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DNA Sequencing and Site-Directed Mutagenesis of the Drug-Metabolizing Enzyme, Cytochrome P450-2B4

Matthew Bidlack

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This project focuses on the development of an undergraduate-level laboratory procedure involving EMU's new automated DNA sequencer. The gene used for this procedure is CYP2B4 from the rabbit genome, which codes for Cytochrome P450-2B4 (CVP450). Cytochrome P450s are an important class of proteins found in many species throughout the animal kingdom, including humans. The wild-type CYP2B4 will be sequenced, and then mutated to replace threonine-302 with alanine. Threonine-302 is suspected to playa key role in P450 function. Thus far, procedures have been developed for site-directed mutagenesis to convert threonine-302 to an alanine residue, transformation of bacteria with the target gene, isolation of the plasmid from the transformed bacteria, harvesting the bacteria to express the gene, and preparation of the protein for spectrophotometric analysis. While development of a viable DNA sequencing protocol has met with limited success, the protocol used for the site-directed mutagenesis of CVP2B4 has brought promising results.

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DNA SEQUENCING AND SITE-DIRECTED MUTAGENESIS OF THE DRUG-METABOLIZING ENZYME, CYTOCHROME P450-2B4

by

Matthew Bidlack

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Abstract

This project focuses on the development of an undergraduate-level laboratory procedure involving EMU's new automated DNA sequencer. The gene used for this procedure is *CYP*2B4 from the rabbit genome, which codes for Cytochrome P450-2B4 (CYP450). Cytochrome P450s are an important class of proteins found in many species throughout the animal kingdom, including humans. The wild-type CYP2B4 will be sequenced, and then mutated to replace threonine-302 with alanine. Threonine-302 is suspected to play a key role in P450 function. Thus far, procedures have been developed for site-directed mutagenesis to convert threonine-302 to an alanine residue, transformation of bacteria with the target gene, isolation of the plasmid from the transformed bacteria, harvesting the bacteria to express the gene, and preparation of the protein for spectrophotometric analysis. While development of a viable DNA sequencing protocol has met with limited success, the protocol used for the site-directed mutagenesis of CYP2B4 has brought promising results.

Introduction

The main objective of this research activity is to devise an undergraduate-level biochemistry laboratory procedure involving EMU's recently acquired automated DNA sequencer. The ultimate goal is to create a reproducible procedure that undergraduate students can perform to sequence a gene and express the encoded protein, then change the sequence of the gene and express the mutated protein.

The target gene for this project is called CYP2B4, from the rabbit genome. It codes for a protein called Cytochrome P450-2B4. Cytochrome P450 is a family of hemoproteins found in many plant and animal species, including humans. Its sheer abundance throughout the animal kingdom indicates its importance to life. Cytochrome P450s are found in virtually every mammalian cell type and are particularly abundant in the liver and kidneys. These enzymes serve the organisms in which they are found by metabolizing toxic substances to water-soluble products that allow them to be excreted from the organism in the urine. Much has been learned about this family of enzymes over the past 50 years. Perhaps the most notable discovery was that functional P450s in their reduced form and in the presence of carbon monoxide exhibit an absorbance peak at 450 nm (Parke). Also, genetic studies have revealed that while all P450s have similar fundamental function and tertiary structure, their primary structures often differ significantly. Despite this, some amino acid residues remain common to all P450s. These amino acids are believed to play key roles in enzyme function and tertiary structure. One such amino acid is threonine-302 (T302) whose hydroxyl group is believed to have special importance.

Cytochrome P450-2B4 is examined in order to gain a more complete understanding of the many metabolic functions of the P450 family of enzymes. One of the most highly conserved primary structure segments in the family of P450s consists of several polar residues forming an internal solvent channel. This channel gives solvent water molecules direct access to the active site allowing for proton transfer and substrate hydroxylation (Raag, *et al.*; Fig. 1). The recently acquired crystallographic structure of Cytochrome P450-2B4 suggests that T302 is situated in a critical position within the solvent channel and near the heme portion of the enzyme (Fig. 2). It is hypothesized that if this residue were changed to alanine, an amino acid without a hydroxyl group, the overall activity of Cytochrome P450 would be greatly reduced.

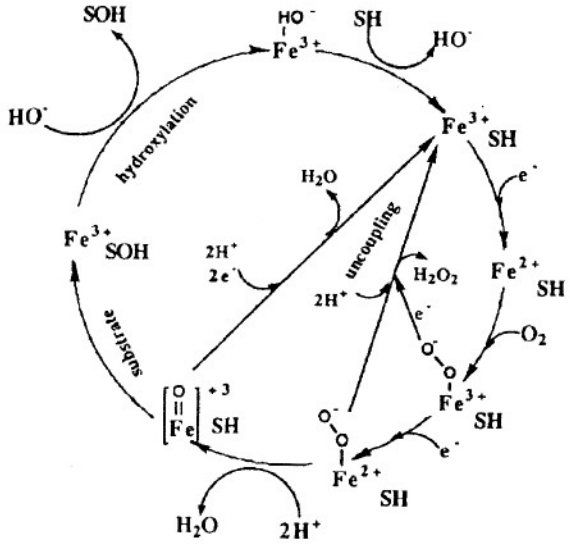

Fig. 1. Cytochrome P450 Reaction Cycle. SH and SOH represent the substrate and product, respectively. Threonine 302 may provide a polar residue that, along with water molecules in the active site, allow access of a proton, which is crucial in promoting oxygen-oxygen bond cleavage to produce the reactive oxygen atom that is inserted into the substrate (Raag, *et al.*).

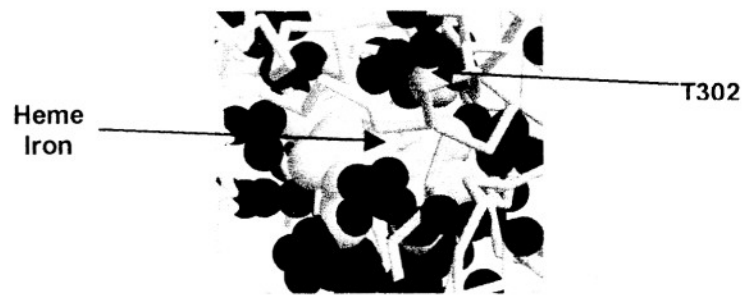

Fig. 2. Crystallographic Structure of Cytochrome P450-2B4 and the Target Site of the T302A Mutation. As shown, T302 is situated very close to the heme group in the active site of the enzyme. Protein Explorer (http://molvis.sdsc.edu/protexpl frntdoor.htm) was used to view the structure of P450-2B4, whose PDB code is 1PO5.

To test this hypothesis, the wild-type CYP2B4 gene must first be sequenced and the Cytochrome P450-2B4 enzyme expressed. Then attention is turned to the site-directed mutagenesis of CYP2B4 in order to convert T302 to alanine (T302A). DNA sequence determination of the mutated gene and comparison with the wild-type gene sequence will confirm that the T302A mutation was successful (Fig. 3). With confirmation, the mutated gene can then be expressed. Activities of the mutated enzyme and the wild-type enzyme can then be compared.

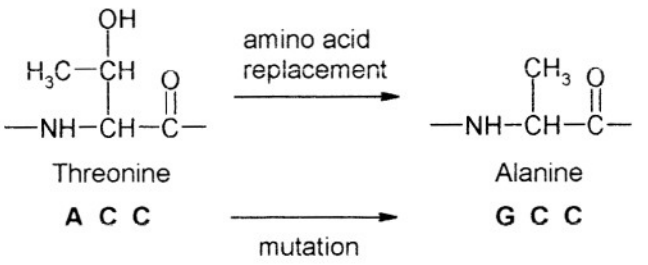

Fig. 3. The T302A Mutation. The codon for Threonine 302 is A·C·C. Converting adenine to guanine results in a codon for alanine. The site of mutation is at base-pair position 904 in the CYP2B4 gene.

In accordance with the main project objective of creating a viable novice-level laboratory procedure, sound procedures have so far been developed for site-directed mutagenesis, transforming bacteria with either the wild-type or mutagenic plasmid containing both the target CYP2B4 gene and an ampicillin resistance gene, isolating the plasmid from these bacteria, harvesting these bacteria to express the CYP2B4 gene, and cell lysis for the separation and analysis of the expressed protein. Although the procedure for automated DNA sequencing has been problematic and continues to be developed, promising results have been obtained with the procedure for site-directed mutagenesis of CYP2B4.

At the current stage of this project, plasmids containing the wild-type and mutagenic CYP2B4 have been isolated. In future stages, two different innocuous modifications of the 2B4 gene will be examined with the aim of determining the most efficient and reliable protein purification process. A GST-tagged protein coded by the gene, GSTΔ2B4, can be purified with a GSH-agarose affinity chromatography. Running the GST-tagged P450-2B4 through the GSH column followed by a thrombin-containing solution will allow for efficient purification. The thrombin proteolytically cleaves

between the GST and P450 proteins to yield purified P450 in the eluate. A His-tagged protein coded by the gene, HisΔ2B4, will be purified in a much different process, which does not require detergent. The two processes will be tested and compared for protein quality, yield, and overall efficiency. It can then be determined which protein purification method is most appropriate for the novice-level laboratory.

Materials And Methods

Calcium Chloride Transformation

For sequencing the wild-type CYP2B4 gene, competent MV1304 *E.coli* cells were transformed with the target CYP2B4 plasmid. The calcium chloride transformation procedure was followed (Appendix I). Transformed cells were then cultivated in an ampicillin medium. Since the target plasmids contain an ampicillin resistance gene in addition to CYP2B4, only the cells that have been successfully transformed can flourish and form colonies.

Plasmid Isolation and Sequencing

Bacterial cells were then lysed and DNA was separated for sequencing by ion-exchange chromatography in small cartridges (Qiagen, Appendix II). Automated DNA sequencing uses the Sanger sequencing method with different colored dyes attached to each of four dideoxynucleotide terminators (ddNTP). The isolated plasmid is mixed with the following sequencing reagents: a primer, all four deoxynucleotides (dNTP), four dye-labeled ddNTP, and *Taq* polymerase. The primer used was 17 bases long (bp 269-285, 5' AAA GGC CTC CGC TTG GT 3') with 59% GC content and was synthesized by Biomedical Research Core Facilities (University of Michigan). The reactants were submitted to thermocycling under conditions shown in Table 1 to produce DNA fragments ending with a colored fluorescent dideoxynucleotide terminators. The fluorescence of each dye-labeled terminator corresponds to a particular nucleotide base. The dye-labeled fragments were then purified via ethanol precipitation, loaded into a single well, and resolved by capillary electrophoresis in an automated DNA sequencer.

Fragments resulting from the sequencing reaction are separated by size by capillary electrophoresis (Applied Biosystem DNA sequencer). Fluorescence from the dye-labels is then detected upon excitation with laser light. Since each base is represented by one specific color, the sequence of colors detected as the fragments pass through the detector corresponds to the gene sequence (Fig. 4; Appendix III).

Table 1. Theromocycling conditions for sequencing CYP2B4.

Temperature	Time	Cycles
96°C	10 seconds	
50°C	5 seconds	25
60°C	4 minutes	
4°C	∞	Hold

Protein Expression and Cell Lysis

After sequencing CYP2B4, cells transformed with the target plasmid were cultivated and induced to express Cytochrome P450-2B4 with isopropylthiogalactoside (IPTG). After incubation at 24 °C for 2 days, cells were harvested. The cell walls of harvested cells were lysed with lysozyme. DNase was applied to degrade the DNA contained in broken cells. The remaining organelles and cellular debris were physically homogenized and a detergent was added to facilitate protein solubilization and to clarify the solution. Cytochrome P450-2B4 was then isolated by centrifugation, and its integrity determined by spectrophotometric analysis (Appendix IV).

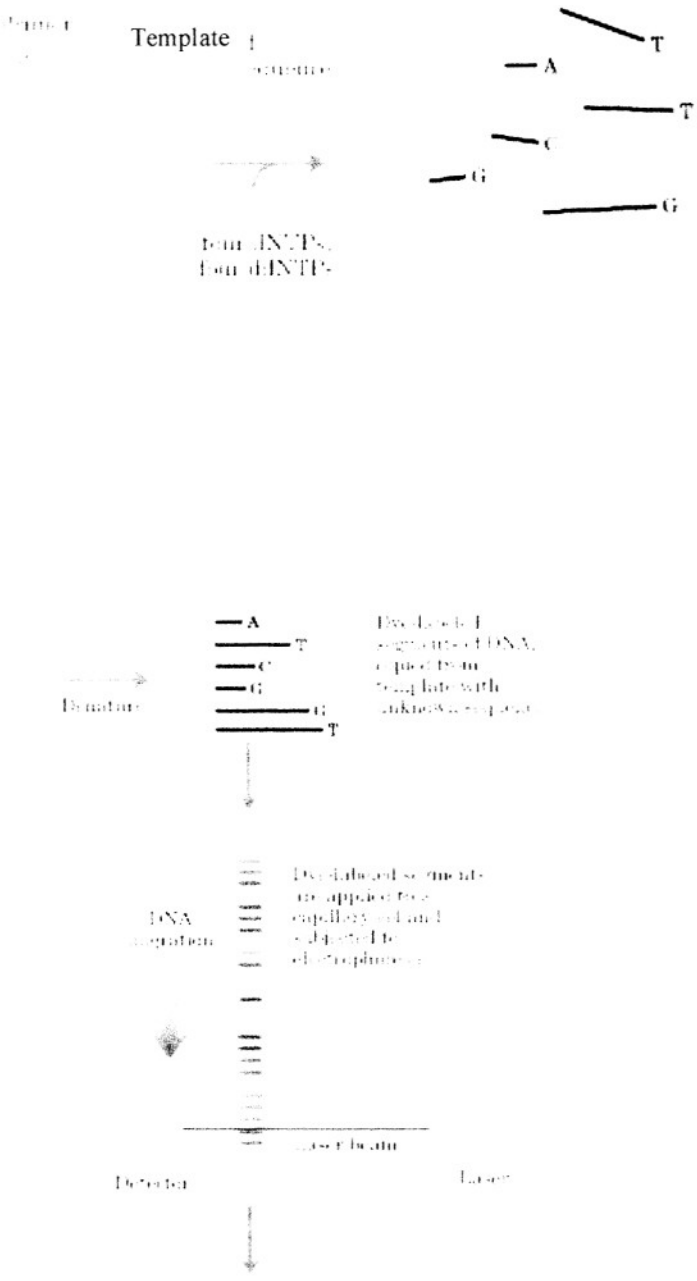

Fig. 4. Automated DNA Sequencing. Template DNA, Primer, dNTPs, ddNTPs, and DNA polymerase are reacted and thermocycled. The resulting dye-labeled fragments are applied to a capillary gel and undergo electrophoresis. As the dye-labeled fragments pass through a laser beam, their fluorescence is detected and the sequence of colors are analyzed to determine the DNA sequence (Nelson *et al.*, 2003).

Site-Directed Mutagenesis

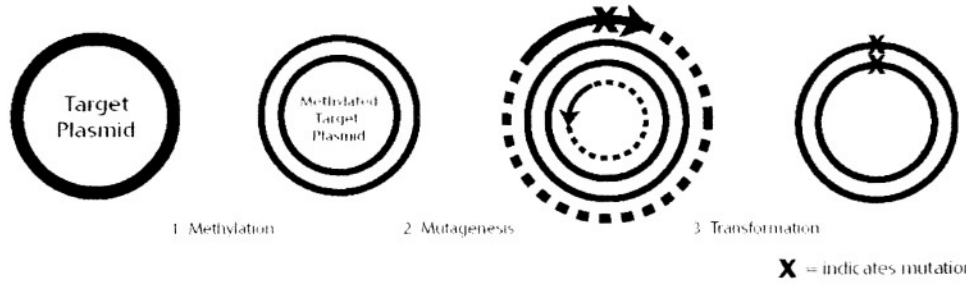

Fig. 5. Site-directed mutagenesis was performed in three main steps (Invitrogen).

Adenine located at position 904 on CYP2B4 was substituted with guanine in order to convert threonine-302 to alanine. The GeneTailor Site-Directed Mutagenesis Kit, manufactured by Invitrogen Life Technologies, was used to induce this mutation. The protocol consisted of three main steps: methylation, mutagenesis, and transformation (Fig. 5). In the first step, methylation, the CYP2B4 plasmid was mixed with S-adenosylmethionine (SAM) and DNA methylase in an aqueous solution (Fig. 6). This was done to tag the non-mutant DNA for later degradation. The methylated plasmid was then mixed with two overlapping primers (one containing the mutation), all four deoxy-nucleotides (dNTP), and Taq polymerase.

To change base 904 from adenine to guanine, two overlapping primers were used. The forward primer, T302AF, is 31 bases long and extends from 884 - 914 and contains the point mutation. The reverse primer, T302AR is 28 bases long and extends from 876 - 903 and contains no base change. The overlapping region is 10 bases long, stretching from 884 to 903 (Fig 7).

Then these reactants were thermocycled to produce a mixture of *un*methylated, mutant CYP2B4 and methylated *non*mutant CYP2B4 (Table 2). Competent DH5α-T1

E.coli cells were transformed with the thermocycled products and cultivated in an ampicillin medium. DH5α-T1 cells express *Mcr*BC endonuclease, which digests the methylated template DNA, thus retaining the mutant CYP2B4 only (Invitrogen; Appendix V). Transformed cells were lysed and the mutant plasmid was isolated by ion-exchange chromatography in small cartridges (Life Technologies; Appendix II).

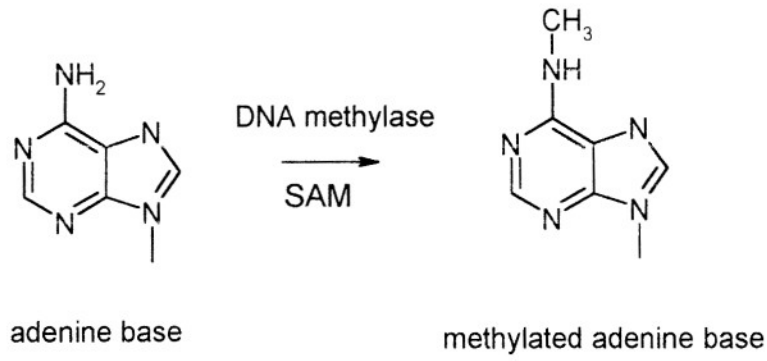

Fig. 6. Methylation involves reacting the plasmid DNA with S-adenosylmethionine (SAM) and DNA methylase in an aqueous solution. This is done to tag the non-mutant DNA for later degradation.

```
5'                TC  TTC TTC GCC GGC ACC GAG GCC ACC AGC AC   3'
                                          ↑
3'   C GAG AGC GAG AAG AAG CGG CCG TGG CTC                      5'
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Fig. 7. Primers for mutagenesis. Overlapping forward and reverse primers synthesized by Integrated DNA Technologies. Forward primer (top) contains the mutation (A becomes G) indicated by arrow and has a melting temperature of 75.0°C. Reverse primer has a melting temperature of 71.1°C.

Table 2. Thermocycling conditions for mutagenesis of CYP2B4

Temperature	Time	Cycles
94°C	2 minutes	1
94°C 55°C 68°C	30 seconds 30 seconds 5 minutes	20
68°C 4°C	30 minutes ∞	1 Hold

Results and Discussion

Transformation

The transformation by the calcium chloride procedure was successful in transforming the MV1304 *E. coli* bacteria with both His- and GST-tagged plasmids.

Isolating the plasmid

Both experimental plasmids were successfully isolated and purified from overnight cultures of *E.coli* in LB medium with the Qiagen protocol. These were analyzed spectrophotometrically to assess DNA quality and concentration (Table 3). DNA concentrations were determined from Equation 1 (below).

$$[\text{DNA}] = A_{260} \times 50\ \mu\text{g/ml} \qquad (1)$$

Table 3. Spectrophotometric Analysis of Plasmids

	A_{260}	A_{280}	A_{260}/A_{280}*	Final DNA concentration (μg/ml)
pHisΔ2B4	0.388	0.203	1.91	19.4
pGSTΔ2B4	0.905	0.517	1.75	45.2

* A_{260}/A_{280} ratio indicates DNA purity. Pure DNA has a ratio in the range of 1.8 – 2.0.

DNA Sequencing

Several attempts have been made to sequence both the pHisΔ2B4 and the pGSTΔ2B4 genes with our automated sequencer. Unfortunately, despite rigorous efforts to minimize error, none of these attempts were successful. Sequencing reaction trials with two different thermocyclers and three different clean-up procedures were performed and all results were of the same poor quality. Later research in the winter term Biochemistry Laboratory (CHEM 453) indicated that the quality of the DNA for sequencing was still not sufficient for sequencing even though the absorbance ratios were appropriate. Continued research will focus on improving this protocol.

Three unique purification protocols were used to resolve unincorporated nucleotides from dye-labeled fragments: Centrisep Spin Columns, ethanol/sodium acetate precipitation, and ethanol/EDTA precipitation (Appendix III).

Protein Expression and Cell Lysis

Protein expression and cell lysis procedures were successful. Prominent peaks were observed at 450 nm upon spectrophotometric analysis of both the His- and GST-tagged P450Δ2B4 enzymes. A large peak at 420 nm ($A_{420} = 0.023$) was observed for the His-tagged protein while a negligible peak was observed in that region for the GST-tagged protein. This suggests that a significant amount of the His-tagged enzyme was denatured during homogenization. If this is the case, adjustments, such as using a smaller Dounce homogenizer, can easily be made to reduce denaturation and increase yield (Table 4).

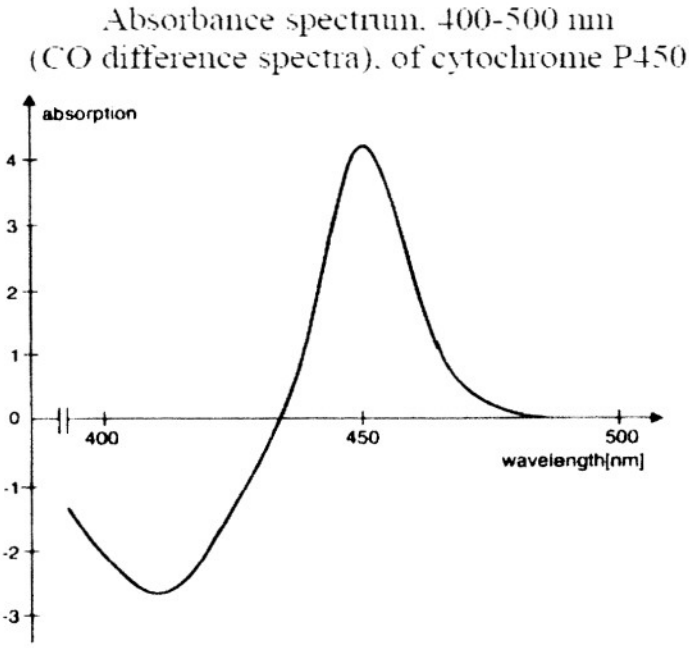

Fig. 8. Typical Ferrous-Carbonyl Difference Spectrum of Cytochrome P450 (Parke 9). CYP450 was reduced with sodium dithionite in both the reference and sample cuvets, and carbon monoxide was bubbled into the sample cuvet.

Table 4. Spectrophotemetric Analysis of P450 Expression.

	A_{450}	A_{490}	Peak Absorbance ($A_{peak} = A_{450} - A_{490}$)	P450 Concentration in Lysate (μM)	Lysate Volume (mL)	Amount of P450 in Bacterial Cells (nmol/g)
HisΔ2B4	0.042	0.016	0.026	0.571	2.75	1.570
GSTΔ2B4	0.017	-0.001	0.018	0.396	1.50	0.594

The absorbance difference between 450 nm and 490 nm is used to calculate the concentration of expressed P450 in the cells using Beer's Law using the ferrous carbonyl difference spectrum (Fig. 8). The Beer's Law absorption coefficient is 91 mM^{-1}. One gram of the cultured cells was analyzed.

Site-Directed Mutagenesis

Methylation, mutagenesis, and transformation were all successfully completed. Mutagenesis products were visualized electrophoretically on a 0.7% agarose gel with ethidium bromide (Fig. 9). Mutagenic CYP2B4 was isolated with Concert High Purity mini-preps (Life Technologies) and spectrophotometrically analyzed. The A_{260}:A_{280} ratio indicates poor DNA quality. The low peak absorbance at 260 nm further indicates low DNA yield. When the wild-type plasmids were isolated earlier, Qiagen mini-prep purification columns were used and the DNA purity and yield were excellent.

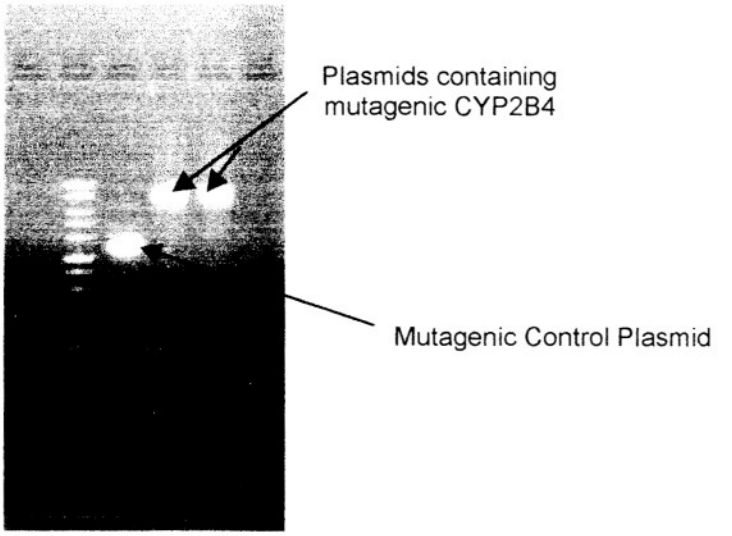

Fig. 9. Mutagenesis Results Visualized by Agarose Gel Electrophoresis (1.5%) Against a 1-kb Ladder. The control plasmid is ≈ 5 kb while the experimental CYP2B4 plasmids are ≈ 7 kb.

Conclusion

Regarding the main objective of developing an undergraduate-level laboratory experiment, great progress has been made during the 2003-2004 academic year. The Invitrogen GeneTailor site-directed mutagenesis kit proved to be a relatively simple and efficient method and appears to be well suited for novice-level laboratory work. Transformation, plasmid isolation, and protein purification protocols were also quite successful. However, our automated DNA sequencing protocol has brought limited success and requires further experimentation and adjustments. In addition, it was found that column purification (Centrisep cartridges) following PCR amplification allows no greater amount of DNA sequence to be determined than ethanol precipitation. It also appears that, for CYP2B4 plasmid isolation, the Qiagen mini-prep cartridges outperform those of the Concert (Life Technologies) variety.

This project is ongoing, but, in accordance with the main project goal, many of the procedures successfully developed this year have already been incorporated into Eastern Michigan University's undergraduate biochemistry laboratory curriculum. Future goals are to adjust the sequencing protocol to improve results and to streamline the overall experimental procedure. In addition, plans include the development of an assay for purifying and comparing the activities of mutant and wild-type P450s to assess how the T302A mutation has disrupted P450 activity.

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Appendix: Detailed Materials and Protocols

I. CALCIUM CHLORIDE TRANSFORMATION

A. Materials

- 500 ml LB (Luria-Bertani) Broth
- Several LB Agar Plates
- Several LB Agar Ampicillin Plates
- Overnight Bacterial Culture
- 250 ml 50 mM $CaCl_2$
- 250 ml Erlenmeyer flask (autoclaved)

1. LB Broth (500 ml)

   5 g Bacto Tryptone
   2.5 g Bacto Yeast Extract
   5g NaCl

   Combine all three ingredients in a 1000 ml Erlenmeyer flask. Dilute to 500 ml with deionized water. Adjust pH to 7.5 with NaOH. Autoclave.

2. LB Agar Plates (100 ml)

   1 g Bacto Tryptone
   0.5 g Bacto Yeast Extract
   1 g NaCl
   1.5 g Bacto Agar

   Combine all ingredients in a 250 ml Erlenmeyer flask. Include a stir bar because the agar solidifies. Dilute to 100 ml with deionized water. Autoclave. Cool solution to room temperature on a stir plate with constant stirring. Pour solution into petri plates so that the bottom of the plate is covered over with solution. The plates are sealed with parafilm and stored upside down in a refrigerator. Storing upside down will prevent any accumulated condensation from falling down onto the plates.

3. LB Agar Ampicillin Plates (100 ml)

    1 g Bacto Tryptone
    0.5 g Bacto Yeast Extract
    1 g NaCl
    1.5 g Bacto Agar
    1.5 g Ampicillin

    Combine all ingredients in a 250 ml Erlenmeyer flask. Include a stir bar because the agar solidifies. Dilute to 100 ml with deionized water. Autoclave. Cool solution to room temperature on a stir plate with constant stirring. Pour solution into petri plates so that the bottom of the plate is covered over with solution. The plates are sealed with parafilm and stored upside down in a refrigerator. Storing upside down will prevent any accumulated condensation from falling down onto the plates.

4. Overnight Bacterial Culture

    a. Cells from -80°C Mv1304 E.coli stock were streaked onto LB-agar plate; dilute to single colonies by quadrant streaking. Grow overnight at 37°C until colonies appear.

    b. Pick single colony and add to 3 mL of LB broth in a culture tube and incubate with shaking at 37°C overnight. This is the "overnight" culture.

B. Protocol

1. Inoculate 100 ml of LB broth in a 250 ml flask with 1 ml of an overnight bacterial culture. Grow cells with vigorous shaking at 37°C for 2 hours. The solution should be cloudy.

2. Chill $CaCl_2$ solution. Centrifuge the cell suspension at 3000 g for 4-5 minutes at 4°C.

3. Discard the supernatant. Re-suspend the cells in 50 ml of chilled 50 mM $CaCl_2$ solution.

4. Place the cell suspension in ice for 15 minutes and centrifuge the suspension at 3000 g for 5 minutes at 4°C.

5. Discard the supernatant. Re-suspend the cells in 1/15 of the original volume of an ice cold, sterile solution of $CaCl_2$. Dispense 0.2 ml aliquots into 3 pre-chilled tubes.

6. Add DNA in a plasmid solution. Mix and store on ice for 30 minutes.

7. Heat shock in a 42°C water bath for 2 minutes.

8. Add 1 ml of LB broth to each tube and incubate for 60 minutes without shaking. This period allows bacteria to recover and begin to express antibiotic resistance.

9. Spread 100 µl of cells onto an LB agar ampicillin plate.

10. Leave plates at room temperature until liquid has absorbed.

11. Invert the plates and incubate at 37°C. Colonies should appear in 12-16 hours.

II. ISOLATION OF PLASMID

A. Materials

- 20 ml LB ampicillin
- Overnight culture
- Qiagen QIA-prep Spin Miniprep Kit

1. LB broth-ampicillin (20ml)

   20 ml LB broth
   40 µl Ampicillin (25 mg/ml stock)

   Combine ingredients in a sterile tube.

2. Overnight Culture

   Choose a colony from your cell culture using a sterile wooden applicator. Add to 5 ml of LB broth-ampicillin, swirling. Let grow overnight at 37°C.

B. Protocol

1. Harvest cells from 5 ml of overnight solution by centrifuging at maximum speed for 30 seconds.
2. Resuspend pelleted bacterial cells in 250 µl Buffer P1with pipetman and transfer to a microcentrifuge tube.
3. Add 250 µl Buffer P2 and gently invert the tube 4-6 times to mix.
4. Add 350 µl Buffer N3 and invert the tube immediately but gently 4-6 times.
5. Centrifuge for 10 minutes and maximum speed in a microcentrifuge. A white flocculent pellet will form.
6. Apply the supernatants from step 5 to the QIAprep column by pipetting.
7. Wash the QIAprep spin column by adding 0.5 ml Buffer PB and centrifuging for 30-60 seconds. Discard the flowthrough.
8. Wash the QIAprep spin column by adding 0.75 ml Buffer PE and centrifuging for 30-60 seconds. This washed all except the DNA out of the column.

9. Discard the flow-through, and centrifuge for an additional 1 minute to remove residual was buffer.

10. Place the QlA-prep column in a clean 1.5 ml microcentrifuge tube. To elute DNA, add 50 µl Buffer EB to the center of each QlAprep column, let stand for 1 minute, and centrifuge for 1 minute.

III. DNA SEQUENCING

A. Materials

- Experimental plasmids
- Control plasmid
- Big Dye Sequencing Kit
- Oligonucleotide primer (3pmol/µl)
- Sterile deionized water
- Thermocylcer
- Applied Biosystems Centri-Sep™ Spin Columns
- 3 M sodium acetate (NaAc), pH 4.6
- 95% non-denatured ethanol (EtOH)
- 70% EtOH
- 100% EtOH
- 125mM EDTA
- Applied Biosystems ABI Prism Automated DNA Sequencer

B. Sequencing Reaction Protocol

The following steps are performed for each experimental plasmid and one control plasmid.

1. Mix the following ingredients in a PCR tube:

Plasmid (100 – 400 ng)	1-5 µl
Big Dye Mix	4.0 µl
Primer (3 pmol/µl)	1.0 µl
Sterile Water	3.5 µl
Total Volume	10.0 µl

2. Centrifuge briefly (a few seconds) just to bring contents together at the bottom of the tube. Place tubes in thermocycler. Thermocycling conditions are outlined in Table 1 on page 9.

C. DNA Cleanup Protocol (3 methods attempted)

1. Spin Column Purification with Centri-Sep™ Columns (3 Hours)

   a. Gently tap the column to cause the gel material to settle to the bottom of the column.

   b. Remove the upper end cap and add 0.8 ml of deionized water.

   c. Replace the upper end cap and vortex or invert the column a few times to mix the water and gel material.

   d. Allow the gel to hydrate at room temperature for at least 2 hours.

      Note: Hydrated columns can be stored for a few days at 2-6°C. Allow stored columns to warm to room temperature before use. Longer storage is not recommended.

   e. Remove any air bubbles by inverting or tapping the column and allowing the gel to settle.

   f. Remove the upper end cap first, and then remove the bottom cap. Allow the column to drain completely.

      Note: If the column does not begin immediately, apply gentle pressure to the column with a pipette bulb.

   g. Insert the column into the wash tube provided.

   h. Spin the column in a microcentrifuge at 730 x g for 2 minutes to remove the interstitial fluid. Note the alignment of the column in the centrifuge. [$g = 11.18 \times r \times (rpm/1000)^2$ where g = relative centrifugal force, r = radius of the rotor in cm, and rpm = revolutions per minute. In our case, 730 x g = 500 RCF.]

      Note: Spin the column for no more than 2 minutes. Do not allow column to dry out.

   i. Remove the column from the wash tube and insert it into a sample collection tube.

   j. Remove the extension reaction mixture from its tube and load it carefully onto the center of the gel material.

k. Spin the column again at 730 x g for 2 minutes. Ensure that the column is aligned as noted in step 8. This is important because the surface of the gel will be at an angle in the column after the first spin.

l. Discard the column. The plasmid to be sequenced is now in the sample collection tube.

m. Dry the sample in a vacuum centrifuge for 10-15 minutes, or until dry. Do not over-dry.

2. Ethanol/Sodium Acetate Precipitation In Microcentrifuge Tubes

Note: Use 95% ethanol rather than absolute (100%) ethanol.

a. Prepare the ethanol/sodium acetate solution by combining the following for each sample.
   - 3.0 µl of 3 M sodium acetate (NaAc), pH 4.6
   - 62.5 µl of non-denatured 95% ethanol (EtOH)
   - 14.5 µl of deionized water

   Note: The final volume should be 80 µl for each sample.

b. Add 80 µl of this EtOH/NaOAc solution to 20 µl of the reaction mixture.

c. Close tubes and vortex briefly.

d. Leave the tubes at room temperature for 15 minutes to precipitate the extension products.

e. Place the tubes in a microcentrifuge and mark their orientations. Spin the tubes for 20 minutes at maximum speed. IMPORTANT: Proceed to the next step immediately. If this is not possible, then spin the tubes for 2 minutes more immediately before performing the next step.

f. Carefully aspirate the supernatants with a separate pipette tip for each sample and discard. Pellets may or may not be visible. IMPORTANT: The supernatants must be removed completely, as unincorporated dye terminators are dissolved in them. The more residual supernatant left in the tubes, the more unincorporated dye terminators will remain in the samples.

g. Add 250 µl of 70% EtOH to the tubes and mix briefly.

h. Place the tubes in the microcentrifuge in the same orientation as step 5 and spin for 5 minutes at maximum speed.

i. Aspirate the supernatants carefully, as in step 6.

j. Dry the samples in a vacuum centrifuge for 10-15 minutes or to dryness. Do not over-dry.

3. Ethanol/EDTA Precipitation

   Note: This procedure uses 100% rather than 95% ethanol.

   a. Add 5 µl of 125 mM EDTA to reaction mixture.

   b. Add 60 µl of 100% ethanol to each tube.

   c. Finger vortex and incubate at room temperature for 15 minutes.

   d. Carefully aspirate off the supernatant.

   e. Add 60 µl of 70% ethanol.

   f. Spin samples in a microcentrifuge at maximum speed at 4°C for 20 minutes.

   g. Aspirate off the supernatant.

   h. Air-dry sample for 20 minutes.

D. Preparing and Loading Samples for Capillary Electrophoresis

1. Add 25 µL of TSR to to each sample pellet.

2. Mix thoroughly on a vortex mixer and heat for 2 minutes at 95 °C.

3. Chill on ice, vortex thoroughly, and then spin briefly in a microcentrifuge.

4. Hold on ice until ready to load on the instrument.

5. Transfer the samples to 0.5 mL or 0.2 mL sample tubes and cover with tube septa.

IV. PROTEIN EXPRESSION AND CELL LYSIS

A. Materials

- 2 x 900 ml Terrific Broth
- 1000 ml Phosphate Buffer
- Lysozyme
- Lysis Buffer
- Storage Buffer
- Dithionite
- Carbon Monoxide Gas
- BioSpec 1601 Spectrophotometer

1. Terrific Broth (2 x 900 ml batches) each containing:

   12 g Bacto Tryptone
   24 g Bacto Yeast Extract
   4 ml of 100% Glycerol

   Combine the three ingredients into two 3 L Fernbach Flasks. Dilute to 900 ml with deionized water. Dissolve completely. Autoclave.

2. Phosphate Buffer (1000 ml)

   23 g KH_2PO_4 (monobasic-not hydrated)
   164 g $K_2HPO_4 \cdot 3H_2O$ (dibasic-trihydrated)

   Combine both ingredients in a 2 L Erlenmeyer flask. Dilute to 1000 ml with deionized water. Autoclave.

   Note: Autoclave the two Fernbachs and the buffer separately.

3. Lysozyme (20 mg/ml)

   20 mg Lysozyme
   1 ml sterile deionized water

   Add water to lysozyme and vortex to dissolve.

4. Lysis Buffer (200ml)

   100 ml Tris-Cl (50mM stock; pH 8.0)
   1 ml EDTA (200 mM stock)
   1.17 g NaCl (0.1 M)

   Combine all ingredients in a 250 ml flask. Dilute to 200 ml with deionized water. Adjust pH to 8.0.

B. Protocol for Cells Harvesting

1. Grow minicultures in 2 separate tubes (one for each plasmid), each containing the following:

   - 10 ml LB broth
   - 20 µl Ampicillin (25 mg/ml stock)

   Incubate on orbital shaker at 37°C for 4 hours.

2. At inoculation, add the following to the 3 L Fernback flasks containing 900 ml TB:

   - 100 ml phosphate buffer (final 17 mM monobasic, 72 mM dibasic)
   - 2 ml Ampicillin (25 mg/ml stock)
   - 2 ml ALA (0.25 M stock – 2.14 g/50ml; final 0.5 mM)
   - 5 ml cells

   Incubate with shaking at 27°C, 130 rpm, overnight.

3. Next morning, induce with:

   - 4 ml IPTG (stock 0.125 M; final 0.5 mM)
   - 2 ml ALA (0.25 M stock – 2.14 g/50ml; final 0.5 mM)
   - 5 ml Ampicillin (25 mg/ml stock)

   Incubate with shaking for 2 days at 24°C; 54 rpm

4. Harvest cells: Transfer cultures from the Fernbach flasks to bottles for GSA centrifuge rotors. Make sure bottles are weighted similar for centrifugation.

5. Spin at 5000 rpm for 10 minutes at 4°C.

6. Discard supernatant.

7. Wash cells by resuspending in 10 mM Tris HCl buffer (pH 8.0).

8. Spin as above, discard supernatant.

9. Store pelleted cells at -20°C.

C. Cell Lysis Protocol

1. Weigh out 1 g of bacterial cells in a test tube or 15 ml Falcon tube.

2. Add 3 ml lysis buffer to resuspend the pellet.

3. Add 40-µl lysozyme solution (20 mg/ml stock solution).

4. Incubate at room temperature stirring occasionally.

5. Add 20 µl DNAase solution (3 mg/ml stock solution)/gram of E.coli.

6. Incubate the mixture at 37°C for 30 minutes.

7. Break up cells with a Dounce Homogenizer on ice for 20 passes. Keep on ice and do not create bubbles; this will denature the protein.

8. Add 10% Tergitol NP-10 detergent to a final concentration of 0.5%.

9. Centrifuge at 12,000 rpm for 10 minutes at 4°C. Save the supernatant, which can be stored at -20°C.

D. Spectrophotometric Analysis Protocol

1. Dilute 1.5 ml of bacterial cell lysate (supernatant from last step of series above). With an equal volume of Glycerol-containing Storage buffer (50 mM KPO4; pH 7.4, 1 mM EDTA, 20% glycerol). Save the remainder of the preparation for SDS-PAGE.

2. Split your sample into a Reference (rear of spec) and Sample (front of spec) cuvette, place the cuvettes into the split beam spectrophotometer (Shimadzu), and run a "Baseline Correction". This will zero the absorbance from 400 to 500 nm.

3. Remove the cuvettes, and transfer the contents of the cuvettes back to the test tube used to make the dilution.

4. Add a small amount of dithionite to the sample, once again transfer the contents to the Reference and Sample cuvettes, and bubble carbpn monoxide (CO) into the Sample cuvette.

5. Place the cuvettes into the appropriate positions in the spectrophotometer, and begin scanning from 400 nm to 500 nm. Look for a prominent peak at 420 nm.

V. SITE DIRECTED MUTAGENESIS

A. Materials

- Experimental Plasmids
- 2 Overlapping Oligonucleotides (T302AR and T302AF)
- Invitrogen GeneTailor Site Directed Mutagenesis System

B. Methylation Protocol

1. In a microcentrifuge tube combine the following reagents:

   1 µl plasmid DNA
   1.6 µl Methylation Buffer
   1.6 µl 10x SAM (diluted from a 200x SAM stock).
   1.0 µl DAN Methylase (4 U/ul).
   Dilute to 16 µl Sterile, distilled water.

2. Incubate at 37°C for 1 hour.

C. Mutagenesis Protocol

1. pGSTΔ2B4 plasmid: In a tube combine the following reagents:

   5 µl High Fidelity PCR Super Mix
   1.5 µl 10 mM dNTP
   1.5 µl 10 mM T302AR Primer
   1.5 µl 10 mM T302AF Primer
   5 µl Methylated DNA
   35.5 ml Autoclaved, distilled water
   TOTAL VOLUME: 50 µl

2. Control Plasmid: In a tube combine the following reagents:

   5 µl High Fidelity PCR Super Mix
   1.5 µl 10 mM dNTP
   1.5 µl Control Primers (10mM each)
   5 µl Methylated DNA
   37 µl Autoclaved, distilled water
   TOTAL VOLUME: 50 µl

3. Thermocycling conditions are outlined in Table 2 on page 12.

4. Prepare a 1.5% agarose gel:

   1.5 g Agarose
   2.0 ml 50X TAE
   Dilute to 100 ml with deionized water
   Microwave the solution until boiling
   Cool to about 50°C-while stirring
   Add 5 µl Ethidium Bromide

5. After the reaction, analyze 10-20 µl of the product on a 1.5% agarose gel.

D. Transformation Protocol

For a Single Reaction:

1. Thaw on ice one 50-µl vial of DH5_-T1 cells for each transformation. Thaw for approximately 5-7 minutes (no more than 20 minutes).

2. Pipet 2 µl from each mutagenesis reaction mixture directly into each vial of cells and mix by tapping gently. Do not mix by pipetting up and down. Store the remaining mutagenesis reaction at –20°C.

3. Cap the vials, cover completely with ice, and incubate for 7-10 minutes.

4. Transfer the vials to a test tube rack and incubate entire rack at once for exactly 30 seconds in the 42°C water bath. Do not mix or shake.

5. Remove rack of vials from the 42°C bath and cover with ice for 1 minute.

6. Remove from ice, de-cap, and add 200 µl of pre-warmed SOC medium to each vial.

7. Recap vials and place sideways in a microcentrifuge rack. Secure the vials with tape and shake at 37°C for exactly 1 hour at 225 rpm in a shaking incubator.

8. While the vials are shaking, label the LB agar plates and warm them for about 30 minutes in a 37°C incubator.

9. Aliquot 125 µl from each transformation vial onto a labeled plate. Do not allow cells to settle before removing an aliquot. If cells settle, mix by tapping the tube.

10. Gently spread the transformation reaction on the plate. Store the remaining transformation reaction at +4°C.

11. Invert the plates and incubate at 37°C for 16-20 hours.

12. Select colonies and analyze the plasmid isolation, PCR, for sequencing.