Phage Selection Against Antibiotic Resistance or Virulence in Opportunistic Bacterial Pathogens

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Abstract
Phage Selection Against Antibiotic Resistance or Virulence in Opportunistic Bacterial Pathogens
Kaitlyn E. Kortright
2021

Bacteriophages (phages) are prolific, ubiquitous viruses that infect bacterial cells. Phages have proven to be invaluable tools of the biological sciences enabling the discovery and description of some of the foundations of molecular biology and genetics; excellent systems for the study of evolutionary dynamics; and their seemingly limitless diversity promises to produce interesting new biology for many years to come. As natural predators of bacteria, phages might additionally be employed for phage therapy, the therapeutic use of phages to treat bacterial infections. Lytic phages impose strong selective pressure on bacteria to evolve resistance to phage infection. Under certain environmental conditions this selection pressure can result in the evolution of phage resistance at reduced fitness; this is an example evolutionary trade-off, or the evolution of a certain trait at the detriment of the fitness. In particular, it is possible that phage-imposed selection on bacterial surface expressed molecules may result in reduced antibiotic resistance and/or attenuated virulence of opportunistic bacterial pathogens. In this thesis, we will examine the idea of phage-imposed trade-offs on opportunistic bacterial pathogens.

A published review paper serves as the first chapter and an introduction to phage biology and phage therapy. We present the history of the discovery of phages as well as the early reports of phage therapy. We highlight recent animal studies, case reports and clinical trials that have investigated the therapeutic use of phage. Finally, we discuss new approaches to phage therapy as
well as identify interesting question and potential hurdles that will likely underscore a modern approach to phage therapy.

In chapter 2, transposon insertion sequencing (INSeq) is used as a high-throughput method to identify phage receptors to find new phage that use antibiotic resistance or virulence factors as receptors. Preliminary experiments using characterized phages T2, T4, T6 and T7 demonstrate that the top results of INSeq screens with phage are involved in phage binding. These screens were extended to enable receptor identification for five newly isolated phages, R3, U115, EC35, EC14 and 8S. Adsorption assays and efficiency of plaquing assays to demonstrate phage binding and infection are used to validate the results of the INSeq screens with uncharacterized phage. In summary, this chapter establishes the use of INSeq screens as a high-throughput method to determine phage receptors which should allow for identification of phage that target virulence factors or molecules contributing to antibiotic resistance.

Evolutionary dynamics between Pseudomonas aeruginosa and a lytic phage, OMKO1 that selects against antibiotic resistance, are examined in a short-term coevolution experiment in chapter 3. Time shift assays showed that coevolution of the three treatment populations followed arms race dynamic. Interestingly, only one treatment population demonstrated the predicted trade-off between phage resistance and antibiotic resistance. Sequencing of bacterial population allowed for identification of mutations underlying bacterial evolution and the trade-off. Results from this study demonstrate that evolution may not be as repeatable or predictable as previous experimental evolution studies have suggested.

In chapter 4, we identify a new Shigella flexneri phage that selects for phage-resistant bacteria that are attenuated for virulence. We isolate and characterize a new Myoviridae phage, A1-1 which uses OmpA of S. flexneri as a receptor. Assays to interrogate membrane permeability
including, live-dead staining, minimum inhibitory concentrations to various antibiotics and measurements of total lipopolysaccharide (LPS) quantity demonstrated that phage A1-1 selects for two different phenotypes of resistant mutants: OmpA deficient and altered LPS. Whole genome sequencing revealed that the five phage-resistant mutants examined in this study have mutations either in \( ompA \) or in genes involved in LPS biosynthesis genes. Using bacterial plaque assays, we showed that all five resistant mutants are attenuated for intercellular spread, indicating that in the case of phage A1-1, phage resistance trades off with bacterial virulence.

While the previous two chapter highlight phage selection resulting in a trade-off, the final chapter involves a system in which phage selection resulted in a trade-up with antibiotic resistance. Tsx is a nucleoside porin in \textit{Escherichia coli} which also serves as a receptor for phage T6, phage U115 and albicidin, a DNA gyrase inhibitor. We showed that selection for resistance to any of these three antibacterials resulted in cross-resistance to the other two. Competition assays showed resistance to these antibacterials does not result in a fitness cost relative to wild-type. In all 29 of the resistant mutants observed in this study, mutations in \textit{tsx} were found. The trade-up between phage resistance and antibiotic resistance in this study highlights the need for rigorous studies of phage-bacteria interactions prior to deployment of phage therapy.
Phage Selection Against Antibiotic Resistance or Virulence in Opportunistic Bacterial Pathogens

A Dissertation
Presented to the Faculty of the Graduate School
Of
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In Candidacy for the Degree of
Doctor of Philosophy

by
Kaitlyn E. Kortright
Dissertation Director: Paul Turner, PhD
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Chapter 1: Introduction to Phages and Phage Therapy
Preface

Chapter 1 has been adapted from a review article that has previously published: Kortright, K. E., Chan, B. K., Koff, J. L., & Turner, P. E. (2019). Phage therapy: a renewed approach to combat antibiotic-resistant bacteria. Cell host & microbe, 25(2), 219-232. Kaitlyn Kortright and Dr. Paul Turner planned, conceptualized and prepared the manuscript. Dr. Benjamin Chan and Dr. Jon Koff provided useful feedback, comments and edits. We thank Caeul Lim and three anonymous reviewers for helpful comments on the manuscript. This work was supported by a Pilot Award from the Cystic Fibrosis Foundation.
Abstract

Phage therapy, long overshadowed by chemical antibiotics, is garnering renewed interest in Western medicine. This stems from the rise in frequency of multidrug resistant bacterial infections in humans. There also have been recent case reports of phage therapy demonstrating clinical utility in resolving these otherwise intractable infections. Nevertheless, bacteria can readily evolve phage resistance too, making it crucial for modern phage therapy to develop strategies to capitalize on this inevitability. Here, we review the history of phage therapy research. We compare and contrast phage therapy and chemical antibiotics, highlighting their potential synergies when used in combination. We also examine the use of animal models, case studies and results from clinical trials. Throughout, we explore how the modern scientific community works to improve the reliability and success of phage therapy in the clinic, and discuss how to properly evaluate the potential for phage therapy to combat antibiotic resistant bacteria.
Chapter Introduction

Soon after Alexander Fleming’s 1928 discovery of penicillin and Western medicine’s widespread use of antibiotics since the 1940’s, Fleming himself warned that misuses of these drugs could result in antibiotic resistant bacteria \(^1\). As predicted, clinical reports of antibiotic resistance followed, such as the evolution of resistant *Mycobacterium tuberculosis* in early clinical trials for streptomycin efficacy in treating tuberculosis \(^2\). Nevertheless, the discovery and development of novel antibiotics flourished for many decades \(^3\). However, in the latter 20\(^{th}\) century, antibiotic discovery slowed and the alarming increase in rates of antibiotic resistance signaled that the golden age of antibiotics had perhaps ended. Indeed, aside from three new antibiotic classes discovered between 2005 and 2018, no novel drug classes have been developed since the 1980s \(^4\)–\(^6\). Similar mechanism of action among these newer drugs has led to potential evolution of cross resistance in bacteria. While synthetic modifications to some pre-existing antibiotics have temporarily extended their clinical usefulness \(^7\), this approach has also selected for broader resistance mechanisms such as extended spectrum beta lactamases \(^8\), adaptive changes that are perhaps more easily evolved compared to *de novo* resistance mechanisms.

In 2017, the World Health Organization (WHO) highlighted the particular threat of Gram-negative pathogens resistant to multiple antibiotics \(^9\). Discovery, design, and development of new and alternative antibacterial therapies are crucial. This review concerns the therapeutic use of bacteriophages (phages): viruses that exclusively infect bacteria and can act as bactericidal agents. This approach of ‘phage therapy’ is an old idea that is recently regaining popularity. Efforts are buoyed by development of easier methods for engineering phages for different purposes in biotechnology \(^10\). Also, the extreme biodiversity of phages in nature \(^11\), \(^12\) can be leveraged for ‘bioprospecting’: discovery and development of naturally evolved phages with properties that are
ideal for phage therapy use. Below we examine the past, present and future uses of phage therapy, especially addressing how this newly energized field may proceed with modern, rational therapeutic approaches.

**Phage Biology**

During a lytic infection cycle (Figure 1.1) a phage will: (1) attach to receptor(s) on the surface of a bacterium; (2) deliver the genomic content into the bacterium; (3) undergo viral replication in the cytosol via bacterial transcription, translation and replication; and (4) upon formation of new phage particles, escape the cytoplasm through lysis of the bacterium. This process is then repeated by the new phage particles as they infect additional susceptible cells. This highlights a long-understood benefit of phage therapy: utilizing lytic viruses as self-amplifying ‘drugs’ that target and kill susceptible cells may be more efficient than applying antibiotics that are incapable of self-amplification.

Obligately lytic (or ‘virulent’) phages seem to be the best candidates for development of phage therapy (hereafter we refer to such phages simply as ‘lytic’). However, for completeness we briefly remind that lysogenic (or temperate) phages are also prevalent in nature. Lysogenic phages integrate into the host genome, and are inherited by daughter cells during binary fission; however, at a later time, under environmental perturbation or other physiological stressor, lysogenic phages excise from the bacterial genome and enter a lytic infection cycle. While lysogenic phages might be preferred in certain biotechnology applications, lytic phages are more akin to antibiotic drugs lethal to bacteria, which suggests an easier path to approval for treating bacterial infections.

In the mid-20th century bacteria and lytic phages were used in classic experiments to elucidate fundamentals of biology and genetics, including demonstration of the spontaneous nature of mutations, DNA as hereditary material, and triplet nature of the amino-acid code. Beyond
these studies, it is increasingly recognized that phage biodiversity is immense. Importantly, whereas some phages are highly specific to a single species or even genotypic strain of bacteria\textsuperscript{17}, other phages have naturally broad host ranges or can easily mutate to infect bacterial genotypes and species other than the typical host\textsuperscript{18}. The aforementioned first step of lytic phages infection is attachment to the receptor(s) on the cell surface. These receptor binding sites are most commonly proteins or sugar moieties on the bacterial cell, which are recognized by phage proteins that are effectively responsible for phage host specificity (Figure 1.2). There are many well characterized examples of phage binding, especially the morphological structures used for these purposes. For instance, phage T4 uses two sets of tail fibers, long and short, to bind to susceptible \textit{Escherichia coli} bacteria\textsuperscript{19}; phage SPP1 uses a tail spike to attach to \textit{Bacillus subtilis} hosts\textsuperscript{20}; the \textit{Siphoviridae} family of phages generally use baseplate proteins to initiate infection of \textit{Lactococcus lactis} bacteria\textsuperscript{21}; and \textit{Cystoviridae} phages such as phi-6 infect \textit{Pseudomonas syringae} via attachment proteins embedded within an envelope that surrounds the nucleocapsid\textsuperscript{22}. However, the immensity of phage biodiversity suggests that the basic biology of phage binding to bacterial proteins remains a vastly untapped science, ripe for new discoveries. The presence of phage-binding sites and their possible structural variation should affect specialized versus generalized ability for phage attachment. Nevertheless, in the following sections we highlight that historical efforts in phage therapy have not always investigated and characterized the receptor binding site(s) used by phages to initiate infection. Clearly, current development of phage therapy candidates could include: an investigation of the receptor binding site(s) used by phages when infecting bacteria; whether phage-imposed selection for changes in these structures alters therapy success; and how the
evolution of bacterial resistance to therapeutic phages may affect bacterial fitness components, such as rates of cell division and expression of pathogenicity traits.

**Early Phage Therapy**

The discovery of phages is attributed to the independent work of two microbiologists: Frederik Twort in 1915 and Félix d’Hérelle in 1917. While Twort was the first to observe and describe the effects of a ‘transparent material’ that inhibited bacterial growth, it was not until 1917 when d’Hérelle isolated an anti-Shigella microbe, that the idea of an obligate parasite of bacteria was termed bacteriophage or “bacteria-eater.” Almost immediately after his discovery, d’Hérelle recognized the therapeutic potential of phages as a treatment for bacterial diseases. In 1919, he successfully used phages to treat chickens infected with *Salmonella gallinarum*. This success in animals soon led d’Hérelle to attempt treating human infections with phages. In 1921, five patients with bacillary dysentery were successfully treated with a phage that infects *Shigella dysenteriae*. In 1927, clinical trials treating cholera in India showed that mortality decreased from 62.8% in control groups to 8.1% in phage-treated groups. Furthermore, d’Hérelle noted that introducing anti-cholera phages into drinking wells of villages during an outbreak prevented additional infections from occurring.

**Early-identified challenges**

Soon after d’Hérelle’s initial successes, many other scientists recognized the therapeutic and prophylactic potential of phages and began to target other infections, though with varying success. Among criticisms surrounding the design and quality of early phage therapy trials, scientists started identifying some potential challenges of phage therapy. (1) The possible drawback of extreme phage specificity was recognized early on, indicating that a phage may not be useful without prior characterization of bacterial susceptibility. For example, in 1923, Beckerish and
Hauduroy used phages successfully to reduce bacterial load in the blood of patients with typhoid fever \(^2^9\), whereas a year later Smith (1924) unsuccessfully used phages on a similar patient population; Hadley (1928) speculated Smith’s failure stemmed from unknowingly using phages with a narrow host-range. d’Hérelle himself acknowledged this weakness, attributing the success of his early trials to careful choice of phages capable of infecting the causative bacterial agent \(^2^6\).

(2) The early methods used to bulk-manufacture therapeutic phages were likely heavily contaminated with lysed bacteria. With limited and unreliable filtering and purification steps, the possible beneficial effects of phages were difficult to separate from the confounding effects of contaminating bacterial antigens \(^3^0\). (3) Early pharmacokinetic experiments showed that phages were rapidly removed from the body via the spleen, calling into question the sustained efficacy of phages over time \(^3^1\). (4) In 1943, Luria and Delbruck used selection by lytic phages to calculate spontaneous mutation rates of bacteria, and in doing so, demonstrated that bacteria are readily capable of evolving resistance to lytic phage attack \(^1^6\). (5) Lastly, early studies showed that in vitro laboratory experiments with phages and bacteria did not always match with experimental outcomes observed in vivo \(^3^2-^3^4\). As these perceived problems were identified, in Western medicine, the interest in phage therapy waned relative to newly discovered chemotherapeutics, antibiotics, and the trend away from phage therapy in the West was firmly cemented through the 1970s. This sentiment was in stark contrast to vested interests of physicians and scientists in the then USSR, Poland and elsewhere who continued to develop phage therapy in earnest; the legacy continues to be evident in locales such as the G. Eliava Institute of Bacteriophages in Tbilisi, Georgia. This important work has been the focus of numerous prior reviews and will not be discussed \(^3^5\). However, as new antibiotic resistance mechanisms arose for every novel class and compound, and
the incidence of antibiotic resistant infections increased globally, phage therapy was re-considered by the West.

**Smith and Huggins’ pioneering studies**

This interest was propelled forward in the 1980s with a series of well-designed experiments by Smith and Huggins. These experiments addressed many of the historic criticisms of phage therapy described above, while demonstrating safety and efficacy in animal models. Smith and Huggins began by showing that phage effectiveness *in vitro* could correlate with *in vivo* efficacy and ultimately chose phage R, which demonstrated the greatest *in vitro* virulence, for further characterization. Phage R appears limited in host range, only infecting K1* E. coli* and likely uses the K1 capsule as a receptor. Through a series of lethal bacterial challenges in mice, Smith and Huggins demonstrated that a single dose of phage R was as effective as eight doses of streptomycin. In the same experiment, they showed that bacterial lysate, free of phages, provided no therapeutic effect. After intramuscular inoculation of mice with no bacterial challenge, they observed that phages persisted in the inoculated muscle and spleen 28 days after the original inoculation while it was cleared relatively rapidly from the liver and blood at 16 and 20 hours post inoculation, respectively. Smith and Huggins noted that all phage-resistant mutants, observed at a frequency of ~0.01, were K1* variants that had been previously shown to be avirulent. In a single paper, Smith and Huggins were able to address many previous criticisms. Furthermore, they observed phage therapy to be potentially more effective than chemical antibiotics.

Smith and Huggins further investigated factors that could influence the effectiveness of phage therapy in an *E. coli* diarrhea model in calves. They cleverly employed a rational multiphage approach to combat the emergence of resistant mutants. Specifically, after choosing lytic phage B44/1 that only infected K85* strains of *E. coli*, they isolated phage-resistant mutants *in*
vitro. Subsequently, they chose a second phage B44/3 for its ability to infect bacteria that were resistant to the first phage B44/1, and additionally selected for B44/3-resistant mutants that were susceptible to phage B44/1 infection. By examining phage resistance prior to the therapeutic use of these phages, Smith and Huggins were able to anticipate the evolution of phage resistance and use a dual-phage approach that might limit the emergence of phage-resistant bacteria. In 1987, Smith and Huggins examined the stability of phages during orally administered therapy and observed that poor phage stability in the acidic environment of the stomach could be countered by administering calcium carbonate prior to phages. These revolutionary studies by Smith and Huggins determined that the many perceived criticisms of phage therapy were unfounded or less concerning than believed, paving the way for new and rational approaches to phage therapy.

Other scientists have since re-examined and expanded on Smith and Huggins’ studies. Rapid clearance of phages in vivo was originally deemed a negative aspect of phage therapy, but Merril et al. (1996) demonstrated that it is possible to select for phage variants that are long circulating in blood. Following Smith and Huggins favorable results in phage treatment of mice infected with E. coli, Soothill et al. (1992) demonstrated efficacy of phage treatment in mice infected with either Pseudomonas aeruginosa or Acinetobacter baumannii. In 2002, Bull and Levin et al. revisited Smith and Huggins’ original experiments comparing the efficacy of K1 antigen targeting phages versus a non-K1 targeting phages against E. coli in mice. The K1-antigen targeting phage was observed to protect 100% of mice treated immediately, while the non-K1 targeting phage resulted in 60% mortality. In a second experiment, phage therapy resulted in 9% mortality compared to streptomycin which resulted in 54% mortality. These data confirm Smith and Huggins results that K1-capsule targeting phages are more effective in treatment than non-K1 targeting phages or antibiotic treatment.
**Phage Therapy: A Renewed Approach**

The ability to characterize and test phages as antibacterial therapies has advanced immensely. The current era offers inexpensive whole-genome sequencing, automated technology for measuring the growth of microbes, and efficient high-throughput methods for screening hundreds or even thousands of samples simultaneously. Meanwhile, it is increasingly recognized that modern clinical trials should be carefully designed to be safer, more inclusive, and (if possible) to generate valuable data, compared to earlier attempts. An appropriately conducted phage therapy trial should be double-blinded and placebo-controlled with large diverse cohorts, and perhaps designed to generate relevant longitudinal data from clinical isolates. For example, researchers could conduct follow-up lab studies and whole-genome sequencing of phages and/or bacteria taken during treatment, to test a myriad of basic and clinical microbiology as well as evolutionary hypotheses. Also, our increased understanding of the human microbiome and its interactions with human immunology warrant closer investigation of possible phage and immune system interactions in clearing infections.

However, one obvious limitation to phage therapy is the inevitable evolution of phage resistance in bacteria. Modern approaches to phage therapy should both acknowledge and capitalize on this certainty. Evolutionary biology describes how genetic trade-offs should be widely observed in biological systems; organisms sometimes evolve one trait that improves fitness (a relative advantage in survival or reproduction), while simultaneously suffering reduced performance in another trait. Phage therapy would thus benefit from utilizing certain phages, which select for the target bacterial pathogen to suffer specific genetic trade-offs. In particular, if the proximate binding of a lytic phage is known to associate with a virulence factor or mechanism for antibiotic resistance in the target bacteria, this should exert strong selection for the
bacteria to mutate or down regulate the phage-binding target(s). This approach should be especially useful in the case of opportunistic bacterial pathogens, because the bacteria could evolve reduced virulence or antibiotic resistance and still thrive in a different ecological setting (e.g., soil) as opposed to ‘arms-race’ selection for escalating virulence in an obligate pathogen such as in response to vaccine pressure (e.g., Marek’s Disease virus in chickens 50). Thus, this approach to phage therapy should be doubly effective; success is achieved when phages lyse the target bacterium, but also when bacteria evolve phage resistance because they suffer reduced virulence or increased sensitivity to antibiotics. In the following sections we return to this paradigm of phage-imposed genetic trade-offs.

A phage that requires a virulence factor to attach to and infect a bacterium may select against the expression of that virulence factor (Figure 1.3). Selection against virulence factors could be multiply effective as some virulence factors such as capsules have been shown to hide antigenic sites 51, provide some degree of antibiotic resistance 52, and prevent phagocytosis by macrophages 51. Phages that use components of LPS as receptors select against the expression of these components typically resulting in ‘rough’ colony-forming mutants through phase variable expression of LPS, point mutations or even large chromosomal deletions in LPS biosynthesis genes 53-57. Bacterial mutants that evolve resistance to LPS targeting phages are typically have both reduced fitness and virulence 58. Selection against other virulence factors that can serve as phage receptors such as adhesins, pili or secretion systems could prevent bacterial attachment and invasion of epithelial cells 59-62.

Similarly, phages that attach to an antibiotic efflux pump to infect may select against the expression of the efflux pump, rendering the bacteria more sensitive to antibiotics that were previously effluxed (Figure 1.3). For example, phage TLS selected for \textit{tolC} and \textit{rfa} mutants in \textit{E. coli}. 


coli at a typical frequency of $10^{-5}$ to $10^{-6}$ \cite{63}. The TLS-resistant mutants with altered TolC were hyper-sensitive to novobiocin. Additionally, when phage-resistant mutants were selected in the presence of novobiocin, the frequency of recovered mutants decreased 1000-fold. More recently, it was demonstrated that phage OMKO1 associates with the outer membrane protein M (OprM) of MexAB- and Mex-XY-OprM efflux pumps of the opportunistic pathogen *P. aeruginosa* \cite{64}. This interaction selects for phage-resistant mutants that are sensitive to antibiotics, as a ‘genetic trade-off’. Chan *et al.* demonstrated that phage-resistant mutants, in both lab strains and clinical isolates of *P. aeruginosa*, were more sensitive to antibiotics including ceftazidime \cite{64}. This was likely due to mutations or deletions in the operon encoding for the multidrug efflux pump resulting in nonfunctional gene products. Hypothetically, this promising result might also occur in other bacterial pathogens with similar modes of achieving broad antibiotic resistance via homologous or convergent efflux pump mechanisms. Overall, thoughtful consideration of the inevitable evolution of phage resistance during treatment could greatly benefit phage therapy efforts.

**Animal Models for Efficacy**

Animal studies can help bridge the gap between *in vitro* studies and actual clinical application of phage therapy. Unfortunately, most animal models investigate acute infections, which may not be the ideal analog for phage therapy targeting chronic infections in humans. Many of these studies observe best results when phages are applied simultaneously with the bacterial challenge, which will not necessarily be applicable in the clinic. In many cases, no measures were taken to check for the *in vivo* evolution of phage resistance by bacteria. Also, the comparison of phage treatment to antibiotic treatment or even a combination of phage and antibiotic treatments are only beginning to be investigated in animal models. Nevertheless, animal models provide vitally useful data on
efficacy and safety of phage therapy in living hosts, and are crucial for further development of the approach.

**Systemic Infections**

Several studies have investigated the efficacy of phage therapy for treatment of systemic infections. In a gut-derived model of *P. aeruginosa* sepsis, Watanabe *et al.* (2007) observed 67% survival of infected mice when phage therapy was administered orally one day post infection. Capparelli *et al.* (2007) observed that successful protection of mice with a systemic *Staphylococcus aureus* infection depended on phage dose; Biswas *et al.* (2002) observed similar results of dose dependent success in a mouse model of vancomycin-resistant *Enterococcus faecium* bacteremia. In a systemic disease model of *Vibrio vulnificus*, successful control of disease was only achieved when bacterial infection and phage treatment were administered simultaneously.

The determinants of success for phage therapy to treat systemic infections are likely dependent on multiple factors which need to be thoroughly examined prior to the widespread use of phages as a treatment for sepsis in humans.

**Local Infections**

Phage therapy for localized infections (e.g., otitis, urinary tract infections, infected burns) is recognized for its potential to entirely circumvent the use of chemical antibiotics. Furthermore, use of chemical antibiotics for surgical and hospital acquired infections is limited as these often constitute the strains with greatest antibiotic resistance. Watanabe *et al.* (2007) observed 92% survival of mice with an intraperitoneal *P. aeruginosa* infection treated simultaneously with phages. A similar study of *S. aureus* abscesses in mice by Capparelli *et al.* (2007) enumerated the reduction in bacterial load resulting from phage therapy, and observed that phages applied concurrently with bacteria prevented the formation of abscesses. When administered four days
after bacterial challenge, a single dose of phages resulted in a 100-fold reduction in bacterial load, whereas multiple doses of phages resulted in a 10,000-fold reduction. In a mouse model of *P. aeruginosa* infection of burn wounds, phage treatment improved survival rate from 6% in the untreated controls to 88% when phages were administered via intraperitoneal injection 72 hours post infection. In contrast, phage treatment only resulted in 22% or 28% survival when administered subcutaneously or intramuscularly. Further pharmacokinetic studies demonstrated that phages delivered intraperitoneally persisted at higher levels in the liver, spleen and blood than phages delivered intramuscularly or subcutaneously. Finally, a murine model was used to investigate the ability of phages to treat an *E. coli* urinary tract infection. Phages administered intraperitoneally 24 hours after bacterial challenge resulted in a 100-fold reduction in bacterial load in the kidneys 48 hours after phage treatment. The same phages resulted in a significant reduction in bacterial load in an *E. coli* pneumonia model, but was ineffective in an *E. coli* model of sepsis.

**Gastrointestinal Infections**

Applying phage therapy to gastrointestinal bacterial infections could potentially reduce or prevent colonization of virulent bacteria without disrupting the natural gut flora. Galtier et al. (2017) observed that a preventative treatment of phages, four days after an adherent-invasive *E. coli* challenge, was able to reduce bacterial colonization in the gut of dextran sodium sulfate treated mice and prevented the progression of colitis symptoms. In an insect model of *Clostridium difficile* colonization, prophylactic treatment with phages two hours prior to bacterial challenge resulted in 100% survival, while simultaneous administration of phages and bacteria resulted in 72% survival, and phage administration two hours post bacterial challenge resulted in 30% survival. Yen et al. (2017) observed that prophylactic treatment with a phage cocktail was able
to reduce *V. cholerae* colonization in the small intestine of infant mice when phages were provided three and six hours prior to bacterial challenge. However, phage-resistant bacterial mutants were recovered after treatment, and effects of phage treatment were reduced when administered more than six hours before bacterial challenge and when mice were challenged with a higher dose of *V. cholerae*. While the result of prophylactic treatment of gastrointestinal infections with phages are generally favorable, more studies that provide treatment after bacterial challenge, such as Galtier et al. (2017), are needed as prophylactic treatment is not always possible in the clinic.

**Lung Infections**

Phage therapy for the treatment of lung infections, particularly chronic lung infections which are common in those with cystic fibrosis (CF), has seen renewed interest recently with the increase in MDR bacteria associated with the lung. Waters et al. (2017) observed complete eradication of a CF strain of *P. aeruginosa* in mice when two doses of phages were administered intranasally to infected mice 24/36 or 48/60 hours after infection. Treatment at 144/156 hours post infection resulted in complete eradication of infection in 70% of mice and a significant reduction in the remaining 30%. In another CF lung infection model, phage treatment significantly improved the survival rate of mice when administered intranasally at two hours post infection. Interestingly, a high dose of phages administered 4 days prior to bacterial challenge provided complete protection to mice, indicating that prophylactic treatment with phages could prevent chronic infections. Semler et al. (2014) investigated different routes of administration of phages in a mouse model of *Burkholderia cepacia* complex respiratory infection. A 100-fold decrease in bacterial load was observed when phages were administered via nebulization while no decrease was observed when administered via intraperitoneal injection. Promising results for both
prophylactic and curative treatment of lung infections with phages indicate that these types of infections may be a reliable target for effective phage therapy.

**Antibiotic and Phages in Combination**

While there have been many *in vivo* studies on the efficacy of phage therapy, not many recent studies have compared the *in vivo* efficacy of phage therapy to that of antibiotics or even combined phages and antibiotic treatment. Huff *et al.* (2004) investigated the efficacy of traditional antibiotics, phage treatment, or a combination of both in a head-to-head trial in an *E. coli* challenge in broiler chickens. The standard of care treatment, enrofloxacin (fluoroquinolone), reduced mortality from 68% in untreated birds to 3%, while phage treatment alone reduced mortality to 15%. A combination therapy of phages and enrofloxacin resulted in no mortality. Similarly, Oechslin *et al.* (2016) observed that phages in combination with ciprofloxacin resulted in a 10,000-fold greater reduction in bacterial load as compared to phages or ciprofloxacin treatment alone in rats with experimental endocarditis due to *P. aeruginosa*. Furthermore, they noted that this particular combination of phages and antibiotics resulted in synergistic killing of *P. aeruginosa* both *in vitro* and *in vivo*. As the future of phage therapy will likely be that of combined therapy with chemical antibiotics, additional studies examining potential synergy between phages and antibiotics both *in vitro* and *in vivo* are needed.

Compared to phage therapy studies *in vivo* animal models, there have been relatively few reports on the clinical use of phages and even fewer controlled clinical trials. As summarized in Table 1.1, below we describe some notable case studies and clinical trials that have been performed; the lists are not exhaustive and other examples can be found in the literature.

**Case Reports of Emergency Phage Therapy**
Case 1: Pseudomonas Sepsis Case Report

A child with DiGeorge syndrome and congenital heart disease presented with *P. aeruginosa* bacteremia following multiple surgeries that included insertion of a pacemaker 81. Anti-pseudomonal antibiotics initially controlled the infection but ultimately failed. Adverse reactions to cephalosporins and fluoroquinolones further limited antibiotic options. Phages provided by the US Navy were screened for lytic activity against the infectious strain and a cocktail of two phages was created. After intravenous phage administration, blood cultures fluctuated between positive for *P. aeruginosa* and below the limit of detection for several days. Phage therapy was resumed on day 11, following a temporary cessation due to decompensation attributed to progressive heart failure, which coincided with four days of blood cultures negative for *P. aeruginosa*. In this case, phage therapy appeared to reduce the infection in the blood, though was apparently ineffective at source control as blood cultures reverted to positive upon termination of therapy.

Case 2: Urinary Tract Infection Case Report

Khawaldeh et al. (2011) reported treating a *P. aeruginosa* urinary tract infection associated with a bilateral ureteral stent 82. Following cessation of antibiotic therapy, the infection consistently recurred within a week. Libraries of phages from the Eliava Institute were screened against the bacterial isolate and a suitable commercial phage product identified. This phage cocktail contained phages with activity against *Streptococcus pyogenes, S. aureus, E. coli, P. aeruginosa, Proteus vulgaris* and *Proteus mirabilis*. On day six of the treatment, antibiotic therapy with meropenem and colistin was initiated. Khawaldeh et al. (2011) reported 10-fold reduction of bacteria in the urine after five days of phage treatment. Two days of subsequent antibiotic treatment resulted in apparent clearance of the infection at which point culturable *P. aeruginosa* was below the limit of detection 82. Following completion of the 30-day course of meropenem, both stents were removed.
and one was replaced. Urine samples remained sterile for one year after treatment, at which point observations were concluded.

**Case 3: Surgical Site Infection Case Report**

LaVergne et al. (2018) reported a postoperative *A. baumannii* infection in an individual following a craniectomy. Strains of *A. baumannii* isolated from the infection were resistant to almost all antibiotics and antibiotic combination therapy. Phages provided by the US Navy were screened for lytic activity against the infectious strain and a cocktail of five phages was chosen for therapeutic use. Five minutes after phage administration, the concentration of phages in blood was approximately 100 PFU/mL. This was approximately 100-fold lower than expected if the phages were simply diluted into an average human blood volume 10 minutes after administration, phages were undetectable. Despite initial improvements, the lack of significant improvement led to withdrawal of care. Unfortunately, bacterial load was not measured during the course of treatment and it is difficult to attribute any clinical improvement to the administration of phages. It appears that in this case, phages were actively removed from the blood, either through adsorption to bacteria *in vivo*, or active removal by the body.

**Case 4: Pancreatitis Case Report**

Phage therapy was utilized in a case of necrotizing pancreatitis complicated by an MDR *A. baumannii* infected pseudocyst. Phages from several institutions were screened against the isolated infectious strains and two cocktails each consisting of four different phages were formulated. The first cocktail was administered via lavage at the site of the pancreatic pseudocyst. The following day, the second cocktail was administered intravenously. Resistance emerged against all of the phages used in both cocktails by eight days after the first administration of phages, suggesting phage selection occurred *in vivo*. A third phage cocktail was formulated that was active
against the resistant strains and was again administered intravenously. Resistance to the original cocktails correlated with increased presence of encapsulated bacteria, further suggesting bacterial response to phage administration. Phage therapy was continued for approximately 12 weeks, over the course of which clinical improvement was observed and the infection was eventually resolved.

**Case 5: Aortic Graft Infection Case Report**

Surgical intervention to repair an aortic aneurysm with a Dacron graft resulted in a *P. aeruginosa* infection that was refractory to standard treatment. This chronic infection resulted in the formation of an aorto-cutaneous fistula with purulent discharge. Infection control was attempted with intravenous ceftazidime followed by oral ciprofloxacin. Resistance to ciprofloxacin evolved during the course of treatment, debridement and irrigation were unsuccessful in resolving the infection, and surgical replacement of the graft was not an option. After three years of suppressive chemical antibiotic therapy which failed to eradicate the infection other options for infection control were considered. A recent report of *P. aeruginosa* phage OMKO1 that had demonstrated synergy when used in combination with ceftazidime was screened for lytic activity against the strain. Instillation of a single dose of phage OMKO1 and ceftazidime was applied topically at the site of fistular discharge while continuing the existing therapy of intravenous ceftazidime. Four weeks after the administration of phages, partial graft excision and replacement was required following bleeding from the fistula. Cultures taken at the time of surgery were negative for *P. aeruginosa* and the course of ceftazidime was discontinued. Two years after phage treatment, there was no recurrence of the infection in the absence of any antibiotic therapy. The favorable outcome of this case underscores the rational choice of phages and route of administration for this particular infection; thoughtful selection of a phage that had previously demonstrated synergism with the
clinically relevant antibiotics applied in proximity to the source of infection undoubtedly contributed to the positive outcome.

**Clinical Trials of Phage Therapy**

**Trial 1: Otitis Clinical Trial**

In 2009, a clinical trial was designed to investigate the safety and preliminary efficacy of phage therapy for treating chronic *P. aeruginosa* otitis \(^8^5\). Wright et al. (2009) utilized a cocktail of six phages with lytic activity against *P. aeruginosa* in individuals with chronic otitis \(^8^5\). While the authors report a significant accumulated reduction of bacterial counts in the phage treatment group and no significant accumulated change of bacterial counts in the placebo group, three individuals from each group had undetectable levels of *P. aeruginosa* by day 42. Phages were isolated for an average of 23 days from individuals in the phage treatment group suggesting that phages were either cleared upon resolution of the infection, phages were unable to reach the site of infection, or the bacteria became phage-resistant. There were no serious adverse events reported in either group, indicating the safety of phage therapy for the treatment of otitis.

**Trial 2: Diarrheal-Disease Clinical Trial**

A clinical trial was conducted in Bangladesh to test safety and efficacy of two different phage cocktails that target pathogenic *E. coli* in diarrheal diseases \(^8^6\). Individuals presenting with acute onset of dehydrating diarrhea were admitted to the study. Phage treatment consisted of oral administration of one of two different phage cocktails that had been previously characterized: a T4-like phage cocktail (T) containing 11 phages and a commercial phage cocktail (M) from Russia consisting of at least 17 different phages in oral rehydration solution. Standard treatment of oral rehydration solution was given to the placebo group. There were no adverse effects reported and no significant differences between the phage treatment and placebo groups. It was unclear if the
phages were lytic against the specific *E. coli* strains causing disease and no actions were taken to buffer the stomach prior to the administration of phages. It is possible, therefore, that significant numbers of phage particles were unable to survive the low-pH environment of the stomach, and that the surviving particles were unable to amplify due to the lack of an appropriate host.

**Trial 3: PhagoBurn Burn Wound Clinical Trial**

The most recent clinical trial to date, PhagoBurn, evaluated the safety and efficacy of phage therapy to treat *P. aeruginosa* infected burn wounds. A phage cocktail of 12 phages with lytic activity against *P. aeruginosa* were added to an alginate template that was applied directly to the wound. The control group received standard of care treatment which consisted of 1% sulfadiazine silver applied topically. The average time to sterilization for the phage treated group and control group was 144 hours and 47 hours, respectively. These unexpectedly poor results could be explained by administration of a lower phage dose than intended. The concentration of the phage cocktail had dropped over the course of the study and a dose of 200 to 2000 PFU was used instead of the expected $2 \times 10^7$ PFU dose. With concentrations of phages 4-5 orders of magnitude lower than expected it is possible, if not probable, that the lack of efficacy can be attributed to this unintended change in the treatment protocol.

**Perspectives and Future Directions**

**Will Phages Ever Replace Antibiotics?**

A rational approach to phage therapy has many potential advantages over a traditional chemical antibiotic approach (Table 1.2). (1) The non-lethal nature of some bacteriostatic antibiotics may permit antibiotic resistance to evolve more easily, as well as accommodate the emergence of persister cells that are genotypically equivalent to wild-type bacteria yet physiologically capable of withstanding antibiotic exposure. On the other hand, lytic phages are always bactericidal,
lysing cells at the completion of their replication cycle. Furthermore, phages (unlike antibiotics) hijack most essential cellular processes including DNA replication, transcription and translation upon infection and are perhaps harder targets for evolution of bacterial resistance. (2) The popular broad-spectrum antibiotics may disrupt the normal balance of the microbiome which might otherwise provide a protective effect by occupying niche sites that prevent or constrain bacterial pathogens from invading the body. (3) Phages can be specific to species and even single strains of bacteria, making them an ideal therapeutic to selectively target and kill pathogens. The clinical use of phages will likely require preliminary laboratory assays to identify sensitivity of strains to therapeutic phages. Phage sensitivity could be determined in parallel with antibiotic sensitivity to ensure a better match between the proposed drug (phage) and the target bacterial strain. Relatedly, this may explain why the above-described case reports have been generally more successful than clinical trials to date (see case studies and clinical trials summarized in Table 1.1). (4) In addition, while antibiotics must be continually dosed to clear infection, phages are able to amplify at the site of infection (suggesting fewer doses should be needed) and will be cleared from the body when the susceptible bacteria are gone. (5) Resistance to antimicrobials is inevitable. However, unlike antibiotic therapy, phage therapy can take advantage of this outcome via careful choice of therapeutic phages that select for resistant bacterial mutants with lower fitness, especially reduced virulence or impaired antibiotic efflux (see above section on Phage Therapy: A Renewed Approach). (6) Furthermore, as phages co-evolve with bacteria over time, it is possible that the administered phage population will evolve to infect the phage-resistant bacteria (an arms race), which is not possible for antibiotics. (7) Phage treatment of biofilms may prove more promising than antibiotic treatment of biofilms; however, this difference may depend on the target bacterium and the general benefits of using phages in treating biofilms merits further investigation.
Finally, while novel antibiotic discovery has stagnated in recent years, discovery of new phages has proven expeditious due to the vast biodiversity of phages in nature that have useful properties in biotechnology.

However, even with this more rational phage therapy approach, phages still have some limitations compared to traditional chemical antibiotics that need to be addressed before phage therapy can be fully accepted in modern clinical practice (Table 1.2). (1) Phages will likely not be an appropriate therapeutic for all infections. While some antibiotics are capable treating intracellular bacterial pathogens, phages do not have a reliable mechanism of entry into eukaryotic cells. (2) Also, there has been decades of research performed on antibiotics and their interactions with the immune system, whereas abundant analogous research with phages has yet to be completed. (3) Since phages can be found everywhere, including within the human microbiome, neutralizing antibodies against certain phages typically associated with humans may be a general obstacle for phage therapy. (4) Because a phage population can undergo rapid exponential growth, widespread lysis of target bacteria can potentially release bacterial antigens that could be dangerous, particularly if the phages are administered internally; thus, endotoxin removal from preparations of phage lysates intended for therapy, as well as generation of endotoxins during therapy are valid concerns. (5) Finally, regulatory hurdles represent a significant barrier to the implementation of phage therapy in modern medicine; unlike the well-established path to approval of antibiotics, this path is currently being paved for phages and therefore very little useful precedence exists.

Realistically, therapeutic use of phages may never completely replace administration of chemical antibiotics and may be inappropriate under some clinical conditions, suggesting that adjuvant approaches should be closely studied. A mixed therapy of phages and antibiotics could
be an ideal combination that capitalizes on each treatment’s differing strengths (Table 1.2). Bedi et al. (2009) observed an additive effect when phages and antibiotics were used to treat a Klebsiella pneumoniae biofilm. Knezevic et al. (2013) investigated the potential for phage-antibiotic synergism and observed synergy between P. aeruginosa phages and subinhibitory concentrations of ceftriaxone, but not with gentamicin, ciprofloxacin or polymyxin B. They proposed that the mechanism of action of the antibiotic must not interfere with critical processes in phage replication to see a synergistic effect. While not all phage and antibiotic combinations appear to be synergistic and the mechanisms behind synergism are still being explored, precision medicine is currently in vogue, and phage therapy shows promise as a ‘personalized’ approach for at least some infected individuals. As with any drug, the ideal circumstance is that phage therapy should be developed to reduce off-target effects and to minimize disruption of helpful microbiome communities to the extent possible.

Other Considerations for Phage Therapy

One method employed to expand phage host-range and subvert the criticism of narrow spectrum is to combine multiple phages to create phage cocktails. Traditionally, this has been perceived as a benefit, allowing the cocktail to be used against different strains or species of bacteria, and presumably decreasing the likelihood that mutations against all of the phages will simultaneously occur. Recent case studies show that in principle either a single phage or phage-cocktail approach might work (see Clinical Cases). However, a conceivable drawback of phage cocktails is their ability to select for “broad-spectrum” mechanisms of phage resistance, such as the production of a capsule that surrounds the cell, preventing phage binding. For these reasons, a rational approach to designing cocktails is warranted, involving consideration of: mechanism(s) whereby phage resistance can evolve; potential for bacteria to develop cross-resistance to multiple phages; and
confirmation that the various phages in a cocktail do not compete with one another to reduce the overall efficacy. At the least, we see relevance for a more dedicated merger between phage-cocktail formulation and core principles and theories of evolution and ecological competition.

A related subject is the mode of delivery for phages in therapy, and whether this choice would impact relative ratios of phages to bacteria at the site(s) of infection, and/or ratios among phages in a cocktail as they are administered versus the times when they actually encounter the infecting bacteria. The self-amplifying nature and lethality of lytic phages suggest that this therapy should often avoid the analogous consequence of bacterial evolution of resistance to chemical antibiotics delivered at too low of concentration. However, the density of phage particles relative to target bacterial cells may greatly impact timing and quality of the phage therapy outcome, and mode of delivery necessarily affects these key ratios. Therefore, the route of administration for phage therapy should consider the most likely method to deliver the highest concentration of phage particles to the site of infection, and therapeutic approaches might consider low initial doses of phages that are adjusted over time. Phage studies have not focused very closely on the therapy benefits versus costs of changes in the multiplicity of infection (ratio of phage particles to target susceptible bacterial cells) over time, either via administration or by estimating changes within the treated human or animal model; this seems like a relevant focus for phage therapy research moving forward.

**Summary**

Renewed interest in phage therapy in the West, and its continued development in countries such as Poland, Russia, and Georgia, mark a time for optimism for a viable alternative (or adjunct) to antibiotic therapy. Phage therapy like any medical treatment has benefits, costs and limitations in usefulness that merit close scrutiny. Overall, we can identify several intriguing questions and
topics that should be addressed, especially as greater numbers of clinical trials on phage therapy are planned and executed. Is it possible to discover or engineer individual phage strains that broadly infect genotypes of a target pathogen, meriting their approval as standalone ‘drugs’? Or, would additional experiments on phage cocktails provide convincing evidence that phage mixtures should be the standard of care? Bioprospecting for phages is likely to continue yielding candidates with useful biologically properties, such as ability to select for reduced virulence and re-sensitization of bacteria to antibiotics. But what is the probability that bacteria can evolve simultaneous resistance to both phages and antibiotics at no cost? How can the process of bioprospecting for phages be made more efficient? Could developing genomics and bioinformatics analyses as well as computer algorithms help identify potential phage candidates and predict phage-binding to cell receptors, strictly through high-throughput sequencing? Would reliable models such as non-pathogenic *E. coli* yield sufficiently useful data to predict utility of phage therapy in pathogens that are much harder to culture in the laboratory or where we lack animal models for acute and chronic bacterial diseases? How is phage therapy observed or theoretically predicted to interact with the immune system and human microbiomes, to either enhance or suppress this approach in resolving systemic and biofilm infections? How would these interactions differ in sites as different as the respiratory and gastrointestinal systems? This non-exhaustive list highlights many hypotheses regarding phage therapy that should be the focus of future basic research. Fortunately, this is an opportune time for basic researchers, clinicians, and physicians to work together to address open questions through rigorous experiments in the laboratory, *in vivo* models and clinical cases to reward phage therapy with the renewed interest and greater examination that it deserves.
Figure 1.1 Lytic phage infection cycle

A cycle of lytic phage replication begins when the virus recognizes and irreversibly binds to a receptor (protein or sugar) on the surface of a bacterial cell. The phage delivers its genomic content into the cytoplasm of the bacterial cell. Typically, host resources, including proteins and genomes are repurposed to fuel phage replication. Replication, transcription and translation of the phage genome begins usually through redirecting host metabolism to the production of new phage particles. Upon assembly of new phage particles, lysis of the bacterial cell allows newly replicated phage particles to escape the cytoplasm and go on to infect other phage-sensitive bacterial cells.
Phage encode binding proteins that recognize and attach to sites on the surface of a bacterial cell. Many phage bind to protein structures on the bacteria such as pili (depicted in red)\textsuperscript{22}, flagella (yellow)\textsuperscript{60}, porins (blue)\textsuperscript{19} or efflux pumps (purple)\textsuperscript{64}. Phage have also been reported to bind to specific sugar moieties in LPS (green)\textsuperscript{22}.
Certain lytic phage may be more effective in phage therapy, because they kill target bacteria while simultaneously imposing strong selection against bacterial virulence or antibiotic resistance when bacteria mutate to avoid phage attack. Phage that use antibiotic efflux pumps as receptors (red) can select for phage-resistant bacterial mutants with impaired efflux pumps; these phage-resistant bacterial mutants are more sensitive to antibiotics. Phage that bind to structural virulence factors such as a capsular antigen (purple) can select for phage-resistant bacterial mutants that lack the capsule; these non-capsulated phage-resistant mutants are less virulent bacteria because they are more easily engulfed by phagocytic cells.
### Table 1.1 Case Reports and Clinical Trials

<table>
<thead>
<tr>
<th>Case 1</th>
<th>Pseudomonas aeruginosa bacteremia</th>
<th>DiGeorge syndrome and congenital heart disease with pacemaker</th>
<th>MEM, TOB, ATM, PMB and CST</th>
<th>MEM, TOB, ATM, PMB, CST</th>
<th>3.5 × 10^5 PFU delivered intravenously every six hours</th>
<th>Initial PT for 36 hours (6 doses total), PT resumed 11 days later</th>
<th>Blood cultures negative after PT but reverted to positive upon cessation of PT</th>
</tr>
</thead>
<tbody>
<tr>
<td>Case 2</td>
<td>Pseudomonas aeruginosa urinary tract infection (2 years)</td>
<td>Intra-abdominal resection and irradiation for adenocarcinoma, bilateral ureteral stent placement</td>
<td>GEN, CAZ, CIP and MEM</td>
<td>None reported</td>
<td>2 × 10^7 PFU directly instilled into the bladder every 12 hours</td>
<td>10 days (MEM and CST treatment initiated on day 6)</td>
<td>Urine samples sterile following a 30-day course of MEM</td>
</tr>
<tr>
<td>Case 3</td>
<td>Acinetobacter baumanii surgical site infection</td>
<td>Craniectomy</td>
<td>Combination of CST, AZM and RIF</td>
<td>Intermediate sensitivity to CST with resistance to all other tested antibiotics</td>
<td>8.56 × 10^7 PFU delivered intravenously every two hours</td>
<td>8 days (98 doses total)</td>
<td>Initial improvements observed; bacterial load not measured</td>
</tr>
<tr>
<td>Case 4</td>
<td>Acinetobacter baumanii infected pseudocyst (3 months)</td>
<td>Necrotizing pancreatitis</td>
<td>AZM, CST, RIF</td>
<td>Cephalosporins, MEM, GEN, AMK, SXT, TET, CIP and CST</td>
<td>5 × 10^9 PFU delivered intravenously every six hours</td>
<td>84 days (336 doses total), MIN added on day 2</td>
<td>Clinical improvement and resolution of infection</td>
</tr>
<tr>
<td>Case 5</td>
<td>Pseudomonas aeruginosa infected aortic graft (3 years)</td>
<td>Aorto-cutaneous fistula</td>
<td>CAZ and CIP</td>
<td>CIP</td>
<td>1 × 10^8 PFU delivered topically on fistula</td>
<td>Single dose</td>
<td>Cultures negative at four weeks post treatment</td>
</tr>
</tbody>
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<table>
<thead>
<tr>
<th>Clinical Trials</th>
<th>Infection</th>
<th>Trial</th>
<th>Treatment group</th>
<th>Placebo group</th>
<th>Phage Dose and Application</th>
<th>Outcome</th>
</tr>
</thead>
<tbody>
<tr>
<td>Trial 1</td>
<td>Pseudomonas aeruginosa otitis</td>
<td>Placebo-controlled, double blind for safety and efficacy</td>
<td>12 individuals received phage cocktail</td>
<td>12 individuals received single dose of glycerol-PBS buffer</td>
<td>10^9 PFU delivered intra aurally (single dose)</td>
<td>Three patients from each group had undetectable levels of P. aeruginosa at the end of the trial</td>
</tr>
</tbody>
</table>

Details and summaries are provided for each of the case reports and clinical trials discussed in the main text.
**Table 1.1 Continued**

![Table 1.1](image)

- **Trial 2**
  - *Escherichia coli* diarrheal diseases
  - Placebo-controlled, double blind for safety and efficacy
  - 40 individuals received phage cocktail M, 39 individuals received phage cocktail T
  - 41 individuals received oral rehydration solution
  - 1.4 × 10⁹ PFU cocktail M or 3.6 × 10⁸ PFU cocktail T delivered orally in oral rehydration solution 3 times per day for 4 days (12 doses total)
  - No significant difference between PT group and placebo group

- **Trial 3**
  - *Pseudomonas aeruginosa* burn wound infection
  - Placebo-controlled, blinded trial for safety and efficacy
  - 12 individuals received a phage cocktail
  - 13 individuals received standard of care 1% sulfadiazine silver
  - 2 × 10⁷ PFU (expected) 200-2000 PFU (actual) applied topically 1 time per day for 7 days (7 doses)
  - Trial was stopped early due to insufficient efficacy of PT as compared to the standard of care (placebo) treatment. This was likely due to significantly lower applied dose of phage than expected.

MEM, meropenem; TOB, tobramycin; ATM, aztreonam; PMB, polymyxin B; CST, colistin; GEN, gentamicin; CAZ, ceftazidime; AZM, azithromycin; RIF, rifampin; AMK, amikacin; MIN, minocycline; SXT, trimethoprim-sulfamethoxazole; TET, tetracycline; CIP, ciprofloxacin; PT, phage treatment
A rational approach to phage therapy has many potential benefits that cannot be achieved with antibiotics alone. However, there are also limitations to phage therapy, in comparison to traditional antibiotics. While many of these differences historically have been considered limitations to using phage therapy, in some circumstances the perceived drawbacks may instead be leveraged as benefits. Both types of therapies historically have been administered alone; however, with many identified differences, a combination approach utilizing both therapies may prove to be the most efficacious in the long run.

**Table 1.2 Comparing and contrasting antibiotics and phage**

<table>
<thead>
<tr>
<th>Activity &amp; Mechanism of Action</th>
<th>Lytic (‘Virulent’) Phage</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bacteriostatic or bacteriacidal</td>
<td>Bactericidal</td>
</tr>
<tr>
<td>Typically disrupts ONE bacterial process</td>
<td>Disrupts MANY/ALL bacterial processes</td>
</tr>
<tr>
<td>Broad-spectrum more common than narrow-spectrum</td>
<td>High degree of species or strain specificity</td>
</tr>
<tr>
<td>Disruption of microbiome</td>
<td>Only disrupts target bacteria</td>
</tr>
<tr>
<td>Not very effective against biofilms</td>
<td>Penetration and destruction of biofilms</td>
</tr>
</tbody>
</table>

**Clinical Use**

<table>
<thead>
<tr>
<th>Minimal identification of bacteria</th>
<th>Phage tested against target bacteria</th>
</tr>
</thead>
<tbody>
<tr>
<td>Short time between diagnosis and treatment</td>
<td>Longer time between diagnosis and treatment</td>
</tr>
<tr>
<td>Constant dosing to maintain inhibitory concentrations</td>
<td>Self-amplifying while target bacteria are present</td>
</tr>
<tr>
<td>Potential for immune recognition</td>
<td>Potential for immune recognition</td>
</tr>
<tr>
<td>Widely accepted; used as treatment for infections</td>
<td>Push back on clinical application; only used on a compassionate care basis</td>
</tr>
<tr>
<td>Diffusion through membranes allows for treatment of intracellular bacteria</td>
<td>Unable to penetrate eukaryotic cells</td>
</tr>
</tbody>
</table>

**Discovery, production & manufacturing**

<table>
<thead>
<tr>
<th>Slow discovery process</th>
<th>Rapid discovery process</th>
</tr>
</thead>
<tbody>
<tr>
<td>Production methods in place to ensure safety of products</td>
<td>Steps must be taken to ensure removal of contaminating bacterial antigens generated during production</td>
</tr>
<tr>
<td>Regulations for production &amp; manufacturing in place</td>
<td>Require new regulations for production &amp; manufacturing</td>
</tr>
</tbody>
</table>

**Resistance**

<table>
<thead>
<tr>
<th>Resistance inevitable</th>
<th>Resistance inevitable but phage can coevolve to infect resistant bacteria</th>
</tr>
</thead>
<tbody>
<tr>
<td>Resistance frequently accompanied by compensatory mutations</td>
<td>Resistant mutants may result in lower fitness via reduced virulence or antibiotic sensitivity</td>
</tr>
</tbody>
</table>
Chapter 2: High-throughput discovery of phage receptors using transposon insertion sequencing of bacteria
Preface

Chapter 2 has been adapted from an article that is published: Kortright, K. E., Chan, B. K., & Turner, P. E. (2020). High-throughput discovery of phage receptors using transposon insertion sequencing of bacteria. Proceedings of the National Academy of Sciences, 117(31), 18670-18679. The study was designed by Kaitlyn Kortright, Dr. Paul Turner and Dr. Benjamin Chan. Kaitlyn Kortright preformed all experiments and analyzed data. The manuscript was prepared and edited by Kaitlyn Kortright, Dr. Paul Turner and Dr. Benjamin Chan.

Raw sequencing is publicly available at NCBI Sequence Read Archive Project # PRJNA637562 and supplemental datasets have been deposited on Dryad (doi:10.5061/dryad.t76hdr7z3). We thank J. Wertz for strains; A. Goodman, B. Kazmierczak, S. Shames and D. Weinberger for valuable discussions regarding this study; and J. Bull and A. Hatoum-Aslan for helpful comments on the manuscript.
Abstract

As the most abundant microbes on earth, novel bacteriophage (phage; bacteria-specific viruses) are readily isolated from environmental samples. However, it remains challenging to characterize phage-bacteria interactions, such as the host receptor(s) phage bind to initiate infection. Here, we tested whether transposon insertion sequencing, INSeq, could be used to identify bacterial genes involved in phage binding. As proof of concept, results showed that INSeq screens successfully identified genes encoding known receptors for previously characterized viruses of *Escherichia coli* (phage T6, T2, T4 and T7). INSeq screens were then used to identify genes involved during infection of six newly isolated coliphage. Results showed that candidate receptors could be successfully identified for the majority (five of six) of the phage; furthermore, genes encoding the phage receptor(s) were the top hit(s) in the analyses of the successful screens. INSeq screens provide a generally useful method for high-throughput discovery of phage receptors. We discuss limitations of our approach when examining uncharacterized phage, as well as usefulness of the method for exploring the evolution of broad versus narrow use of cellular receptors among phage in the biosphere.

Chapter Introduction

At an estimated $10^{31}$ particles, the number of bacteriophage (phage; bacteria-specific viruses) in the global biosphere comprises a vast and uninvestigated microbiological diversity. This largely untapped “bioprospecting” resource allows for newly discovered, naturally-occurring phage, to be developed for numerous applications. In stark contrast to this biodiversity are the relatively few phages that have been well-characterized (e.g., lambda, ΦX174, T4, T7, Φ6). These well-studied phages have been valuable for elucidating fundamental aspects of molecular biology and evolutionary genetics. However, there is increasing interest in utilizing phage for a myriad of
purposes, including alleviating current medical challenges posed by antibacterial resistance, exemplified by recent cases where phages were used to treat antibiotic-resistant bacterial infections in humans\textsuperscript{13,99}. It is therefore crucial to design approaches whereby newly discovered phage are characterized prior to any intended applications.

Isolating phage strains from environmental sources can be straightforward, though uncovering detailed characteristics of phage biology can be challenging. Sequencing approaches and molecular techniques are increasingly affordable, allowing for easier determination of genome size and nucleic acid content (DNA vs. RNA; single- vs. double-stranded) used to classify newly discovered phages into known or proposed virus families\textsuperscript{100}. Electron microscopy is typically sufficient to describe basics of phage morphology and capsid structure\textsuperscript{101}. Classic laboratory methods are useful for estimating host range (infectability across bacterial genotypes or species) and to determine whether a phage has a temperate versus strictly lytic replication cycle\textsuperscript{100}. However, other details of phage-bacteria interactions can be more difficult to ascertain. In particular, identifying the specific cell-surface receptor(s) that a phage uses in host binding, and the bacterial genes that are involved in resistance to phage infection are not as readily determined using the above methods.

To initiate an infection, a phage particle must first recognize and bind one or more receptors on the surface of a susceptible bacterial cell. Many of the phage specific to Gram-positive bacteria use carbohydrate moieties of peptidoglycan or techoic acid as their receptors\textsuperscript{102}. Whereas others, such as phage $\gamma$ which infects \textit{Bacillus anthracis} and phage SPP1 that infects \textit{Bacillus subtilis}, use protein receptors GamR and YueB, respectively\textsuperscript{61,103}. Thus far, receptors used by phage of Gram-negative bacterial hosts appear limited to specific proteins and/or lipopolysaccharide (LPS) moieties present on the outer-cell membrane\textsuperscript{104-111}. Overall, these binding targets are often highly-
conserved structures of bacteria, indicating that phage have been evolutionarily selected to exploit essential features of their hosts. In the simplest cases, a phage may use a single outer-membrane protein as a receptor, such as phage T6 attachment to the Tsx nucleoside channel of *Escherichia coli*. Other phage bind to a specific LPS motif, as seen in several members of the *Podoviridae* virus family. In further examples, phage use a primary receptor to facilitate attachment but require a secondary receptor to irreversibly bind to a host cell. Finally, phage such as T2 can use two or more receptors on the surface of a bacteria interchangeably to bind and initiate infection.

Elucidating molecular details of phage binding is fundamental to understanding phage biology because the receptors confer host-specificity of a virus, by allowing phage to differentiate between species or strains of bacteria. Nevertheless, it is challenging to determine phage receptors, especially when host bacteria themselves are not well characterized. Previously, knock-out libraries such as the Keio collection (single-gene deletion mutants of *E. coli* K-12) have been utilized to identify specific bacterial genes involved in phage T7 infection, whereas similar resources do not exist for other non-model bacteria. Even in the case of *E. coli*, however, such methods can be laborious, involving the screening of individual phage samples on very large numbers of mutant bacterial strains if genes for phage receptors are unknown. Transposon mutant libraries have been used to identify bacterial mutants that are resistant to phage infection. While this method requires relatively less effort, it is limited by the number of transposon mutants chosen for follow-up study, making it potentially challenging to identify the receptor(s) used by a single phage. One other common method to identify possible phage receptors is to characterize spontaneous phage-resistant mutants of bacteria. However, this approach typically necessitates comparing whole-genome sequences of phage-resistant mutants to a well-annotated reference.
genome to identify candidate mutations, which are not guaranteed to be in genes encoding phage receptors.

Advances in high-throughput sequencing have facilitated the development of new sequencing-based transposon mutant screens, which enable the rapid identification of genes that contribute to bacterial fitness in a particular selective environment\textsuperscript{118,119}. This method makes use of a modified transposon to create a diverse transposon mutant library, where each bacterial genome contains a single randomly- incorporated insertion mutation. The sites of transposition in the mutant population can be easily identified by sequencing, and the relative abundances of specific insertions can be quantified. Comparison of the relative abundances of transposon mutants pre- and post- selection can be used to identify genes contributing to fitness (bacterial growth) under the selective environment of interest.

Here, we describe a novel high-throughput method to identify phage receptors by querying the entire repertoire of bacterial genes that are critical for survival during selection by lytic phage (Figure 2.1A). The disruption of a gene involved in phage resistance would result in a fitness disadvantage under the selective pressure of phage; consequently, these mutants would be represented at a lower frequency in the output transposon mutant pool (TMP) (Figure 2.1B). Conversely, disrupting a bacterial gene required for phage infection and replication (e.g. genes encoding phage receptors), would provide a fitness advantage under the selective pressure of phage and these mutants should be enriched in the output TMP.

We hypothesized that this approach could successfully identify bacterial genes responsible for receptor-binding in four well-characterized viruses capable of infecting \textit{E. coli} K12: phages T2, T4, T6 and T7. Results of our high-throughput method agreed with the published literature in all cases. We further verified these conclusions using classic adsorption (phage attachment) assays,
demonstrating that phages fail to attach to bacterial knockout mutants lacking the gene(s) encoding phage receptor(s). We then tested our second hypothesis that this method could be used to identify receptor-binding genes in each of six newly discovered and previously uncharacterized phage, similarly capable of infecting \emph{E. coli} K12. Our approach, along with confirmatory adsorption assays, was successful in discovering the binding targets for five out of six of these viruses, demonstrating both the general utility of the method as well as the enduring challenge of developing an infallible technique for discerning phage receptors in bacteria.

**Results**

**INSeq transposon mutant library covers 80% of non-essential genes**

The final pooled transposon mutant library contained 17,100 independent transposon insertions in 3,253 bacterial genes. Visual inspection of results (Figure 2.2) showed genome coverage did not appear to be affected by the inherent GC bias of \emph{mariner} transposons \footref{120}. However, there was an increased number of transposition events near the \emph{E. coli} K12 origin of replication (Figure 2.2). This result likely was attributed to multiple copies of the origin present during DNA replication \footref{120}. While the final library was not completely saturated (i.e., not every non-essential gene was represented by a transposon mutant), approximately 80\% of non-essential \emph{E. coli} genes had at least one independent transposon insertion event in the final transposon mutant library. We used EcoCyc \footref{121} to compile a list of 137 genes expressed at the cell outer membrane (Table 2.1); of these genes, 31 were not represented in the TMP used for our screens.

The INSeq screen was initiated with a multiplicity of infection (MOI; ratio of phage to bacteria) roughly equaling 0.01 (see Methods). We noted that a lower MOI could allow for the occurrence of additional spontaneous (non-transposon-insertion) mutations that could experience subsequent positive selection in the phage environment. Nevertheless, low MOI was essential to
allow the TMP to undergo multiple rounds of cell division under phage selection, which was necessary for transposon mutant enrichment in the designed INSeq screen (Figure 2.1). Furthermore, we reasoned that the reduced initial selective pressure at low MOI would allow us to observe transposon-disrupted genes with subtle (i.e., low to moderate) fitness effects.

**INSeq identifies receptors for phages T6, T2, T4 and T7**

As a proof of concept for our method, we tested whether an INSeq screen could accurately identify the bacterial receptors for four well-characterized phages: T6, T2, T4 and T7. Controls consisted of INSeq screens using ‘mock infections’ that were performed identically, but in the absence of test phage. Results (Figure 2.3 A-D) were plotted to depict all insertions in bacterial genes on a logarithmic scale, as the ratio of input normalized (to counts per million, CPM) reads to output normalized reads in CPM. As compared to the mock infected control (Supporting Information Appendix: Figure S2.1 A), each screen in the presence of a test phage resulted in an observed shift of the TMP with many transposon mutants becoming under-represented in the output population. This result was consistent with phage imposing strong selection pressure on the TMP. As the entire TMP shifts towards under-representation in the output population due to declining abundance of bacteria, it becomes difficult to distinguish truly significant hits that are at a fitness disadvantage in the presence of phage. However, this does not impair our ability to identify genes encoding phage receptors, as mutants in these genes should become over-represented in the output population. Therefore, candidate receptor hits were considered if they were statistically significant, had a positive log ratio, A, of output (A_o) to input (A_i) relative abundance (A = \log(A_o/A_i)), and were genes encoding a membrane protein (Figure 2.3 E).

The screen in the presence of phage T6 (Figure 2.3 A) yielded 144 significant hits (Chapter 2 Appendix: Supplemental Data 1), of which 59 were positively enriched in the presence of phage.
T6. The log ratio of output to input relative abundance (A) of a subset of the TMP, transposon mutants in genes encoding membrane proteins (Figure 2.3 E), were queried for candidate receptors. Transposon mutant \( tsx::\text{Tn} \) (A = 3.09), was the top hit of these screens. This result was consistent with previous observations of phage T6 using the Tsx nucleoside channel as a primary receptor. Eight other genes encoding membrane proteins were enriched in the output: \( lamB::\text{Tn} \) (A = 1.84), \( btuB::\text{Tn} \) (A = 1.94), \( ompX::\text{Tn} \) (A = 0.90), \( rhsD::\text{Tn} \) (A = 0.55), \( ybgQ::\text{Tn} \) (A = 0.36), \( ynfB::\text{Tn} \) (A = 0.22), \( ompW::\text{Tn} \) (A = 1.08), and \( pgaB::\text{Tn} \) (A = 0.68). However, A-values of these hits were far lower than that of \( tsx::\text{Tn} \), indicating that in future screens, only the hits with the highest A-values should be considered as candidate receptors. Nonetheless, a subset of these other eight hits were assayed in further experiments to demonstrate that these genes were not involved in the binding of phage T6 (see below).

The phage T2 screen (Figure 2.3 B) resulted in 143 significant hits, of which 86 (Chapter 2 Appendix: Supplemental Data 1) were positively enriched in the presence of phage T2. Candidate receptors \( fadL::\text{Tn} \) (A = 2.60) and \( ompF::\text{Tn} \) (A = 2.53) were identified (Figure 2.3 E) as the top two hits of these screens. This was a key result, as it was unclear if our approach could successfully identify both receptors used by a phage when the presence of only one was necessary and sufficient for phage adsorption. Thus, these results were consistent with previous observations of OmpF and FadL as interchangeable receptors for phage T2. There were two other enriched hits in genes encoding membrane proteins: \( rhsD::\text{Tn} \) (A = 1.00) and \( ynfB::\text{Tn} \) (A = 0.55). These two hits also came up in the screen with phage T6, indicating that they might be commonly required for phage infection. However, as above, their A-values were far below those of \( fadL::\text{Tn} \) and \( ompF::\text{Tn} \), and neither were considered for follow-up validation.
The phage T4 screen (Figure 2.3 C) resulted in 177 significant hits, of which only 20 (Chapter 2 Appendix: Supplemental Data 1) were positively enriched in the presence of phage T4. The top hit (Figure 2.3 E) was \textit{ompC::Tn} (A = 2.35); additionally \textit{envZ::Tn} was significantly enriched in the output TMP (Figure 2.2 C). EnvZ is part of a two-component regulatory system that increases the expression of OmpC\textsuperscript{123}. Two other identified hits (Figure 2.3 E) were \textit{yjbF::Tn} (A = 1.04) and \textit{yjbH::Tn} (A = 0.34). Another gene in the same operon, \textit{yjbE::Tn} (A=1.36), was significantly enriched in the output TMP. The Yjb operon is involved in production of extracellular polysaccharide and biofilm formation\textsuperscript{124}. Previous literature suggests that phage T4 is not spatially inhibited by extracellular matrixes and is instead able to associate with the surface of biofilms\textsuperscript{125}. While it is interesting that multiple genes in this operon are significantly enriched in the output, further investigations of the importance of phage T4 with products of the Yjb operon is reserved for future studies, as previous results have demonstrated that phage T4 uses both OmpC and LPS as co-receptors\textsuperscript{110}. When OmpC is present, phage T4 can infect regardless of the terminal sugar residue of LPS; however, in the absence of OmpC, phage T4 is able to attach to LPS chains with an exposed, terminal glucose residue\textsuperscript{110}. Exposure of this terminal glucose residue in LPS is conferred by a deletion of \textit{waaU}, a gene that was not included in the TMP. Validation assays, therefore, were conducted on the top hit from this screen, OmpC (see below).

The phage T7 screen (Figure 2.3 D) resulted in 188 significant hits, of which 44 (Chapter 2 Appendix: Supplemental Data 1) were enriched in the output TMP. A previous screen of the Keio collection identified 10 genes important for phage T7 infection; however, none of these single-gene deletions were sufficient to abolish infection\textsuperscript{114}. Phage T7 is predicted to use LPS, specifically lipid A or a keto-deoxyoctulosonate (KDO) sugar moiety, as a receptor\textsuperscript{126}; therefore, it is unlikely that deletion of a single bacterial gene would eliminate phage adsorption as many of
the genes involved in synthesis of the inner core regions of LPS are essential. Therefore, instead of querying the 44 significantly output-enriched hits from the INSeq screen for a specific gene, these hits were subjected to a gene ontology (GO) enrichment analysis. The results of GO Term analysis indicated that the significant, output-enriched transposon mutants from the phage T7 screen were enriched for 22 genes involved in cellular biosynthetic processes (pyrI, yjbH, mutT, accA, uvrD, mmuP, yaeI, mmuM, hisA, metG, yegS, carB, sufS, opgH, waaF, gmhB, trpS, waaG, yebK, speB, hldD, and dnaQ). Upon closer inspection, approximately one third of these genes appeared to be involved in LPS metabolism: yaeI, waaF, gmhB, waaG, hldD, and yegS. Phosphodiesterase, YaeI, is annotated as a protein involved in lipid A biosynthesis and YegS is an annotated lipid kinase; while waaF, gmhB, waaG, and hldD are four out of five genes that make up Functional Gene Cluster 91 which consists of genes involved in synthesis and polymerization of the core heptose region of LPS. This result was encouraging, as it was unclear if our screen would be successful in identifying LPS as a receptor due to the complex nature of LPS biosynthesis and the essentiality of many of the genes involved.

We note that false positives are expected to occur during our screen, especially since previous studies demonstrate that altering expression of one outer-membrane protein affects the expression of other outer-membrane proteins. While we observed some false positives (e.g., see phage T6 results above), identification of phage receptors is still expected to succeed with this method in combination with confirmatory assays described below. In each of the screens conducted with phages T2, T4 and T6, the known receptors were all the top hit(s) from the short list of candidate receptors. The use of LPS as a receptor, as is the case for phage T7, is likely to be successfully identified using GO Term with the overrepresented hits.
Efficiency of plating and adsorption assays confirm known receptors for characterized phages

To verify that the top identified hits in the INSeq analyses were genes important for phage infection, we conducted efficiency of plating (EOP) assays to measure phage ability to productively infect bacterial strains lacking expression of the candidate gene relative to productive growth on WT bacteria for a subset of the top hits. Since EOP does not directly measure phage attachment, adsorption assays were used to confirm that the putative genes were phage receptors for a subset of the top hits (Figure 2.4). We concluded that EOP and adsorption assays were successful in confirming that the top hit(s) from the screens with phage T6, T2, and T4 were the known receptor(s) (Chapter 2 Appendix: Supplemental Results). In addition, results from GO Term enrichment for phage T7 were confirmed by reduction in titers upon adsorption with LPS.

INSeq identifies candidate receptors for newly isolated phage

Next, uncharacterized phage were screened for receptors using our validated methods. Six phages (EC14, EC35, R3, P2, U115 and 8S) were chosen randomly from a large library of environmental phage isolates, and subjected to an INSeq screen for receptor identification. Prior to the current study, no traits for these phage strains were determined, other than their ability to infect E. coli K12. Screens with these six coliphage were performed as described above; hits that were statistically significant, had a positive log ratio of output to input relative abundance, and were in genes encoding membrane proteins were considered for further validation.

The phage R3 screen resulted in 161 significant hits (Supplemental Figure S2.3 A), of which, 67 (Chapter 2 Appendix: Supplemental Data 1) were enriched in the presence of phage R3. Only two hits (Figure 2.5) were identified, ompA::Tn (A = 3.44) and rhsD::Tn (A = 0.96). As the top hit, OmpA was considered the candidate receptor for phage R3.
The phage U115 screen resulted in 150 significant hits (Supplemental Figure S2.3 B), of which, 58 (Chapter 2 Appendix: Supplemental Data 1) were enriched in the presence of phage U115. The top hit (Figure 2.5) of these screens was tsx::Tn (A = 3.17). There were four other hits: lamB::Tn (A = 1.41), rhsD::Tn (A = 0.88), ompX::Tn (A = 0.45) and ybgQ::Tn (A = 0.06). The top two hits from these screens, Tsx and LamB, were considered candidate receptors for phage U115.

The phage P2 screen resulted in 143 significant hits (Supplemental Figure S2.3 C), of which, 59 (Chapter 2 Appendix: Supplemental Data 1) were enriched in the presence of phage P2. Three hits (Figure 2.5) were slightly enriched: rhsD::Tn (A = 0.92), ynfB::Tn (A = 0.18), and yjbH::Tn (A = 0.84). However, none of these hits were promising, as receptors for the well characterized phages T6, T2 and T4 were enriched to a log ratio greater than 1. Furthermore, all three of these transposon mutants were background hits in other screens. Therefore, it is likely that either phage P2 uses LPS as a receptor (as in phage T7), or that the receptor for phage P2 is one of the 31 membrane proteins not included in the TMP.

The phage 8S screen resulted in 164 significant hits (Supplemental Figure S2.3 D), of which, 49 (Chapter 2 Appendix: Supplemental Data 1) were enriched in the presence of phage 8S. The top two hits (Figure 2.5) were ompC::Tn (A = 2.09) and lamB::Tn (A = 1.36). Other hits included rhsD::Tn (A = 0.64) and ynfB::Tn (A = 0.08). Additionally, OmpC regulators, envZ::Tn (A = 2.50) and ompR::Tn (A = 2.57), were enriched in the output TMP. The top two hits from this screen, OmpC and LamB, were considered candidate receptors for phage 8S infection.

The phage EC35 screen resulted in 160 significant hits (Supplemental Figure S2.3 E), of which, 69 (Chapter 2 Appendix: Supplemental Data 1) were enriched in the presence of phage EC35. The top two hits (Figure 2.5) were lamB::Tn (A = 2.47) and ompC::Tn (A = 2.33). Other
hits included \textit{rhsD::Tn} \((A = 0.50)\) and \textit{loiP::Tn} \((A = 2.42)\). Additionally, OmpC regulators, \textit{envZ::Tn} \((A = 2.38)\) and \textit{ompR::Tn} \((A = 3.03)\), as well as LamB regulators, \textit{malT::Tn} \((A = 2.61)\) and \textit{malK::Tn} \((A = 0.31)\), were enriched in the output TMP. The top three hits from this screen were LamB, OmpC and LoiP; however, \textit{loiP::Tn} was only enriched in one out of three replicate screens. Therefore, LamB and OmpC were considered candidates for phage EC35 receptors.

The phage EC14 screen resulted in 177 significant hits (Figure 2.5), of which, 32 (Chapter 2 Appendix: Supplemental Data 1) were enriched in the presence of phage EC14. Similarly to phage EC35, the top two hits from the screen with phage EC14 were \textit{lamB::Tn} \((A = 2.47)\) and \textit{ompC::Tn} \((A = 2.33)\). The other hit was \textit{rhsD::Tn} \((A = 0.48)\). Additionally, OmpC regulators \textit{envZ::Tn} \((A = 2.09)\) and \textit{ompR::Tn} \((A = 2.77)\), LamB regulator \textit{malT::Tn} \((A = 2.60)\), and \textit{malK::Tn} \((A = 0.003)\) were enriched in the output TMP. LamB and OmpC were considered candidates for phage EC14 infection.

**Receptors for five uncharacterized phages identified with EOP and adsorption assays**

EOP and adsorption assays were used to validate the candidate receptors for uncharacterized phage which were identified in each INSeq screen.

Outer membrane protein OmpA was the candidate with the highest A-value in the screen with phage R3 (Figure 2.5), and was selected as the likely receptor for further analysis. In an EOP assay, phage R3 had an EOP (Figure 2.6 A) below the limit of detection on BW25113Δ\textit{ompA}. Plaquing was restored upon plasmid complementation of the \textit{ompA} gene; however, plaquing was only restored to 46% of the phage growth on the parental strain. This result was likely due to differences in expression levels between chromosomally-encoded \textit{ompA} and exogenous \textit{ompA}. Furthermore, phage R3 did not adsorb (Figure 2.6 B) to strain BW25113Δ\textit{ompA}. Similar to plaquing, adsorption was restored upon complementation with OmpA. Results of both the EOP
and adsorption assays strongly indicated that OmpA was the primary receptor for the previously uncharacterized phage R3.

The screen of phage U115 identified Tsx, a nucleoside transporter, as well as LamB as potential receptors for phage U115 (Figure 2.5). Phage U115 had an EOP (Figure 2.6 C) below the limit of detection on BW25113Δtsx and plaquing was restored to wild-type levels upon complementation with Tsx. Deletion of lamB had no significant effect on the EOP of phage U115. Phage U115 did not adsorb (Figure 2.6 D) to BW25113Δtsx or to the empty vector control, BW25113Δtsx. However, complementation with Tsx not only restored phage U115 adsorption, but allowed U115 to adsorb better than wild-type; 90% of phage U115 has adsorbed to BW25113Δtsx::pSF-tsx by 15 minutes while only 46.4% of phage U115 had adsorbed to WT by 20 minutes. This result was likely due to the overexpression of exogenous tsx resulting in more Tsx on the outer membrane, relative to basal expression in WT bacteria. Phage U115 adsorbed to BW25113ΔlamB at levels comparable to wild-type. Results of both the EOP and adsorption assays indicated that phage U115 did not require LamB as a receptor to adsorb or infect E. coli, and that Tsx was the primary receptor for phage U115.

The INSeq screen of phage 8S identified OmpC, an outer membrane porin, and LamB as potential receptors for the virus (Figure 2.5). EOP assays (Figure 2.6 E) revealed a 2.3-fold decrease in EOP of phage 8S on BW25113ΔompC and a 1455-fold decrease in EOP on BW25113ΔlamB. Phage 8S was unable to infect the double knockout, BW25113ΔompCΔlamB, and infection was restored upon complementation with either ompC or lamB. Phage 8S appeared to adsorb (Figure 2.6 F) to BW25113ΔompC at levels similar to wild-type. However, phage 8S did not significantly adsorb to BW25113ΔlamB or to BW25113ΔompCΔlamB. Adsorption was restored upon complementation of BW25113ΔompCΔlamB with lamB but not ompC. This result
indicated that LamB was necessary for adsorption of phage 8S. However, since phage 8S was able to infect BW25113ΔlamB but unable to infect BW25113ΔompCΔlamB, it likely used OmpC as a secondary receptor. Adsorption assays indicated that LamB was the primary receptor for phage 8S as it adsorbed well to BW25113ΔompCΔlamB:pSF-lamB but did not adsorb appreciably to BW25113ΔompCΔlamB complemented with ompC over the course of the assay.

Similar to the screen for phage 8S, the screen for phage EC35 identified OmpC and LamB (Figure 2.5). There was a 1.7-fold decrease and a 6-fold decrease in EOP (Figure 2.6 G) of phage EC35 on BW25113ΔompC and BW25113ΔlamB, respectively. Phage EC35 was unable to infect BW25113ΔompCΔlamB and infection was restored upon complementation with either ompC or lamB. Phage EC35 was unable to adsorb (Figure 2.6 H) to BW25113ΔlamB or BW25113ΔompCΔlamB, but was able to adsorb to BW25113ΔompC and BW25113ΔompCΔlamB upon complementation with lamB, and not with ompC. Again, these data indicated that phage EC35 used LamB as a primary receptor but likely required OmpC as a secondary receptor, as only deletion of both ompC and lamB abolished infection in an EOP assay.

INSeq screens for a third phage, EC14, also identified OmpC and LamB as potential receptors (Figure 2.5). There was a 1.2-fold decrease and a 2.6-fold decrease in EOP (Figure 2.6 I) of phage EC14 on BW25113ΔompC and BW25113ΔlamB, respectively. Phage EC35 was unable to infect BW25113ΔompCΔlamB. Complementation with either ompC or lamB was sufficient to restore infection. Phage EC14 was unable to adsorb (Figure 2.6 J) to BW25113ΔlamB and BW25113ΔompCΔlamB but was able to adsorb to BW25113ΔompC and BW25113ΔompCΔlamB upon complementation with lamB or ompC. This result indicated that, unlike phage 8S or EC35, EC14 was able to use either LamB or OmpC as a receptor.
Unlike the screens above, the screen with phage P2 resulted in no statistically significant hits (Figure 2.5) with a log ratio of output CPM to input CPM above 1.0 in genes expressed at the outer membrane; the top two hits were \textit{rhsD} (A = 0.92) and \textit{yjbH} (A = 0.84). Transposon mutant \textit{rhsD::Tn} is likely background noise as it came up in every screen and follow up assays were not done. EOP and adsorption assays were performed with BW25113\textDelta yjbH. There is no decrease in EOP of phage P2 on BW25113\textDelta yjbH (Supplemental Figure S2.4 A). Phage P2 is able to adsorb to BW25113\textDelta yjbH (Supplemental Figure S2.4 B). It is possible that similar to phage T7, phage P2 uses LPS as a receptor. However, incubation with purified LPS did not result in a significant drop in titer (Supplemental Figure S4 C). Furthermore, GO term analysis did not uncover any significant enrichment for any biological processes. Another possibility is that similar to phage T2, phage P2 has multiple receptors. However, successful identification of both receptors of phage T2 indicates that our method should allow for the identification of multiple receptors. Therefore, it is likely that the receptor for phage P2 is one of the 31 outer membrane genes that was not represented in the original TMP; it is possible that a transposon mutant library with higher saturation would allow for the identification of the phage P2 receptor. However, it is also possible that an essential gene encodes the receptor for phage P2 which would not be included in a transposon mutant library.

In all screens performed, whether well-characterized phage or newly-isolated ones, \textit{lon::Tn} was consistently the top hit (Figure 2.3 and Supplemental Figure S2.3). As \textit{lon} encodes the Lon protease which functions in the cytoplasm, it was not considered a candidate receptor hit and no follow up was conducted. However, mutations in \textit{lon} that disrupt the proteolytic function result in accumulation of \textit{rcsA}, an activator of capsular polysaccharide biosynthesis \textsuperscript{130}. Therefore, it is likely that deletion of \textit{lon} generally results in phage resistance due to overexpression of capsule.
Based on these screens, it is possible that mutations in *lon* result in a smaller fitness cost than mutations in genes that code for phage receptors. This result will be explored further in future studies.

**Discussion**

INSeq screens were successful in identifying candidate phage receptors for five out of six previously uncharacterized phage and further analysis of specific hits confirmed that the top hit(s) identified in the screens were the phage receptor(s). We note that our approach is designed to identify the receptor(s) used by a phage when interacting with the single bacterial strain. However, this method could be employed in multiple different strains of bacteria in order to identify receptors for different hosts and phage that encode multiple tailspikes that permit infection across multiple bacterial hosts.\(^{131}\)

Interestingly, each of the phage receptors identified in this study have been identified as receptors for other phage.\(^ {132}\) With 137 annotated membrane proteins and a branching LPS chain consisting of 10 sugar monomers with various phosphate groups in *E. coli* BW25113, it is surprising that previous reviews on phage receptors reported that only 10 membrane proteins and various sugar linkages were exploited as phage receptors.\(^ {132, 133}\) Furthermore, three out of five phages chosen randomly from our coliphage library used OmpC as a receptor in some capacity. These observations are interesting because the vast biodiversity of lytic phage suggests that these viruses may have evolved to exploit most, if not all, possible receptors on the surface of bacteria, implicating selection by these ‘predatory’ phage as a possible key driver of genomic diversity in bacteria.\(^ {134}\) However, this expectation assumes that all cell-surface structures are equally suitable to serve as phage receptors in the eyes of natural selection. Rather, selection for phage to use a particular structure on the surface of a bacterium is likely influenced by the abundance of that
molecule on the surface and whether the protein is well-conserved among bacterial genotypes. Both OmpA and OmpC are reported to be among the top 20 most highly expressed proteins in *E. coli*, with 207,618 and 163,538 molecules per cell respectively \(^{135}\). On one hand, with so many potential binding sites on the surface (~1 OmpA or OmpC molecule per 30 nm\(^2\)), a bacterium is extremely vulnerable to attack by phage that bind to either OmpA or OmpC, suggesting that phage should be strongly selected to target these structures \(^{136}\). However, on the other hand this creates the possibility for intense competition among phage that use common binding sites such as OmpA or OmpC, which likely results in selection for phages to exploit a less common receptor to reduce competition for hosts. Previously, phage receptors have been vastly understudied, likely because of the large effort required to screen phage on individual bacterial mutants. Thus, the higher-throughput INSeq screen presented here demonstrates a method to rapidly identify phage receptors, fostering a broader possibility to efficiently explore the evolution of phage binding sites and inter-phage competition to use these receptors. This could be a key first approach in the ultimate study of whether virus evolution has resulted in some binding targets to be over- versus under-exploited in the phage world.

Around the time of submission, both a paper came out and a preprint was posted describing similar methods for identifying genes involved in phage infection in *Enterococcus faecalis* and *E. coli* respectively \(^{137,138}\). However, there were some differences in outcomes among these studies which may reflect differences in the efficacies of the approaches. It would be interesting to compare these various methods in future work.

**Conclusion**

Transposon insertion sequencing is a powerful technique that allows for identification of bacterial genes that contribute to fitness in particular selective conditions. Here, we used INSeq screens to
identify phage receptors for multiple *E. coli* phage. Proof of concept experiments validated this approach by confirming known phage receptors. In particular, this method was successful in confirming receptors for previously well-characterized phage that use a single protein receptor, dual-protein receptors, a primary LPS receptor and secondary protein receptor, and LPS as receptor. This method was then extended to identify receptors for five out of six previously uncharacterized randomly-chosen phage. We expect that a more saturated transposon mutant library would allow for identification of the receptor for newly-discovered phage P2. Our approach was particularly promising, because for each screen that resulted in the successful protein receptor identification, the genes encoding the receptors were among the top hits for each phage.

In addition to identifying phage receptors, our screens could provide information about the fitness of other bacterial genes in the presence of phage, such as bacterial genes that were involved in phage replication or resistance to phage. Thus, our approach might allow for identification of a subset of bacterial genes that are universally important during phage infection or resistance.

While this study focused on phage receptors for viruses that infect *E. coli*, transposon-insertion sequencing provides information for any transposon mutant in the TMP, and has been employed in many different species of bacteria. Our new method for the high-throughput identification of phage receptors should allow for rapid characterization of newly isolated phage that target various different species of bacteria. If enough phage receptors are identified for sequenced phage, bioinformatic approaches might be extended to allow for receptor identification based on the genomes of phage alone. Furthermore, in addition to using INSeq screens to identify phage receptors, our screens provide information about the fitness of other bacterial genes in the presence of phage, potentially allowing for the identification of a subset of bacterial genes that are universally important during phage infection. Therefore, in addition to allowing for a high-
throughput identification of phage receptors, INSeq screens may provide insight into generalized mechanisms of phage resistance.

**Materials and Methods**

**Bacterial strains, plasmids and phage**

Bacteria used in this study were obtained from the Coli Genetic Stock Center at Yale University, and phages T2, T4, T6 and T7 were kindly provided by J. Wertz (Yale U). The six uncharacterized phages in the study were isolated from sewage or environmental water samples. All bacteria, plasmid and phage strains are listed in Supplemental Table S2.1 of the Chapter 2 Appendix. Bacteria were cultured at 37°C with shaking (200 rpm) in Luria Broth (LB) and on LB agar (15%) plates, where dilutions in LB followed by plating were used to estimate bacterial densities as colony-forming units (CFU) per mL. Carbenicillin (Cb; 100µg/mL), gentamicin (Gm; 10 µg/mL), kanamycin (Km; 50 µg/mL), arabinose (0.1%) and isopropyl β-D-1-thiogalactopyranoside (IPTG; 1mM) were added when appropriate. Phage strains were amplified in shaking liquid culture overnight on the amplification host, BW25113ΔicdC (hereafter referred to as wild-type, WT), and filter sterilized with a 0.22µm filter to obtain a cell-free lysate. Titers were estimated as plaque-forming units (PFU) per mL, and determined by plaques formed via dilution plating in ‘soft’ agar (7.5%) overlays on lawns of WT grown on agar plates, unless otherwise noted.

**INSeq transposon mutant library preparation**

The insertion-sequence (INSeq) transposon mutant library was made via conjugation. Donor bacterial strain S17 λ pir118 containing plasmid pSAM_PA142 was conjugated with recipient strain WT on plates containing arabinose, at a ratio of 1:1 donor to recipient. After 3 hours incubation at 37°C, conjugation mixtures were plated for single colonies on plates with Gm and Km to isolate transconjugants that were resistant to both markers (Gm+, Km+). Following 24 hours incubation at
37°C, colonies were scraped into LB medium using a sterile spatula and stored in 20% glycerol at -80°C.

**INSeq screen**

Aliquots of the library were thawed on ice, washed once in LB and resuspended to a concentration of approximately 10^7 CFU/mL in LB and incubated at 37°C for 1 hour, with shaking. A sample of test phage was added, in triplicate, to a final concentration of approximately 10^5 PFU/mL, for MOI~ 0.01. Following overnight incubation, cultures were harvested and genomic DNA was extracted and prepared for sequencing. DNA libraries were pooled and sequenced at the Yale Center for Genome Analysis via the Illumina HiSeq2500 system. Sequences were analyzed using scripts modified from Goodman et al (2009). Briefly, using Python scripts adapted from analysis packages previously described, sequencing reads were indexed by barcode, and transposon sequence was trimmed leaving 16 base pairs of adjacent genomic DNA. These 16 base pair sequences were aligned to the reference genome (Genbank accession # CP009273.1) using Bowtie2, by counting the number of reads for each insertion site, normalizing to counts per million reads and binning by gene. Transposon insertions mapping to the distal 5% ends of any coding region as well as transposon insertions mapping to intergenic regions were filtered out during analysis. A Z-test was performed using the log ratio of normalized output count to normalized input count, and Q-values from false discovery rate (FDR) correction of < 0.05 were considered significant for further analysis.

**Efficiency of plating, and adsorption assays**

Efficiency of plating (EOP) was measured as the ratio of the test phage titer on the experimental strain to its titer on the WT strain. Phage binding to cells (adsorption) was measured over a 20 minute adsorption assay, which estimates the rate of ‘disappearance’ of phage particles (i.e.,
reduction in titer) in liquid LB medium over time when viruses are challenged to attach to bacterial cells.
Design of INSeq screen. **A** Experimental setup for selection of pooled transposon mutant library (TMP) with phage indicating enrichment for phage-resistant mutants. **B** Mutants in genes involved in resistance to phage will become underrepresented in
Figure 2.2 Genome plot of *E. coli* strain BW25113

Genome plot of *E. coli* strain BW25113. Track 1 (inside) is a heat map of GC content, where red represents high GC content and blue represents low GC content. Track 2 plots the abundance of transposon insertions in a coding region (green) or intergenic region (blue). Track 3 shows intergenic regions (red) and Track 4 shows coding regions (multi-color).
Results of INSeq screen with well-characterized phage. Each point represents the normalized relative abundance of all transposon mutants in a specific gene. All statistically significant transposon mutants are grey (P < 0.05). Certain hits are colored; other mutants in regulatory genes are unfilled circles in the same color as the gene. A Results of a screen with phage T6. Significantly over-represented hits *tsx::Tn*, *btuB::Tn*, *lamB::Tn*, and *ompX::Tn* are in blue, orange, red and yellow respectively. B Results of a screen with phage T2. Significantly over-represented hits *ompF::Tn* and *fadL::Tn* in green and blue respectively. C Results of a screen with phage T4. Significantly over-represented hit *ompC::Tn* is in purple. D Results of screen with phage T7. E Log ratio (A) of relative abundance of 106 transposon mutants in genes encoding membrane proteins for each screen. The outlier in the control screen is *lamB::Tn*. 
Figure 2.4 Assays validating phage receptors of well-characterized phage

Assays validating phage receptors of well-characterized phage. A Efficiency of plating (EOP) assays for phage T6 on various E. coli strains. EOP was calculated as a ratio of titer on test strain to the titer on WT (BW25113 ΔicdC). EOPs are plotted as mean and standard deviation of three independent experiments. Significance was calculated using T-test as significantly different from 1. “<200” indicates that the titer on the test strain was below the limit of detection for this assay which was 200 PFU/mL. B Adsorption assays of phage T6 on various E. coli strains plotted as a log of the percent of free phage. Dashed lines indicate stains with an empty vector (pSF) or a complement vector (pSF-tsx) grown in the presence of 100 mg/L carbenicillin with induction by 1mM IPTG. Percent free phage was determined as a ratio of free phage at the time point divided by total phage added at the beginning of the assay and is plotted as a mean and standard deviation of three independent experiments. Linear regression was performed for each strain. Regression lines with a significantly non-zero slope indicate that the particular strain is able to support phage T2 adsorption. C EOP assays for phage T2 on various E. coli strains. D Adsorption assays of phage T2 on various E. coli strains. E EOP assays for phage T4 on various E. coli strains. F Adsorption assays of phage T4 on various E. coli strains. G LPS phage inactivation assays for phage T2, T4 and T7. Titers for each phage was determined after a 20 minute incubation with either 80 ng/mL LPS or water 61. Titers are plotted as means with standard deviation from
three independent experiments. A T-test was used to determine significant differences between the mock condition or the LPS condition. (* indicates $P < 0.05$, ** indicates $P < 0.01$, *** indicates $P < 0.001$)
Results of INSeq screen with uncharacterized phage. Log ratio (A) of relative abundance of 106 transposon mutants in genes encoding membrane proteins for each screen with uncharacterized phage. Grey represents statistically significant hits, other top hits $ompA::Tn$, $tsx::Tn$, $ompC::Tn$, and $lamB::Tn$ are in green, blue, purple and red respectively. The outlier in the control screen is $lamB::Tn$. ($P < 0.05$).
**Figure 2.6** Assays validating phage receptors of newly isolated phage

Assays validating phage receptors of newly isolated phage. **A** Efficiency of plating (EOP) assays for phage R3 on various *E. coli* strains. **B** Adsorption assays of phage R3 on various *E. coli* strains plotted as a log of the percent of free phage. **C** EOP assays for phage U115 on various *E. coli* strains. **D** Adsorption assays of phage U115 on various
E. coli strains. E Efficiency of plating (EOP) assays for phage 8S on various E. coli strains. F Adsorption assays of phage 8S on various E. coli strains plotted as a log of the percent of free phage. G EOP assays for phage EC35 on various E. coli strains. H Adsorption assays of phage EC35 on various E. coli strains. I EOP assays for phage EC145 on various E. coli strains. J Adsorption assays of phage EC14 on various E. coli strains. (* indicates p < 0.05, ** indicates p < 0.01, *** indicates p < 0.001).
Table 2.1 List of genes encoding membrane proteins

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List of genes encoding membrane proteins. Bolded genes are not included in the TMP. A * indicates essential genes.
**Chapter 2 Appendix**

**Supplemental Results**

**Efficiency of plating and adsorption assays confirm known receptors for characterized phages**

Plaques of phage T6 were below the limit of detection (200 PFU/mL) on bacterial strain BW25113\(\Delta\)tsx and complementation with plasmid pSF-tsx restored plaquing to similar levels as on WT (Figure 2.4 A). Phage T6 did not adsorb to BW25113\(\Delta\)tsx, however adsorption was restored upon complementation (Figure 2.4 B). While these results, along with previous literature, demonstrate the ability of phage T6 to use Tsx as a receptor, EOP and adsorption assays were run on the other enriched membrane genes from the screen. As expected, phage T6 had an EOP similar to WT and adsorbed to BW25113\(\Delta\)ompX, BW25113\(\Delta\)lamB and TA563\(\Delta\)btuB::Tn (Figure S2.2 A,B), indicating that these genes were not involved in phage binding.

Both BW25113\(\Delta\)ompF and BW25113\(\Delta\)fadL were able to support phage T2 infection and replication at a similar level as WT (Figure 2.4 C). However, on a double knockout (BW25113\(\Delta\)ompF\(\Delta\)fadL), phage T2 plaques were below the limit of detection, indicating that host bacteria lacking both ompF and fadL genes were unable to support phage infection and replication. Infection of phage T2 was restored through complementation of either OmpF or FadL in BW25113\(\Delta\)ompF\(\Delta\)fadL (Figure 2.4 C). Phage T2 did not adsorb to BW25113\(\Delta\)ompF\(\Delta\)fadL (Figure 2.3 D) in the time allowed in the assay. In contrast, adsorption was restored upon complementation with either OmpF or FadL (Figure 2.4 D). These results, combined with the aforementioned inability to detect phage T2 plaques on strain BW25113\(\Delta\)ompF\(\Delta\)fadL, strongly suggested that phage T2 could use either OmpF or FadL as a receptor to initiate productive lytic infection.
We similarly analyzed whether our approach accurately identified known binding mechanisms for phage T4. Results for phage T4 (Figure 2.4 E) showed virus infection of BW25113ΔompC was below an EOP of 1.0, but was not significantly different from the complemented strain or the complemented control. Adsorption of phage T4 (Figure 2.4 F) to strain BW25113ΔompC was diminished and could be rescued upon exogenous expression of OmpC. However, phage T4 could use LPS as a co-receptor and therefore could still infect BW25113ΔompC as seen in the EOP assay. The results of the EOP and adsorption assays corresponded with previous observations in the literature that phage T4 uses OmpC to reversibly bind cells and increase efficiency of adsorption while irreversibly binding LPS a co-receptors110, 130.

Last, we conducted experiments to verify that our method produced results consistent with known receptor usage of phage T7. Instead of traditional EOP or adsorption assays with isogenic strains of E. coli, purified LPS was used to determine if LPS was a receptor for phage T7. Incubation of phage T7 with 80 ng/mL of LPS resulted in observed reduction in phage titer (Figure 2.4 G) as compared to incubation of phage with no LPS, which indicated that phage T7 could use LPS alone as a receptor. Interestingly, there was no observed drop in titer when phage T4 was incubated with LPS even though phage T4 could infect cells in the absence of OmpC (Figure 2.4 G). The results of the adsorption assay indicated that phage T4 did not appreciably adsorb to BW25113ΔompC over a 20 minute period. Therefore, perhaps longer incubation of phage T4 with LPS would result in a reduction of titer.
**Supplemental Datasets**

Supplemental Data 1 can be found on Dryad (doi:10.5061/dryad.t76hdr7z3). Statistically significant hits for each phage screen with the Q-value, log ratio of output to input counts (A), Gene ID, and gene name. X indicates that the hit was either enriched in the output or in a gene that encodes a membrane protein, respectively.
Results of a control INSeq screen. Each point represents the normalized relative abundance of all transposon mutants in a specific gene. All statically significant transposon mutants are grey.
Supplemental Figure S2.2 Additional assays validating phage T6 receptor

**A** Efficiency of plating (EOP) assays for phage T6 on various *E. coli* strains. **B** Adsorption assays of phage T6 on various *E. coli* strains plotted as a log of the percent of free phage. (* indicates p < 0.05, ** indicates p < 0.01, *** indicates p < 0.001)
Supplemental Figure S2.3 Results of INSeq screen uncharacterized phage

Results of INSeq screen uncharacterized phage. Each point represents the normalized relative abundance of all transposon mutants in a specific gene. All statically significant transposon mutants are grey (P < 0.05). Certain hits are colored; other mutants in regulatory genes are triangles in the same color as the gene they regulate. **A** Results of a screen with phage R3. Significantly over-represented hit ompA::Tn is in green. **B** Results of a screen with phage U115. Significantly over-represented hits txs::Tn and lamB::Tn in blue and red respectively. **C** Results of a screen with phage P2. **D** Results of screen with phage 8S. Significantly over-represented hits ompC::Tn and lamB::Tn in blue and red respectively. **E** Results of screen with phage EC35. Significantly over-represented hits ompC::Tn and lamB::Tn in blue and red respectively. **F** Results of screen with phage EC14. Significantly over-represented hits ompC::Tn and lamB::Tn in blue and red respectively.
Assays examining phage receptors of newly isolated phage. 

A Efficiency of plating (EOP) assays for phage P2 on various *E. coli* strains. 

B Adsorption assays of phage P2 on various *E. coli* strains plotted as a log of the percent of free phage.

C LPS phage inactivation assays for phage R3, U115, P2, 8S, EC35 and EC14. Titers are plotted as means with standard deviation from three independent experiments. A T-test was used to determine significant differences between the mock. (***) indicates $p < 0.001$
### Supplemental Table S2.1 Bacterial strains, plasmids and phage used in this chapter

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GmR, gentamicin resistant; KmR, kanamycin resistant; CbR, carbenicillin resistant; TetR, tetracycline resistant; AmpR, ampicillin resistant
Chapter 3: Phenotypic variation among *Pseudomonas aeruginosa* populations when coevolving with phage OMK01
Preface

Chapter 3 has been adapted from a manuscript that is intended for submission to Journal of Evolutionary Biology in Summer, 2021.

This study was designed by Kaitlyn Kortright and Dr. Benjamin Chan. Kaitlyn Kortright and Dr. Benjamin Chan preformed experiments. Kaitlyn Kortright analyzed data. The manuscript was prepared and edited by Kaitlyn Kortright, Dr. Benjamin Chan and Dr. Paul Turner. Raw sequencing will be publicly available at NCBI Sequence Read Archive Project # PRJNA713810. This project was supported by an award from Cystic Fibrosis Foundation, and a portion of the resources to conduct genome-sequencing came from an award to Felix Biotechnology.
Abstract

Experimental evolution studies have long been used to examine coevolutionary dynamics between phages and bacteria; however, here we extend traditional coevolution studies to examine both coevolutionary dynamics of phage OMKO1 and *Pseudomonas aeruginosa* and the evolution of an anticipated trade-off between phage resistance and antibiotic resistance in the bacteria. We observed that lytic phage OMKO1 and *P. aeruginosa* are able to coexist over a 10-passage evolution experiment, and ‘time-shift’ assays revealed that coevolutionary dynamics of the two microbes followed an arms-race pattern of coevolution with slight differences in dynamics across all three experimental populations. Measurements of antibiotic resistance at both clonal and population levels demonstrated that one experimental population evolved phage resistance at the expense of decreased resistance to tetracycline and erythromycin antibiotics (evolutionary trade-off), whereas the other two *P. aeruginosa* treatment populations did not suffer the predicted trade-off. Whole genome sequencing of bacterial populations revealed that treatment populations accrued greater mutational differences relative to the wild-type ancestor, compared to a control where bacteria evolved in absence of phage selection. In each treatment population, mutations were found in genes involved in biosynthesis of flagella, type-IV pilus and lipopolysaccharide; no mutations in these genes were observed in the control. In the bacterial population that showed a trade-off, results indicated three mutations in *fleQ, wzz1* and *wzz2*, that swept to fixation at the same passage when the phage-resistance/antibiotic-resistance trade-off was observed. Results of this coevolution experiment remind that predicted evolutionary trade-offs may not always occur, and that in-vitro experimental studies of phage-bacteria interactions are valuable for elucidating possible adaptive trajectories.
Chapter Introduction

It is estimated that Earth contains a staggering $10^{30}$ bacterial cells, and these are even outnumbered roughly tenfold by bacteriophages (phages; viruses of bacteria) that specifically infect them $^{145}$. Although these ubiquitous microbes form the bases of ecosystems, details of their coexistence and antagonistic coevolutionary interactions remain understudied. Nevertheless, the limited studies demonstrate that phage-bacteria associations have significant and widespread implications, including biogeochemical cycling of carbon, nitrogen, and phosphorus in oceans; regulating primary production in ecosystems; dictating flamingo population crashes $^{146}$; and developing antibacterial therapies. Phage therapy, the therapeutic use of phages to treat bacterial infections, is increasingly being considered as an adjunctive approach to treat multi-drug resistant bacterial infections. However, without rigorous studies of the coevolutionary details of phage-bacteria interactions, such treatments may yield unanticipated outcomes. Experimental evolution studies could benefit the re-emerging field of phage therapy, by describing the evolutionary dynamics that occur when bacteria are subjected to phage selection pressures. Additionally, experimental evolution studies might inform both the repeatability and predictability of the outcomes of these treatments. If phage therapy is to be used successfully, it is crucial to better understand the dynamics of phage-bacteria coevolution.

Lytic phages exert selection pressure on bacteria to evolve resistance; in response, resistant bacteria select for phages to evolve improved infectivity. This reciprocal selection can produce two types of antagonistic coevolution: arms race dynamics (ARD) versus fluctuating selection dynamics (FSD). ARD follows a classic ‘gene-for-gene’ model where one bacterial genotype has a broad resistance (and one phage genotype has very broad infectivity), meaning that the bacteria evolves to resist all phage genotypes (and the phages coevolve to infect all bacterial genotypes) in
the phage-bacteria relationship. In contrast, coevolution via cyclical fluctuations in allele frequencies is the basis of FSD, which follow matching-allele models of coevolution. Here, phage infectivity (or bacterial resistance) requires a specific match between the bacterial and phage genotypes, and polymorphism is maintained by negative frequency-dependent selection. Theory predicts that environments with weaker costs of resistance/infectivity should produce ARD, whereas those with stronger costs should foster FSD; ARD are seen in many phage-bacteria coevolution studies with fewer showing FSD suggesting that the cost of evolution of phage resistance tends to be weak under laboratory conditions. However, if a trade-off exists between phage resistance and another phenotypic trait, there might be a greater cost to phage resistance than previously observed.

Phage OMKO1 is a ‘jumbo phage’ of Pseudomonas aeruginosa that selects for phage resistant bacteria with increased sensitivity to traditional antibiotics. This observed trade-off between phage resistance and antibiotic resistance may be explained by the binding of phage OMKO1 to the surface exposed outer membrane component, OprM, of one of the multi drug efflux pump systems (MexAB-OprM) in P. aeruginosa. However, recent evidence suggests that OMKO1 is either plastic or highly evolvable in its binding, perhaps using the type-IV pilus or flagellum as a receptor instead of OprM (unpublished data). Here, we conducted a short-term (10 passage) laboratory coevolution experiment with replicate communities of phage OMKO1 and P. aeruginosa strain PA01, to determine (1) if phage OMKO1 and the host bacteria can coexist over approximately 100 bacterial generations, (2) whether ARD or FSD govern the coevolutionary trajectories, (3) if a trade-off between phage resistance and antibiotic resistance occurs, and (4) which loci in the bacterial genome undergo changes relative to the wild-type sequence.

**Results**
**Bacteria and lytic phages coexist for the duration of a short-term coevolution experiment**

A short-term (10-passage) serial-transfer experiment (as described in the Methods) was conducted with *P. aeruginosa* strain PA01 and lytic phage OMK01, to test whether the microbes could coexist and to look for evidence that coevolution occurred (Figure 3.1). All three treatment communities (bacteria plus phages) presented generally lower bacterial densities in the first half of the study (days 1 to 5), compared with maximum densities achieved by bacteria in the one control containing bacteria alone, which was numerically stable throughout (Figure 3.2A). Furthermore, all of the treatments experienced decreases in bacterial density that were not evident in the control; the bacteria fell to a low density in all treatments at passage 2; in treatments 1 and 2 at passage 5; and in treatment 3 at passage 6. Interestingly, the lower bacterial densities in treatment 1 at passages 2 and 5 correlate with higher phage titers at these same time points (Figure 3.2B), with phage titer becoming stable around $10^9$ PFU/mL for the remainder of the study. The phage titers of the other two treatments tracked with each other; after a large increase in titer at passage 1, phage titers in treatments 2 and 3 steadily decreased until passage 9, such that all three treatments ended with $10^9$ PFU/mL (Figure 3.2B). We concluded that PA01 bacteria and phage OMK01 were capable of coexisting throughout the 10-passage study, indicating that reciprocal coevolution was at least possible during the short-term experiment.

**Coevolutionary timeline of PA01 and OMK01 occurs differently in three populations**

To determine if bacteria in experimental treatments were evolving to resist phages, the fitness ($\text{fitness} = \frac{\text{OD600}_{\text{challenge}}}{\text{OD600}_{\text{LB}}}$) of 96 randomly-sampled bacterial clones was assayed as described in the Methods. As expected, bacterial clones from the control showed lower fitness in the phage challenge than bacterial clones from the treatments, which were subjected to phage selection (Figure 3.3 and Supplemental Figure S3.1). Furthermore, bacterial clones from earlier
passages of each treatment population were less-fit in the phage challenge, than bacterial clones from the same treatment isolated during later passages (Figure 3.3A-C). Fitness of bacterial clones from treatment 1 and 2 increased markedly from passages 2 to 3 (Figure 3.3A,B). While the fitness of bacterial clones from treatment 1 remained stable for the rest of the experiment, the fitness of bacterial clones from treatment 2 seemed to decrease slightly in the later passages (Figure 3.3A,B). The fitness of bacterial clones from treatment 3 increased gradually over the 10 passages, without the dramatic ‘jump’ upwards in fitness observed in treatments 1 and 2 (Figure 3.3C). We concluded that fitness of treatment bacteria in a phage challenge increased over time, indicating evolution of phage resistance in *P. aeruginosa* tended to occur in the presence of phage OMK01 selection.

To examine whether phages in the treatments changed in terms of their host range, phages at each passage were assayed for their ability to infect the ancestral PA01 used to initiate the experiment. Impact of phages on the growth curves measured for test bacteria were used to examine whether viruses changed in their killing efficiency. Results showed that phages in the earlier passages were better able to suppress (negatively-impact) bacterial growth, compared to those isolated in later passages (Figure 3.4A-C). The maximum OD600 value of the test bacteria was extracted from each growth-curve dataset, to better-visualize the reduced fitness of passaged phages when allowed to infect the ancestral bacteria. Phages from passage 1 of treatment 1 suppressed bacterial growth (OD600 of ~0.5), while later-passage phages allowed the ancestral bacteria to achieve OD600 of approximately 0.9 (Figure 3.4D). Phages from treatments 2 and 3 caused less-dramatic fitness reductions on ancestral bacteria, with earlier passages (1 through 4) suppressing bacterial growth at OD600 of approximately 0.35, and later passages (5 through 10) allowing bacteria to reach maximum OD600 of 0.45 (Figure 3.4D). We concluded that phage
fitness, as measured by the ability to infect ancestral PA01, decreased over time in all treatments, indicating that phage evolution had occurred.

Coevolutionary dynamics were investigated using time shift assays, where the bacteria obtained at each passage were cross-streaked against the phages from each passage to ascertain bacterial resistance and phage infectivity. In general, bacteria from each treatment were more resistant to phages of the past, and more sensitive to phages from future passages (Figure 3.5A). Correspondingly, phages were more infective on bacteria of the past, and less infective on bacteria of the future (Figure 3.5B). Some bacteria from treatments 2 and 3 were sensitive to phages from the past, specifically 6, 7 or 8 passages prior to the contemporary phages; whereas, in treatment 1 some bacteria were resistant to phages of the future (Figure 3.5A). Time-shift assays for each of the treatments were consistent with expectations of ARD coevolution.

**Trade-off between phage resistance and antibiotic resistance occurs in one population**

To determine if the evolution of phage resistance resulted in a trade-off with resistance to antibiotics, the 96 bacterial clones from each passage that were assayed for fitness in the presence of contemporary phages (Figure 3.3) were similarly assayed for fitness in 3mg/L of tetracycline. Over the course of ten passages, fitness in tetracycline of treatment 2, treatment 3 and the control either didn’t change or increased slightly as seen by a positive slope of the regression line (Figure 3.6B-D). However, fitness of treatment 1 in the presence of tetracycline appeared to decrease over the ten passages; more specifically, bacterial clones taken from passages 8, 9 and 10 have a lower fitness than clones taken from earlier passages (Figure 3.6A). These results were confirmed using both Etest strips and two-fold dilutions to measure the minimum inhibitory concentrations (MIC’s) of various antibiotics for bacteria taken from each passage. MIC measurements made using Etest strips show that later passages (days 7-9) of the bacteria taken from treatment 1 have a slightly
lower MIC to tetracycline and erythromycin (Supplemental Figure S3.2A,B) but remain resistant to ampicillin (Supplemental Figure S3.2C). Additionally, to determine the MIC needed to kill 90% of bacteria (MIC90), bacterial from each treatment were plated on a range of two-fold dilutions of tetracycline (4 and 8 mg/L), erythromycin (64, 128 and 256 mg/L), meropenem (0.25 and 0.5 mg/L) and gentamicin (0.5, 1 and 2 mg/L). MIC90 of gentamicin and meropenem (Supplemental Table S3.1) remain unchanged over the 10 days of passages, however, MIC90 of tetracycline and erythromycin (Supplemental Table S3.1) trend down for treatment 1 at the latter passages (days 7-10) as compared to the control. Taken together, these results show preliminary evidence that a trade-off between phage resistance and antibiotic resistance may have occurred for treatment 1 but not for treatment 2 or 3.

**Genomics reveals likely loci undergoing change**

Deep sequencing (DS) of bacterial populations from each passage was used to examine the genetics underlying the observed ARD coevolution. Frequencies of high-quality variants in coding regions that differed from the common ancestor’s genome were plotted as heat maps for each of the treatments and control (Supplemental Figure S3.3). Treatment 1 showed the greatest number of alleles that differed from the ancestor (n=288); furthermore, the number of mutations was elevated at passages 1 and 2 (n=84 and 87, respectively), while the number of such mutations decreased in future passages (Supplemental Figure S3.3A). Treatments 2 and 3 had fewer sites that changed (n=125 and n=163 respectively) and the number of mutations did not deviate throughout the experiment (Supplemental Figure S3.3B,C). As expected, the control had the fewest number of allele changes (n=115) and the number of mutations did not vary across passages (Supplemental Figure S3.3D). The majority of the mutations observed in each treatment and the control were polymorphic non-synonymous (P<sub>n</sub>) or polymorphic synonymous (P<sub>s</sub>) (Table 3.1). Treatments 1
and 2 did not have any fixed synonymous mutations (Ds) while treatment 3 and the control had 2 Ds mutations each (Table 3.1). Treatment 1 and 3 had 9 mutations each that were fixed and non-synonymous (Dn) while treatment 2 and the control had 7 Dn mutations each (Table 3.1).

Additionally, DS and analysis was used to identify a subset of the genetic variations, specifically variants in genes common to the treatments but not observed in the control. Treatments 1 and 2 had mutations in genes involved in type-IV pilus biogenesis; these bacterial populations shared 4 point mutations (three of which were non-synonymous) in pilB that were low frequency variants at passages 1 and 2 (Figure 3.7A,B and Supplemental Table S3.2). Treatment 2 had an additional two non-synonymous point mutations in fimU that fixed at passage 3 and 4, but were subsequently lost from the population (Figure 3.7B and Supplemental Table S3.2). No mutations in type-IV pilus biogenesis genes were observed in treatment 3 or control bacteria (Figure 3.7C and Supplemental Table S3.3).

All three treatments showed bacteria with mutations in flagellar biosynthesis genes. Treatment 1 had two non-synonymous mutations in fleQ, three indels in fleS, and two point mutations in fliI (Figure 3.7A and Supplemental Table S3.2). Treatment 2 bacteria had a single point mutation in fleQ that fixed at passage 3 and remained at high frequency until passage 8 (Figure 3.7B and Supplemental Table S3.2). Bacteria from treatment 3 had two mutations in fleQ and an insertion in fleR, all three of which rose independently to high frequency for a single passage (Figure 3.7C and Supplemental Table S3.2). No mutations in flagella biosynthesis genes were observed in the control.

Finally, each treatment population showed mutations in multiple genes involved in both A-band and B-band lipopolysaccharide (LPS) biosynthesis. Treatment 1 bacteria had a high frequency mutation in wzy at passage 2, a high frequency deletion in wbpL for passages 3 and 4.
and low frequency mutations in \( wbpX \) and \( wbpZ \) towards the end of the experimental evolution (Figure 3.7A and Supplemental Table S3.2). Treatment 2 had mutations in \( wbpX \) and \( gmd \) that were observed during passages 6 and 7-8 respectively (Figure 3.7B and Supplemental Table S3.2). Treatment 3 bacteria had mutations in \( wbpY \) (passage 6) and \( wzt \) (passage 9-10) (Figure 3.7C and Supplemental Table S3.2). All three treatments had bacteria with multiple non-synonymous \( wzz_1 \) and \( wzz_2 \) mutations that occurred over the course of the experiment. No mutations were observed in any genes involved in LPS synthesis in the control (Supplemental Dataset S1). A complete list of mutational variants observed in each population can be found in Supplemental Dataset S1.

**Discussion**

Bacterial densities and phage titers (Figure 3.2) showed that it was possible for lytic phage OMKO1 and \( P. \) aeruginosa host bacteria to coexist for the entirety of a short-term experiment. Fitness challenges in the presence of phages (Figure 3.3) showed that bacteria from treatment populations were able to evolve resistance (achieve high fitness) to contemporary phages, while bacteria from the control remained highly susceptible (low fitness) to phage infection. Furthermore, decreases in phage titers (Figure 3.2B) for each treatment population seemed to correlate temporally with the evolution of phage resistance in each bacterial population (Figure 3.3). These population dynamics are most clearly seen in treatment 1; phages from treatment 1 monotonically decreased from passages 1 to 4 (Figure 3.2B, blue) while bacteria from treatment 1 shift from phage-susceptible at passages 1 and 2 to phage-resistant at passages 3 and 4 (Figure 3.3A). However, the titer of treatment 1 phages rebounded after passage 4, likely indicating that the phage population had evolved to infect the phage-resistant bacteria. Taken together, these results indicated that bacteria from the treatments containing phages evolved resistance to phages. Since phages were observed in the final passage in each of the treatments, this indicated that either
phages were evolving in response to bacterial evolution, or that bacteria had not evolved complete phage resistance. Results (Figure 3.4) seemed to indicate that phages had evolved decreased infectivity on ancestral PA01 bacteria. This was likely due to phage evolution of increased infectivity on coevolving bacterial populations (phages were evolving to infect bacteria that were evolving to resist phages). Time shift assays (Figure 3.5) helped illuminate the dynamics of the coevolution that appears to have occurred in the experimental treatments; bacteria were more resistant to phages of the past and more sensitive to future phages (Figure 3.5A); and phages were more infective to past bacteria and less infective to those of the future (Figure 3.5B). These results were consistent with predictions of ARD. However, there were some instances in treatment 1 where bacteria were resistant to future phages, and in treatments 2 and 3 there were phages that could successfully infect future bacteria. While the coevolutionary dynamics seemed to follow patterns generally indicative of ARD, during some passages, FSD may have occurred instead.

All three treatments contained bacterial populations that became resistance to phages; however, the previously observed trade-off between phage-resistance and antibiotic-resistance was only observed in treatment 1 (Figure 3.6, Supplemental Figure S3.2 and Supplemental Table S3.1). This result is curious as each population originated from the same ancestral bacterial clone, experienced the same selection pressures, and evolved phage-resistant bacteria over time. Previously, reduced MIC was observed for individual clones of bacteria that evolved resistance to phage OMK01, originating both from phage selection on standing genetic variation in the bacteria and when P. aeruginosa was subjected to short-term experimental evolution. This offers two possible explanations for the non-observed trade-off in treatments 2 and 3. In this study, instead of measuring MIC using individual clones, MIC was measured at the population level. It is possible that minority variants present in treatments 2 and 3 did exhibit a trade-off that was missed when
making measurements at the population level. Additionally, during the short-term coevolution study, individual bacterial clones in each experimental population experienced selection by co-evolving phages, but also contended with other bacterial competitors in the population. Different bacterial genotypes present in the population likely had differing relative fitnesses to each other. Perhaps the trade-off is less likely to occur when bacteria are forced to contend with multiple selection pressures, due to differences in relative fitness of individual bacterial clones.

Genomics may also help explain why the trade-off was only observed in one bacterial population. Results showed that there was more genetic variance in the treatment populations than in the control. Furthermore, bacteria in treatment 1, the only population where the trade-off was observed, had the most variants, particularly at passages 1 and 2 prior to the evolution of phage resistance in that treatment (Figure 3.3A). This greater genetic variation likely contributed to a higher likelihood of mutations present that increased individual fitness in the local environment; it is possible that this greater genetic variation, early in the experiment for treatment 1, contributed to the evolution of the observed trade-off between phage resistance and antibiotic resistance.

The observed changes in allele frequency over the course of the experiment demonstrated that all three treatment populations, as well as the control, underwent microevolution. A higher ratio of $D_n/D_S$ than $P_n/P_S$ for each treatment as well as the control indicated that these populations were likely undergoing positive selection. During positive selection, also known as Darwinian selection, certain genotypes are beneficially advantaged and can sweep to fixation, as opposed to negatively selected (i.e., purifying selection) where deleterious genotypes removed from the population. In the context of coevolution, positive selection is indicative of ARD while negative section is indicative of FSD. This supports the conclusions made from the time-shift assays (Figure 3.5); ARD seems to dominate the coevolutionary dynamics of this experiment.
Additionally, bacterial genomics revealed the loci that changed and likely contributed to phage resistance and to the trade-off observed in treatment 1. The treatment populations showed variants in genes involved in the biosynthesis of type-IV pilus, flagella and LPS, while the control presented no variants in these genes (Figure 3.7, Supplemental Table S3.2 and Supplemental Dataset S1). The treatment populations did not have any high frequency variants in multi-drug efflux genes such as oprM, the predicted receptor for phage OMKO1. Unpublished data suggests that unselected variants of phage OMKO1 use the type-IV pilus or the flagella of PA01 as receptors. This is interesting considering the convergence of variants with type IV pilus and flagella changes observed multiple times in the treatments. Transcriptional activator FleQ regulates FleRS a two-component regulatory system that controls flagellar biosynthesis; each population has a mutation in at least one of these genes that is fixed for one or more passages at some point over the course of the experiment. Treatments 1 and 2 had low frequency variants in pilB, mutants of which are defective in pili biogenesis, at early passages. Treatment 2 had an additional mutation in fimU, part of the prepilin operon, which was fixed at passage 3 and 4. Together, these mutations indicate that perhaps more variants in the phage population were using pili or flagella as receptors instead of the expected receptor, OprM. Further sequencing and analysis of phage genomes could uncover tail-fiber mutations that confirm this hypothesis. All three treatment populations had many mutations in various genes involved in LPS biosynthesis, particularly in genes involved in both A-band and B-band O-antigen synthesis and chain length regulation (wzz1, wzz2, wzy, wbpX, wbpY, wbpL and wbpZ). While phage OMKO1 is not expected to use LPS as a receptor, defects in LPS have been shown to result in a loss of both flagellar- and pili-based motility in P. aeruginosa.
Many of the mutations discussed above were observed transiently in treatments 2 and 3. However, treatment 1 had three mutations (A1188191G, G3546149A, and C1028191CG in \textit{fleQ}, \textit{wzz}_1, and \textit{wzz}_2 respectively) that rose to high frequency or fixed in passages 7 through 10 (Figure 3.7A), making it possible that these mutations are linked together on a single genotype that is at high frequency in the population. Furthermore, the timing of these mutations corresponds with the observation of the trade-off between phage resistance and resistance to tetracycline and erythromycin (Figure 3.5A), indicating that these mutations might be responsible for the trade-off in treatment 1. Loss of the O-antigen has been previously implicated in alteration of membrane permeability, due to the hydrophilic nature of the O-antigen \(^{168}\). Therefore, it is possible that the mutations in \textit{wzz}_1 and \textit{wzz}_2 are contributing to the trade-off observed between phage resistance and sensitivity to erythromycin, a hydrophobic antibiotic. This is in contrast to gentamicin where loss of the O-antigen has been shown to increase resistance to gentamicin; however, no change in resistance to gentamicin was observed in this study \(^{169}\). It is not surprising that the trade-off was not observed for ampicillin, as resistance is usually driven by beta-lactamases present in the cytoplasm of the bacteria \(^{170}\) and we did not expect phage selection to affect the expression of these enzymes. Resistance to meropenem and tetracycline is usually conveyed by the upregulation of multi-drug efflux pumps \(^{170}\); as no efflux mutations were observed in treatment 1 at passages 7 through 10, it is unsurprising we see no trade-off between phage resistance and meropenem resistance. However, resistance to tetracycline has also been reported to change with alterations in membrane permeability; therefore, it is possible that the same high-frequency mutations in \textit{wzz}_1 and \textit{wzz}_2 are contributing both to the trade-off between phage resistance and erythromycin resistance, as well as the trade-off observed between phage resistance and tetracycline \(^{168, 171}\).
Further experiments, using genotyped clones from later passages of treatment 1, could confirm this observation and will be the goal of future studies.

While the experimental evolution study presented here demonstrates that bacteria and lytic phages can both coexist and coevolve over a short time period, it also shows that there is more work to be done if we are to predict the outcomes of such interactions. Each treatment was initiated using identical starting material: media was taken from the same bottle, bacteria were taken from the same culture, and phages were taken from the same stock. We see the bacterial evolution of phage resistance, and convergence on a similar set of variants at the population level in the treatment populations. However, the bacterial phenotypes at the end of the 10-day passaging are not the same for each treatment population. Based on prior knowledge of phage OMKO1 selection resulting in a trade-off between phage resistance and antibiotic resistance, we expected to see this trade-off evolve in each treatment population. Instead, we observe this trade-off occurring only in bacteria from treatment 1. Although it is possible that the trade-off would have occurred in each treatment population if we had let the coevolution experiment play out for longer, the evolutionary dynamics occurring in treatments 2 and 3 seem to be occurring at a different pace than in treatment 1, demonstrating that experimental evolution may not be as repeatable as previously thought. The population level approach combined with the high temporal and genomic resolution used in this study likely contributed to the observations that highlighted the irreproducibility of evolution seen between the three experimental populations. This underscores the complexities involved in predicting evolutionary outcomes; even though the evolution that occurred in this study was repeatable to some extent, the predicted outcome of the evolution of phage resistance, namely the trade-off between phage resistance and antibiotic resistance, did not always occur. Clearly there is more to investigate before we will be able to accurately predict evolutionary outcomes.
Materials and Methods

Strains and Culture Conditions

*P. aeruginosa* strain PA01 and phage OMKO1 were the only microbes used in this study. All bacteria and phages were grown in Lysogeny Broth (LB) medium (10g tryptone, 5g yeast extract, 10g NaCl per L). Bacteria were grown on 1.5% LB agar plates and phages were grown with bacterial lawns in 0.75% LB top agar.

Experimental Evolution Design

Three treatments (PA01 bacteria plus phage OMKO1) were established. At the start of the experiment, phages and bacteria were mixed together in a sterile flask at multiplicity of infection (MOI; ratio of phage particles to bacterial cells) of ~0.0001 initial concentration (i.e., $10^4$ plaque-forming units [PFU] of phage OMKO1 and $10^8$ colony forming units [CFU] of PA01 bacteria per mL), in a final volume of 10 mL LB medium. One control (bacteria alone) was similarly established, without input phages. After overnight incubation at 37°C with 200 rpm shaking, each treatment and control was passaged 1:100 into a new sterile flask with 9.9mL fresh LB medium. This process of 1:100 serial transfer was repeated for a total of 10 experimental passages.

Bacterial Phenotypic-Challenge Assays

At each passage of the experimental evolution study, a sample from each overnight treatment and control was diluted and plated on LB agar; after overnight incubation at 37°C visible colonies formed. At random, 96 individual colonies were picked from each plate and resuspended in 200µL LB medium. The goal was to compare how 96 randomly-isolated colonies (clones) from each treatment and control grew across three different challenge environments, throughout the experiment; 3840 clones total were examined (96 clones from 4 treatments/control over 10 passages). To do so, an equal volume of each resuspended colony was used to inoculate a single
well in each of three 96-well plates, which contained either LB medium, LB plus phage OMKO1 at MOI greater than 10, or LB with 3mg/L tetracycline. The 96-well plates were then placed in a static 37°C incubator. After 18 hours, plates were removed from incubation and optical density at wavelength 600 nm (OD$_{600}$) was measured for the bacteria in each well, using a spectrophotometer (Tecan Infinite F500 microplate reader). Fitness of a bacterial clone in the challenge environment (phage OMKO1; tetracycline) was defined as the observed OD$_{600}$ in that environment divided by observed OD$_{600}$ in the control environment (LB medium). Ratio estimates greater, equivalent and less than 1.0 indicated that the clone was more fit, equally fit or less fit in the challenge environment, respectively.

Antibiotic Resistance Measurements
The minimum inhibitory concentrations (MIC) of tetracycline, erythromycin and ampicillin antibiotics that were effective in impacting growth of a bacterial strain/population were determined using Etest strips (Biomerieux). Briefly, an overnight culture of bacteria was diluted to a McFarland standard of 0.5 and spread on Mueller-Hinton agar plates. The bacterial lawn was allowed to dry and an Etest strip containing was placed on top of the lawn. After overnight incubation at 37°C the plate was scored by recording the lowest concentration of antibiotic that inhibited growth (zone of clearing) of the bacterial lawn.

The MIC necessary to inhibit the growth of 90% of a bacterial population (MIC90) was determined for tetracycline, erythromycin, gentamicin and meropenem using a direct plating method. Briefly, serial dilutions of a test bacterial culture were plated on LB plates and on LB plates containing a range of two-fold dilutions of each antibiotic (tetracycline: 4 and 8 mg/L; erythromycin: 64, 128 and 256 mg/L; gentamicin: 0.5, 1 and 2 mg/L; and meropenem: 0.25 and
0.5 mg/L). After overnight incubation at 37°C, CFU were counted for each plate and MIC90 was recorded as the antibiotic concentration that reduced the number of CFUs to 10% or less of the CFU observed on LB without antibiotics.

**Sequencing and Variant Calling**

Bacterial genomes were extracted using a previously described protocol \(^{141}\) and sequencing libraries were made using Nextera XT library preparation kits (FC-131-1096, Illumina). Paired-end 150 base pair reads were generated on an Illumina HiSeq 2500 rapid run. High-quality reads were aligned to the PA01 reference genome (Genbank Accession # AE004091.2) using Bowtie2 \(^{143}\). Variants were called using GATK \(^{174}\). Only high-quality variants (GATK quality score > 200) that were not in intergenic or prophage regions were considered for further analysis in this study.
Experimental setup of short-term coevolution experiment. All populations were started from a clonal PA01 culture. Three treatment populations of phages and bacteria (blue, purple and green) were initiated at an MOI of ~0.0001. One control of bacteria only (grey) was started at the same time. All populations were passaged 1:100 every 24 hours into fresh media.
Figure 3.2 Bacterial density and phage titer

Bacterial density and phage titer A Bacterial density measured as OD600 at the time of each passage for 10 days for treatment 1 (blue), treatment 2 (purple), treatment 3 (green) and the control (grey). B Phage titers of OMK01 from treatment 1 (blue), treatment 2 (purple) and treatment 3 (green) were measured on the contemporary control.
Figure 3.3 Bacterial fitness in the presence of contemporary phages

Bacterial fitness in the presence of contemporary phages for approximately 96 randomly selected bacterial clones from A treatment 1, B treatment 2 and C treatment 3. Bacterial clones from D the control were challenged to grow in the presence of contemporary phages from treatment 1. Here fitness is defined as the ratio of bacterial density in the phage challenge to the bacterial density in media. For each population, a linear regression was performed to determine the change in fitness overtime.
Impact of phage populations on ancestral bacteria. **A-C** Bacterial growth curve of ancestral PA01 alone (grey dotted line) or in the presence of phage populations at each passage from treatment 1, treatment 2, and treatment 3 respectively. Each growth line represents the average of three technical replicates. **D** The first maximum OD600 measurement for ancestral PA01 grown in the presence of phage populations at each passage from treatment 1 (blue), treatment 2 (purple) and treatment 3 (green). Error bars represent standard deviation of three technical replicates.
Figure 3.5 Coevolutionary dynamics

Coevolutionary dynamics **A** Time shift assays to determine the proportion of phage resistant bacterial populations. The dashed line at 0 are assays of bacterial populations with contemporary phage populations. Negative numbers along the x-axis represent assays of bacterial populations with past phage populations. Positive numbers along the x-axis represent assays of bacterial populations with future phage populations. **B** Time shift assays to determine the proportion of infective phage populations to time shifted bacterial populations.
Bacterial fitness in the presence of 3mg/L of tetracycline for approximately 96 randomly selected bacterial clones from A treatment 1, B treatment 2, C treatment 3 and D the control. Here fitness is defined as the ratio of bacterial density in the tetracycline challenge to the bacterial density in media. For each population, a linear regression was preformed to determine the change in fitness overtime.
Figures 3.7 Frequencies of new alleles

Frequencies of new alleles of genes involved in type IV pilus biogenesis (grey), flagellar synthesis (grey), and LPS biosynthesis (B-band genes in solid line and A-band genes in dotted line) of A treatment 1 (blue), B treatment 2 (purple), and C treatment 3 (green).
Table 3.1 Types of mutations

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<th>Control</th>
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The total number of synonymous & fixed (D_s), non-synonymous & fixed (D_n), synonymous & polymorphic (P_s) and non-synonymous & polymorphic (P_n) mutations in coding regions for treatments 1, 2 and 3 and the control.
Chapter 3 Appendix

Supplemental Figure S3.1 Fitness in the presence of contemporary phages

Fitness in the presence of contemporary phages for approximately 96 randomly selected bacterial clones from the control challenged to grow in the presence of contemporary phages from A treatment 2 or B treatment 3. Here fitness is defined as the ratio of bacterial density in the phage challenge to the bacterial density in media. A linear regression was preformed to determine the change in fitness overtime.
Supplemental Figure S3.2 Minimum inhibitory concentrations of each population

Minimum inhibitory concentration for each passage of treatment 1 (blue), treatment 2 (purple), treatment 3 (green) and the control (grey) of A tetracycline, B erythromycin, and C ampicillin. Measurements were made at the population level using Etest strips.
**Supplemental Figure S3.3** Heat maps of new allele frequencies at each passage for A treatment 1, B treatment 2, C treatment 3 and D the control.
**Supplemental Table S3.1** MIC90 of tetracycline, erythromycin, meropenem and gentamicin

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Minimum inhibitory concentration to kill 90% of bacteria (MIC90) of tetracycline, erythromycin, meropenem and gentamicin made in duplicate for each passage of treatment 1, treatment 2, treatment 3, and the control. (> indicates the MIC90 was higher than the range of concentrations examined and ≤ indicates the lowest concentration examined was sufficient to kill 90% of the colony forming units, but the actual MIC90 could be lower)
**Supplemental Table S3.2** Location, mutation and frequencies of new alleles

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105
**Supplemental Table S3 cont.** Location, mutation and frequencies of new alleles

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Location, gene, mutation type and frequencies of new alleles found in treatment 1, treatment 2 and treatment 3 plotted in Figure 3.7.
**Supplemental Datasets**

Supplemental Dataset 1 can be found on Dryad (doi: 10.5061/dryad.1g1jwstvp). This is a table of the location, mutation, and frequencies of all high quality (Q>200) found in treatment 1, treatment 2, treatment 3 and the control.
Chapter 4: Selection for phage resistance reduces virulence of Shigella flexneri
Preface

Chapter 4 has been adapted from a manuscript that is intended for submission to PLOS Pathogens in Spring, 2021.

This study was designed by Kaitlyn Kortright and Dr. Benjamin Chan. Kaitlyn Kortright and Rachel Done preformed experiments. Kaitlyn Kortright analyzed data. The manuscript was prepared and edited by Kaitlyn Kortright, Dr. Benjamin Chan and Dr. Paul Turner. Raw sequencing will be publicly available at NCBI Sequence Read Archive Project # PRJNA713796. We thank Kristen Parent and John Wertz for kindly providing strains used in this study. Additionally, we would like to thank Valeria Souza for water samples and Dylan Sloan and Akshay Mody for technical support and assistance with experiments conducted in the early stages of this study. This project was supported by an award from Project High Hopes Foundation, and a portion of the resources to conduct genome-sequencing came from an award to Felix Biotechnology.
Abstract

There has recently been an increasing interest in phage therapy as an alternative option for infection management, especially when phages have been shown to cause an evolutionary trade-off between phage resistance and bacterial fitness traits such as virulence. A vast repertoire of virulence factors allows *Shigella flexneri* to invade gut epithelial cells, replicate intracellularly, and evade the immune system through intercellular spread. In particular, the intercellular spread stage of *S. flexneri* infection causes tissue damage, contributing to mortality. It has previously been shown that the porin OmpA is necessary for the polar localization of virulence factor IcsA, which polymerizes host actin and allows the bacterium to be motile inside host cells. We hypothesize that a phage which uses OmpA as a receptor to recognize, bind and infect *S. flexneri*, will select for phage-resistant bacteria that are attenuated for intercellular spread. Here we show that a naturally isolated *Myoviridae* phage, A1-1, requires OmpA as a receptor. Using phenotypic traits observed from efficiency of plaquing assays, membrane integrity assays, total LPS measurements, MIC measurements as well as phage cross resistance profiles, five A1-1 resistant *S. flexneri* mutants can be placed into two phenotypic groups. R1 and R2 phenotypically resemble *ompA* knockouts and R3, R4 and R5 phenotypically resemble LPS-deficient strains. Whole genome sequencing confirms that R1 and R2 have mutations in *ompA*, while R3, R4 and R5 have a mutation in *gmhA*, a mutation in *gmhC* and a 30,000 base pair deletion spanning *gmhA*, respectively. Bacterial plaque assays confirm that all five phage-resistant mutants are unable to spread intercellularly. Thus, selection for phage A1-1 resistance results in a trade-off between phage resistance and bacterial virulence.
Chapter Introduction

As a facultative, intracellular pathogen and a causative agent of bacillary dysentery, *Shigella flexneri* is a Gram-negative bacterium of medical importance. Fecal-oral transmission of these bacteria is common and usually occurs via contaminated drinking water \(^{175}\). Bacteria rapidly traverse the gastrointestinal tract to invade colonic epithelial cells. Upon invasion, both chromosomally-encoded and plasmid-encoded virulence factors enable *S. flexneri* to overcome host immune responses, replicate intracellularly, and spread to neighboring epithelial cells \(^{176}\). In particular, intercellular spread of *S. flexneri* is a virulence trait that damages colonic epithelial cells, destroying barrier function and resulting in severe shigellosis and dysentery.

Shigellosis is a major public health problem in low-income countries that lack dependable water sanitation, and this disease contributes to approximately 1.3 million deaths annually \(^{177}\). Children under the age of five account for almost 70% of the total mortality, primarily for cases in sub-Saharan Africa and southern Asia \(^{178}\). Antibiotic treatment for shigellosis can rapidly improve patient outcomes \(^{178}\). However, many of the recommended antibiotics are expensive and increasingly ineffective due to bacterial resistance to antibiotics, making them difficult to implement with limited health services \(^{178}\). Thus, shigellosis remains a persistent cause of mortality, particularly for young children, with insufficient practical options for disease management. Therefore, alternatives to expensive and logistically challenging antibiotic treatments for shigellosis could valuably reduce disease burden, especially in children.

As the rates of antibiotic resistance in bacteria continue to increase alarmingly, complementary treatments to control bacterial infections are quickly being considered. One such treatment is phage therapy, the clinical use of bacteriophages (phages), viruses that specifically infect bacteria, to treat bacterial infections \(^{179}\). In particular, lytic phages are considered good
candidates for phage therapy due to their predator-like effect on susceptible host bacteria. The lifecycle of a lytic phage involves attachment to one or more receptors on the surface of a bacterium, introduction of genomic material to the cytoplasm, intracellular replication, transcription and translation of phage genes, assembly of new phage particles, and lysis of the bacterial host to repeat the cycle. There are recent reports of implementation of phage therapy to treat refractory bacterial infections, particularly those that are resistant to multiple antibiotics. While phage therapy has demonstrated potential and promise as a complementary therapy to antibiotics, it is expected that these viruses should select for evolution of phage-resistance in target bacteria. Therefore, it is crucial to design rational phage treatments, so that evolution of phage resistance is leveraged as a possible benefit, rather than necessarily constituting a barrier to effective treatment.

The concept of evolutionary trade-offs permeates evolutionary biology. Trade-offs occur when an organism evolves a new phenotype that improves fitness in a certain environment at the cost of decreased fitness in another environment. In the context of phage therapy, it should be possible to identify phages that direct the evolution of their host bacteria, such that evolution of phage resistance, on average, results in a clinically-useful fitness trade-off. In particular, by choosing a lytic phage that exerts selection pressure on an antibiotic-resistance and/or virulence mechanism, the phage-susceptible bacteria are killed while the remaining population is enriched for phage-resistant variants with reduced drug resistance and/or virulence. In either case, the phage treatment would cause evolution of phage resistance to be a benefit, rather than a liability.

To implement a rationally designed phage treatment that selects for a virulence trade-off in bacteria, a surface-expressed virulence factor must be identified that the phage uses for binding. Intercellular spread of *S. flexneri* contributes greatly to tissue damage and its accompanying
symptoms, while providing the bacteria refuge from immune system detection and from antibiotics that poorly transit across eukaryotic cell membranes. One of the plasmid-encoded virulence factors of *S. flexneri*, IcsA, recruits and polymerizes host cell actin on one end of the bacterium to create actin tails. These actin tails move the bacterium around the host cytoplasm, occasionally propelling the bacterium into the membrane, causing protrusions which can lead to spread of the bacterium to a neighboring host cell. IcsA is both surface-expressed and essential for *S. flexneri* virulence, suggesting its potential usefulness as a binding receptor for a phage therapy candidate. However, IcsA is plasmid-encoded, indicating that this virulence factor might be spontaneously lost from target bacteria during cell division, and may be capable of horizontally transferring into cells of non-target bacteria. For this reason, a better strategy would be to discover a phage that interacts with a known chromosomally-encoded virulence factor of *S. flexneri* bacteria, rather than targeting a genetic element that could be more easily gained or lost. Recent reports have implicated other outer membrane proteins as necessary for intercellular spread of *S. flexneri*, including OmpA, a highly conserved outer-membrane porin: a pore or channel in Gram-negative bacteria that translocates small molecules across the membrane. In *S. flexneri*, OmpA is surface-expressed, required for virulence, and chromosomally encoded; these three properties make it an attractive target for testing our hypothesis due to its potential as a phage receptor that could be leveraged to select against virulence in this pathogen.

In this study, we describe the recently-isolated lytic phage A1-1, a dsDNA virus discovered in a waste water sample obtained in Cuatro Cienegas, Mexico, a geographic region renowned for its extreme microbial diversity. Characterization of phage A1-1 revealed that it was a member of the *Myoviridae* family, and that the virus naturally used OmpA as a binding receptor to infect susceptible *S. flexneri* bacterial cells. We hypothesized that phage A1-1 would kill its host bacteria
while selecting for evolution of resistance in the bacterial population, to cause a trade-off in decreased virulence (i.e., reduced capacity for phage-resistant mutants to undergo intercellular spread). We randomly isolated five spontaneous phage-resistant mutants, and results of our phenotypic and genotypic characterizations revealed that these strains could be placed generally into two different groups. Nevertheless, our data confirmed that all isolated mutants suffered the predicted trade-off: these bacteria could invade and replicate in mammalian-derived cells in a tissue culture model of *S. flexneri* infection, but the evolved phage resistance was always associated with attenuated virulence because the mutants were incapable of intercellular spread.

**Results**

**Novel lytic phage A1-1 is a Myoviridae virus that binds to OmpA of Shigella flexneri**

We predicted that naturally-occurring lytic phages of *S. flexneri* have evolved to exploit OmpA as a receptor. We sought to isolate such a phage from water samples, assuming that these contained high levels of microbial biodiversity. From waste water obtained in Cuatro Cienegas, Mexico, phage A1-1 was purified on *S. flexneri* strain PE577 using classical phage-isolation techniques, and was then grown using *S. flexneri* strain M90T as a host in the below experiments.

We used SPAdes\(^{188}\) for *de novo* assembly of the phage A1-1 genome, and results showed a tentative genome size of 104,552 base pairs. Phage A1-1 was observed to have a GC content of 35.89%, whereas the host bacteria had a GC content of 50.9%. Preliminary annotation of phage genes using PHASTER\(^{189}\) revealed 139 coding regions (Figure 4.1A) with no indication that genes for lysogeny (temperate phage life cycle) were present. Sequence alignment suggested that phage A1-1 was similar to myoviruses known to infect *Escherichia coli*. Transmission electron microscopy (TEM) revealed that phage A1-1 had a long, contractile tail (Figure 4.1B); this
morphology is consistent with the typical structure of virus particles of the *Myoviridae* family of dsDNA viruses.

We then conducted phage growth curves, in triplicate, that estimated key features of the phage A1-1 lytic reproductive cycle, and tested its ability versus inability to infect wild-type and OmpA knockout strains of *S. flexneri*, respectively. Results showed that phage A1-1 had a latent and eclipse period of approximately 30 minutes, and a burst size of roughly 12 phage particles per lysed bacterial cell (Supplemental Figure S4.1A). Furthermore, these data confirmed that phage A1-1 was able to grow on M90T bacteria, but was unable to infect M90TΔompA lacking the putative OmpA binding site.

To determine conclusively whether phage A1-1 required OmpA to bind and infect *S. flexneri* cells, we performed adsorption assays to measure cell binding, and efficiency of plaquing (EOP) assays to measure infectivity of phage A1-1. Results of replicated (*n* = 3) adsorption assays showed that phage A1-1 was unable to adsorb to both M90TΔompA and M90TΔompA:pSF-vector (empty vector control); in both cases, the slope of the linear regression for observed plaque forming units (PFU) over time was not statistically significantly different than zero. However, we observed that phage A1-1 was able to adsorb to wild-type M90T as well as M90TΔompA:pSF-ompA, (Figure 4.1C). Similarly, EOP assays performed in triplicate (Supplemental Figure S4.1B) showed that phage A1-1 was able to infect M90TΔompA:pSF-ompA with an EOP of 0.365±0.15 (mean ± sem), but unable to infect M90TΔompA or M90TΔompA:pSF-vector with EOPs below the limit of detection (200 PFU/mL) on both strains. Finally, we examined growth kinetics of *S. flexneri* in the presence of phage A1-1 using bacterial growth curves. As expected, growth of the susceptible wild-type strain, M90T, was completely suppressed in the presence of phage over the 15-hour assay (Supplemental Figure S4.1C). Conversely, growth of M90TΔompA and M90TΔompA:pSF-
vector strains were both unimpacted by phage A1-1 (Supplemental Figure S4.1C). While growth of M90TΔompA:pSF-ompA was suppressed by phage presence, the reduced growth was less severe compared to the M90T wild-type, likely due to differences in chromosomal versus exogeneous expression of OmpA (Supplemental Figure S4.1C). Taken together, the above results showed that phage A1-1 was a naturally-occurring virus that could be isolated and characterized as requiring OmpA to bind and initiate infection of *S. flexneri* host bacteria.

**Phage A1-1 selects for two different phenotypes of phage-resistant mutants**

Since the above results showed phage A1-1 used OmpA to infect *S. flexneri*, we hypothesized that phage resistance would involve modification of the phage receptor and that phage-resistant mutants would be phenotypically similar to M90TΔompA. Fluctuation assays revealed that phage A1-1 selected for phage-resistant mutants of M90T at a frequency of $4.18 \cdot 10^{-7} \pm 1.22 \cdot 10^{-7}$ (mean ± sd). Five spontaneous phage-resistant mutants (hereafter, R1, R2, R3, R4 and R5) were independently isolated and randomly chosen for further characterization.

Using similar assays as above, we sought to confirm that all five mutants showed traits associated with phage resistance. As expected, phage A1-1 had an EOP below the limit of detection (200 PFU/mL) on each resistant mutant (Figure 4.2A). Similar to data for M90TΔompA presented above, all five resistant mutants did not support phage adsorption, indicated by the slopes of linear regressions that were not statistically significantly different than zero (Figure 4.2B). Also, growth of the phage-resistant mutants was not visibly altered by the presence of phage A1-1, while growth of wild-type M90T was completely suppressed by phage A1-1 (Figure 4.2C). We noted that mutants R1 and R2 grew to final densities similar to the M90T ancestor, but mutants R3, R4 and R5 reached final densities that were roughly two-fold less than the wild-type (Figure 4.2C). Furthermore, R1 and R2 produced phenotypically ‘smooth’ colonies on agar, while R3, R4, and
R5 grew as ‘rough’ colonies on agar, indicating that the latter mutants were potentially altered for lipopolysaccharide (LPS) production. In summary, these results confirmed that all of the mutants had phenotypic traits consistent with resistance to phage A1-1 infection, but the strains could be separated into two groups (R1 and R2; R3, R4 and R5).

To further characterize the five phage-resistant mutants, we used assays that investigated membrane permeability, LPS quantity per cell and resistance to several antibiotics, because changes (relative to wild-type) in these traits were anticipated if phage resistance was conferred by altering OmpA and/or LPS. Using membrane-integrity assays as proxies for cell permeability, results showed that permeability of resistant mutants R1, R3 and R5 was statistically significantly lower than that of the M90T wild-type (Figure 4.3A). Total LPS measurements revealed that while R1 and R2 had similar amounts of LPS per colony forming unit (CFU) compared to M90T and M90TΔompA, R3, R4 and R5 showed almost an order of magnitude more LPS per CFU (Figure 4.3B). It was expected that alterations to OmpA and LPS would result in changes in minimum inhibitory concentration (MIC) for certain antibiotics. Mutants R3, R4 and R5 showed a significant decrease in mean fold change (as compared to wild-type) in MIC of erythromycin (R3: 0.042±0.015 sem; R4: 0.056±0.009 sem; R5: 0.071±0.012 sem) (Figure 4.3C). Mutant R5 presented a significantly decreased fold change in ciprofloxacin MIC, 0.667±0.083 sem (Figure 4.3D). Mutant R1 showed a significantly decreased fold change in vancomycin MIC, 0.438±0.188 sem, while mutants R3 – R5 had significantly increased fold changes in vancomycin MICs (R3: 2.33±0.417 sem; R4: 2.25±0.334 sem; R5: 2.25±0.334 sem) (Figure 4.3E). Fold change in MIC of tetracycline was not different from wild-type for all five mutants (Figure 4.3F). We further concluded that phage A1-1 tended to select for phage-resistant mutants in two groups; R1 and R2 likely have mutations affecting OmpA, while R3, R4 and R5 likely have mutations altering LPS.
To further investigate the potential role for LPS in phage resistance, EOPs were measured on *E. coli* knockout strains for genes involved in LPS biosynthesis. The EOP of phage A1-1 was below the limit of detection when the following fifteen *E. coli* genes were knocked out: *ompA*, *galU*, *waaC*, *gmhA*, *gmhB*, *gmhC*, *gmhD*, *waaQ*, *waaP*, *waaY*, *waaG*, *waaO*, *waaJ*, and *waaB* (Supplemental Figure S4.2). EOP measurements for phage A1-1 on BW25113ΔicdC and BW25113ΔompC controls, as well as on BW25113ΔwaaS and BW25113ΔwaaL knockouts, were not statistically different from phage ability to grow on wild-type BW25113 (Supplemental Figure S4.2). In contrast, the EOP of phage A1-1 on BW25113ΔwaaZ was slightly improved, compared to wild-type BW25113 (Supplemental Figure S4.2). These results suggested that LPS biosynthesis genes *waaS*, *waaL*, and *waaZ* were not determinants of phage A1-1 growth. From these results, we concluded that portions of *S. flexneri* LPS were required somehow for phage A1-1 infection.

Since our earlier results showed that phage A1-1 used OmpA to bind and initiate infection of *S. flexneri*, we anticipated that the genetics underlying phage resistance should be governed by alterations of OmpA, and not by changes in LPS. While results above (Figure 4.1) hinted that OmpA might be the only receptor for phage A1-1, EOP data from the *E. coli* LPS knockout strains implied that further tests were needed to examine whether LPS also served as a receptor. To that end, we tested whether resistance to phage A1-1 additionally altered susceptibilities of the resistant mutants to previously-characterized phages 60B and T7. Phage 60B putatively requires OmpC as a receptor. Our results showed that wild-type M90T, and mutants R1 and R2 were susceptible to phage 60B infection, indicating that this constituted the ancestral phenotype, whereas our data found that mutants R3, R4 and R5 displayed resistance to phage 60B (Supplemental Table S4.1). *E. coli* phage T7 uses the inner core of LPS as a receptor. Results showed that wild-type M90T,
R1 and R2 were resistant to phage T7, indicating this was the ancestral phenotype; while R3, R4 and R5 were susceptible to phage T7 (Supplemental Table S4.1). Differences in phage T7 resistance again suggested that there were putative LPS changes conferring phage A1-1 resistance in mutants R3, R4, and R5. Moreover, the differences in phage 60B resistance for these strains compared to wild-type suggested that LPS changes may be broadly affecting the structure of different outer membrane porins. Therefore, we hypothesized that while LPS might be necessary for A1-1 infection, it was not serving as a receptor; rather, LPS could serve to maintain the appropriate structure of the phage A1-1 binding receptor OmpA.

Western blots were used to determine whether the five resistant mutants expressed OmpA. Blots for OmpA of whole cell lysates showed that resistant mutants R1 and R2 did not express OmpA; however, R3, R4 and R5 expressed OmpA to a similar degree as the wild-type control (Figure 4.4A). Furthermore, western blots of fractioned cell lysates revealed that R3, R4 and R5 expressed OmpA at the membrane (Figure 4.4B). These results supported our hypothesis that LPS was likely not a receptor for phage A1-1, but instead was maintaining a conformation of OmpA that was required for phage A1-1 binding.

Based on differing results of observed colony morphologies, MICs of various antibiotics, phage A1-1 infection ability on LPS mutants of E. coli, and western blots for OmpA, we concluded that phage-resistant mutants R1 and R2 phenotypically resembled the M90TΔompA knockout and did not express this OmpA, while R3, R4 and R5 appeared to be phage A1-1 resistant due to LPS deficiencies.

**Whole genome sequencing reveals underlying mutations conferring resistance**

Whole genome sequencing was used to determine whether the genotypes of the resistant mutants matched our inferences based on the above-described phenotypes. Genomes of all five resistant
mutants were sequenced, aligned to the reference genome for the wild-type M90T ancestor (GenBank # CM001474.1), and GATK was used to identify variants. Results showed that both R1 and R2 had a nonsense mutation in *ompA*, resulting in a premature stop codon after amino acid 172 (Figure 4.5). R3 had a nonsense mutation in *gmhA* that resulted in a premature stop codon after amino acid 176 (Figure 4.5). R4 had a transversion at base pair 787 of *gmhC*. Finally, R5 had a 29,645 base pair deletion between two insertion sequence 1 (IS1) elements at positions 303,277 and 332,873; this deletion included 27 genes, one of which was *gmhA* (Figure 4.5). The EOP of phage A1-1 was restored to levels observed on wild-type *S. flexneri*, when R1 and R2 were complemented with plasmid pSF-*ompA*, R3 and R5 harbored plasmid pSF-*gmhA*, and R4 contained plasmid pSF-*gmhC* (Figure 4.2A). Altogether, whole genome sequencing and EOP assays confirmed that R1 and R2 were *ompA* mutants, while R3, R4 and R5 were LPS-deficient mutants.

**Phage-resistant mutants are attenuated for intercellular spread**

We originally sought to test the prediction that a phage requiring OmpA to bind and infect *S. flexneri* would select for phage-resistant mutants that were attenuated for intercellular spread. Virulence of phage-resistant mutants was assayed using intracellular replication assays and plaque assays in primate-derived Vero cells grown in laboratory tissue culture. As expected, M90T and M90TΔ*ompA* bacteria were capable of invading and replicating in Vero cells, while the negative control, M90TΔ*mxIH*, a knockout for the type III secretion system needle, was unable to invade (Figure 4.6A). All five of the phage-resistant mutants were able to invade and replicate intracellularly; R1, R2, R3 and R5 replicated to levels similar to M90T at the 7-hour timepoint while R4 replicated 10-fold less than M90T by this time (Figure 4.6A). Plaque assays revealed that wild-type M90T (Figure 4.6B) could spread intercellularly; in contrast, neither M90TΔ*ompA*
nor M90TΔmxiH (Figure 4.6B,C) could spread intercellularly, and none of the phage-resistant mutants (Figure 4.6D-I) could spread between cells. Therefore, although all five resistant mutants were able to invade and replicate intracellularly, as predicted they were unable to spread intercellularly, indicating that resistance to phage A1-1 led to evolution of reduced virulence in S. flexneri.

**Discussion**

Classical phage biology assays, including adsorption assays, EOPs and growth curves suggest that phage A1-1 uses OmpA as a receptor. A1-1 selected for two different phenotypes of phage-resistant mutants. R1 and R2 formed smooth colonies, exhibited decreased membrane permeability as compared to wild-type, and were more sensitive than wild-type to vancomycin; R3, R4 and R5 formed rough colonies, exhibited decreased membrane permeability as compared to wild-type, were more sensitive than wild-type to erythromycin, were more resistant than wild-type to vancomycin, had greater total LPS per CFU than wild-type, and showed altered phage resistance profiles to phages 60B and T7. Whole genome sequencing revealed that A1-1 selected for resistant mutants with mutations in *ompA* (R1 and R2). However, A1-1 also selected for resistant mutants in *gmhA* (R3 and R5) and *gmhC* (R4), two genes involved in the biosynthesis of the heptose sugars that make up the inner core of LPS. Resistant mutants R3, R4 and R5 all express OmpA on the membrane and phage A1-1 fails to adsorb to these resistant mutants. This leads to two possibilities: phage A1-1 both OmpA and LPS as co-receptors, or phage A1-1 uses OmpA as a receptor and LPS is somehow required for binding but is not a receptor.

While the first possibility cannot be ruled out in this study, it is worth noting that plaques of A1-1 were never observed on M90TΔ*ompA* even when high titers of A1-1 were plated. If both OmpA and LPS are receptors for A1-1, selective pressure to make more progeny and standing
genetic variation would likely lead to variants of phage A1-1 that could plaque on M90TΔompA, using only LPS as a receptor. This was not observed; instead, it seems likely that A1-1 uses OmpA as a primary receptor and that LPS is required for binding either as a secondary receptor or to maintain the proper conformation of OmpA for A1-1 binding. Since resistant mutants R3, R4 and R5 all express OmpA and no phage adsorption to these resistant mutants was observed, it seems unlikely that LPS is being used as a secondary receptor by phage A1-1. Therefore, perhaps LPS is involved in maintaining a particular conformation of OmpA that is necessary for phage binding. Indeed, it has been previously observed that complex interactions between LPS and outer membrane proteins (OMPs) are involved in both OMP biogenesis and stabilization of particular conformations of various OMPs. Three different conformations of *E. coli* OmpA have been proposed: a monomeric narrow pore conformation with two domains (an eight stranded β-barrel and a periplasmic domain), a monomeric large pore conformation with a single domain (sixteen stranded β-barrel), and a dimer of the two-domain narrow pore structure. OmpA in M90T appears to be localized to both the periplasm and membrane fractions (Figure 4.3B), indicating that perhaps the two-domain narrow pore structure with the periplasmic domain is the main conformation of OmpA in *S. flexneri*. Interestingly, OmpA in resistant mutants R3, R4 and R5 is localized more in the membrane fraction than in the periplasmic fraction (Figure 4.3B), possibly indicating that the single domain large pore structure dominates the conformation state of OmpA in these LPS-deficient mutants. Indeed, it has been previously suggested that core LPS plays a role in maintaining the conformation of OmpA. Experiments in the current study seem to support this hypothesis.

When *S. flexneri* invades and replicates within human host cells, it is known to spread intercellularly via actin-based motility which occurs through polymerization of host actin by
unipolar IcsA on the bacteria. It is previously demonstrated that O-antigen null mutants (i.e., those with truncated LPS) are attenuated for intercellular spread\textsuperscript{197}. Therefore, it is perhaps unsurprising that the phage-resistant LPS mutants R3, R4 and R5 are capable of intracellular invasion and replication but not intercellular spread. However, the mechanism behind why O-antigen confers the ability for \textit{S. flexneri} to spread intercellularly has not been fully elucidated. Furthermore, prior work shows that OmpA is required for intercellular spread\textsuperscript{184}; as expected, our isolated phage-resistant mutants with OmpA changes, R1 and R2, are similarly defective for this virulence trait. Interestingly, the shared \textit{ompA} mutation that likely confers phage resistance in R1 and R2 is in the region that likely composes the final predicted extracellular loop of the eight-stranded $\beta$-barrel conformation and truncates OmpA at position 174. It is observed that OmpA residues 188-190 interact with a periplasmic protein, PhoN2, to maintain polarity of IcsA\textsuperscript{198}. Therefore, if these two resistant mutants express a partial peptide of OmpA, where residues 1-169 are wild-type and 170-173 are frameshifted, this partial protein likely is neither suitable for phage A1-1 adsorption nor has the region predicted to interact with PhoN2 to maintain polarity of IcsA. This perhaps explains the observed defect in intercellular spread observed for phage-resistant mutants R1 and R2.

It is unsurprising that \textit{ompA} mutants and rough mutants of \textit{S. flexneri} in the current study are attenuated for intercellular spread based on the previous literature, but it is noteworthy that these mutants were obtained strictly via selection for resistance to phage A1-1. In all five randomly-chosen mutants, phage resistance led to attenuation for intercellular spread; thus, selection for resistance to phage A1-1 in \textit{S. flexneri} consistently traded off with maintenance of a key virulence factor in this biomedically-important pathogen. While this interesting result yields insights into possible \textit{S. flexneri} interactions with lytic phages, expectations for phage-bacteria interactions in natural environments and in the context of clinical infection are harder to predict.
The epidemiology of *S. flexneri* typically involves colonization of an index human case (presumably via consumption of contaminated food or water) which then can be fecal-orally transmitted to household members and other nearby individuals. As a facultative intracellular pathogen, *S. flexneri* is capable of growth inside of human host cells, but can also exist as “free-living” bacteria in the natural environment. During clinical infections, bacteria are presumably protected from phage attack while replicating inside colonic epithelial cells; however, during fecal-oral transmission, bacteria are free-living and once again vulnerable to phage. This is an example of an alternating environment with intermittently-changing selection pressures experienced by the bacteria. That is, aside from other possible selection pressures such as escape from host immunity, a population of *S. flexneri* may sometimes experience selection to avoid phage infection, and at other times can experience absence of this selection if intracellular invasion provides a refuge from phage exposure. Under such complex conditions, selection would positively favor evolution of phage resistance in free-living bacteria; in this ecology, the mutations underlying phage resistance would be beneficial and positively selected. However, inside intestinal epithelial cells, the ecology presents a different target for selection; here, the mutations for phage resistance observed in this study would be deleterious because of the trade-off we observed between phage resistance and intercellular spread.

This scenario stimulates two predictions: (1) evolution of phage resistance (i.e., positive vs. negative selection for this trait) should occur differently for facultative intracellular pathogens such as *S. flexneri*, depending whether the bacterial population is growing inside versus outside of the human host; and (2) the particular phage-resistant mutants in the current study should experience a net fitness cost if the frequency of environmental fluctuations necessitate intracellular growth and spread during infection of human host cells. The first prediction is the subject of
ongoing studies in our laboratory to examine spontaneous phage-resistant mutants of *S. flexneri* selected under simultaneous selection pressures of phage A1-1 and intracellular growth constraints in Vero cells. The second prediction can be examined by subjecting the phage-resistant mutants in the current study to experimental evolution that toggles between batch culture and tissue culture environments. Here, the outcome can be informed by previous studies of microbial evolution in fluctuating environments. For example, it is observed that phages subjected to elevated-temperature selection (i.e., heat shocks) outside of bacterial cells results in evolution of greater thermotolerance (increased particle stability) to withstand degradation in elevated temperatures, despite the deleterious nature of these mutations for phage reproduction in benign temperature environments. Thus, antagonistically-pleiotropic mutations can evolve in microbes, even though these changes confer high fitness in only a portion of the selective environment. We might expect that a fluctuating environment would cause the particular phage-resistant mutants in our study to undergo further evolution, such as compensation for their complete inability to spread intercellularly, to perhaps maintain some degree of phage resistance while also achieving minimal intercellular spread (a compromise). In contrast, our study may reveal that evolution of phage resistance creates a strong evolutionary constraint, whereby acquisition of phage A1-1 resistance prevents *S. flexneri* from readily undergoing compensatory evolution, thus effectively confining the bacteria to the free-living ‘ecology’ when faced with selection to avoid phage attack. If this result holds true for the system presented in the current study, it might be possible to select for long-lasting phage-resistant *S. flexneri* strains that are attenuated for virulence, an important step toward developing phage treatments for this important human pathogen.

**Conclusion**
Phage A1-1 is a newly isolated Myoviridae phage that uses OmpA as a receptor to infect S. flexneri. Mutants resistant to phage A1-1 have altered membrane permeability, changes in antibiotic resistance profiles, and do not support adsorption of phage A1-1. A combination of whole genome sequencing and targeted Sanger sequencing reveal that two of these resistant mutants have a mutation in *ompA*, two resistant mutants have mutations in *gmhA* and one has a mutation in *gmhC*. EOP assays with complemented strains confirm that these mutations confer resistance to phage A1-1. While phage A1-1 does not appear to use LPS as a receptor, we hypothesize that interactions between OmpA and LPS are required to maintain a particular confirmation of OmpA to allow for phage attachment and will investigate this further in future studies. Finally, all five phage-resistant mutants tested in this study are attenuated for virulence; while they can still invade and replicate intracellularly, they are unable to spread intercellularly in a tissue culture model of infection. This study demonstrates that it is possible to find phage that select for a trade-off between phage resistance and virulence in bacteria.

**Materials and Methods**

**Strains and culture conditions**

Bacteria, phage and plasmid strains used in this study are listed in Supplemental Table S4.2. *S. flexneri* was grown in 0.1% congo-red tryptic soy (CR-TS) medium (10g tryptone, 5g soytone, 10g NaCl, 0.1g congo red dye per L) unless otherwise noted. *E. coli* was grown in Lysogeny Broth (LB) medium (10g tryptone, 5g yeast extract, 10g NaCl per L). Phage A1-1 was amplified to high titer on M90T unless otherwise noted. Bacteria were grown on 1.5% agar plates and phages were grown on plates in 0.75% agar top layer. Plasmids were maintained using 100ug/mL of carbenicillin and expression was induced with 1mM IPTG. Lambda-red recombination 200 was used to engineer bacterial strains M90TΔompAKan r and M90TΔmxiHKan r, and plasmid pCP20
was used to remove the Kanr cassette from each of these strains to create strains M90TΔompA and M90TΔmxiH.

**Assays to characterize phage**

Adsorption assays to estimate phage cell-binding ability were performed as described by Kropinski (2009) 144. Efficiency-of-plating assays to estimate phage infectability on challenge bacteria relative to that on a permissive host strain, and phage growth curves to estimate virus traits (i.e., latent period, exponential growth rate, burst size) were performed as previously described 100. Bacterial growth curves were performed at multiplicity of infection (MOI; ratio of phage particles to bacterial cells) of approximately 100. Transmission electron microscopy (TEM) was performed using uranyl acetate-stained phages on 300 mesh carbon film copper grids, and phage particles were visualized on a FEI Tecnai Biotwin microscope.

**LPS extractions**

Lipopolysaccharide (LPS) was extracted from bacteria using an LPS extraction kit (Abcam ab239718) and LPS was quantified using a carbohydrate quantification assay (Abcam ab155891). LPS was extracted following the instructions provided in the kit. Briefly, bacteria were grown overnight on LB agar, scraped into PBS, centrifuged for 5 min at 5,000g at 4°C, resuspended in extraction buffer, sonicated and incubated on ice for 10 minutes to allow for full lysis. Any unlysed cells were removed via centrifugation (5 min at 5,000g at 4°C) and the supernatant was treated with proteinase K at 60°C for one hour. Any remaining debris was removed via centrifugation (5 min at 5,000g at 4°C). Total carbohydrate content (grams/CFU) in the supernatant was quantified following the instructions of the carbohydrate quantification assay.
**MIC measurements**

Minimum inhibitory concentration (MIC) was measured using Etest strips (Biomerieux). Briefly, overnight cultures of bacteria were spread on Mueller Hinton Agar (MHA, 2g beef extract, 17.5g casein hydrolysate, 1.5g starch, 17.0g agar per L) plates and test strips were plated. After overnight incubation, MIC values were determined by the lowest concentration at which the bacterial growth was inhibited. These measurements were conducted with three-fold replication.

**Membrane permeability assay**

Membrane permeability of cells grown in LB was assayed using the Live/Dead BacLight kit (ThermoFisher L34856) using a microplate reader (Tecan Infinite F500) following the manufacturer instructions.

**Cell fractionation, protein gels and western blots**

Whole cell lysates were made by centrifuging cells (5 minutes at 5,000g), resuspending in 1x Laemmli buffer (Biorad) and boiling at 100°C for 15 minutes. Cell fractions were made following the previously published protocols. Briefly, late log cultures were centrifuged at 5,000g for 5 minutes and gently resuspended in 200 µL of TES buffer (200mM Tris-HCl pH 8.0, 0.5mM EDTA, 0.5M sucrose). Lysozyme (10 µg/mL final) was added to the resuspended cells followed by 720 µL of TES buffer that had been diluted 1:1 in water. The lysate was incubated for 30 minutes on ice with gentle inversion to mix and then centrifuged at 5,000g for 5 minutes at 4°C. The supernatant was saved as the periplasmic fraction. The spheroplast fraction, the pellet, was resuspended in TES buffer diluted 1:1 in water containing 2mM phenylmethylsulfonyl fluoride (PMSF), 2mM MgCl₂ and 10 µg/mL of DNAseI and lysed via four cycles of freeze thaws from -273°C to 37°C. Unlysed spheroplasts were removed via centrifugation at 2,000g for 5 minutes at 4°C. Finally, the supernatant was ultracentrifuged at 120,000g for 45 minutes at 4°C. The
supernatant was saved as the cytoplasmic fraction, and the pellet was resuspended in TES buffer diluted 1:1 in water and saved as the membrane fraction. Whole cell lysates and fractions were run at 200V for 45 minutes on a 10% Tris-Glycine polyacrylamide gel and transferred to a PVDF membrane (90V for 60 minutes) and blotted with an anti-OmpA antibody (Biorbyt orb241331).

**Sequencing and analysis**

Genomes were extracted as previously described. Sequencing libraries were made using NexteraXT library preparation kits (FC-131-1096, Illumina). Samples were run paired-end 150bp reads on the Illumina HiSeq 2500 platform. Reads were aligned to reference genomes for M90T (Genbank Accession # CM001474.1) and the virulence plasmid, pWR501 (Genbank Accession # NC_002698.1) using Bowtie2 and variants were called using GATK.

**Bacteria intracellular growth curves**

Intracellular growth curves of *S. flexneri* were performed as described by Hale and Formal (1981) with some modifications. Briefly, 24 hours prior to assay, Vero cells were plated in Dulbecco’s Modified Eagle Medium (DMEM) with 5% fetal bovine serum (FBS) to 90% confluency in a 24-well dish. On the day of the assay, overnight bacterial cultures were diluted 1:100 and grown to mid-late log. Cultures were centrifuged (5 minutes at 5,000g) and resuspended in DMEM with 5% FBS. Vero cells were washed once with PBS prior to infection. Bacteria were added to each well to an MOI of 10 and the plate was centrifuged at 200 rpm for 5 minutes. Plates were incubated for 30 minutes at 37°C and 5% CO₂. Wells were washed three times with PBS, DMEM with 5% FBS and 100 µg/ml gentamicin was added to each well, and plates were incubated at 37°C and 5% CO₂ for the duration of the assay. At five time points (0, 1, 3, 5 and 7 hours), plates were destructively sampled, supernatant was aspirated, wells were washed 3 times with PBS, Vero cells were lysed
with sterile, deionized water and gentle pipetting, and bacterial CFU was enumerated via plating on CR-TS agar plates.

**Bacterial plaque assays**

Plaque assays were performed as described by Oakes et al. (1985) with some modifications. Briefly, 24 hours prior to infection, Vero cells were plated to 90% confluency in a 6-well dish in DMEM with 5% FBS. On the day of the assay, overnight bacterial cultures were diluted 1:100 and grown to mid-late log. Cultures were centrifuged (5 minutes at 8,000g) and resuspended in DMEM with 5% FBS to achieve a range of MOI’s between 100 and 0.1. Vero cells were washed once with PBS. Bacterial suspensions were plated on Vero cells and allowed to attach and invade for one hour at 37°C and 5% CO₂ with gentle rocking every 15 minutes. After the incubation, 2x Minimum Essential Media (MEM) complete (10X MEM, 100X L-Glutamine, 100xVitamins, 100X NEAA, 7.5% sodium-bicarbonate added to sterile distilled water to a final concentration of 2x with 200 µg/ml gentamicin) was mixed with an equal volume of molten 1% agarose in water and FBS to 5%, added to each well, and allowed to solidify. Plates were incubated for 48 hours at 37°C and 5% CO₂. An additional top layer of 2% neutral red stain (MilliporeSigma N2889) in PBS with 0.5% agarose was added to each well. After 24 hours of incubation, plates were visualized for plaques.
**Figure 4.1** Basic characterization of newly-discovered lytic phage A1-1

Basic characterization of newly-discovered lytic phage A1-1 on host, *Shigella flexneri*. A. Genome annotation of phage A1-1 using PHASTER. Plate proteins are shown in purple, fiber proteins shown in yellow, coat proteins shown in blue, phage-like proteins shown in red and hypothetical proteins are shown in grey. B. TEM image of phage A1-1 (200 nm scale bar). C. Adsorption assay of phage A1-1 on bacterial hosts: M90T (purple), M90T△ompA (yellow), M90T△ompA:pSF_vector (grey) and M90T△ompA:pSF_ompA (black). Error bars show standard deviations of the mean of three biological replicates. Significance determined by testing whether slope of the linear regression line deviates from zero. (* indicates p < 0.05, ** indicates p < 0.01, *** indicates p < 0.001).
Figure 4.2 Characterization of phage resistance

A. Efficiency of plaquing (EOP) assay of phage A1-1 on various strains. Dashed line at 1 indicates EOP on M90T. DL indicates the EOP was below the limit of detection (~10^{-9}).

B. Adsorption assay of phage A1-1 on bacterial hosts: M90T (purple), R1 (yellow), R2 (grey), R3 (black), R4 (blue) and R5 (red). Error bars show standard deviations of the mean of three biological replicates. Significance determined by testing whether slope of the linear regression line deviates from zero.

C. Growth curves of M90T (purple), R1 (yellow), R2 (grey), R3 (black) R4 (blue), and R5 (red). Solid lines indicate bacteria growing in absence of phage; dashed lines indicate phage A1-1 presence (MOI=10). (* indicates p < 0.05, ** indicates p < 0.01, *** indicates p < 0.001).
Phenotypic characterization of five spontaneous phage-resistant mutants of *S. flexneri*. 

**A.** Membrane permeability measured as a linear regression of the ratio of green to red fluorescence of different suspensions of live and dead cells stained with SYTO 9 dye and propidium iodide. Error bars show standard deviations of the mean of three biological replicates. Significance determined using a student’s t-test comparing the slopes of the linear regressions to that of wild-type bacteria. 

**B.** Total LPS measured as µg per CFU. Error bars show standard deviations of the mean of three biological replicates. 

**C-F.** Fold change in MIC compared to MIC of wild-type M90T for erythromycin, ciprofloxacin, vancomycin (shaded region indicates limit of detection) and tetracycline. Error bars show standard errors of the average of three biological replicates. Unless otherwise noted significance determined using a student t-test. (* indicates p < 0.05, ** indicates p < 0.01, *** indicates p < 0.001).
**Figure 4.4** OmpA expression in phage-resistant mutants

OmpA expression in phage-resistant mutants of *S. flexneri*, compared to controls. **A.** Western blot for OmpA of whole bacterial cell extract of M90T, M90TΔompA, R1, R2, R3, R4 and R5. **B.** Western blot for OmpA of cytoplasmic (C), periplasmic (P) and membrane (M) fractions of M90T, R3, R4 and R5.
Diagram of mutations identified via whole-genome sequencing. The chromosome of wild-type M90T is 4,580,866 base pairs. Mutations were identified in all five phage-resistant mutants. R1 and R2 have identical single base pair insertions at position 507 in \textit{ompA} (yellow). R3 has a single base pair insertion in \textit{gmhA} (blue) at position 400. R4 has a transversion at position 787 of \textit{gmhC} (red). R5 has a 29,645 base pair deletion spanning 27 genes, including \textit{gmhA} (blue) between two IS1 elements (black).
**Figure 4.6 Virulence of phage-resistant mutants**

Virulence of phage-resistant mutants, relative to controls, in a tissue-culture model of *S. flexneri* pathogenicity. **A.** Representative intracellular growth curves of wild-type M90T (purple), M90TΔompA (yellow), M90TΔmxiH (red), R1 (black circle), R2 (black square), R3 (black triangle), R4 (black inverted triangle), and R5 (black diamond) bacteria. Error bars show standard deviations of the mean of three technical replicates. **B-I.** Representative plaque assays of wild-type M90T, M90TΔompA, M90TΔmxiH, R1, R2, R3, R4 and R5, respectively, on monolayers of Vero cells at MOI = 5 (bacteria CFU relative to Vero cells) after 72 hour incubation.
Further characterization of newly-discovered lytic phage A1-1 on host, *Shigella flexneri*. **A.** Phage growth curve of A1-1 on M90T (purple) and M90TΔompA (yellow). Titers of filtered and chloroformed conditions are indicated with solid and dashed lines respectively. Error bars show standard deviation of three biological replicates. **B.** Efficiency of plaquing (EOP) assay of phage A1-1 on various strains. Dashed line at 1 indicates EOP on M90T. DL indicates that the EOP was below the limit of detection (~10⁻⁹). **C.** Bacterial growth curves of M90T (purple), M90TΔompA (yellow), M90TΔompA:pSF_vector (grey), M90TΔompA:pSF_ompA (black) and M90TΔompA:pSF_ompA171 (blue). Solid lines indicate bacteria growing in absence of phage; dashed lines indicate phage A1-1 presence (MOI=10).
Efficiency of plaquing of phage A1-1 on LPS knockouts. A. EOP assay of A1-1 on various strains. Dashed line at 1 indicates EOP on M90T. Yellow shaded knockouts are genes involved in synthesis of KDO sugars. Red shaded knockouts are genes involved in synthesis of the inner core LPS. Purple shaded knockouts are genes involved in synthesis of outer core LPS and O-antigen. DL indicates that the EOP was below the limit of detection (~10^{-9}). Significance determined using a student t-test between B25113 and test strains. (* indicates p < 0.05, ** indicates p < 0.01, *** indicates p < 0.001) B. Diagram of M90T LPS with lipidA (grey), KDO region (yellow), inner core LPS (red) and outer core LPS (purple). Abbreviations as follows phosphoryl group (P), N-acetylglucosamine (GlcN), KDO sugar (Kdo), heptose sugar (Hep), glucose (Glc), and galactose (Gal).
**Supplemental Table S4.1** Phage resistance profile of various strains to phages 60B and T7

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S, phage sensitive; R, phage resistant
**Supplemental Table S4.2** Bacterial strains, phage strains and plasmids used in this chapter

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KanR, Kanamycin resistance; SmR, Streptomycin resistance; AmpR, Ampicillin resistance; CGSC, Coli Genetic Stock Center
Chapter 5: Evolution of bacterial cross-resistance to lytic phages and albicidin antibiotic
Preface

Chapter 5 has been adapted from an article that has been accepted pending minor revisions to Frontiers.

This study was designed by Kaitlyn Kortright and Dr. Benjamin Chan. Kaitlyn Kortright and Simon Doss-Gollin preformed experiments. Kaitlyn Kortright analyzed data. The manuscript was prepared and edited by Kaitlyn Kortright, Dr. Paul Turner and Dr. Benjamin Chan. Raw sequencing is publicly available at NCBI Sequence Read Archive Project # PRJNA693868 and supplemental datasets have been deposited on Drayad (doi:10.5061/dryad.ghx3ffbm). We thank Dean Gabriel for kindly providing *X. albilineans* strains used in this study. Additionally, we would like to thank editor Robert Czajkowski and two anonymous reviewers for helpful comments on the manuscript.
Abstract

Due to concerns over the global increase of antibiotic-resistant bacteria, alternative antibacterial strategies, such as phage therapy, are increasingly being considered. However, evolution of bacterial resistance to new therapeutics is almost a certainty; indeed, it is possible that resistance to alternative treatments might result in an evolved trade-up such as enhanced antibiotic resistance. Here, we hypothesize that selection for *Escherichia coli* bacteria to resist phage T6, phage U115, or albicidin will result in a pleiotropic trade-up in the form of cross-resistance, because all three antibacterial agents interact with the Tsx porin. Selection imposed by any one of the antibacterials resulted in cross-resistance to all three of them, in 29 spontaneous bacterial mutants examined in this study. Furthermore, cross-resistance did not cause measurable fitness deficiencies under normal growth conditions for any of the bacterial mutants. A combination of whole-genome and targeted sequencing confirmed that mutants differed from wild-type *E. coli* via change(s) in the *tsx* gene. Our results indicate that evolution of cross-resistance occurs frequently in *E. coli* subjected to independent selection by phage T6, phage U115 or albicidin. This study cautions that deployment of new antibacterial therapies such as phage therapy, should be preceded by a thorough investigation of evolutionary consequences of the treatment, to avoid the potential for evolved trade-ups.
**Chapter Introduction**

As global concerns grow over the widespread emergence of antibiotic-resistant bacteria, attention has increasingly turned to antibiotic alternatives such as phage therapy: the use of bacteria-specific viruses, bacteriophages (phages), to treat bacterial infections. Although phage therapy is frequently seen as a novel medical technology, the approach originated in the early 20th century soon after phages were discovered\(^{24,28}\). Less than a decade after the discovery of phages, penicillin was discovered and focus shifted to research and deployment of new antibiotics\(^ {204}\). Recently, interest in phage therapy has resurfaced as a tool for treating antibiotic resistant infections\(^ {179}\); however, just as antibiotics select for the evolution of antibiotic resistance, phages select for the evolution of phage resistance\(^ {16,205}\). To avoid the historical mistakes that resulted in multi- and even pan-drug resistant strains, the evolutionary consequences of phage therapy need to be considered and investigated prior to its widespread use\(^ {206}\).

While the evolution of phage resistance in bacteria is perhaps inevitable, phage therapy strategies can be devised to leverage the evolution of phage resistance as an asset, rather than a limitation\(^ {179}\). In particular, by using lytic phages that interact with bacterial mechanisms of virulence and antibiotic resistance, these phages should kill bacteria while selecting for phage resistance that may compromise – or pleiotropically “trade-off” with – virulence or antibiotic-resistance traits\(^ {179}\). Theory, experiments and emergency patient treatment provide evidence that certain phages can direct favorable “trade-off” outcomes, where the therapy kills the target pathogen while selecting for reduced virulence or increased antibiotic sensitivity in the remaining bacterial population\(^ {13,207,208}\). This approach exemplifies one goal of evolutionary medicine: applying “evolution thinking” to improve the effectiveness of therapy\(^ {209,210}\).
However, not all bacterial mutations underlying the evolution of phage resistance result in genetic changes with extreme fitness costs that result in a trade-off\textsuperscript{211}. Instead, the opposite may happen whereby evolution of phage resistance results in an additional fitness gain, also known as a “trade-up”. Indeed, it is possible that the fitness effects of evolved phage resistance might pleiotropically trade-up with virulence or antibiotic resistance\textsuperscript{212,213}. To examine this possibility, here we studied the interactions of *Escherichia coli* with two lytic phages and an antibiotic that all require the Tsx porin to enter the cell. Tsx is a substrate-specific outer-membrane porin, which uptakes nucleosides and deoxynucleotides into the periplasmic space\textsuperscript{214}. Phages T6 and U115 are shown to use the Tsx porin as a receptor for binding to *E. coli*\textsuperscript{215}. Additionally, the Tsx porin uptakes the antibiotic albicidin, a phytotoxic DNA-gyrase inhibitor produced by *Xanthomonas albilineans*, an agriculturally important pathogen that causes leaf scald in sugar cane grasses\textsuperscript{216-219}. We hypothesized that selection for *E. coli* resistance to phage T6, phage U115, or albicidin should tend to produce cross-resistance to the other antibacterial agents, due to converging selection at the *tsx* locus.

Our results confirmed that the predicted pleiotropic trade-ups evolved frequently; selection exerted by any one of the antibacterials led to perfect (100%) cross-resistance of *E. coli* mutants to all three antibacterials. Moreover, we observed that cross-resistance was generally “cost-free”, evidenced by equivalent growth of bacterial mutants relative to their wild-type ancestor in the absence of the phages and albicidin. In addition, we used sequence analysis to show that a wide variety of mutations at the *tsx* locus of *E. coli* may govern cross-resistance. Our study suggests that prior characterization of evolutionary consequences of antibacterial treatments, particularly the mechanistic interactions of lytic phages and antibiotics with target bacteria, can be used to inform treatment strategies that potentially avoid the evolution of undesired trade-ups.
Results

Phages T6 and U115 each select for cross-resistance to the other phage and to albicidin

Because phages T6 and U115 both rely on the Tsx porin for cell-binding, we predicted that evolution of resistance to one phage should often lead to cross-resistance against the other phage. To test this idea, we used a classic fluctuation analysis (see Methods) to obtain a collection of spontaneous mutants of *E. coli* that were resistant to each phage individually. We used a paired approach, where each of 10 independently-grown bacterial cultures were used to isolate one T6-resistant and one U115-resistant strain (20 mutants total). Results for efficiency of plaquing (EOP) assays (see Methods) confirmed that growth of phage T6 on each of the 10 T6-resistant mutants (T6R1 through T6R10) was below the limit of detection, compared to normal infectivity of the phage on wild-type bacteria (Supplemental Table S5.1). Similarly, EOP experiments showed that phage U115 was unable to grow on each of the 10 U115-resistant mutants (U115R1 through U115R10), relative to expected infectivity on the wild-type (Supplemental Table S5.1). Furthermore, we found positive support for our hypothesis; in all 20 cases, evolved bacterial resistance to phage T6 provided cross-resistance to phage U115 using EOP assays, and *vice versa* (Supplemental Table S5.1). We concluded that independent selection for *E. coli* resistance to one phage provided cross-resistance to the other phage.

We then used antibiotic-resistance assays (see Methods) to test the prediction that evolution of phage resistance would lead to cross-resistance to the antibiotic albicidin. We first used *E. coli* strains BW25113 and BW25113Δ*icdC* as two positive controls, to confirm that these albicidin-sensitive bacteria fail to form confluent lawns (i.e., they show zones of inhibited growth) when exposed to albicidin-producing *X. albilineans* strain XA23, but grow normally on *X. albilineans* strain LS126 which does not produce albicidin. Results (Figure 5.1) showed that the controls
behaved as expected, with zones of growth inhibition around XA23 indicating sensitivity of both bacterial strains to albicidin. We estimated that wild-type BW25113 had a mean clearing ratio (a ratio of the zone of clearing divided by the area of the *X. albilineans* spot) of $4.88 \pm 0.47$ s.d. in the presence of XA23 albicidin-producing bacteria, and that BW25113ΔicdC bacteria showed a mean clearing ratio of $5.094 \pm 0.524$ s.d. (Supplemental Figure S5.1 A). As a negative control, the Tsx knockout, BW25113Δtsx, had no zone of inhibited growth on either XA23 or LS126 (Figure 5.1); in both cases the mean clearing ratio of BW25113Δtsx was $1.0 \pm 0.0$ s.d. (Supplemental Figure S5.1 A). A test of the hypothesis confirmed our prediction was correct; all 20 phage-resistant mutants showed no zones of growth inhibition around XA23 or LS126 (Figure 5.1 and Supplemental Figure S5.1 B) and presented mean clearing ratios of $1.0 \pm 0.0$ s.d. on XA23 and LS126 (Supplemental Figure S5.1 A), indicating that all the phage-resistant mutants were completely resistant to albicidin as well.

**Selection for albicidin resistance confers cross-resistance to phages T6 and U115**

We used albicidin selection (see Chapter 5 Methods) to isolate 9 independent spontaneous mutants of *E. coli* (albR1 through albR9). Using the above-described growth challenges on *X. albilineans* strains XA23 and LS126, our results confirmed that each mutant was albicidin resistant (Figure 5.1 and Supplemental Figures S5.1 A,B). We then tested whether the albicidin-resistant mutants were cross-resistant to infection with phage T6 and phage U115. Results showed that in all 9 strains tested, acquisition of albicidin resistance conferred cross-resistance to both phages T6 and U115 (Supplemental Table S5.1). We concluded that the evolution of cross-resistance was absolute in this study system, such that evolution of resistance to one of the three antibacterials provided perfect cross-resistance to all of the selective agents.
Cross-resistance to albicidin and phages T6 and U115 is cost-free for *E. coli*

To further examine the evolution of antibacterial cross-resistance, bacterial growth assays (see Methods) were performed with replication (*n* = 3) in LB medium. Controls confirmed that wild-type *E. coli* strain BW25113 showed no discernable growth in the presence of either phage T6 or U115 (Figure 5.2 A), and that growth of the *tsx*-knockout strain, BW25113Δtsx, was similar in the presence and absence of each phage (Figure 5.2 B). In contrast, all 10 of the T6-resistant mutants grew normally in the presence and absence of phage T6 (representative data shown in Figure 5.2 C,D; see Supplemental Figure S5.2 B-I for all results). Similarly, growth of all 10 U115-resistant mutants was unaffected by presence or absence of phage U115 (representative data in Figure 5.2 E,F; see Supplemental Figure S5.2 J-Q for all results). Lastly, the 9 albicidin-resistant mutants were capable of approximately equivalent growth in the presence and absence of either phage T6 or U115 (representative data in Figure 5.2 G,H; see Supplemental Figure S5.2 R-X for all results).

A visual comparison of the growth-curve results suggested that all the resistant mutants grew similarly to wild-type strain BW25113 in the absence of each phage (Figure 5.2 and Supplemental Figure S5.2). To examine this outcome more closely, we analyzed the growth data with GrowthCurver to estimate intrinsic growth rate (*r*) as a proxy for bacterial fitness (Figure 5.2 I). None of the resistant mutants had an *r* that was differed significantly from that of BW25113. These results suggested that there are no appreciable growth costs associated with bacterial evolution of resistance to phage T6, phage U115 and albicidin. While resistance to any of the antibacterials did not affect bacterial fitness under the tested conditions, we could not eliminate the possibility that our growth assays failed to detect minor fitness differences.

Thus, we conducted additional experiments, in an attempt to measure more subtle fitness differences among bacterial strains. To do so, we performed replicated (*n* = 3) competition assays
(see Chapter 5 Methods) for each test strain to gauge its fitness relative to a genetically-marked wild-type strain (BW25113∆icdC) under normal growth conditions. Competitive indexes were calculated as the ratio of resistant-mutant colony forming units (CFU) to CFU of the common competitor strain, BW25113∆icdC, to the ratio of BW25113∆icdC CFU to wild-type, BW25113, CFU. Each ratio was normalized by the starting ratio each competing strain. Results showed no significant differences in competitive indexes of the resistant mutants as compared to the common competitor (Figure 5.3). Therefore, it appeared that all T6-resistant, U115-resistant and albicidin-resistant mutants suffered no fitness deficits, compared to non-resistant bacteria under normal growth conditions.

All phage-resistant and albicidin-resistant mutants have mutations in tsx

Owing to the importance of the Tsx porin in the interactions of all three antibacterials with cells, evolution of E. coli cross-resistance suggested that genetic changes in the tsx gene were likely involved. To examine this idea, we conducted whole genome sequencing (WGS) of all 29 strains. While some strains showed some single nucleotide variants (SNVs) and short insertions or deletions (indels) in tsx, many strains had no observable mutations in any genes using multiple variant calling pipelines including GATK and breseq. While WGS and variant calling pipelines are amenable for detecting SNVs and short indels, structural variants (SVs), including movement of transposable elements, are not easily detected. Therefore, we conducted targeted Sanger sequencing of tsx in all 29 resistant mutants. As expected, results for the wild-type showed no mutations in tsx; however, mutations in tsx were identified in all 29 resistant mutants. For the 10 T6-resistant strains we observed the following mutations in the tsx gene: 3 insertion sequence (IS) elements, 3 deletions, 3 nonsense mutations and 1 missense mutation (Figure 5.4 A, Supplemental Table S5.2). For the 10 U115-resistant strains, we documented the following in tsx:
4 IS elements, 2 deletions, 1 insertion, 2 missense mutations, and 2 nonsense mutations (Figure 5.4 A, Supplemental Table S5.2). Interestingly, of the 10 pairs of phage-resistant mutants isolated from the same parent culture, only 3 pairs (T6R4/U115R4; T6R8/U115R8; T6R10/U115R10) showed identical mutations in tsx (Figure 5.4 B, Supplemental Table S5.2). For the 9 albicidin-resistant mutants we observed 6 IS elements, 2 deletions, and 1 missense mutation in tsx (Figure 5.4 A). In addition, our data showed that eight of the 29 mutations in tsx clustered within the region spanning base pairs 375 to 435 (Figure 5.4 B). While it is likely that these mutations are conferring resistance to phage T6, phage U115 and albicidin, this was not experimentally confirmed via recombinant genetics because the work fell outside the scope of the current study.

**Discussion and Conclusion**

The phage life cycle relies on surface-exposed molecules to initiate infection and bacterial metabolism to replicate inside cells. Since phages T6 and U115 both require Tsx as a primary receptor, it is not surprising that spontaneous mutants to one phage confer resistance to the other phage. However, it is interesting that all 20 phage-resistant mutants are also cross-resistant to albicidin and that all 9 albicidin resistant mutants are cross-resistant to both phages. It was previously observed that T6-resistant mutants maintained their sensitivity to colicin-K, another antibiotic that enters via the Tsx porin. While all three antibacterials used in the current study require Tsx as a receptor, it is unclear whether replication of phages T6 and U115 and the mechanism of action of albicidin also converge on the same gene to confer resistance. Albicidin is a DNA gyrase inhibitor that blocks topoisomerase II, an enzyme which cleaves both strands of DNA to modulate supercoiling during DNA replication, gene regulation, and transcription. Phages require the DNA replication machinery of the host bacterial cell to make viral progeny within the cell; however, many phages, including T6, encode their own topoisomerasess.
Furthermore, the minimum inhibitory concentrations (MICs) of ciprofloxacin (another DNA gyrase inhibitor) and ampicillin for wild-type BW25113 bacteria and for each of the 29 resistant mutants did not differ statistically (Supplemental Table S5.3). These observations of unchanged phenotypes imply that selection by phage T6, phage U115, and albicidin seems to evolutionarily converge on the tsx locus, rather than involving changes at other loci. Indeed, the results of the targeted sequencing indicated this possibility, because each mutant was observed to undergo a change in the tsx gene (i.e., as opposed to no mutation at this locus, suggesting resistance occurred via a different mechanism). While the identified mutations were not validated as the causative mutations of resistance, it seems likely that the 29 different mutations found in tsx were conferring resistance to phage T6, phage U115, and albicidin. The combination of tsx mutations observed in all 29 resistant mutants and the lack of detectable fitness defects in these strains indicate that Tsx may be superfluous for E. coli in the laboratory environment.

The 29 observed mutations in tsx fall within the promoter region, as well as various other locations along the gene. However, there seems to be a cluster of ‘hotspots’ for mutational changes in the region spanning 332 to 435 base pairs. These residues make up a surface exposed alpha helix and a beta sheet that spans the outer membrane of the bacteria. All of the mutations in this region were observed in phage-resistant strains, indicating that perhaps amino acid residues 88 through 123 are important for phage binding, but not albicidin entry.

Moreover, of the 10 “paired” phage-resistant mutants, only 3 of the pairs (T6R4/U115R4; T6R8/U115R8; T6R10/U115R10) showed the same tsx mutation. This means that even in a clonal population of E. coli, there are often multiple spontaneous mutants with different changes in tsx. This finding indicates that the number of resistant mutants observed to arise during fluctuation
assays cannot be used to compute a rate of spontaneous mutation; instead, these data should only be used to calculate a rate of spontaneous phenotypic resistance.

It is also interesting that the profile of *tsx* mutations was different for the phage-selected resistant mutants, compared with the antibiotic-selected resistant mutants. There were 6 albicidin-resistant mutants with an IS element inserted in *tsx*, while 3 of each of the phage-resistant mutants showed IS-element changes in *tsx*. This observation could be due either to a difference in selection pressures of phages versus antibiotics, or to a difference in the method of selection to amass these mutants. Underlying these possibilities are two opposing ideas. It has been previously assumed that insertion and excision of IS elements are stochastic events. However, recent evidence suggests that IS movement may actually be a form of directed mutagenesis in which IS elements target specific chromosomal loci to reduce stress under certain environmental conditions. The isolation of phage-resistant mutants in this study relied on spontaneous mutations accumulated in an overnight culture, while the isolation of antibiotic-resistant mutants allowed for both selection of spontaneous resistant mutants as well as evolution in the presence of albicidin. This difference suggests that the larger proportion of IS element insertions in *tsx* found in the set of albicidin-resistant mutants might be caused by direct targeting of *tsx* by IS elements under stressful conditions. Without further experiments that use the same method to select for spontaneous phage and albicidin resistant mutants, we cannot rule out that a difference in selection pressures between phages and the antibiotic resulted in the skew of IS-element-insertion changes in *tsx* found in the albicidin-resistant mutants.

Finally, it is worth noting that all 29 resistant mutants have gained fitness relative to wild-type in the presence of albicidin or phage, with no appreciable fitness cost in an ordinary environment. Antibiotic cross-resistance has extreme clinical relevance as selected resistance to a
single antibiotic can also result in evolution of multi-drug resistance. Here, we showed that phage resistance can result in antibiotic resistance, and *vice versa*, at no fitness cost. This highlights a potential pitfall of the future therapeutic use of any antimicrobial whether it be phage or antibiotics. Without a clear understanding of the evolutionary implications of treatment, antimicrobial selection can result in both resistance and cross-resistance at no fitness cost to the bacteria.

**Materials and Methods**

**Strains and culture conditions**

Strains in this study are listed in Supplemental Table S5.4. *E. coli* strains were cultured for 24 hours with shaking incubation at 37°C in lysogeny broth. The Tsx knockout, BW25113Δtsx, was used as a negative control in many experiments. A pseudogene knockout, BW25113ΔicdC, was used as a control for the KanR cassette and as a marked competitor in the competition assays. Where appropriate, LB was supplemented with kanamycin at 30 µg/mL (LB Kan30). *X. albilineans* strains (provided by D. Gabriel, U Florida) were cultured for 48 hours with shaking at 30°C in Modified Wilbrinks (MW) medium. Lysates of phages T6 and U115 were obtained by mixing each phage with *E. coli* strain BW25113 in LB medium and culturing for 24 hours at 37°C, followed by filtration (0.22 µm) to remove bacteria.

**Isolation of phage-resistant bacterial mutants**

Ten cultures of *E. coli* strain BW25113 were grown independently as described above, and diluted samples were spread on LB agar (1.5% agar) plates, pre-saturated with either phage T6 or phage U115. After overnight incubation at 37°C, one colony was chosen randomly from each plate and colony-purified three times. For each of the 20 isolated mutants, phage resistance was confirmed via efficiency of plaquing (EOP) assays, which compared plaquing ability of phage T6 or U115 on the test mutant relative to growth on wild-type strain BW25113.
Isolation of antibiotic-resistant bacterial mutants

*X. albilineans* strain XA23 was cultured as described above, and a 10 µL sample was spotted onto each of ten Sucrose Peptone agar (SPA) plates that were incubated for 5 days at 30ºC. A sample from each of ten independently-grown cultures of BW25113 was then overlaid on one of the SPA plates, and incubated overnight at 37ºC. Colonies that appeared in the zone of growth inhibition around the XA23 spot represented spontaneous *E. coli* mutants that were resistant to albicidin. One plate was lost due to contamination; from each of the remaining 9 plates, one colony was randomly chosen and colony-purified three times. Albicidin resistance of each mutant was verified by plating a sample of a grown-up culture of the isolate on an XA23 spot, as described above.

Antibiotic-resistance assays

*X. albilineans* strains XA23 and LS126 were each cultured for 48 hours at 30ºC, and their optical densities at wavelength $\lambda = 600$ nm (OD$_{600}$) were estimated via spectrophotometry. Each culture was then diluted in MW medium, to obtain OD$_{600} = 0.25$. Then, a 10 µL sample of each diluted culture was spotted on a SPA plate, allowed to dry completely and then incubated for 5 days at 30ºC. SPA plates were overlaid with 4 mL of LB top agar (0.75%) with 1 mL of a test *E. coli* strain at OD$_{600} = 0.25$, and incubated overnight at 37ºC. The next day, plates were imaged. Images were quantified using ImageJ, by thresholding until either the *X. albilineans* spots or *E. coli* clearings were outlined and the area within the outline was quantified. The ratio of the area of the zone of clearing to the area of the *X. albilineans* spot was used to define albicidin sensitivity: a ratio of greater than 1.0 indicates albicidin sensitivity, while a ratio of 1.0 indicates albicidin resistance. Minimum inhibitory concentrations (MICs) of ampicillin and ciprofloxacin were determined using the two-fold dilution method.

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**Bacterial growth curves**

*E. coli* bacteria were cultured overnight as described above. Dilutions of a test strain in LB medium were placed in wells of a flat-bottomed 96-well plate, in the absence of phage or mixed with phage at a multiplicity-of-infection (MOI; ratio of phage particles to host cells) of ~10. Plates were incubated with shaking for 18 hours in an automated spectrophotometer at 37°C, and OD$_{600}$ measurements were obtained every 10 minutes. Growth curve data were fit to a logarithmic curve using GrowthCurver and growth parameters were extracted $^2$. All growth curve data will be deposited on Dryad (doi:10.5061/dryad.ghx3ffbmn).

**Bacterial competition assays**

Replicated ($n=3$) competition assays were conducted in LB medium by mixing two bacterial strains at a 1:1 initial ratio, and serially passaging (1:100 dilution) every 24 hours for 72 hours total. At the end, a diluted sample of each competition was plated on LB agar to measure viable bacterial density (colony-forming units; CFU), and on LB Kan30 agar to estimate the CFU of a Kan$^R$ competitor; the density of the Kan$^S$ competitor was estimated by subtraction. Competitive indexes were calculated as the ratio of resistant-mutant CFU to the CFU of BW25113ΔicdC divided by the ratio of BW25113ΔicdC CFU to BW25113 CFU. Each ratio was normalized by the starting ratio each competing strain.

**Sequencing**

Sequencing libraries were prepared using Illumina’s Nextera prep kit. Whole genome sequencing was done using paired-end 150bp reads on the Illumina NextSeq platform or paired-end 300bp reads on the Illumina HiSeq platform. For targeted sequencing, primers (5’-CTGTGAAACGAAACATATTGG-3’ and 5’-CGTGCTTTTGGTAG-3’) were designed ~100 base pairs upstream and downstream of *E. coli* gene *tsx*, and used to amplify *tsx* of each
resistant mutant and the ancestral BW25113 strain. Amplicons were Sanger sequenced at the Yale DNA Analysis Facility on Science Hill. Mutations were identified by comparing sequencing reads to *tsx* loci of the reference strain GenBank CP009273.1
Tests of albicidin resistance for representative spontaneous mutants of *E. coli*, selected for resistance to phage T6, phage U115, or albicidin. Wild-type BW25113 and the positive-control BW25113Δicdc showed inhibited growth on agar plates with albicidin-producing *X. albilineans* (XA23), and normal growth on the non-producer, LS126. In contrast, mutants (T6R1, T6R2, U115R5, U115R6, albR4, albR7) and negative-control BW25113Δtsx grew equally well in the presence/absence of albicidin-producing bacteria.
Growth dynamics of representative *E. coli* mutants selected for resistance to phage T6, phage U115, or albicidin, in environments with and without each phage. **A-B** Wild-type BW25113 bacteria grew only in phage absence (solid line), whereas Tsx knockout strain BW25113 Δtsx grew similarly in phage-free as well as T6 (dashed line) and U115 (dotted line) environments. **C-H** Each of the representative resistant mutants grew similarly in the presence/absence of phages T6 and U115, regardless of their prior selection for phage or albicidin resistance. **I** Intrinsic growth rate (*r*; proxy for fitness) estimates from growth-curve data (**A-H** and Supplemental Figure S5.2) showed that each strain did not differ statistically from BW25113 (P > 0.05; unpaired t-tests).
Competition assays showed that *E. coli* mutants resistant to phage T6 A phage U115 B or albicidin C did not measurably differ in fitness, relative to a non-resistant wild-type strain. Each point represents results from independent competition assays (*n* = 3), and statistical significance was determined using a t-test comparing competitive index of the resistant mutant relative to the competitive index of BW25113ΔicdC.
Figure 5.4 Mutations in \textit{tsx}

Mutations in \textit{tsx} A Proportions of \textit{tsx} mutations observed in sets of \textit{E. coli} strains, selected for spontaneous resistance to either phage T6, phage U115 or albicidin. Mutations were due to insertion sequence elements (blue), deletions (red), insertions (yellow), missense mutations (purple) and nonsense mutations (orange). B Locations of \textit{tsx} mutations for each resistant mutant.
Tests of albicidin resistance for spontaneous mutants of *E. coli* not described in main text, selected for resistance to phage T6, phage U115 or albicidin antibiotic. **A** Quantification of albicidin resistance (ratio of cleared zone to area of *X. albilineans* growth) showed that each mutant and the Tsx-knockout strain, BW25113Δtsx, were albicidin-resistant (ratio = 1.0), whereas wild-type BW25113 and positive-control BW25113ΔicdC bacteria were albicidin-sensitive (ratio > 1.0). **B** In all cases, mutant growth on agar plates was not inhibited by albicidin-producing *X. albilineans* (XA23), and growth was normal on non-producer strain LS126 controls.
Growth dynamics of *E. coli* mutants selected for resistance to phage T6, phage U115, or albicidin, in environments with phage T6 (dashed line), with phage U115 (dotted line), and without phages (solid line). A-X In all cases, mutants grew similarly in presence/absence of phages T6 and U115, regardless of their prior selection for phage or albicidin resistance.
**Supplemental Table S5.1 Efficiency of plaquing**

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<thead>
<tr>
<th>Phage T6</th>
<th>Phage U115</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>BW25113 ΔicdC</strong></td>
<td><strong>1.11 ± 0.55</strong></td>
</tr>
<tr>
<td>BW25113 Δtsx</td>
<td>0*</td>
</tr>
<tr>
<td>T6 R1</td>
<td>0*</td>
</tr>
<tr>
<td>T6 R2</td>
<td>0*</td>
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<tr>
<td>T6 R3</td>
<td>0*</td>
</tr>
<tr>
<td>T6 R4</td>
<td>0*</td>
</tr>
<tr>
<td>T6 R5</td>
<td>0* ^</td>
</tr>
<tr>
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<td>0*</td>
</tr>
<tr>
<td>T6 R7</td>
<td>0*</td>
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<tr>
<td>T6 R8</td>
<td>0*</td>
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<tr>
<td>T6 R9</td>
<td>0*</td>
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<tr>
<td>alb R8</td>
<td>0*</td>
</tr>
<tr>
<td>alb R9</td>
<td>0*</td>
</tr>
</tbody>
</table>

Efficiency of plaquing (EOP) for phages T6 and U115 on challenge strains of *E. coli*, selected for spontaneous resistance to each phage or to albicidin. EOP is defined as phage titer (PFU/mL) on the test strain divided by that on wild-type BW25113; EOP = 0 indicates phage growth on the challenge is below the limit of detection (<100 PFU/mL). Positive (BW25113ΔicdC) and negative (BW25113Δtsx) controls confirm expected susceptibility and resistance to each phage in replicate (*n* = 3) assays, whereas, all 29 mutants show cross-resistance to phage infection. Statistical significance (*; *P* < 0.05) indicates result of an unpaired t-test comparing three replicate measures to the EOP on BW25113ΔicdC. The "^" indicates a phage with observable turbid clearing of the challenge-bacteria lawn, without making visible plaques.
### Supplemental Table S5.2 Mutations in *tsx*

<table>
<thead>
<tr>
<th>Strain</th>
<th>Mutation Type</th>
<th>Position</th>
<th>Notes</th>
</tr>
</thead>
<tbody>
<tr>
<td>T6 R1</td>
<td>Deletion</td>
<td>291-321</td>
<td>31 base pair deletion, new stop codon at residue 101</td>
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<td>Insertional Element</td>
<td>-75</td>
<td>IS2 with sequence [AGGCTCCTC] repeated</td>
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<tr>
<td>T6 R3</td>
<td>Deletion</td>
<td>402-733</td>
<td>332 base pair deletion</td>
</tr>
<tr>
<td>T6 R4</td>
<td>Nonsense</td>
<td>773</td>
<td>* G773A (W258Stop)</td>
</tr>
<tr>
<td>T6 R5</td>
<td>Insertional Element</td>
<td>403</td>
<td>IS2 with sequence [GGTCGTA] repeated</td>
</tr>
<tr>
<td>T6 R6</td>
<td>Nonsense</td>
<td>435</td>
<td>G435A (W146Stop)</td>
</tr>
<tr>
<td>T6 R7</td>
<td>Insertional Element</td>
<td>165</td>
<td>IS2 with sequence [GGAAC] repeated</td>
</tr>
<tr>
<td>T6 R8</td>
<td>Missense</td>
<td>725</td>
<td>* T725G (L242R)</td>
</tr>
<tr>
<td>T6 R9</td>
<td>Nonsense</td>
<td>535</td>
<td>G535T (E179Stop)</td>
</tr>
<tr>
<td>T6 R10</td>
<td>Deletion</td>
<td>410-420</td>
<td>* 11 base pair deletion, new stop codon at residue 24</td>
</tr>
<tr>
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<td>Insertional Element</td>
<td>400</td>
<td>IS3 with sequence [ATG] repeated</td>
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<td>Insertion</td>
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<td>1 base pair insertion at position 53, new stop codon at residue 24</td>
</tr>
<tr>
<td>U115 R3</td>
<td>Insertional Element</td>
<td>774</td>
<td>IS1 with sequence [GACAGCGGT] repeated</td>
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<tr>
<td>U115 R4</td>
<td>Nonsense</td>
<td>773</td>
<td>* G773A (W258Stop)</td>
</tr>
<tr>
<td>U115 R5</td>
<td>Missense</td>
<td>31</td>
<td>T31A (V11E)</td>
</tr>
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<td>Insertional Element</td>
<td>161</td>
<td>IS2 with sequence [GCAAC] repeated</td>
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<td>C375G (Y125Stop)</td>
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<tr>
<td>U115 R8</td>
<td>Missense</td>
<td>725</td>
<td>* T725G (L242R)</td>
</tr>
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<td>Deletion</td>
<td>410-420</td>
<td>* 11 base pair deletion, new stop codon at residue 24</td>
</tr>
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<td>IS1 with sequence [CATATGAAA] repeated</td>
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</table>

* Indicates paired phage resistant mutants with the same mutation

Putative resistance mutations in the *tsx* gene, for each *E. coli* mutant selected for spontaneous resistance to either phage T6, phage U115, or albicidin.
**Supplemental Table S5.3** Minimum inhibitory concentrations of ciprofloxacin and ampicillin

<table>
<thead>
<tr>
<th>Strain</th>
<th>Ampicillin (µg/mL)</th>
<th>Ciprofloxacin (ng/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>BW25113</td>
<td>20 ± 0</td>
<td>11.67 ± 7.64</td>
</tr>
<tr>
<td>BW25113 (\Delta icdC)</td>
<td>20 ± 0</td>
<td>13.33 ± 5.77</td>
</tr>
<tr>
<td>BW25113 (\Delta tsx)</td>
<td>20 ± 0</td>
<td>20 ± 0</td>
</tr>
<tr>
<td></td>
<td>20 ± 0</td>
<td>6.67 ± 2.89</td>
</tr>
<tr>
<td></td>
<td>26.67 ± 11.55</td>
<td>10 ± 0</td>
</tr>
<tr>
<td>T6 R1</td>
<td>20 ± 0</td>
<td>6.67 ± 2.89</td>
</tr>
<tr>
<td>T6 R2</td>
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<td>10 ± 0</td>
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<tr>
<td>T6 R3</td>
<td>20 ± 0</td>
<td>8.33 ± 2.89</td>
</tr>
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<td>T6 R4</td>
<td>20 ± 0</td>
<td>8.33 ± 2.89</td>
</tr>
<tr>
<td>T6 R5</td>
<td>20 ± 0</td>
<td>10 ± 0</td>
</tr>
<tr>
<td>T6 R6</td>
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<td>T6 R8</td>
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<td>U115 R2</td>
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<tr>
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**Supplemental Table S5.4** Strains used in this chapter

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<th>Species</th>
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<th>Reference or Source</th>
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<td><em>E. coli</em></td>
<td>-</td>
<td>CGSC</td>
</tr>
<tr>
<td>BW25113 ∆icdC</td>
<td><em>E. coli</em></td>
<td>KanR</td>
<td>CGSC</td>
</tr>
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<td>BW25113 ∆tsx</td>
<td><em>E. coli</em></td>
<td>KanR</td>
<td>CGSC</td>
</tr>
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<td>This study</td>
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**Phage** | **Reference or Source**
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T6 | J. Wertz (Yale U)
U115 | 215

KanR, Kanamycin resistance; StR, Streptomycin resistance; CGSC, Coli Genetic Stock Center
References


78. Oechslin, F.; Piccardi, P.; Mancini, S.; Gabard, J.; Moreillon, P.; Entenza, J. M.; Resch, G.; Que, Y. A., Synergistic Interaction Between Phage Therapy and Antibiotics Clears


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