4, the catalytic triad functions to hydrolyze or cleave the ester bonds in cutin. This is possible due to the interaction of the catalytic triad. The reaction begins with a negatively charged Aspartate causing the deprotonation of Histidine. The deprotonation of Histidine causes a negative charge which consequently deprotonates the Serine. The serine is then very nucleophilic and forms a tetrahedral intermediate with the cutin (Figure 1.5). The

reaction continues with the OR group on the cutin leaving, hydrolyzing the ester bond. Water removes the Serine from the substrate and protonates it such that the reaction could take place again.

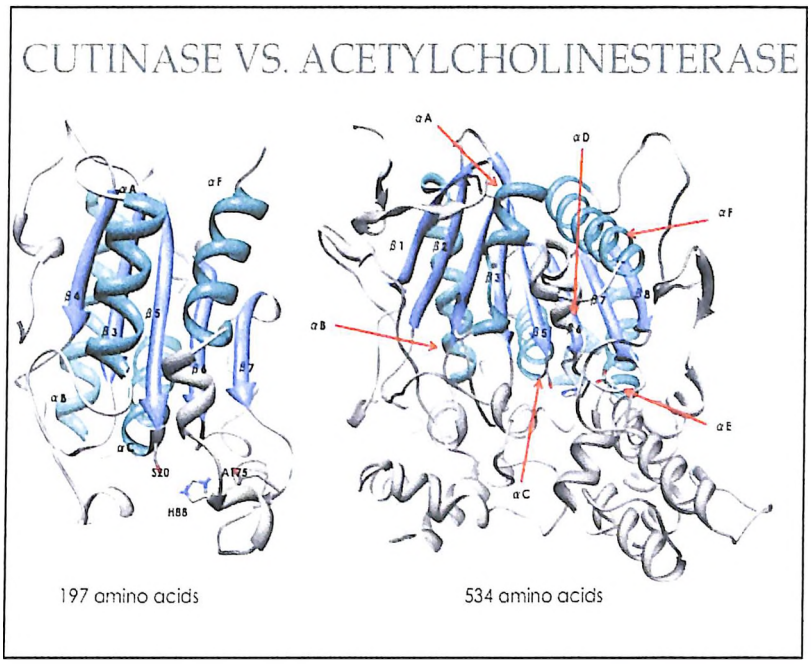

Figure 1.3 A Comparison of Cutinase and Acetylcholinesterase

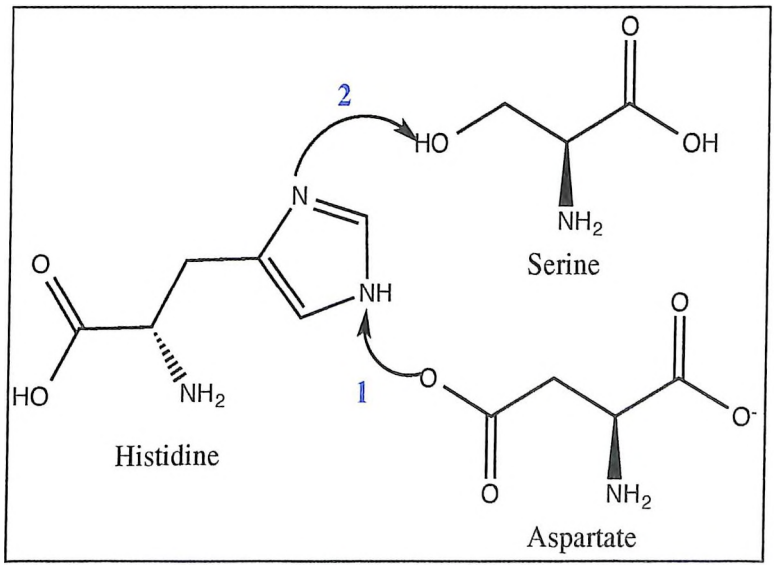

Figure 1.4 Catalytic Triad of Cutinase Ping Pong Reaction

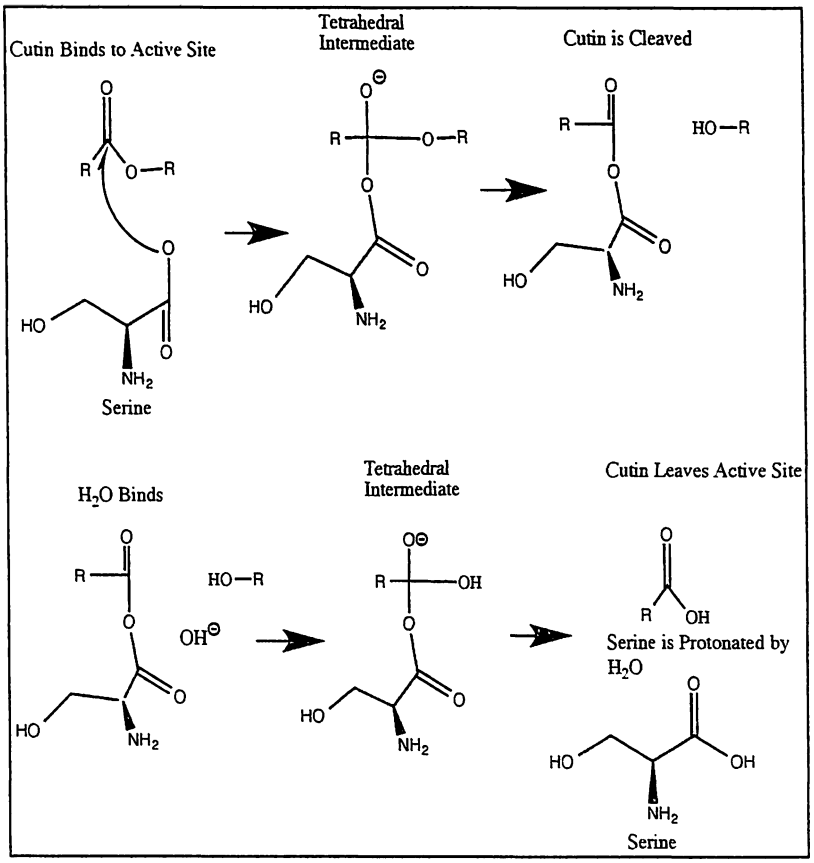

Figure 1.5 Hydrolysis of Cutin by Cutinase

1.3 THESIS FOCUS

When cutinase is compared to the typical alpha/beta hydrolase fold, it is missing two alpha helices and three beta sheets. This led us to wonder how cutinase is still able to function as a hydrolase with so many sections of the gene missing compared to its larger counterparts. This work addresses the question of whether or not cutinase is the smallest possible functioning alpha/beta hydrolase. How is it possible that with all the sections deleted from the characteristic canonical fold that cutinase still is stable and can catalyze reactions? This raises the question that if cutinase can still be stable and functional with all of these components of the parental canonical folds missing, then can there be an even

smaller alpha/beta hydrolase? Could nature make an even smaller alpha/beta hydrolase or does cutinase represent the core structure that every alpha/beta hydrolase relies on? We asked how deleting some of the major alpha helices and beta sheets of cutinase affected the catalytic function of the deletion mutants to better understand the cutinase stability and if there is anything smaller possible. Given the wide variation in sizes between different members of the alpha/beta hydrolase family, there is a question of exactly what part of the alpha/beta hydrolase fold is essential for the structure and catalytic activity for this family of enzymes.

CHAPTER 2: BACKGROUND OF METHODS

2.1 POLYMERASE CHAIN REACTION

Kary B. Mullis created polymerase chain reaction (PCR). PCR is used to amplify a small amount of DNA. A PCR reaction requires a buffer, polymerase, forward and reverse primers, template DNA, and dNTPs. The first step of PCR is denaturing the DNA. Denaturing the DNA separates it into two pieces of single-stranded DNA. The second step of PCR is annealing. The primers anneal to the template DNA as the thermocycler cools the mixture down. Once the primers are annealed and while the DNA strands are separated, the polymerase enzyme creates two new DNA strands using the original strands as the template. This step is called extension and it is the third and final step of the cycle. The new DNA will have one strand from the original sample and one newly synthesized strand. This can be done multiple times resulting in a huge amplification of DNA (Figure 2.1). Thirty cycles of a PCR reaction will result in 2^{30} amplification of DNA. The reaction takes place in a short amount of time and is completed in a machine called a thermocycler. With PCR, a small amount of DNA can be easily amplified for further analysis. In this experiment a variation of PCR was used to create a deletion mutant plasmid.

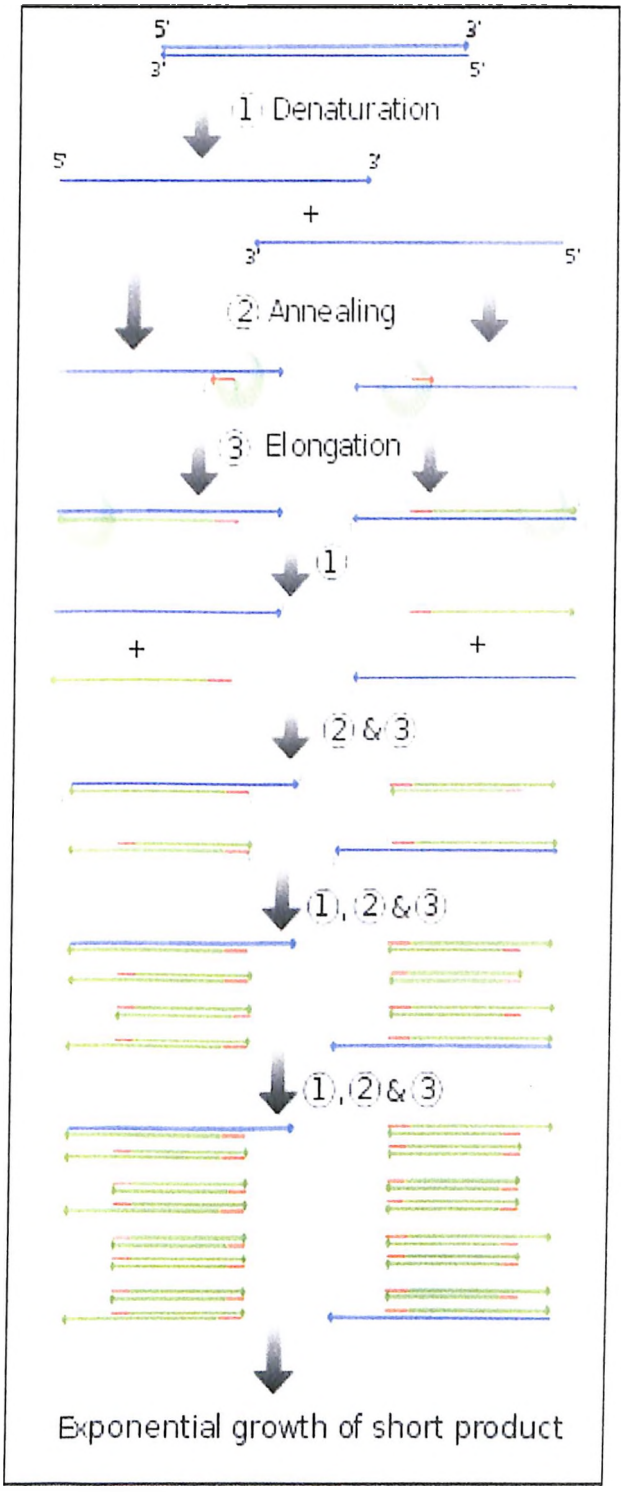

Figure 2.1 Exponential Amplification of PCR Product*

*Exponential Amplification of PCR used under Commons License from Wikipedia

2.2 INVERSE POLYMERASE CHAIN REACTION

In this experiment, inverse PCR was used to create a deletion mutant plasmid. Inverse PCR is a little variation on the original PCR method. The difference is the primers are designed to anneal to the template just adjacent to the sequence to be deleted so that everything gets amplifies but the deleted sequence. Using specifically designed primers (see Appendix), a section of the gene was not amplified, thereby deleting the gene from the final PCR product as well as amplification of the deletion plasmid (Figure 2.2). Using the pUC57 plasmid as a template, the primers are designed to anneal to the plasmid directly adjacent to the sequence that will be deleted. The remaining fragment gets amplified. This linear PCR product can be ligated resulting in a mutated plasmid [15].

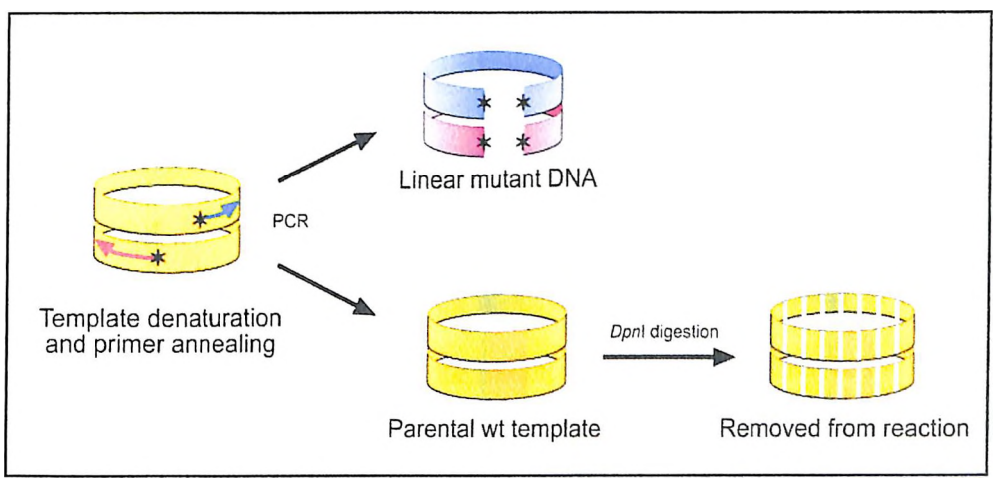

Figure 2.2 Inverse PCR*

*Inverse PCR figure from Williams *et al.* *Malaria Journal* 2007

2.3 DNA GEL ELECTROPHORESIS

Gel electrophoresis involves creating an electric field in order to separate molecules based on size, shape, and difference in charge. There are two techniques used for gel electrophoresis, agarose gel electrophoresis and polyacrylamide gel electrophoresis (PAGE). In this experiment, agarose gel electrophoresis was used to analyze DNA samples. PAGE was not used because PAGE is normally used for very small fragments and proteins. The DNA fragments being analyzed in this experiment were approximately 0.6 to 3.7 kb long, therefore too large to use PAGE. The samples are loaded into an agarose gel and the gel is immersed in an appropriate buffer. In this experiment, TAE buffer was used. When an electric field is applied to the buffer, the DNA (negatively charged due to the phosphate backbone) will migrate towards the positive cathode through the porous agarose gel. As the molecules travel through the gel, they will separate by size due to the agarose gel. The higher the concentration of agarose in the electrophoresis buffer, the smaller the pore size. Smaller fragments of DNA are able to travel further because they have an easier time navigating through the pores in the agarose gel.

The distance that the linear DNA bands travel is directly related to the size. To determine size of an unknown band the distance traveled is compared to the distance of a DNA band of known size. Multiple bands of known size make up a DNA ladder, which is used to calculate the size of the unknown bands. For quantitative analysis, a standard graph is created with the distance the band travelled in millimeters on the x-axis and the size of the DNA fragment in kb on the y-axis. Using the equation of the line, measuring the distance travelled of any band in the gel and plugging it in to the formula will

determine the size of that fragment in kb.

In an agarose gel DNA is initially not visible. There is no way to visualize where the bands are on a gel. Staining the DNA bands with Ethidium bromide stain allows for the DNA bands to be seen under a UV light. Ethidium bromide is used because, when exposed to UV light it fluoresces. Ethidium bromide is also especially good for staining DNA because once bound to DNA the florescence of the Ethidium bromide is increased. Ethidium bromide works by intercalating in between the two strands of the double helix.

2.4 BACTERIAL TRANSFORMATION

Bacterial transformation involves introducing a plasmid into a bacterial host cell in order to have that bacterial cell produce the desired protein that was inserted into the plasmid. Plasmids are small circular pieces of exogenous DNA that bacterial cells will accept and express as if it was their own DNA (Figure 2.3).

Plasmids contain the gene that is desired for expression as well as a gene for antibiotic resistance. Having a gene for an antibiotic resistance in the plasmid allows the bacteria to grow on selective media such as Lysogeny Broth. This also prevents the growth of unwanted bacteria or bacteria that did not take up the plasmid.

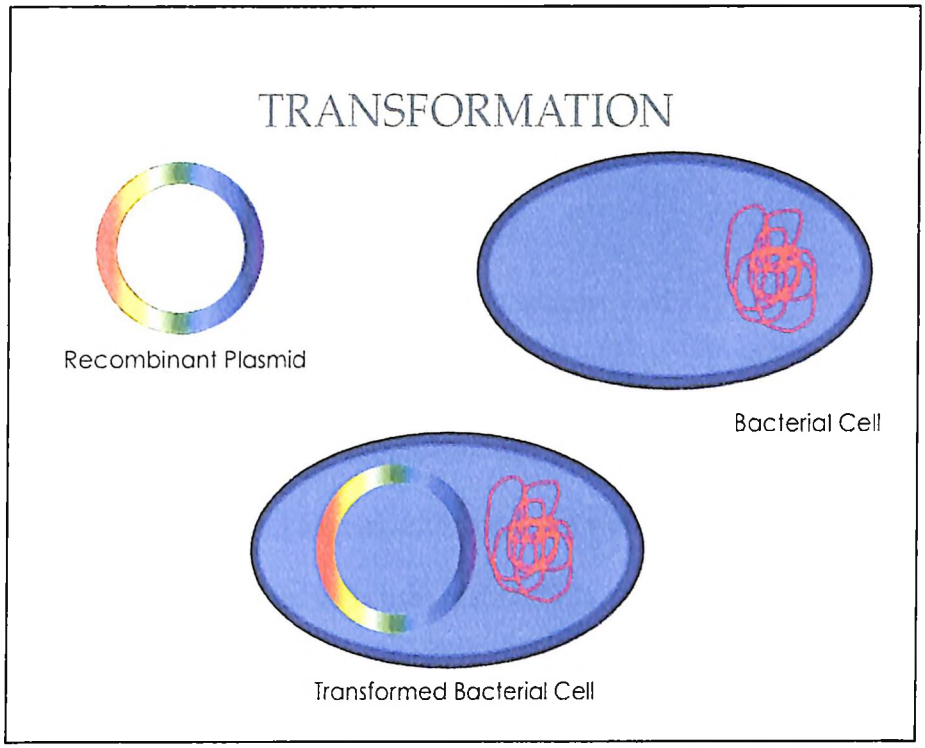

Figure 2.3 Transformation of Bacterial Cells

There are several types of bacterial lines that are engineered to accept plasmids including XL-1 Blue, *Origami B*, BL21(DE3), and XL-10 gold. In this experiment XL-10 gold cells were used because these cells grow very quickly and are easy to transform even with small amounts of DNA. The protocol for XL-10 gold used in this experiment is listed below.

1. Pre-chill 14 mL FalconBD round bottom tubes on ice
2. Thaw XL-10 gold cells on ice
3. Pipet 100 μL – 300 μL of XL-10 gold cells into pre-chilled tubes
4. Add 0.2 μl of β-mercaptoethanol to each tube
5. Place tubes on ice for 10 minutes, swirl every 2 minutes
6. Add 50 ng of DNA the appropriate DNA to their corresponding tubes
7. Gently swirl tubes then incubate on ice for 30 minutes

8. Heat tubes for 30 seconds in a 42°C water bath
9. Place tubes back on ice for 2 minutes
10. Add 900 μL of room temperature SOC medium to each tube
11. Incubate cells in at 37°C for 1 hour
12. Plate ≤ 200 μL of the bacteria onto selective media

As seen in Figure 2.3, the recombinant plasmid is transformed into the competent bacterial cell. A competent bacterial cell is a cell that will take up a plasmid. The protocol for transforming the bacterial cells uses heat shock to get the competent bacterial cell to take up the plasmid. The bacterial cells are cooled down in the same tubes as the plasmid DNA. The solution cools and this causes the cell membrane of the bacterial cell to cool and partially solidify. Then heat shocking the bacteria involves quickly placing the tubes in warm water as described above, which causes the bacterial plasma membrane to become porous and the plasmid DNA is taken up by the cell.

2.5 RESTRICTION ENZYME DIGESTION

Restriction enzymes (RE) are enzymes that hydrolyze the phosphodiester linkages between DNA base pairs. Some examples of RE's include BamH I, Hind III, EcoR I, and Dpn I. These restriction enzymes recognize specific sections of base pairs (Table 2.1).

Restriction Enzyme	Cleavage Sites
BamH I	G/GATCC
Hind III	A/AGCTT
EcoR I	G/AATTC
Dpn I	GA/TC

Table 2.1 List of Restriction Enzymes and Cleavage Sites*

*Cleavage sites from New England Bio Labs

Their discovery has been extremely useful in DNA technology for plasmid generation and gene insertion into plasmids. If a both a plasmid and a gene are cleaved with the same restriction enzyme, corresponding ends are created. Using DNA ligation, the phosphodiester linkages can be reconnected to create a hybrid plasmid that has the desired gene to be expressed.

2.6 DNA LIGATION

DNA ligases are enzymes that catalyze the formation of the phosphodiester bond between the 3' hydroxyl and 5' phosphate of neighboring DNA bases. In this experiment, T4 DNA ligase was used to ligate linear PCR product into a circular plasmid for transformation into bacterial cells. The linear PCR product was subjected to a blunting reaction to fix the 5' and 3' overhangs then ligated according to NEB (New England Bio Lab) protocol.

CHAPTER 3: "IS CUTINASE THE SMALLEST POSSIBLE ALPHA/BETA HYDROLASE? INSIGHTS INTO STRUCTURE AND FUNCTION USING DELETION MUTANTS."

3.1 INTRODUCTION

3.1.1 CUTINASE STUCTURE

Cutinase is the smallest alpha/beta hydrolase whose structure has been resolved so far. Cutinase consists of 197 amino acids and has the alpha/beta hydrolase fold that is characteristic of alpha/beta hydrolases. The active site of cutinase has a catalytic triad consisting of Ser120, Asp175, and His188. Additionally, there are two important disulfide bonds present in cutinase that play a role in stabilizing the protein [8]. Cutinase contains only 4 alpha helices and 5 beta sheets from the canonical fold characteristic of alpha/beta hydrolases. Cutinase contains alpha helices A, B, C, and F and beta sheets 3, 4, 5, 6, and 7. With so much of the original fold missing, this raises the question of how cutinase is still able to function as a catalytic molecule. Studying the structure of cutinase will show us how cutinase remains a catalytic molecule. With cutinase being the smallest alpha/beta hydrolase, we also can ask the

question of whether or not there could possibly be another smaller alpha/beta hydrolase out there that hasn't been found yet. If, after certain sections of the gene are deleted, enzyme activity doesn't change, it can me assumed that the section of the gene did not contribute to the stability or function of the enzyme. Studying the subsequent deletion mutants will give insight to the function of substructure that was deleted.

3.1.2 COMPUTATIONAL EXPERIMENTS

In a previous experiment, computer generated models from *Chimera* for cutinase mutations were studied. Homology models were made based on the template wild-type cutinase and the predicted motions of the protein with certain deletions were recorded. The results suggested that the deletion of alpha helices would reduce catalytic activity in the enzyme and deletion of beta sheets would not create a significant change [14]. Our lab would like to study these deletion mutations to determine substructure protein relationships. Because there is evidence that the deletion of beta sheets would not cause any damage to the protein, creating the mutations and testing the hypotheses will give great insight to the substructure of cutinase. The first step in studying the catalytic activity of the mutated proteins is to create the deletion mutant plasmid. Thus, the goal of this work is to create a plasmid with the appropriate deletions that can be used to induce mutated cutinase protein for study.

3.2 RESULTS AND DISCUSSION

3.2.1 DELETIONS

Three deletions were chosen for this experiment. Deletion 1, or mutant X, has residues 92-108 deleted. The removal of these resides will delete alpha helix B. The homology models for Deletion 1 show that the deletion of this alpha helix creates an odd twisting motion within the protein (Figure 3.2). The homology models show that the binding loop does not fold towards the catalytic triad as dynamically as it did in the wild type cutinase (Figure 3.1). We hypothesize that this deletion mutant may or may not have catalytic activity. The only way to confirm the hypothesize that there will be no or diminished activity is to actually express the mutated protein.

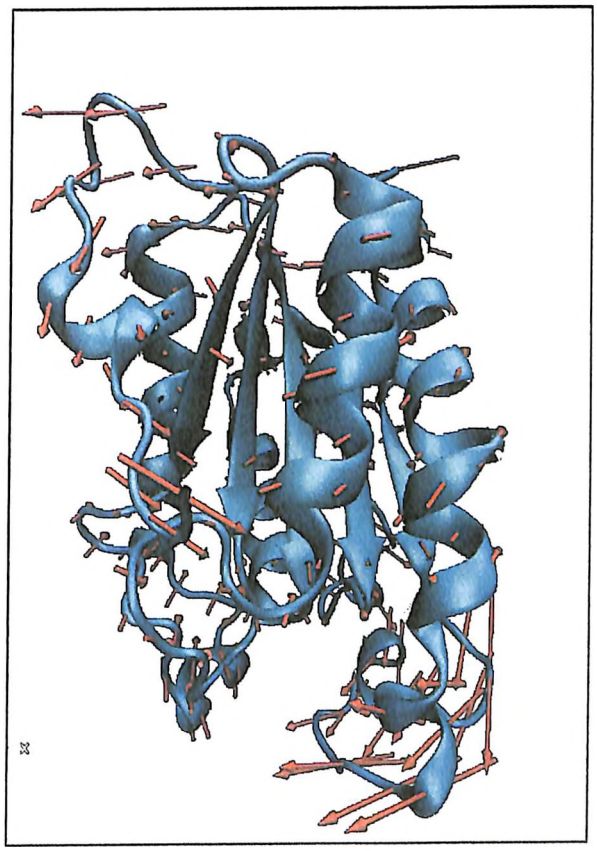

Figure 3.1 Arrow Plot of Wild-Type Cutinase

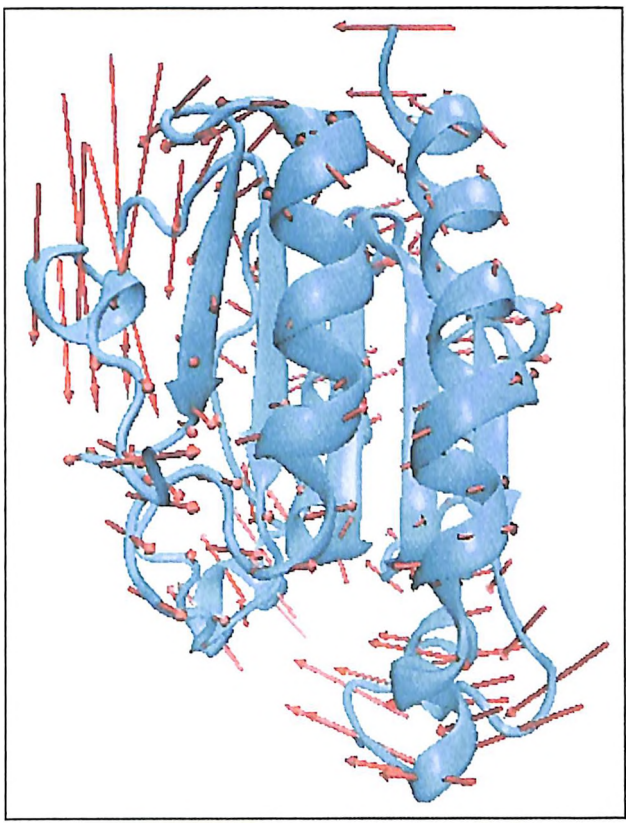

Figure 3.2 Arrow Plot of Deletion of Residues 92-108 or Alpha Helix B

Deletion 2, or mutant Y, has residues 49-63 removed. This caused the deletion of alpha helix A. According to the homology model (Figure 3.3), there is a flapping or sandwiching motion as a result of the deletion and once again the scooping motion of the binding loop into the catalytic triad is diminished. Therefore with this homology model we hypothesize that there would be a decrease or a complete diminish in catalytic activity of this mutant enzyme.

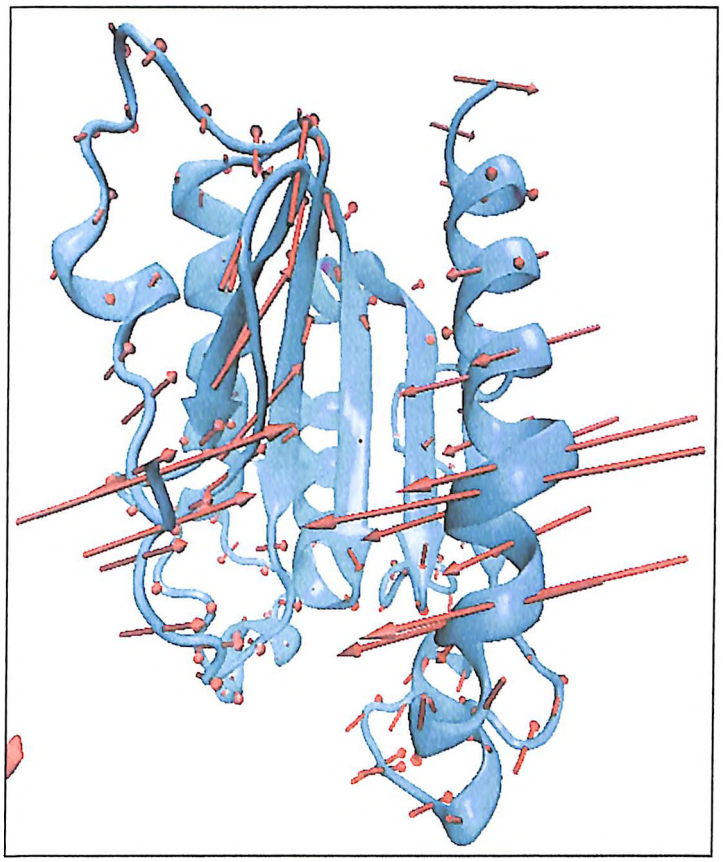

Figure 3.3 Arrow Plot of Deletion of Residues 49-63 or Alpha Helix A

Deletion 3, or mutant Z, has residues 68-72 removed. This caused the deletion of beta sheet 3. According to the homology model (Figure 3.4), normal motion is mostly maintained in this model so we hypothesized that there would still be catalytic function in this mutation. Catalytic function might be diminished but the only way to tell is to study the actual mutated enzyme. This suggests beta sheet 3 is more of a structural support than part of the catalytic activity.

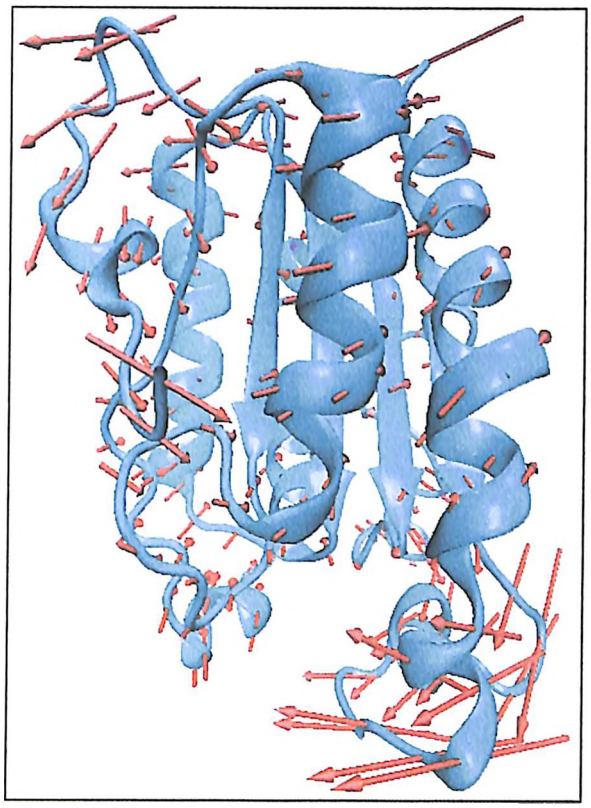

Figure 3.4 Arrow Plot of Deletion of Residues 68-72 or Beta Sheet 3

The following figures show the results of plasmid development of mutant X, Y, and X.

3.2.2 INVERSE PCR

The first step in creating the mutated plasmid is inverse PCR. Below are the results of a PCR reaction for mutations X and Y (Figure 3.5).

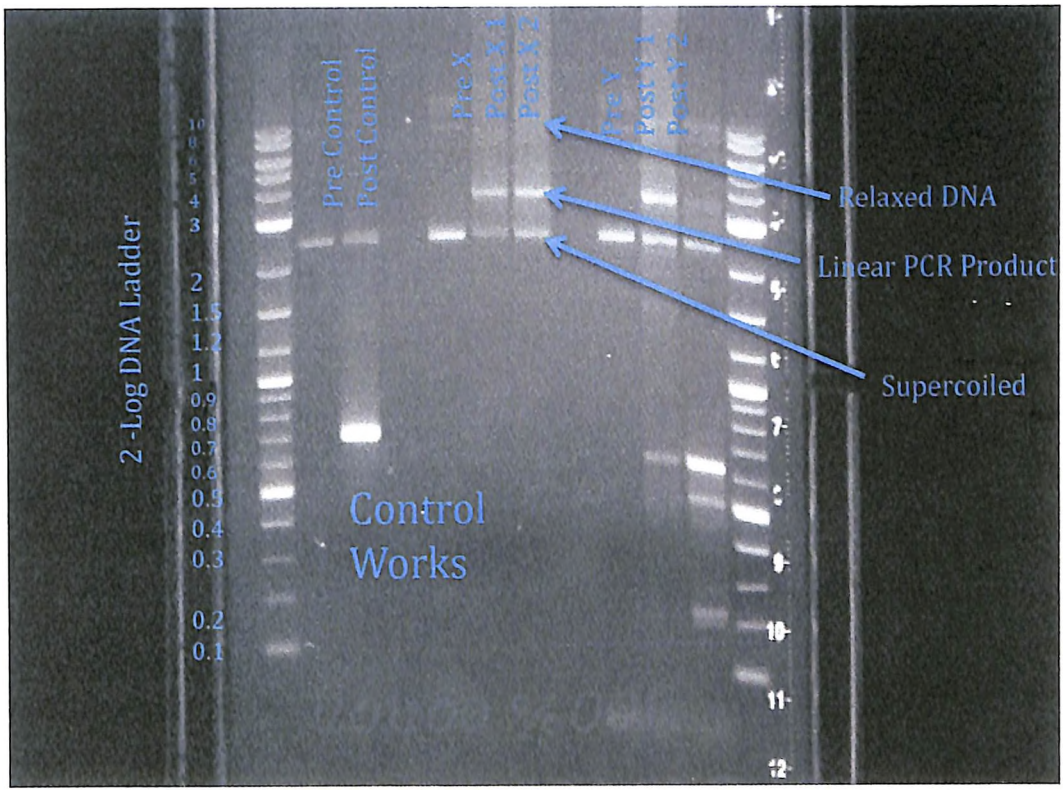

Figure 3.5: Post PCR Gel Results

Figure 3.5 shows a post PCR gel analysis. The far left lane of the gel and the far right lanes contain DNA ladders. DNA ladder is a mixture of DNA bands of known size that are used to qualitatively analyze gels. The first two lanes starting from the right are the pre and post control samples. The control is used to determine if a protocol is successful or not because the control is something that is confirmed to work properly. The control contained primers that were designed to amplify the cutinase sequence. The cutinase sequence is approximately 693 base pairs long. Therefore the control product is the cutinase gene so the control was a success.

The template DNA appears in the gel in two forms: relaxed and supercoiled. Relaxed DNA is circular so it is very bulky and takes up a lot of space. This prevents

it from making it very far down the gel; thus, relaxed DNA is expected to be seen towards the top of the gel. Supercoiled DNA is tightly wound so that it takes up much less space so it is able to travel much further down the gel. Both forms of DNA are seen and labeled on the gel.

The pUC57 plasmid is 2,710 base pairs long. pUC57 plasmid with cutinase gene insertion is 3,403 bp. Linear DNA product should be seen between the 3.0 kb marker and the 4.0 kb marker. Figure 3.4 shows that the results of the PCR reaction were a success. There is linear DNA product at approximately 3.5 kb, where product is expected to be. This means the mutated plasmid was successfully made. The next step in the process is a Dpn I digestion to remove template DNA.

3.2.3 DPN I DIGESTION

Dpn I cleaves at methylated DNA sites only and since the template DNA is methylated, a Dpn I digestion ensures that template DNA will be removed.

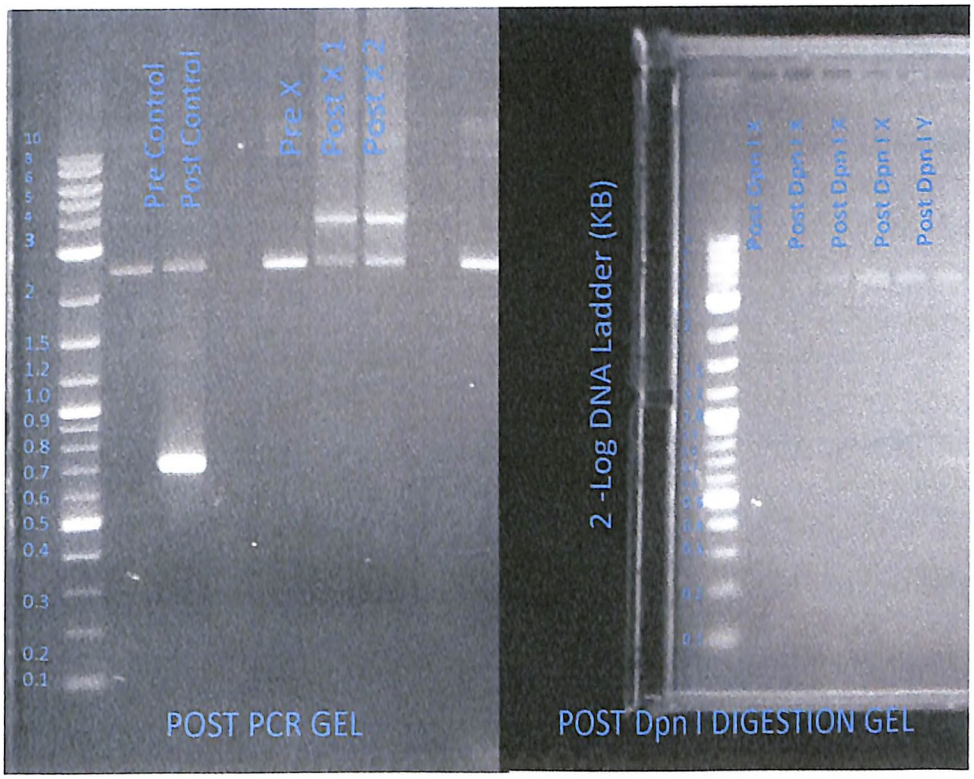

Figure 3.6 Post PCR results Compared to Post Dpn I Results

Figure 3.6 shows a comparison of a post PCR gel and a post Dpn I gel to show the digestion of the template DNA. The gel clearly shows that the Dpn I enzyme digested the template DNA. There are no DNA bands at 2.7 kb which is where the template DNA falls on the gel in the supercoiled form. There is also no template DNA around the 10 kb marker where the relaxed template DNA form falls on the gel. Blunting, ligation, and transformation followed.

3.2.4 TRANSFORMATION

There were no successful transformations with the mutated plasmid DNA. There was no growth on the negative (-) control plate or the positive (+) control plate. There was no growth on the plates with the bacteria containing the mutated plasmid.

The fact that nothing grew on the positive (+) control indicates that the cells used in the transformation protocol were not competent, or able to take up a plasmid. This means it is not necessarily the mutated plasmid DNA preventing successful transformation.

3.3 CONCLUSION

Cutinase is the smallest known alpha/beta hydrolase. The purpose of this experiment was to create plasmids with mutated cutinase DNA. Studying the mutated cutinase would provide insight to the substructure and function of each substructure in the molecule. Deleting certain alpha helices and beta sheets and studying the catalytic activity after deletion would provide knowledge on the purpose of each of those substructures. Creating the mutant plasmid was the first step in being able to express the mutated cutinase for kinetic studies. Mutated plasmids were not successfully transformed, but there is reason to believe that the deletion mutant plasmid has been created.

3.4 EXPERIMENTAL

3.4.1 LB BROTH

Lysogeny broth (LB) is a medium primarily used for the growth of bacteria. Selective LB broth for growing bacteria was prepared by placing 10 g of NaCl, 10 g of Tryptone, and 5 g of yeast extract in a 1-liter flask. The final volume was brought to 1 liter by adding deionized H_2O. Following the addition of deionized H_2O, the pH was adjusted to approximately 7.0 with 5 M NaOH. The solution was autoclaved at

121 °C for 30 minutes. In order to prevent undesired bacteria from growing in the LB broth, 100 mg of ampicillin was added once the solution cooled to 55 °C.

3.4.2 LB AGAR PLATES

Petri dishes containing LB Agar for plating bacteria was prepared by placing 10 g of NaCl, 10 g of Tryptone, and 5 g of yeast extract, and 20 g of agar in a 1-liter flask. The final volume in the liter was brought to 1 liter by adding deionized H_2O. Following the addition of deionized H_2O, the pH was adjusted to approximately 7.0 with 5 M NaOH. The solution was autoclaved at 121 °C for 30 minutes. In order to prevent undesired bacteria from growing on the LB Agar plates, 100 mg of ampicillin was added once the solution cooled to 55 °C. The solution was then poured into 100 mm petri dishes, cooled until solid, wrapped in Para film, and stored in 4 °C until use.

3.4.3 INVERSE PCR

In order to make the plasmid DNA for the mutants, inverse PCR was used. The total volume for the PCR reaction was 70 μL. PCR reactions required 10-20 ng template DNA, 0.2 mM of each dNTP, 0.5 final concentrations of forward and reverse primers, one unit of *Taq* polymerase in 2.5 units of *Taq* polymerase buffer [15]. Before placing the PCR tubes in the PCR machine, a 10 μL sample was taken for a pre PCR sample for comparison and analysis. The following table and figure shows the PCR condition (Table 3.1 and Figure 3.3). Because of the temperature range in the primers for mutations X, Y, and Z, a gradient was applied. Denaturation, annealing, and extension were repeated 30 times.

Initial Temp	**Denaturation**	**Annealing**	**Extension**	Final Step
95 °C	95 °C	62 °C	72 °C	72 °C
2 minutes	30 seconds	52 °C	3.5 minutes	10 minutes
		40 °C		
		30 seconds		

Table 3.1 Conditions of PCR

After completion the PCR, tubes are either stored at 4 °C overnight or continue to analysis of product. For Mutant X (deletion of bases 192-212) T_m-5°C is 52°C. For Mutant Y (deletion of bases 49-63) T_m-5°C is 40°C. For Mutant Z (deletion of bases 92-108) T_m-5°C is 62°C.

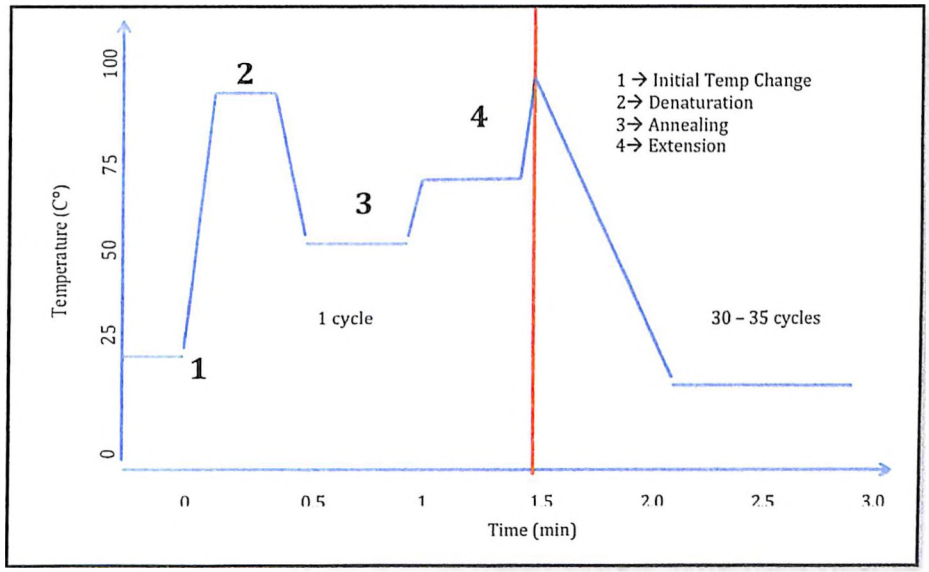

Figure 3.6 Polymerase Chain Reaction Temperature Changes

3.4.4 ANALYSIS OF INVERSE PCR REACTION BY GEL ELECTROPHORESIS

A 1% agarose gel was used with 1X TAE running buffer with 50 μL of ethidium bromide. Samples were run at 150 V for 90 minutes.

3.4.5 DPN I DIGEST

After confirmation of PCR product, a Dpn I digestion was performed on the samples in order to remove the template DNA. Thirty five μL of inverse PCR product, 5 μL of Dpn I digest buffer, 9.8 μL of H_2O, and 0.2 μL of Dpn I enzyme were added to a sterile micro centrifuge tube with a total volume of 50 μL. This was done for each sample of inverse PCR. The tubes were then places in an incubator overnight 37 °C. Gel electrophoresis was performed to verify digestion of the template DNA.

3.4.6 QIAQUICK PCR PURIFICATION

A QIAquick PCR Purification kit was used to purify PCR product. The protocol was followed according to the directions given by the Qiagens protocol. PCR Purification was performed in order to purify the DNA and get rid of primers and dNTPs.

3.4.7 BLUNTING PROTOCOL

In a sterile micro centrifuge tube 19 μL of purified DNA, 2.5 μL of 10X blunting buffer, 2.5 μL 1mM dNTP mix, 1.0 μL blunt enzyme mix and 2.5 μL of deionized

H_2O was mixed (total volume of 30 μL). The mixture was incubated at room temperature (16 °C) for 30 minutes then the enzyme was inactivated by heating at 70 °C for 10 minutes. Samples were then cooled to room temperature and submitted to a ligation reaction. Blunting was performed to fix the 3' and 5' overhanging's of the mutated plasmid.

3.4.8 T4 LIGATION

1 μL of T4 DNA Ligase and 2 μL of T4 DNA Buffer were added to cooled samples from blunting reactions with a total volume of 20 μL. The mixture was then incubated at room temperate overnight.

3.4.9 TRANSFORMATION OF BACTERIA

In order to have a positive control 100 μL of XL-1 gold ultra competent cell were transferred to a 14 mL BDFalcon tube along with 1 μL of pUC57-cutinase template DNA. In this protocol, the DNA was added after the XL-10 gold cells and the BME incubate. A negative control (no DNA), was 100 μL of XL-1 gold ultra competent cells transferred to a 14 mL BDFalcon tube with the absence of DNA. For the samples with mutant DNA, 300 μL of XL-1 gold ultra competent cell were transferred to a 14 mL BDFalcon tube with the entire purified DNA sample left over from the Blunting Protocol and T4 Ligation. The following protocol was done to each tube. Two hundred μL of β-mercaptoethanol (BME) was added to the tube and incubated at room temperature for 10 minutes. The solution was carefully swirled every 2 minutes. The DNA described earlier was added to the appropriate tubes. The

tubes were then incubated on ice for 30 minutes. The tubes were then heat pulsed in a 42 °C hot water bath for 30 seconds. The tubes were then incubated on ice again for 2 minutes. In the positive and negative control tubes, 0.9 mL of pre heated (42 °C SOC media) was added and incubated at 37 °C for 1 hour while shaking at 250 rpm. In the tubes with mutant DNA, 2.0 mL of pre heated (42 °C SOC media) was added and incubated at 37 °C for 1 hour while shaking at 250 rpm. After incubation, approximately 200 μL of the transformation mixture was plated onto the LB agar (+ ampicillin) plates. The plates were incubated overnight and bacteria were given time to grow.

3.4.10 GROWING BACTERIA CULTURES

To begin, 5 mL of LB broth containing ampicillin was inoculated with one isolated colony of bacteria in a 14 ml BD Falcon tube. One colony is procured from the plate using a sterile inoculating loop under a sterile hood. The tubes were placed in an incubator at 37 °C overnight while shaking at 250 rpm. After the bacteria had grown in the BD Falcon tubes, the 5 mL sample was decanted into a 1 Liter volumetric flask of LB Broth and ampicillin. The 1 Liter flask was placed in an incubator at 37 °C overnight while shaking at 250 rpm.

3.4.11 QIAPREP SPIN MINI PREP

A QIAprep Spin Mini Prep Kit was used to complete this protocol. The protocol was followed according to the directions given by the Qiagens protocol. This

protocol was completed to isolate the DNA from the bacteria grown in culture. This protocol was performed to extract template DNA from transformed bacteria.

REFERENCES

[1] Brissos, V., T. Eggert, J.M.S. Cabral, and K.-E. Jaeger. "Improving Activity and Stability of Cutinase towards the Anionic Detergent AOT by Complete Saturation Mutagenesis." *Protein Engineering Design and Selection* 21.6 (2008): 387-93. Print.

[2] Carvalho, Cristina M. L., Maria Raquel Aires-Barros, and Joaquim M. S. Cabral. "Cutinase Structure, Function and Biocatalytic Applications." *Electronic Journal of Biotechnology* 1.2 (1998): 160-73. Print.

[3] Chen, S., X. Tong, R. W. Woodard, G. Du, J. Wu, and J. Chen. "Identification and Characterization of Bacterial Cutinase." *Journal of Biological Chemistry* 283.38 (2008): 25854-5862. Print.

[4] Ettinger, William F., Sushil K. Thukral, and Pappachan E. Kolattukudy. "Structure of Cutinase Gene, CDNA, and the Derived Amino Acid Sequence from Phytopathogenic Fungi." *Biochemistry*26.24 (1987): 7883-892. Print.

[5] Fett, W. F., C. Wijey, R. A. Moreau, and S. F. Osman. "Production of Cutinase by Thermomonospora Fusca ATCC 27730." *Journal of Applied Microbiology* 86.4 (1999): 561-68. Print.

[6] Holmquist, Mats. "Alpha Beta-Hydrolase Fold Enzymes Structures, Functions And Mechanisms." *Current Protein & Peptide Science* 1.2 (2000): 209-235. *Academic Search Premier*. Web. 17 Apr. 2013.

[7] Kim, Kyeong Kyu, Hyun Kyu Song, Dong Hae Shin, Kwang Yeon Hwang, Senyon Choe, Ook Joon Yoo, and Se Won Suh. "Crystal Structure of Carboxylesterase from Pseudomonas Fluorescens, an α/β Hydrolase with Broad Substrate Specificity." *Structure* 5.12 (1997): 1571-584. Print.

[8] Longhi, Sonia, and Christian Cambillau. "Structure-activity of Cutinase, a Small Lipolytic Enzyme." *Biochimica Et Biophysica Acta* 1441 (1999): 185-96. Print.

[9] Nicolas, Anne, Maarten Egmond, C. Theo Verrips, Jakob De Vlieg, Sonia Longhi, Christian Cambillau, and Chrislaine Martinez. "Contribution of Cutinase Serine 42 Side Chain to the Stabilization of the Oxyanion Transition State." *Biochemistry* 35.2 (1996): 398-410. Print.

[10] Ollis DL, Cheah E, Cygler M, Dijkstra B, Frolow F, Franken SM, Harel M, Remington SJ, Silman I, Schrag J, et al. "The alpha/beta hydrolase fold." *Protein Eng.* 1992:197-211.

[11] Prompers, Jeanine J., Anneke Groenewegen, Cornelis W. Hilbers, and Henri A. M. Pepermans. "Backbone Dynamics OfCutinase Probed by Nuclear Magnetic Resonance: The Lack of Interfacial Activation Revisited." *Biochemistry* 38.17 (1999): 5315-327. Print.

[12] Prompers, Jeanine J., Anneke Groenewegen, Rene C. Van Schaik, Henri A.M., Pepermans, and Cornelis W. Hilbers. "1H, 13C, and 15N Resonance Assignments of Fusarium Solani Pisi Cutinase and Preliminary Features of the Structure in Solution." *Protein Science* (1997): 2375-384. Print.

[13] Soliday, C.L., W.H. Flurkey, T.W. Okita, and P.E. Kolattukudy. "Cloning and Structure Determination of CDNA for Cutinase, and Enzyme Involved in Fungal Penetration of Plants." *Proc. Natl. Acad. Sci, USA* 81 (1984): 3939-943. Print.

[14] Stoddard, Erica M., Austin E. Pernell, Shana V. Stoddard, Dawn Wilkins, and Randy M. Wadkins. "Structural alignment and normal mode analysis of deletion mutants of cutinase to determine substructure function in alpha/beta hydrolases." (in preparation)

[15] Stoynova, Ramona Solorzano, and Elaine D. Collins. "Generation of Large Deletion Mutants from Plasmid DNA." *BioTechniques* (2004): 402-06. Print.

APPENDIX

Primer	Sequence
49-63 Forward	5' GGCAAGGACGGTGTCTGGATT 3'
49-63 Reverse	5' CAAGTTGCCCGTCTC 3'
68-72 Forward	5' GTTGGCGGTGCCTACCGAGCCACTCTT 3'
68-72 Reverse	5'ACCGTCCTTGCCGAAGGCGGATCAAG 3'
92-108 Forward	5' TGCCCTGACGCGGACTTTGATC 3'
92-108 Reverse	5' AGAGGTTCCGCGAGGGAGAGCATT 3'

Table A: Primer Sequences for Deletion Mutations

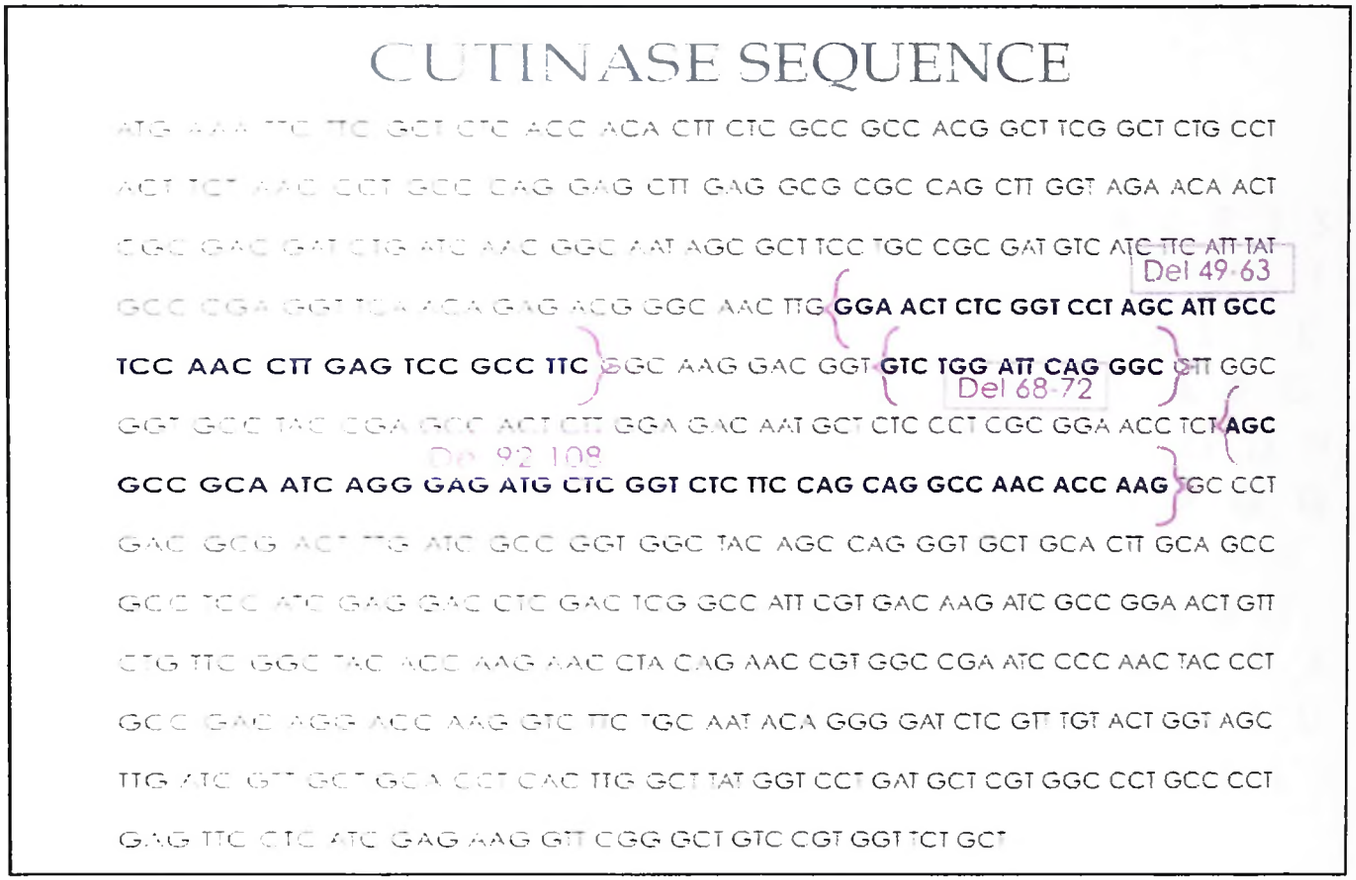

Figure A: Cutinase Sequence

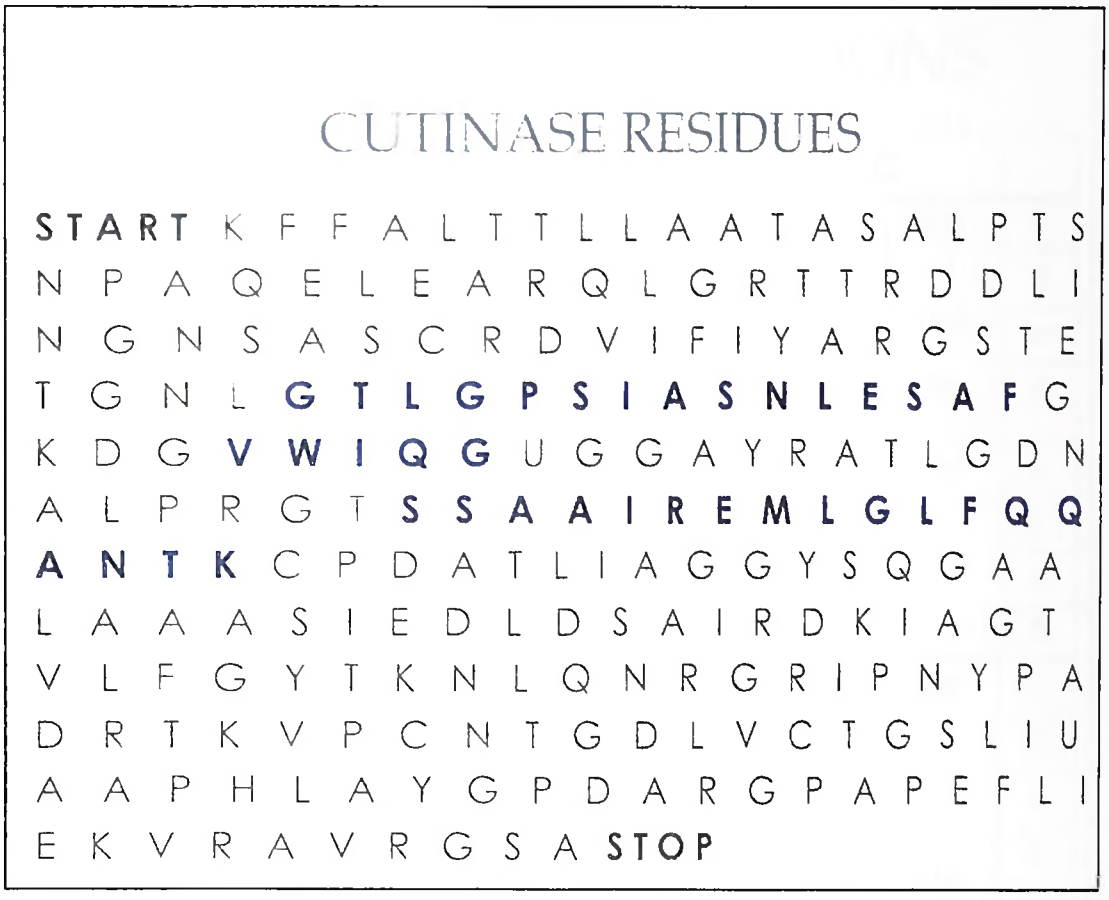

Figure B: Amino Acid Residues of Cutinase

AMINO ACID CODONS

	U		C		A		G		
U	UUU UUC	PHE	UCU UCC UCA UCG	SER	UAU UAC	TYR	UGU UGC	CYS	U C
	UUA UUG	LEU			UAA UAG	STOP	UGA UGG	STOP TRP	A G
C	CUU CUC CUA CUG	LEU	CCU CCC CCA CCG	PRO	CAU CAC	HIS	CGU CGC CGA CGG	ARG	U C A G
					CAA CAG	GIN			
A	AUU AUC AUA	ILE	ACU ACC ACA ACG	THR	AAU AAC	ASN	AGU AGC	SER	U C
	AUG	MET			AAA AAG	LYS	AGA AGG	ARG	A G
G	GUU GUC GUA GUG	VAL	GCU GCC GCAG CG	ALA	GAU GAC	ASP	GGU GGC GGA GGG	GLY	U C
					GAA GAG	GLU			A G

Table B: Amino Acid Codons

AMINO ACID ABBREVIATIONS

Amino Acid	3 letter abbr	1 letter abbr
G	GLY	GLYCINE
P	PRO	PROLINE
A	ALA	ALANINE
V	VAL	VALINE
L	LEU	LEUCINE
I	ILE	ISOLEUCINE
M	MET	METHIONINE
C	CYS	CYSTEINE
F	PHE	PHENYLALANINE
Y	TYR	TYROSINE
W	TRP	TRYPTOPHAN
H	HIS	HISTIDINE
K	LYS	LYSINE
R	ARG	ARGININE
Q	GLN	GLUTAMINE
N	ASN	ASPARAGINE
E	GLU	GLUTAMIC ACID
D	ASP	ASPARTIC ACID
S	SER	SERINE
T	THR	THREONINE

Table C: Amino Acid Abbreviations