Characterization of a Mutant Bacteriophage that Overcomes An Antitermination Defect in E. coli RNA

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CHARACTERIZATION OF A MUTANT BACTERIOPHAGE THAT OVERCOMES AN ANTITERMINATION DEFECT IN E.COLI RNA POLYMERASE

A Capstone Experience/Thesis Project

Presented in Fulfillment of the Requirements for
the Degree Bachelor of Science with
Honors College Graduate Distinction at Western Kentucky University

By
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*****

Western Kentucky University
2014

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Department of Biology
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ABSTRACT

Mutations in the highly conserved zinc-binding domain of the β’ subunit of *E.coli* RNA polymerase prevent the growth of phage that use a unique RNA-based mechanism of transcription antitermination. Here, we describe the isolation and characterization of a mutant phage that overcomes this block. The genome of the mutant only differs from the parental phage by 2 nucleotides. Close inspection of the sequences surrounding the mutation suggested that a new promoter had been created. This was confirmed by cloning the potential promoter sequences into a promoter probe expression vector. We hypothesize that the new promoter permits the expression of phage genes that are essential for growth on the mutant bacterial host.

Keywords: Antitermination, Mutant Phage, Escherichia coli, RNA Polymerase, Reporter Plasmids, Genomics
Dedicated to my family and friends
ACKNOWLEDGMENTS

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CHAPTER 1

INTRODUCTION

Gene expression is the process by which DNA information is transformed into functional products, such as proteins. The model that summarizes this process is known as the Central Dogma: DNA is transcribed to mRNA and mRNA is translated into protein [1]. Gene expression is a highly regulated event and regulation often occurs at the transcriptional level. The enzyme responsible for all transcription is RNA polymerase. Proper functioning RNA polymerase is essential for cell viability [2].

For viruses, whose survival depends on the utilization of the host’s replication, transcription, and translation machinery, it is especially important that RNA polymerase functions properly. Because of this dependence, viruses can be used as a model to understand how mutations in RNA polymerase affect its function [2].

Viruses that infect bacteria are called bacteriophages or phage. Temperate phages can adopt two different life cycles: lysogenic or lytic. The phage life cycles have been extensively studied in order to understand viral growth properties, gene expression and virus-host interactions. [2]

The Lytic Cycle

Phage infection begins when it attaches to host cell surface receptors. The genomic material is then injected into the host cell cytoplasm. Upon successful entry, the DNA circularizes and phage gene expression begins. Phage genes are expressed in a
specific order to ensure that key components of the phage are produced as needed. Once the phage has generated all of its structural components, the DNA is packaged and the particles are assembled. At the end of the infection cycle, lysin, an enzyme that directs cell lysis, is made. This enzyme causes degradation of the cell wall, which weakens it, allowing it to succumb to osmotic pressure and resulting in cell lysis. New phage particles erupt from the cell to continue to the cycle by infecting other bacterial hosts. [2] See Figure 1.

**Lysogenic Cycle**

The lysogenic cycle is begins similarly to the lytic cycle. The phage attaches to the bacterial cell and injects its DNA into the host. However after injection and circularization of the phage DNA, the phage genome is integrated into the host chromosome and becomes a “prophage”. Due to the activity of the phage repressor protein, most phage genes are not expressed in the prophage. The phage can exist in this stage through multiple cellular replications. A prophage can become a lytic phage either spontaneously or in response to a stimulus such as DNA damage. This causes the phage genome to excise and enter the lytic cycle. See Figure 1.
Figure 1. Lytic vs. Lysogenic cycles. Part A shows the initial encounter between a phage and a host bacterium. Part B shows the attachment by the phage and injection of its DNA. Part C. The phage DNA circularizes. Parts D and E show the replication of the phage inside the cell and rupture of the cell in the lytic cycle. Parts F and G show the insertion of the phage DNA into the chromosome and continual replication of the bacterial chromosome with the prophage. The red represents bacteria chromosomal DNA while the green represents phage DNA. [3]

Gene Expression

As previously mentioned, gene expression is the process by which information found in DNA is transformed into products such as RNA and proteins. It is the expression of these genes that give rise to the phenotype of the organism. This occurs in two general steps: transcription (the process of copying DNA into RNA) and translation (the process of making proteins from the RNA message). [2]

Transcription

Bacterial transcription begins when RNA polymerase binds to the DNA at a promoter sequence. *E.coli* RNA polymerase is comprised of five subunits to recognize components of a promoter: $\beta$, $\beta^\prime$, $\alpha$, and $\omega$ subunits (shown in Figure 2; $\omega$ is not pictured). An additional subunit called $\sigma$ gives RNA polymerase its sequence specificity by recognizing the promoter consensus sequence, which is the most commonly recognized sequence for that promoter. There are many $\sigma$ factors that recognize different consensus sequences. The $\sigma^{70}$ subunit confers sequence specificity to the RNA
polymerase by recognizing a TTGACA hexameric sequence at the -35 region and TATAAT hexameric sequence at the -10 region relative to the start of transcription (designated +1). [4, 5]

![Figure 2. E. coli RNA Polymerase bound to a bacterial promoter. The RNA polymerase is represented in red and different subunits are labeled. The orange represents the sigma-70 factor that recognizes the promoter. The direction of transcription is indicated along with the -35, -10, and +1 regions. [2, 6](image)](image)

Promoters can vary in strength depending on how closely they match the consensus promoter sequence and the distance between the -35 and -10 elements. The optimal distance is 17 base pairs. Deviation from the consensus spacing typically results in a weaker promoter. [4, 5] Sequences such as an UP element can compensate for nonconsensus promoter sequences.

**UP element**

An UP element is a sequence found upstream of the -35 region in some bacterial promoters. This sequence increases transcription by interacting with the α subunit of polymerase. The standard consensus sequence of an UP element is shown in Figure 3 [7].
Figure 3. UP Element consensus sequence. The bottom line shows the UP element consensus sequence. The top shows the frequency of nucleotides occurring at each position in the UP element sequence; taller nucleotides correspond to an increased frequency of occurrence. [7]

The UP element sequence is typically located between -59 and -38 relative to the start site of transcription. Published experiments have shown that presence of an UP element sequence can increase transcription by as much as 326-fold. The size of the letter in the top row of Figure 3 indicates the frequency of the nucleotide occurring in the UP element sequence. The bottom row is a consensus sequence for UP elements, with the most common nucleotides indicated. Positions marked with an “n” can be any nucleotide. Eleven matches out of 15 are sufficient to confer activity. [7]

Termination

Transcription terminators are sites that cause the RNA polymerase to stop transcribing. At these sites, the newly formed RNA is released and enzyme dissociates from the DNA. *E.coli* transcription terminators are divided in to two categories: rho-independent (or intrinsic) and rho-dependent. [2]

Rho-Independent Termination

Intrinsic terminators do not require additional proteins to assist in the termination process, but they do require structured RNA. These terminators contain a region that is rich in G-C bases that will allow it to fold upon itself into a structure called a hairpin, due to base complementarity. In addition to the hairpin, the RNA must also contain a region of seven to nine consecutive uracil residues at the base of the hairpin. Biochemical evidence has shown that the hairpin causes RNA polymerase to pause. This is thought to
provide time for the weak uracil bonds in the U-rich region of the transcript to disassociate from the DNA template and terminate transcription. Approximately half of all *E. coli* genes have intrinsic terminators at their 3’ end, especially those found in operons. [2] Figure 4 illustrates the general features of an intrinsic terminator.

![Figure 4](image)

**Figure 4.** Rho-independent terminator. The hairpin loop forms as a result of complementary base pair interactions in the G-C rich regions of the transcript. This structural feature interacts with RNA polymerase, shown faintly in purple, causing it to slow. The U-rich region, composed of weakly interacting uracil bases, dissociates from the DNA leading to transcription termination. [8]

**Rho-dependent Termination**

Rho-dependent termination requires the activity of a protein called “Rho”. This factor is found in *E. coli* and catalyzes transcription termination at rho-dependent terminators. Rho binds to the *rut* (*rho utilization*) site in the RNA transcript, which is a C-rich region approximately 70 bases long. It then moves along the RNA until it catches up with the stalled RNAP molecule. Once Rho catches up to the polymerase, its helicase activity dissociates the RNA-DNA hybrid and the transcript is released [2]. Figure 5 illustrates the events that occur during rho-dependent termination.
Figure 5. Mechanism of Rho-dependent transcription termination. RNA Polymerase binds promoter on the DNA and initiates transcription (A). The Rho protein (red) binds at the rut site on the mRNA (B), it then moves along the mRNA until it reaches the stalled polymerase (C), and causes dissociation of the ternary complex (D). [9]

Antitermination

Transcription antitermination was first discovered in experiments performed with bacteriophages and led to the discovery of phage-encoded antitermination proteins that promoted terminator read through. In this process, the final RNA product is elongated because polymerase is modified to ignore the terminator and continue transcription. The first antitermination mechanism was discovered in the phage Lambda.

Lambda phage

Two proteins mediate antitermination in bacteriophage Lambda: N and Q. The N protein binds to a specific 15-nucleotide RNA element called boxB, which is encoded at two different locations on the Lambda genome. One is located near the starting point of the P_L operon and the other is located after the first translated gene of the P_R operon. [10] The boxB sequence is located adjacent to a second critical sequence called boxA. These sequences constitute the N utilization site, or nut site. Binding of the N protein to the nut site in RNA, is stabilized by several host-encoded proteins called the Nus factors. This complex of proteins converts RNAP into a termination resistant form. [10]
The Q protein is responsible for antiterminating late gene expression that initiates at the late gene promoter \( P_R' \). Q is a DNA binding protein that specifically recognizes the \( qut \) site located just downstream of \( P_R' \). Q interacts with a stalled RNA polymerase and stabilizes the elongation complex. [10, 11] Figure 6 shows a simplified mechanism for antitermination.

![Figure 6](image_url)

**Figure 6.** Antitermination of transcription. The top half of the figure shows the normal progression of a termination event that results in the disassociation of the RNA polymerase (purple), from the DNA and the release of the transcript. The bottom half shows the antitermination protein in green interacting with the enzyme to allow transcription to continue into downstream genes allowing extension of the original nascent mRNA transcript. [12]

**Bacteriophage HK022**

HK022 is a lambdoid phage that antiterminates early gene expression in an unusual way. It does not encode an N-like antitermination protein. Instead, it promotes antitermination directly through the action of RNA molecules called \( put \) (polymerase utilization) sites. This type of antitermination is called factor-independent antitermination. [10] After the \( put \) regions are transcribed, they fold into a structure that contains two stem and loops separated by an unpaired base. (Figure 7) The folded RNA interacts with RNA polymerase to make it highly processive and termination resistant [7]. HK022 antiterminates late gene expression using a Q-protein dependent mechanism similar to that of the Lambda phage. [10]
Lambda and HK022 suppress both categories of terminators found in *E. coli*. This suggests the mechanisms of intrinsic and Rho-dependent terminators share common steps. Antitermination may be achieved by preventing destabilization of the RNA-DNA hybrid and/or accelerating RNA polymerase past termination sites [10].

**A Host Mutation that Blocks Antitermination**

The β’ subunit of RNA Polymerase is encoded by the *rpoC* gene. Mutations in this gene that block HK022 growth change amino acid residues in the highly conserved zinc-binding region (Figure 8). [14] These mutations block HK022 growth by preventing the interaction of the *put* sites with RNA polymerase [14].

---

**Figure 7.** RNA stem-loop structures of HK022 *putL* and *putR* [13].
Figure 8. Mutations in the $\beta'$ subunit of RNA Polymerase that prevent HK022 antitermination. Diagram of cysteine cluster that binds zinc in the $\beta'$ subunit. The arrows indicate the changes to the amino acids, which are number according to their appearance in the protein sequence [14].

**Reporter Vectors**

Plasmids are small, circular, self-replicating, extra-chromosomal DNAs. Although they are nonessential, they often contain genes that provide special capabilities to a cell, such as antibiotic resistance. Plasmids are a critical tool in molecular biology. [2] Reporter vectors are plasmids that are useful for identifying promoters. These vectors contain selectable markers and unique restriction sites for DNA insertion. Selectable markers are genes that allow for selection of transformed cells, such as antibiotic resistance. In a promoter probe vector, the reporter gene will only be expressed if the inserted DNA contains promoter activity and thus provides a way to quantify the activity of the promoter [2].

The reporter vector used in this study was the pRS415 plasmid. This vector contains the $lacZ$ reporter gene and the Ampicillin resistance gene used for selection. The plasmid has four tandem copies of the T1 terminator, upstream of the multiple cloning site (MCS) [15]. These serve to block transcription originating from upstream promoters. The MCS contains EcoR1, BamH1, and Sma1 restriction sites, which can be used to
clone DNA fragments that are suspected to contain promoter sequences [15]. The reporter gene is the lacZ gene and does not contain a promoter. The lacY and lacA are also part of the lac operon, where lacY encodes for permease, which is a molecule that brings lactose into the cell, and lacA encodes for an enzyme that transfers an acetyl group from acetyl-CoA to β-galactoside [15]. These genes are necessary for lactose catabolism [16]. Figure 9 illustrates important features of the pRS415 plasmid.

Figure 9. pRS415 plasmid. The lacZ reporter gene and the ampicillin resistance, Amp’, gene are labeled. The MCS region (gray) contains unique EcoR1, BamH1, and Sma1 restriction sites. The series of four strong terminators, T1, is denoted in orange [15, 17].
CHAPTER 2

MATERIALS AND METHODS

Bacteria and Phages used in this Study

*E. coli* strain MC1000 is the bacterial strain used in electroporation. This strain contains the $\Delta(lac)X74$ mutation, which makes it in defective in the metabolism of lactose [18]. Bacteriophage O276 originates from the study by Oberto, et. al [19] and contains the Lambda $b519$ and $b515$ deletions with the HK022 genes *nun*, *cl*, *cro* and *cII*. Bacteriophage O367 is a derivative of O276 and originates from this work.

Plasmid

The pRS415 reporter plasmid shown in Figure 9 was used to quantify promoter strength.

Plasmid DNA Isolation

Cells containing the pRS415 plasmid were grown in 125ml of TB (Appendix A) in an Erlenmeyer flask at 37°C with shaking (250 rpm) for 24 hours before isolation. Two 50ml aliquots were each placed into a 50ml culture tube (Fisherbrand Cat. No. 06-443-18) and centrifuged at 15500xg for 15 minutes at 4°C to pellet the cells. For DNA isolation, the protocol from the Qiagen Qiafilter Plasmid Midi Kit (Cat. No. 1243) was followed. The final volume for the plasmid preparation was 50μl. A NanoDrop Spectrometer was used to determine the purity and concentration of the sample.
To recover plasmids from the various clones of pRS415 made in this study, the QIAGEN QIAprep Spin Mini Prep Kit was used as recommended by the manufacturer (Cat. No. 27104).

**Preparing the pRS415 vector for cloning**

Restriction enzyme reactions for preparing the pRS415 vector for cloning DNA sequences amplified from phages O276 and O367 are shown in Table 1.

<table>
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<tr>
<td><strong>BamH1</strong></td>
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<td>20µl DNA</td>
</tr>
<tr>
<td>(780.2ng/µl)</td>
</tr>
<tr>
<td>5µl Buffer #3</td>
</tr>
<tr>
<td>(New England BioLabs; 10X Concentration; #B7003S)</td>
</tr>
<tr>
<td>20µl nanopure H₂O</td>
</tr>
<tr>
<td>3µl BSA buffer</td>
</tr>
<tr>
<td>(New England BioLabs; 10mg/ml; #B9001S)</td>
</tr>
<tr>
<td>2µl Enzyme</td>
</tr>
<tr>
<td>(New England BioLabs; 20000 u/ml; #R0136S)</td>
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<tr>
<td>Total reaction volume: 50µl</td>
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Digests were incubated at 37°C for 2-2.5 hours. A 5μl aliquot of each digest was analyzed by gel electrophoresis to ensure proper cutting. The expected fragment size was 10,746 base pairs.

To generate both cloning sites in the pRS415 vector, the digests were first purified using the chloroform-phenol extraction method to remove the enzyme and the buffer solution. After purification, the DNA was digested with the opposite enzyme. The double digested vector was analyzed for complete digestion via gel electrophoresis. Double digested vectors were further purified by gel purification and the phenol/freeze method.[21]

**Restriction Digest of PCR Amplified Phage DNA**

Phage PCR products were double digested with EcoR1 and BamH1 in a single reaction. A typical 50 μl reaction consisted of the following:

30 μl of DNA (456-2061 ng)

7μl of EcoR1 buffer (New England BioLabs; 10X Concentration; #B0101S)

2μl of BSA buffer (New England BioLabs; 10mg/ml; #B9001S)

9μl of H2O

1μl of EcoR1 enzyme (New England BioLabs; 20,000 u/ml; #R0101S)

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

50μl Total reaction volume

These reactions were incubated at 37°C for 2-2.5 hours and purified using the Qiagen QIAquick PCR Purification Kit (Cat. No. 28104) according to the manufacturer’s instructions.
Agarose Gel Electrophoresis

Gel electrophoresis was used to separate DNA molecules based on differences in size. Agarose (Fisher BioReagents: BP160-100) is a linear polymer of α- and β-glycosidic linkages of alternating residues of D- and L-galactose and is derived from a species of seaweed. [20]

Agarose gel provides a matrix through which DNA molecules can pass and be differentially separated based upon their length. Movement of the DNA is due to the electrical current that is passed through the gel. DNA moves from the negatively charged pole to the positively charged pole because of DNA’s overall negative charge. The size of the DNA fragment and the porosity of the gel affect the speed at which DNA moves through the gel. A higher concentration of agarose achieves better resolution of small DNA fragments, and lower concentrations of agarose allow for better resolution of larger fragments. Additionally, the applied voltage also affects the resolution of DNA fragments. [20]

Preparation of agarose gels

Agarose solutions used in this study were made by dissolving 1.2 grams of agarose in 100ml of 1X TAE buffer (Tris-base, acetic, acid, EDTA buffer; Appendix A). After boiling the solution, the molten agarose was cooled to 55°C before pouring 30ml into a gel mold. A comb was used to create the wells for sample loading. After cooling, the comb was removed from the hardened agarose and the gel was placed into an electrophoresis tank and submerged in 1X TAE buffer. Electrophoresis was carried out at 90V for 1 hour. Agarose gels with a higher concentration were run for 75 minutes.
Before being loaded into the wells of an agarose gel, DNA samples were first mixed with a loading dye (Appendix A). This dye facilitates sample loading in two ways. The color makes it easier to see the sample as it is pipetted into the wells of the translucent gel. It also contains glycerol, which increases the density of the sample. This ensures that the sample sinks into the well as it is being loaded. DNA ladders containing fragments of known size were included on all gels. The markers used in this study included the Lambda HindIII ladder (Fermentas; 0.5 mg/ml; #SM0101) and the 100 base pair ladder (Axygen Product No. M-DNA-100bp 500 μl).

**Visualization of DNA in agarose gels**

After electrophoresis, agarose gels were stained with ethidium bromide (0.5 μg/ml final concentration in 1X TAE buffer; Sigma-Aldrich 10 mg/ml stock; E1510-10 ml) for 10 minutes. The ethidium bromide inserts into the DNA and fluoresces when exposed to UV light. Photodocumentation was done with Alpha Innotech FluorChem HD2 (S/N: 504332; EPI UV Lights: Dual 254/365nm).

**Purification of Plasmids after Digestion**

Proteins were removed from the restriction digests using chloroform-phenol extraction (Chloroform: Fisher Chemical, C298500; Phenol: Fisher BioReagents. BP1750’-100). Briefly, an equal volume of chloroform and phenol were mixed in a microcentrifuge tube (total volume = 1ml). To facilitate pipetting, the restriction digest volumes were increased to 100μl with the DNA elution buffer (EB; QIAGEN; Mat. No. 1014608). An equal volume of the chloroform-phenol mixture was then added and vortexed until the solution turned milky white. The mixture was then centrifuged at 12000xg for ~15-20 seconds at room temperature. The aqueous (top) layer, which
contained the DNA, was transferred to a fresh microcentrifuge tube. The organic phase was back extracted once with 100μl of EB buffer to recover any remaining DNA and the aqueous phase was combined with the first extraction. The combined aqueous sample was extracted with an equal volume of phenol/chloroform twice more. Three extractions with an equal volume of chloroform were performed to remove any traces of phenol. The chloroform was removed by ultrafiltration.

**Ultrafiltration**

Buffer exchanges were accomplished using Amicon Ultra Centrifugal Filter Units (0.5ml; UFC 510096). A sample was placed into a filtration unit and diluted with nanopure H₂O (total unit volume = 500 μl). The sample was centrifuged at 6600xg for 10 minutes at room temperature in the microcentrifuge. The flow through was discarded. The filter cup was refilled with 500μl of nanopure H₂O and the centrifugation was repeated. The flow through was again discarded. The filter unit was then transferred to a fresh collection tube. A small amount (20μl) of nanopure H₂O was typically used to wash the filter surface before sample recovery. The sample was recovered from the unit by placing the inverted filter cup in a collection tube then centrifuging at 6600xg for 3 minutes. The final recovered volume was approximately 40μl.

For ligation reactions, the entire 20μl heat inactivated reaction was transferred to a filter unit. To maximize recovery of the ligation products, the reaction tube was washed with 80μl of nanopure H₂O, and this volume was added to the filter cup. Nanopure water was added (final volume = 500μl) and filtration was carried out as outlined above. For sample recovery, no additional water was added. In this case, the final recovered volume was approximately 20μl.
Gel Purification of DNA

Specific DNA fragments were recovered from agarose gels using the phenol/free method [21]. After electrophoresis and staining, the gels were placed on UV light box. Upon illumination, the desired DNA bands were cut from the gels using a razor blade. This was done as quickly as possible to minimize exposing the DNA to UV light. The gel slice was cut in half and placed into separate 1.5 microcentrifuge tubes. These larger pieces of gel were then minced with a needle to increase the surface area. Eight hundred μl of phenol was added and the mixture was vortexed thoroughly. The tubes were then incubated for 10 minutes at -80°C. The frozen samples were then centrifuged at 12000xg at room temperature for 10 minutes to separate the agarose and phenol from the aqueous layer. The aqueous (top layer), containing the DNA, was pipetted into a fresh tube and extracted three times with an equal volume of chloroform to remove any traces of phenol. The aqueous layer was then transferred to a fresh microcentrifuge tube and the DNA was recovered by ethanol precipitation.

Concentration of DNA by Ethanol Precipitation

Ethanol precipitation was used to concentrate DNA samples and remove residual agarose after the gel purification process. A 1/10th volume of 3M Sodium acetate (pH 5.2) was added to the DNA containing solution and mixed well. This was followed by the addition of two volumes of cold ethanol. After mixing, the samples were stored overnight at -20°C.

After overnight storage, the solution was centrifuged for 15 minutes at 16000xg at 4°C. The supernatant was removed quickly by inversion. The tubes were kept in an inverted position while the alcohol was allowed to evaporate. The precipitated DNA was
resuspended in 100μl of EB buffer and transferred to an Amicon Centrifugal Filtration device. The tube that contained the precipitate was washed with an additional 100μl of EB buffer and this was combined with the sample in the filter cup. Three hundred μls of nanopure H₂O was added to the filter cup (total volume 500μl) and the samples were centrifuged as outlined in “Ultrafiltration”. Washings with nanopure H₂O were repeated twice before the concentrated sample was recovered. The final volume of the DNA solution was 50μl in nanopure H₂O. Five μls of this purified sample was analyzed by gel electrophoresis.

**PCR**

**Amplification of Phage DNA**

PCR was used to specifically amplify the region of DNA suspected to contain a new promoter. Four primer pair combinations were designed to amplify the desired regions from the parental and mutant phages. Table 2 contains the list of the primers. Table 3 contains the strain numbers assigned to the amplified segments of DNA with the expected fragment size.

<table>
<thead>
<tr>
<th>Table 2. Primers</th>
</tr>
</thead>
<tbody>
<tr>
<td>PRIMER</td>
</tr>
<tr>
<td>RK765</td>
</tr>
<tr>
<td>RK767</td>
</tr>
<tr>
<td>RK770</td>
</tr>
<tr>
<td>RK1</td>
</tr>
</tbody>
</table>

*EcoR1 (GAATTC) and BamHI (GGATCC) restriction enzyme cut sites underlined.*
Table 3. Amplicon Strains and Sizes

<table>
<thead>
<tr>
<th>Primer pair</th>
<th>Strain Number</th>
<th>Fragment Size</th>
</tr>
</thead>
<tbody>
<tr>
<td>RK765 x RK770</td>
<td>1362 (parental*)</td>
<td>567 base pairs</td>
</tr>
<tr>
<td></td>
<td>1364 (mutant*)</td>
<td></td>
</tr>
<tr>
<td>RK765 x RK767</td>
<td>1359 (parental*)</td>
<td>74 base pairs</td>
</tr>
<tr>
<td></td>
<td>1360 (mutant*)</td>
<td></td>
</tr>
</tbody>
</table>

*indicates the DNA used as template

A master mix solution was created to set up multiple PCR reactions quickly and accurately. Master mixes included:

- 1.5μl primer 1 (100pmol/μl)
- 1.5μl primer 2 (100pmol/μl)
- 45μl PCR mix (Buffer A, dNTPs; Appendix A)
- 0.75μl Taq enzyme (Fisher Scientific; 5000 u/ml; FB600015)
- 96.75μl nanopure H₂O
- 145.5μl for 3 tubes total volume
- 48.5μl volume per tube

See Table 4 for thermocycling settings.
Table 4: Polymerase Chain Reaction Thermal Cycler Settings

<table>
<thead>
<tr>
<th>Step</th>
<th>Temperature (°C)</th>
<th>Time (Minutes)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Step 1</td>
<td>94</td>
<td>2:00</td>
</tr>
<tr>
<td>Step 2</td>
<td>94</td>
<td>0:30</td>
</tr>
<tr>
<td>Step 3</td>
<td>55</td>
<td>0:30</td>
</tr>
<tr>
<td>Step 4</td>
<td>72</td>
<td>1:00</td>
</tr>
<tr>
<td>Step 5</td>
<td>Go to step 2 and repeat cycle 30 times</td>
<td></td>
</tr>
<tr>
<td>Step 6</td>
<td>4</td>
<td>Forever</td>
</tr>
<tr>
<td>Step 7</td>
<td></td>
<td>End</td>
</tr>
</tbody>
</table>

Amplicons were examined by gel electrophoresis prior to purification to ensure successful amplification of the desired products. PCR reactions were purified using the column-based Qiagen QIAquick PCR Purification Kit (Cat. No. 28104) according to the manufacturer’s instructions. The recovered volume was 50 μl in nanopure H₂O. After purification, the samples were analyzed on a NanoDrop Spectrophotometer to determine concentration.

**Whole Cell PCR**

Whole cell PCR was conducted on clone cultures containing the proper plasmids with the correct fragment insert. A larger volume of cells was used in this reaction than the template DNA used previously to ensure an adequate amount of DNA was present for amplification. A typical reaction consisted of the following:

- 0.5μl primer 1 (100pmol/μl)
- 0.5μl primer 2 (100pmol/μl)
- 15μl PCR mix (Appendix A)
- 0.25μl Taq enzyme (Fisher Scientific; 5,000u/ml; FB600015)
- 31.75μl nanopure H₂O
- 2μl of whole cell suspension (in 10mM MgSO₄)
- 50μl total volume

Cycling conditions are shown in Table 5. After PCR, samples were analyzed by gel electrophoresis.

<table>
<thead>
<tr>
<th>Table 5. Whole colony PCR amplicon sizes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Strains</td>
</tr>
<tr>
<td>---------------</td>
</tr>
<tr>
<td>1362, 1364</td>
</tr>
<tr>
<td>1359, 1360</td>
</tr>
</tbody>
</table>

**Ligations**

Each double digested PCR amplicon was ligated into the double digested pRS415 reporter vector (see “Preparing the pRS415 vector for cloning”). A typical ligation reaction consisted of the following:

- 50ng of plasmid vector
- 50ng of amplified phage DNA
- 2μl 10X buffer (New England BioLabs #B0202S; 10X stock)
- Xμl H₂O
- 1μl of ligase enzyme (New England BioLabs #M0202S; 400000 u/ml)
- 20μl total volume
The concentration of the double digested, purified PCR amplicons was estimated from the band intensity on agarose DNA gels. To avoid multiple inserts, a 1:10 dilution of the amplicon was typically used.

Ligations were incubated at 14°C overnight in a PCR machine. After incubation, the enzyme was heat inactivated at 65°C for 20 minutes. After heat inactivation, the reactions were purified by ultrafiltration using npH₂O. Final recovered volume was 20 μl.

**Preparation of Electrocompetent Cells**

*E. coli* MC1000 cells were grown overnight at 37°C in TB broth. The overnight culture was diluted 1:100 in 100ml of LB (Appendix A) in a 250ml Erlenmeyer flask. The culture was incubated at 37°C, 250 rpm until the OD₆₀₀ reached ~0.5. The culture was divided into 2-50ml conical centrifuge tubes and cooled on ice at least 15 minutes. After chilling, the samples were centrifuged in a swinging bucket rotor at 2500xg, 15 minutes, at 4°C. The supernatant was removed and the pellets were suspended in 25ml each of ice cold 10% glycerol. The sample was centrifuged again and the supernatant was discarded. This cell-washing step was repeated once. After the second 25ml wash, the cells were pelleted again and suspended in 1.5 of 10% glycerol and transferred to a cold 1.5ml centrifuge tubes. The cell suspensions were centrifuged at 4°C, 3400xg for 10 minutes and the supernatants were removed. Each cell pellet was suspended in 300μl of 10% glycerol. Aliquots (40μl) were transferred to sterile microcentrifuge tubes. The electrocompetent cells were used immediately or stored at -80°C.

For a single tube of electrocompetent cells, a 5ml culture was grown overnight and diluted as described above. Once the culture reached an OD₆₀₀ ~0.5, the culture was centrifuged in a swinging bucket rotor at 2500xg, 15 minutes, at 4°C. The supernatant
was removed and the pellet was resuspended with 5ml of 10% ice-cold glycerol and centrifuged again. The supernatant was removed and the pellet was resuspended with 1.5ml of 10% ice-cold glycerol and transferred to a microcentrifuge tube. The cells were centrifuged at 3400xg for 10 minutes. The supernatant was removed and the pellet was resuspended in 40μl of ice-cold 10% glycerol. This was used immediately or stored at -80°C.

**Electroporation**

Electrocompetent cells were freshly prepared or thawed on ice immediately prior to use. For electroporation of a purified ligation reaction, an aliquot of cells (40-50ul) was mixed with approximately half of the purified ligation products. This mixture was transferred to a pre-chilled electroporation cuvette (2 mm gap; BioExpress; Cat. No. E-5010-2) and kept on ice. A Bio-Rad GenePulser Xcell machine was used for all electroporation. The electroporation settings were: 2.5 kV, 25 μF, 200 Ω. After electroporation, 1 of Super Optimal Broth, SOC (Appendix A), was added to the cuvette and thoroughly, but gently, mixed. This broth maximizes the efficiency of cell transformations. The cell suspension was transferred to a 15ml culture tube (Fisherbrand; Cat. No. 05-539-12) and incubated for 1 hour at 37°C with shaking (250rpm). Different amounts (0.1ml or 0.15ml) of cells were plated onto prewarmed MacConkey-Lactose–Ampicillin plates (Appendix A) and incubated at 37°C overnight. A control electroporation in which no DNA was added was performed on each new batch of electrocompetents cells. These cells were plated on the same selective media and incubated under the same conditions as the cells that received ligated plasmid DNA.
Purification and culturing of clones

The desired clones containing amplified DNA from the parental (O276) and mutant (O367) phages were selected on MacConkey-Lactose ampicillin plates. If a promoter is inserted in the reporter vector, the lac operon will be expressed, causing transformants to turn red on the plates. A white colony indicates the uptake of the plasmid, but no expression of the lac operon. After the initial selection, candidates were colony purified by streaking for isolation on the same type of media. Plates were incubated at 37°C overnight. After overnight growth, single colonies were picked and transferred to 5ml of TB broth supplemented with ampicillin. The broth cultures were incubated overnight at 37°C with shaking at 250 rpm. The next day, the overnight cultures were centrifuged at 9500xg at 4°C. The supernatant was discarded and the cell pellet was suspended in 10mM MgSO4. These cell suspensions were stored in the refrigerator. This maintains cell viability while additional tests were performed to verify the desired clones.

Sequencing

All clones were verified by DNA sequencing. A typical sequencing reaction consisted of the following:

X μl DNA (100ng/μl); Do not exceed 5μl volume
1μl primer (100pmol/μl)
2μl 5X sequencing buffer
2μl “Sequencing Juice” (Applied Biosystems BigDye® Terminator v3.1 Cycle Sequencing Kit; Part No.: 4336917)
(5-X)μl nanopure H₂O
10µl final volume

Reactions were completed in a PTC-200 Peltier Thermal Cycler using the cycling conditions outlined in Table 6.

<table>
<thead>
<tr>
<th>Step</th>
<th>Temperature (°C)</th>
<th>Time (Minutes)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Step 1</td>
<td>96</td>
<td>0:30</td>
</tr>
<tr>
<td>Step 2</td>
<td>50</td>
<td>0:15</td>
</tr>
<tr>
<td>Step 3</td>
<td>60</td>
<td>4:0</td>
</tr>
<tr>
<td>Step 4</td>
<td>72</td>
<td>1:00</td>
</tr>
<tr>
<td>Step 5</td>
<td></td>
<td>Go to 1 for 24 times</td>
</tr>
<tr>
<td>Step 6</td>
<td>10</td>
<td>Forever</td>
</tr>
<tr>
<td>Step 7</td>
<td></td>
<td>End</td>
</tr>
</tbody>
</table>

Purification of Sequencing Reactions

The SigmaSpin Sequencing Reaction Clean-up Kit (Sigma-Aldrich; S5059-70EA) was used to remove unincorporated nucleotides and enzyme from the reaction products. The purified samples were dried using a CentriVap Concentrator (LABCONCO; Cat. No. 7810000). The dried material was suspended in 15 µls of formamide before loading onto the DNA sequencer.

Analysis of Sequencing Products

DNA sequencing reaction products were examined on an ABI 3130 Automated Capillary Sequencer.
Bioinformatic Analysis

Geneious® R6 (Biomatters Ltd.; Version 6.1.7) software was used to analyze DNA sequences, perform alignments and generate figures.

β-Galactosidase Assay

Quantitative β-Gal assays were performed as described in *Experiments in molecular genetics* by Jeffery H. Miller [22].

Five ml cultures of the confirmed clones were grown overnight at 37°C in TB broth. The following day, the cultures were diluted 1:100 in 25ml of LB broth with ampicillin in a 125ml Erlenmeyer flask while shaking at 250 rpm. The cultures were incubated at 37°C until the OD$_{650}$ reached approximately 0.2. Samples (3-5ml) were then taken at an approximate OD$_{650}$ of 0.4, and again at an approximate OD$_{650}$ of 0.6, and kept on ice until all samples had been collected. [22]

Five ml cultures of the confirmed clones were grown overnight at 37°C in TB broth. The following day, the cultures were diluted 1:100 in 25ml of LB broth with ampicillin in a 125ml Erlenmeyer flask while shaking at 250 rpm. The cultures were incubated at 37°C until the OD$_{650}$ reached approximately 0.2. Samples (3-5ml) were then taken at an approximate OD$_{650}$ of 0.4, and again at an approximate OD$_{650}$ of 0.6, and kept on ice until all samples had been collected. The OD$_{650}$ gives a standard measure of cell density. By taking this value at multiple time points, cell growth can be monitored for the exponential phase of cell division. This value also allows for the normalization of enzyme readings for that specific density. [22]

Reactions were started by adding 0.2ml of the substrate O-Nitrophenyl-β-galactoside, ONPG (4mg/ml stock solution in water), to each tube and mixed quickly.
The time of addition of the substrate was immediately noted and the reaction was closely monitored for the appearance of a light yellow color. Reactions were stopped by adding 0.5 ml of 1M Na$_2$CO$_3$ and the time was recorded. The absorbance at 420nm and 550nm was measured for each reaction tube. Equation 1 shows how Miller Units of β-Galactosidase activity were calculated. [22]

\[
\text{Miller Units} = 1000 \times \left( \frac{OD_{420} - 1.75 \times OD_{550}}{OD_{650} \times \text{time}_{\text{seconds}} \times \text{volume}_{\text{milliliters}}} \right)
\]  

(1)
CHAPTER 3

RESULTS

This study worked with a parental hybrid phage (O276) whose growth was blocked by the \textit{rpoCY75N} mutation in the \( \beta' \) subunit of RNA polymerase. We wanted to find a mutant phage derivative that could grow on this antitermination defective strain. To do this, the phage was mutagenized and forced to grow on the antitermination defective host. Plaques formed and phage was harvested from these plaques. A mutant phage (O367) was selected and its genome was sequenced, along with the parental strain. Comparison of the two sequences revealed a 2-base pair mutation in O367 and we set out to characterize this mutation.

\textbf{Hybrid phage}

The parental phage used in this study is a hybrid of bacteriophages HK022 and Lambda, (phage O276). The hybrid contains the HK022 immunity region with the remaining part of the genome originating from Lambda. This phage was generated by crossing a Lambda phage mutant carrying the \textit{b519} and \textit{b515} deletions with an HK022 prophage that carried a \textit{cIts12 P :: Km}\(^R\) mutation [19]. The HK022 genes present in O276 include \textit{nun}, \textit{cl}, \textit{cro} and \textit{cII} [19]. Figure 10 shows the parental phage genome. It also shows classic Lambda mutations \textit{nin} and \textit{byp} for comparison of location relative to the \textit{orc} mutation; however, these mutations (\textit{nin} and \textit{byp}) do not occur in the parental or mutant phage.
Figure 10. Parental phage genome. Terminators are denoted in red. The mutation characterized in this study is labeled “orc mutation” for “overcomes rpoC” and is shown between TR3 and TR4. The location of the Lambda phage byp mutation is downstream of TR4 and before TR’. The sites of the nin3 and nin5 deletions in the Lambda phage are shown. pl, pR, and Pmun promoters are denoted in blue. The putR and putL RNA products are shown in purple. The ctsI2 was used as a marker during the construction of the hybrid phage.
The parental phage was mutagenized via growth on a mutagenic bacteria strain and UV irradiated. Phage was harvested from plaques and forced to grow on the antitermination defective host carrying the *rpoCY75N* mutation. A mutant derivative of this hybrid (O367) was recovered from plaques and sequenced.

**Phage Sequence Analysis**

From the genomic sequence, it was determined that the mutation did not occur in a gene or in a transcription terminator, meaning this mutation had a different function. Analysis of the sequence around the mutation in O367 revealed that it created a potential -10 hexamer by changing two sequential bases (T and C at positions 35,702 and 35,703 in the genome to an A and T respectively) between the TR3 and TR4 terminators in the phage genome (Figure 10). Sequence alignment of the mutation with an *E.coli* promoter consensus sequence supported the hypothesis that a new promoter may have been created. (Figure 11)

![E.coli promoter consensus sequence alignment](image)

*Figure 11. E.coli promoter consensus sequence alignment.* Alignment of parental and mutant phage around the mutation with the consensus sequence in the 5’ to 3’ direction. The -35 and -10 hexamers are underlined. The original sequence in the parental phage is denoted in blue. In the mutant phage, five out of 6 bases match in the -10 region and 3 out of 6 bases matches in the -35 region, denoted in red [4, 5].

Additional sequence inspection revealed a possible UP element near the -35 region [7] (Figure 12). The presence of an UP element may contribute to the strength of
the promoter.

![Figure 12](image1.png)

**Figure 12.** UP element alignment. Alignment of UP element sequence with the phage sequence upstream of the -35 region. This region is homologous in the parental phage. There are 14 matches with the consensus indicated in red.

**Amplification of potential promoter sequences from phage DNA**

To determine if this new mutation resulted in promoter activity, template DNA was first amplified using the primer combinations shown in Table 3. Amplified DNA for strains RK1362 and RK1364, analogous DNA from the parental and mutant phage respectively and also containing the T_R4 terminator, were analyzed on a 1.2% agarose gel. (Figure 13)

![Figure 13](image2.png)

**Figure 13.** 1.2% Agarose gel of amplified phage DNA from RK1362 and RK1364. Lane 1: Lambda HindIII Ladder. Lane 2: 100-bp ladder. Lane 4: amplification from RK1362. Lane 7: amplification from RK1364. Both were expected to be 567 base pairs in length.

Amplified DNA for strains RK1359 and RK1360, analogous DNA from the parental and mutant phage respectively without the T_R4 terminator, were resolved on a
2% gel due to the smaller size of the amplicon. Figure 14 is the gel image of amplified DNA.

![Figure 14](image.png)

**Figure 14.** 2% Agarose Gel of Amplified phage DNA from RK1359 and RK1360. Lane 1: Lambda HindIII. Lane 2: 100-bp ladder. Lane 3: RK1359. Lane 6: RK1360. Both were expected to be 74 base pairs in length.

Following amplification of phage DNA as shown above, the phage DNA was digested as described in the materials and methods. It was then purified and ligated into digested pRS415 plasmid and prepared for electroporation as described in the materials and methods to monitor the expression of the *lac* operon by the putative promoter on the pRS415 plasmid.

**Electroporation Results**

MacConkey-Lactose plates supplemented with ampicillin were used to select for the desired recombinants after electroporation. The average colony number recovered from each transformation is reported in Table 7.
Table 7. Transformants recovered on MacConkey-Lactose-Ampicillin plates

<table>
<thead>
<tr>
<th>Strain</th>
<th>White Colonies</th>
<th>Red Colonies</th>
</tr>
</thead>
<tbody>
<tr>
<td>RK1359</td>
<td>T.N.C.</td>
<td>0</td>
</tr>
<tr>
<td>RK1360</td>
<td>0</td>
<td>T.N.C.</td>
</tr>
<tr>
<td>RK1362</td>
<td>60</td>
<td>5</td>
</tr>
<tr>
<td>RK1364</td>
<td>T.N.T.C.</td>
<td>40</td>
</tr>
</tbody>
</table>

*T.N.T.C. is the abbreviation of “too numerous to count”, which signifies colony numbers greater than 300.

A transformation plate of strain RK1360 is shown in Figure 15 as an example of the phenotype of clones that display promoter activity on a MacConkey-Lactose-Ampicillin plates.

![Transformation plate of strain RK1360](image)

**Figure 15.** Transformation plate of strain RK1360 exhibiting red colonies indicating promoter activity.

A transformation plate of strain RK1359 is shown in Figure 16 as an example of the white phenotype on a MacConkey-Lactose-Ampicillin plate.
Figure 16. Transformation plate of strain RK1359 exhibiting too numerous to count white colonies indicating no promoter activity.

Streak Plate Purification

Single colony transformants were picked from the transformation plates and purified by the streak plate method on MacConkey-Lactose-Ampicillin plates. The number selected along with their color after growth are outlined in Table 8. Purified colonies from these plates were used to inoculate broth cultures.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Number Selected for Isolation</th>
<th>Phenotype After Growth</th>
</tr>
</thead>
<tbody>
<tr>
<td>RK1359</td>
<td>8 white</td>
<td>All White</td>
</tr>
<tr>
<td>RK1360</td>
<td>8 red</td>
<td>All Red</td>
</tr>
<tr>
<td>RK1362</td>
<td>9 red, 7 white</td>
<td>All White</td>
</tr>
<tr>
<td>RK1364</td>
<td>8 red, 16 white</td>
<td>All Red</td>
</tr>
</tbody>
</table>
PCR Confirmation of candidate clones

After colony purification, single colonies were grown in LB broth media with ampicillin. To confirm that cell cultures were transformed with the correct plasmid, whole cell PCR was performed with the primer pairs from Table 5.

PCR products amplified from pRK1362 were resolved on a 2% gel. Three cultures were correct as shown in Figure 17.

Figure 17. 2% Agarose Gel of Amplified RK1362 from transformants. Lane 1 is the 100-bp ladder. Lanes 6-8: RK1362. The expected size was 724 base pairs.

PCR products amplified from pRK1364 were resolved on a 2% gel. Five cell cultures were correct as shown in Figure 18.

Figure 18. 2% Agarose Gel of Amplified RK1364 from transformants. Lane 1 is 100-bp ladder. Lanes 4-8: RK1364. Expected size was 724 base pairs.
PCR amplified products from pRK1359 and pRK1360 were imaged on a 2% gel due to the small fragment size. Six cell cultures were correct for RK1359 and 8 cell cultures were correct for RK1360 as shown in Figure 19.

Figure 19. 2% Agarose Gel of Amplified RK1359 and RK1360 from transformants. Lane 1: 100-bp ladder. Lanes 2, 5-9: RK1359. Lanes 11-18: RK1360. Expected size was 231 base pairs in length for both.

**Sequencing**

Plasmids were isolated from cultures confirmed by Whole Cell PCR and sequenced using the RK1 primer (Table 5). This analysis confirmed that all the clones were correct and no mutations were introduced by the PCR amplification process. Figure 20 shows alignment of pRK1362 and pRK1364 with emphasis on the cloned region and the mutation difference between the two.

Figure 20. Sequence Alignments of amplified phage DNA. The parental strain is pRK1362A and the mutant strain is pRK1364A. The green bar at the top represents homologous sequence alignment. The gap in the green bar reflects the differences between the two sequences. The mutation is located upstream of the Lambda T4 terminator.
β-Galactosidase Assays

The pRS415 plasmid contains a promoterless *lacZ* gene whose expression can be assayed to determine the activity of cloned promoter sequences. Quantitative β-gal assays were performed on cells containing pRK1359, pRK1360, pRK1362, and pRK1364. pRK1359 and pRK1360 are equivalent clones of DNA amplified from the parental and mutant phages respectively containing only the region with the putative promoter element. pRK1362 and pRK1364 are equivalent clones of DNA amplified from the parental and mutant phages respectively and include the region containing the putative promoter and the T<sub>R</sub>4 terminator.

Strain RK1359 exhibited only 9 Miller Units of activity, which is consistent with the idea that this region of the parental phage does not contain a promoter. Strain RK1360 exhibited 16400 Miller Units of activity, which is consistent with the idea that this region contains promoter like sequences. Strain RK1362 exhibited only 65 Miller Units of activity, which is consistent with the idea that this region from the parental phage does not contain a promoter. Strain RK1364 exhibited 4042 Miller Units of activity, which is consistent with the idea that this region from the mutant phage contains the putative promoter. The decreased level of activity reflects the activity of the T<sub>R</sub>4 terminator. Table 9 shows the assay results.
<table>
<thead>
<tr>
<th>Strain</th>
<th>Features</th>
<th>Average Miller Units</th>
<th>Standard Error of the Mean (+/-)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1359</td>
<td>No promoter; No terminator</td>
<td>9</td>
<td>1.39</td>
</tr>
<tr>
<td>1360</td>
<td>Putative promoter; No terminator</td>
<td>16400</td>
<td>1889.44</td>
</tr>
<tr>
<td>1362</td>
<td>No promoter; terminator</td>
<td>65</td>
<td>4.75</td>
</tr>
<tr>
<td>1364</td>
<td>Putative promoter; terminator</td>
<td>4042</td>
<td>251.21</td>
</tr>
</tbody>
</table>

This assay also provides a measure of the $T_{R4}$ terminator’s efficiency by taking the ratio of the values obtained for RK1360 and RK1364. This calculation shows that the $T_{R4}$ terminator is 75.4% effective.
CHAPTER 4

DISCUSSION

An *E.coli* host containing the *rpoCY75N* mutation in the zinc-binding region of the β’-subunit of RNA polymerase blocks antitermination and, therefore, prevents the growth of the bacteriophage that use antiterminator RNAs as the mechanism of antitermination. A mutant phage was discovered that had the ability to grow on this defective *E.coli* strain, and sequence comparison with the parental strain revealed a two base-pair change. This mutation was named “orc” for “overcomes rpoC”.

In this study, we have presented evidence that this mutation occurs between two terminators, *T*R3 and *T*R4, in the hybrid phage and creates a new promoter. β-Galactosidase assays of plasmids with phage DNA inserts showed that the mutant strains exhibited significance increases in activity over the wild type strains. This mutation is analogous to the byp mutations in the Lambda phage [23].

**Transcription terminators of bacteriophage Lambda**

The genome of bacteriophage Lambda contains a number of well-characterized terminators. These terminators exist to block unwanted phage gene expression, In the *λ* phage, there are at least 4 terminators in the right operon (*T*R1, *T*R2, *T*R3, and *T*R4) that are biologically significant [24].
At $P_R$ initiated transcription, transcription continues through the $T_R1$ terminator with 50% efficiency due to the $N$-protein encoded in the $P_L$ operon [24]. However, transcription halts in the $nin$-region due to the presence of multiple terminators before reaching the gene that encodes for the $Q$-protein (Figure 21) [24]. $T_R2$ was the first terminator to be characterized in the $nin$ region because it shares common features with the Rho-independent termination mechanism [24, 25].

The characterization of the $T_R3$ and $T_R4$ terminators occurred via the analysis of a Lambda derivative with the $roc$ mutation. The $roc$ region occurs downstream of $T_R2$ and is a sub-region of the $nin$-region and is characterized by a 1900 base deletion [25]. This analysis showed that simple point mutations in this region did not lead to an elimination of termination in that region, leading to the conclusion that additional terminators existed in this region. [24]. Analysis of this region via mutagenesis experiments showed that two biologically significant terminators existed, $T_R3$ and $T_R4$, and were found to be Rho dependent terminators [24].

**Similarities to Lambda Phage**

There are two types of mutations that occur in Lambda that allows for different degrees of N-independent gene expression: the $nin5$ deletion and the $byp$ mutation. The $nin5$ mutation is a deletion that removes 2,805 base pairs, which includes the $T_R2$ and $T_R3$ terminators (Figure 21) [23, 24]. This deletion removes the need for the N protein and Nus host factors. This is called N-independence [23, 24].

The $byp$ mutation, characterized in the early 1970s [26, 27], creates a new promoter that drives the expression of the $Q$ protein. Consequently, $Q$ can be transcribed independently of the antitermination in the $P_R$ operon (Figure 21) [23]. However, this
only confers partial N-independence. [24] In Lambda, the *byp* mutation occurs after the
*Tr*2 and *Tr*3 terminators [23]. The *orc* mutation in the hybrid phage O367 creates a -10
hexamer similar to the Lambda *byp* mutation. The *orc* mutation occurs after *Tr*3 but
before *Tr*4. Therefore, *orc* and *byp* occur at different locations in the genome.

Figure 21. Classic Lambda Mutations. This image shows the critical region of the hybrid phage genome with the
classic *nin* and *byp* Lambda mutations in relation to the position of our *orc* mutation. The transcription terminators are
denoted in red. The Lambda *byp* mutation is shown downstream of *Tr*4, but before the Q gene for the Q protein. The
*nin* deletions are shown in relation to the transcription terminators that are removed [28].

Broader Implications

In our study of gene expression, the phage has served as a model system in an
attempt to gain knowledge that is applicable to other fields. In the study of mammalian
viruses, the human immunodeficiency virus, HIV, is one of the viruses at the forefront of
research. The expression of HIV genes has been found to behave in a similar way to that
of bacteriophage antitermination [29].

In HIV, a *tat* gene exists, which encodes for a protein named Tat. This protein
binds at a trans-activating region (TAR) site in the RNA transcript to form a stable stem-
loop RNA structure. This structure interacts with RNA polymerase II with the assistance
of several co-factor proteins to accelerate the polymerase and to lead to the expression of
HIV genes [29]. The Tat RNA-binding protein is similar to the Lambda RNA-binding
protein N. Tat utilizes co-factor proteins to interact with RNA polymerase II to allow for
HIV gene expression; whereas Lambda N interacts with host Nus factors to bind to RNA
polymerase to antiterminate and allow for phage gene expression. By understanding bacteriophage gene expression and the model system bacteriophage can be, the HIV Tat-TAR interaction could be more closely studied to allow for a greater understanding of HIV gene expression.
CHAPTER 5

CONCLUSION

The two-base pair orc mutation that is found between T_R3 and T_R4 of the mutant phage creates a promoter that allows for phage growth on the antitermination defective *E. coli*. Qualitatively, we showed that the putative promoter in the mutated phage functions to express the *lacZ* gene in the promoter probe vector, while the comparable region in the parental phage does not express the *lacZ* gene. This was demonstrated by the phenotypes exhibited by the respective strains on the MacConkey-Lactose-ampicillin plates. In quantitative β-Galactosidase assays, the putative promoter exhibited significantly higher levels of activity over the comparable parental phage DNA region, in the presence or absence of the terminator sequence. A reduction in *lacZ* gene expression was observed in the β-Galactosidase assays between RK1360 and RK1364. This reduction was expected because of the activity of the terminator. However, the activity of the single terminator is not sufficient to prevent growth of the phage. Approximately 25% of the transcripts originating from the new promoter read through the T_R4 terminator.
A detailed analysis of the possible UP element sequence could be performed to determine if it increases RNA polymerase recognition at the promoter and, therefore, increases the promoter’s strength. The sequence has 14 matches to the UP element consensus sequence and could possibly serve as an UP element.

A second study could be performed to analyze the changes in mutant phage growth if it was grown on the \textit{rpoCY75N} mutant host that was further mutated to also be Rho deficient. A previous study suggests that N-mediated antitermination is not required for \textit{Q} expression if the host bacterium has reduced Rho production because termination at T\textsubscript{R}3 and T\textsubscript{R}4 is prevented [21]. The absence of Rho dependent termination may allow for better phage growth on the \textit{rpoCY75N} host bacterium, possibly leading to the formation of larger plaques. This would support the idea that terminators are efficient at restricting gene expression unless they are suppressed or bypassed. This study could be extended to the parental phage to determine if the growth of the parental phage could be restored based on the appearance of plaques on the mutant host.
REFERENCES

APPENDIX

The recipes for the types of media used in this lab are as follows: Luria Broth (LB), Lennox recipe: NaCl (Fisher BioReagents; BP358-212) 5g, Yeast Extract (BD; 212750) 5g, Tryptone (Fisher BioReagents; BP9726-500) 10g, per liter in diH₂O. Aliquot 100ml to 125ml screw cap bottle then autoclave. Terrific Broth (TB): Bacto-Tryptone (BD; 211705) 10g, NaCl (Fisher BioReagents; BP358-212) 5g, adjust pH to 7.4 with 5N NaOH (Sigma-Aldrich; 221465-500G) per liter. Aliquot 100ml to 125ml screw cap bottles then autoclave. Super Optimal Broth (SOC): Tryptone (Fisher BioReagents; BP9726-500) 20g, Yeast Extract (BD; 212750) 5g, NaCl (Fisher BioReagents; BP358-212) 0.5g, 2M KCl (Acros; CAS: 744-40-7) 1.25ml, 1M MgCl₂ (Fisher Chemical; M-13448) 10ml per liter. Aliquot 100ml to 125ml screw cap bottles then autoclave. Cool then add 1ml 1M MgSO₄ (Sigma; M-2773) and 2ml glucose (Fisher Scientific; D16500). MacConkey-Lactose Plates with Ampicillin: Difco MacConkey Agar (Difco; 281810) 50.0g per liter. Autoclave then cool before adding 100µg/ml of Ampicillin (Fisher BioReagents; BP-1760-25). Pour into petri plates (USA Scientific; 100x15mm; 8609-0010).

The recipe for the PCR mix used in this protocol is as follows: 6µl of 10mM dATP, 6µl of 10mM dGTP, 6µl of 10mM dCTP, 6µl of 10mM dTTP, 330µl of 10X Buffer A (Fisher Scientific Kit; FB-6000-10). 316µl of nanopure H₂O. Mix in a 1.5 microcentrifuge tube and keep on ice if used immediately or store at -20°C.
The recipe for the TAE buffer used is as follows: 50X TAE: Tris-base (Fisher BioReagents; M-11645) 242g, Glacial Acetic Acid (Amresco; 0714-4L) 57.1ml, 0.5M EDTA (Sigma; E-5134) 100ml. Dilute with diH₂O to 1 liter. Aliquot 20ml to a 1-L screw cap bottle and fill to 1-L line to make 1X TAE. Store at room temperature.

The recipe for the loading dye used in this lab 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 500µl to a 1.5µl centrifuge tube. Store at room temperature.

The recipes for the solutions used in the β-Galactosidase assay are as follows: Z-Buffer: 60 mM Na₂HPO₄ * 7H₂O (Sigma-Aldrich; 7782-85-6) 16.1g, 40 mM NaH₂PO₄ * H₂O (Sigma-Aldrich; 10049-21-5) 5.5g, 10mM KCl (Acros; CAS: 744-40-7) 0.75g, 1 mM MgSO₄ * 7H₂O (Sigma-Aldrich; 10034-99-8) 0.246g, 50 mM β-mercaptoethanol (Sigma-Aldrich; M-3148) 2.7ml. Do not autoclave. Adjust pH to 7.0. Store in refrigerator. 10% SDS: Mix 1g SDS (Sigma-Aldrich; L6026) in 10ml. Equilibrate in 55°C water bath to facilitate dissolving. Dilute 1ml into 9ml of nanopure H₂O to create a 0.1% solution.