Amplification of the Phenol Hydroxylase Promoter Region of Pseudomonas Putida CF600 as a Precursor for Transcriptional Fusion

Priya Bhat

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Amplification of the phenol hydroxylase promoter region of Pseudomonas putida CF600 as a precursor for transcriptional fusion

Submitted by

Priya Bhat

The University of Alabama in Huntsville
Department of Biological Sciences

Project Advisor: Dr. Joseph Leahy

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Honors Senior Project
Approval

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Name of candidate: Priya Bhat

Department: Biological Sciences

Degree: Bachelor of Science

Full title of project: Amplification of the promoter region of Pseudomonas putida CF600 as a precursor for transcriptional fusion

Approved by:

[Signature]
Project Advisor
Date: 5/5/99

[Signature]
Department Chair
Date: 5/7/99

Honors Program Director for Honors Council
Date
Amplification of the phenol hydroxylase promoter region of *Pseudomonas putida* CF600 as a precursor for transcriptional fusion

Abstract

Benzene, toluene, ethylbenzene and xylene are monoaromatic hydrocarbons that comprise the BTEX group of chemical compounds. Some bacteria are capable of degrading these compounds as well as phenol by the action of their monooxygenase or hydroxylase enzymes. The bacterial genes coding for these enzymes have different promoter regions; these differences may play a role in transcription rates of the genes as well as enzyme kinetics. In the present project, the promoter region of the hydroxylase (*dmp*) gene of *Pseudomonas putida* CF600, a phenol degrader, was isolated and identified. Primers were selected and designed in order to amplify the quantity of DNA using polymerase chain reaction (PCR). Gene sequences were verified by agarose gel electrophoresis. The region of interest was cloned into a plasmid, which was then used to transform competent *E. coli* cells. The cells were then lysed, and their DNA extracted and screened using restriction digests. Difficulties with the PCR procedure led to the repeated failure of the subsequent ligation, transformation and mini lysis procedures. PCR was repeated frequently at different annealing temperatures with a variety of primers to obtain a singular product. Ultimately, the PCR procedure itself most likely failed because of inappropriate binding of primers, too many similar sequences within the promoter region, and difficulty arriving at the optimal annealing temperature. Once the ideal primers are selected and PCR and subsequent procedures are performed successfully, future research involving the mating of the transformed *E. coli* cells with the native *Pseudomonas putida* CF600 strain as well as a β-galactosidase assay to study enzyme activity will be performed.

Introduction

The harmful effects of phenol on humans have long overshadowed its use as a source of nourishment for microbes. The use of phenol as an antiseptic for treating wounds and wound dressings was instrumental in establishing the importance of sterile technique in medicine and saved many lives. However, its contemporaneous use as an orally administered suicide agent spoke strongly of its toxicity. While use of phenol as an antiseptic agent diminished by the turn of the century, it continued to be used as a general disinfectant, and new uses for phenol and its derivatives were rapidly found. Presently, about 1700 million
kilograms of phenol are synthesized per annum in the United States alone. Furthermore, massive quantities of phenol and its derivatives are produced as industrial waste. Because this toxic compound is produced in such large quantities, its degradation is of the utmost interest. Both physico-chemical and biological agents contribute to phenol degradation, limiting its persistence in the environment. However, like any metabolic process, biodegradation is affected by numerous factors including the presence of alternative carbon sources or substituents on the phenol ring. Although these limitations can be overcome by genetic manipulation of the pathway of interest, the mechanisms and their regulation must first be understood.¹

Pseudomonas putida CF600 has the ability to utilize phenol, cresols (monomethyl phenols) or 3,4-dimethylphenol as its sole source of carbon and energy because of its pVI150 plasmid-encoded dmp system. This system is comprised of the dmpR gene and the dmp operon, which are closely linked but divergently transcribed. The dmp operon encodes all of the enzymes required for catabolism of the substrates to central metabolites.² The sequences of all of the fifteen structural genes required to encode the nine enzymes of the catabolic pathway have been determined and the corresponding proteins have been extracted and purified. The first step of this pathway, which is the conversion of phenol to catechol, is catalyzed by a multicomponent phenol hydroxylase.¹
Location of the dmpR gene. The upper part of the figure is the restriction map of pV1150 DNA derived from *Pseudomonas* sp. Strain CF600. The lower part of the figure represents the restriction map of an overlapping EcoRI fragment cloned from PB2701 (pV1150).

The transcription of the dmp operon is regulated in a positive manner by the dmpR gene product and results in the expression of these specialized catabolic enzymes only when the appropriate substrates are present. DmpR belongs to the NtrC family of bacterial transcriptional activators, which includes NtrC, NifA, XylR, DctD, FhIA, FlbD, HoxA, HupR, HydG, HrpS, AcoR, PilR, and TyrR. Members of this family regulate genes that are involved in a variety of physiological processes and do so in response to an assortment of environmental cues. The transcriptional activators bind to enhancer-like elements and regulate transcription from a distinct set of promoters recognized by σ^54_ holoenzyme RNA polymerase. The Pu promoter of the upper TOL operon and the Po promoter of the dmp operon are two examples of σ^54^-dependent promoters.

Members of the NtrC family of regulators are made up of distinct functional domains, as is the case with eukaryotic enhancer-binding proteins. The central C domain is the most conserved one within the family. Likely roles for this domain include interaction with σ^54_ RNA polymerase and the binding and
hydrolysis of ATP to form open transcriptional complexes. The C and carboxy-terminal D domains are separated by a region of variable length. The D-domain exhibits a helix-turn-helix DNA-binding motif that is found in numerous transcriptional activators and repressors. The B domain (Q linker), which is a short hydrophobic region rich in Gln (Q) residues, functions as an interdomain linker between the central and amino-terminal regulatory A domains.²

Many questions surround the mechanisms by which the activity of constitutively expressed transcriptional factors is controlled. Several members of the NtrC family are part of two-component regulatory systems made up of a sensory protein that activates the expressed response protein by transfer of a phosphate group to the regulatory A domain. However, like the pWWO-encoded XylIR transcriptional regulator of toluene and xylene metabolism, DmpR seems to be activated by aromatic effector molecules without the involvement of additional sensor proteins. The A domain of DmpR has a 64% homology with the corresponding domain of XylIR but has no homology with any other members of the NtrC family or other proteins. That the homologous region is involved in the activation of DmpR and XylIR by aromatic compounds is suggested by the unique homology of DmpR and XylIR in this region in conjunction with the signal receptor function of the A domains in other members of the family.² Both XylIR and DmpR can cross-activate each other’s promoter in spite of the different organizations of the Pu and Po regions; this data supports the notion that genes for σ⁵⁴-dependent activators may have evolved along with discrete binding sequences in
the guise of regulatory modules that ultimately control the transcription of unrelated pathways.³

The long-term implications of the present research are three-fold. First, research on the types of promoter regions of various genes and their effects on enzyme kinetics will provide insight into the mechanisms of gene regulation. Secondly, knowledge thus gained about the degradative capabilities of microorganisms could be used to remedy environmental problems. Finally, in patients with cystic fibrosis, *Pseudomonas aeruginosa* infects the lungs and causes overproduction of polysaccharides, particularly alginate which is the gene product of the alginate gene. The alginate gene has a $\sigma^{54}$ promoter. Such infections frequently result in the death of patients with cystic fibrosis; perhaps research on the mechanisms of action of genes with $\sigma^{54}$-dependent promoters could provide physicians with a means to save numerous lives that are lost too soon.

**Methods**

1) The desired DNA region was identified in internet data banks and a primer pair list and a restriction enzyme map were generated using the MacVector® program. A pair of primers were selected and ordered from Research Genetics (Forward primer = 5'-GAGATCGAGGAAAGCCTGATGC-3'; Reverse primer = 5'-CGAGTGAGCTGATGAAAGTCG-3'). The amount of each primer to be used in PCR was calculated (Forward primer = 2.9 µL; Reverse primer = 2.9 µL). PCR was performed using Deep Vent Polymerase enzyme. Gel
electrophoresis revealed no product, so PCR was repeated with fresh enzyme. In order to obtain fewer PCR products, the procedure was repeated at higher annealing temperatures (T_m=53°C, 55°C, 57°C). Because PCR at 55°C was the most successful, PCR was repeated at that temperature to yield enough product for Gene Clean. However, when the DNA was run out on the large gel, the presence of too many bands made Gene Clean impossible, and this line of experimentation was abandoned.

II) New primer pairs were selected and ordered from Research Genetics (Forward primer 1 [F1] = 5'-ATCGAGGAAAGCCTGATGCG-3'; Forward primer 2 [F2] = 5'-GAAGAAAAATCGGCATCGAAGG-3'; Reverse primer 1 [R1] = 5'-AAAGTCGGTGTGGGGGTATTG-3'; Reverse primer 2 [R2] = 5'-GGATGTAACGAGCTGAGCTGAG-3'; Reverse primer 3 [R3] = 5'-AAAGTCGGTGAGCTGAGCTGTC-3'). The amounts of each primer to be used in PCR were calculated to be F1/R1 = 1.7 μL/1.5 μL; F2/R2 = 1.8 μL/1.6 μL; F2/R3 = 1.8 μL/2.8 μL; F1/R3 = 1.7 μL/2.8 μL. PCR was performed using the mentioned combinations of primers, and gel electrophoresis was used to stain the results. The QIAquick PCR Clean up Kit was used to clean up the PCR product obtained using the F1/R3 combination of primers. A restriction digest of pKRZ1 (the vector plasmid) was set up as follows:

40 μL DNA  
45 μL H2O  
10 μL Buffer B  
5 μL of BamHI restriction enzyme
Another pKRZ1 digest was set up as follows:

8 μL pKRZ1
9 μL H2O
2 μL Klenow enzyme buffer
1 μL BamHI restriction enzyme

The digests were run overnight at 37°C. The Klenow enzyme reaction was performed. To the reaction tube with the Klenow enzyme buffer, the following ingredients were added:

1 μL of 1 mM dATP
1 μL of 1 mM dGTP
1 μL of 1 mM dCTP
1 μL of 1 mM dTTP
1 μL of 1 mM Klenow enzyme

The tube was allowed to stand at room temperature for thirty minutes and incubated in a 70°C water bath for 10 minutes. A ligation reaction was set up as follows:

2 μL H2O
6 μL Buffer
1 μL T4 DNA ligase
24 μL DNA (21 μL PCR product + 3 μL Klenow pKRZ1)

The reaction was incubated at 16°C for 8 hours. The mixture was then used to transform competent E. coli SM10 λ pir cells, which were streaked onto Amp100 plates and incubated at 37°C for approximately 44 hours. All colonies obtained were subcultured using the short streak technique. The cells were screened for transformed plasmids using the mini lysis procedure. This line of experimentation was unsuccessful.
III) Fresh primers were ordered from Research Genetics (F1/R3 = 1.4 µL/2.2 µL) and PCR was repeated. A restriction digest of pKRZ1 was set up and allowed to run overnight at room temperature.

- 40 µL DNA
- 10 µL Buffer A
- 45 µL H₂O
- 5 µL SmaI enzyme

The PCR product was cleaned and pooled, and Gene Clean was performed unsuccessfully. PCR was repeated at an annealing temperature of 55°C and cleaned using the QIAquick procedure. The DNA was quantitated, Gene Clean was performed, and DNA was quantitated again. Reaction tubes were set up as follows for the ligation procedure:

<table>
<thead>
<tr>
<th>Tube</th>
<th>µL H₂O</th>
<th>µL Buffer</th>
<th>µL Vector</th>
<th>µL Insert</th>
<th>µL Ligase</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>VECTOR = Klenow / pKRZ1</td>
<td>22</td>
<td>3</td>
<td>1.5</td>
<td>1.5</td>
</tr>
<tr>
<td>2</td>
<td>VECTOR = Klenow / pKRZ1</td>
<td>19</td>
<td>3</td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td>3</td>
<td>VECTOR = Klenow / pKRZ1</td>
<td>16</td>
<td>3</td>
<td>4.5</td>
<td>4.5</td>
</tr>
<tr>
<td>4</td>
<td>VECTOR = 1:100 Smal / pKRZ1</td>
<td>13</td>
<td>3</td>
<td>6</td>
<td>6</td>
</tr>
<tr>
<td>5</td>
<td>VECTOR = 1:100 Smal / pKRZ1</td>
<td>22</td>
<td>3</td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>6</td>
<td>VECTOR = 1:100 Smal / pKRZ1</td>
<td>19</td>
<td>3</td>
<td>2</td>
<td>4</td>
</tr>
<tr>
<td>7</td>
<td>VECTOR = 1:100 Smal / pKRZ1</td>
<td>16</td>
<td>3</td>
<td>3</td>
<td>6</td>
</tr>
<tr>
<td>8</td>
<td>VECTOR = 1:100 Smal / pKRZ1</td>
<td>13</td>
<td>3</td>
<td>4</td>
<td>8</td>
</tr>
<tr>
<td>9</td>
<td>VECTOR = 1:100 Smal / pKRZ1</td>
<td>22</td>
<td>3</td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>10</td>
<td>Vector = 1:100 Smal / pKRZ1</td>
<td>19</td>
<td>3</td>
<td>2</td>
<td>4</td>
</tr>
<tr>
<td>11</td>
<td>Vector = 1:100 Smal / pKRZ1</td>
<td>16</td>
<td>3</td>
<td>3</td>
<td>6</td>
</tr>
<tr>
<td>12</td>
<td>Vector = 1:100 Smal / pKRZ1</td>
<td>13</td>
<td>3</td>
<td>4</td>
<td>8</td>
</tr>
</tbody>
</table>

The cells were cultured on Amp100 (ampicillin) and KM75 (kanamycin) plates that were incubated overnight at 37°C.
IV) An overnight restriction digest using pBluescript (pBS) as the vector was set up and run at room temperature:

- 40 µL pBluescript
- 45 µL H₂O
- 10 µL Buffer J
- 5 µL Smal enzyme

**Ligation reaction mixes were set up as follows:**

<table>
<thead>
<tr>
<th>Tube</th>
<th>µL Vector</th>
<th>µL insert</th>
<th>µL buffer</th>
<th>µL ligase</th>
<th>µL H₂O</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1</td>
<td>2</td>
<td>3</td>
<td>2</td>
<td>22</td>
</tr>
<tr>
<td>2</td>
<td>2</td>
<td>2</td>
<td>3</td>
<td>2</td>
<td>19</td>
</tr>
</tbody>
</table>

A 1:10 dilution of pBS was used. The ligation reactions were run overnight at 17°C. 10 Amp100 plates were each treated with 40 µL of 20 mg/ml of X-gal and 4 µL of IPTG. The mixtures from the ligation tubes were used to transform competent *E. coli* DH5α which were streaked onto the X-gal plates. White colonies were picked and subcultured using short streak technique onto Amp100 plates. All plates were incubated overnight at 37°C. Mini lysis was performed repeatedly to screen for transformed cells. Restriction double digests were set up as follows:

- 1 µL BamHI
- 1 µL EcoRI
- 1 µL DNA
- 6 µL H₂O
- 1 µL Buffer

The reaction was run overnight at 37°C. Mini lysis products were digested with PvuII enzyme to screen for orientation of the PCR product in transformants. The following digest was allowed to run overnight at 37°C:

- 4 µL H₂O
- 4 µL DNA
Because PvuII results were unsatisfactory, this line of experimentation was discontinued.

V) Maxi preps were performed to obtain fresh pKRZ1. QI Amp procedure was used to obtain fresh CF600 template. Fresh primers were received from Research Genetics (Forward primer = 5'-ATGGAGGAAAGCCTGATGCG-3'; Reverse primer = 5'-AAAGTCTGGTGTGGGGGTATTGGTC-3'). 7.6 μL of forward primer and 3.2 μL of reverse primer were used to perform PCR at annealing temperatures of 57°C, 56°C and 59°C. However, since too many products were obtained, this line of experimentation was halted.

Note: Please refer to Appendix for detailed descriptions of laboratory procedures.

Results

I) 

Small 1.5% agarose gel. PCR products obtained at annealing temperatures of 53°C and 55°C. The expected fragment is 469 base pairs long.
The desired PCR product is 489 base pairs long. Evidently, there are numerous bands in the products obtained at both annealing temperatures. Because there are fewer bands in the product obtained at 55°C, the DNA used for subsequent Gene Clean procedure is obtained at 55°C.

![Large 1.5% agarose gel. PCR product at 55°C.](image)

Evidently, there are too many bands near the 500 base pair mark to efficiently cut a specific band out of the gel to isolate the 489 base pair long PCR product.

II)
Small 1.5% agarose gel. PCR performed with new primer pairs.

No product was obtained using primers F1/R1 and F2/R3. There are numerous bands in the product obtained using F2/R2. Consequently, the F1/R3 combination, which produced the fewest bands, was used for subsequent PCR reactions during this phase of experimentation.

![Image of gel](image)

Small 1% agarose gel. Results of mini lysis. Expected fragments should have been located at 10.3 kb and 3.9 kb (these belong to the lysed pKRZ1) and at 572 bp (the insert).

Mini lysis itself was performed correctly because there are bands at 10.3 kb and 3.9 kb; these bands belong to the lysed vector. However, if transformation had been performed successfully, a band at 572 bp denoting the presence of the insert would have been present.

III) DNA Quantitation results following 1st Gene Clean procedure:

<table>
<thead>
<tr>
<th>Absorbance (260 nm)</th>
<th>0.011 AU</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ratio</td>
<td>2.321</td>
</tr>
</tbody>
</table>
dsDNA concentration | 14.0 μg/ml

Large 1.5% agarose gel. 2nd attempt at Gene Clean. The band near the 500 bp mark is the desired PCR product.

Small 1.5% agarose gel. The desired PCR bands obtained by Gene Clean. Bands 1 and 3 were first extractions while bands 2 and 4 were second extractions (discarded).

DNA Quantitation results following 2nd Gene Clean procedure:

<table>
<thead>
<tr>
<th></th>
<th>Band 1</th>
<th>Band 3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Absorbance (AU)</td>
<td>0.012</td>
<td>0.010</td>
</tr>
<tr>
<td>Ratio</td>
<td>2.381</td>
<td>9.042</td>
</tr>
<tr>
<td>dsDNA (μg/ml)</td>
<td>25.6</td>
<td>19.4</td>
</tr>
</tbody>
</table>
The ligation and transformation procedures following Gene Clean were unsuccessful. No growth was obtained on either the KM75 plates or the Amp100 plates.

IV) Medium 1.5% agarose gel. The PCR product was inserted into a new vector, pBluescript, and digested with PvuII.

Transformation of *E. coli* DH5α cells and streaking onto X-gal plates yielded white colonies that contained the insert and blue colonies that did not. The DNA from the transformed bacteria was digested with PvuII to determine the orientation of the insert.

V)
Medium 1% agarose gel. Results of maxi prep and QIAmp procedures. EcoR1/pKRZ1 and HindIII/pKRZ1 samples in gel are 1:100 dilutions.

Maxi preps and QIAmp procedures were performed to obtain fresh pKRZ1 and CF600 template, respectively. The fresh products are for use in PCR and subsequent procedures.

Small 1.5% agarose gel. PCR was performed with fresh stock of primers at the high annealing temperature of 59°C.

PCR was repeated with a fresh batch of primers. The annealing temperature began at 55°C and was raised each time PCR was repeated in hopes of getting fewer products. Even at 59°C, however, several products are present because of non-specific binding of primers to the template.

Discussion

1) When primers were selected, they had to bind to the DNA around the region of interest without binding too far upstream or downstream. When PCR was first performed, no product was obtained because the Deep Vent Polymerase enzyme had gone bad. When PCR was repeated with fresh enzyme, many bands were obtained. For every primer pair, there is an optimal annealing temperature ($T_m$) at which primers best bind to the
desired sequences on the template. At too high a temperature, the primers are rendered ineffective. At too low a temperature, the primers bind to the DNA and to each other in too non-specific a manner. Although no PCR product was obtained at $T_m=57^\circ C$, and several bands were present at $T_m=55^\circ C$, the $55^\circ C$ product was the best result of all attempts. The product, when run out on an agarose gel, was muddy. But there were fewer distinct bands in the $55^\circ C$ product than in any other; therefore, it was the best candidate for Gene Clean.

When Gene Clean is performed, the DNA is separated on a large gel at a lower current. This longer process allows for optimal separation of all bands in the DNA. Upon running the large gel, it was discovered that more bands were present than initially assumed. Gene Clean is most effective when a single, relatively isolated band is cut out of the gel. The desired band was near the 500 bp mark; unfortunately, so were numerous others. As a result, Gene Clean was abandoned, and the primers used for PCR were deemed unworthy.

In order to select the most efficient primer, PCR was performed using various combinations of different forward and reverse primers. The primer pair that yielded the fewest bands was used for subsequent PCR reactions. The QIAquick PCR purification kit was effective in removing undesirable, low molecular weight substances from the selected PCR product. pKRZ1 was used as the vector for the ligation and transformation procedures because it possesses the $lac z$ operon, which is necessary
during the β-galactosidase assays performed upon successful completion of ligation, transformation and mating procedures. The circular vector was cleaved with BamHI restriction enzyme to create an opening where the PCR product could be inserted. However, digestion with BamHI resulted in “sticky” ends or uneven ends in pKRZ1. Because the PCR product was "blunt" or even ended, it could only be joined with blunt-ended DNA. Therefore, the Klenow enzyme was used to fill in the sticky ends of pKRZ1 to make them blunt ends. Theoretically, the PCR product would now be able to be inserted into the vector in the presence of ligase enzyme.

However, mini lysis revealed that ligation had failed. A likely reason is that the Klenow reaction, which had never before been performed in the present laboratory, was an inefficient one and probably did not render enough pKRZ1 physically able to accept an insert. Furthermore, although only two bands were visible upon electrophoresis of the PCR product, other nearly indistinct bands may have been present, indicating the presence of low concentrations of other competing products. Ultimately, a BamHI digest of the post-Klenow pKRZ1 revealed that the Klenow reaction had failed.

The Klenow reaction was repeated, and fresh primers were obtained in order to generate more PCR product. Fresh primers were used because primers, like the template, can degrade if stored frozen for too long. However, this time, PCR was not as efficient as the previous attempt, and Gene Clean had to be performed. The first attempt at Gene Clean failed,
most likely due to some procedural error. If DNA concentration upon quantitation is less than 25 μg/ml, Gene Clean is usually considered a failure. When Gene Clean was performed for the second time, DNA concentrations of 25.6 μg/ml and 19.4 μg/ml were obtained. Although low, these concentrations were enough to deem Gene Clean a success because initial DNA concentrations were also low (66.0 μg/ml, 56.4 μg/ml), and some loss of product upon Gene Clean is to be expected.

For the ligation procedure, some additional pKRZ1 was digested with Smal restriction enzyme, a blunt-ended cutter. Along with the Klenow/pKRZ1, the Smal/pKRZ1 was mixed with quantities of the insert in the presence of ligase. Because the 17°C water bath was not turned on for most of the ligation, the reaction failed. pKRZ1 confers bacterial resistance to kanamycin, so any data from the KM75 plates was discarded. No growth was observed on the Amp100 plates because ligation had failed. Because only a small quantity of PCR product remained for experimentation, direct insertion of PCR product into pKRZ1 was halted. Instead, the PCR product would now first be inserted into pBluescript (pBS), and then the area of pBS around the insert would be cut and that new insert would be cloned into pKRZ1 for subsequent procedures.

IV) pBS was treated with Smal restriction enzyme to obtain blunt ends. Ligation of pBS and the insert, as well as transformation, were performed, and X-gal plates were streaked with the E. coli DH5α cells. When pBS-
containing cells are plated onto X-gal plates, colonies that are white contain pBS+insert while colonies that are blue only contain pBS. This is an efficient and simple primary screening for desired cells. Mini lysis ultimately revealed the presence of inserts in the DNA of the selected white colonies. However, upon electrophoresis, the band of the insert appeared to correspond more closely to the 1 kb marker instead of the expected 0.5 kb marker. This observation was temporarily ignored.

In order to screen for the orientation of the insert in pBS, samples of successfully ligated pBS and insert were digested with Pvull restriction enzyme. The differences in band sizes obtained following the digest would reveal the orientation of the insert. However, Pvull digestion yielded products of very low molecular weight that could not easily be separated or distinctly viewed upon electrophoresis. Furthermore, the fact that the bands did not correspond with their expected positions could no longer be ignored. Too many different products of roughly the same molecular weight were most likely obtained during PCR. These products then competed with the desired product for insertion into pBS. Ultimately, the competing products, and not the PCR product of interest, were inserted into the vector. Consequently, this line of experimentation had to be abandoned.

Maxi preps and the QIAmp procedure were performed in order to obtain fresh pKRZ1 and CF600 template respectively. Fresh primers were obtained from Research Genetics. In order to get fewer PCR products,
the PCR procedure was performed at increasingly higher annealing temperatures because, theoretically, a higher annealing temperature would, to a limit, result in more specific binding of primers to the template. However, PCR performed at annealing temperatures as high as 59°C continued to yield numerous products. Analysis of the DNA sequence of and around the desired dmp region revealed the presence of several sequences that strongly resembled the complementary sequence to the primers (i.e. the binding site for the primers). Essentially, these similar sequences served to "confuse" the primers. In addition to the correct binding sites, the primers attached to these other sequences. Consequently, when PCR was performed, several products were synthesized; therefore, use of the present primers was discontinued.

Ultimately, new and more selective primers will be chosen for PCR. Once ligation and transformation are successfully completed, the transformed E. coli cells will be mated with the native Pseudomonas putida CF600 strain. A β-galactosidase assay will be performed to study transcription and enzyme kinetics of phenol hydroxylase. The data obtained from the dmp gene with its σ^{54} promoter will be compiled with data from organisms with σ^{70} promoter genes for a comparative analysis of transcription rates and enzyme kinetics.

References


4Shingler, Victoria; Bartilson, Magdalena; Moore, Terry (March 1993). Cloning and Nucleotide Sequence of the Gene Encoding the Positive Regulator (DmpR) of the Phenol Catabolic Pathway Encoded by pV1150 and Identification of DmpR as a Member of the NtrC Family of Transcriptional Activators. *Journal of Bacteriology*. 175 (6):1596-1604.

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APPENDIX

**Protocols (in alphabetical order)**

**Agarose Gel Electrophoresis**

The desired size and concentration of gel was prepared as deemed by the following information:

<table>
<thead>
<tr>
<th>Gel Type</th>
<th>Concentration</th>
<th>Agarose</th>
<th>Buffer Volume</th>
<th>Buffer Type</th>
</tr>
</thead>
<tbody>
<tr>
<td>Small gel</td>
<td>1.5%</td>
<td>0.226g</td>
<td>15 ml</td>
<td>0.5X TBE buffer</td>
</tr>
<tr>
<td></td>
<td>1%</td>
<td>0.15g</td>
<td>15 ml</td>
<td>0.5X TBE buffer</td>
</tr>
<tr>
<td>Medium gel</td>
<td>1.5%</td>
<td>0.75g</td>
<td>50 ml</td>
<td>0.5X TBE buffer</td>
</tr>
<tr>
<td></td>
<td>1%</td>
<td>0.50g</td>
<td>50 ml</td>
<td>0.5X TBE buffer</td>
</tr>
</tbody>
</table>

The agarose/TBE mixture was heated to dissolve the agarose. The solution was then poured into a casting tray of the desired size and a comb was inserted. The gel was allowed to cool at room temperature for at least 30 minutes. Once
prepared, the samples were prepared (described below) and pipetted into the wells. A marker (Hi-Lo) was also prepared and run in the gel. A current of 150 volts was applied to a small gel for 30 minutes and to a medium gel for 45 minutes. 0.5X TBE was used as the running buffer. A bath of 70 μL ethydium bromide dissolved in 500 ml dH2O was prepared. The gel was then placed in the bath for 15 minutes to stain the DNA. Finally, the gel was exposed to ultraviolet light to illuminate the DNA bands and photographed.

**Gel Samples:**
- DNA: 2μL
- Sterile H2O: 6μL
- Blue-Orange loading dye: 2μL

**DNA Quantitation Using GeneQuant II Spectrophotometer**

5 μL of DNA was added to a clean microcentrifuge tube containing 245 μL of sterile water, making a 1:50 dilution of the DNA. Another 1.5 ml microcentrifuge tube was filled with 250 μL of sterile water. The following parameters were set on the machine:
- cell path length = 10
- printer = ON
- 320 nm background comp. = YES
- dilution factor = 50
- factor = dsDNA

A cuvette was rinsed out with deionized water. 250 μL of sterile water was pipetted into the cuvette. The “Set ref” button on the machine was pushed and the cuvette was inserted into the sample compartment and removed at the tone. This zeroes the instrument. The cuvette was rinsed out again and the DNA sample was pipetted into it. The “Sample” button on the machine was pushed and the cuvette was inserted into the compartment and rapidly removed. The buttons for Absorbance, Ratio and Concentration measurements were pushed, and the printer printed out these values. The cuvette was rinsed out again, and all tubes were disposed off.

**Gene Clean**

PCR was performed to produce an adequate quantity of substrate for Gene Clean. The DNA thus obtained was pooled. A large 1.5% agarose gel was prepared and combs were taped off as needed. After the gel was cooled for 30 minutes, the pooled DNA + Blue-Orange Dye (10% of volume) was added to the large well. Hi-Lo marker was added to a well that was at least 2 lanes away from the PCR DNA. A 100 volt current was applied to the gel for 3.5 hours after which the gel was stained in an ethydium bromide bath for 15 minutes. The gel was photographed, keeping exposure to UV light to a minimum. The desired band of DNA was cut out of the gel with a razor blade. The gel slice was weighed to approximate volume (0.1 g = 100 μL). The agar was cut into small cubes with a razor blade and transferred to a reusable 12 ml sterile polypropylene tube. ¼ volume of TBE modifier and 4.5 volumes of NaI were added to the volume of agarose. The mixture was incubated at 55°C to melt the
gel. Glassmilk was vortexed prior to use, and 20 µL was added to the mixture. The mixture was allowed to sit at room temperature for 15 minutes while mixing frequently. The tube was spun in the centrifuge for 2 minutes at 10000 rpms and 4°C. The supernatant was aspirated, and the pellet was resuspended in 500 µL NEW Wash and transferred to a 1.5 ml microcentrifuge tube. The tube was spun for 5 minutes in the microcentrifuge, and the supernatant was removed. The pellet was resuspended in 500 µL of NEW Wash and spun and aspirated as before. The procedure was repeated again with 500 µL of NEW Wash. The tube was spun one last time to remove all traces of liquid, and the pellet was allowed to vacuum dry for 5 minutes. The pellet was resuspended in 40 µL of sterile, dH₂O and centrifuged for 30 seconds. The supernatant containing the DNA was carefully removed and transferred to a fresh 1.5 ml microfuge tube. The pellet was discarded, and the DNA was quantitated. A sample was used for analysis by agarose gel electrophoresis while the rest of the DNA was stored frozen.

Maxi preps

2 X 250 ml LB broth + 2 X 0.5 ml ampicillin were placed in two 500 ml Erlenmeyer flasks and incubated overnight at 37°C and 100 rpms. The solution was centrifuged at 7K rpms for 15 minutes at 4°C in large 250 ml plastic centrifuge bottles. The large centrifuge rotor (-1.5 comp) was used. The supernatant was removed. 5 ml of pre-chilled P1+RNase was added to the tube and the pellet was resuspended completely. The mixture was transferred to a small O.R. tube. 10 ml of pre-chilled P3 was added to the solution, which was mixed 6 times and incubated on ice for 20 minutes. The mixture was then centrifuged at 19K rpms for 30 minutes at 4°C. The tube was removed from the centrifuge and the white flakes on the surface were removed with a Pasteur pipette. The supernatant was immediately transferred to a new opaque O.R. tube. The mixture was centrifuged again at 19K rpms for 30 minutes at 4°C (comp= -7). When five minutes were left on the spin, a large Quiagen column was equilibrated with 10 ml of QBT. 50 ml conical tubes were used to collect the flow through and were emptied as needed into the waste beaker. All remaining white flakes on the surface were removed with a Pasteur pipette. Immediately, a 10 ml pipette was used to transfer supernatant to the Quiagen column. The column was washed with 2X30 ml QC. The eluate was collected in a new O.R. tube which was marked for the pellet. The column was eluted with 15 ml QF. The column was disposed off. 10.5 ml room temperature isopropanol was added directly to the O.R. tube and mixed six times. The centrifuge tube was marked as to where the pellet would be. The tube was centrifuged immediately at 19K rpms for 30 minutes and the liquid was aspirated. 5 ml of cold 70% ethanol was gently added and immediately re-centrifuged at 19K rpms for 30 minutes. The liquid was immediately aspirated and the pellet was allowed to vacuum dry for 10 minutes. 250 µL of sterile H₂O was added to the pellet and the mixture was pipetted up and down several times. The O.R. tube was placed in a basket into the 37°C incubator and incubated for 30 minutes with the cap off. The mixture was transferred to a clean, labeled 1.5 microcentrifuge tube and pipetted up and
down five times. A small quantity of the solution was used for quantitation purposes and another sample was analyzed by agarose gel electrophoresis. The rest of the mixture was frozen.

**Mini lysis**

A sample of the transformed *E. coli* cells was used to inoculate a large, wide-bore test tube with Kaput cap containing 2 ml LB broth + 4 μL ampicillin. The tube was incubated overnight at 37°C and 300 rpms. 1 ml of the broth was transferred to a 1.5 ml microfuge tube and microfuged for 3 minutes at room temperature. All of the supernatant was aspirated, and 100 μL GTE solution (no lysozyme) was added. The mixture was vortexed for 30 seconds. 200 μL of freshly prepared alkaline SDS (described below) was added and the solution was mixed gently 6 times. The mixture was incubated on ice for 5 minutes and then microfuged for 10 minutes at 4°C. 350 μL of the supernatant was transferred to a fresh 1.5 ml tube. 400 μL of cold phenol:chloroform (24:1 isoamyl alcohol) was added, and the mixture was vortexed for about 30 seconds. Then, it was microfuged for 5 minutes at 4°C. 2X125 μL of the top layer was transferred to a fresh 1.5 ml tube and 400 μL of isopropanol at room temperature was added to the solution, which was then gently mixed. The mixture was allowed to stand at room temperature for 10 minutes and then microfuged for 15 minutes at 4°C. All traces of liquid were immediately aspirated. 500 μL of cold 70% ethanol was gently added and the mixture was microfuged for 15 minutes at 4°C. Immediately, all traces of liquid were aspirated, and the pellet was vacuum dried for 5 minute. A stock solution of BM RNase was prepared (described below) and 40 μL was added to the tube, which was then placed in a 37°C water bath for 15 minutes. A small sample of the solution was removed for restriction digests and the rest was frozen.

**Alkaline SDS solution:**

- 2000 μL 1M NaOH
- 1000 μL 10% SDS
- 7000 μL sterile H2O

**BM RNase solution**

- 5 μL BMRNase stock solution
- 400 μL sterile H2O

**Polymerase Chain Reaction (PCR)**

The following items were placed under ultraviolet light for at least 24 hours: tube of sterile water, PCR reaction tubes and caps, PCR pipette tips (non-aerosol), microcentrifuge tube rack, P20 and P200 pipetman. A 1:5 dilution of fresh template was prepared. A table indicating amounts of all reaction mixes was prepared as follows:

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>μL</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>1st STEP</strong></td>
<td></td>
</tr>
<tr>
<td>10X Thermopol Buffer</td>
<td>2.5</td>
</tr>
<tr>
<td>dNTPs (nucleotides)</td>
<td>2.0</td>
</tr>
<tr>
<td>dH2O</td>
<td>0.5</td>
</tr>
</tbody>
</table>
The tubes were spun in the microcentrifuge after the ingredients in each step were added. After the first step was completed, a ball of Ampliwax was placed above the mixture, which was heated at 80°C for 5 minutes and 6°C for 1 minute in the thermal cycler. This melted the wax and resolidified it above the ingredients. After the final step was completed, the tube(s) were placed in the thermal cycler for Touchdown PCR. The programs (described below) were linked and run. After the programs were completed, the contents of each PCR tube were transferred to a labeled 1.5 ml microcentrifuge tube. A sample of the products of PCR were analyzed using agarose gel electrophoresis; the remaining product was stored in a frost-free freezer until further use.

<table>
<thead>
<tr>
<th>Program</th>
<th>Step</th>
<th>Temperature</th>
<th>Time</th>
</tr>
</thead>
<tbody>
<tr>
<td>Program 94:</td>
<td>Window 1</td>
<td>95°C</td>
<td>5 min</td>
</tr>
<tr>
<td></td>
<td></td>
<td>62°C</td>
<td>1 min 30 sec*</td>
</tr>
<tr>
<td></td>
<td></td>
<td>72°C</td>
<td>1 min 30 sec</td>
</tr>
<tr>
<td></td>
<td></td>
<td>25°C</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>2 cycles</td>
<td>95°C</td>
<td>1 min</td>
</tr>
<tr>
<td></td>
<td></td>
<td>61°C-59°C</td>
<td>1 min 30 sec*</td>
</tr>
<tr>
<td></td>
<td></td>
<td>72°C</td>
<td>1 min 30 sec</td>
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<tr>
<td></td>
<td></td>
<td>25°C</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>2 cycles per window</td>
<td>95°C</td>
<td>1 min</td>
</tr>
<tr>
<td></td>
<td></td>
<td>58°C-55°C</td>
<td>1 min 30 sec*</td>
</tr>
<tr>
<td></td>
<td></td>
<td>72°C</td>
<td>1 min 30 sec*</td>
</tr>
<tr>
<td></td>
<td></td>
<td>25°C</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>2 cycles per window</td>
<td>95°C</td>
<td>1 min</td>
</tr>
<tr>
<td></td>
<td></td>
<td>54°C-51°C</td>
<td>1 min 30 sec*</td>
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<tr>
<td></td>
<td></td>
<td>25°C</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>2 cycles per window</td>
<td>95°C</td>
<td>1 min</td>
</tr>
<tr>
<td></td>
<td></td>
<td>50°C</td>
<td>1 min</td>
</tr>
<tr>
<td></td>
<td></td>
<td>72°C</td>
<td>1 min 30 sec</td>
</tr>
</tbody>
</table>

*annealing temperature drops 1°C after each window.

<table>
<thead>
<tr>
<th>Primer 1 (Forward)</th>
<th>Variable</th>
</tr>
</thead>
<tbody>
<tr>
<td>Primer 2 (Reverse)</td>
<td>Variable</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>2nd STEP</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Template (1:5)</td>
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</tbody>
</table>

<table>
<thead>
<tr>
<th>3rd STEP</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>dH2O</td>
<td>Variable</td>
</tr>
<tr>
<td>10X Theremopol Buffer</td>
<td>10.0</td>
</tr>
<tr>
<td>Deep Vent Polymerase</td>
<td>1.0</td>
</tr>
<tr>
<td>Final Mix Volume</td>
<td>102.5</td>
</tr>
</tbody>
</table>
QIAmp Blood and Tissue Kit Protocol

A TNA plate was streaked with a sample of frozen *Pseudomonas putida* CF600 and incubated for 2 days at 30°C. A large test tube with Kaput cap containing 2000 µL LB broth was inoculated with a sample of CF600 and incubated for 18 hours at 30°C and 100 rpms. 1 ml of the bacterial culture was placed in a 1.5 ml microcentrifuge tube and centrifuged for 5 min at 7500 rpms (5000 X g). The supernatant was aspirated, and the volume of the pellet was estimated. Buffer ATL was added to produce a total volume of 180 µL. 20 µL of refrigerated Proteinase K stock solution was added to the solution, which was vortexed and incubated at 55°C for approximately 3 hours. 200 µL of Buffer AL was added to the sample, which was mixed and incubated at 70°C for 10 minutes. 210 µL of cold, 100% ethanol was added to the sample, which was vortexed. A QIAmp spin column was placed in a 2 ml collection tube. The mixture from the previous step was applied to the column and centrifuged at 6000 X g (8000 rpms) for 1 minute. The spin column was placed in a fresh collection tube. 500 µL of Buffer AW was added and the mixture was centrifuged for 1 minute at 6000 X g (8000 rpms). The spin column was placed in a fresh 2 ml collection tube. Another 500 µL of Buffer AW was added to the mixture, which was centrifuged at full speed for 3 minutes. The spin column was placed in a fresh 2 ml microcentrifuge tube. 200 µL of Buffer AE (preheated to 70°C) was added to the spin column and the mixture was allowed to stand for 1 minute at room temperature. The mixture was then centrifuged at 6000 X g (8000 rpms). 200 µL more of Buffer AE was added and the subsequent steps were repeated. A small sample of the solution was used for agarose gel electrophoresis, and the rest was frozen.

QIAquick PCR Purification Kit Protocol

5 volumes of Buffer PB were added to 1 volume of the PCR reaction mixture and mixed. A QIAquick spin column was placed in a 2 ml collection tube. The sample was applied to the column and centrifuged for 1 minute. The flow-through was discarded and the QIAcolumn was placed back into the same tube. 750 µL of Buffer PE was added to the column and centrifuged for 1 minute. The flow-through was discarded and the column was returned to the collection tube and spun for an additional one minute at maximum speed. The column was placed in a clean, labeled 1.5 ml microcentrifuge tube. 50 µL of sterile H2O was added to the column and centrifuged for one minute to elute the DNA. A sample of the mixture in the microcentrifuge tube was used for DNA quantitation and analysis by agarose gel electrophoresis. The rest of the mixture was stored frozen.
Transformation

A frozen (-80°C) sample of *E. coli* cells (either DH5α or SM10 λpir as dictated by choice of vector) was thawed in a wet ice bath for approximately 15 minutes. The cells were transferred to a cold, labeled 1.5 ml microcentrifuge tube. 10 μL of the ligation mixture was added and mixed gently. The mixture was placed on ice for 30 minutes and then heat shocked in a 37°C water bath for 5 minutes. 500 μL of pre-warmed LB broth were added which was transferred to labeled falcon tubes. The cells were allowed to grow for 1.5 hours in a 37°C incubator for 1.5 hours at 100 rpms. 100 μL aliquots of cells were plated onto 5 plates containing selective media. The plates were incubated overnight at 37°C.

Note: All protocols were designed according to manufacturer's instructions OR Sambrook, Joseph *et al.* Molecular Cloning. Cold Spring Harbor Laboratory Press, 1989.