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Developing a Diagnostic Tool for Acyl Carrier Proteins Through Trypsinolysis, Reverse-Phase Chromatography and Native Chemical Ligation

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Developing a Diagnostic Tool for Acyl Carrier Proteins through Trypsinolysis, Reverse-Phase Chromatography and Native Chemical Ligation

by

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Report

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Abstract

Developing a Diagnostic Tool for Acyl Carrier Proteins through Trypsinolysis, Reverse-Phase Chromatography and Native Chemical Ligation

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Polyketide biosynthesis is a field that has had tremendous advances in the past 50 years. The understanding of the mechanisms is updated as investigations delve into domain interactions of these microbial natural products. Although numerous polyketides are known, similarities in the sequence of product generation can be used as templates for further exploration of enzymatic activity. The focus of studies recently has been towards developing protocols to manipulate the natural products resulting in medicinally important manufactured products. This investigation examined the mechanism of the acyl carrier protein (ACP) module involved in biosynthesis.

Table of Contents

List of Tables

List of Figures

CHAPTER 1 INTRODUCTION

1.1 PURPOSE OF STUDY

Polyketide biosynthesis (PKS) is a field that has had tremendous advances in the past 50 years. The understanding of the mechanisms constantly updated as investigations delve into domain interactions of these microbial natural products. Although numerous polyketides are known, similarities in the sequence of product generation can be used as templates for further exploration of enzymatic activity. The focus of studies recently has been towards developing protocols to manipulate the natural products resulting in medicinally important manufactured products. The technologies are being developed for the genetic engineering of PKS genes. There are still some obstacles to overcome. The need for development of novel polyketides is spurred on by the resistance exhibited by more and more bacteria to whole classes of antibiotics.

1.2 RESEARCH OBJECTIVES

This purpose of this investigation is to examine the mechanism of the acyl carrier protein (ACP) domain involved in biosynthesis. The research is to ascertain which testing method could identify manipulation of biosynthesis through the attachment of a phosphopantetheinyl (p-pant) arm from Coenzyme A (CoA) to the acyl carrier protein (ACP) via 4´-phosphopantetheinyl transferase (Sfp) followed by Trypsin digestion. Reaction results will be analyzed by high performance liquid chromatography (HPLC).

The method of native chemical ligation will also be used to determine the extent to which the ACP domain can be manipulated.

1.3 STUDY SCOPE

This research project was begun by a review of current literature of polyketides synthesis and similar biosynthetic processes. Next, existing protocols were used as templates for the development of a new or novel antibiotic. Finally, experimental results are to be analyzed by use of the HPLC.

1.4 ORGANIZATION OF REPORT

Chapter 2 summarizes the current literature on polyketides synthesis as a biological process and in comparison with mammalian fatty acid synthesis. Methodologies are discussed in Chapter 3. The results and discussion are found in Chapter 4. Finally, Chapter 5 includes the experimental conclusions along with the relation of the research project experience to science education at the secondary level.

CHAPTER 2 BACKGROUND

2.1 POLYKETIDES

A brief overview of an acyl carrier protein (ACP) begins with a description of the protein and its function. ACPs can be described as two helixes bound with a loop. According to one definition, ACPs are an α-helical bundle negatively charged proteins similar in structure to amino acids. NMR and crystallography techniques have been used to identify the structures of various ACPs, (Alekseyev, et al, 2007), (Menzella, et.al, 2005). Figure 1 represents several views of ACP using CHIME software.

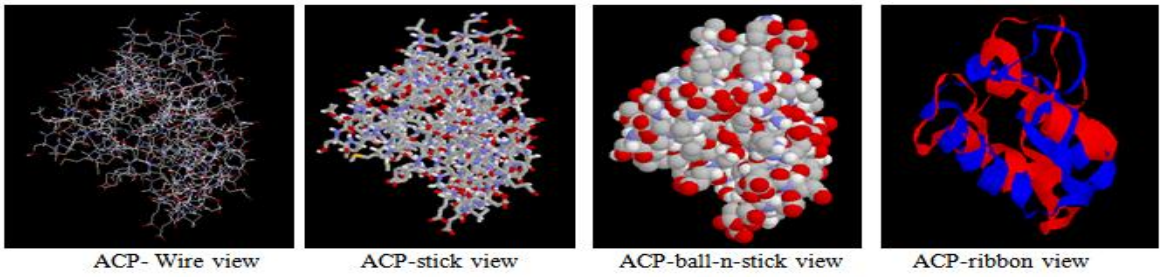

Figure 1: CHIME software models of ACP

. To analyze the attachment of the p-pant arm from Coenzyme A (CoA) to the ACP, several software programs have been used. One such program, called the WHATIF homology model, calculates electrostatic potential surfaces. Using these software programs can show similarities among the ACP domains as well as being able to note differences specifically in the side chains. Methods such as HPLC help "generate the chromatogram on its display and to identify and quantitate the concentration of the

sample constituents," according to the Waters Corporation website. The procedures for preparing samples for internal sequencing use Trypsin digestion resulting in totally cleaved peptide fragments which can be analyzed on the HPLC.

2.2 BIOSYNTHESIS

The acyl carrier protein is involved in the biosynthesis of both fatty acids and polyketides. The focus of this discussion is its role in polyketides synthesis (PKS). "Understanding the mechanistic basis for this phenomenon could provide a valuable route for engineering novel functional groups into aromatic polyketides produced by Type II PKSs." (Meadows and Khosla, 2001) The role of the acyl carrier protein in polyketides synthesis is to elongate or connect modules to form a chain resulting in the formation of polyketides. The study of polyketide biosynthesis is exemplified by 6-Deoxyerythronolide B synthase (DEBS). This particular PKS produces the equivalent of the backbone of the erythromycin antibiotic. The structure of DEBS has three major components each subdivided into two modules. "Each module is responsible for catalysis of one complete cycle of polyketide or polypeptide chain elongation and associated functional group modifications."(Cane, Walsh, Khosla, 1998) PKS can vary in composition, but the general structure shown in Figure 2. The are six DEBS modules(M) containing a minimum of three types of domains with designations- ketosynthase(KS), acyl carrier protein(ACP), and acyl transferase(AT), each having a specific function in the polyketide synthesis. Other catalytic domains with their specific functions are the ketoreductase (KR), dehydratase (DH), enoyl reductase (ER), and thioesterase (TE).

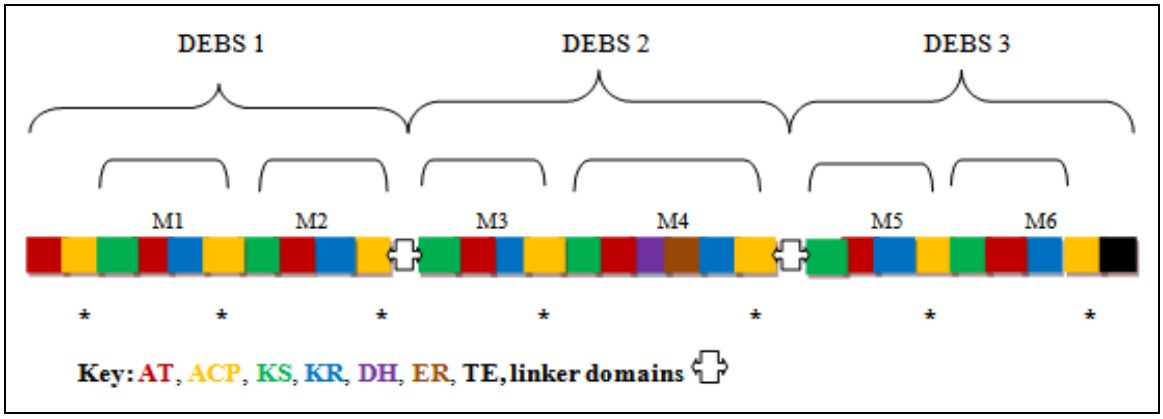

Figure 2: PKS drawn on Paint program

In PKS, ACP will initially "accept the extender unit from the AT, then collaborates with the KS domain in chain elongation, and finally anchors the newly elongated chain" (Keatinge-Clay, 2008) into the β-keto position. The growing polyketide is elongated by a two carbon unit at each ACP. The process of biosynthesis can be detailed by the steps beginning with the transference of a two carbon acetyl group from CoA to a side chain of PKS. Next an acetyl group from a malonyl group is transferred by the CoA to the ACP. The reactions following this step are a condensation, reduction, and dehydration. Another reduction completes the conversion of the condensation product to an acyl group. The acyl group next is transferred the enzyme Cys group from the ACP to be condensed again. These steps follow six more rounds of extension to build the polyketide. The last step is the thioester bond hydrolysis to release the polyketide. The obstacles for PKS gene engineering are: 1)“natural polyketide producers are not readily amenable to a complete range of genetic methodologies introducing DNA into unstudied

organisms"(Menzella, et al, 2005), and 2) the length of the PKS gene (35 to +200 kb) with limited number of "restriction sites to facilitate domain or module exchange". (Menzella, et al, 2005) Polyketides are important because of their medicinal properties, such as: immunosuppressants, antibacterials, antituberculosis, anticholesterol, anticancer, and chemopreventatives.

CHAPTER 3 EXPERIMENTAL METHODOLOGY

3.1 OVERVIEW

The assays performed for the research were to "create testable hypotheses for identifying components displaying the highest flexibility or promiscuity for future combinatorial biosynthesis" as referred to in earlier research (Cane, et, al, 2005). Previous research had ascertained the homology model for the first ACP domain structure. This model was then used as a template for the other ACP domains. The knowledge of the reactions to build the polyketide is being used in this research to allow for manipulation. A reaction employing 4′-phosphopantetheinyl transferase (Sfp) was selected to be the first assay. A Trypsin digestion followed the Sfp reaction to test cleavage of the p-pant arm. Finally, Native chemical ligation was also selected to verify the success of the ACP manipulations.

3.2 PROTOCOLS

The first reaction was the Sfp Synthase (4′-phosphopantetheinyl transferase) reaction. The reaction could be written as: EryACP + ppant + Sfp → holo-ACP. Reagents, concentrations and volumes of reagents were identified and calculated (Table 1). Glycerol, HEPES (pH 7.5), and nanopure water were placed in microcentrifuge reaction tube. The salts magnesium chloride and sodium chloride were then added. The ACP, p-pant, and Sfp were added next to the reaction vessel. This master mix was incubated at room temperature for six hours. The resultant mixture was flash frozen in liquid nitrogen for later analysis in the HPLC.

Table 1: Reagent Mix for Sfp reaction

Stock reagents	Final Concentrations	Reaction Volume (μL)
ACP 15 mg/mL	1.0 mg/mL	1.0
P-pant 10 mM		0.5
Sfp 20 mg/mL	0.01 mg/mL	1.0
MgCl2 1 M	10 mM	5.0
NaCl 5 M	150 mM	15
HEPES (pH 7.5) 1 M	100 mM	50
Glycerol 100%	10 %	50
Nanopure water		377.5
Total Volume		500

The second reaction was the Trypsinolysis reaction. The reaction could be written as: holo-ACP + Trypsin → fragments. The digestion reagents, concentrations and volumes of reagents were identified and calculated (Table 2). 80% of the Sfp reaction mixture was used as the stock solution. The product of the Sfp reaction and the Trypsin were placed in microcentrifuge reaction tube. This master mix was incubated at room temperature. Samples were taken at intervals of 1 hour, 2 hours, 4 hours and then overnight. The samples were flash frozen in liquid nitrogen for later analysis in the HPLC.

Table 2: Reagent Mix for Trypsinolysis reaction

Stock reagents	Final Concentrations	Reaction Volume (μL)
Holo-ACP/Sfp 1.0 mg/mL		400
Trypsin 0.20 mg/mL	0.02 mg/mL	34
Total Volume		**434**

The native chemical ligation reaction was next. The Keatinge-Clay lab has engineered an EryACP with a N-terminal histidine tag referred to Novagen's pET 28b vector diagram shown in Figure 3. The intention is to mutate a Glycine to a Cysteine to determine if Thrombin can cleave at this point. The process began with designing the

primers to initiate the mutagenesis of Glycine to Cysteine. For the single point mutation, 15 residues on either side of the target site are selected. In this case, the original sequence including the codon for Glycine was selected as follows: GGCCTGGTGCCGCGCGGCA GCCATATGGCTA. The website for the Center for Biological Sequence Analysis allowed for selection of the proper codon to change G to a C as GGCCTGGTGCCGCGCTGCAGCCATATGGGGC. The website at San Diego State University, College of Sciences gave "an expanded codon table showing the relative frequency that different codons are used in E. coli genes". The Sequence Manipulation Site of the website Bioinformatics allowed for the reverse sequence to be formatted as GCCCCATATGGCTGCAGCGCGGCACCAGGCC.

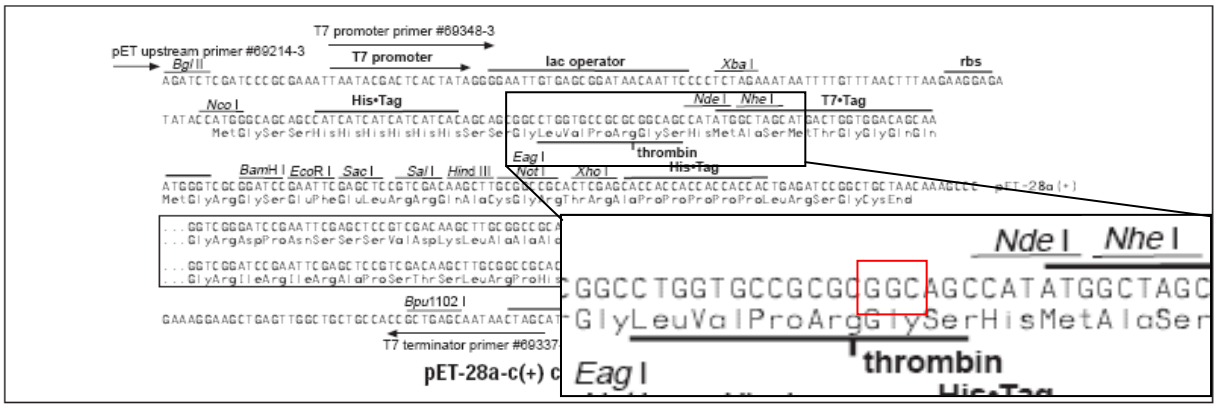

Figure 3: pET28b vector diagram

The primers were then ordered from Sigma-Aldrich, Inc. Upon arrival, the primers designated as EryACP Gly to Cys F and EryACP Gly to Cys R, were suspended in nanopure water to make the stock solution. The concentrations were obtained from the Nano Drop (ND1000) program. Once the concentrations were determined, the reaction

required solutions could then be prepared. At this point, an overnight transformation using Top10 cells was performed to obtain the plasmid to be used in this reaction. Concentrations and volumes of reagents were identified and calculated (Table 3). 10X AD Buffer, EryACP5/pET 28b (plasmid), EryACP Gly to Cys F, EryACP Gly to Cys R, dNTP, (0%, 5%, 10%) DMSO, nanopure water, and PF ultra were placed in mini microcentrifuge reaction tube. These master mixes were incubated in the Thermocycler overnight using CDF method. DPN1 is added to the reaction tubes and left to incubate overnight. The prescribed volume of solution is added to Top 10 cells. The Heat Shock protocol was followed next to produce the mutant colonies. The bench protocol, QIAprep Spin Miniprep Kit was used to isolate the mutated colonies to be DNA sequenced at the Molecular Biology Building at UT-Austin.

Table 3: Reagent Mix for Mutagenesis reaction

Stock reagents	**Volume (µL)**	**Volume (µL)**	**Volume (µL)**
10xAD Buffer	5.0	5.0	5.0
EryACP5/pET28b	1.0	1.0	1.0
EryACP Gly to Cys F	1.0	1.0	1.0
EryACP Gly to Cys R	1.0	1.0	1.0
dNTP	2.0	2.0	2.0
DMSO	0	2.5	5
Nanopure H2O	40.0	37.5	35
PF ultra	1.0	1.0	1.0
Total Volumes	**51**	**51**	**51**

A check digestion (Table 4) was performed simultaneously to verify that the reaction has the correct plasmid. The diagram Figure 4 from Novagen vector diagram indicated which restriction enzymes to use in this reaction.

Table 4: Reagent Mix for Check Digestion reaction

Reagents	Volume(μL)
DNA (Miniprep)	3
10x BSA	2
NEBuffer 4	2
Xho l	0.5
Nde l	0.5
Nanopure H_2O	12
Total volume	**20**

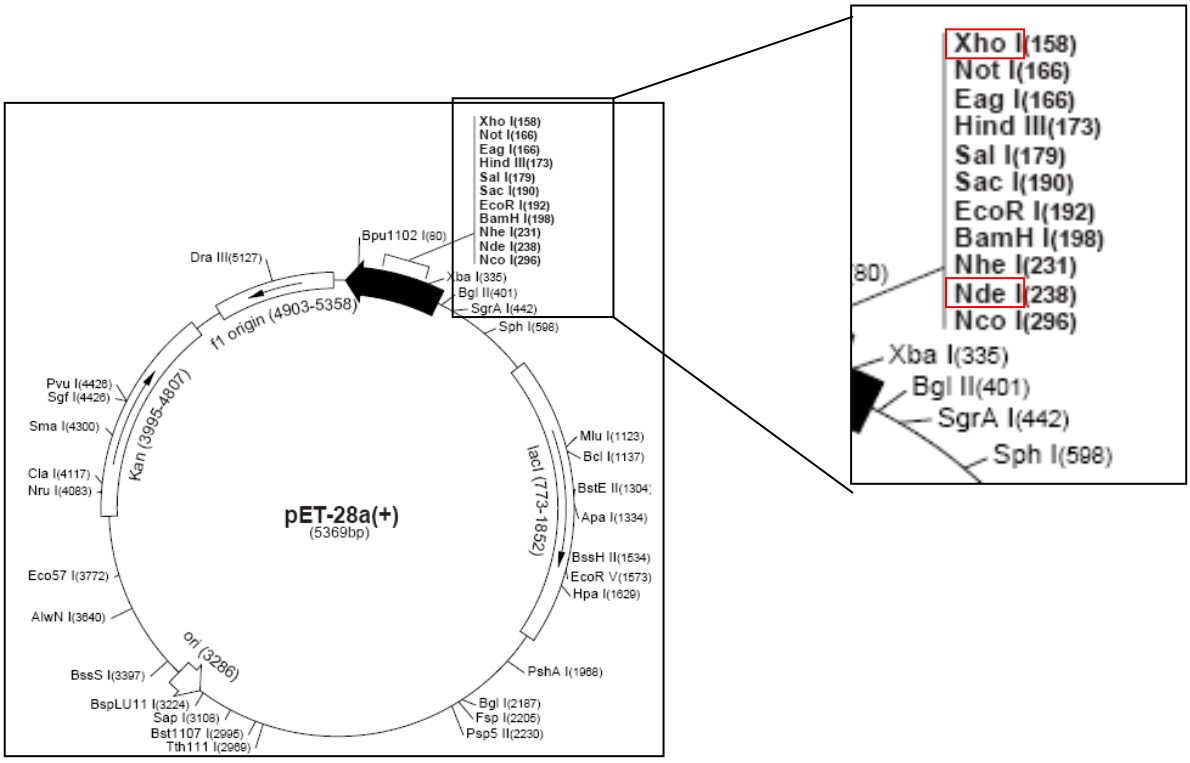

Figure 4: Novagen vector diagram with restriction enzymes labeled

The resultant mutagenesis reaction would possibly allow a Thrombin cleavage at the new site of LeuValProArgCysSer, meaning that a cysteine residue would now occupy the N-terminus of the ACP. Thermally or chemically denaturing the ACP would lead to a transfer from the acyl group to the phosphopantetheinyl arm to the N-terminus through

native chemical ligation. Another cleavage of four residues with cyanogens bromide results in a peptide able to be analyzed with the HPLC.

3.3 ANALYSIS

The experiment called for analysis by the HPLC. Graphical printouts of peaks and retention times of experimental samples could be compared to standard graphs of enzymes. Analysis method 1(100/50/100%A) used for analysis is shown in Table 5 below. Analysis method 2(50/100% A), a modification of the concentrations and time was required due to the results peaks becoming apparent after minute 15 as shown in Table 6 below.

Table 5: HPLC 100/50/100% A

TIME(min)	PUMP A (%) Water	PUMP B (%) Methanol
0	100	0
15	50	50
15.01	50	50
18	100	0
23	100	0

Table 6: HPLC 50/100% A

TIME(min)	PUMP A (%) Water	PUMP B (%) Methanol
0	50	50
18	100	0
23	100	0

CHAPTER 4 RESULTS AND DISCUSSION

Figure 5 represents the HPLC graphical results for the EryACP with Sfp using analysis method 2 indicated in Table 6. Concentrations of the product amounts were not calculated at the time.

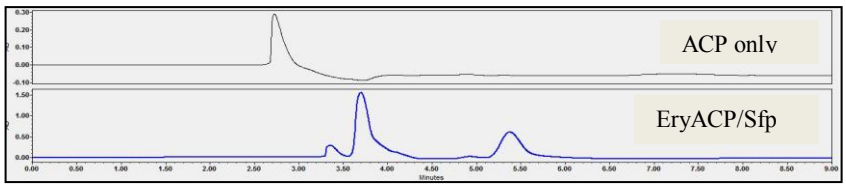

Figure 5: HPLC results for Sfp reaction with ACP

Once the solution was digested with the Trypsin, the samples drawn at the 1, 2, 4 and overnight times were analyzed on the HPLC. The graphical results using concentrations in Analysis Method 1 are shown below in Figure 6. Since the first analysis method had a long retention time and did not show the peaks until after minute 15, the samples were analyzed using Analysis Method 2. The results for Analysis Method 2 are shown in Figure 7 below.

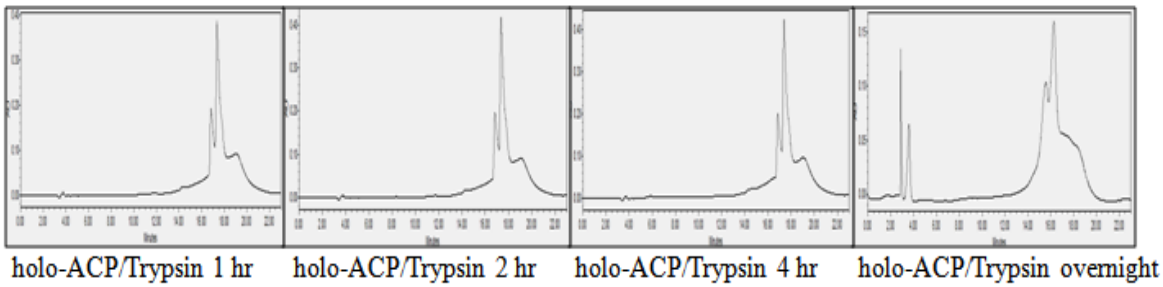

Figure 6: HPLC Trypsinolysis results (100/50/100%A)

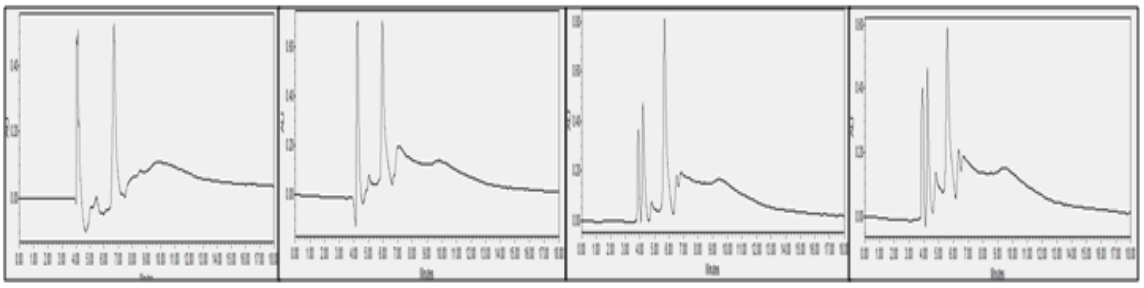

Figure 7: HPLC Trypsinolysis results (50/100%A)

The three samples of the first mutagenesis reaction were sent for DNA sequencing at the Molecular Biology Building. Result viewing options from the University of Texas at Austin DNA Sequencing ICMB Core Facilities website were shown as a chromatogram or text as shown in Figure 8. The text was translated at the website-ca.expasy.org/tools/dna. The mutation was determined to have been unsuccessful. The second mutagenesis reaction was performed and yielded similar results.

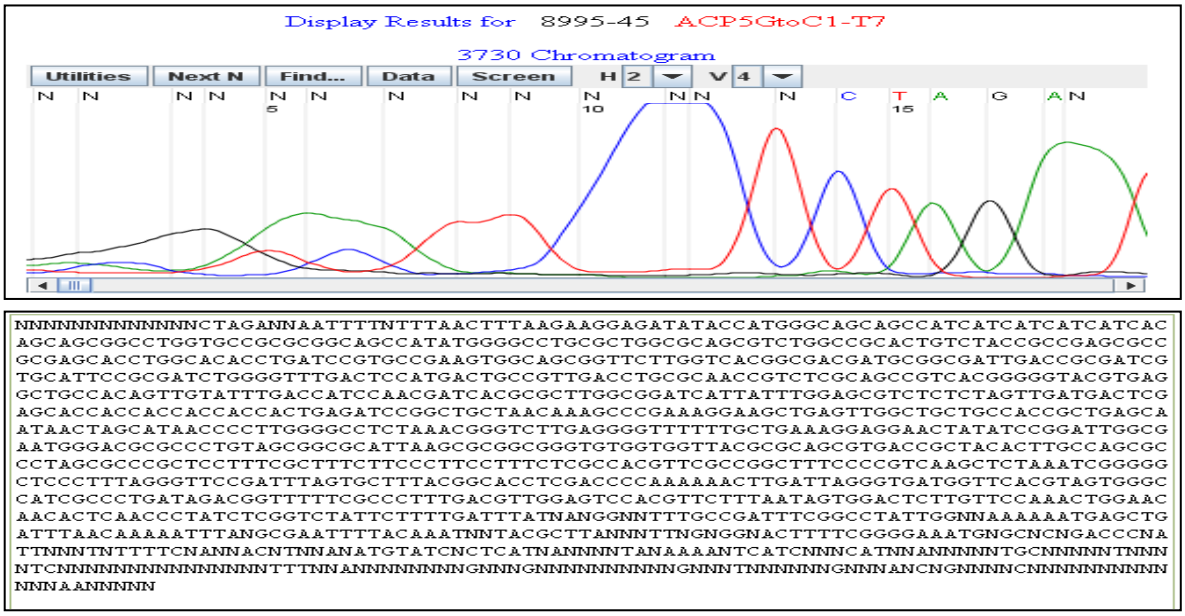

Figure 8: DNA Sequencing Chromatogram and text views of mutagenesis reaction

CHAPTER 5 CONCLUSIONS AND DISCUSSION

5.1 CONCLUSIONS

Numerous trials of the Sfp, Trypsinolysis and mutagenesis reactions were performed. Although the HPLC analysis revealed indications of products, a definitive statement as to success of either reaction cannot be made at present time. Further, more extensive studies are required before a determination can be made as to the validity of present data collected so far.

5.2 DISCUSSION OF APPLICATION TO EDUCATION

Research experience for a biochemical project was the goal of this summer. Research methods from lab safety to various techniques not available at the high school level provided insights to the rigor required of students choosing this as their career choice. The major limiting factor for the completion of this project was time. Repeated trials and revisions of protocols to ensure product formation which are inherent in research depleted the time allotted for the research. The experience will lead to modifications in instruction of both the AP and Chemistry I courses. The depth and complexity of the scientific method will be emphasized along with the need for good scientific recordkeeping.

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Center for Biological Sequence Analysis http://www.cbs.dtu.dk/staff/dave/roanoke/fig13_18.jpg

Codon usage in E. Coli http://www.sci.sdsu.edu/~smaloy/MicrobialGenetics/topics/in-vitro-genetics/codon-usage.html

Reverse complement http://www.bioinformatics.org/sms/rev_comp.html

Vita

Graciela Reyes was born in Brownsville, Texas. She attended a private Catholic school her primary years then transferred to the public school system in Brownsville. She graduated from Homer Hanna High School, Brownsville, Texas. Her post secondary education included Texas Southmost College, Brownsville, Texas, Pan American University-Edinburg, Texas, University of Texas-Brownsville, Texas. In 1996, she received a Bachelor of Science from UT-Brownsville, and TEA certification in Composite Science. Her employment has been with the Brownsville Independent School District where she has taught Biology, Science III, Anatomy & Physiology, Aquatic Science, Integrated Physics and Chemistry, and currently, Chemistry. In June of 2008, she entered the UTEACH Masters Program at the University of Texas at Austin.

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