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Contribution of a Putative UP Element DNA Sequence to the Activity of a Newly Identified Phage Promoter

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CONTRIBUTION OF A PUTATIVE UP ELEMENT DNA SEQUENCE TO THE ACTIVITY OF A NEWLY IDENTIFIED PHAGE PROMOTER

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By

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ABSTRACT

In transcription, a universal step in gene expression, information from a DNA sequence is copied into RNA. A key component in gene expression is the promoter sequence, a region of DNA to which RNA polymerase binds during the initiation of transcription of downstream genes. Most bacterial promoters contain a -10 and a -35 sequence that are bound by the RNA polymerase. Some promoters also contain an Upstream Promoter (UP) element. UP elements have been shown to boost promoter activity. We recently identified a new promoter in a mutant bacteriophage that grows on a bacterial host that prevents antitermination of phage transcription. Close inspection of the promoter sequence suggested it had a putative UP element. The goal of this project was to determine if the UP element-like sequence contributes to promoter activity and, if so, to what degree it might influence growth on the restrictive bacterial host. To test the effect of the putative UP element, reporter gene fusions were created in which the UP element-like sequence was deleted. Our results show that the deletion of the UP element-like sequence decreased promoter activity two-fold. These results suggest that the sequence is an UP element. This research has enhanced our understanding of how the phage mutation suppresses the effect of the antitermination defective host.

Keywords: Transcription, Antitermination, RNA Polymerase, UP Element, Promoter Sequence, *Escherichia coli*

Dedicated to Mum, Dad, Justin, Emily, Barrett & Khaleesi

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CHAPTER ONE

INTRODUCTION

Transcription

Transcription is a process that occurs in all organisms and is the first step in gene expression. The process of transcription entails the generation of a ribonucleic acid (RNA) sequence from a deoxyribonucleic acid (DNA) template by an enzyme known as RNA polymerase. RNA polymerase recognizes promoter regions in DNA and begins the production of RNA transcripts that can include messenger RNA (mRNA) [1]. RNA polymerase then elongates the transcript processively before terminating at a transcription termination site; it then releases both the single-stranded primary RNA transcript and the DNA. In *E. coli*, the rate of the transcription process is not uniform. Instead, the transcription process may pause and the enzyme may backtrack. Such disruptions in transcription are potential control points, and are often caused by a particular DNA sequence or RNA structure. Pausing and backtracking are resolved either spontaneously or by transcription factors such as GreA/B [12]. Several other transcriptional factors, proteins that modulate gene transcription, regulate the rate of transcription in *E. coli*. Factor NusG, for instance, increases the rate of transcription by suppressing both pausing and backtracking. The NusA protein, on the other hand, slows the rate of transcription by promoting pausing and transcription termination [12].

E. coli RNA polymerase consists of six subunits—two alpha (α) subunits, the beta (β) and beta prime (β') subunits, a sigma (σ) subunit, and an omega (ω) subunit (Figure 1). The central function of the α subunits, which are 329 amino acids in length, is the coordination of the assembly of the various subunits into the RNA polymerase holoenzyme. The α subunits also activate transcription by recognizing promoter sequences in DNA [1]. The β subunit assists in transcription initiation, elongation, and termination. Encoded by the *rpoC* gene, the positively-charged β' subunit of RNA polymerase can bind to negatively-charged DNA in the absence of all other subunits. The β' subunit participates in transcription elongation and termination [1]. Several subunits play a role in initiation of transcription, but the σ subunit, usually in the form of sigma 70 (σ^{70}) in *E. coli*, plays the central regulatory role in transcription initiation [1]. The ω subunit is the only subunit not required for transcription. It restores denatured RNA polymerase in case of damage to the enzyme [1].

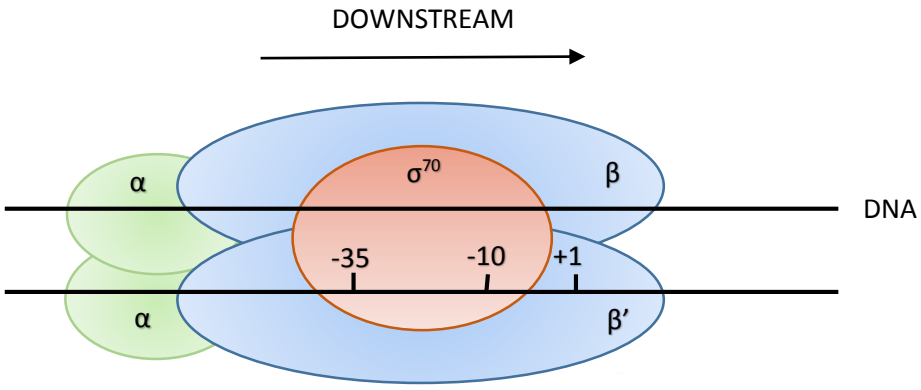

Figure 1. Subunits of *E. coli* RNA polymerase bound to a promoter region. RNA Polymerase consists of two α subunits, a β subunit, a β' subunit, a σ subunit, and an ω subunit. The σ^{70} recognizes the bacterial promoter sequence. The ω subunit is not pictured.

Bacterial Promoters

Promoter regions in bacterial DNA are the areas to which RNA polymerase binds in order to initiate transcription. Additional proteins associated with DNA promoter regions, such as activators and repressors, affect the efficiency of the promoter region and therefore the rate at which transcription initiation occurs [11]. The σ subunit of the *E. coli* RNA polymerase confers the enzyme's specificity to bind promoter sequences [11]. Specifically, σ^{70} recognizes two particular regions of bacterial promoter sequences, the -10 region (consensus sequence TATAAT) and the -35 region (consensus sequence TTGACA), allowing RNA polymerase to bind to the DNA. Optimal activity is achieved when the regions are separated by 17 base pairs [6, 11]. Promoter strength is correlated with how closely the promoter region resembles the consensus sequence [11].

The -10 element is a highly conserved region of the promoter sequence [6]. When RNA polymerase binds to the promoter at the -10 sequence, it lines up at the transcription start site [6]. The -35 region, although not as highly conserved as the -10 region, serves as the initial binding site to which σ^{70} directs RNA polymerase [6, 11].

Transcription Termination

RNA polymerase continues the process of transcription until encountering a transcription termination signal, after which the transcript is released from the DNA. In this way, transcription terminators delineate the 3' end of the RNA transcript [12]. *E. coli* has two different types of transcription terminators: Rho-dependent and Rho-independent.

Rho-dependent Terminators

Rho is a highly conserved protein in bacteria [12]. The Rho protein contains ATPase and helicase actions that are dependent on RNA [3]. Rho binds to mRNA at its Rho utilization (*rut*) site, and then moves along the RNA strand from the 5' to the 3' end until it reaches the RNA polymerase that is paused at a release site. At this point, Rho releases the RNA from the DNA, thereby terminating transcription [12]. The rate of transcription in *E. coli* can affect the efficiency of Rho-dependent terminators—the slower the rate of transcription, the greater the efficiency of Rho-dependent termination, and the faster the rate, the lower the efficiency [12].

Rho-independent Terminators

Rho-independent, or intrinsic, termination only requires certain RNA sequence elements and does not require the Rho protein. The DNA sequence at which intrinsic termination occurs is an inverted repeat followed by a run of adenine bases [12]. The inverted repeat forms a hairpin structure in the RNA that destabilizes the transcription elongation complex and leads to transcription termination [13]. Rho-independent terminators are heavily dependent upon the coordination of the hairpin folding and the arrival of RNA polymerase at the transcription termination site. Following the RNA hairpin is a series of uridine residues that serves to destabilize the RNA-DNA transcription complex, and promote transcription termination [13]. The intrinsic termination complex is illustrated in Figure 2.

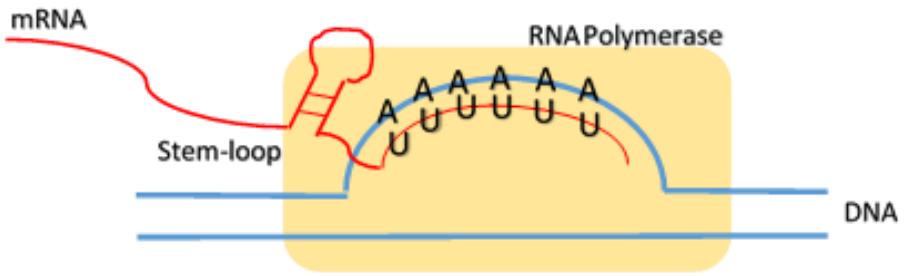

Figure 2. Intrinsic termination complex. RNA forms a stem-loop structure due to an inverted repeat in the sequence. The stem-loop structure is followed by a series of uridine residues (U) whose weak base pairing with the DNA template helps destabilize the transcription complex and promote transcription termination.

Antitermination of Lambdoid Bacteriophages

The process of transcription is halted when RNA polymerase reaches a transcription termination signal on the DNA sequence. Transcription termination can be prevented, however, by a process known as antitermination. Antitermination promotes continued transcription of mRNA by converting the transcription elongation complex to a terminator-resistant form that allows read-through of the transcription termination signals [8]. Antitermination is thus a regulatory mechanism that extends the number of genes expressed by allowing continued transcription of mRNA.

Transcription terminators precede some gene sequences of lambdoid phages, blocking gene expression in the absence of antitermination. In bacteriophage λ, antitermination occurs upon modification of RNA polymerase after reaching one of two promoter-proximal antiterminator loading sites, located in the left and right operons of the bacteriophage [8]. These two regions, known as N utilization sites (*nut*L and *nut*R), interact with the N protein. The N protein is a member of the RNA-binding proteins that are rich in the amino acid arginine [2]. This protein functions by binding to the *nut*-

encoded RNA stem-loop structure [2], which, in conjunction with a set of host proteins known as Nus factors, convert RNA polymerase to a terminator-resistant form [8] (Figure 3).

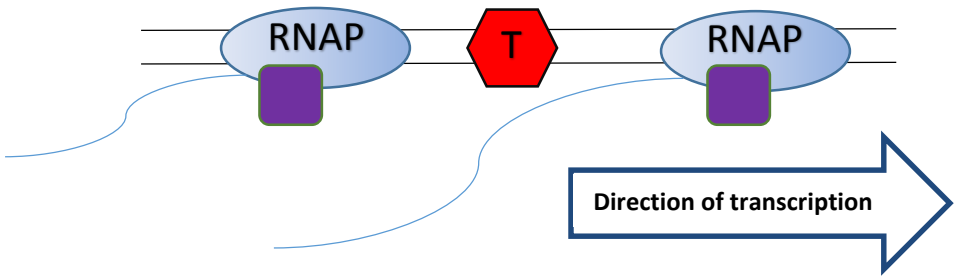

Figure 3. Antitermination of Lambda Phage. The N protein with associated Nus factors is shown in purple interacting with RNA polymerase. This interaction promotes read-through of transcription terminators, shown in red, by converting RNAP to a terminator-resistant form.

Antitermination of Lambdoid Bacteriophage HK022

Bacteriophage HK022 is a relative of lambda. Despite the close relationship to other lambda bacteriophages, the antitermination mechanism of HK022 is unique. Antitermination of HK022 requires neither the N protein nor the bacterial Nus factors. Instead, the RNA transcript itself modifies RNA polymerase. These antiterminator RNA transcripts are encoded by polymerase utilization (*put*) sites that increase the efficiency of the read-through of transcription terminators [8]. The *put* transcripts form stem-loop structures that, although they contain different arrangements of nucleic acid residues, exhibit similar antitermination efficiency (Figure 4). The folded *put*L and *put*R transcripts interact directly with RNA polymerase, conferring termination resistance [9].

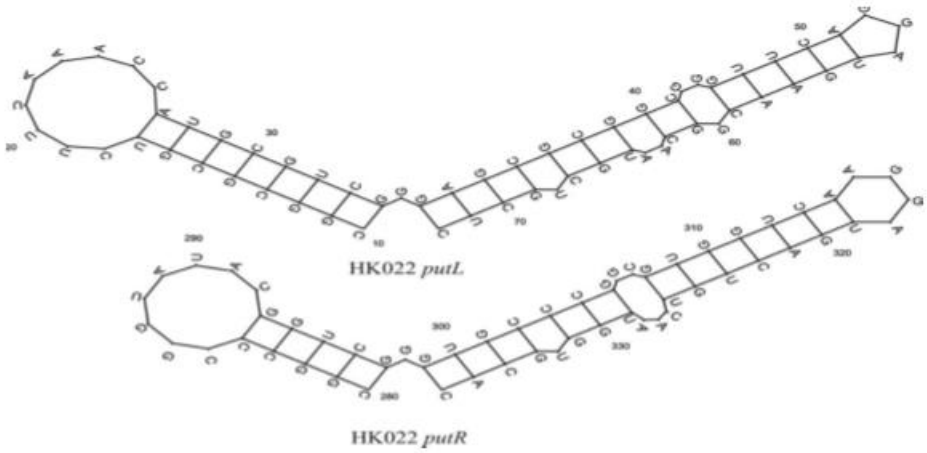

Figure 4. HK022 Antitermination RNAs. Pictured are the stem-loop structures of the transcripts of HK022 *putL* and *putR* [9].

Host Mutants that Block RNA-mediated Antitermination

In 1995, Michel Clerget, et al., conducted an experiment to locate *E. coli* host mutants that block HK022 antitermination [4]. The experimenters expected to locate analogs to the Nus factors involved in antitermination of bacteriophage lambda. Rather than discovering such analogs, the experimenters located mutations in the host RNA polymerase itself that inhibited phage growth. These host mutations occurred in the *rpoC* gene that encodes the β' subunit of the polymerase [4]. The results showed that a single amino acid substitution within the zinc finger of the *rpoC* protein resulted in bacteriophage resistance. This mutation, *rpoC*Y75N, was found to prevent antitermination of early transcription in bacteriophage HK022, which inhibited the completion of the phage life cycle and subsequent plaque formation (Figure 5).

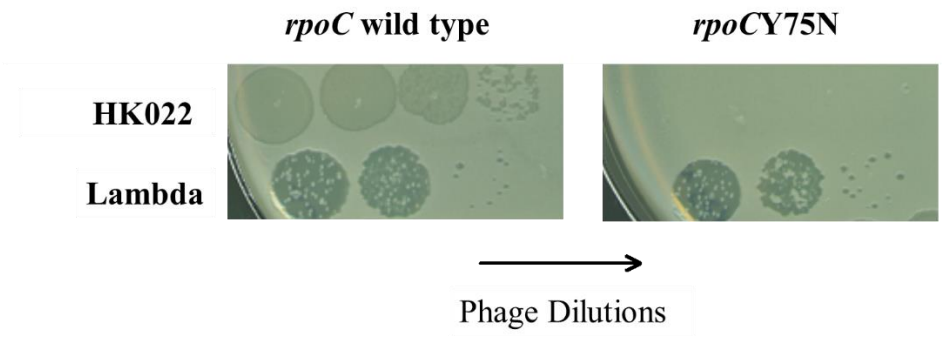

Figure 5. The *E. coli rpoC*Y75N mutation prevents the growth of bacteriophage HK022. The lack of growth of HK022 can be seen from the absence of plaques on the plate containing the *rpoC*Y75N mutant bacterial host. The *rpoC* mutation does not affect the growth of bacteriophage lambda.

Bacteriophage Mutants that Circumvent the Antitermination Defect of the rpoCY75N Mutant Host

In order to find bacteriophage mutants that could grow on the restrictive *E. coli* host, a hybrid phage of lambda and HK022 was utilized. Bacteriophage O276 is mostly a lambda phage but contains the immunity region of HK022 (Figure 6). The hybrid phage recapitulates the plating phenotype of HK022—it grows as well as the parental phages on a wild type *E. coli* host but does not grow on the *rpoCY*75N mutant. Because lambda is very well characterized, substituting a small portion of the HK022 phage for the same region of the lambda phage allows for a better understanding of phage HK022 and its antitermination mechanism.

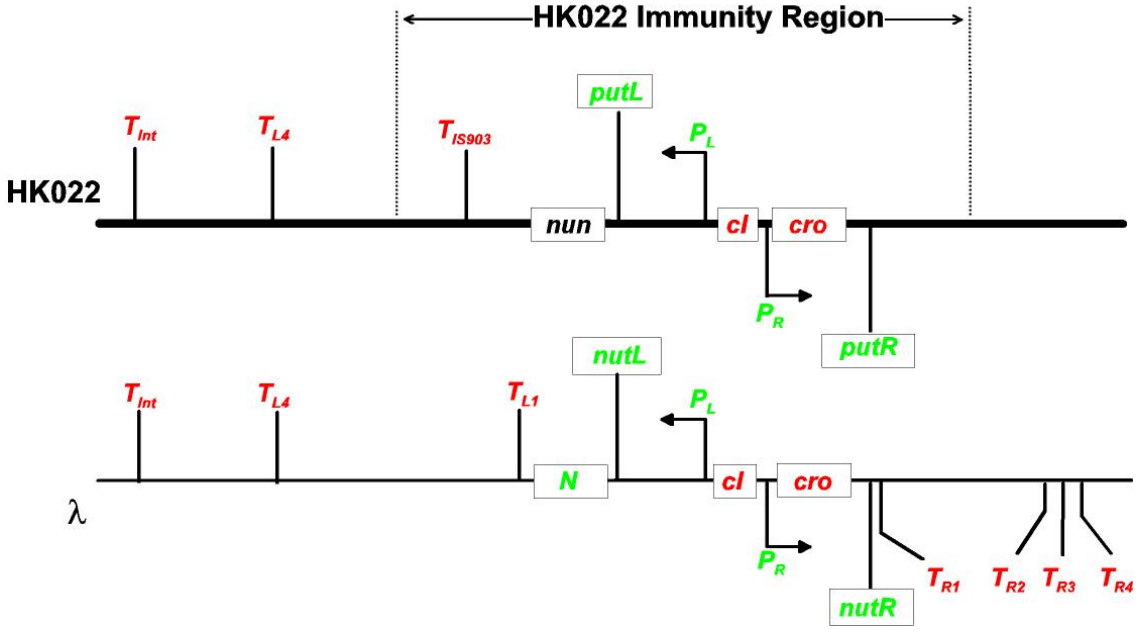

Figure 6. Alignment of HK022 and λ immunity regions. Phage O267 is mostly a lambda phage but contains the immunity region of HK022 pictured above.

Bacteriophage capable of growing on a host carrying the *rpoC*75N mutation were recovered by mutagenizing the parental phage O276 with UV light and looking for plaque formation. The resulting mutants were referred to as "orc" (overcoming *rpoC*) mutations. Genomic sequencing revealed that one bacteriophage containing the "orc" mutation, phage O367, differed from the original phage O276 by only two base pairs (Figure 7).

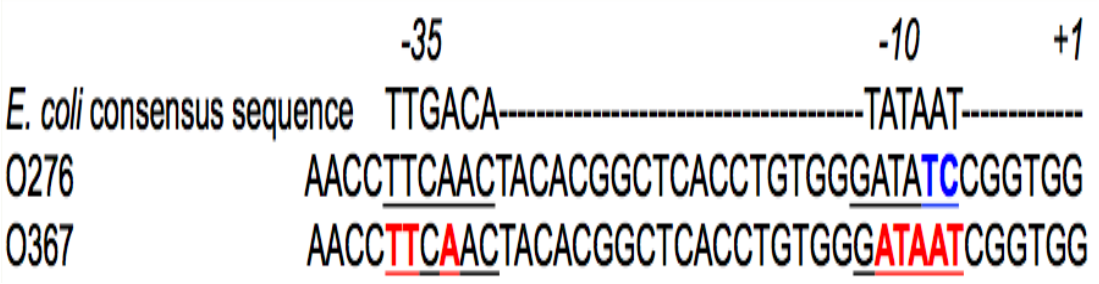

Figure 7. The "orc" mutation in phage O367 creates a new promoter sequence, differing from phage O276 by only two base pairs, bringing the region in O367 closer to the consensus sequence of the *E. coli* -10 region.

Sequence comparisons showed that the two base pair change created a -10 promoter element in the bacteriophage genome that is located downstream of three lambda transcription terminators. It was hypothesized that transcription initiation at this new promoter allows RNA polymerase to bypass the transcription termination sites and express the downstream genes that are needed to complete the phage life cycle. In this way, the creation of a new promoter element allows RNA polymerase to circumvent the three lambda terminators rather than being converted to a terminator-resistant form (Figure 8).

Figure 8. The sequence between and including primers RK774 and RK767 constitutes the promoter sequence of phage O367 amplified by PCR. The sequence just upstream of RK774, beginning with the run of "A" residues, is the UP element-like sequence to be tested. The orange arrow denotes the third lambda transcription terminator circumvented by the new promoter region.

Upstream Promoter Elements

An upstream promoter (UP) element is a sequence of DNA located upstream from the -35 sequence of a bacterial promoter. These sequences have been shown to boost promoter activity by interacting with the α subunits of RNA polymerase [5]. The consensus sequence—the most common order of nucleic residues found in a particular alignment—for UP elements is rich in adenine and thymine bases (Figure 9). The interaction between the UP element sequence and the α subunits of RNA polymerase

increases promoter activity, sometimes by as much as 30-fold [5]. UP elements, while increasing promoter activity, are not essential for promoter activity [5].

The sequence upstream of the newly created promoter region of bacteriophage O367 closely resembles the consensus sequence for bacterial UP elements which contain many adenine and thymine residues. The putative UP element-like sequence in phage O367 matches the consensus sequence in 14 out of 22 bases, highlighted in red below (Figure 10).

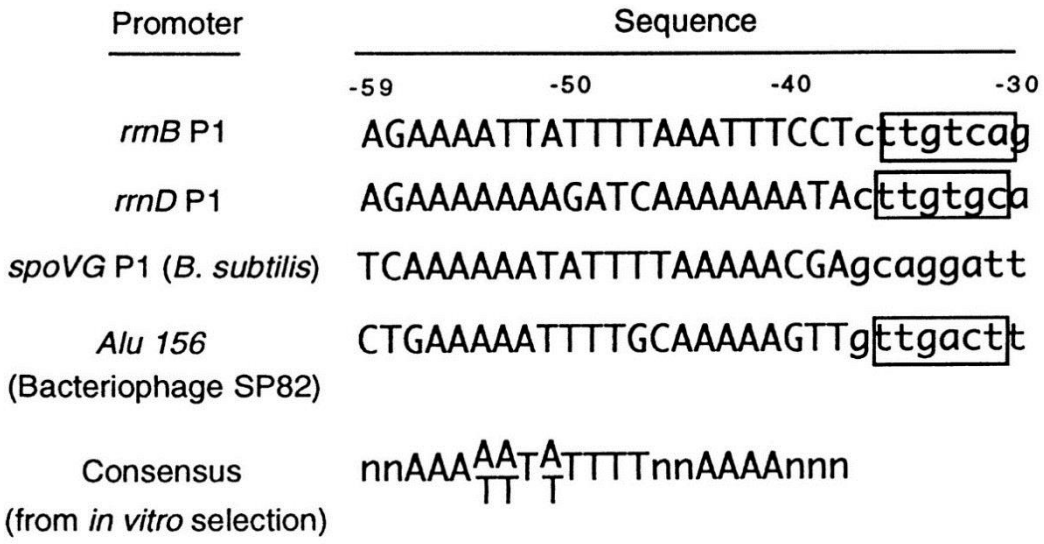

Figure 9. Examples of UP elements aligned by the adjacent -35 sequence. The -35 sequence is boxed. The "n" in the consensus sequences denotes any base [5].

The hypothesis of this project is that the sequence located upstream of the newly identified promoter in phage O367 is an UP element. To test this hypothesis, reporter gene fusions were created with and without the UP element-like sequence in order to determine its effect on promoter activity. This effect was analyzed qualitatively using differential growth media and quantitatively using β-galactosidase assays.

Figure 10. The region upstream of the -35 region of the promoter region of O367 resembles the consensus sequence for bacterial UP elements. The residues in common between the O367 region and the consensus sequence are highlighted in red. The top sequence in the figure demonstrates the frequency of each base pair at each position. Larger letter size corresponds to increased frequency of the base.

Promoter Probe Vector

A plasmid is small, circular, nonessential DNA that may, in spite of its nonessential nature, confer special properties to a cell such as antibiotic resistance. The plasmid utilized in this study, pRAK31, contained its own promoter, which was deleted to prepare it for the desired DNA insert of this project (Figure 11). Expression of the *lacZ* promoter gene is evidence of promoter activity. This activity can be measured both qualitatively and quantitatively. The transcription terminators found in the promoter probe vector serve to block transcription originating from sources other than the desired one—namely, the DNA insert containing the promoter sequence of O367.

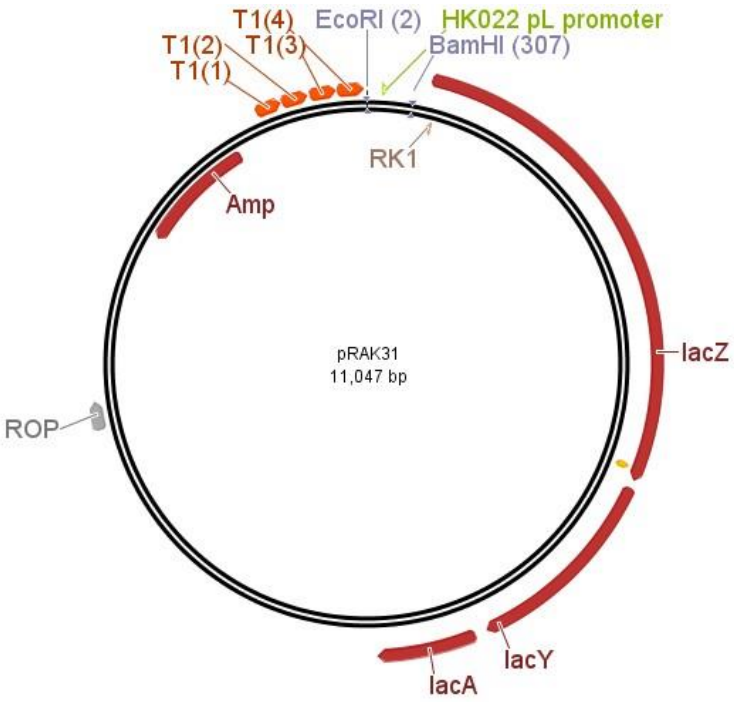

Figure 11. Promoter Probe Vector. Plasmid pRAK31 contains the *lacZ* operon for analysis of promoter activity. The HK022 pL promoter was deleted by digesting the plasmid with EcoRI and BamHI and purifying the resulting vector backbone.

CHAPTER TWO

MATERIALS AND METHODS

Plasmid

Plasmid pRAK31 was the promoter probe vector used in this study. It was utilized to determine the efficiency of the promoter region of phage O367 before and after deleting the UP element-like sequence. Plasmid pRAK31 contains the *lacZ* reporter gene as well as a location to clone the desired promoter sequence into the vector. The cloned promoter sequence from phage O367 drove the expression of the *lacZ* reporter gene. The activity of the promoter was quantitatively measured using a β-galactosidase assay as described below.

Bacterial Strains

The bacterial strains used in this study are listed in Table 1. RK1365 and RK1367 are independent isolates of the same annealed oligonucleotide construct.

Table 1. *E. coli* Strains

STRAIN	GENOTYPE	SOURCE
RK898	MG1655, wild-type *E. coli*	Singer *et al.* (1989) [14]
RK899	*rpoCY*75N mutation	Robert Weisberg, NIH [4]
RK1359	No promoter sequence, derived from phage O276	This study

RK1360	Promoter sequence, derived from phage O367	This study
RK1365	Promoter without putative UP Element (cloned DNA was made by annealing oligonucleotides RK775 and RK776)	This study
RK1366	Promoter without putative UP Element (cloned DNA amplified using oligonucleotides RK767 and RK774)	This study
RK1367	Promoter without putative UP Element (cloned DNA was made by annealing oligonucleotides RK775 and RK776)	This study

Plasmid Isolation

A 100 mL overnight culture of MC1000 *E. coli* cells containing the plasmid pRAK31 was grown in LB media supplemented with Ampicillin. Plasmid DNA was isolated using the Qiagen QIAfilter Midi Kit (Cat. No. 1243). The bench protocol for the Qiagen Kit is as follows:

I. Bacterial harvesting and lysis

One hundred milliliters of the MC1000/pRAK31 cell culture was split into two tubes and centrifuged at 3,500 x g for 10 minutes at 4°C. After removing the supernatant, the remaining cell pellet was re-suspended in 4 mL of Buffer P1. Four milliliters of Buffer P2 was then added, followed by inversion of the tube 4-6 times to mix until the solution was cloudy. The solution was incubated at room temperature for 5 minutes. After incubation,

4 mL of chilled Buffer P3 was added and mixed by inversion 4-6 times. A QIAfilter Midi Cartridge was prepared by attaching a cap onto its outlet nozzle, then placing the assembly in a test tube.

II. Bacterial lysate clearing

The lysate from the previous step was poured into the QIAfilter Midi Cartridge and incubated at room temperature for 10 minutes. A QIAGEN-Tip 100 was equilibrated by pipetting 4 mL of Buffer QBT into the column and allowing the column to empty by gravity flow. The QIAGEN-Tip was transferred to a clean tube. The cap from the QIAfilter Midi Cartridge was then removed, and a plunger was inserted into the Midi Cartridge in order to filter the lysate into the QIAGEN-Tip.

III. Binding, washing, and eluting on the QIAGEN-Tip

The cleared lysate was allowed to enter the resin by gravity flow. The QIAGEN-Tip was washed twice, using 10 mL of Buffer QC for each wash. The Tip was then moved to a new collection tube and the plasmid DNA was eluted with 5 mL of Buffer QF.

IV. Precipitating, washing, and re-dissolving the plasmid DNA

This portion of the plasmid isolation process deviates from the Qiagen protocol. Plasmid DNA was precipitated by adding 3.5 mL of room temperature isopropanol to the DNA. After mixing this solution, 1 mL aliquots were transferred into microcentrifuge tubes. The tubes were centrifuged at 13,400 x g for 30 minutes at 4°C. The pellets were re-suspended after centrifuging by transferring DNA elution buffer (EB; QIAGEN; Mat. No. 1014608) between the tubes. The addition of the elution buffer was repeated with a fresh aliquot of buffer. These volumes were combined (200 µL final volume).

Spectrophotometric Determination of Concentration

Purified DNA was quantified using a NanoDrop spectrophotometer. The NanoDrop pedestal was cleaned with nanopure water and dried with a Kimwipe. Two microliters of the DNA sample were added to the pedestal and the DNA concentration (ng/µL) was measured.

Bacteriophage Stock Titer

A series of ten-fold dilutions (10^{-1} to 10^{-8}) of bacteriophage stocks O276 and O367 were made to titer the stocks. The phage stocks were diluted in Tris-Magnesium-Gelatin (TMG) medium. To make the dilution series, ten microliters of stock O276 or O367 were added to 90 µL of TMG in the 10^{-1} dilution blank. Ten microliters of the resulting mix were added to 90 µL of TMG in the 10^{-2} dilution tubes and mixed, and so on until the 10^{-8} dilution tubes were completed. From each dilution, 10 µL was mixed in a tube with 90 µL of the *Escherichia coli* host (RK898 or RK899) (Table 1). In preparation for plating, tubes of 4 mL microwaved top agar (supplemented with $MgSO_4$; final concentration = 10 mM) were placed in a heat block and then mixed with the phage and host mixtures. These mixtures were then poured onto pre-warmed (37°C) LB plates. The top agar was allowed to harden, and the plates were inverted prior to incubation at 37°C. Plates were placed inside an unsealed plastic bag to prevent drying.

Preparation of the Promoter Probe Vector for Cloning

To prepare the promoter probe vector to receive insert, it was sequentially digested with EcoRI and BamHI. These enzymes cut the plasmid upstream of the reporter gene. The

restriction enzymes used in the double digestion created "sticky ends"—overhangs that enable ligation—on the DNA. The EcoRI digestion consisted of the following:

X µL nanopure water

X µL DNA (promoter probe vector pRAK31, approximately 4 µg)

3 µL EcoRI buffer (New England BioLabs; 10X Concentration; #B0101S)

1 µL EcoRI (New England BioLabs; 20000 u/ml; #R0101S)

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30 µL total

The digestion reaction was incubated for 2 hours at 37°C. After incubation, the vector was purified using the phenol-chloroform procedure described below. The purified DNA was then digested with BamHI. The BamHI digestion reaction consisted of the following:

X µL nanopure water

X µL pRAK31 (DNA previously digested with EcoRI; approximately 4 µg)

3 µL buffer (New England BioLabs; 10X Concentration; #B7003S) (1X final concentration)

3 µL bovine serum albumin (BSA; New England BioLabs; 10mg/ml; #B9001S)

1 µL BamHI (New England BioLabs; 20000 u/ml; #R0136S)

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30 µL total

The digest was incubated at 37°C for 2 hours and then placed in a freezer overnight at -20°C until further processing. After freezing, the digestion was purified using the phenol-chloroform method below.

Purification of Digested DNA

Because double digestions of DNA were performed sequentially, the first enzyme had to be removed before cutting the DNA with the second enzyme. Restriction enzymes were removed from the reactions by the following procedure. Seventy microliters of EB buffer (10mM Tris-HCl pH 8) was added to each of the digestion reactions to achieve a total volume of 100 µL. An equal volume of 1:1 phenol-chloroform (Chloroform: Fisher Chemical, C298500; Phenol: Fisher BioReagents. BP1750'-100) was added to each of the digestion tubes, and the tubes were then mixed by inversion until cloudy. The tubes were centrifuged at 10,000 x g for 30 seconds, and the top aqueous layers from each tube were transferred to new tubes. A back extraction of the organic layer was performed by adding 70 µL EB buffer. After centrifugation, the aqueous phases containing the DNA were recovered and transferred to fresh tubes. In the fresh tubes, an equal volume of chloroform was added, and, after mixing, the tubes were centrifuged at 10,000 x g for 30 seconds. The aqueous top layer was added to two new tubes, and the chloroform extraction was repeated once more.

The aqueous material was then filtered through microconcentrator filters (Amicon Ultra Centrifugal Filter Units; 0.5ml; UFC 510096) into two new tubes. Three hundred microliters of nanopure water were added to each of the microconcentrators containing the filtered aqueous material, followed by centrifugation for 3 minutes at 10,000 x g. The filters were then inverted and placed into new tubes, and the pure DNA was recovered by centrifugation for 3 minutes at 10,000 x g. The DNA (approximately 30 µL per tube) was then digested with BamHI, incubated for two hours at 37°C, and purified as above after overnight freezing.

Agarose Gel Electrophoresis

Agarose gel electrophoresis is often used for the visualization and separation of DNA fragments. To make a 1% agarose gel solution for electrophoresis, one gram of agarose (Fisher BioReagents: BP160-100) was added to 1X TAE buffer (Appendix A) and heated in the microwave until the agarose was completely dissolved. The molten agarose was then cooled in a 55°C water bath. Once cool, 30 mL of the molten agarose was poured into the gel mold containing the appropriate well-forming comb and allowed to solidify for 10 minutes until opaque. Once the gel solidified, the mold was placed in the electrophoresis unit. 1X TAE buffer was poured over the agarose gel until covered.

Two different electrophoresis ladders were used in this study. The λHindIII ladder (Fermentas; 0.5 mg/ml; #SM0101) was used for DNA of a large size, and the 100bp ladder (Axygen Product No. M-DNA-100bp 500 µL) was used for DNA of smaller size determination. Before DNA was loaded into the wells, it was mixed with a loading dye (Appendix A)—the blue color of the loading dye makes the DNA easier to see, and the glycerol contained in the dye increases the density, allowing the DNA to sink into the wells. Once the gels were loaded with DNA samples, the electrophoresis lids were placed on the units, and the attached electrodes were connected to an electrical current. The positively charged electrode was attached to the end of the gel opposite the side containing the wells; since DNA is negatively charged, it will migrate towards this positive pole.

Unless otherwise noted, gels were run at 100V for approximately 80 minutes. After electrophoresis was completed, the gels were placed in an ethidium bromide staining gel solution (0.5 microgram/ml final concentration in 1X TAE buffer; Sigma-Aldrich 10

mg/ml stock; E1510-10 ml) for 10 minutes. Ethidium bromide staining solution allows visualization of the DNA in the gel when exposed to UV light. After the completion of the staining process, the gels were carefully removed from the solution and photographed using Alpha Innotech FluorChem HD2 (S/N: 504332; EPI UV Lights: Dual 254/365nm).

Gel Purification of Promoter Probe Vector DNA

After separating DNA fragments by gel electrophoresis, the desired fragments were cut from the gel using a razor blade. The gel slices were placed in microcentrifuge tubes and minced to increase surface area using a needle. Phenol (800-900 µL) was added and the tubes were placed in a freezer for 10 minutes. After freezing, the tubes were centrifuged at 13,400 x g for 10 minutes. The aqueous layer was recovered and transferred to new tubes, which were then treated with an equal volume of chloroform. These tubes were then centrifuged for 30 seconds at 10,000 x g. The aqueous layer was transferred to a new tube. The process of chloroform addition, centrifugation, and aqueous layer extraction was repeated once more. The aqueous layers were pooled and the DNA was ethanol-precipitated by adding one tenth the volume of 3M sodium acetate (pH 5.2) and 2 volumes of ethanol. After mixing well, the tubes were placed in the freezer overnight at -20°C.

Recovery of DNA with Microconcentrator Devices

The ethanol precipitated DNA was collected by centrifugation at 13,000 x g for 20 minutes. The aqueous layer containing the DNA was recovered and transferred to new tubes. The DNA was purified as follows. The pellets of DNA were resuspended in EB buffer by sequentially transferring 50 µL of EB buffer between the tubes. These solutions were transferred into microconcentrator devices, and 250 µL of EB buffer were added to

each of the tubes to ensure complete recovery of DNA. The microconcentrators containing the DNA were then centrifuged at 10,000 x g for 7 minutes. The resulting filtrate was removed and 500 µL of EB buffer was added back to each filtered tube containing the DNA. These tubes were again centrifuged for 7 minutes at 10,000 x g. The filtrate was removed, and after addition of 500 µL of EB buffer, the filtered tubes containing the DNA were centrifuged a third time. The remaining EB buffer was carefully poured out, and the filters were inverted into new tubes, which were centrifuged for 5 minutes at 10,000 x g. The DNA was removed by pipette from these tubes and transferred to new tubes.

Annealing of Oligonucleotides RK775 and RK776 to Create DNA Insert

Equivalent quantities (in µL) of complementary oligonucleotides RK775 and RK776 which contain the cut sites for EcoRI and BamHI were mixed with 30 µL of TE buffer. This mixture was then incubated starting at 94°C for 5 minutes to facilitate denaturation of the synthetic DNA strands. The temperature was then decreased in 5°C increments, with a 5 minute incubation period at each temperature, until 40°C was reached. The decreases in temperature allowed the synthetic DNA strands to hybridize. All of the oligonucleotides used in this study were synthesized by Integrated DNA Technologies (Table 2).

Table 2. Oligonucleotides

Oligonucleotide	SEQUENCE
RK767	5’-GATC**ggatcc**GACGTCTTAGCCACCG-3’
RK774	5’-CGATC**gaattc**CTTCAACTACACGGCTC-3’
RK775	5’-GATC**gaattc**CTTCAACTACACGGCTCACCTGTGGG ATAATCGGTGGCTAAGACGTC**ggatcc**GATC-3’
RK776	5’-GATC**ggatcc**GACGTCTTAGCCACCGATTATCCCACAG GTGAGCCGTGTAGTTGAAG**gaattc**GATC-3’

*****G/AATTC is the cut site of EcoRI, and G/GATCC is the cut site of BamHI, denoted in bold.

Polymerase Chain Reaction using Oligonucleotides RK767 & RK774

The orc promoter region was amplified by PCR using oligonucleotides RK767 and RK774 as primers (Table 2). To prepare a PCR reaction, a template and two primers are required. The template pRK1360A was derived from phage O367, and contained the target sequence of DNA [15]. This amplicon was designed to contain the promoter region of O367 without the putative UP element-like sequence. The thermocycler settings for the PCR reaction are found in Table 3. The PCR reaction setup was as follows.

1 µL 1360A template (contains the promoter created by the orc mutation; 1:10 dilution)

1 µL RK767 (primer 1: 100 pmol/µL stock)

1 µL RK774 (primer 2: 100 pmol/µL stock)

30 µL PCR mix (Buffer A, dNTPs; Appendix A)

66.5 µL nanopure water

0.5 µL Taq (Fisher Scientific; 5000 u/ml; FB600015)

100 µL total

Table 3. Polymerase Chain Reaction Conditions

	Temperature (°C)	Time (minutes)
Step 1	94	2.0
Step 2	94	0.5
Step 3	55	0.5
Step 4	72	1.0
Step 5	Return to step two and repeat 30 times	
Step 6	4	Forever
Step 7	End	

Double Digestion of PCR Product with EcoRI & BamHI

The PCR product created by using oligonucleotides (RK767 x RK774) was digested with the restriction enzymes EcoRI and BamHI to prepare it for cloning into the promoter probe vector. The reaction conditions were as follows.

20 µL PCR product (RK767 x RK774; 50.0 ng/µL)

20 µL nanopure water

3 µL BSA (New England BioLabs; 10mg/ml; #B9001S)

5 µL EcoRI buffer (New England BioLabs; 10X Concentration; #B0101S)

1 µL EcoRI (New England BioLabs; 20,000 u/ml; #R0101S)

1 µL BamHI (New England BioLabs; 20,000 u/ml; #R0136S)

---

50 µL total

After mixing thoroughly, the reaction was incubated for 3 hours at 37°C. The digested PCR product was then purified using Zymo Research's DNA Clean and Concentrator -5 protocol (Zymo Research; D4004) below. A 2% agarose gel was run to check the digested PCR product.

Double Digestion of Annealed Oligonucleotides RK775 & RK776 with EcoRI & BamHI

The double-stranded synthetic DNA made by annealing complementary oligonucleotides RK775 and RK776 was double-digested with EcoRI and BamHI to prepare it for cloning into the promoter probe vector. The reaction conditions were as follows.

15 µL annealed oligonucleotides RK775 & RK776 (100 pmol/µL stocks)

25 µL nanopure water

3 µL BSA (New England BioLabs; 10mg/ml; #B9001S)

5 µL EcoRI buffer (New England BioLabs; 10X Concentration; #B0101S)

1 µL EcoRI (New England BioLabs; 20,000 u/ml; #R0101S)

1 µL BamHI (New England BioLabs; 20,000 u/ml; #R0136S)

---

50 µL total

After mixing thoroughly, the reaction was incubated for 3 hours at 37°C in a PCR machine.

Purification of Double-Digested Synthetic Promoter DNA

Zymo Research's DNA Clean and Concentrator -5 (Zymo Research; D4004) was used to purify the double-digested synthetic DNA. The kit protocol is as follows. The reagents were prepared according to the manufacturer's instructions. Five volumes of DNA Binding Buffer was added to the annealed DNA. This mixture was loaded into the Zymo-Spin column. The tube was centrifuged at 13,400 x g for 30 seconds. Two hundred microliters of DNA Wash Buffer was added to the column and the tube was centrifuged again at 13,400 x g for 30 seconds. This step was repeated once more. The Zymo-Spin column was then placed into a new microcentrifuge tube, and 15 µL of DNA Elution Buffer was added to the column. This was centrifuged briefly to collect the eluted DNA. The eluted products were examined on a 2% agarose gel.

Ligation Reactions

A general ligation reaction contained the double-digested promoter probe vector, 1X buffer (New England BioLabs #B0202S; 10X stock), either the synthetic DNA (annealed and digested oligonucleotides RK775 x RK776) or the PCR product (amplified with oligonucleotides RK774 x RK767), nanopure water, and ligase (New England BioLabs #M0202S; 400000 u/ml) for a total volume of 20 µL (Table 4).

Table 4. Ligation Reactions

REACTION 1	REACTION 2	REACTION 3	REACTION 4	REACTION 5
5 µL double digested promoter probe vector	5 µL double digested promoter probe vector	5 µL double digested promoter probe vector	5 µL double digested promoter probe vector	5 µL double digested promoter probe vector
2 µL 10X buffer	2 µL 10X buffer	2 µL 10X buffer	2 µL 10X buffer	2 µL 10X buffer
2 µL synthetic DNA (8pmol/µL) (1:25 dilution)	5 µL synthetic DNA (8pmol/µL) (1:25 dilution)	2 µL PCR product (50.0 ng/µL) (1:10 dilution)	5 µL PCR product (50.0 ng/µL) (1:10 dilution)	N/A
10 µL nanopure H_2O	7 µL nanopure H_2O	10 µL nanopure H_2O	7 µL nanopure H_2O	12 µL nanopure H_2O
1 µL ligase	1 µL ligase	1 µL ligase	1 µL ligase	1 µL ligase

After overnight incubation in the PCR machine at 14°C, ligations were heat-inactivated by incubation at 65°C for 20 minutes. Four hundred microliters of water were added to each of five microconcentrator devices, through which the ligations were

filtered. Each ligation reaction tube was washed with 80 µL of sterile water, and this volume was transferred to the appropriate microconcentrator device. The DNA was recovered using the microconcentrator procedure above. The purified DNA (typical volume approximately 20 µL in each tube) was transferred to microcentrifuge tubes and stored at -20°C until further use.

Preparing Electrocompetent Cells

One hundred microliters of an overnight culture of MC1000 *E. coli* cells were added to 100 mL of LB media and then transferred to a baffled Erlenmeyer flask using aseptic technique. The flask was placed in the incubator at 37°C with shaking at 250 rpm. The MC1000 cells were grown to an OD_{650} of approximately 0.5, and were transferred to tubes and placed on ice. After chilling, the tubes were centrifuged for 10 minutes at 4,400 x g and 4°C, and the supernatant was discarded. Five milliliters of ice cold 10% glycerol was added to each of the tubes and mixed thoroughly for resuspension of the pellet of cells. The cells were centrifuged again using the same conditions, and the supernatant was discarded. The process of adding glycerol, mixing, centrifuging, and removing the supernatant was repeated once more. After the final spin and removal of the supernatant, approximately 1.4 mL of 10% glycerol was added to each of the tubes to resuspend the pellet of cells. The mixtures were then transferred to microcentrifuge tubes. These tubes were centrifuged for 10 minutes at 6,000 x g and 4°C. The supernatant was discarded, leaving about 40 µL behind in the tubes to resuspend the pellets. These tubes were stored in a -80°C freezer or used immediately.

Electroporation

Ten microliters of each purified heat-inactivated ligation reaction were added to the ice cold electrocompetent cells. The mixtures were added to chilled electroporation cuvettes (2 mm gap; BioExpress; Cat. No. E 5010-2), and electroporated (settings 2 mm gap, 2.5 kV). The electric shock of electroporation allows the cells to take up DNA. After electroporation, 900 µL of Super Optimal Broth with Catabolite repression (SOC; Appendix A) was added to each of the cuvettes. The broth and cells were mixed well and then transferred to 15 mL conical tubes. The tubes were incubated with 250 rpm shaking at 37°C for one hour.

After incubation, different aliquots (in 50, 100, and 150 µL quantities) of the electroporated cells were spread onto MacConkey-lactose plates supplemented with ampicillin (Appendix A). The plates were then incubated overnight at 37°C. The transformants were then streaked onto MacConkey-lactose-ampicillin plates to verify the phenotype. The plates were placed in an unsealed plastic bag and incubated overnight at 37°C.

Growth of Transformants

Five milliliters of LB media supplemented with ampicillin (100 µg/mL final concentration) was added to each of four test tubes. Two colony-purified clones containing synthetic DNA insert and two colony-purified clones containing PCR product insert were inoculated separately into four individual tubes containing media. All tubes were incubated with shaking overnight at 37°C at 250 rpm.

The overnight cultures of the 4 purified reporter fusion constructs were centrifuged for 10 minutes at 13,400 x g. The cell pellets were resuspended in 2.5 mL of 10 mM $MgSO_4$. One milliliter of cells from one colony-purified clone containing synthetic DNA was transferred into a microcentrifuge tube, and a second milliliter was transferred to a separate microcentrifuge tube for a total of 2 tubes. This process was repeated with the three remaining overnight cultures containing the colony-purified clones for a total of 8 microcentrifuge tubes (2 for each purified reporter fusion construct) each containing 1 milliliter of clones.

The Zyppy Plasmid Miniprep Kit (Zymo Research; D4036) was used to purify the clones. The Zyppy protocol is as follows. The cells were centrifuged for 5 minutes at 13,400 x g, and the supernatant was discarded. Each pellet of cells was then resuspended in 600 µL of water. One hundred microliters of 7X Lysis Buffer was added to each of the tubes, which were each inverted four to six times to mix. Three hundred fifty microliters of Neutralization Buffer were added to each of the tubes, which were mixed thoroughly by inverting two to three times. The tubes were centrifuged at 11,000 – 16,000 x g for two minutes. The supernatants were transferred to Zymo-Spin IIN columns that were placed in collection tubes and centrifuged as above for 15 seconds. After the flow-through was discarded, each column was returned to its collection tube. Two hundred microliters of Endo-Wash Buffer was added to each of the columns, which were centrifuged as above for 30 seconds. Four hundred microliters of Zyppy Wash Buffer was then added to each of the tubes, which were centrifuged as above for one minute. The columns were transferred to clean microcentrifuge tubes, to which 50 µL of Zyppy Elution Buffer was added. The tubes

were allowed to stand for one minute, and were then centrifuged as above for 30 seconds to collect the purified DNA.

DNA Sequencing

The purified plasmid DNA containing synthetic DNA insert or PCR product insert were sequenced using dideoxy sequencing technology. The amount of DNA and the amount of water in each reaction depended upon the original concentration of DNA as determined by spectrophotometric analysis.

X µL DNA (100 ng/µL)
1 µL RK1 primer (100 pmol/µL stock)
2 µL 5X sequencing buffer
X µL water
2 µL Sequencing Juice (Applied Biosystems BigDye® Terminator v3.1 Cycle Sequencing Kit; Part No.: 4336917)

10 µL total

The sequencing juice was added last in each of the reactions, which were then mixed thoroughly. After purification of the samples (protocol below), the reactions were run according to the thermocycler settings in Table 5.

Table 5. DNA Sequencing

	Temperature (°C)	Time (minutes)
Step 1	96	0.5
Step 2	50	0.25
Step 3	60	4.0
Step 4	72	1.0
Step 5	Return to step one and repeat cycle 24 times	

Step 6	10	Forever
Step 7	End	

Purification of DNA Sequencing Reaction

To purify the DNA sequencing reactions the SigmaSpin Sequencing Reaction Clean-up Kit (Sigma-Aldrich; S5059-70EA) was used according the manufacturer's protocol. The purified samples were then dried using a CentriVap Concentrator (LABCONCO; Cat. No. 7810000). After drying, the samples were suspended in 15 µL of formamide prior to sequencing.

β-Galactosidase Assays

Bacterial cultures containing the plasmid constructs were grown to an OD_{650} between 0.2 and 0.6 in LB media at 37°C and 250 rpm shaking. The three constructs used, all of which lacked the putative UP element-like sequence, were 1365 (contains the reporter plasmid with synthetic DNA made by annealing oligonucleotides RK775 and RK776), 1366 (contains PCR product made by using RK767 and RK774), and 1367 (contains the reporter plasmid with the cloned synthetic DNA made by annealing oligonucleotides RK775 and RK776). The assay tubes contained the following mixture:

0.1 mL culture (strain)
0.9 mL Z buffer
50 µL 10% SDS
100 µL chloroform

Chloroform was utilized to permeabilize the cells in preparation for addition of substrate. After adding cells, the tubes were vortexed and then placed in a 28°C water bath for five

minutes. The assays were initiated by adding 200 µL of substrate ONPG (4 mg/mL) to a tube, and incubating at 28°C until a light yellow color developed. At this point, reactions were stopped by adding 500 µL of 1M sodium carbonate, and the time was recorded. After all the reactions were completed, the absorbance values at 420 nm and 550 nm were measured for each sample. Miller units of β-galactosidase activity were calculated using the following equation:

$$Miller\ units = \frac{1000\ x\ (OD_{420} - 1.75\ x\ OD_{550})}{time\ x\ volume\ x\ OD_{650}}$$

CHAPTER THREE

RESULTS

This study focused on a recently identified mutant bacteriophage, phage O367, that was selected to grow on an antitermination-defective *E. coli* host (*rpoCY*75N). It was shown that the "orc" mutation of phage O367 created a new promoter sequence that allowed the phage to overcome the *rpoCY*75N host mutation. It was observed that the sequence upstream of the newly discovered promoter region resembled a bacterial UP element, which has been shown to increase promoter activity [5]. The purpose of this study was to determine whether this sequence functions as an UP element by determining its contribution to promoter activity. To accomplish this task, reporter gene fusions were created in which the UP element-like sequence was deleted. Promoter activity was analyzed qualitatively on differential bacteriological media and quantitatively by β-galactosidase assays. The results demonstrated an approximately two-fold decrease in *lacZ* gene expression without the UP element-like sequence. Previous research indicates that, in order to be characterized as an UP element, the sequence must have at least a 1.5-fold effect on promoter activity [10]. Our results show that the UP element-like sequence in phage O367 satisfied this minimum requirement and can thus be classified as an UP element.

Efficiency of Plating

Dilutions of phage O276 (phage titer: 6.25 x 10^9 pfu/mL) and O367 (phage titer: 1.32 x 10^{10} pfu/mL) were plated onto *E. coli* strains RK898 (wild type *E. coli* host) and RK899 (restrictive host containing the *rpoC*Y75N mutation) to determine their plating efficiencies. As expected, strain 898 permitted growth of both phages. Phage O276 exhibited no growth on strain 899, while the mutant bacteriophage O367 produced individual countable plaques. The plaque sizes of phage O367 on the restrictive bacterial host were much smaller than the plaques of phage O367 on the wild type 898 host (Figure 12). Table 6 shows the results of the phage plating.

Table 6. Efficiency of Plating

***rpoC* Allele**	**Phage**	**Phage Titer**	***Relative EOP**
RK898	O276	1.2 x 10^9 pfu/mL	1.00
RK898	O367	2.8 x 10^8 pfu/mL	1.00
RK899	O276	0	$<10^{-9}$
RK899	O367	8.2 x 10^7 pfu/mL	0.3

* The efficiency of plating (EOP) for phage O276 on host 899 is relative to phage O276 titer on host strain 898 which was set to 1.00. The EOP for phage O367 on host 899 is relative to phage O367 titer on host 899 which was set to 1.00.

These results show that the O367 *orc* mutation allows the phage to partially overcome the effect of the host *rpoC*Y75N mutation.

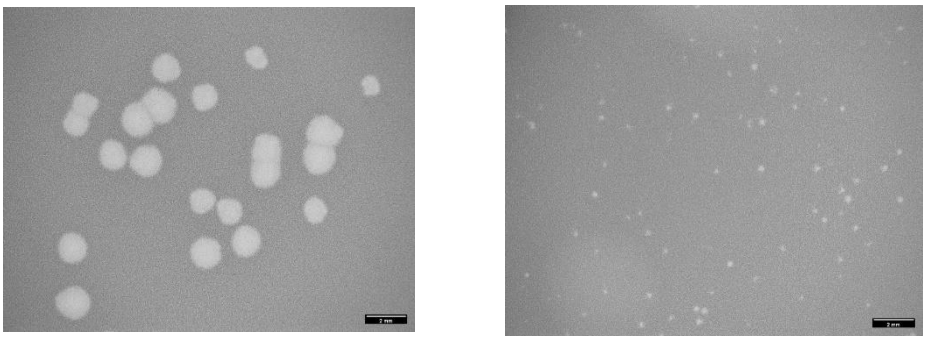

Figure 12. The figure on the left demonstrates the plaque sizes of phage O367 on the wild-type 898 *E. coli* host. The figure on the right demonstrates the plaque sizes of phage O367 on the restrictive 899 *E. coli* host. Scale bar in the bottom right is 2 mm.

Agarose Gel Confirmation of Double Digestion of Plasmid pRAK31 with EcoRI & BamHI

To prepare the promoter probe vector pRAK31 for the DNA insert, it was double-digested with the restriction enzymes EcoRI and BamHI. To determine if the promoter probe vector was successfully cut by EcoRI and BamHI, the DNA was run on 1% agarose gel. A razor blade was used to cut out the desired plasmid pRAK31 band, visualized under UV light (Figure 13). Band B shown on the gel is the excised promoter region of plasmid pRAK31. Because it is a separate band from the plasmid itself, the gel shows that the region was successfully separated from the plasmid backbone. Successful removal of this region prepared the promoter probe vector for the desired DNA insert. The vector backbone was extracted from the gel using the phenol-chloroform freezing method described in the materials and methods above.

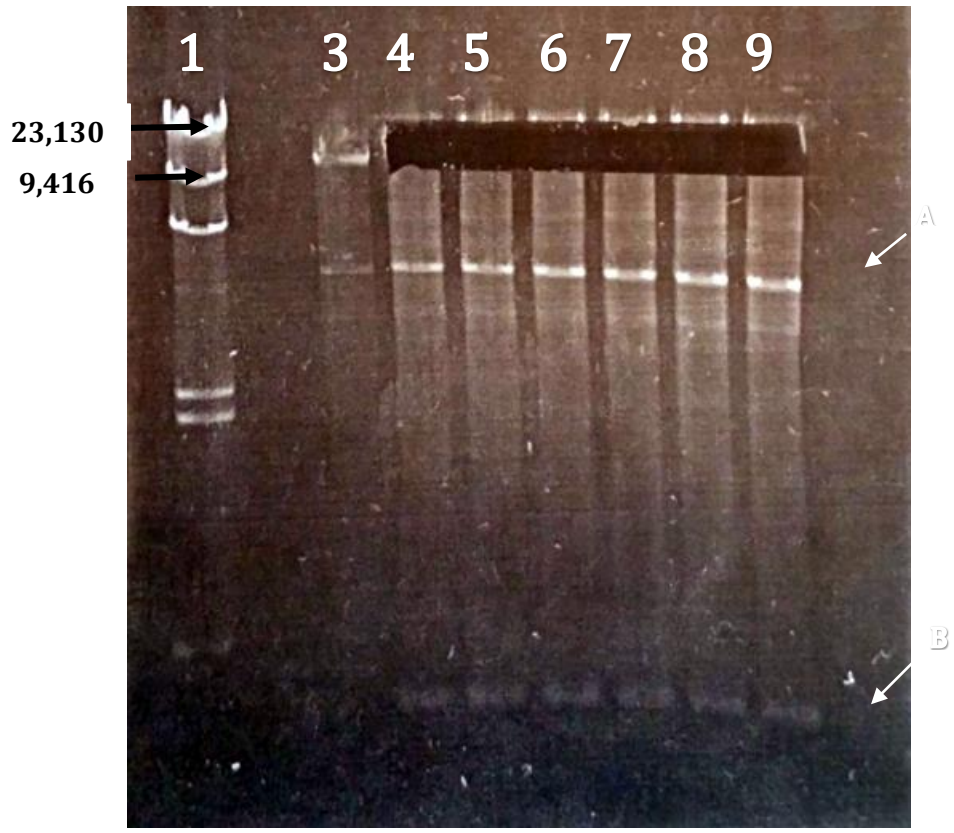

Figure 13. A 1% agarose gel of double digested promoter probe vector pRAK31. Lane 1: Lambda HindIII ladder. Lanes 3-9: double digested promoter probe vector. The cut segment is the vector without its promoter region. Band A is a second plasmid, pMOC170, that is present in the host from which the pRAK31 plasmid was isolated. Band B is the HK022 pL promoter region that has been excised from plasmid pRAK31.

Agarose Gel Confirmation of Plasmid pRAK31 Purification

A 1% gel was run on the purified promoter probe vector to confirm that the vector was purified appropriately with the phenol-chloroform purification method described above. Successful purification indicates that all restriction enzymes were removed. The gel shows only one segment of DNA of an expected size of approximately 13,000 base pairs (Figure 14). This result indicates that the plasmid DNA was successfully purified.

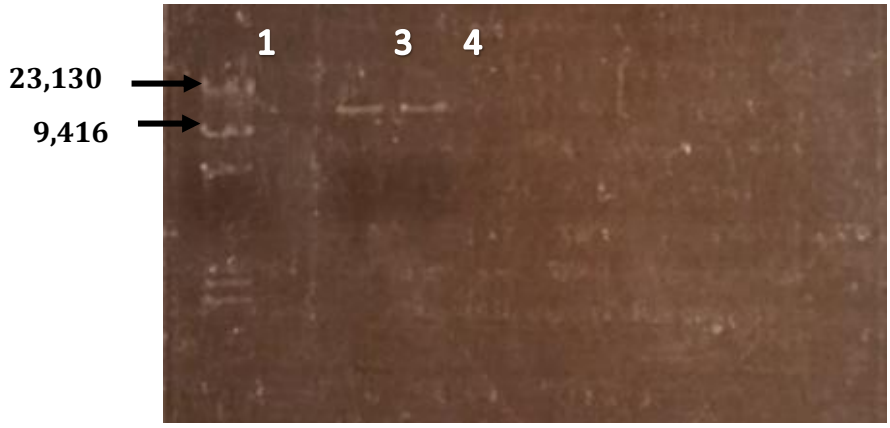

Figure 14. A 1% agarose gel of purified promoter probe vector. Lane 1: Lambda HindIII ladder. Lanes 3 and 4: purified promoter probe vector.

Agarose Gel Confirmation of Annealed Complementary Oligonucleotides RK775 and RK776

The annealed oligonucleotides RK775 and RK776 contain the promoter sequence created by the "orc" mutation in phage O367 but lack the putative UP element. This synthetic DNA was designed to help determine the role of the UP element in promoter activity. To test for the successful annealing and purification of the resulting double-stranded DNA, a 2% agarose gel was run (Figure 15). Because the annealed DNA fragment was small, a 100bp size marker was used to verify its size. The expected size of the fragment was approximately 100 base pairs.

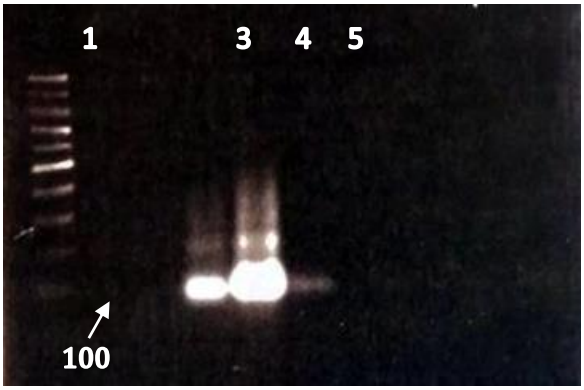

Figure 15. A 2% agarose gel of annealed oligonucleotides RK775 and RK776. Lane 1: 100bp ladder. Lane 3: purified annealed, eluted oligonucleotides. Lane 4: unpurified annealed oligonucleotides. Lane 5: PCR product amplified with oligonucleotides RK767 and RK774.

Deletion of the UP Element-like Sequence using Targeted PCR Amplification

PCR was a second approach used to delete the UP element-like sequence from the newly identified promoter in phage O367. Oligonucleotides RK767 and RK774 were used to generate the desired amplicon from a template that contained the newly discovered promoter. The PCR amplicon, just like the synthetic DNA construct, was designed to determine the effect of the UP element on promoter activity. A 2% agarose gel was run to confirm the success of the reaction. The expected size of the DNA fragment was approximately 100 base pairs (Figure 16).

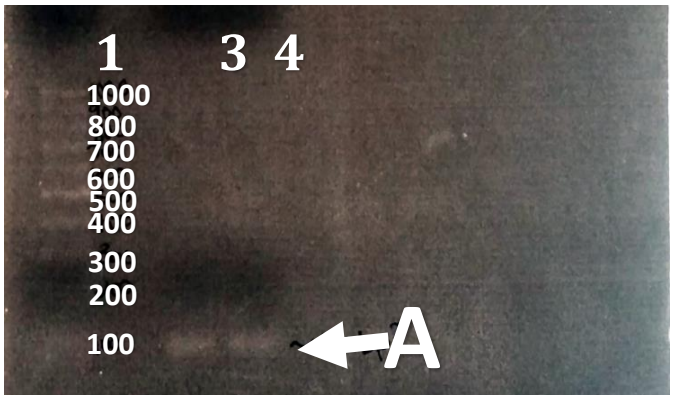

Figure 16. A 2% agarose gel of the PCR product amplified with primers RK767 x RK774. Lane 1: 100bp ladder. Lanes 3 and 4 (Band A): PCR product, approximately 100 base pairs in length.

Phenotypes of MacConkey-lactose Plates

To qualitatively determine promoter activity in constructs lacking the UP element-like sequence, transformants were plated on MacConkey-Lactose plates supplemented with ampicillin. The expression of the *lacZ* operon found in the promoter probe vector is evidenced by the fermentation of lactose in the bacteriological medium. As lactose is fermented, the pH decreases—MacConkey-Lactose plates contain an indicator dye that turns red in an acidic environment. Therefore, red colonies are evidence of lactose fermentation and, thus, promoter activity. Conversely, white colonies signify a lack of promoter activity.

A positive control and a negative control were used in the analysis of promoter activity using the MacConkey-Lactose indicator plates. The negative control construct, construct 1359, contains no promoter sequence and exhibited no evidence of fermentation activity on the indicator plates, appearing as white colonies. Construct 1360, the positive control, contained both the promoter sequence and UP element-like sequence of phage O367. The colonies on these plates were red, indicating gene expression (Figure 17).

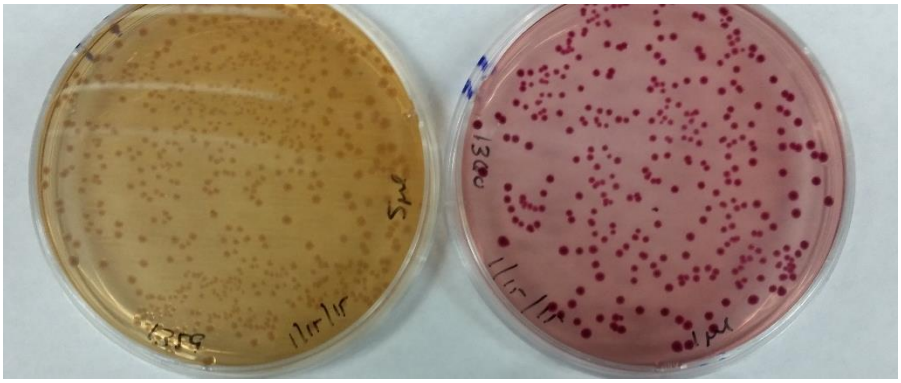

Figure 17. MacConkey-Lactose indicator plates. The plate on the left contains construct 1359, which appears white, indicating no reporter gene expression. The plate on the right contains construct 1360, which appears red because of expression of the *lacZ* gene.

Plates containing the synthetic DNA fusion (annealed oligonucleotides RK775 and RK776) exhibited red colonies, indicating promoter activity in the absence of the UP element-like sequence. Similarly, plates containing the fusion of PCR product amplified with RK774 and RK767 had red colonies, again demonstrating promoter activity in the absence of the UP element-like sequence (Figure 18). These results qualitatively indicate the existence of promoter activity even in the absence of the UP element-like sequence.

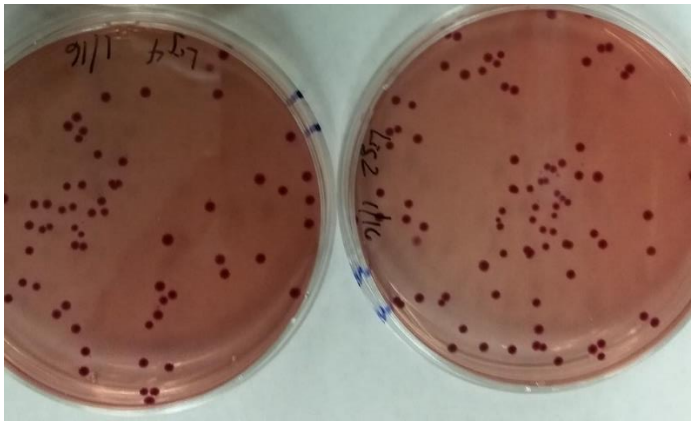

Figure 18. The left plate contains the construct containing the PCR product amplified with RK767 and RK774. The right plate contains the construct containing annealed oligonucleotides RK775 and RK776. Both constructs lack the UP element. The red colonies demonstrate *lacZ* gene expression and therefore promoter activity.

β-Galactosidase Assays

β-galactosidase assays were used to quantitatively determine the effect of the putative UP element-like sequence on the promoter activity of phage O367. The constructs utilized in the assays were 1359, 1360, 1365, 1366, and 1367. All constructs were created in the King lab. Construct 1359, derived from phage O276, has no promoter sequence and served as a negative control (9 Miller units of activity). Construct 1360, derived from mutant phage O367, contained both a promoter region and the UP element-like sequence and generated an average of 16,400 Miller units. This construct served as a positive control.

Constructs 1365, 1366, and 1367 did not contain the UP element-like sequence. The difference in Miller Units between Construct 1360 and the three constructs lacking the UP element-like sequence demonstrates the degree that the sequence affects promoter activity. Construct 1365 exhibited an average of 6,958 Miller units of activity, construct 1366 exhibited an average of 7,615 Miller units of activity, and construct 1367 exhibited an average of 7,590 Miller units of activity. The results of the assays are summarized in Table 7.

Table 7. β-galactosidase Assays

STRAIN	**Features**	**Average Miller Units**	**Standard Error of the Mean (+/-)**
1359	No promoter	9	1.4
1360	Promoter with putative UP element	16,400	1889
1365	Promoter without putative UP element (cloned DNA was made by annealing oligonucleotides RK775 and RK776)	6,958	533
1366	Promoter without putative UP element (cloned DNA amplified using oligonucleotides RK767 and RK774)	7,615	637
1367	Promoter without putative UP element (cloned DNA was made by annealing oligonucleotides RK775 and RK776)	7,590	665

These results demonstrate approximately a two-fold difference in promoter activity between constructs containing both the promoter region and the UP element-like sequence and similar constructs without the UP element-like sequence. The results show that the putative UP element-like sequence has a significant effect on promoter activity and can be classified as an UP element. Published studies show that a 1.5-fold effect on promoter activity is the minimum required effect for characterization as an UP element [10]. Because some UP elements affect promoter activity by as much as 326-fold, the UP element sequence of phage O367 appears to have only a weak effect on promoter activity (Table 8) [5, 10].

CHAPTER FOUR

DISCUSSION

The phage that is the focus of this work, phage O367, contains a two base pair change from its parent, phage O276. This two base pair change created -10 promoter hexamer in phage O367, effectively creating a new promoter region. Upstream of this new promoter region is a sequence that has homology with an UP DNA sequence element, a sequence known to boost promoter activity [5]. The goal of this project was to determine if this UP element-like sequence does, in fact, affect the activity of the newly created promoter. This task was accomplished by deleting the UP element and measuring the effect of the deletion on promoter activity. A comparison of promoter activity in the presence and absence of the UP element-like sequence was analyzed qualitatively using indicator plates and quantitatively using β-galactosidase assays.

The quantitative analysis of the β-galactosidase assays suggest that the UP element-like sequence of bacteriophage O367 stimulated promoter activity by approximately two-fold, demonstrating a positive role of the sequence in promoter activity. Although some UP elements increase promoter activity up to 326-fold, the effect can be significantly less pronounced—for instance, the removal of the UP element in *E. coli* promoter *merT* causes only a 50% reduction in promoter activity [10]. The removal of the UP element from phage O367 caused a 55% reduction in promoter activity. Our

results thus show that the UP element-like sequence of phage O367 can be classified as an UP element. The following table lists various UP elements and their effect on promoter activity.

Table 8. UP Elements and Their Effect on Promoter Activity [10]

E. coli Promoter containing UP Element	Degree of Similarity to Consensus Sequence	Effect on Promoter Activity
rrnD P1	18 out of 22	93.0-fold
rrnB P1	18 out of 22	33.3-fold
rrnB P2	14 out of 22	12.8-fold
RNA II	13 out of 22	4.9-fold
O367	**14 out of 22**	**2.2-fold**
merT	17 out of 22	2.0-fold
lac	12 out of 22	1.5-fold

The above survey of UP elements and their effects on promoter activity confirm that the UP element-like sequence in phage O367 fits the definition of an UP element. The UP element sequence of O367 contains a significant number of matches to the UP element consensus sequence, but is lower in matches with the consensus sequence and has a weaker effect on promoter activity than other UP elements that have been characterized.

Plaque Sizes on Restrictive Bacterial Host

Although bacteriophage O367 can grow on the restrictive *E. coli* host while the parent phage O276 cannot, the plaques that formed were significantly smaller, as shown

in Figure 11. The efficiency of plating (Table 6) shows that O367 is about half as efficient on the restrictive host as on the non-restrictive host. The difference in plaque size demonstrates that while the mutation in bacteriophage O367 can overcome the restrictive bacterial host, the mutation does not completely restore a wild type phenotype in terms of plaque size.

Synthetic DNA versus PCR Product

The synthetic double-stranded DNA formed by annealing complementary oligonucleotides RK775 and RK776 and the PCR product amplified with RK767 and RK774 primers were two approaches used to generate the desired UP element deletion constructs. The main difference between the synthetic DNA (RK775 and RK776) sequence and the PCR product (RK767 and RK774) is that the synthetic DNA sequence has one additional upstream base pair. The two different methods were utilized to increase the likelihood of success. The synthetic DNA method can generate a low yield of double-stranded DNA product; on the other hand, PCR tends to produce large amounts of double-stranded DNA. The efficiency of PCR can, however, be decreased due to poor priming or multiple priming sites. The two methods ultimately produced nearly identical results in terms of promoter activity, demonstrating that both methods were effective.

CHAPTER FIVE

CONCLUSION

The red colony phenotype on the MacConkey-Lactose-Ampicillin plates showed qualitatively that the deletion of the putative UP element-like sequence did not eliminate promoter activity, but the β-galactosidase assay demonstrated quantitatively that there is a two-fold difference in activity between the original promoter fusion and the fusion which lacks the putative UP element sequence. These results suggest that the deleted sequence contributes to the overall activity of the promoter, and that the sequence behaves like other characterized UP elements [10].

CHAPTER SIX

RECOMMENDATIONS

The results of this study show that the UP element sequence identified in phage O367 contributes to promoter activity. However, the study does not reveal if this UP element sequence is required for growth of phage O367 on a non-permissive bacterial host. Determining whether the sequence is essential for growth could be a direction for future work.

Because removal of the UP element caused only an approximately 55% decrease in promoter activity, it can be classified as an UP element with a fairly weak effect on promoter activity. It is likely that the effect is not more pronounced because the sequence only matches the consensus sequence for UP elements in 14 out of 22 base pairs. An experiment could be conducted to produce an UP element more closely resembling the UP element consensus sequence to determine if these alterations increased the effect on promoter activity.

The location of the new promoter region of phage O367 is downstream of three lambda transcription terminators. The promoter sequence thus allows RNA polymerase to bypass these terminators and initiate transcription downstream from these sites. However, there is a single terminator located downstream of the promoter. Another direction for

future work could be to remove this terminator and determine if its removal restores the efficiency of transcription and plaque size of phage O367 on the restrictive host.

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APPENDIX A

The recipes for the various media used in the above procedures are as follows:

1. Luria Broth (LB): 5g NaCl (Fisher BioReagents; BP358-212), 5g Yeast Extract (BD; 212750), 10 g Tryptone (Fisher BioReagents; BP9726-500), 15 g Agar (Difco; 214530), per liter in H_2O. Autoclave. Let cool and pour into petri plates (USA Scientific; 100x15mm; 8609-0010).
2. Luria Broth Top Agar (LB Top Agar): 5g NaCl (Fisher BioReagents; BP358-212), 5g Yeast Extract (BD; 212750), 10 g Tryptone (Fisher BioReagents; BP9726-500), 7 g Agar (Difco; 214530), per liter in H_2O. Aliquot 100 mL Top Agar to 125 mL screw cap bottles. Autoclave.
3. Luria Broth (LB), Lennox Recipe: 5g NaCl (Fisher BioReagents; BP358-212), 5g Yeast Extract (BD; 212750), 10 g Tryptone (Fisher BioReagents; BP9726-500) per liter in H_2O. Aliquot 100 mL LB broth to 125 mL screw cap bottles. Autoclave.
4. Super Optimal Broth (SOC): 20 g Tryptone (Fisher BioReagents; BP9726-500), 5 g Yeast Extract (BD; 212750), 0.5 g NaCl (Fisher BioReagents; BP358-212), 1.25 mL 2M KCl (Acros; CAS: 744-40-7), 10 mL 1M $MgCl_2$ (Fisher Chemical; M-13448), per liter in H_2O. Aliquot 100 mL SOC to 125 mL screw cap bottles and autoclave. Let cool then add 1 mL 1M $MgSO_4$ (Sigma; M-2773) and 2 mL glucose (Fisher Scientific; D16500)

5. MacConkey-Lactose-Ampicillin: 50 g Difco MacConkey Agar (Difco; 281810) per liter in H_2O. Autoclave. Let cool to 45°C then add 100 µg/mL of Ampicillin (Fisher BioReagents; BP-1760-25). Pour into petri plates (USA Scientific; 100x15mm; 8609-0010).

The recipes for the TAE buffer are as follows:

1. 50X TAE: 242 g Tris-base (Fisher BioReagents; M-11645), 57.1 mL Glacial Acetic Acid (Amresc; 0714-4L), 100 mL of 0.5M EDTA (Sigma; E-5134). Dilute to 1L with H_2O. Store at room temperature.
2. 1X TAE: Aliquot 20 mL of 50X TAE to a 1L screw cap bottle and fill to 1L line with H_2O. Store at room temperature.

The recipe for the loading dye is as follows: mix 0.25% bromophenol blue (Sigma-Aldrich; CAS: 115-39-9), 0.25% xylene cyanol FF (Research Organics; 7113X), and 30% glycerol (Fisher Chemical; CAS; 56-81-5) in a 15 mL tube. Aliquot 500 µL to a 1.5 mL microcentrifuge tube. Store at room temperature.

The recipe for the PCR mix is as follows: 6 µL of 10mM dATP, 6 µL of 10mM dGTP, 6 µL of 10mM dCTP, 6 µL of 10mM dTTP, 330 µL 10X Buffer A (Fisher Scientific Kit; FB-6000-10), 316 µL nanopure H_2O. Mix in 1.5 mL microcentrifuge tube and keep on ice if using immediately or freeze at -20°C.

The recipes for the solutions used in the Beta-Galactosidase assay are as follows:

1. Z Buffer: 16.1 g $Na_2HPO_4 * 7H_2O$ (Sigma-Aldrich; 7782-85-6), 5.5 g $NaH_2PO_4 * H_2O$ (Sigma-Aldrich; 10049-21-5), 0.75 g KCl (Acros; CAS: 744-40-7), 0.246 g

$MgSO_4$ * $7H_2O$ (Sigma-Aldrich; 10034-99-8), 2.7 mL β-mercaptoethanol (Sigma-Aldrich; M-3148). Do not autoclave. Adjust pH to 7.0 and store in refrigerator.

2. 10% SDS: 1 g SDS (Sigma-Aldrich; L6026) in 10 mL H_2O. Equilibrate in 55°C water bath to dissolve.