2020

Identifying Essential Viral Genes through Genomic Engineering

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IDENTIFYING ESSENTIAL VIRAL GENES THROUGH GENOMIC ENGINEERING

A Capstone Experience/Thesis Project Presented in Partial Fulfillment

of the Requirements for the Degree Bachelor of Science

with Mahurin Honors College Graduate Distinction at

Western Kentucky University

By

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December 2020

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ABSTRACT

Bacteria developed resistance to penicillin a mere four years after the ground-breaking antibiotic was first mass produced (Casadevall, 2010). Since then, the development of antibiotic-resistant bacteria has steadily risen, causing millions of difficult to treat infections annually. The challenge is to identify ways to combat these menacing microbes. Bacteriophages are viruses that infect bacteria and can potentially be used to eliminate deadly antibiotic-resistant bacteria. The number of sequenced bacteriophage genomes has increased tremendously over the past 10 years, but little is known about the function of most bacteriophage genes. The purpose of this study was to expand our understanding of bacteriophage gene function by identifying genes that are essential for lytic growth of bacteriophage HK639. Using recombineering, a genome engineering technique, five HK639 genes and one regulatory sequence were individually replaced with a kanamycin gene. The recombinants were selected on kanamycin plates, and the gene replacements were confirmed by Polymerase Chain Reaction (PCR). To determine if the replaced genes and regulatory sequence were essential for lytic growth, the recombinants were tested for the release of viable phage. Phage production was unaffected by four of the gene replacements, which indicates the replaced genes are not essential for lytic growth. However, replacement of ORF 40 prevented phage growth. These results indicate that ORF 40 performs an essential function. Biochemical analysis of these proteins will be necessary since sequence analysis did not reveal any clues about the potential function of ORF 40.

I dedicate this thesis to my parents, Jennifer Bedel and Doug Carroll, who are a great inspiration to me and have always supported me in all of my endeavors. I also dedicate this to my grandfather, Dr. Alvin Bedel, who is the reason behind much of my success, and the reason I chose to complete my undergraduate degree at Western Kentucky University. I dedicate this work to all of my friends who have supported me along my thesis journey, and most notably, Olivia Urso with whom I had the pleasure of working alongside with in the lab. Most importantly, I dedicate this work to my research advisor, Dr. Rodney King, who has greatly impacted my life since I took his class my first semester here five years ago. I want to thank him for igniting a passion in me for science and for taking the time to mentor me during my time here at Western Kentucky University. Lastly, I want to dedicate this work to anyone who has been affected by a virus, such as COVID-19 or HIV, or has been infected by an antibiotic-resistant strain of bacteria such as tuberculosis or E. coli. Those who have fought against these viruses, or against antibiotic-resistant bacteria, inspired me greatly to contribute to this work.

ACKNOWLEDGEMENTS

I would like to express how truly grateful I am for each and every person that has assisted me throughout this journey. I will forever appreciate the support and the guidance that each and every person gave me. Without the selflessness of others, this project would not be what it is today.

First and foremost, I would like to acknowledge the mentorship of Dr. Rodney King. This paper would not have been possible without his guidance, assistance, and patience. His knowledge on this subject is unmatched, and it is something I greatly admire about him. I would also like to acknowledge my second reader and advisor, Dr. Claire Rinehart. His advice on this project and in my academic career has been invaluable, and for that, I thank him. I also extend a thank you to Dr. Galindo for her support and advice as my third reader.

I would like to acknowledge Olivia Urso, with whom I collaborated on this project. Our combined effort on parts of this project truly created a wonderful experience. Our combined work expanded and diversified this project further than I could have done alone. Additionally, I would like to thank all of my professors in the Biology and Chemistry Departments for always challenging me to stive for greatness and to go above and beyond. Finally, I would like to thank the Carol Martin Gatton Academy for inspiring my passion for research and allowing me to fall in love with Western Kentucky University. The Carol Martin Gatton Academy is the reason I met Dr. King, and for that, I am forever grateful

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INTRODUCTION

Antibiotic-resistant bacteria are an increasing threat to public health. *Escherichia coli*, *Mycobacterium tuberculosis*, and *Neisseria gonorrhoeae* are among several pathogenic bacteria that frequently do not respond to previously effective antibiotics. It is estimated that antibiotic-resistant bacteria infect two million Americans every year (CDC, 2020). By 2050, nearly ten million deaths will occur globally every year due to antibiotic-resistant bacterial infections (O'Neill, 2014). A solution to this impending disaster must be identified.

Bacteriophages, or viruses that infect bacteria, are natural bactericidal agents that could be used to destroy antibiotic-resistant bacteria. Bacteriophages exterminate their hosts by producing enzymes that attack the cell wall. In some cases, these enzymes have been purified and shown to eliminate targeted bacteria (Roach *et al*., 2013, Fischetti, 2008). In order to be effectively used to fight human bacterial infections, it is crucial to understand the life cycle of bacteriophages. In this study, genes required for the growth of a unique *E. coli* bacteriophage, phage HK639, were identified. Knowing which genes are essential for bacteriophage growth will offer important insight into phage biology and increase their usefulness as therapeutic agents.

Bacteriophages infect their host by attaching to a receptor located on the bacterial cell's surface. Once attached, the phage injects its genetic material into the host cell. Temperate phage can adopt one of two life cycles: lytic or lysogenic (Figure 1). In the lytic life cycle, the phage hijacks the host's macromolecular synthesis machinery to create new viral particles, which ultimately escape by lysing the host cell. In the lysogenic life cycle, the bacteriophage integrates its DNA into the host cell's chromosome, becoming a prophage (a dormant bacteriophage). When the bacterial host replicates, the integrated phage genome is also replicated as part of the host genome. Therefore, each cell division creates an expanding population of infected bacterial cells. The bacteriophage remains quiescent within the host bacterium until an appropriate signal reactivates the virus. DNA damage is an event that often leads to the reactivation of prophages, causing the phage to enter the lytic cycle and exit the bacterial host (Little, 2005).

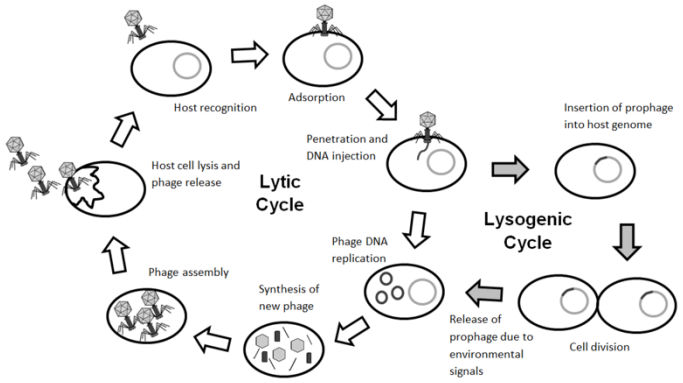

Figure 1: Lytic and lysogenic life cycles (Doss *et al.*, 2017). The phage genomic DNA is colored black. The bacterial (host) chromosome is colored light gray.

Phage **λ** is the best-characterized temperate phage. Since its discovery in 1951, phage **λ** has been studied extensively and has provided important insight into our understanding of how bacteriophage genes are regulated. For example, a unique mechanism of gene regulation, called transcription antitermination, was first discovered in this phage. During this process, the host RNA polymerase (RNAP) is modified to read

through transcription terminators or "stop" sequences. Most lambdoid phages utilize protein-mediated antitermination. This occurs when proteins produced by both the phage and the host cell modify the host RNA polymerase and convert it into a termination resistant form. However, a rarer method of antitermination, called RNA-mediated antitermination, was discovered in the lambdoid phage HK022. In this case, there is not a protein that interacts with RNAP. Instead, the RNA transcript of the *put* (polymerization utilization) sites folds into a highly organized, two stem-loop structure that directly converts RNAP into a terminator-resistant form (King *et al.*, 1996). *E. coli* bacteriophage HK639 was chosen for this study because it also uses this poorly understood mechanism of gene regulation and because the genes essential for phage growth are unknown.

Only a small portion of viral genes have known functions. In fact, it is unknown whether a large portion of genes in most *E. coli* bacteriophages are even necessary for lytic growth. The early genes of lambdoid phages are characteristically organized into two divergently transcribed operons; named "left" and "right" to signify the direction of transcription. Bacteriophage HK022 has no genes in the left operon that are essential for lytic growth (Cam *et al*., 1990). This was shown by deleting the left operon promoter and demonstrating that this did not prevent phage production. In contrast, a similar mutation in phage HK639 prevented phage release from an HK639 lysogen (Seaton, 2013). To determine which HK639 left operon genes were essential for phage growth, large sections were systematically replaced (Seaton, 2013). The replacement of the first five genes, after the terminator, of the HK639 left operon (Figure 2) prevented phage growth and suggested that at least one of these five genes plays a vital role in lytic phage growth.

In Figure 2, ORF 39, ORF 40, ORF 41, ORF 42, and ORF 43 were replaced by the kanamycin gene, as defined in the previous study.

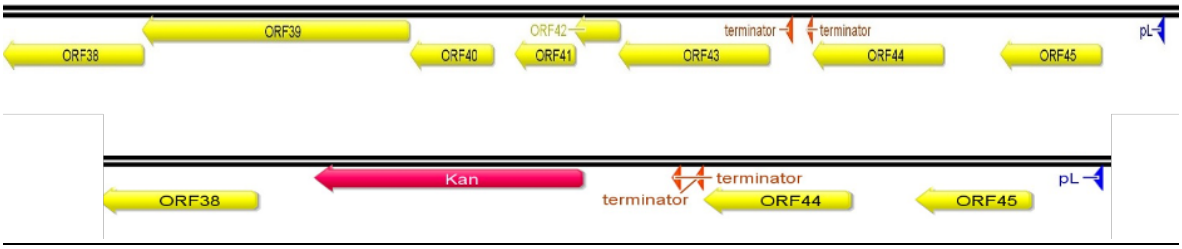

Figure 2: The top map shows the original HK639 genome and the bottom shows the replacement of five early genes with the kanamycin gene (colored in pink). The combined replacement of the first five genes of the HK639 left operon prevented phage growth. Maps were generated using Geneious software.

Recombineering is a powerful chromosomal engineering technique. It involves inserting or deleting DNA sequences *in vivo* using natural recombination functions within cells (Marinelli *et al*., 2012). To successfully replace a genetic sequence in vivo, two essential components must be prepared: a pure DNA fragment specifically designed to recombine with the desired sequence and bacterial cells competent for completing the recombination event. Electroporation, a brief electric shock, is used to introduce the substrate DNA into host cells where the proteins responsible for mediating recombination catalyze the reaction. The specifically designed DNA fragment recombines at identical sequences flanking the replacement location in the genome, effectively replacing the desired sequence (Figure 3).

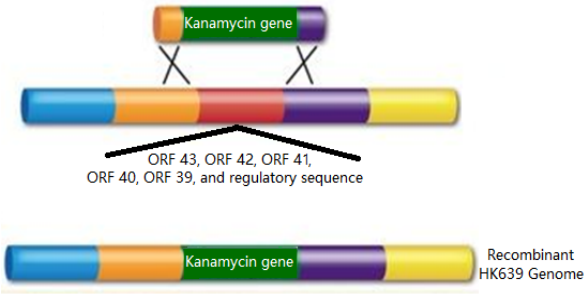

Figure 3: In this example, recombineering is used to replace genes and regulatory sequences with the kanamycin gene. The orange and purple regions flanking the kanamycin gene are homologous to the corresponding regions in the bacteriophage HK639 genome. This homology allows the kanamycin gene to recombine into the chromosome and replace the targeted sequences.

Our knowledge of how bacteriophage control their gene expression is very limited. To serve as effective tools against antibiotic-resistant bacteria, it is vital to understand how these viruses work. This study focuses on understanding gene expression in bacteriophage HK639. HK639 is unusual in that it uses RNA-mediated antitermination to promote the full expression of its early genes and it must express genes in the left operon to successfully complete the lytic growth cycle. The purpose of this project was to identify essential genes and regulatory sequences in the left operon of bacteriophage HK639 using recombineering (Figure 4). After confirming the recombinants, the effect of each substitution on phage growth was determined.

Figure 4: Maps showing the individual replacement of genes or regulatory sites in the HK639 left operon. Maps were generated using Geneious software (Biomatters, Inc., San Diego, CA).

MATERIALS AND METHODS

Summary:

Several steps were required to locate essential genes in HK639 for lytic growth. First, the DNA substrate for recombination was prepared; the kanamycin gene, flanked by regions of homology to the desired chromosomal target, was amplified by Polymerase Chain Reaction (PCR). Next, electrocompetent *E. coli* cells containing the HK639 prophage were prepared. These strains carried a plasmid, pRK1006, that allowed the recombination proteins to be expressed after a heat induction event. After moving the DNA substrate into the cells and allowing the recombination reaction to take place, the recombinants were selected for by growing them in the presence of kanamycin. PCR was performed to ensure the kanamycin gene had recombined in the correct location using the oligonucleotide list in Table 2. These PCR products were visualized on an agarose gel and sequenced to verify the recombination junctions. Lastly, the recombinant cells were tested for phage release. If phage were released, PCR was performed to verify they were indeed recombinant. A total of five genes and a regulatory sequence in the left operon were individually replaced.

Bacterial Strains, Plasmids, and Bacteriophage:

The bacterial strains, plasmids, and bacteriophage used during this experiment are shown in Table 1.

Media for Plates:

The recipes for the bacteriological media used to make the plates are given: LB plates (per liter): 10 g Tryptone, 5 g yeast extract, 5 g NaCl, and 15 g agar. TB top agar (per liter): 10 g Tryptone, 5 g NaCl, and 7 g agar.

Table 1: Bacterial strains, plasmids, and bacteriophage used in this study.

Strains	**Relevant Characteristics/Genotype**	**Reference**
MC1000	Δ(lac)X74	20
RK1097	MG1655(HK022 @ tRNA*gly* at 10Kb) rpoC+/pLUXam; SB1424/pSB1326; *amp*^{R}, *cam*R, *kan*R	21
RK1145	MC1000 (HK639) transformed with pRK1006; *amp*^{R}	King, R.
RK898	RW4198 = MG1655; wild-type, *rpoC*+	Weisburg, R. unpublished
RK899	RW4200 = MG1655; *rpoC*Y75N	Weisburg, R. unpublished
RK1372	RK1145 Δ33,095-33,200::*kan*^{R}, kanamycin replacing two terminators in left operon	Carroll, A. and Urso, O. This study
RK1373	RK1145 Δ32,178-32,383::*kan*^{R}, kanamycin replacing ORF 41	Urso, O. This study
RK1374	RK1145 Δ32,384-32,535::*kan*^{R}, kanamycin replacing ORF 42	Urso, O. This study
RK1377	RK1145 Δ31,811-32,096::*kan*^{R}, kanamycin replacing ORF 40	Carroll, A. This study
RK1379	RK1145 Δ32,532-33,050::*kan*^{R}, kanamycin replacing ORF 43	Carroll, A. This study
RK1385	RK1145 Δ30,886-32,906::*kan*^{R}, kanamycin replacing ORF 40 and ORF 39	Urso, O. and Carroll, A. This study
RK1386	RK1145 Δ30,866-31,808::*kan*^{R}, kanamycin replacing ORF 39	Carroll, A. and Urso, O. This study
Plasmid		
pRK1006	pSim6 [= ori-pSC101repA(ts) (cI857 PL-*exo-bet-gam*;*amp*^{R})]	18
Phage		
HK639		4

Table 2: Oligonucleotide list in which the oligos were ordered from Integrated DNA Technologies (IDT)

Oligo	Sequence	Use
290	TTgCCATgTTTCAgAAACAACTC	Verification of Terminators, ORF 39, 40, 41, and 43.
291	gATCTTgCCATCCTATggAAC	Verification of Terminators, ORF 39, 40, 41, 42, and 43
568	CGGCAACATCGACAACTTTG	Verification of ORF 39
570	CGCGGCAAGCCAAAATATAG	Verification of Terminators and ORF 43
729	GAATCAGTCAGCTTTGCGAAATCATTGG CGATCT GGTAAAGAGCTGTCATgtggaccagttggtgatttt g	Recombineering for ORF 39
748	TGGCCCCAAAACTGTATCTCCTCGAATA CGCAGC CTCACTAATTAAGTAAtccgacaaccgatgaaagc	Recombineering for Terminators
749	CGCTTAAGGCCGCGCCGCCGAACGTTAA ACAAG ACTTCTGCGCTTGTGCGgtggaccagttggtgatttt g	Recombineering for Terminators
750	AAGCGCAGAAGTCTTGTTTAACGTTCGG CGGCG CGGCCTTAAGCGCGGAGtccgacaaccgatgaaa gc	Recombineering for ORF 43
751	TCGTTGAGCTTTTTGGCGGTGTACTGCTT GCCGT TGTGGGTGACTGTCATgtggaccagttggtgattttg	Recombineering for ORF 43
752	ACATTGACGCCATCAATGCGGCAAATGC CAAAG CCAGGGAGGCATCATGAtccgacaaccgatgaaa gc	Recombineering for ORF 42
753	AAAACCTCGCTACGTGGCACTGATGCGG TGCCATAGTGCATTCCGATCATgtggaccagt tggtgattttg	Recombineering for ORF 42
754	GGCAGATGCATATCGCTGGCCTCCTGAA ACAGG TTGAGGTGAAGGTATGAtccgacaaccgatgaaa gc	Recombineering for ORF 41
755	GATCCCGCCCGCAAAAAGCCAGGCCGA TCGGTT GAATAGGGTGGTTAATAgtggaccagttggtgatt ttg	Recombineering for ORF 41

756	CTGGCTTTTTGCGGGCGGGATCTGCACA TCCAAA TTTCAGGAGTTCAGCCtccgacaaccgatgaaagc	Recombineering for ORF 39 and 40
757	GGCTCCTGCTCCTTTACGAACTGAATCA GATCGA AGCTCATAATCACTCCgtggaccagttggtgattttg	Recombineering for ORF 40
758	CATGCTGGACCATCTCAACC	Verification of Terminators
760	GTGGTGTTGCAGCATTGTGC	Verification of ORF 42 and 43
761	CTTAACTGGCACTGCACTTAAC	Verification of ORF 41 and 42
762	GTATGGGCTGGAGTCAATCG	Verification of ORF 41
763	CATCATCAAAAGTGATTCCGTCG	Verification of ORF 39 and 40
764	ACGTTGATAATCGCGTTCTGG	Verification of ORF 40
804	ACGCCCAGGCGGAATACGACTGGGAAC ACCGTA CCGGCTGCCCGTTTTAAtccgacaaccgatgaaag c	Recombineering for ORF 39
805	GAAATCATTGGCGATCTGGTAAAGAGCT GTCAT TGCGTAACCTCTTCGAATTTTGgtggaccagtt ggtgattttg	Recombineering for ORF 39

Generating the Recombination Substrate:

To generate the substrate for recombineering, the kanamycin gene was amplified using PCR. The kanamycin gene was flanked by oligonucleotides (oligos) that were designed to incorporate approximately 50 bp of sequence homology to the targeted region on the phage genome (Table 2). The Phusion High Fidelity PCR Kit (NEB Cat. No. E0553L) was used to amplify the kanamycin resistant gene since high-fidelity was required for this reaction. The Phusion enzyme is a high-fidelity polymerase and creates products with blunted ends. The error rate for this enzyme is nearly >50 fold lower than that for Taq polymerase.

A typical 100 µL reaction contained the following components: 73 µL water, 20 µL HF buffer (1X final concentration), 100 pmol of each oligo primer from IDT (Table

2), 2 µL 10mM deoxynucleotide mix (dNTP), 2 µL of cell suspension, and 1 µL Phusion enzyme (2,000 units/mL). The reactions were placed into a Peltier Thermal Cycler (PTC). An individual cycle included heating the samples to 98°C for 10 seconds to denature the DNA, cooling to 57°C for 30 seconds for annealing, and raising to 72°C for two minutes to allow the DNA polymerase (Phusion enzyme) to add the appropriate nucleotides. After 34 cycles, the temperature was maintained at 72°C for ten minutes to allow for full extension of the products. PCR products were then held at 4°C until further processing.

Agarose Gel Electrophoreses:

To ensure success, PCR products were visualized using gel electrophoresis. This was performed using the Fisher brand electrophoresis units (Fisher Cat. No. FB-SB-710 or FB-SB R-1316). Electrophoresis was completed using 1.25% agarose gels consisting of 1.25 g of agarose dissolved in 100 mL of 10X TAE buffer (per liter: 942 g Trizma base, 150 mL 500mM EDTA, 67.1 mL Glacial Acetic Acid). The DNA size standards used were a λ DNA/Hind III ladder (Fisher Cat. No. PR- G1711) and a 100 bp ladder (Axygen Cat.No. M-DNA-100BP). The ladder sizes are labeled in the figures in the results. Samples were prepared for electrophoresis by mixing 2 µL of Sambrook's loading dye (Sambrook & Russell, 2001) with 5 µL of PCR reaction. The expected lengths of the PCR products were predicted using Table 2 and the Geneious Prime software package (Biomatters, Inc., San Diego, CA). If the band sizes matched the predicted size of the amplified fragment, the products were purified and prepared for electroporation.

Purification of PCR Products:

The Sigma-Aldrich GenElute PCR clean-up kit (Cat. No. NA1020) was used to purify the PCR products. The directions from the kit were followed and the purified DNA was recovered in elution buffer.

Buffer Exchange using a Centrifugal Filter Unit:

An additional round of purification was performed on the DNA substrate used for recombineering to ensure the removal of salts. The DNA sample was mixed with water (600 µL total volume) in an AmiconUltra 0.5 mL centrifugal filter unit (Fisher Cat. No. UFC510096). The device was centrifuged for 10 minutes at 4,000 X g. The filtration device was then flipped upside down, placed in a fresh 1.5 mL microcentrifuge tube, and centrifuged for two minutes at 4,000 X g. The retentate contained approximately 20 µL of the purified DNA in water. Purity and concentration were determined by spectrophotometry.

Preparation of Electrocompetent Cells:

Host cells containing the HK639 prophage were prepared for electroporation for each recombineering experiment. First, 5 mL of Luria Bertani (LB) broth (per liter: 10 g Tryptone, 5 g yeast extract, and 5 g NaCl) was placed in a 15 mL conical tube and inoculated with a loop full of RK1145 culture (Table 1). This culture was grown overnight at 30°C with shaking at 250 rpm. The next day, 200 µL of the overnight culture was transferred into a flask containing 30 mL of LB media. This subculture was grown at 30°C while shaking at 250 rpm until the optical density at wavelength 650 nm (OD_{650})

reached 0.4-0.5. At this point, the culture was shifted to a 42°C water bath while shaking at 250 rpm for 20 minutes to activate the expression of the recombination proteins. Strain RK1145 contains plasmid pRK1006, from phage γ, that possesses a temperature-sensitive repressor. Heating the culture to 42°C causes the repressor to denature and allows for the promoter to transcribe the recombination function proteins which are then released into the cell.

After the induction period, the culture was quickly cooled in an ice water bath to allow for the repression of the recombination functions in the plasmid. It is important to place the cells in an ice water bath to stop the transcription of the recombination functions. The cooled culture was divided evenly into two 15 mL tubes and centrifuged at 1,000 X g at 4°C for 10 minutes. The pelleted cells were re-suspended in ice cold 10% glycerol to remove any excess salts. The centrifugation and washing with ice cold 10% glycerol were repeated twice. After the final centrifugation, the supernatant was poured off and the cells were re-suspended in 40 µL of ice cold 10% glycerol. The electrocompetent cells were always used immediately after preparation.

All electrocompetent cells and DNA were kept on ice during this part of the experiment. One sample of cell suspension was used as a control (no substrate DNA added). The other cell sample was combined with 200 ng of recombineering DNA substrate. The cells were transferred by pipette to 2 mm electroporation cuvettes (Fisher Cat. No. FB102) and subjected to a 2.5 kV electric shock. The Bio-Rad Gene Pulser xCell was used to perform the electroporation.

After the electroporation event, 1 mL of Super Optimal broth with Catabolite repression (SOC) broth (per liter: 20 g Tryptone, 5 g yeast extract, 0.5 g NaCl, 1.25 mL

of 2M KCl, 5 mL 1M $MgCl_2$, 1 mL 1M $MgSO_4$, and 2 mL 1M glucose) was then added to the cuvettes to provide the cells with a nutrient-rich medium. This mixture was transferred into a 15 mL tube and was incubated at 30°C with shaking at 250 rpm for 1 hour to allow the cells to recover from the electroporation. The cells were then transferred to 1.5 mL microcentrifuge tubes and spun at 1,000 X g until cells were pelleted. The cells were re-suspended in 200 µL of LB media.

Using the spread plating technique, the cells were evenly distributed onto two LB plates containing kanamycin (20 µg/mL) and incubated for 24 hours at 30°C. The recovery of kanamycin-resistant colonies suggested that the recombination was successful since the original strain is unable to grow in the presence of kanamycin. Individual colonies were picked and streaked onto kanamycin plates (20 µg/mL) and incubated at 30°C for 24 hours to purify the recombinants. Independent colonies were then inoculated into 5 mL of LB media supplemented with kanamycin (20 µg/mL) and grown overnight at 30°C with shaking at 250 rpm. The overnight cultures were centrifuged at 1,000 X g for 10 minutes at 4°C. The supernatant was discarded, and the cell pellet was re-suspended in 2.5 mL of 10mM $MgSO_4$ and stored at 4°C.

Confirmation of Recombination Junctions:

To ensure that the kanamycin gene was inserted into the correct location in the HK639 genome, the recombination junctions were amplified using PCR. Oligonucleotides were designed to prime at each recombination junction (Table 2). Taq polymerase was used to amplify the recombination functions (Fisher Cat. No. FB-6000-15). A typical 100 µL PCR reaction consisted of the following: 2 µL of the recombinant

cell suspension (from an overnight culture), 30 µL 1.8 mM PCR mix, 1 µL Taq enzyme (5,000 Units/µL), 64 µL water, and 100 pmole of each oligonucleotide. The amplification products were run on a 1.25% agarose gel, as described above. A correctly sized product (estimated using Geneious software and Table 2) indicated that the kanamycin gene recombined in the appropriate location in the HK639 genome. The same analysis was performed on purified phage stocks to confirm the released phage were indeed recombinant.

Testing Recombinant Cells for Phage Release:

Lysogenic cells often spontaneously release phage as the release has an on and off rate. Occasionally, the repressor falls off long enough for the genes to be transcribed and expressed. This was verified to occur with the starting strain RK1145 (Table 1). To determine if specific gene replacements affect phage production, supernatants from overnight cultures were assayed for lytic activity. In addition, many prophages can be induced to enter the lytic cycle by treating the cells with mitomycin-C, a DNA damaging agent. Mitomycin-C has an indirect effect on the prophage. Once mitomycin-C has been introduced, the bacterial cell releases a protein, rec-A, which stimulates the repressor of the prophage to cleave itself and fall off. This allows for the expression of genes that could cause the phage to grow lytically.

The recombinant cells were tested for both spontaneous phage release and induced phage release by mitomycin-C. To perform these tests, two 5 mL stocks of recombinant cells were grown in LB, supplemented with kanamycin (20 µg/ml), at 30° C until the OD_{650} reached 0.3-0.4. At this point, 20 µL of a 50 mg/mL stock solution of

mitomycin-C were added to one of the two cultures to induce phage release. Both cultures were incubated at 30° C with shaking at 250 rpm overnight.

After overnight incubation, 50 µL of chloroform were added to each culture to kill the cells and promote lysis. The cultures were thoroughly mixed and then centrifuged for10 minutes at 12,000 X g. One mL of the supernatant was collected from each tube; the lysed bacterial cells in the pellet and the chloroform were carefully avoided. An indicator plate was prepared by mixing 4 mL of molten LB top agar with 80 µL of overnight RK898 culture (Table 1). This mixture was poured onto an LB plate and evenly distributed by gently swirling the plate. After solidifying, 10 µL of each purified supernatant was spotted onto the plate. After the spots soaked in, the plate was incubated at 37° C for 24 hours and then examined for plaque formation. If plaques appeared, they were picked and suspended in 100 µL of 1X Phage Buffer (PB) (per liter: 10 mM Tris Stock, 10mM $MgSO_4$, 0.4% NaCl, and 1 mM $CaCl_2$ stock).

The phage samples were diluted from concentrations of 10^0 to 10^{-5} and each dilution was plated onto a lawn of indicator cells (as mentioned above). The plate was incubated at 37°C for 24 hours. A plate containing confluent plaques (displaying a "webby" pattern of plaques) was then flooded with 5 mL of 1X PB. The phage were allowed to diffuse into the PB for 24 hours. The phage-rich stock was collected using a 10 mL syringe and was filtered through a Fisherbrand 25 mm syringe filter with 0.2 µm pores. The titer of the phage lysate was determined by making serial dilutions (10^{-5} to 10^{-9}), using the methods described above. After incubation, the plates were examined, and plates with 20-200 isolated plaques were counted. The titer was then determined using the formula:

$$\frac{\text{phage forming units (pfu)}}{\text{mL}} = \frac{\text{number of plaques}}{\text{dilution} \times \text{volume plated}}$$

DNA Sequencing:

The recombination junctions were confirmed by DNA sequencing using the BigDye Terminator v3.1 Cycle Sequencing Kit (ABI, Catalog No. 4337457). A typical 10 µL reaction contained 3 µL of the whole cell PCR reactions (see *Confirmation of Recombination Junctions*), 2 µL of BigDye Terminator sequencing juice, 2 µL of 5X BigDye Terminator sequencing buffer, and 100 pmol of a sequencing primer (Table 2). The thermocycler settings were as follows: 25 cycles of 96°C for 30 seconds, 50°C for 15 seconds, 60°C for 4 minutes followed by a 10°C hold.

Upon completion, the sequencing reactions were purified using a SigmaSpin Sequencing Reaction Clean-Up kit (Sigma Catalog No. S5059) following the manufacturer's instructions. The purified DNA was dried in a centrivap concentrator. The sequencing products were then suspended in 20 µL of formamide and loaded into a 3130 ABI sequencer. The resultant sequences were then compared to the published HK639 genome in GenBank (Accession NC_016158) (King *et al*., 2011).

RESULTS

Summary:

To identify essential genes in the left operon of HK639, five genes (ORF 39, ORF 40, ORF 41, ORF 42, and ORF 43) and a pair of transcription terminators were systematically replaced. In each case, recombineering, a powerful chromosome engineering technique, was used to replace the targeted sequences with a kanamycin resistance gene.

Phage Recombineering:

Recombineering is a technique that allows for the precise replacement of genes inside of cells. For this study, it was used to replace five genes and a regulatory sequence in the left operon of HK639 with the kanamycin resistance gene. After the amplified DNA substrate was electroporated into the cells and recombination occurred, the recombineering substrate was verified. All recombineering experiments generated recombinants as evidenced by the growth of kanamycin-resistant colonies (Figure 5). Selecting for recombinants on a kanamycin plate is effective because cells that did not successfully recombine are unable to grow in the presence of kanamycin.

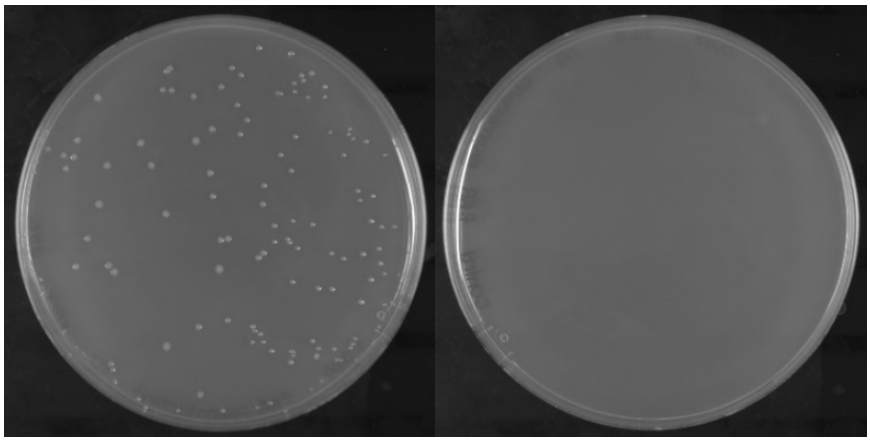

Figure 5: The plate on the right is the control kanamycin plate (20 μg/ml) and shows no colony formation. The plate on the left depicts an example of recombinant colonies growing in the presence of kanamycin (20 μg/ml). The ORF 43 recombinant is shown here.

The recombination junctions were verified by amplifying the regions of interest using PCR, and if the kanamycin gene was in the correct location, phage release was observed. To test for the viability of the recombinant phages, the culture containing the recombinants were place on a lawn of indicator cells. Clear plaques indicate lytic activity as the bacteriophage has successfully lysed the bacterial cell. Most of the recombinants released phage, and the morphology of the plaques stayed consistent throughout the replacements. A typical plate exhibiting HK639 phage release on the indicator strain RK898 (Table 1) can be seen in Figure 6. Details of each replacement are given below.

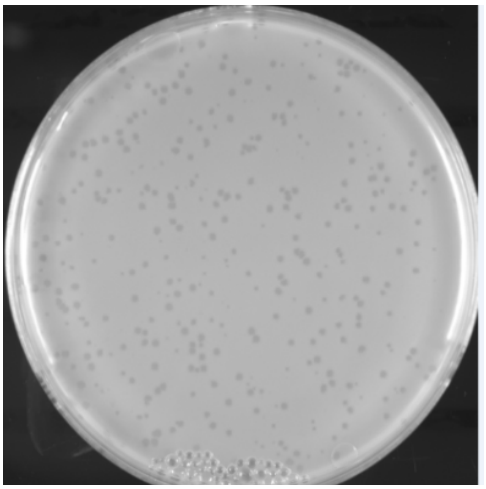

Figure 6: Example of HK639 phage release on an indicator strain RK898 (Table 1). Clear areas (plaques) indicate lytic activity. Plaque morphology remained consistent throughout this study.

Terminator Sequence Replacement:

Two terminators in the left operon of HK639 were replaced with the kanamycin resistance gene (Figure 4). The recombineering substrate was successfully generated on a 1.25% agarose gel (Figure 7) and kanamycin resistant recombinants were recovered. The size band of 1,300 bp corresponds to the size of the kanamycin gene. The recombination junctions were amplified by PCR and visualized using a 1.25% agarose gel (Figure 8). In this replacement, the predicted sizes of the junctions (using Geneious and Table 2) were 540 bp and 586 bp.

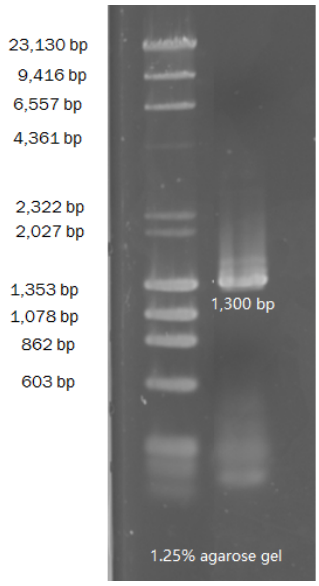

Figure 7: Recombineering substrate for the terminator replacement. The 1,300 bp band shown in Lane 2 is the expected size product. The product was run on a 1.25% agarose gel. This figure has been cropped to only show the lanes in question.

Lane	Oligos	Size
A-C	758 x 291	540 bp
D-F	570 x 290	586 bp

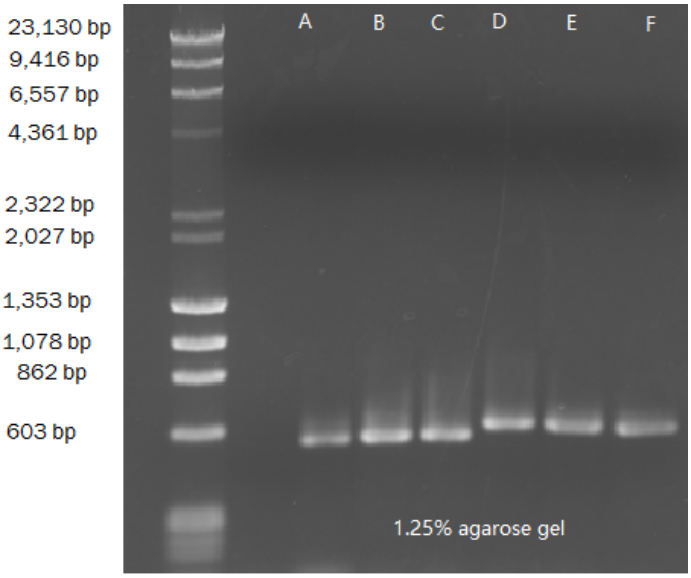

Figure 8: Verification of terminator replacement recombination junctions. Lanes A-C are amplification products from the upstream (Kan gene N-terminus) recombination junction. All three candidates generated a band of the expected size (540 bp). Lanes D-F represent amplification products from the downstream (Kan gene C-terminus) recombination junction. All three candidates generated a band of the expected size (586 bp).

Sequencing also verified the recombination junctions and that no unwanted mutations were introduced through the amplification steps. Spontaneous release of phage was not observed. However, when this recombinant was induced with mitomycin-C, a single plaque was recovered. The single plaque was picked and subjected to several rounds of purification. A high titer lysate (1.84×10^{10} pfu/mL) of the phage was generated and tested by PCR to confirm that the phage contained the kanamycin gene in the correct location.

ORF 43 Replacement:

ORF 43 was replaced with the kanamycin resistance gene (Figure 4) as outlined above. The recombineering substrate was successfully generated on a 1.25% agarose gel (Figure 9) and kanamycin resistant recombinants were recovered. The size band of 1,300 bp corresponds to the size of the kanamycin gene. The recombination junctions were amplified by PCR and visualized using a 1.25% agarose gel (Figure 10). In this replacement, the predicted sizes of the junctions (using Geneious and Table 2) were 600 bp and 540 bp. Sequencing also verified the recombination junctions.

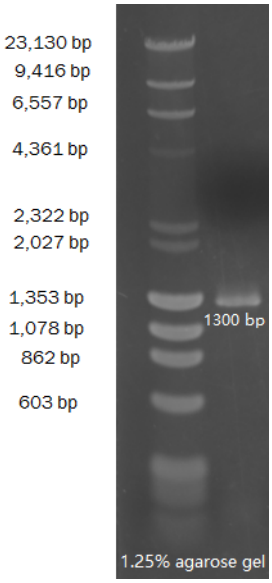

Figure 10: Recombineering substrate for the ORF 43 replacement. The 1,300 bp band shown in Lane 2 is the expected size product. The product was run on a 1.25% agarose gel. This figure has been cropped to only show the lanes in question.

Lane	Oligos	Size
A-D	759 x 291	600 bp
E-H	760 x 290	540 bp

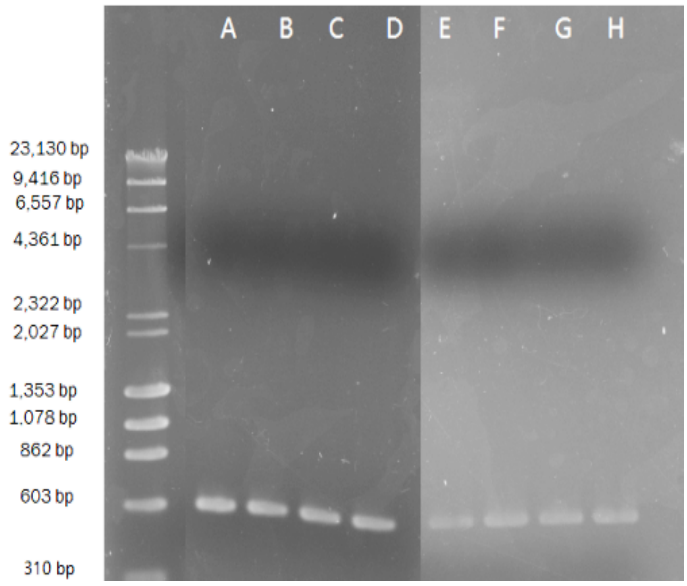

Figure 9: Verification of ORF 43 replacement recombination junctions. Lanes A-D are amplification products from the upstream (Kan gene N-terminus) recombination junction. All four candidates generated a band of the expected size (600 bp). Lanes E-H represent amplification products from the downstream (Kan gene C-terminus) recombination junction. All four candidates generated a band of the expected size (540 bp).

The ORF 43 recombinant cells did not release phage spontaneously but did release phage when induced with mitomycin-C. A single plaque was picked and subjected to several rounds of purification. A high titer lysate (1.94 x 10^{11} pfu/mL) of the phage was generated and tested by PCR to confirm that the phage contained the kanamycin gene in the correct location.

ORF 42 Replacement:

ORF 42 was replaced with the kanamycin resistance gene (Figure 4). The recombineering substrate was successfully generated on a 1.25% agarose gel (Figure 11) and kanamycin resistant recombinants were recovered. The size band of 1,300 bp corresponds to the size of the kanamycin gene. The recombination junctions were amplified by PCR and visualized using a 1.25% agarose gel (Figure 11). In this replacement, the predicted sizes of the junctions (using Geneious and Table 2) were 521 bp and 384 bp. Sequencing also verified the recombi nation junctions.

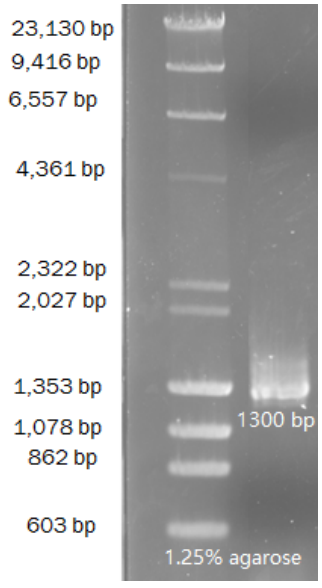

Figure 11: Recombineering substrate for the ORF 42 replacement. The 1,300 bp band shown in Lane 2 is the expected size product. The product was run on a 1.25% agarose gel. This figure has been cropped to only show the lanes in question.

Lane	Oligos	Size
A, C, E, G	760 x 291	521 bp
B, D, F, H	761 x 290	384 bp

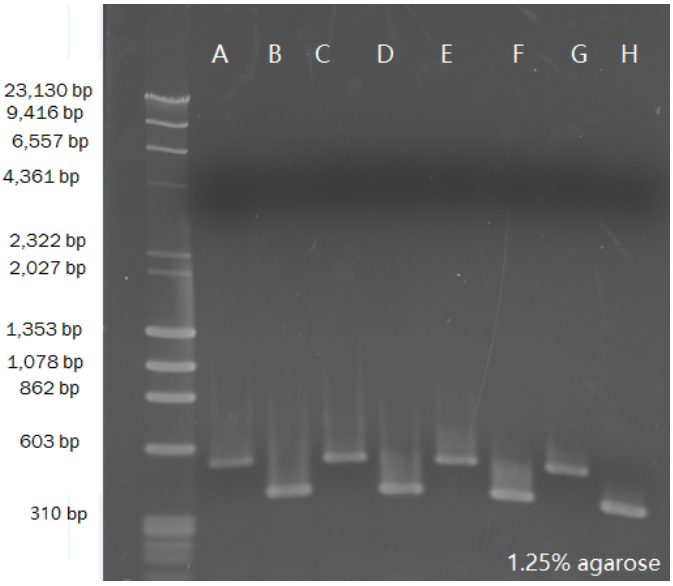

Figure 12: Verification of ORF 42 replacement recombination junctions. Lanes A, C, E, and G are amplification products from the upstream (Kan gene N-terminus) recombination junction. All four candidates generated a band of the expected size (521 bp). Lanes B, D, F, and H represent amplification products from the downstream (Kan gene C-terminus) recombination junction. All four candidates generated a band of the expected size (384 bp).

The ORF 42 recombinant cells released phage spontaneously and release was stimulated by mitomycin-C treatment. A single plaque was picked and subjected to several rounds of purification. A high titer lysate (1.44 x 10^9 pfu/mL) of the phage was generated and tested by PCR to confirm that the phage contained the kanamycin gene in the correct location.

ORF 41 Replacement:

ORF 41 was replaced with the kanamycin resistance gene (Figure 4). The recombineering substrate was successfully generated on a 1.25% agarose gel (Figure 13) and kanamycin resistant recombinants were recovered. The size band of 1,300 bp corresponds to the size of the kanamycin gene. The recombination junctions were amplified by PCR and visualized using a 1.25% agarose gel (Figure 14). In this

replacement, the predicted sizes of the junctions (using Geneious and Table 2) were 577 bp and 462 bp. Sequencing also verified the recombination junctions.

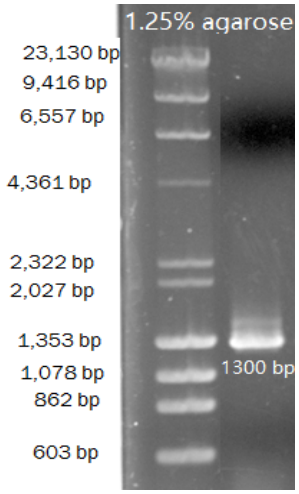

Figure 14: Recombineering substrate for the ORF 41 replacement. The 1,300 bp band shown in Lane 2 is the expected size product. The product was run on a 1.25% agarose gel. This figure has been cropped to only show the lanes in question.

Lane	Oligos	Size
A, C, E	761 x 291	577 bp
B, D, F	762 x 290	462 bp

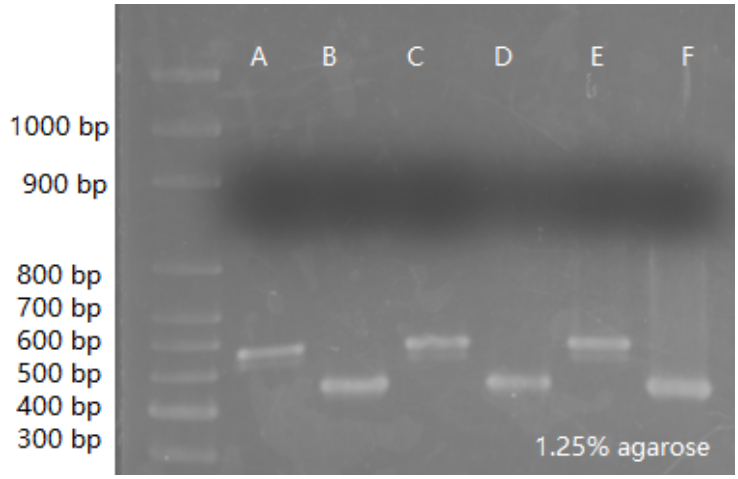

Figure 13: Verification of ORF 41 replacement recombination junctions. Lanes A, C, and E are amplification products from the upstream (Kan gene N-terminus) recombination junction. All three candidates generated a band of the expected size (577 bp). Lanes B, D, and F represent amplification products from the downstream (Kan gene C-terminus) recombination junction. All three candidates generated a band of the expected size (462 bp).

The ORF 41 recombinant cells released phage spontaneously and release was stimulated by mitomycin-C treatment. A single plaque was picked and subjected to several rounds of purification. A high titer lysate (1.44×10^8 pfu/mL) of the phage was generated and tested by PCR to confirm that the phage contained the kanamycin gene in the correct location.

ORF 40 Replacement:

ORF 40 was replaced with the kanamycin resistance gene (Figure 4). The recombineering substrate was successfully generated on a 1.25% agarose gel (Figure 15) and kanamycin resistant recombinants were recovered. The size band of 1,300 bp corresponds to the size of the kanamycin gene. The recombination junctions were amplified by PCR and visualized using a 1.25% agarose gel (Figure 16). In this replacement, the predicted sizes of the junctions (using Geneious and Table 2) were 492 bp and 504 bp. Sequencing also verified the recombination junctions.

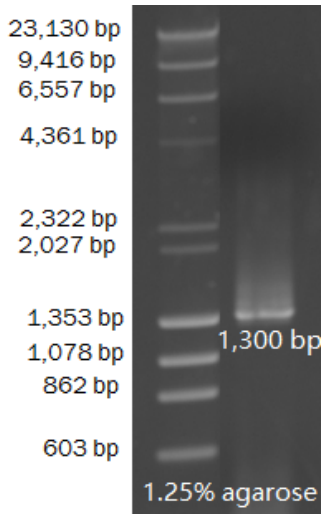

Figure 15: Recombineering substrate for the ORF 40 replacement. The 1,300 bp band shown in lane 2 is the expected size product. The product was run on a 1.25% agarose gel. This figure has been cropped to only show the lanes in question.

Lane	Oligos	Size
A-D	763 x 291	492 bp
E-H	764 x 290	504 bp

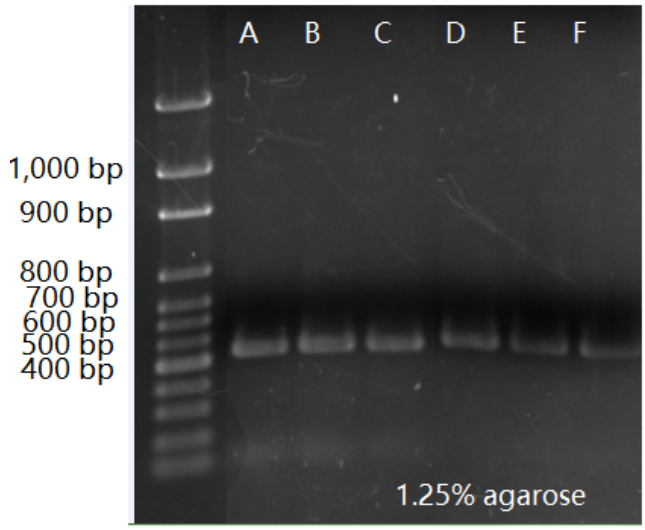

Figure 16: Verification of ORF 40 replacement recombination junctions. Lanes A-C are amplification products from the upstream (Kan gene N-terminus) recombination junction. All three candidates generated a band of the expected size (492 bp). Lanes D-F represent amplification products from the downstream (Kan gene C-terminus) recombination junction. All three candidates generated a band of the expected size (504 bp).

ORF 40 recombinants did not release phage under any condition. This test indicated that ORF 40 was essential for either lytic phage release or phage replication. We also cannot rule out the possibility that the ORF 40 knockout affected the expression of a potentially critical downstream gene. To determine if ORF 40 itself was necessary, two additional recombineering experiments were performed: one removing the adjacent downstream gene (ORF 39) and one removing ORF 39 and ORF 40 together. The ORF 39 and ORF 40 replacement did not release phage. However, the ORF 39 replacement released phage. In addition, the ORF 40 recombination junctions were amplified and confirmed through sequencing. The results of sequencing indicated that the sequence of the downstream gene was not affected by the knockout/insertion.

DISCUSSION

The rise of antibiotic resistant bacteria has created a medical crisis. Bacteriophages are a potential solution to this problem; however, very little is known about bacteriophage gene function and biology. By identifying essential genes for lytic growth in the left operon of HK639, this study adds to the knowledge of bacteriophage gene expression and phage biology.

Bacteriophages HK022 and HK639 are similar in that they both use RNA-mediated antitermination, a unique and poorly understood mechanism to control the expression of their early genes. Surprisingly, HK022 can grow lytically even in the absence of gene expression from its left operon. A similar result was expected with HK639, but a previous study showed that the replacement of the HK639 left operon genes prevented phage release from an HK639 lysogen (Seaton, 2013).

It was assumed that the phage integrase gene, which plays a role in phage excision from the host genome, was the only gene in the left operon essential for lytic growth, therefore preventing phage release when the left operon expression was blocked. However, the results of this study demonstrate that a(n) additional left operon gene(s) is also crucial for phage release. The necessity of this gene was initially shown by the lack of plaque formation when ORF 40 was replaced with the kanamycin resistance gene.

The lack of phage release indicates that either the expression of this gene is essential for lytic growth and release, or the replacement of this gene interfered with the expression of essential downstream genes. It was possible that the ORF 40 knockout

interfered with the expression of the adjacent downstream gene, ORF 39 (phage-related recombinant protein) (Accession NC_016158) (NCBI, 2020). Additional knockouts, however, showed that when ORF 39 and ORF 40 were replaced together, phage release was blocked, but a replacement of ORF 39 alone allowed for the release of phage. These results suggest that the replacement of ORF 40 did not interfere with ORF 39, and that ORF 40 may perform an essential function for phage release.

In addition, the kanamycin promoter is not identical to the promoter of the gene it replaced, which may have altered the expression of downstream genes critical to phage release. If ORF 40 contains a promoter for a downstream essential gene, and the kanamycin promoter does not promote for the expression of this same gene, this could prevent phage release. This may mean these genes (and possibly additional downstream genes) work together to promote phage growth and release, and that interference with any single component prevents phage release.

When ORF 40 was "BLAST-ed" (searched against all documented phage genes in the national DNA sequence database), there was no significant matches. The lack of known function further illustrates that that little is known about bacteriophage genetics, even genes that may be crucial for phage release.

Whether ORF 40 is essential for phage release or the knockout interfered with the expression of crucial downstream genes, our results demonstrate that the alteration of specific genes of the left operon has detrimental effects on HK639 lytic growth. Based on studies of other phages, the replacements may have interfered with the excision of the phage from the host's genome or the replication of the phage. This study has identified

the players, but further work is now needed to identify the way in which phage excise and replicate. Additional studies are needed to distinguish between these possibilities.

Lytic phage do not need to express genes used for excision from the host cell's genome. Lytic phage can simply infect the bacterium, replicate and assemble the viral parts, and lyse the cell. This process does not involve the phage integrating its DNA into the host's genome. However, with current technology and recombineering, bacteriophage genes are nearly impossible to alter unless they are in the lysogenic state inside the host's genome. If the replication of the phage is inhibited, then lytic phage truly cannot grow without the expression of that gene because it cannot synthesize more viral parts. If the gene replacement affects excision from the host genome, however, then a crucial step of the lysogenic life cycle is inhibited, not necessarily lytic growth.

The HK639 left operon contains two adjacent transcription terminators located downstream of the antiterminator RNA element (*putL*). It was expected that the removal of these terminators would result in increased phage release since it would no longer be necessary to promote the readthrough of the terminator pair. Surprisingly, phage release was significantly reduced compared to the other recombinants generated in this study; only 1-3 plaques recovered per phage release experiment. Interestingly, the recombinant phage was perfectly capable of growing to high titers. This suggests that antitermination is important for phage excision and that the recombinant is deficient in this step of the life cycle. After excision, the recombinant phage appeared to grow normally.

The replacement of ORF 39, ORF 41, ORF 42, and ORF 43 did not prevent phage release. These results clearly demonstrate that these genes are not essential for lytic growth in a lab environment. However, organisms tend not to maintain useless functions.

Perhaps these genes are necessary for a different step in the phage life cycle (e.g. lysogeny), or perhaps they are critical under conditions that are different from a lab. Historically, understanding basic phage biology has led to the discovery of novel regulatory mechanisms (e.g. lysis-lysogeny decisions, RNA-mediated antitermination, etc.) and it has provided revealing insights into virus-host interactions. Expanding our understanding of these simple viruses will provide the insight needed to exploit these natural parasites to benefit human health and help us deal with the menace of antibiotic resistant microbes.

In this study, modifications in the DNA sequence were achieved using phage mediated recombineering. A cloned plasmid, from bacteriophage λ, catalyzed the reaction and allowed for recombination functions to be expressed. However, there is a new mechanism of chromosome engineering that is on the frontier of microbiology and has been used to manipulate the genomes of plants and animals. The CRISPR-Cas 9 system is not only easy to use but is also cost effective (Doudna & Charpentier, 2014).

CRISPR is short for "Clustered regularly interspaced short palindromic repeats", and Cas 9 is an associated protein to CRISPR that acts as an endonuclease. CRISPR-Cas 9 takes advantage of a naturally occurring mechanism. Originally, CRISPR was identified in bacteria as an immune system, but this newly discovered mechanism has since been modified and exploited to perform controlled, site-specific modifications in DNA. It functions by forming base pairs with targeted DNA sequences which then allows the endonuclease to perform two site-specific breaks in the DNA (Doudna & Charpentier, 2014).

To do this, CRISPR-Cas 9 works in two parts: the chimera (guide) RNA and the Cas 9 protein. To cut a specific sequence of DNA, the target sequence is identified. The guide RNA is then manipulated to contain homologous pairs that will bind to the target sequence. The DNA is then fed through the complex, and CRISPR contains helicases that unwind the DNA. When the guide RNA aligns with the target sequence, it holds it there while Cas 9 cleaves the DNA at two site-specific locations. This is shown in Figure 17.

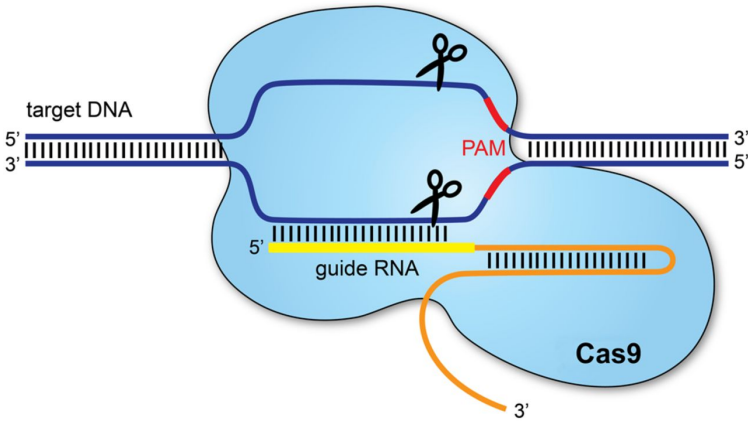

Figure 17: Displays how CRISPR-Cas 9 functions (Redman *et al*., 2016). The guide RNA (yellow) is specifically designed to be homologous to a certain sequence of the target DNA (blue). Once these sequences align, the Cas 9 protein then cleaves the target DNA in two site-specific locations (indicated by the scissors).

In other words, at the 5' side, there is a DNA target site, and at the 3' side, there is a structure that binds to Cas 9 (Ran *et al*., 2014). The associated proteins to CRISPR can ultimately be modified to contain different functions. For example, instead of just simply cutting the DNA, a gene can be removed and replaced with a new gene, or, a site-specific base pair change can be generated. This genomic engineering technique is more specific, extremely versatile, easier to construct, and is very efficient. The use of this genomic engineering technique is also much quicker than the recombineering used in this lab.

CONCLUSION AND FUTURE WORK

During this experiment, recombineering was used to replace six genetic sequences in the left operon of lysogenic *E. coli* bacteriophage HK639. After replacing the six genetic sequences, phage release tests were conducted to determine the importance of each genetic sequence. Out of the six different replaced genetic sequences, five of the sequences released phage: the terminator replacement, ORF 39, ORF 41, ORF 42, and ORF 43. The plaques formed by the released phage were characterized and compared. Our results show that these genes and regulatory sequence are thus non-essential for the lytic growth of bacteriophage HK639.

No phage were released from the ORF 40 knockout. Gene replacements were performed to determine whether ORF 39 or ORF 40 was important or if the knockouts were affecting downstream genes. Because ORF 39 and ORF 40 replacements did not release phage, but ORF 39 did, it can be determined that ORF 39 is not essential for lytic release but ORF 40 is. Whether this gene itself is crucial for lytic release or if this replacement affects the expression of crucial downstream genes is inconclusive, however.

Further research is necessary to identify additional essential genes that are necessary for supporting phage growth in bacteriophage HK639. There may be additional genes in the left operon that are essential for lytic growth. The remaining genes in the left operon should be knocked out and tested for phage release as was done in this study. Additionally, further biochemical analysis of ORF 40 needs to be conducted. This may

reveal its function and determine why it is essential and if it affects the replication or excision of the phage.

In the future, there are two particular experiments that can be performed to determine if ORF 40 affects excision or replication, and if it does, which one. A real time PCR could be used to determine if the bacteriophage replicates in the absence of ORF 40. If the number of copies of the phase increases, this would suggest that the phage is in fact replicating. A complementation assay could be generated to determine if excision is affected by the removal of ORF 40. An excision gene can be cloned and placed into the cell. If this replacement of the excision gene ultimately allows for phage release, then it can be concluded that ORF 40's function is related to excision.

Ultimately, this information provides the groundwork for viral genetics. In order to combat anti-biotic resistant bacteria, bacteriophage and their gene functions must first be understood. For example, a cytotoxicity assay could be completed for each of the genes to determine if they have an impact on cell growth. Knowing which genes code for certain functions under certain conditions can eventually allow for them to be manipulated so that bacteriophages can act as therapeutic agents.

It is also worth noting that in future experiments, a new genomic engineering technique, CRISPR-Cas 9, could be used. The technique used in this lab is somewhat limited. For recombineering, a strain with a plasmid with recombination functions had to be used. Many overnight cultures had to be obtained and there were several hours of preparation work. However, with CRISPR-Cas 9, the opportunities are endless. If CRISPR-Cas 9 was used for this experiment, it would be a lot quicker and require less downtime, would require less resources, and would be more efficient.

Without a solution, antibiotic-resistant bacteria will have detrimental effects on humanity. However, bacteriophages may be a useful weapon to control the threat. Further investigation of bacteriophage gene function brings us one step closer to exploiting their use as bactericidal agents to control human infections. In addition, analyses such as this deepen our understanding of viruses in general, and this could help us control global pandemics like COVID-19.

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