Characterization of *Escherichia coli* Single-Gene Deletion Mutants Impaired in Bacteriophage Reproduction

by

Kelly Ryan Reimer

A thesis submitted in conformity with the requirements for the degree of Master of Science

Graduate Department of Molecular Genetics

University of Toronto

© Copyright by Kelly Ryan Reimer 2012
Characterization of *Escherichia coli* Single-Gene Deletion Mutants Impaired in Bacteriophage Reproduction

Kelly Ryan Reimer

Master of Science

Graduate Department of Molecular Genetics
University of Toronto

2012

Abstract

An assay was designed to measure the sensitivity of *Escherichia coli* mutants to bacteriophage infection via growth curves, using a Tecan temperature controlled plate reader. I screened 3985 single-gene deletion strains in *Escherichia coli* K12 from the Keio collection and identified 43 strains displaying varying degrees of resistance to four different phages, three non-contractile tailed phages (*λ*cI857, HK97, and HK243) and the myoviridae T6, including 20 genes not previously implicated in phage infection. Additional assays, such as adsorption and tests of DNA-injection, were designed to further characterize resistant strains. The use of these assays helped identify varying sensitivities to LPS structure and LamB receptor concentration in the three non-contractile tailed phages, showing HK97 is the most sensitive to changes and HK243 the least. I also found that the periplasmic chaperone, FkpA, is required for HK97 DNA-injection.
Acknowledgments

This thesis would not have been possible without my supervisors. Their hard work and
guidance was instrumental in my development into a critical scientist. I am grateful to Dr. Karen Maxwell, Dr. Al Edwards, and Dr. Alan Davidson for all of their support, direction, and
for allowing me the opportunity to do my work in such a wonderful and interesting lab.

In addition, I would like to extend my gratitude to my committee members, Dr. Jack
Greenblatt and Dr. John Parkinson, for their critical advice and scientific input, and for helping
to ensure my mind remained sharp. I would also like to show my appreciation to Dr. Paul
Sadowski for providing the pDV64 plasmid, and for the benefit of his experience and guidance
during my research. Additionally, thanks to Greg Brown for providing the ASKA plasmids
used in this study, and for his assistance in this aspect of my project.

A huge thanks to all the current and former members of the Davidson and Maxwell labs
for creating an amazing work environment and being available for help with anything I needed.
In particular I would like to thank Lemieux for his work with the HK97 screen, and to Diane for
her technical support, and for teaching me how to do basically everything that made this project
possible. A special thanks to my colleagues Lisa, Nichole, Senjuti, Mostafa, Kris, and Nasim
for all of their assistance and critiquing, as well as making sure there was never a day with a dull
moment.

I would like to extend my deepest gratitude to all of my friends and family, for their help
and comfort throughout the writing process. In particular, thanks to Christine for her patience in
putting up with me throughout this process, and for being an amazing source of encouragement,
advise, and moral support. To Kelly, for dealing with a difficult roommate during this time, and
helping make sure I still took some time for fun aside from work. And finally, thanks especially
to my parents, for all of their love and unconditional support in everything I’ve done. Without
them I would not have made it to where I am today!
# Table of Contents

Acknowledgments .................................................................................................................. iii

Table of Contents .................................................................................................................. iv

List of Tables .......................................................................................................................... vi

List of Figures .......................................................................................................................... vii

Chapter 1 Introduction .......................................................................................................... 1

1.1 Thesis Overview ............................................................................................................... 1

1.2 Background and Bacteriophage Diversity ...................................................................... 1

1.3 The Typical Gram Negative Bacteriophage Life Cycle .................................................. 2

1.3.1 Host Adsorption and DNA-Injection ........................................................................ 2

1.3.2 The Lysis/Lysogeny Decision in Temperate Phages ................................................. 5

1.3.3 Entry into the Lytic Cycle, and DNA-Replication ..................................................... 9

1.3.4 Virion Assembly ........................................................................................................ 10

1.3.5 Host Cell Lysis ......................................................................................................... 11

1.4 Methods of Characterizing Bacteriophage Resistances .............................................. 12

1.5 Goals of this Thesis ....................................................................................................... 13

Chapter 2 Identification of genes in *Escherichia coli* required for bacteriophage replication ..... 15

2.1 Overview ........................................................................................................................ 15

2.2 Bacteriophages Used in this Study .............................................................................. 16

2.3 Materials and Methods ............................................................................................... 19

2.3.1 Keio Collection Growth Curve Screen for Resistances ........................................... 19

2.3.2 Keio Collection Plating Screen for Resistances ......................................................... 19

2.3.3 Adsorption Assay ..................................................................................................... 20

2.3.4 Maltose Dependency Assay - Plating ................................................................... 21

2.3.5 Maltose Dependency Assay – Growth Curve ...................................................... 21
2.3.6 Potassium Efflux Assay as a Measure of DNA-Injection ........................................... 22
2.3.7 Lysogen Formation Assay ........................................................................................................ 22
2.3.8 Removal of Kanamycin Resistance Cassette .............................................................................. 23

2.4 Results .......................................................................................................................................... 24

2.4.1 Tecan Screening Reveals 43 Genes Involved in λcI857, HK97, HK243, or T6 Reproduction ........................................................................................................................................... 24
2.4.2 Spotting Reveals 34 Genes Involved in Phage Reproduction .............................................. 27
2.4.3 LPS Mutations Lead to an Adsorption Defect in λcI857 that Correlates with its Ability to Reproduce .................................................................................................................................................. 30
2.4.4 HK97 Shows a High Sensitivity to Maltose Levels, while HK243 is Unaffected .................... 32
2.4.5 Potassium Efflux is a Sensitive Measure of λcI857 DNA Injection .......................................... 37
2.4.6 Removal of Kanamycin-Resistance Cassette from E14 Prophage Genes Restores Infectivity to ymfO− and ymfE− ........................................................................................................................................... 39

2.5 Discussion .................................................................................................................................. 41

2.5.1 The Liquid Growth Curve Screen Detects Subtle Phenotypes Missed by the Plating Screen .................................................................................................................................................. 41
2.5.2 Downregulation of the mal-Regulon Leads to Resistance to λcI857, HK97, and HK243 .................................................................................................................................................. 42
2.5.3 λcI857, HK97, and HK243 have Differing Dependencies on LPS Structure for Infection .................................................................................................................................................. 46
2.5.4 Different Strains of the Same Phage Can Have Differing Requirements for Host Factors .................................................................................................................................................. 48
2.5.5 The Defective Lambdoid E14 Prophage Plays a Role in T6 Infection ....................................... 50

Chapter 3 Summary and Future Directions .................................................................................. 53

3.1 Summary .................................................................................................................................. 53
3.2 Future Directions ......................................................................................................................... 54

References ....................................................................................................................................... 57
List of Tables

Table 1: Characteristics of phages used in this study. ................................................................. 18

Table 2: Lysis timing in hours from Tecan growth curves screen for four phages and E. coli deletions causing phage resistance................................................................. 25

Table 3: Functions of E. coli genes whose deletions caused phage resistance in the Tecan growth curve screen. ................................................................. 26

Table 4: Changes in apparent titer from spotting-based resistance screen ................................. 29

Table 5: Relative strengths of λcl857 resistance from growth curve screen and measured ability to adsorb by 20 minutes. ................................................................. 32

Table 6: Increases in observed titer by order of magnitude from 0.4% glucose to 0.4% maltose containing minimal media plates showing maltose partially rescues HK97 and λcl857 infectivity ................................................................. 35
List of Figures

Figure 1: Typical life cycle of a temperate bacteriophage ................................................................. 4

Figure 2: Genomic layout of bacteriophage λ ......................................................................................... 8

Figure 3: Sample plate from spotting screen for phage resistance ......................................................... 28

Figure 4: Representative graphs from adsorption assay ........................................................................... 31

Figure 5: Representative plating results from maltose-dependence assay ............................................. 35

Figure 6: Representative growth curves showing HK243’s ability to infect rfaD- strains compared with λcI857 ........................................................................................................................................ 36

Figure 7: Potassium efflux is observed for E. coli after λcI857 infection ..................................................... 38

Figure 8: FLP-recombinase curing of the kanamycin resistance cassette partially restores T6 infectivity ........................................................................................................................................ 40

Figure 9: Schematic diagram showing λcI857 resistant strains relating to the mal-regulon ......... 43

Figure 10: Basic structure and synthesis of E. coli K12 LPS ................................................................. 47

Figure 11: Overview of the e14 genome and T6 resistance ..................................................................... 52
1.1 Thesis Overview

In this thesis I describe a method of discovering and characterizing *Escherichia coli* K12 genes that are involved in bacteriophage reproduction. By detecting mutations in genes that lead to the inability for a bacteriophage to reproduce, as measured by uninhibited bacterial growth, we can gain knowledge into the function of those genes. Studies on these mutants and their effect on different bacteriophages have led to the discovery of many important systems in molecular genetics, such as restriction modification/systems\(^1\). The use of bacteriophages in research, in general, led to the birth of molecular genetics, with many early studies unraveling the basic principles of life, including the culmination of a series of experiments showing that DNA itself is the material responsible for passing along genetic information\(^2\); \(^3\). With the tools available now, including the capacity for large-scale growth screens, whole genome sequences, and comprehensive gene knockout libraries, the study of bacteriophage replication should continue to prove a valuable source of new insights in the field.

1.2 Background and Bacteriophage Diversity

*Escherichia coli* is one of the most well-studied and important model organisms in biological research, with the first genome sequence completed in 1997\(^4\). Despite being one of the first fully-sequenced genomes, with over a decade of analysis, 1081 of its approximately 4465 genes remain poorly characterized. Of the 3384 characterized genes, only 2941, or 66%, have had their functions assigned experimentally\(^5\). The human genome, consisting of approximately 20,300 protein-encoding genes, includes 6000 which have no experimental evidence at the protein level, with many more lacking information on protein abundance, functions, and other information\(^6\). If we ever hope to be able to fully understand the genome of a complex organism such as a human, we first need to be able to understand a less complex one such as *E. coli*. One of the ways we can obtain information about the functions of these genes is by the use of bacteriophages (also referred to as phages), bacterial viruses which rely on a variety of gene products of their host organism in order to reproduce.
Bacteriophages are viruses that infect and kill bacteria, including *E. coli*. Discovered in 1915 by Frederick Twort, an English bacteriologist in London, during work with Staphylococcus, and independently in 1917 by Felix d’Herelle, a French Canadian at the Pasteur Institute, by observing the destruction of Shigella in broth, they have since been instrumental in developing our understanding of biological organisms at the molecular level. The study of one phage in particular, phage λ, has led to major contributions to the study of transcription regulation, recombination mechanisms, mechanisms of DNA replication, viral evolution, and DNA sequencing and cloning technologies, amongst many others. Comparisons of growth inhibition in phages infecting various bacterial hosts led to the discovery of the bacterial restriction/modification systems, consisting of restriction endonucleases and DNA-methylases. This discovery was recognized by the 1978 Nobel Prize.

In addition to their role in deciphering the molecular basis of biology, bacteriophages play an important ecological role, both in the environment and inside the human body. They are estimated to be the most widely distributed and diverse entities in the biosphere, exceeding the number of bacteria tenfold, with approximately $10^{31}$ existing worldwide. They play an important role in ocean ecology, accounting for 20% of marine bacterial mortality and contributing a significant portion of the 50% turnover rate in marine bacteria every 24 hours.

1.3 The Typical Gram Negative Bacteriophage Life Cycle

1.3.1 Host Adsorption and DNA-Injection

Bacteriophages have an intimate relationship with their hosts, as illustrated by the different stages of their life cycles. The work in my thesis focuses on the tailed phages, and in order to provide a better understanding of how dependent the phage is on the many factors within its host, I have provided an overview of the tailed phage life cycle, using λ as a model phage. Because λ has been well characterized, it serves as a good demonstration of how interconnected the phage life cycle is with the various host factors in *E. coli*. Emphasis on host factors that are necessary for phage infection and replication will be highlighted with additional examples drawn from the study of other phage systems where applicable. For a general summary of phages used in this study, see section 2.2.
The life cycle of a phage begins with adsorption to the host (see Figure 1). Specialized tail fibers on the phage make contact with their receptor on the surface of the cell, whether it be a protein such as the maltoporin LamB used by phage λ, or other components such as the lipopolysaccharide making up the outer leaflet of the outer membrane used by phage T7. In some cases, an initial reversible adsorption takes place, such as with the long tail fibers of phage T4, making contact with the OmpC protein in E. coli K-12 strains, before the short tail fibers make irreversible contact with the LPS.

After adsorbing irreversibly to the cell, the phage must get the DNA into the cell in order to reproduce. This carries with it the challenge of bypassing the outer membrane, the highly crosslinked peptidoglycan layer that provides structural support, the periplasmic space filled with nucleases, and finally the cellular membrane before reaching the cytoplasm. In phages with side tail fibers, once contact is made, conformational changes occur to bring the baseplate in contact with the surface. After the phage makes irreversible contact with its target receptor, the central fiber protein is thought to be cleaved or lost, followed by the injection of a tape measure protein through the outer membrane into the periplasm.

The tape measure protein is thought to form a channel from the phage through to the cytoplasm, although the exact mechanism, or other proteins involved have not yet been determined. The tape measure protein gpH and the tail tip protein gpJ are the only structural components of the phage tail tip complex long enough to span the periplasmic space and may serve a role in protecting DNA from nucleases present in the periplasm. The tape measure protein of λ becomes protease-resistant in complex with phage-bound LamB embedded in a liposome, but does not when complexed with soluble LamB. Conversely, gpJ becomes more protease-sensitive when complexed with LamB embedded in a liposome, indicating that it is gpH that must form the channel. Additional studies with T5 tape measure Pb2 also show that the tape measure protein forms channels and mediates DNA injection into liposomes. λ gpH has also been shown to acquire mutations that compensate for DNA-injection defective mutants of E. coli (pel’ mutants) which possess mutant forms of ManY, a component of the phosphotransferase (Pts) system in the inner membrane required for DNA injection. In addition to ManY, the only two other E. coli host proteins definitely shown to play a role in DNA injection are DcrA and DcrB, necessary for phages C1 and C6, respectively. This phenotype was initially discovered as a series of mutations that lead to resistance to the phage C1.
The cycle begins when a phage virion makes contact with its particular receptor on the host cell surface. A poorly understood process occurs in which the phage injects its genetic material through the periplasm and into the cytoplasm, which can involve a variety of periplasmic and inner membrane proteins. At this point a temperate phage can either enter the lysogenic cycle based on environmental conditions, or continue on with the lytic cycle, while lytic phages proceed straight into the lytic cycle. The next step in the lytic cycle consists of phage DNA replication and transcription, followed by translation into the protein components for the mature phage virion. These protein components self-assemble in two separate pathways, creating an unfilled procapsid (head) of the virion, and the tail component. The procapsid is next filled with individual copies of each phage genome and becomes a mature phage capsid containing highly compacted DNA. The tail and the head then join to form a complete mature virion. In the majority of known phages, the production of phage encoded products, called endolysins and holins, causes the cell to lyse by degrading the peptidoglycan layer until osmotic pressure bursts the cell, though some single-stranded DNA phages are released without lysis. In the case of a temperate phage entering the lysogenic cycle, the DNA is first inserted into the host chromosome. The phage DNA is replicated along with the host’s. Cells continue to divide, with each daughter cell possessing a copy of the phage genome integrated into its chromosome. The presence of phage proteins called repressors maintain the lysogenic state of the cell until environmental conditions again become conducive to phage growth. Once this state is detected, the phage genome is excised from the host chromosome, and the cell proceeds along into the lytic cycle.
In order for the DNA to get through to the cytoplasm it also needs to bypass the peptidoglycan layer (murein layer). This is a rigid structure of cross-linked polymers of N-acetylglucosamine and N-acetylmuramic acid which lends strength and structure to E. coli and prevents diffusion of globular proteins larger than about 50 kDa. Under certain physiological conditions, the peptidoglycan can be highly cross linked, making it difficult for the tape measure to form a channel. The peptidoglycan is constantly breaking its cross-linkages and forming new ones in growing cells, and temporary gaps in the network form during peptidoglycan recycling. This process allows for transport of larger molecules and structures, though some, such as those involved in flagellar assembly, actively hydrolyse peptidoglycan with muralytic enzymes. The speed and synchronization of genome insertion following phage adsorption make it unlikely that phages only take advantage of this transient restructuring of the peptidoglycan, and indeed phage virions are known to contain peptidoglycan-degrading activities. For example, peptidoglycan-degrading activities have been characterized for the Pb2 protein of T5, the tape measure protein for mycobacteriophage TM4, and gp16 of E. coli phage T7. Studies on TM4 and T7 also show that while deletion of the peptidoglycan hydrolase motif from these proteins abrogated growth on stationary phase cells, it did not affect their ability to grow on exponentially dividing cells. Gp16 of T7 is part of the internal core structure of the capsid, is known to be injected into the host before the phage genome, and likely functions to enlarge a hole in the peptidoglycan layer. The action of these muralytic enzymes, whether part of the tape measure protein or released into the host separately, could assist DNA injection in conditions of highly cross-linked peptidoglycan and allow the tape measure to form a channel.

1.3.2 The Lysis/Lysogeny Decision in Temperate Phages

Once the phage genome has entered into the cell, and the phage is a temperate phage, the phage may face a choice in its life cycle. Temperate phages are capable of proceeding along the lytic pathway, leading to cell lysis, or integrating into the host genome and replicating along with the host, which a lytic phage cannot do. If the conditions in the cell are not conducive to phage growth, such as poor nutritional status, then temperate phages enter a lysogenic cycle. This decision is brought about primarily in the case of λ by the levels of phage-encoded CI, CII, CIII, and CRO proteins. CII levels control the initial step of the lysis/lysogeny decision by activating transcription of the cI repressor gene from the P_RE promoter, as well as by activating the P_I...
promoter, which drives expression of the integrase. (For a summary of transcriptional control in the λ genome, see Figure 2 at the end of this section). The CI protein is a repressor protein necessary for maintaining the state of lysogeny once it has begun. In addition to activating its own transcription from the \( P_{RM} \) promoter\(^{19} \), CI inhibits transcription from the early promoters involved in lytic development, pL and pR, favouring a lysogenic life cycle\(^{7} \). Integrase is a phage protein encoded by \( int \) that, with assistance from the host-encoded integration host factor (IHF), catalyzes a site-specific reciprocal recombination step between the \( attP \) site on the phage chromosome and the \( attB \) site on the \( E. coli \) chromosome\(^{20}; 21; 22 \). While \( \lambda \) is still capable of forming plaques, or areas of clearing on a lawn of bacteria caused by the lysis and spread of phages, on strains deficient in \( ihf \), these mutants can inhibit the lytic growth of HK022\(^{23} \). Once integrated into the chromosome, the \( \lambda \) genome is maintained in this state by CI and replicates along with the host genome.

Control of the lytic/lysogeny decision depends on the ability of the phage to sense the nutritional conditions inside of the cell and determine the appropriate response. Phages can sense these conditions by a variety of mechanisms, such as through interaction with host factors such as RNaseIII and FtsH/HflB in the case of \( \lambda \), as well as by multiplicity of infection, possibly through gene dosage effects of CII and other phage proteins\(^{24}; 25 \). RNaseIII is a double-strand specific ribonuclease whose expression appears to be tied to cell growth rate, with higher rates leading to higher expression of RNaseIII\(^{26} \). It regulates the expression of three \( \lambda \) genes, \( N \) (required for N mediated transcriptional antitermination initiated at \( P_L \) and \( P_R \) through to the remaining early genes), \( cII \), and \( cIII \)\(^{26}; 27 \). Mutants that cannot express RNaseIII (\( rnc^{-} \)) show an upregulation of \( N \) and, additionally, overexpressing \( N \) from a plasmid results in clear plaques, suggesting a lower probability of lysogenization\(^{26} \).

FtsH/HflB is an essential ATP-dependent protease located in the membrane that associates with HflK and HflC\(^{28}; 29; 30; 31 \). FtsH is thought to affect the stability of \( \lambda \) CII and CIII, which have \textit{in vivo} half-lives of about one-to-two minutes\(^{30}; 32 \) and seven minutes,\(^{33} \) respectively. Loss of function mutations in \( ftsH \), \( hflK \), and \( hflC \) increase the half-life of CII, and mutations in host-encoded \( hflD \), which expresses a membrane protein that recruits CII to the membrane for FtsH-mediated proteolysis, promote lysogeny\(^{30}; 34 \). Overexpression of \( ftsH \) promotes lytic growth, resulting in clear plaques\(^{35}; 36 \), although, curiously, overexpression of HflKC stabilizes CII and promotes lytic growth, which is contrary to what is expected\(^{30} \). CIII is also degraded by
FtsH, with slower kinetics, and limits degradation of CII by binding to FtsH as a competitive inhibitor\textsuperscript{37}, and thus conditions that increase CIII expression also lead to an increased frequency of lysogenization\textsuperscript{35; 38; 40; 41; 42}. FtsH activity is increased at higher temperature, and it has been suggested to be increased at higher levels of nutrition as well\textsuperscript{43}. Low cAMP levels when cells are grown on glucose have been suggested to be linked to FtsH activity, but this idea remains to be tested\textsuperscript{44; 45; 46; 47; 48}.

Once conditions become conducive to lytic growth, λ excises its genome from the host, with the aid of the host-encoded IHF, as well as phage encoded \textit{int} and \textit{xis} (excisionase), and proceeds through the lytic cycle. In order for this to occur, breakdown of the phage repressor must occur. This can occur stochastically in exponentially growing cultures. It can also be controlled experimentally by having a thermally sensitive repressor, such as in the case of \(\lambda cI\textsubscript{857}\), with a heat destabilized CI protein. Another more natural method of induction is through activation of the bacterial SOS response to DNA damage. This global response occurs when single strand oligonucleotide breakdown products are generated by irradiation or other means, which activates the coprotease activity of the host RecA protein (involved in homology-dependent recombination). This activity normally helps cleave and deactivate the host LexA master control protein, which represses genes whose products are involved in DNA repair and damage recovery, leading to an upregulation of these genes. Many phage repressors, including λ’s CI, which mimic LexA and are susceptible to cleavage by active RecA, are deactivated by DNA damage, leading to de-repression of phage-encoded lytic genes\textsuperscript{7}. 
Figure 2: Genomic layout of bacteriophage λ: (Figure and caption are taken from Casjens, S. R. and Hendrix, R. W. 2001. Bacteriophage Lambda and its Relatives. eLS)\textsuperscript{49}. The linear virion chromosome is shown with a scale in kilobase pairs (kbp) below. Rectangles above indicate known genes; with yellow ones transcribed rightward and green ones leftward. Important sites (e.g. P, promoters; t, terminators) on the DNA are indicated below the genes, and arrows below the kbp scale indicate the transcripts made from the λ chromosome: brown, made only in a lysogen; black, made in lysogen and pre-early conditions; orange, pre-early; blue, early; green, late; purple, made in response to high CII levels; grey, made in response to DNA replication.
1.3.3 Entry into the Lytic Cycle and DNA-Replication

Once the phage has entered the lytic cycle several events occur. \( \lambda \) begins transcription from the promoters \( P_L \) and \( P_R \), using the host RNA polymerase. Some phages, such as T7, encode their own polymerase, which they use instead of the host’s. A single gene from each promoter is transcribed, \( N \) from \( P_L \) and \( cro \) from \( P_R \). The protein \( N \) acts as a transcription antiterminator by binding to a specific sequence in the nascent mRNA transcripts from each promoter called the \textit{nut} (\( N \) utilization) site\(^{50}; 51; 52; 53\). In order for \( N \) to render the RNA polymerase insensitive to termination signals, \( N \) interacts with the RNA polymerase and four host proteins (\( NusA, NusB, NusE, \) and \( NusG \)). After \( N \) mediated antitermination takes place, transcription is able to extend through to the remaining early genes, including genes involved in homologous recombination\(^{54}; 55\) and phage DNA replication, followed by 10 genes in the nin region not essential for phage growth in the laboratory. Genes in this region remain poorly characterized and can be removed with no known ill effect on the phage under standard culturing. The last gene transcribed is \( Q \), which is responsible for allowing RNA polymerase to read through a strong terminator downstream of the late promoter \( P_R' \), a function similar to that of \( N \), though \( Q \) recognizes DNA sequences rather than the mRNA\(^{56}\).

The \( \lambda \) lytic cycle exits the “early period” once expression from the late genes at \( P_R' \) begins, around 10-12 minutes post-infection. \( cro \) encodes a repressor protein \( Cro \), which binds to the operators \( O_L \) and \( O_R \), controlling \( P_L \) and \( P_R \), respectively,\(^{57}\) and leads to a decrease in transcription from each promoter. Phage DNA replication begins when the O protein binds to the replication origin\(^{58}; 59\) and recruits the P protein, which in turn recruits the host DnaB Helicase\(^60\). This complex is partially disassembled by host DnaK, DnaJ, and GrpE chaperones, which release the complex from the origin, following which the remainder of the replication apparatus is assembled and DNA replication begins. Replication of \( \lambda \) proceeds bidirectionally to produce daughter circles, and, 10-15 minutes into the lytic cycle, switches to a rolling circle mechanism that generates linear genomic concatemers, which are later cut into single genomes to be packaged into heads. The mechanism for this switch remains unclear. Finally, transcription which had begun early on at the constitutive late promoter \( P_R' \) continues forth downstream due to the action of \( Q \), and transcription of the remaining genes involved in virion assembly and host cell lysis takes place.
1.3.4 Virion Assembly

From P_R’ essentially all of the late genes, including the structural genes, are transcribed. Though RNA polymerase takes over 10 minutes to traverse this operon in λ, thus temporally controlling transcription of these genes, the assembly of these protein products appear to not be dependent on the order transcribed. Levels of each protein are controlled at the level of translation, likely due to translation initiation signals surrounding the beginning of each gene, and proteins self-assemble in two separate pathways to produce phage heads and tails. DNA is packaged into the head, which joins with a tail, and the cell lyses to release mature virions.

The process of head assembly begins with the assembly of a procapsid, an empty shell composed of coat proteins, into which DNA is ultimately packaged. Of the ten genes in λ needed for head assembly, only the products of six end up in the mature head. Twelve subunits of gpB make up the portal structure, a ring at the base of the procapsid where the DNA will enter. In order for this process to produce functional portals, the host GroEL/S chaperone must interact at the level of subunit folding. The discovery of this host chaperone was a result of finding an E. coli mutant that blocked phage λ growth (hence Gro) that was later overcome by a compensatory mutation in λ gene E (thus GroE). The portal then may act as a nucleus for the remainder of prohead assembly, though the details of the pathway are not clearly understood. After gpE forms the initial procapsid, the head maturation protein gpC, along with the putative scaffold protein product of Nu3 which is nested in frame in the last one third of the C gene, are included in the procapsid, with gpNu3 eventually being removed from the inside of the capsid. Ten copies of gpC and gpE form a fusion product, which gets trimmed down proteolytically into either X1 or X2. The locations of these products in the procapsid are not known, but they are thought to be structural adaptors between gpE and the portal.

After assembly and maturation, the concatemeric phage DNA is cleaved at the cos site by a protein complex made up of the products of genes Nu1 and A called the terminase. This generates the DNA substrate to be packaged into each procapsid. The terminase docks with the portal and pumps the DNA into the portal, then cuts at the downstream cos site to release the terminase attached to the beginning of the next chromosome to be packaged. Both of these steps, cutting at the cos site as well as packing the DNA, are aided in vitro by an accessory role of the
bacterial IHF\textsuperscript{72}. In cells lacking IHF, \(\lambda\) is able to still infect \textit{in vivo}, though the burst size is reduced to 25\% of IHF\textsuperscript{+} cells\textsuperscript{73}.

In a separate pathway, the tail is assembled beginning at the tip with gpJ, followed by gpI, gpK, gpL, and gpM\textsuperscript{74; 75; 76}. gpH, gpG, and gpG-T form a complex that interacts with gpV, the major tail protein, and the tip to form the majority of the tail\textsuperscript{77; 78} with the last two proteins, gpU which acts as a cap to stabilize the tail shaft, and gpZ whose absence results in phage deficient in DNA injection\textsuperscript{79}. gpH encodes the tape measure, which determines the length of the tail shaft\textsuperscript{80; 81}.

1.3.5 Host Cell Lysis

The final step of the typical bacteriophage life cycle involves the release of the mature virions by cell lysis, although some single-stranded DNA (ssDNA) phages do not lyse the cell at all\textsuperscript{7}. All bacterial cells possess a peptidoglycan layer, which helps maintain cell integrity and acts as a barrier for the release of phage. Phages are capable of degrading this barrier via one of two different mechanisms\textsuperscript{7; 82}. Some small single-stranded nucleic acid phages are capable of lysing without their genomes encoding a muralytic enzyme. They achieve this through the product of a single gene in each known case. This protein was shown in the cases of phages \(\phi\text{X174}\) and \(Q\beta\) to interfere with specific steps of cell wall synthesis, eventually leading to lysis\textsuperscript{83; 84; 85}. By contrast, all known double-stranded phages utilize a “holin-endolysin” strategy, whereby the peptidoglycan becomes degraded by an endolysin with timing controlled by the holin.

In the case of \(\lambda\), five genes are involved in lysis. \(R\) encodes a transglycosylase that hydrolyzes a 1,4-\(\beta\) linkage between \(N\)-acetyl-D-glucosamine and \(N\)-acetyl muramic acid, weakening the cell wall so that it can be ruptured by osmotic pressure\textsuperscript{86; 87}. \(Rz\) and \(Rz1\) are encoded in the same segment of DNA, but under two different open reading frames\textsuperscript{88}. They encode two proteins that are required for lysis and plaque formation in the presence of high concentrations of divalent cations\textsuperscript{89}. The \(S\) gene encodes both the holin and the antiholin using an alternate translational start site mechanism\textsuperscript{90; 91}. The holins form “rafts” at the cellular membrane and disrupt the membrane, while the anti-holin inhibits the action of the holin. The timing of membrane collapse and subsequent release of endolysin to the peptidoglycan is believed to be controlled by the levels of \(S\) protein in the cell. When a critical concentration is
reached it is thought that a local disruption in the holin raft leads to a conformational change in S that favours dispersion, thus creating a hole.7

The antiholin appears to fine-tune the timing of this event in λ, with knockouts leading to a 5 minute faster lysis timing in a laboratory setting, though mutants of S have been isolated that have this same timing. It appears that rather than being necessary for normal timing, the antiholin may play a role in overriding the evolutionarily selected time schedule under differing conditions, as has been seen in studies of the T4 antiholin RI. In λ, the proportions of holin and antiholin are controlled by RNA structures, and there is evidence that a host-encoded RNA-binding factor plays a role in efficiency of translation of the S mRNA. This host factor may play a role in sensing environmental conditions and overriding lysis timing. Similar structures have been found in other systems containing two translational start sites as well.93 After the release of endolysin and degradation of the peptidoglycan, osmotic forces will lyse open the cell to release mature phage virions, and the life cycle can begin anew.

1.4 Methods of Characterizing Bacteriophage Resistances

Due to the strong dependence on host factors at many points in the bacteriophage life cycle, resistance to infection can result from a defect at many stages in a bacteriophage infection. Mutations in genes encoding proteins affecting the cell envelope can lead to the inability of a phage to properly adsorb to its receptor, or an inability to inject its genome into the cell, or may interfere with the actual lysis process. Possessing knowledge of what stage of this cycle is being affected allows information to be gained about the gene that has been knocked out in the host, and can also help identify new roles for previously uncharacterized or partially characterized genes. In the past, large scale screens in E. coli looking for resistance to bacteriophages have been performed via different mechanisms. In the podoviridae (short-tailed) bacteriophage T7, a screen was performed by spotting bacterial colonies from the Keio collection of single-gene deletions in E. coli K12 onto a nutrient lawn with embedded T7 phages. They then looked for mutants that were able to grow on the lawn of phage. Another recent screen was performed on λ by another mechanism.95 Lawns of each Keio knockout were prepared such that dilutions of λ plated with each lawn formed plaques. The absence or sizes of each plaque were then measured the next day. From each screen, mutants found that were impaired in phage reproduction were subjected to further analysis, including looking at their growth curves under phage infection.
Each of these methods identifies resistant mutants by one endpoint condition, whether it be presence or absence of a bacterial colony or the size of a plaque. In contrast, by measuring the growth curves, you can see defects in phage reproduction right from the initial infection, and mutations that impair but do not entirely eliminate reproduction could be more readily identified.

In addition to simply measuring phage reproduction, numerous techniques exist to measure defects at specific points of the phage life cycle, such as adsorption, injection, the ability of temperate phages to form lysogens, or assembly. Simple centrifugation-based assays have been described to measure adsorption, whereby phages bound to cells are spun down, while those unable to bind remain in the supernatant and can be measured by plating on sensitive cells. Lysogen formation can also easily be measured in temperate phages containing a temperature-sensitive repressor, such as λcI857, due to the ability to control induction through temperature and look for lysis or lack of lysis in non lysogens or defective lysogens. Defects in phage assembly can by identified by viewing lysates under an electron microscope and looking for aberrant or lack of structures such as tails or heads. DNA injection can be measured by exploiting the observation that potassium ions tend to be released in correlation with the amount of DNA entering the cell, and can be measured by use of a potassium-selective electrode.

1.5 Goals of this Thesis

It is clear that while there is a lot known about bacteriophage biology and interaction with its host, many aspects of this relationship remain poorly understood. Additionally, despite being one of the most well-studied model organisms, *E. coli* still contains a large percentage of uncharacterized or poorly characterized genes. Due to the strong dependence on conditions within the host, bacteriophage have been used to great effect in the past to probe and uncover the roles of many genes. Examples include the GroEL/S folding chaperone, discovered using a mutant *E. coli* strain that blocked *λ* growth, and restriction modification systems, discovered studying different bacterial strains which inhibited the growth of phages grown on some hosts, but not others. In addition, as the field of molecular biology has developed, better tools have become at our disposal, including comprehensive gene knockout libraries and the capacity for large scale screens, making large scale studies of the interaction of phages with individual components of the host feasible. Due to the success of phage sensitivity screens in the past, the large number of still relatively poorly understood aspects of the phage life cycle and *E. coli* gene
function, and the powerful tools now available, a comprehensive identification of *E. coli* host factors that play a role in phage reproduction would be of great value in our understanding of these systems. To that end I proposed to:

a) **Develop a rapid, high-throughput, quantifiable assay to measure phage resistance:** This will allow for the aforementioned benefits of a growth curve based assay to be applied to screens easily and quickly performed in the lab

b) **Use the assay to screen 3985 single-gene knockouts of *E. coli* K12 for strains that are resistant to phage infection:** This can be used to validate the utility of the assay, as well as uncover new phenotypes and perform a comparison across a variety of phages

c) **Develop additional rapid assays for use in characterizing these strains:** These will allow for comparatively simple and fast testing of results from high-throughput screens, and to identify the stages of the phage life cycle that are affected
Chapter 2
Identification of genes in *Escherichia coli* required for bacteriophage replication

2.1 Overview

In order to characterize resistance to bacteriophages a screen was designed based on measuring growth curves. Four bacteriophages, including the three *Siphoviridae*, λcl857, HK97, and HK243, as well as one T4-like *Myoviridae*, T6, were screened against 3985 nonessential single-gene deletion mutants in *E. coli* K12 from the Keio knockout library\(^{96, 97}\). By measuring growth curves with a plate reader, resistance phenotypes such as lack of cell lysis or a delay in cell lysis could be observed. There are several advantages over traditional techniques, such as plaque assays or plating colonies on phage-containing lawns. In a traditional plaque-based assay, dilutions of phage are spotted on a lawn of bacteria, and measurements of phage infectivity can be determined by the turbidity of the plaque, the overall size of the plaque, and whether or not a plaque is able to form. While this information can help determine infectivity of a phage and changes in burst size based on plaque diameter, it suffers from the fact that analysis occurs at one endpoint, and any information on the growth of the phage during incubation is lost. A similar technique, whereby bacterial cultures of colonies are spotted onto an agar plate containing phage embedded in the media, and resistance is detected by growth of the colony, suffers from the same lack of growth information. Additionally, the phage are embedded in the media and have less mobility to freely infect cells, leading to difficulty in controlling the effective concentration of phage in the assay. While both of these methods work well to identify strongly resistant bacteria, and have been used with success in other studies, both could have difficulty in picking up partial resistances, which could indicate a given gene product may be involved directly or indirectly in a given phage infection process, but not essential for that process, and thus may have been overlooked in the past. Mutants which would show a delay in lysis, but still lyse eventually, would be determined as nonresistant if only observed at the endpoint, whereas a growth curve would retain this information from the start.

In order to measure a wider range of phenotypes, a 96 well growth curve based screen was developed to examine the impact of phages on the growth of Keio knockouts, and phage-
challenged wells were compared with their uninfected controls. Resistances could be determined in as little as six hours. Three siphoviridae phages were chosen to gain an idea of the diversity in phenotypes amongst morphologically similar bacteriophages, as well as comparing to a more distantly related Myoviridae phage. In order to assess the effectiveness of the assay, and in order to draw comparisons with data previously collected in the lab by spotting studies, these four phages were also screened by a spotting assay. For this assay 3 additional Siphoviridae, phi80, T5, and HK022, were also screened to look for additional resistance phenotypes.

Upon generating a list of resistance phenotypes across the phages, it becomes necessary to determine at what stage of the phage life cycle this resistance occurs. This was accomplished through a series of assays designed to sort positive results into adsorption defects and DNA-injection defects, with additional tests to gather more specific information about the adsorption requirements. In order to accommodate a large scale screen, several assays have been adapted or designed to be rapid and reliable. Genes of interest from the resistance screen were then tested by an adsorption assay in microcentrifuge tubes incubated in a water-filled heating block, by their ability to form lysogens in the case of λcl857, or by an injection assay using a potassium probe, designed to be completed in a half hour with no supervision beyond initial setup. An additional assay was designed as a result of discovering a range of phenotypes across genes involved in lipopolysaccharide synthesis in the three lambdoid phages. This assay made use of maltose as an inducer of lamB, which encodes the receptor for these phages, and could help determine relative binding strengths of the phages under conditions of limited adsorption.

2.2 Bacteriophages Used in this Study

Four different phages were chosen as the main focus of this study, including three siphoviridae, or long-tailed phages, λcl857, HK97, and HK243, as well as one myoviridae, or contractile-tailed phage, T6. The characteristics of each phage are summarized in Table 1 at the end of this section.

The λcl857 strain possesses an allele of the cl repressor gene that produces a temperature-sensitive protein. The repressor is stable at 32 °C, but can be inactivated at higher temperatures, leading to induction of the lytic pathway. This allows for the ability to test lysogen formation in mutants, and additionally can prevent lysogen formation from interfering with assays testing the lytic pathways. The life cycle for λcl857 has been described in detail in section 1.3.
HK97 was originally isolated from pig dung in Hong Kong, has similar morphology, host range, and immunity properties with lambda, and is capable of recombining with λ\(^98\). Many genes share high sequence identity with those in lambda, including the cl, cro, and int genes, which share 97-98% identity, while many others show no significant similarity\(^99\). Because of the cl gene similarity, HK97 will not replicate inside a λ lysogen, and it follows a life cycle similar to λ. The strain of HK97 used in these experiments was originally isolated from a clear plaque unable to form lysogens from a strain of HK97 possessing an amber mutation in gene H. The phage was then plated on a non-suppressor strain of E. coli to select for a revertant of the amber mutation, while still maintaining the clear phenotype.

The third siphoviridae used in the main screen is HK243. Similar to HK97, it was isolated from cow dung by the same group\(^100\). Little is known about HK243, as it remains unsequenced, other than the original two papers describing its characteristics\(^100; 101\). It is a long-tailed phage capable of infecting E. coli C and E. coli K-12 strains, but not B strains, and shows mature virion formation 20 minutes post-infection, with lysis occurring as early as 30 minutes post-infection\(^101\). Despite this time frame, some cells continue to produce phage up to 80 minutes past the end of the 30 minute latent period\(^101\). Some mutants surviving HK243 infection also show resistance to λ infection and are maltose-negative\(^101\), suggesting that HK243 also uses LamB as its receptor. One of the most striking phenotypes of HK243 is that it produces small, 1-2 mm, opaque plaques that drastically clear up and expand slightly under exposure to chloroform vapour. It has been suggested this phenotype is due to a high degree of lysis inhibition\(^100\), as seen in other phages such as T4, where superinfecting phage lead to a delay in lysis\(^102\). This correlates well with the observed high burst size of about 1000 virions per cell\(^100\) and the observation that some cells produce phage well after they were expected to lyse\(^101\).

The final phage used in the main screen is the myoviridae (contractile-tailed) phage T6. Like HK243 it remains unsequenced, though much more work has been done with T6. T6 belongs to a group of structurally, antigenically, and genetically similar phages, including T2, T4, and T6, called the T-Even phages, popularized by Max Delbrück and the Phage Group in the 1940s\(^103\). T6 was most likely obtained from a mixed sample of raw sewage containing the original T4, T5, and T6 isolates\(^103\). One of the key differences between the T-Even life cycle and the λ life cycle is the control of transcription. In T-Even phages, there is often no clear temporal differentiation between early and late transcription, with many genes transcribed from multiple
promoters, and not having clearly separated transcription units such as in the λ genome, but rather with genes from different periods interdigitated, such as with the late genes 26, 25, and the middle gene uvsY. Furthermore, genes for related or interacting proteins are not necessarily clustered, such as with genes 5 and 27, which encode the first interacting baseplate components, nor does transcription direction unambiguously distinguish between early and late genes. Additionally, most T4-related phages, including T6, possess HMC residues instead of cytosine to escape host and other phage restriction enzymes. T6 is also a lytic phage and is incapable of forming a lysogen.

Because all of these phages either possess a temperature-sensitive repressor, are a clear mutant, or are virulent strains to begin with, the resistance assays described in this chapter are only looking for genes that affect the lytic pathway. This was initially chosen so that lysogen formation would not interfere with the growth curve results. An advantage to having three siphoviridae that appear to use the same receptor and one myoviridae using a different receptor is that it will allow me to get a sense of the variation between phages with similar host ranges and morphologies, compared to a phage that differs in both aspects. Additionally, both HK243 and T6 have no published sequence, and sequencing could be a logical future step in this research.

**Table 1: Characteristics of phages used in this study.**

<table>
<thead>
<tr>
<th>Phage</th>
<th>Type</th>
<th>Genome</th>
<th>Sequenced</th>
<th>Receptor</th>
<th>Temperate</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>T6</td>
<td>Myo</td>
<td>~164kbp</td>
<td>no</td>
<td>LPS/Tsx</td>
<td>no</td>
<td>7</td>
</tr>
<tr>
<td>Lambda</td>
<td>Sipho</td>
<td>48kbp</td>
<td>yes</td>
<td>LamB</td>
<td>Yes*</td>
<td>7; 106</td>
</tr>
<tr>
<td>HK97</td>
<td>Sipho</td>
<td>40kbp</td>
<td>yes</td>
<td>LamB</td>
<td>yes†</td>
<td>99</td>
</tr>
<tr>
<td>HK243</td>
<td>Sipho</td>
<td>~72kbp</td>
<td>unpublished</td>
<td>LamB</td>
<td>no</td>
<td>100; 101</td>
</tr>
<tr>
<td>Phi80</td>
<td>Sipho</td>
<td>48kbp</td>
<td>yes</td>
<td>FhuA</td>
<td>yes</td>
<td>107; 108</td>
</tr>
<tr>
<td>HK022</td>
<td>Sipho</td>
<td>41kbp</td>
<td>Yes</td>
<td>FhuA</td>
<td>yes</td>
<td>99</td>
</tr>
<tr>
<td>T5</td>
<td>Sipho</td>
<td>122kbp</td>
<td>yes</td>
<td>FhuA</td>
<td>no</td>
<td>109</td>
</tr>
</tbody>
</table>

*the cI857 strain used does not form lysogens at the nonpermissive temperature  
†the strain of HK97 used was isolated as a clear mutant and cannot form lysogens
2.3 Materials and Methods

2.3.1 Keio Collection Growth Curve Screen for Resistances

The growth curve screen for phage replication inhibition utilized an incubated plate reader (Tecan Infinite 200). Strains from the Keio collection were grown up overnight (approximately 16 hours) in 96 well plates to stationary phase in LB-Lennox (Lysogeny broth containing 5g of NaCl/L, hereafter referred to as LB) containing 0.25 µg/ml kanamycin. The following day 2 µl of stationary phase cells were added to clear 96 well flat-bottom plates containing 10 µl of phage at 10^5 pfu/ml for λcI857 and HK97, 10^4 pfu/ml for T6, and 10^7 pfu/ml for HK243 (MOIs of approximately 10^{-3}, 10^{-4}, and 10^{-1}, respectively). Low MOIs were chosen for the initial screen so that phages must replicate and lyse cells for several rounds before culture lysis can occur. This allows the assay to be more sensitive to subtle factors affecting phage growth. Plates were incubated at 37 °C for 15 minutes to allow for preadsorption of phage. After preadsorption, 100 uL of LB containing 10 mM MgSO_4 was added to each well and the plate was covered with transparent tape to prevent moisture loss due to evaporation. For λcI857 this was done with the plate sitting on a 37 °C hot plate to inhibit lysogen formation. Each plate was incubated with orbital shaking at an amplitude of 3 mm in the plate reader for a total of six hours at 37 °C, with OD_{595} readings being taken every 15 minutes. Using this method, six different plates could be screened each day on the three available plate readers. After the initial screen for each phage, lysis timing was calculated by determining the time of maximum OD_{595} before the subsequent onset of lysis, as indicated by a drop in optical density. Wells with lysis timing two standard deviations above median lysis times for that particular phage were selected for further verification. Hits were verified by repeating the growth assay twice at initial MOI, as well as at higher MOIs. Strength of resistance was determined by length of delays in lysis timing compared with controls, as well as by how high a titer of phage in which cells were still capable of growth.

2.3.2 Keio Collection Plating Screen for Resistances

Strains from the Keio collection selected from a previous screen in the lab were grown overnight at 37 °C in a shaking incubator (approximately 16 hours) in deep-well 96-well plates containing 400 uL of LB with 0.25 ug/ml kanamycin. The following morning, subcultures were
started by inoculating 40 µl of overnight culture into 400 µl of LB in another 96-well plate. Cells were grown to mid-log phase (approximately five hours post inoculation) in a shaking incubator at 37 °C. From these subcultures, 200 µl were added to molten 0.7% top agar at 55 °C, and briefly mixed by rolling the tube between the hands. This mixture was poured onto square Nunc plates containing 50 ml of LB and 1.5% agar to create a uniform lawn of *E. coli*. The top agar was then allowed to cool and solidify for 30 minutes. Plates and top agar were prepared fresh two days prior to each experiment, with media carefully weighed before and after autoclaving and topped up with sterile water to maintain target moisture content, and were set aside at room temperature to cool and partially dry until used.

10-fold serial dilutions of high titer phage stocks were prepared in LB broth, and 100 ul of each dilution was added to a well of a 96-well plate as a dilution stock plate for the spotting assay. A 96-pin metal replicator was sterilized and used to spot dilutions of the phage onto the lawns of *E. coli* knockouts, with each spot containing approximately 1 ul of phage dilution. Plates were allowed to dry for 30 minutes, followed by incubation overnight, upside down, in a 37 °C forced air convection incubator. The following day plates were scored for plaque morphology and titer, as well as photographed for future reference.

### 2.3.3 Adsorption Assay

Overnight cultures of the Keio parental strain and LPS knockouts were grown at 37 °C in LB + 25 µg/ml kanamycin where applicable. The following day, subcultures were started by adding 500 µl of overnights to 5 ml LB and grown at 37 °C with shaking to an OD<sub>600</sub> of ~ 0.5 (approximately 5x10<sup>8</sup> cfu/ml). 1 ml of subculture was then pelleted by spinning at 16000g in a benchtop centrifuge for 60 seconds, washed in 1 ml adsorption buffer (10 mM MgSO<sub>4</sub> adjusted to pH 7.2), pelleted at top speed for 60 seconds, and again resuspended in 1 ml Adsorption Buffer. 1 ml of each cell suspension was then pipetted into sterile microfuge tubes, to which 10 µl of λ<sub>cI857</sub> stock was added to attain a MOI of 0.0001 (approximately 5x10<sup>4</sup> pfu/ml) and vortexed. A blank containing just adsorption buffer and phage was prepared in the same manner. Bacteria plus phage, as well as the blank, were incubated at 37 °C without shaking in a hot plate filled with water. At 0 min, 2 min, 5 min, 10 min, 15 min, and 20 min, tubes were gently mixed by vortexing at low speed, and 100 µl samples were taken and diluted into 900 µl of adsorption buffer in sterile microfuge tubes and pelleted at 16000g for 1 min. This causes any adsorbed
phage to pellet along with the heavier bacteria, with unadsorbed phage remaining in the supernatant. After pelleting, 500 µl samples of the supernatants were taken and stored at 4 °C. Samples were titered on 100 µl of the Keio parental strain in 3 ml top agar on LB plates. The following day, plaques on each plate were counted and used to determine the relative amounts of unadsorbed phage in each supernatant compared to immediately post-addition of phage.

2.3.4 Maltose Dependency Assay - Plating

Minimal medium agar plates were prepared containing 40 ml of M9 minimal medium supplemented with either 0.4% glucose, 0.2% glucose and 0.2% maltose, or 0.4% maltose, and 1.5% agar. Top agar was prepared using the same components, but substituted with 0.7% agar. Overnights of the Keio parental strain and knockouts were grown overnight in each of the three different minimal media solutions, with 0.25 µg/ml kanamycin added for the knockouts. The following day, 150 µl of each overnight was added to 3 ml of molten top agar containing the same sugar content as the overnight, poured over the corresponding M9 plate, and allowed to cool and solidify for 30 minutes. After solidifying, 10-fold serial dilutions of λcI857, HK97, and HK243 were spotted on each lawn of cells. Once dry, the plates were incubated upside down in a 37 °C incubator overnight. The following day, photographs of the plates were taken, and plaquing ability and morphology was recorded.

2.3.5 Maltose Dependency Assay – Growth Curve

Liquid M9 medium was prepared as above, supplemented with either 0.4% glucose, 0.2% glucose and 0.2% maltose, or 0.4% maltose. Overnight cultures of the parental strain and rfaD+ were grown overnight in each of the three different minimal media solutions, with 0.25 µg/ml kanamycin added for the knockouts. The following day, 2 µl of each of each overnight culture was inoculated into wells of 96-well plates containing 100 µL of each minimal media composition. To some of these wells 10 µl of either λcI857 or HK243 was added from a series of 10-fold dilutions in minimal media, creating a range of MOIs from 5x10⁻² to 5x10⁻⁵ for each glucose/maltose combination, as well as uninfected controls containing an additional 10 µL of minimal media. The 96-well plate was then sealed with a clear piece of tape and placed into a preheated Tecan Infinite 200 plate reader at 37 °C. The plate was incubated for 24 hours to accommodate the slower growth in minimal medium, with OD595 readings recorded every 15 minutes.
2.3.6 Potassium Efflux Assay as a Measure of DNA-Injection

Overnight cultures from the Keio collection were grown in LB broth containing 0.25 µg/ml kanamycin at 37 °C. The following day, subcultures were started by preparing 100-fold dilutions of overnight in fresh LB broth and grown up to an OD_{600} of 0.5. Cells were then spun down at 6000g for 5 min at 4 °C and the supernatant discarded. Cells were then washed with SM medium and again spun at 6000g for 5 min at 4 °C. Cell pellets were resuspended in fresh SM to the original volume of the culture, and the OD_{600} was again measured. Cells were always spun down and resuspended immediately prior to potassium readings to limit potassium leakage due to cell damage from centrifugation and from the Tris in SM, which can lead to measurement errors after delays of about an hour.

5 ml of resuspended cells were added to a 50 ml conical tube sitting in a 37 °C water bath and were allowed to equilibrate at 37 °C for about 5 min. The potassium probe was calibrated with 5 ml of potassium standards at 37 °C as per the manufacturer’s instructions before placing into cell solution. After allowing time for stabilization, a baseline reading in which the measured electric potential did not fluctuate for 1 min was recorded before addition of phage. Phage from a 10^{11} stock of CsCl banded λcI_{857} dialyzed into SM was pipette into the cell suspension to create an MOI of approximately 3 (about 150 µl depending on cell OD). The 50 ml conical tube was briefly mixed by swirling without letting the probe emerge from the sample before returning to the water bath. Measurements were recorded every 5 seconds for 20 minutes post phage-addition for each sample and were later analyzed in Excel.

The probe used in the assay was a 9719BNWP electrode (Thermo Scientific) and an Orion Dual Star meter (Thermo Scientific).

2.3.7 Lysogen Formation Assay

100 µL of each overnight was incubated with 10 µL of a stock phage solution containing 10^{10} λcI_{857} particles and incubated without shaking at 30 °C for 15 minutes. Cells were diluted and 50 µL of a 10^{-4} dilution was plated and grown at 30 °C, allowing for lysogen formation. The next day 50 colonies were selected and replica-plated and incubated at 30 °C and 42 °C overnight. The higher temperature inactivates the temperature-sensitive cI repressor in λcI_{857}
responsible for maintaining lysogeny, lysing the colony. The next day the plates were compared and the number of lysogens determined.

2.3.8 Removal of Kanamycin Resistance Cassette

pDV64, an ampicillin resistant plasmid, was obtained from Paul Sadowski, and contains FLP recombinase, derived from the 2-micron plasmid from *Saccharomyces cerevisiae*, under the control of an IPTG-inducible promoter. Knockout cultures were transformed with this plasmid and grown to an OD$_{600}$ of 0.5 and 10mM IPTG was added. The cultures were then incubated at 37 °C for 20 minutes. A culture of the parental strain not containing a kanamycin resistance cassette was mock-treated as a control. Cells from the culture were streaked out on an LB plate and incubated overnight at 37 °C. The following day, about 50 colonies from each plate were replica-plated on LB and LB plus 25 µg/ml kanamycin and again incubated overnight. Colonies were then identified that had lost the resistance cassette and were replica-plated on media containing LB and LB plus 50 µg/ml ampicillin to screen for the loss of the pDV64 plasmid.
2.4 Results

2.4.1 Tecan Screening Reveals 43 Genes Involved in $\lambda cI_{857}$, HK97, HK243, or T6 Reproduction

In order to determine the impact of phage on each Keio mutant I performed a systematic screen based on growth curve dynamics on each of the Keio knockouts in the collection in the presence of four different phages. The growth curve screens identified 43 hits that showed a lysis timing 2 standard deviations above the mean lysis timing from at least one of the four phages (see Table 2 for lysis timing data and Table 3 for a comparison among the four phages, including functions of genes knocked out). These include: 25 hits for $\lambda$, 17 of which involve the cell envelope, as well as six genes previously unidentified to have a role in $\lambda$ phage infection; 19 hits for HK97, including 14 envelope genes and four genes previously not identified as having a role in phage infection; nine hits for T6, including two envelope genes, and six genes previously unidentified; as well as 11 hits for HK243, of which six had not been previously identified as having a role in phage infection and two genes involved the envelope.

Genes involved in the cell envelope, including those involved in biosynthesis of LPS and those encoding inner membrane, outer membrane, and periplasmic proteins, account for 21 of the 43 hits, or 48.8%. In E. coli K12, genes involving the cell envelope account for an estimated 1472 of 4345 genes, or approximately 34% of the genome. Of the envelope genes involved in resistance, five encode inner membrane proteins, three encode periplasmic proteins, two encode outer membrane proteins, and ten are involved in LPS biosynthesis, showing a slight enrichment towards genes involved in the outer membrane.

For $\lambda cI_{857}$ and HK97, the largest number of hits came from the LPS biosynthesis pathway. These included genes involved in sequential addition of sugar residues to the growing LPS molecule ($rfaC$, $rfaP$, $rfaF$, and $rfaG$) and those involved in production of these sugar residue precursors ($rfaD$, $rfaE$, $lpcA$, $galU$, and $gmhB$), as well as the transcriptional regulator of genes involved in LPS production, $rfaH$. Of these hits, $rfaC$, $rfaD$, $rfaE$, $rfaF$, $galU$, and $lpcA$ knockouts showed the highest resistance, with lack of, or delayed, lysis at an MOI of 10 for both phages. Knockouts of $rfaG$ and $rfaH$ showed resistance at an MOI of $10^{-1}$ for both phages,
whereas galU and gmhB knockouts only showed resistance at $10^{-3}$ for $\lambda cI_{857}$, while maintaining resistance against HK97 at the higher MOI of $10^{-1}$.

Knockouts of genes encoding the receptors for each phage, lamB for $\lambda cI_{857}$, HK97, and HK243, and tsx for T6, all lead to full resistance. Additional genes with well-characterized roles in $\lambda$ infection were detected, including dnaK, dnaJ, nusB, manY, malT, cyaA, and ihfA. See Table 3 for details of each gene.

### Table 2: Lysis timing in hours from Tecan growth curves screen for four phages and E. coli deletions causing phage resistance.

<table>
<thead>
<tr>
<th>Phage</th>
<th>$\lambda cI_{857}$</th>
<th>Phage HK97</th>
<th>Phage HK243</th>
<th>Phage T6</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>MOI</td>
<td>$10^{-3}$</td>
<td>$10^{-1}$</td>
<td>10</td>
</tr>
<tr>
<td>WT</td>
<td>3</td>
<td>2</td>
<td>1.5</td>
<td>3.75</td>
</tr>
<tr>
<td>gmhB</td>
<td>3</td>
<td>2</td>
<td>1.5</td>
<td>6</td>
</tr>
<tr>
<td>malT</td>
<td>3.25</td>
<td>2.25</td>
<td>1.5</td>
<td>6</td>
</tr>
<tr>
<td>lamB</td>
<td>3.5</td>
<td>2.5</td>
<td>1.5</td>
<td>6</td>
</tr>
<tr>
<td>cyaA</td>
<td>6.6</td>
<td>6</td>
<td>6</td>
<td>6</td>
</tr>
<tr>
<td>dnaJ</td>
<td>6.6</td>
<td>5.25</td>
<td></td>
<td>6</td>
</tr>
<tr>
<td>entE</td>
<td>5.5</td>
<td>6</td>
<td></td>
<td>6</td>
</tr>
<tr>
<td>manY</td>
<td>6.6</td>
<td>4.5</td>
<td></td>
<td>6</td>
</tr>
<tr>
<td>manZ</td>
<td>6.6</td>
<td>6</td>
<td></td>
<td>6</td>
</tr>
<tr>
<td>nusB</td>
<td>6.6</td>
<td>6</td>
<td></td>
<td>6</td>
</tr>
<tr>
<td>yfD</td>
<td>4.5</td>
<td>2.5</td>
<td>1.75</td>
<td>6</td>
</tr>
<tr>
<td>ygfY</td>
<td>3.75</td>
<td>2.25</td>
<td>1.5</td>
<td>6</td>
</tr>
<tr>
<td>yglM</td>
<td>4.5</td>
<td>2.5</td>
<td>1.75</td>
<td>6</td>
</tr>
<tr>
<td>dnaK</td>
<td>4.75</td>
<td>6</td>
<td></td>
<td>6</td>
</tr>
<tr>
<td>ihfA</td>
<td>5.35</td>
<td>2.25</td>
<td>2.25</td>
<td>6</td>
</tr>
<tr>
<td>lpcA</td>
<td>3.25</td>
<td>2.25</td>
<td>1.25</td>
<td>6</td>
</tr>
<tr>
<td>rfaC</td>
<td>6.6</td>
<td>5.75</td>
<td></td>
<td>6</td>
</tr>
<tr>
<td>rfaE</td>
<td>6.6</td>
<td>6</td>
<td></td>
<td>6</td>
</tr>
<tr>
<td>rfaF</td>
<td>6.6</td>
<td>4.5</td>
<td></td>
<td>6</td>
</tr>
<tr>
<td>rfaG</td>
<td>5.25</td>
<td>1.75</td>
<td>2.75</td>
<td>6</td>
</tr>
<tr>
<td>rfaH</td>
<td>5.75</td>
<td>3.25</td>
<td>2.5</td>
<td>6</td>
</tr>
<tr>
<td>rfaP</td>
<td>6.6</td>
<td>4</td>
<td>2.75</td>
<td>6</td>
</tr>
<tr>
<td>surA</td>
<td>5.5</td>
<td>3</td>
<td>2.25</td>
<td>6</td>
</tr>
<tr>
<td>ubf</td>
<td>3.6</td>
<td>2</td>
<td>1.5</td>
<td>6</td>
</tr>
<tr>
<td>galU</td>
<td>4.75</td>
<td>3.25</td>
<td></td>
<td>6</td>
</tr>
</tbody>
</table>

Lysis Timing

<table>
<thead>
<tr>
<th>0hrs</th>
<th>6hrs</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Table 3: Functions of *E. coli* genes whose deletions caused phage resistance in the Tecan growth curve screen.

<table>
<thead>
<tr>
<th>Type</th>
<th>Keio KO</th>
<th>Function</th>
<th>Resistances seen in Tecan assay</th>
</tr>
</thead>
<tbody>
<tr>
<td>DNA replication and</td>
<td><em>dnaK</em></td>
<td>Involved in λ DNA replication</td>
<td>λI₆₅₇  HK97  HK243  T6</td>
</tr>
<tr>
<td>transcription</td>
<td><em>dnaJ</em></td>
<td>Involved in λ DNA replication</td>
<td>High</td>
</tr>
<tr>
<td><em>nusB</em></td>
<td></td>
<td>Transcription termination</td>
<td>High</td>
</tr>
<tr>
<td><em>malT</em></td>
<td></td>
<td>Regulation of mal-regulon</td>
<td>Low</td>
</tr>
<tr>
<td><em>ihfA</em></td>
<td></td>
<td>Integration host factor, lysogenization, DNA packaging</td>
<td>High</td>
</tr>
<tr>
<td><em>ihfB</em></td>
<td></td>
<td>Integration host factor, lysogenization, DNA packaging</td>
<td>High</td>
</tr>
<tr>
<td><em>hns</em></td>
<td></td>
<td>Silences foreign AT-rich DNA</td>
<td>Med</td>
</tr>
<tr>
<td><em>malI</em></td>
<td></td>
<td>transcriptional repressor of <em>malY</em></td>
<td>High</td>
</tr>
<tr>
<td><em>yneJ</em></td>
<td></td>
<td>putative transcriptional regulator (LysR family)</td>
<td>High</td>
</tr>
<tr>
<td>Membrane and periplasmic</td>
<td><em>lamB</em></td>
<td>λ, HK97 receptor, maltose transporter</td>
<td>High</td>
</tr>
<tr>
<td>proteins</td>
<td><em>manY</em></td>
<td>Mannose pts permease (req’d for λ DNA injection)</td>
<td>High</td>
</tr>
<tr>
<td></td>
<td><em>manZ</em></td>
<td>Mannose pts permease (req’d for λ DNA injection)</td>
<td>High</td>
</tr>
<tr>
<td></td>
<td><em>entE</em></td>
<td>Iron siderophore</td>
<td>Low</td>
</tr>
<tr>
<td></td>
<td><em>yfd</em></td>
<td>Putative membrane protein</td>
<td>Low</td>
</tr>
<tr>
<td></td>
<td><em>ygd</em></td>
<td>Putative membrane protein</td>
<td>Low</td>
</tr>
<tr>
<td></td>
<td><em>surA</em></td>
<td>Periplasmic protein-folding chaperone</td>
<td>Med</td>
</tr>
<tr>
<td></td>
<td><em>ptsG</em></td>
<td>Binds repressor of mal-regulon</td>
<td>Med</td>
</tr>
<tr>
<td></td>
<td><em>fpa</em></td>
<td>Periplasmic protein-folding chaperone</td>
<td>High</td>
</tr>
<tr>
<td></td>
<td><em>dcrB</em></td>
<td>Periplasmic protein, DNA injection in phages c1, c6</td>
<td>High</td>
</tr>
<tr>
<td>LPS biosynthesis</td>
<td><em>galU</em></td>
<td>LPS biosynthesis – Glucose subunit preparation</td>
<td>High</td>
</tr>
<tr>
<td></td>
<td><em>lpcA</em></td>
<td>LPS biosynthesis – Heptose subunit preparation</td>
<td>High</td>
</tr>
<tr>
<td></td>
<td><em>rfcC</em></td>
<td>LPS biosynthesis – Inner core assembly</td>
<td>High</td>
</tr>
<tr>
<td></td>
<td><em>rfcD</em></td>
<td>LPS biosynthesis – Heptose subunit preparation</td>
<td>High</td>
</tr>
<tr>
<td></td>
<td><em>rfcE</em></td>
<td>LPS biosynthesis – Heptose subunit preparation</td>
<td>High</td>
</tr>
<tr>
<td></td>
<td><em>rfcF</em></td>
<td>LPS biosynthesis – Inner core assembly</td>
<td>High</td>
</tr>
<tr>
<td></td>
<td><em>rfcG</em></td>
<td>LPS biosynthesis – Outer core assembly</td>
<td>Med</td>
</tr>
<tr>
<td></td>
<td><em>rfcH</em></td>
<td>LPS biosynthesis – Transcriptional activator</td>
<td>Med</td>
</tr>
<tr>
<td></td>
<td><em>rfcP</em></td>
<td>LPS biosynthesis – Inner core assembly</td>
<td>Low</td>
</tr>
<tr>
<td></td>
<td><em>gmiB</em></td>
<td>LPS biosynthesis – Heptose subunit preparation</td>
<td>Low</td>
</tr>
<tr>
<td>Metabolic proteins</td>
<td><em>cyaA</em></td>
<td>Adenylate cyclase</td>
<td>High</td>
</tr>
<tr>
<td></td>
<td><em>abiF</em></td>
<td>Ubiquinone synthesis, efficient aerobic energy production</td>
<td>Low</td>
</tr>
<tr>
<td></td>
<td><em>pgm</em></td>
<td>Phosphoglucomutase, involved in mal-regulon</td>
<td>Med</td>
</tr>
<tr>
<td></td>
<td><em>purA</em></td>
<td>Adenylosuccinate synthetase, purine synthesis</td>
<td>Low</td>
</tr>
<tr>
<td></td>
<td><em>ygbL</em></td>
<td>putative fuculose phosphate aldolase</td>
<td>High</td>
</tr>
<tr>
<td></td>
<td><em>ycfH</em></td>
<td>putative hydrolase (1st module)</td>
<td>High</td>
</tr>
<tr>
<td>Prophage</td>
<td><em>ymfR</em></td>
<td>e14 prophage</td>
<td>Med</td>
</tr>
<tr>
<td></td>
<td><em>ymfO</em></td>
<td>e14 prophage portal pseudogene</td>
<td>Low</td>
</tr>
<tr>
<td></td>
<td><em>ymfE</em></td>
<td>e14 prophage</td>
<td>Low</td>
</tr>
<tr>
<td></td>
<td><em>JW5167</em></td>
<td>e14 prophage</td>
<td>Med</td>
</tr>
<tr>
<td>Unknown</td>
<td><em>ygY</em></td>
<td>Hypothetical protein</td>
<td>Low</td>
</tr>
<tr>
<td></td>
<td><em>ycaR</em></td>
<td>unknown CDS</td>
<td>High</td>
</tr>
<tr>
<td></td>
<td><em>yodC</em></td>
<td>Unknown</td>
<td>Med</td>
</tr>
</tbody>
</table>

*Indicates not previously implicated in phage infection. Results are grouped into DNA transcription and replication, membrane proteins, LPS biosynthesis, metabolic enzymes, prophage genes, and unknown. Phenotypes are defined as High, Med, and Low for simplicity, based on whether resistance was seen at the highest, medium, or lowest MOI within each phage.
2.4.2 Spotting Reveals 34 Genes Involved in Phage Reproduction

One of the traditional methods for looking at phage resistance is by spotting serial dilutions of phages on a lawn of bacteria. In order to compare the results from the growth curve based assay to a spotting assay, as well as to tie in data from a previous screen in the lab based on a pour plate method with the newly collected growth curve data in one common screen, a traditional spotting assay was performed on all collected hits. A previous screen in the lab resulted in an initial list of 143 putative, but unconfirmed, resistant knockout strains across seven phages, λcI857, HK97, HK243, T6, HK022, φ80, and T5. These were added to the results from the Tecan screen to form a panel of knockouts to compare resistances across phages. This new phage spotting screen for resistance resulted in a total of 34 hits across the seven screened phages (see Figure 3 for a representative plate from the screen and Table 4 for results from the screen). 17 of the 34 hits are genes involved in LPS biosynthesis, or are encoding inner membrane, outer membrane, or periplasmic proteins, representing 50% of all hits, comparable to the proportion in the growth curve screen. Hits were defined as knockouts that cause a 100-fold decrease in titer or a significant change in plaque morphology, such as an increase in turbidity. Tenfold variations in spotting were not uncommon between plates and on separate days, and so a tenfold drop or increase in apparent titer was taken to be due to either biological or methodological variability.

The spotting screen picked up several well-characterized genes involved in λ infection, including dnaJ, nusB, lamB, and several genes involved in LPS biosynthesis, but it failed to detect manY or manZ, malT, dnaK, and ihfA, all of which were detected by the growth curve assay. A previous plating-based screen in the lab with another λ strain, λcIrr, detected lamB, malT, dnaK, dnaJ, and several genes involved in LPS biosynthesis, but failed to detect nusB, manY, manZ, or ihfA.
Figure 3: Sample plate from spotting screen for phage resistance. Phages were spotted using a 96-pin replicator onto lawns containing either WT or a knockout-strain of *E. coli*. Phages were spotted in a series of ten-fold dilutions, from the highest concentration at the top of the plate, to the lowest at the bottom. Shown here are sample results from a spotting experiment on the parental WT strain.
Table 4: Changes in apparent titer from spotting-based resistance screen

<table>
<thead>
<tr>
<th></th>
<th>λcI857</th>
<th>HK97</th>
<th>HK243</th>
<th>T6</th>
<th>HK022</th>
<th>phi80</th>
<th>T5</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>cyaA</td>
<td>&gt;10^6</td>
<td>&gt;10^6</td>
<td>&gt;10^6</td>
<td>10^1</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>rfaE</td>
<td>&gt;10^6</td>
<td>&gt;10^6</td>
<td>10^7*</td>
<td>10</td>
<td>10^*</td>
<td>10^2</td>
<td>10^2</td>
</tr>
<tr>
<td>dnaJ</td>
<td>&gt;10^6</td>
<td>0</td>
<td>10</td>
<td>0</td>
<td>0</td>
<td>10</td>
<td>10^1</td>
</tr>
<tr>
<td>nusB</td>
<td>&gt;10^6</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>10</td>
<td>10</td>
<td>10</td>
</tr>
<tr>
<td>lpcA</td>
<td>10^5</td>
<td>10^6</td>
<td>0</td>
<td>0</td>
<td>10</td>
<td>10^1</td>
<td>0</td>
</tr>
<tr>
<td>rfaC</td>
<td>10^3</td>
<td>10^3</td>
<td>10^2</td>
<td>0</td>
<td>0</td>
<td>10</td>
<td></td>
</tr>
<tr>
<td>gnhB</td>
<td>10^4</td>
<td>10^4</td>
<td>0</td>
<td>10^1</td>
<td>10</td>
<td>0</td>
<td>10^1</td>
</tr>
<tr>
<td>galU</td>
<td>10^5</td>
<td>10^3</td>
<td>10</td>
<td>10^2</td>
<td>0</td>
<td>0</td>
<td>10^1</td>
</tr>
<tr>
<td>rimM</td>
<td>10</td>
<td>10^1*</td>
<td>10</td>
<td>0</td>
<td>10</td>
<td>10^1</td>
<td>10</td>
</tr>
<tr>
<td>hfiC</td>
<td>10</td>
<td>10^2</td>
<td>10^2</td>
<td>&gt;10^3</td>
<td>10^3</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>atpF</td>
<td>10</td>
<td>0</td>
<td>10^1</td>
<td>10^4</td>
<td>0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ymP</td>
<td>10</td>
<td>10^1</td>
<td>10^1</td>
<td>10^2</td>
<td>10</td>
<td>10</td>
<td>10^1</td>
</tr>
<tr>
<td>speB</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>10</td>
<td></td>
</tr>
<tr>
<td>fkpA*</td>
<td>0</td>
<td>&gt;10^6</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>10</td>
<td>10^2</td>
</tr>
<tr>
<td>ptsG*</td>
<td>0</td>
<td>&gt;10^6</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>10</td>
</tr>
<tr>
<td>rfaG</td>
<td>0</td>
<td>10^3</td>
<td>0</td>
<td>0</td>
<td>10^2</td>
<td>0</td>
<td>10</td>
</tr>
<tr>
<td>rnhA</td>
<td>0</td>
<td>10^3</td>
<td>5*10^1</td>
<td>5*10^1</td>
<td>0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pgm*</td>
<td>0</td>
<td>10^3</td>
<td>10^1</td>
<td>0</td>
<td>0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ihfB</td>
<td>0</td>
<td>10^2</td>
<td>10^2</td>
<td>10^2</td>
<td>5*10^3</td>
<td>5*10^3</td>
<td>0</td>
</tr>
<tr>
<td>ihfA</td>
<td>0</td>
<td>10^2</td>
<td>10</td>
<td>10^3</td>
<td>10</td>
<td>5*10^3</td>
<td>0</td>
</tr>
<tr>
<td>ymR*</td>
<td>0</td>
<td>10</td>
<td>10</td>
<td>&gt;10^4</td>
<td>0</td>
<td>5</td>
<td>0</td>
</tr>
<tr>
<td>dcrB</td>
<td>0</td>
<td>0</td>
<td>&gt;10^4</td>
<td>0</td>
<td>10^1</td>
<td>10^1</td>
<td>0</td>
</tr>
<tr>
<td>JW5751</td>
<td>0</td>
<td>0</td>
<td>&gt;10^4</td>
<td>0</td>
<td>10^1</td>
<td>10^1</td>
<td></td>
</tr>
<tr>
<td>JW3133</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>10</td>
<td>&gt;10^1</td>
<td>10^1</td>
</tr>
<tr>
<td>dsbB</td>
<td>0</td>
<td>0</td>
<td>10^1</td>
<td>10^2</td>
<td>0</td>
<td>10^4</td>
<td>10^1</td>
</tr>
<tr>
<td>ymF0*</td>
<td>10^1</td>
<td>10</td>
<td>10</td>
<td>&gt;10^4</td>
<td>0</td>
<td>5</td>
<td>10</td>
</tr>
<tr>
<td>tonB</td>
<td>10^1</td>
<td>10</td>
<td>0</td>
<td>0</td>
<td>10</td>
<td>10^1</td>
<td>10^2</td>
</tr>
<tr>
<td>lmbB</td>
<td>&gt;10^6</td>
<td>&gt;10^6</td>
<td>&gt;10^6</td>
<td>0</td>
<td>0</td>
<td>10^1</td>
<td>0</td>
</tr>
<tr>
<td>tsx</td>
<td>0</td>
<td>0</td>
<td>10</td>
<td>&gt;10^6</td>
<td>10</td>
<td>10^2</td>
<td>5</td>
</tr>
<tr>
<td>fhuA</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>10</td>
<td>&gt;10^6</td>
<td>&gt;10^6</td>
<td>&gt;10^6</td>
</tr>
</tbody>
</table>

Values shown indicate the increase or decrease in titer relative to spotting on the parental strain (WT). Outlined values indicate known receptor for that particular phage. Only the strains that showed a 10^2 fold change or more were classified as hits. All strains from the expanded pool of putative hits were screened (see text for details), though none of the other strains, including many detected by the Tecan (see Table 2), showed any significant change in spotting and are not listed.

* indicates higher plaque turbidity than WT
2.4.3 LPS Mutations Lead to an Adsorption Defect in \(\lambda cl_{857}\) that Correlates with its Ability to Reproduce

Given that LPS lies on the cell surface and may be involved in the folding of LamB, a rapid adsorption assay was modified from existing adsorption assays to rapidly analyze multiple samples in 1.5 ml microcentrifuge tubes, using only a heating block and microcentrifuge. This allowed the assay to be useful for a large number of samples, using the minimum required space. The adsorption assay was then tested on the LPS knockouts, as well as controls lacking \(lamB\) or \(manY\), required for adsorption and DNA-injection, respectively. By the end of the twenty minute time period, 91% of phages adsorbed to the parental strain control, with a standard error of 7% among 3 replicates (Figure 4 and Table 5). In the \(lamB\) knockout, where no adsorption was expected, the percentages of unadsorbed phages showed a large variation between time points ranging from 90% of the original reading, to 147% of the original reading in one case, but did not show any trend towards absorption. In general, samples with a higher percentage of unadsorbed phages showed greater variation than those where phages adsorb well.

\(manY\) was chosen as a knockout of the mannose-permease system required for \(\lambda\) phage DNA-injection, but not adsorption, as a control, and showed a similar adsorption curve to the parental strain, with 89% phage adsorption in the final timepoint as expected. Finally, knockouts in the LPS biosynthesis pathway were chosen with either a strong resistance phenotype or a moderate resistance phenotype to test the sensitivity of the assay. \(rfaD\) showed a fully resistant phenotype in the Tecan assay and had a similar adsorption curve to \(lamB\), indicating a strong adsorption defect. Additionally, \(rfaG\), which showed a low resistance phenotype in the Tecan screen, resulted in an adsorption curve with a final time-point at 36% adsorbed phage, with a 16% error between three trials. In general, the ability to adsorb to each knockout corresponded roughly with the strength of \(\lambda cl_{857}\) resistance seen in the Tecan screen. These results suggest that knocking out various steps in LPS biosynthesis leads to adsorption defects of varying strengths, which correlate with the resistance phenotype.
Figure 4: Representative graphs from adsorption assay. Bacterial cells grown to OD$_{600}$ of 0.5 were incubated in 1.5 ml microcentrifuge tubes in a 37 °C heating block with $\lambda C I_{857}$ added at time zero to an MOI of $10^{-4}$. At 5 minute timepoints, samples were taken, spun down in a benchtop centrifuge, and the supernatant plated on the Keio collection parental strain to obtain a count of unadsorbed phage present.
Table 5: Relative strengths of $\lambda cI_{857}$ resistance from growth curve screen and measured ability to adsorb by 20 minutes.

<table>
<thead>
<tr>
<th></th>
<th>$\lambda cI_{857}$ Resistance</th>
<th>Adsorption at 20 min</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT</td>
<td>None</td>
<td>Full</td>
</tr>
<tr>
<td>lamB</td>
<td>Full</td>
<td>None</td>
</tr>
<tr>
<td>galU</td>
<td>Full</td>
<td>None</td>
</tr>
<tr>
<td>lpcA</td>
<td>Full</td>
<td>Near-Full</td>
</tr>
<tr>
<td>rfaC</td>
<td>Full</td>
<td>None</td>
</tr>
<tr>
<td>rfaD</td>
<td>Full</td>
<td>None</td>
</tr>
<tr>
<td>rfaE</td>
<td>Full</td>
<td>Some</td>
</tr>
<tr>
<td>rfaF</td>
<td>Full</td>
<td>Some</td>
</tr>
<tr>
<td>rfaG</td>
<td>Med</td>
<td>Some</td>
</tr>
<tr>
<td>rfaH</td>
<td>Med</td>
<td>Some</td>
</tr>
<tr>
<td>rfaP</td>
<td>Low</td>
<td>Near-Full</td>
</tr>
<tr>
<td>yaeD</td>
<td>Low</td>
<td>Near-Full</td>
</tr>
</tbody>
</table>

Adsorption defined as follows:

- **Full** = 0-19% of original unadsorbed by 20 min
- **Near-Full** = 20-39% of original unadsorbed by 20 min
- **Some** = 40-69% of original unadsorbed by 20 min
- **None** = 70-100% of original unadsorbed by 20 min

2.4.4 HK97 Shows a High Sensitivity to Maltose Levels, while HK243 is Unaffected

From the growth curve assay, the phages $\lambda cI_{857}$, HK97, and HK243 all required lamB, but each had different requirements for genes involved in LPS biosynthesis. One of the known phenotypes of LPS mutations is a decrease in outer membrane proteins, and so in order to determine whether these resistances could be rescued by an increase in LamB concentration, maltose was used to induce LamB expression\textsuperscript{110}. Additionally, glucose is known to repress LamB\textsuperscript{111; 112; 113}, and thus a gradient of maltose and glucose can be used to generate a gradient of LamB levels. The three phages were screened against 7 knockouts involved in LPS biosynthesis, a knockout of the receptor-encoding gene lamB, as well as the parental strain from the Keio collection as a control (see Table 6 and Figure 5). Knockouts of rfaD, rfaE, rfaG, rfaH, rfaP, and lpcA showed similar relative resistance on the 0.4% glucose/0.0% maltose plates, compared to the growth curve assay, with rfaD, rfaE, and lpcA knockouts showing full resistance to $\lambda cI_{857}$ and near-full resistance for HK97, and rfaG, rfaH, and rfaP showing an intermediate phenotype.
for both phages. *galU* showed very little resistance for HK97, and no resistance for λcI857, which contrasts with the growth curve data. These data are, however, similar to results from spotting experiments, which show the same lack of resistance. None of the LPS knockouts show resistance to HK243 infection on any of the plates. Additionally, knockouts of *lamB* led to full resistance against all three phages as expected.

Increasing the relative amount of maltose vs. glucose had no effect on the visible spotting for HK243, showing no significant changes in titer from 0.4% glucose/0.0% maltose, to 0.0% glucose/0.4% maltose, although on the plates lacking maltose the plaques appear more turbid. This same change in maltose was able to partially rescue plaque formation in λcI857 and HK97 to varying degrees. Phage spotting on *rfad*, *rfae*, and *lpcA* was restored to near-wild type levels in λcI857, with no effect on *galU*, *rfaG*, *rfaH*, or *rfaP*. This same increase in maltose was able to partially rescue plaque formation across all LPS genes excluding *rfaP* for HK97, while at the same time increasing the apparent titer of phage on the Keio parental strain 1000-fold.

In order to better visualize the effect maltose had on infection, a growth curve was generated using the same maltose concentrations as the plating experiment for the parental strain and *rfad*, representative of a strongly resistant knockout (see Figure 6). λcI857 and HK243 were tested at MOIs in 10-fold dilutions from 5x10⁻² to 5x10⁻⁵. In cases where cells lysed, increasing MOI generally led to a faster lysis time and less growth before lysis. In the parental strain, lysis did not occur at any MOI when infected with λcI857 at 0.4% glucose/0.0% maltose, in contrast with the plating experiments. While this was unexpected given the plating results, it is possible that under these conditions of low *mal-regulon* induction λcI857 is unable to bind to its receptor in a more turbulent liquid environment. Lysis did occur in the parental strain between 2.75 and 6 hours for λcI857 in 0.2% glucose/0.2% maltose, and between 3 and 5 hours for 0.0% glucose/0.4% maltose from highest to lowest MOI. In HK243, lysis times ranged from 3-5 hours for 0.4% glucose/0.0% maltose, 3-3.25 hours for 0.2% glucose/0.2% maltose, and 3-4 hours for 0.0% glucose/0.4% maltose, although it was difficult to determine lysis timing for the high maltose condition as very little growth occurred in the HK243 infected wells. HK243 showed almost identical lysis on the *rfad* strain for the maltose containing wells, but no lysis on the 0.4% glucose/0.0% maltose wells, whereas λcI857 showed no lysis for *rfad* in any condition.
Taken together, the plating results suggest HK243 is unaffected by interfering with LPS biosynthesis, or changing maltose content, while increasing maltose can partially rescue lysis for \( \lambda cI_{857} \) and HK97 on a solid medium. This was as predicted would be the case if these phages were weaker binders to LamB due to lower levels in LPS mutants, as suggested by their resistant phenotype, and could be rescued by restoring LamB levels with maltose. Conversely the growth curves show that maltose cannot rescue lysis for \( \lambda cI_{857} \) in liquid culture, possibly due to an increased difficulty in adsorbing inside a turbulent environment, and that HK243 is unable to infect \( rfaD^- \) with only glucose as the carbon source.
Table 6: Increases in observed titer by order of magnitude from 0.4% glucose to 0.4% maltose containing minimal media plates showing maltose partially rescues HK97 and λcI857 infectivity

<table>
<thead>
<tr>
<th></th>
<th>HK97</th>
<th>λcI857</th>
<th>HK243</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT</td>
<td>3</td>
<td>0</td>
<td>-1</td>
</tr>
<tr>
<td>galU</td>
<td>1.5</td>
<td>0</td>
<td>-1</td>
</tr>
<tr>
<td>lpcA</td>
<td>4</td>
<td>4</td>
<td>0</td>
</tr>
<tr>
<td>manY</td>
<td>2</td>
<td>0</td>
<td>-0.5</td>
</tr>
<tr>
<td>rfaD</td>
<td>2</td>
<td>4</td>
<td>0.5</td>
</tr>
<tr>
<td>rfaE</td>
<td>3</td>
<td>4</td>
<td>0</td>
</tr>
<tr>
<td>rfaG</td>
<td>5</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>rfaH</td>
<td>2</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>rfaP</td>
<td>1</td>
<td>0</td>
<td>1</td>
</tr>
</tbody>
</table>

Figure 5: Representative plating results from maltose-dependence assay: 10-fold dilutions of phages HK97, λcI857, and HK243 (from top to bottom on each plate) were spotted on lawns of knockouts grown on M9-minimal medium supplemented with either 0.4% glucose, 0.2% glucose and 0.2% maltose, or 0.4% maltose (top, middle, and bottom plates respectively) to induce expression of lamB. Increasing maltose concentration led to an apparent increase in titer for HK97 on WT cells, and was able to partially restore plaquing for HK97 and λcI857 on both rfaD- and rfaH-. HK243 showed little variation on any of the plates.
Figure 6: Representative growth curves showing HK243’s ability to infect rfaD- strains compared with λcI857. 100µL of minimal medium supplemented with either 0.4% glucose, 0.2% glucose/0.2% maltose, or 0.4% maltose was inoculated with 2µl of overnight culture, and 10µl of phage stock to create an MOI of 0.0005. OD_{595} readings were measured for 24 hours at 37 °C. WT strains showed a decreasing lysis time as the maltose is increased for both phages, suggesting a dependence on LamB concentrations on the cell surface. In the rfaD knockout, increasing maltose did not rescue λcI857 lysis and showed normal growth at all three concentrations. By contrast, normal lysis was restored for HK243 at 0.2% and 0.4% maltose, suggesting HK243 has a lesser reliance on LPS structure and can infect with relatively fewer LamB proteins present.
2.4.5 Potassium Efflux is a Sensitive Measure of $\lambda cI_{857}$ DNA Injection

Potassium efflux during a phage infection correlates with DNA-injection and can be used to measure defects in injection. Therefore a simple and rapid assay utilizing a potassium selective electrode, based on similar experiments in the 1980s on T4, T5, and T7$^{114; 115}$, was used on a series of knockout strains to determine if any of the strains blocked DNA-injection. $lamB^-$ and $manY^-$ were used as negative controls deficient in adsorption and DNA-injection, respectively, while the parental strain was tested in five replicates to show the reproducibility and sensitivity of the assay. Electric potentials from the potassium-selective probe were recorded in 5-second intervals over a twenty minute period post-infection and converted into potassium concentration using a standard curve. In runs which did not show an increase in potassium content in the external medium there was often a slight drift towards increasing potassium, which can be attributed to extended usage of the probe, as well as effects of mixing, and not potassium efflux. The drifted value would partially return to initial levels upon mixing the reaction tube.

The calculated internal potassium content of the five replicate parental strain runs all start to drop beginning around 50 seconds post infection, from 450 nmol/mg of cell dry weight to 315 nmol/mg, with an average standard deviation of 18.3 at each timepoint. By contrast, the two runs of the known DNA-injection deficient $manY^-$ drop to 408 nmol/mg with an average standard deviation of 11.1 at each timepoint. $lamB^-$, which shows no $\lambda cI_{857}$ adsorption and thus no DNA-injection drops to 434 nmol/mg by the last timepoint (see Figure 7).

When the experiment was repeated for the parental strain at room temperature, the calculated internal potassium dropped from 450 nmol/mg to 315 nmol/mg by the last timepoint, matching exactly with the 37 °C result. The onset of potassium efflux, however, begins at a delayed 3 minutes post infection when compared with the 37 °C experiment. Additionally, the potassium levels take much longer to reach 315 nmol/mg, indicative of a full, albeit slower efflux of ions. Taken together, these results show the reproducibility and reliability of the assay, as well as its potential ability to look at the dynamics of DNA-injection under different conditions.
Figure 7: Potassium efflux is observed for *E. coli* after λ*cI*_{857} infection. A 150 µl volume of phage stock was added to a 5 ml culture of cells at an OD_{600} of 0.5 and resuspended in SM at 37 °C, representing an MOI of approximately three. Using a potassium selective electrode, the internal potassium content of the parental strain (WT) from the Keio collection, *lamB*⁻, and *manY*⁻ was calculated based on a standard potassium content of 450 nmol/mg dry weight. WT cells showed an average potassium loss of 135 nmol/mg, with efflux beginning about 50 to 70 seconds post-infection (n=5). WT cells incubated at 25 °C took 3 minutes for efflux to begin, and lost potassium at a slower rate until reaching the same endpoint as at 37 °C. *lamB* and *manY* strains lack the phage receptor and membrane proteins required for DNA-injection, respectively, and consequently show no significant potassium efflux during the timecourse.
2.4.6 Removal of Kanamycin-Resistance Cassette from E14 Prophage Genes Restores Infectivity to \textit{ymfO} and \textit{ymfE}

The finding that several knockouts that exist within the defective lambdaide14 prophage provide resistance to T6 was surprising. In order to determine whether the phenotypes were caused by the gene knockouts, or whether the phenotypes were an artifact caused by the presence of the antibiotic resistance cassette, FLP-recombinase was used to remove the kanamycin resistance cassettes from each knockout. The knockouts were screened by spotting assay against \textit{\lambda}cI_{857} and T6 before and after removal of the resistance cassette to see if the phenotype persisted (see Figure 8). In the case of the \textit{tsx} knockout, both conditions led to full T6 resistance, as expected, with no observable growth after spotting, and likewise, a mock-treated WT strain showed normal plaquing in both cases with an observed titer of around $10^6$. However, removing the cassette from \textit{ymfO} and \textit{ymfE} led to a restoration of normal plaquing with T6 from an observed titer of 0 for \textit{ymfO} and $10^4$ for \textit{ymfE} with the cassette in place, up to around $10^7$ for each when the cassette was removed. The \textit{JW5167} knockout, which encodes an intergenic region upstream of \textit{ymfE} that has since been removed from current releases of the Keio collection, did not show any change in observed titer whether or not it possessed the cassette, but did show that the slightly turbid plaques seen when possessing the cassette cleared up upon cassette removal. Lastly, \textit{ymfR} showed normal plaquing with \textit{\lambda}cI_{857} up to an observed titer of $10^6$, but was reduced to 0 and $10^2$ in knockouts possessing and lacking the resistance cassette respectively. Given that removal of the cassette from all knockouts except \textit{ymfR} restored infectivity, it appears only \textit{ymfR} shows a true phenotype while the others could be due to downstream effects of the kanamycin resistance cassette promoter.
Figure 8: FLP-recombinase curing of the kanamycin resistance cassette partially restores T6 infectivity: FLP-recombinase encoded by the plasmid pDV64 was transformed into each e14 prophage knockout and expressed with 1 mM IPTG for 20 min to remove the kanamycin resistance cassette, and subsequently grown nonselectively to cure each strain of pDV64. λcI857 (a) and T6 (b) were spotted on each knockout and kan-cured knockout strain in 10-fold dilutions with spotting on wild type seen up to a 10^6 dilution, representing approximately 10^6 phage. In λcI857, little change is seen after kan-curing each knockout, but T6 infectivity was rescued for ymfO and partially rescued for ymfE and ymfR, though not up to wild type levels in the case of ymfR. JW5167 showed no change in spotting dilution, but turbid plaques on JW5167::kan became clearer upon removal of the kan-resistance cassette. tsx showed no plaquing in either condition, as expected.
2.5 Discussion

2.5.1 The Liquid Growth Curve Screen Detects Subtle Phenotypes Missed by the Plating Screen

As data had previously been collected by spotting, in order to directly compare with the new data obtained from the growth curve screen, a common spotting assay was used on all preliminary positives from both screens. It was found that of the 43 hits found in the Tecan screen across $\lambda cI_{857}$, HK97, HK243, and T6, only 18 came up as positive from the spotting assay (see Table 2 and Table 4). Amongst these four phages, an additional eight hits were obtained only in the spotting screen, namely *rim*, *hflC*, *atpF*, *ymfP*, *rnhA*, *ihfB*, JW5751, and *dsbB*. *ymfP* was obtained as a preliminary hit for the T6 liquid growth screen, along with *ymfD* and *ymfN*, all found within the E14 prophage in *E. coli* K12. In general, these results show that different methods each pick up a range of results that the other methods may miss, but that the growth curve screen detected 26 additional hits that the spotting screen missed, while conversely the spotting screen picked up 10 additional hits missed by the growth curve screen.

One of the key differences between the screens is that many of the phenotypes picked up by the growth curve screen are quite subtle, yet would be predicted. An example of this lies with the identification of *ihfA*, the integration host factor responsible for catalyzing site-specific reciprocal recombination between *attP* in the phage and *attB* in *E. coli* during viral integration. In addition to its role in lysogenization, it also plays an accessory role during packaging of phage DNA. Mutants lacking IHF are still capable of infecting and lysing *in vivo*, but show a burst size approximately 25% that of WT *E. coli* strains$^{73}$. Since $\lambda cI_{857}$ should not be forming lysogens at the temperature of the screen, it is likely that it is this activity being picked up by the screen. Another example is with *surA*, which encodes a protein-folding chaperone involved in the proper folding of beta-barrel containing outer membrane proteins, such as LamB. In knockouts lacking *surA*, levels of LamB are down to approximately half that of their wild type levels$^{116}$. Other resistance screens for $\lambda$ have not detected SurA in the past$^{95}$, including the spotting screen described in this study. Additionally, it only shows in the growth curve assay a weak phenotype for $\lambda cI_{857}$ and a slightly stronger phenotype for HK97, both of which use LamB, suggesting that the resistance is due to a drop in LamB. Another interesting finding is that *hns* plays a subtle role in T6 infection. One of the factors that may contribute to the initial preference of host RNA
polymerase during early infection by T4 before degradation of host promoters is that host DNA is bound nonspecifically to some protein, such as H-NS. Incoming T4 DNA is largely free from these proteins, making it more accessible.\textsuperscript{7, 117} Due to the similarity between T-even phage promoters, such as those in T4, and host \textit{E. coli} promoters (they resemble host promoters but with extended -10 regions, different -35 regions, and additional information content\textsuperscript{118; 119}), this initial preference is an important factor in the initial infection of T-even phages. Three promoters in particular, \textit{P}_{57}, \textit{P}_{bac}, and \textit{P}_{repE}, more closely resemble major \textit{E. coli} promoters\textsuperscript{120; 121; 122}. It is possible that T6 could use a similar method and that removal of H-NS would make that initial transcriptional advantage less distinct, leading to the subtle delay in the initial stages of the T6 infection process.

\subsection*{2.5.2 Downregulation of the mal-Regulon Leads to Resistance to \textit{\lambda}cl_{857}, HK97, and HK243}

Given that three of the phages tested in the resistance screens use LamB as their receptor, it is unsurprising that several of the hits for each are involved in regulation of the mal-regulon, which includes \textit{lamB}. These hits include genes directly encoded in the regulon, such as \textit{lamB} and \textit{malT}, genes whose products are necessary for transcription from some promoters in the regulon, such as \textit{cyaA}, and other genes whose products indirectly influence transcription of the mal-regulon, including \textit{malI}, \textit{ptsG}, and \textit{pgm}\textsuperscript{110}.

The mal-regulon is responsible for maltose and maltodextrin transport and metabolism. It consists of two main clusters of genes in different regions of the chromosome. The metabolic cluster contains two divergently oriented operons, with \textit{malT} transcribed clockwise and \textit{malPQ} transcribed counterclockwise. The other transport cluster has \textit{malK}, \textit{lamB}, and \textit{malM} being transcribed clockwise and \textit{malEFG} being transcribed counterclockwise. Two additional genes involved in metabolism encoded in two additional separate operons are \textit{malZ} and \textit{malS}. All genes in this regulon are regulated by MalT, the transcriptional activator, including \textit{malT} itself. In addition to \textit{malT}, other genes not in the mal-regulon encode products which control mal gene expression, including \textit{cya}, \textit{crp}, \textit{malI}, \textit{malY}, and \textit{mlc}, among others. Finally, many other genes exist which influence levels of maltotriose, which is the endogenous inducer of the mal-regulon, including \textit{pgm} and \textit{glk}\textsuperscript{110} (see \textbf{Figure 9} for summary diagram).
Figure 9: Schematic diagram showing λcI₈₅⁷ resistant strains relating to the mal-regulon. Proteins highlighted in orange are the products of genes that confer resistance to λcI₈₅⁷ when knocked out. Likewise, proteins highlighted in red, blue, and green are the products of genes that confer resistance to HK97, HK243, and T6, respectively. Transcription of the receptor encoded by lamB is under the control of the activator MalT (activation indicated by blue arrows). Transcription of malT itself can be repressed by mlc (indicated by red lines), a protein that binds to the glucose transporter PtsG. When PtsG is deleted, this leaves mlc free to inhibit malT transcription, as seen in the resistant phenotype to HK97. MalT requires binding of maltotriose, an intermediate in the breakdown of maldextrins to maltose, in order to be activated. This activation can be inhibited by other factors competing for the binding site on MalT, including MalY and MalK. MalY is transcribed from malY, whose transcription is normally inhibited by MalI. Deletion of MalI would allow malY transcription and ultimately inhibit MalT activation. MalK forms the energy-providing part of the MalFGK₂ maltose/maldextrin transporter, and when bound to ATP under conditions of low maltose transport, binds MalT sequestering it from activating the mal-regulon. EIIA forms a subunit of PtsG, which donates a phosphate to glucose to make glucose-6-phosphate during active transport. When not actively transporting glucose, phosphorylated EIIA activates CyaA, which produces cAMP, an activator of the mal-regulon. Maltotriose can be formed endogenously as well in the absence of maltose, from glucose-6-phosphate, created from glucose-1-phosphate through the action of Pgm. Deletion of pgm leads to a low resistance phenotype for HK97 but not λcI₈₅⁷. See details in text.
It is simple to explain that deletion of *lamB* would lead to resistance due to elimination of the receptor for the tested phages, as would deletion of *malT*, the transcriptional activator directly involved in transcribing *lamB*. The situation becomes more complex when looking at the other hits from the screen. In order for MalT to activate transcription of the mal-regulon, it must first be activated by maltotriose, a product formed during the metabolism of maltodextrins to maltose through the enzymatic activities of MalZ, which cleaves glucose units sequentially from the reducing end of maltodextrins. When maltotriose is present, it binds to inactive MalT, thus creating the activated form necessary for transcription\(^{110}\). Maltotriose can also be synthesized endogenously from glucose-6-phosphate produced from glucose or glycogen in the cell, and is responsible for low levels of transcription even in the absence of maltose or maltodextrins. This is possible due to the enzyme phosphoglucomutase, encoded by *pgm*, which converts between glucose-6-phosphate and glucose-1-phosphate. It has been suggested, as well, that MalT has a basal level of transcriptional activity and that there need not be an endogenous inducer at all\(^ {123}\), though this is less able to explain the phenotype seen in the *pgm* knockout with HK97.

In addition to the inducer maltotriose, there are several proteins that interact with and regulate the activity of MalT, including MalK and MalY. MalK forms the energy-providing part of the MalFGK2 maltose/maltodextrin transporter, and under conditions where ATP is bound but unhydrolyzed (for example when no transport is occurring due to low sugar levels), MalK is capable of binding MalT, sequestering it and preventing transcription\(^ {110; 124; 125; 126}\). When transport again becomes active, MalT is released and the mal-regulon is upregulated. MalY works in a similar manner, competing with MalK to bind to MalT and keep it in its inactive form. MalY is thought to form part of an additional transporter of an as yet unknown sugar and is encoded by an operon including *malX* and *malY* located at 36 min\(^ {127}\). This operon is inhibited by an additional product of *malI* which is transcribed in the opposing direction to *malXY*\(^ {128}\). *malI* mutants are known to cause an increase in MalY, and thus inactivation of MalT and a reduced transcription from the mal-regulon\(^ {129}\), which in the case of a *malI* knockout would explain an increase in resistance seen in HK243.
In addition to \textit{malT}, \textit{cyA} is also necessary for transcription of \textit{lamB}. \textit{cyA} encodes adenylate cyclase, the enzyme responsible for production of cyclic adenosine monophosphate (cAMP.) cAMP is used as a global regulator of sugar transport, and is necessary for transcription of genes such as \textit{lamB} and \textit{malT} by activating cAMP receptor protein (CRP)\textsuperscript{130, 131}, which together with activated MalT allows transcription of the mal-regulon. During sugar transport, such as glucose through the EIIBC\textsuperscript{glc} complex (encoded by \textit{ptsG}), glucose is taken in by an active process as glucose-6-phosphate, with a phosphate being donated by EIIA\textsuperscript{glc} (encoded by \textit{crr}), which in turn obtains the phosphate from phosphoenolpyruvate during glycolysis. Under high glucose conditions, EIIBC\textsuperscript{glc} is continuously accepting phosphates from EIIA\textsuperscript{glc} which, in addition to its role as a phosphate donor, is responsible for upregulating adenylate cyclase in its phosphorylated form. Since phosphates are being donated to the incoming glucose, and EIIA\textsuperscript{glc} exists primarily in the unphosphorylated form, adenylate cyclase is not activated and cAMP levels drop\textsuperscript{132}. This drop in cAMP represses transcription of transporters such as LamB, as maltose transport would not be needed in high glucose conditions.

From this interaction of two sugar transport systems it can be suggested that knocking out \textit{cyA} would also lead to a reduction of cAMP, leading to a lack of \textit{lamB} transcription, and lack of receptor on the cell surface. Indeed, this knockout leads to a fully resistant phenotype to \textit{λcI\textsubscript{857}} and HK243. An additional interaction between the \textit{ptsG}-encoded EIIBC\textsuperscript{glc} and two additional proteins, Mlc and GLK, helps explain its role in \textit{lamB} transcription. Mlc is an inhibitor of \textit{malT} transcription and is capable of binding unphosphorylated EIIBC\textsuperscript{glc}, as would exist in a situation where there are high glucose levels as described earlier. In that case, Mlc would be sequestered away from \textit{malT} and transcription would be derepressed\textsuperscript{123}. Interestingly, this is the opposite effect from catabolite repression by cAMP levels, and the reason for this system is not well understood. In a situation where \textit{ptsG} is knocked out, however, Mlc could remain bound primarily to the \textit{malT} operon, leading to decreased transcription and a drop in receptor levels.

The differences seen between the three phages primarily affected by mutations in the mal-regulon indicate that they have varying reliance on this pathway, likely due to the effect mutations would have on LamB levels on the surface. \textit{λcI\textsubscript{857}} and HK243 appear to use the same components of the pathway as they are affected by mutations in \textit{cyA}, \textit{lamB}, and \textit{malT}. HK97 appears to be affected by all these as well, and is additionally affected by mutations in \textit{pgm} and
ptsG, suggesting that HK97 is more reliant on the mal-regulon, possibly due to differing sensitivities to LamB levels, the endpoint of the pathway.

2.5.3  \(\text{\lambda cI}_857\), HK97, and HK243 have Differing Dependencies on LPS Structure for Infection

Of the 43 hits in the growth curve screen, 21 involved the cell wall, including ten genes in the LPS biosynthesis pathway (see Figure 10). These findings show 48.8\% of the hits involved envelope genes, forming the biggest category of hits from the growth curve resistance screen. This could be explained due to the heavy dependence phages have on the envelope across many periods of their life cycles, including adsorption, DNA-injection, and lysis. In the case of \(\text{\lambda cI}_857\), the relative strengths of the LPS phenotypes correlated with the phages’ ability to adsorb, with the most resistant mutants showing a strong adsorption defect, suggesting that LPS mutations are involved in either limiting expression of the LamB receptor on the cell surface, blocking access to the receptor, or playing some additional role in adsorption. One of the known physiological conditions brought about by changes near the LPS core region is called the deep rough phenotype\(^{133}\).

Rough phenotypes are caused by LPS that lacks an O-antigen. Deep rough mutants lack the core oligosaccharides and were originally named due to the rough colony morphology observed in these mutants. In the deep rough phenotype, it is known that concentrations of outer membrane porins can be reduced 50-90\%, and that, on average, all outer membrane proteins are reduced as much as 50-60\%, possibly due to the impaired ability to properly form and stabilize porin trimers, as seen with outer membrane porin F (OmpF) and LamB\(^{134}\), or to the loss of improperly anchored proteins to the external media\(^{135}\). Under these conditions a lack of available receptor would lead to the resistance seen across the three lambdoid phages. Interestingly, the three phages are affected to different extents by the LPS mutations, with HK97 and \(\text{\lambda cI}_857\) showing the strongest dependence and HK243 not being affected by LPS at all. This leads to the possibility that each phage has a different binding strength for the receptor, with HK243 being able to bind readily even under conditions of low concentrations of LamB, and could explain some of the different susceptibilities of each phage to knockouts of the mal-regulon. This is further supported by the findings that addition of maltose increases phage infectivity for HK97 in both WT and LPS knockout strains, increases \(\text{\lambda cI}_857\) infectivity to a lesser extent in the LPS knockouts, and does not affect HK243 at all.
Figure 10: Basic structure and synthesis of *E. coli* K12 LPS. The lipopolysaccharide of *E. coli* K12 strains consists of the membrane-anchored lipid A moiety, two keto-deoxyoctulosonate (Kdo) residues and three heptoses in the inner core, followed by three glucose residues that possess bound galactose and heptose residues. To the terminal glucose residue in other strains of *E. coli* an O-antigen may be attached, consisting of a polymer of oligosaccharides that can vary by strain, but is lacking in K12. Biosynthesis of LPS occurs from the inner core outward, with each enzyme labeled catalyzing the addition of the residue, or phosphate group, distal to itself, with the exceptions of RfaP, RfaQ, and RfaY, which require the addition of the first glucose residue by RfaG before proceeding. The oligosaccharide subunits are prepared for assembly in two separate processes involving a series of reactions catalyzed by enzymes located in the boxes. Proteins which affect both λcI857 and HK97 growth are labeled in red. Proteins which affect λcI857, HK97, and HK243 are labeled in blue. Proteins which affect λcI857, HK97, and T6 are labeled in green. This figure represents data from both Table 2 and Table 4.
The deep rough phenotype can be recreated with only a deletion of \( rfaP \), encoding an enzyme that phosphorylates the first core heptose in the LPS structure, or by deletion of \( rfaG \), which catalyses addition of the first hexose in the core structure\(^{133}\; ^{136} \). Interestingly, neither of these knockouts leads to a strong resistance phenotype in any of the lambdoid phages tested. More recent studies have shown that specific deletion of \( rfaP \) and \( rfaG \) by insertion mutations did not lead to a decrease in OmpA, OmpC, or OmpF, which would support the concentration model. However, despite this finding, the same study showed hypersensitivity to hydrophobic agents such as sodium dodecyl sulfate (SDS), matching other well characterized deep rough phenotypes\(^{139} \). The results of this study have been called into question due to the difficulty in reconciling them with a large body of evidence to the contrary\(^{140} \). This complicates matters if only the concentration of LamB on the surface were affecting adsorption. The findings from this study suggest that \( rfaP \) and \( rfaG \) indeed may not have as great an effect on LamB concentrations as was suggested, and this would explain the decreased resistance seen. Otherwise, they hint that LPS is playing some additional role in phage adsorption that remains to be determined. Some phages such as T7 are known to use LPS as the receptor, and some studies in the past have suggested an accessory role for LPS in helping guide \( \lambda \) to its receptor\(^{141} \). Additional studies in our lab have shown that immunoglobulin-like domains found in phages, such as one found in the \( \lambda \) major tail protein GpV, may serve this function\(^{141};^{142} \). It remains to be determined what exact role LPS structure plays with regards to phage infection, and particularly how \( \lambda cI_{857} \), HK97, and HK243 all show differing dependencies on this structure.

### 2.5.4 Different Strains of the Same Phage Can Have Differing Requirements for Host Factors

During the screening process, another group from Stanford published a similar study looking at \( E. \ coli \) host factors involved in \( \lambda \) reproduction\(^95 \). This group used the Keio collection as well. By plating phages on lawns of \( E. \ coli \) and looking for strains showing a change in plaque size, they found 57 genes involved in \( \lambda \) growth. One of the key differences between this study and my own is that the strain used in theirs was different (ATCC, 23724-B2), because it contains the wild type CI repressor protein and was capable of forming lysogens at the 37 °C used in their screening. My screen found all of the expected hits involved in the lytic pathway, including some not present in their screen. Two genes involved in LPS synthesis, \( galU \) and \( rfaG \), one of the genes encoding a subunit of mannose permease, \( manY \), needed for DNA
transport, and dnaK, which plays a role in λ DNA replication, were not picked up in their screen (see Table 3 for my results and brief descriptions of each gene). Additionally, their screen detected malI, which plays a role in LamB regulation, while mine did not for λcI857, but did for HK243.

These differences could be due to differing sensitivities of the two screens. My initial screen looking at growth curves may have picked up subtle phenotypes missed by just looking at the endpoint plaque size. Another possibility is that the two strains of λ used in the screens have diverged during laboratory cultivation, such that differing results represent truly different requirements for processes such as host adsorption, as suggested by the differing LPS findings, or ability to inject DNA, seen in the differing mannose permease findings. One example of an established difference is the finding that most strains of λ used in labs lack the side tail-fibers found in the original isolate, which have a dramatic effect on its ability to adsorb to the cells and infect\textsuperscript{143}.

Additionally, their screen detected hflC, hflD, and hflK, whose products are involved in CII degradation\textsuperscript{30, 34}, and ihfB, involved in genome integration\textsuperscript{20, 21, 22}. These play a role in the lysis/lysogeny decision and would not be detected by growing the λcI857 strain at 37 °C. Their screen also revealed a further 37 genes involved in a variety of cell processes, including cell structure, information transfer, regulation, metabolism, and transport, as well as four genes encoding iron-sulfur cluster containing products and four unannotated genes. One of these genes was pmg, which plays a role in LamB levels, which was not picked up by my screen for λcI857 but was picked up in my screen for HK97. While many of these genes show a significantly lower degree of growth inhibition than the others found by both our screens when they analyzed their growth curves, it is possible that many of these genes are involved in the lysogenic life cycle and would not be detected by my screen. It would be interesting to compare each strain of λ in side by side studies to see if these represent truly differing requirements, which would further broaden the variation I see even between related strains. There have been recorded differences in different isolates of the same phage having changes in host range susceptibility, such as the presence of side tail fibers found in the original λ isolate, but not commonly found on later derived lab strains\textsuperscript{143}. As very few large scale screens such as this one have been performed to date, it is possible that more differences will be uncovered as different groups look more closely at their own phage isolates.
2.5.5 The Defective Lambdoid E14 Prophage Plays a Role in T6 Infection

Perhaps one of the more surprising findings from the resistance screens is the resistance seen for phage T6 when replacing a number of genes in the e14 prophage with a kanamycin resistance cassette. The e14 prophage is a defective lambdoid prophage, one of eight prophages found in *E. coli* K12. λ itself was first discovered as a prophage, with six of the seven others, including e14, being considered lambdoid in nature. E14 is a 15.4 kbp element lying between 1195432 bp and 1210646 bp on the K12 chromosome and includes an estimated 21 open reading frames (ORFs)\(^{144}\). Of these 21 ORFs, 4 gave resistance to T6 when knocked out, including *ymfE*, *ymfO*, *ymfR*, and *JW5167* (see Figure 11). These four verified phenotypes occur in gene clusters located in two distinct regions of the prophage element, between base pairs 1325 and 2486, as well as between base pairs 9341 and 9934\(^{144}\). However, when the kanamycin resistance cassette in each of the four verified hits was knocked out by FLP recombinase, normal infectivity was returned to *ymfO* and *ymfE*. *ymfR* showed little change in spotting results when the cassette was removed compared to the plating screen, and JW5167 wasn’t originally picked up by the plating screen but rather the growth curve screen, and thus showed no change in observable spotting in the kan-cured strain. A mock kan-cured sample of the parental strain showed no change in spotting compared to the uncured parental strain, and the tsx knockout remained fully resistant after kan-curing, suggesting that any changes seen are not a side effect of the procedure.

Taken together, these results suggest that the phenotypes seen for *ymfE* and *ymfO* are due to the presence of the kanamycin resistance cassette rather than the deletion of the gene, since flipping out the cassette returns normal infectivity to both. JW5167 does not display a drop in titer in the plating screens, though it does show a higher turbidity, that disappears after excision of the kanamycin resistance cassette. JW5167 encodes an intergenic segment and is no longer present in current releases of the Keio collection\(^{97; 145}\), suggesting the effect is not due to the production of a gene product, but rather the presence of the antibiotic resistance cassette. This leaves only *ymfR* as having a real knockout phenotype. This gene encodes a small protein of unknown function containing two transmembrane domains, similar to ones encoded in the same genetic location by phages ST64B, SfV, and phiP27\(^{144}\).
It is also possible that downstream targets of the promoter for the kanamycin resistance cassette could be upregulated in the case of ymfE, ymfO, and possibly JW5167, which lead to T6 resistance. One gene that lies immediately downstream of both ymfE and JW5167 is lit, which encodes the T4 exclusion protein. The protein is activated by the 29-amino-acid Gol peptide, produced by phage T4 late in the infection cycle as part of the major capsid protein. Activated Lit can cleave elongation factor-Tu between Gly59 and Ile60, which subsequently puts an end to protein synthesis, a bacteriostatic condition leading to bacterial death and abortion of phage infection. As T4 and T6 are morphologically similar and share sequence homology over 88% of their genomes as determined by electron microscopy studies, it is possible that expression of this gene may also have some effect on T6 if overproduced. This would further explain why resistance would be seen in a shorter growth curve time course before cells die, and turbid zones of clearing seen in the plating experiments with an overnight incubation. Additionally, lying several genes downstream of ymfR and ymfO and across from an invertible region is mcrA, which encodes a restriction endonuclease specific for methylated DNA. T4 and other T4-like phages contain hydroxymethylcytosine (HMC) in place of cytosine, which confers protection against most R-M systems that recognize sequences with a cytosine. It is possible that upregulation of this gene could lead to some restriction enzyme cleavage of T6 DNA, leading to resistance.
Figure 11: Overview of the e14 genome and T6 resistance. (Modified from Mehta et al: BMC Microbiol. 2004 Jan 20;4:4). Shown is the overall layout of the defective lambdoid e14 prophage found in the *E. coli* K12 genome, inserted within the icd gene encoding isocitrate dehydrogenase. Regions highlighted in yellow indicate homology with a typical lambdoid phage, showing significant overlap with the e14 genome. Genes coloured in red indicate host genes, in black represent genes known to be functional, in blue represent genes whose functionality remain to be characterized, and in red represent genes unlikely to be functional. Predicted transcriptional start sites are represented by arrows, and predicted transcriptional terminators by solid lines. Along the bottom is a bar graph showing the relative resistances to T6 seen in each knockout, with the largest bars indicating resistance seen at an MOI of $10^2$, and medium-sized bars indicating resistance seen at the initial MOI of $10^4$. The names of genes hit by the resistance screen are included below the resistance bars, and correspond to the Blattner number names given in the original diagram. Highlighted in blue boxes are *lit*, which encodes a T4 exclusion protein, and *mcrA*, encoding a methylation-dependent restriction endonuclease that targets T-even phages.
3.1 Summary

As a result of this study, 43 genes in *E. coli* K12 were identified to play a role in phage infection across four phages, namely λcI857, HK97, HK243, and T6, including 20 genes not previously stated to have a role in phage infection based on available literature at the time of this study. Of these 20, 11 were either previously uncharacterized or had only putative functions assigned to them. It is important to note that even with λ, which is one of the most well-studied model systems, several new genes were discovered to play a role in its growth. These include subtle roles for *ygfY, ubiF*, and a previously underappreciated role for subtle effectors of LamB production, including LPS structure, the mal-regulon transcriptional activator MalT, and the periplasmic folding chaperone SurA. With regards to previously known genes involved in phage infection across these phages, all were picked up by the growth curve assay, including known weak phenotypes such as the accessory role of *ihfA* in packaging DNA into λ proheads, which lyse, but show a reduced burst size.

One of the interesting outcomes of this study is the diversity seen even in siphoviridae phages using the same receptor, which differ greatly with regards to reliance on host LPS, possibly as a result of their relative differences in binding strength to their receptors. Even among the same species, when λcI857 from this study is compared with λcI857 isolated from ATCC 23724-B in the Stanford study on host factors affecting λ growth, there are differences in the previously uncharacterized hits, though one hit from that study, *yneJ*, which was missed by my study, turns up as a strongly resistant phenotype for HK243. This diversity amongst even highly related phages suggests that even studies with one of the most well-studied phages contains some surprises, and that these variations could lead to an unexpectedly higher number of variations from lab to lab depending on which strain is used.

The importance of other genes found in the screens have also been tested using the assays described in this study. A role for the periplasmic chaperone FkpA in DNA injection by HK97 was identified using the potassium assay (work by Nichole Cumby), in addition to its previously
established role in folding colicin M. The adsorption ability of λcI857 was shown to correlate with the resistance seen in a variety of LPS mutants, with the strongest phenotype occurring with modification of the first core heptose in the structure. Interestingly, the deep rough phenotype, in which concentrations of outer membrane proteins such as LamB are drastically reduced, has been shown to depend on phosphorylation of this core region, and mutants in rfaP show a physiological deep rough phenotype, whereas the rfaP knockout in this screen had relatively low resistance and appeared normal in adsorption. This suggests that it is not only the reduced levels of LamB found in LPS mutants that cause a resistance phenotype, and that LPS may play an additional accessory role in adsorption.

Finally, the finding that genes within the defective e14 lambdoid prophage have an effect on T6 is interesting. Using FLP recombinase to remove the kanamycin resistance cassette from three of these pseudogenes also removed the resistance phenotype, leaving the possibility that perhaps upregulation from the Kan promoter of some downstream gene could be responsible for the phenotypes. _lit_ is a gene lying downstream of ymfE and JW5167 that encodes the T4 exclusion protein, leading to a shutdown of late gene expression during T4 infection. It is possible that _lit_ is also affecting the related T6 in this manner. The other gene, _ymfR_, is predicted to encode a small protein of unknown function which does not appear to be expressed under normal conditions, and yet it retains a resistant phenotype even after removal of the resistance cassette. The role this gene from a lambdoid phage is playing during infection with a Myoviridae remains to be determined.

The addition of new roles for 20 genes in screens with only four phages, as well as the range of phenotypes seen in related phages, suggest that screening a variety of phages for resistance, in particular the subtle resistances picked up by these sensitive screens, would be of great use for any lab working with either bacteria or phages.

### 3.2 Future Directions

One of the findings of this study was that with every additional screen, a significant number of genes not previously implicated in phage infection were discovered, even amongst three similar lambdoid phages. It is clear that it would be beneficial to continue screening additional phages, and other phage-like particles such as bacteriocins, against the knockout collection to further expand the database of phage-host interactions. My screens can additionally
be adapted to create an extremely rapid screen using only the positive hits from the various screens pooled together, as these hits have been shown to play a role in infection already and are likely to be involved in other phage life cycles as well. This could be used to rapidly compare a selection of phages on a very small number of plates, and could be used to create a database of resistance “fingerprints” that can be used to identify unknown phages. One interesting comparison between phages would be with the \( \lambda cI_{857} \) strain used in my study and the \( \lambda \) strain used in the Stanford study\(^9\).

Additionally, knowing this pattern of resistance for a given phage can be used to study mutants or gene-deletions within that phage to help determine their functions. One example is with the nin region containing ten nonessential \( \lambda \) genes. This region can be deleted with no observable deleterious effect under laboratory conditions. This strain would be screened against the pool of knockouts shown previously to have effects with the growth curve screen, and results would be compared to wild type \( \lambda cI_{857} \). If differences are found in the nin-deleted strain, the individual nin genes could be tested by a single-gene deletion protocol for bacteriophage developed in our lab by Senjuti Saha, and could be verified by testing whether complementation with a plasmid restores the wild-type phenotype. This same gene-deletion method could be used to test other \( E. \ coli \) phage genes of interest as well against the pooled results. In a similar experiment, the \( \lambda cI_{857} \) strain could be compared with a phage thought to be more similar to the original \( \lambda \) isolate called \( ur\lambda \) which possesses side tail fibers that have since become lost in the \( \lambda cI_{857} \) strain and other laboratory strains\(^{143} \). Additionally, the side tail fibers could be restored to \( \lambda cI_{857} \) via a plasmid. It would be possible to test whether these tail fibers alter any of the resistances seen, particularly with regards to the LPS deletions and the dependency on LamB concentrations seen in the maltose assay. One of the findings from the maltose assay was that \( \lambda cI_{857} \) unexpectedly did not lyse in the 0.4% glucose condition at any MOI, in contrast to the lysis seen in the spotting assay. It is possible that the side tail fibers seen in the original isolate may help with adsorption in a more turbulent, liquid environment, and that including these tail fibers may restore normal lysis in these conditions.

Additional verification and characterization of certain hits remains to be completed as well, including the hits for T6 from the E14 prophage. It was shown that removal of the kanamycin-resistance cassette restored normal growth in \( JW5167, ymfE, \) and \( ymfO, \) but not in \( ymfR \). One of the possibilities is that the promoter for the resistance cassette is leading to an
upregulation of downstream genes that are responsible for the resistances seen. One of these genes is *lit*, which leads to resistance to T4 when upregulated. Testing whether upregulation of *lit* using the ASKA overexpression plasmid leads to T6 resistance could help explain the phenotype seen for *JW5167*. This sequence lies just upstream, does not appear to code for a gene at all, and has been removed from more recent versions of the Keio collection. *ymfE* lies upstream of *JW5167*, but is transcribed in the opposite direction, as is the Keio deletion mutant.

Additionally, downstream of *ymfO* lie two structural genes encoding proteins similar in structure to phage baseplates, an invertible region encoding several genes similar to tail fiber genes, a gene encoding the invertase for the previous region, and finally *mcrA*, which encodes a methylation-dependent restriction endonuclease, responsible for restriction of T-even phages. It could be that upregulation of these structural proteins may interfere with T6 assembly in some way, or that upregulation of *mcrA* is leading to the resistance. There is a predicted transcriptional terminator just prior to the *mcrA* gene that would limit this possibility, but the terminator has not been experimentally verified. Upregulation of each of these genes using ASKA plasmids could help determine which, if any, are the cause of the resistance to T6 seen, and electron microscopy could help determine any assembly defects.
References


111. Dean, D. A., Reizer, J., Nikaido, H. & Saier, M. H., Jr. (1990). Regulation of the maltose transport system of *Escherichia coli* by the glucose-specific enzyme III of the


