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UNIVERSITY OF CALIFORNIA SAN DIEGO

SAN DIEGO STATE UNIVERSITY

Formulation, Purification, and Application of Phages to Combat

Pseudomonas aeruginosa

A dissertation submitted in partial satisfaction of the requirements for the degree Doctor
of Philosophy

in

Biology

by

Tiffany Luong

Committee in charge:

University of California San Diego

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Professor Forest Rohwer

2024

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Chair

University of California San Diego

San Diego State University

2024

DEDICATION

To my 婆婆 (maternal grandma), 媽咪 (mom), 爹哋 (dad), 麻麻 (paternal grandma), Landon, and Ninja. Thank you for all your love and support.

EPIGRAPH

"We're so busy watching out for what's just ahead of us that we don't take out time to enjoy where we are."

-Bill Watterson, *Calvin & Hobbes*

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LIST OF ABBREVIATIONS

AMR	antimicrobial resistant
ATP	adenosine triphosphate
AUC	area under the curve
BSC	biosafety cabinet
CDC	Center for Disease Control
CF	Cystic Fibrosis
CFF	cross-flow filtration
CFTR	cystic fibrosis transmembrane conductance regulator
CFU	colony-forming unit
CsCl	cesium chloride
CSS	steady state concentration
CT	computed tomography
DEG	differentially expressed genes
DF	diafiltration
DGU	density gradient ultracentrifugation
DOI	Digital Object Identifier
dsDNA	double-stranded Deoxyribonucleic acid
ECS	extracapillary space
eIND	emergency Investigational New Drug
EOP	efficacy of plating
EPS	exopolysaccharide
EU	endotoxin unit

FDA	Food and Drug Administration
fDNA	free DNA
FEV ₁	forced expiratory volume in 1 second
FVC	forced vital capacity
GMP	good manufacturing practices
GO	gene ontology
h	hour
HFIM	hollow fiber infection model
IFN	interferon
IL	interleukin
IO	intraoperative
IR	intrarectal
IV	intravenous
kDa	kilodalton
KEGG	Kyoto encyclopedia of genes and genomes
LAL	<i>Limulus</i> amoebocyte lysate
LB	Luria Bertani
LOD	limit of detection
LPS	lipopolysaccharide
LTA	lipoteichoic acids
LVAD	left ventricular assist device
MDR	multidrug resistance
MIC	minimum inhibitory concentration

min	minutes
μL	microliter
μm	micrometer
mL	milliliter
mM	millimolar
MMEF	maximum mid-expiratory flow
MOI	multiplicity of infection
MWCO	molecular weight cut-off
NCBI	National Center for Biotechnology Information
OD	optical density
PAK_P1	P1A
PAMP	pathogen associated molecular pattern
PBS	phosphate buffered saline
PCA	principal component analysis
PCR	polymerase chain reaction
PD	pharmacodynamics
PEG	polyethylene glycol
PES	polyethersulfone
PFU	plaque-forming units
PK	pharmacokinetics
PTFE	polytetrafluoroethylene
pwCF	persons with Cystic Fibrosis
qCT	quantitative computed tomography

qPCR	real-time quantitative polymerase chain reaction
RT	room temperature
SDS	sodium dodecyl sulfate
SDS-PAGE	sodium dodecyl-sulfate polyacrylamide gel electrophoresis
SEC	size exclusion chromatography
SEM	standard error of the mean
SOC	standard of care
TKA	time-kill assay
UF	ultrafiltration
USP	United States Pharmacopeia
vol	volume
WHO	World Health Organization
wt	weight

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LIST OF SUPPLEMENTAL FILES

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SUPPLEMENTAL FILE 2: LUONG_CH5_CF_SUPPLEMENTARY1.PDF

SUPPLEMENTAL FILE 3: LUONG_CH5_CF_SUPPLEMENTARY2.XLSX

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PUBLICATIONS

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ABSTRACT OF THE DISSERTATION

Formulation, Purification, and Application of Phages to Combat

Pseudomonas aeruginosa

by

Tiffany Luong

Doctor of Philosophy in Biology

University of California San Diego, 2024

San Diego State University, 2024

Professor Dwayne Roach, Chair

Bacteriophages are viruses of bacteria that are among the leading alternative antimicrobials for treatment of the multidrug resistant (MDR) and opportunistic pathogen *Pseudomonas aeruginosa*. Although phage therapy is now generally considered safe, access to the treatment remains limited and its efficacy remains unproven. The goal of this dissertation is to develop innovative phage processes and intravenous treatment regimens to combat MDR *P. aeruginosa*. In the third chapter, I evaluate how to select phages to combat *P. aeruginosa* and study the interactions between different phage

strains during combination treatment. In two-phage cocktail treatment, I found that mixing phages with unequal activities diminishes initial treatment potency. Sequential treatment with the same phages improved potency but generated unique patterns of resistance. This study provides new insight on how to formulate and administer phage combinations based on the infectivity of each phage to its bacterial host. In the fourth chapter, I develop and optimize the standardized production of phages by introducing cross-flow filtration to improve the removal of endotoxin from phage preparations. The simultaneous washing and concentration of phage particles was found to improve endotoxin removal across multiple Gram-negative species. Leveraging the kinetics of each phage, the accumulation of phage-resistant bioburden could then be reduced in an improved purification process. In the fifth chapter, I validate the targeting and replication of clinical phages during the treatment of patients infected by multidrug resistant *P. aeruginosa*. Phages suitable for therapy were determined by screening across a multi-infection isolate library. The efficacy of clinical phage treatments were then validated using a combination of microbiological and metagenomic markers of failure or success. In conclusion, this thesis provides insights into current barriers for phage therapy. Identifying and characterizing individual phage properties will have important implications on how to treat and reduce the mortality of MDR *P. aeruginosa* infections.

CHAPTER 1

GENERAL INTRODUCTION

This work describes the development of innovative phage processes and therapeutic regimens for infections caused by the opportunistic pathogen *Pseudomonas aeruginosa*. Considered a priority pathogen by the World Health Organization (WHO) [1], the Gram-negative, facultative aerobe causes severe disease in humans with consistently high rates of multidrug resistance (MDR) that range from 11.5% to 24.7% in the United States [2, 3]. The bacterium is strongly associated with mortality and morbidity in immunocompromised patients, with 7% prevalence among healthcare-associated infections and chronically infecting >40% of persons over age 40 with cystic fibrosis (CF) disease [4, 5]. In different circulatory or respiratory organ environments, *P. aeruginosa* overcomes osmotic stress, intraspecies bacterial competition, antibiotic treatment, and oxidative stress by undergoing an array of metabolic, phenotypic, and genotypic changes to evade host defenses and antimicrobials [6, 7]. For patients with MDR infections, a mortality rate of 44.6% is nearly doubled that of 24.8% in non-MDR *P. aeruginosa* [8]. There is an urgent need to develop alternative antimicrobials for MDR infections which were associated with 4.95 million deaths in 2019 [9].

Bacteriophages (or phages) are viruses of bacteria that have been used sporadically to fight bacterial infectious diseases for over a century and are considered a top alternative antimicrobial [10]. Phages are the apex predators of the microbial world able to dominantly infect, replicate within, and lyse bacterial cells within all biospheres [11]. This parasitic cycle of phage replication also ensures, in most environments, that small numbers of phages can continually self-replicate so long as host cells are available.

By all accounts, phages are nature's most abundant bacteria killers and thus are attractive candidates for mechanistically novel antimicrobial therapy. Furthermore, phages have demonstrated synergy with existing antibiotic and host immune responses during bacterial infection making them well-suited for combination treatment [12, 13]. Moreover, the ability of phages to infect more specifically than chemical antimicrobials make them compatible for personalized treatment approaches that do not interfere with beneficial commensal bacteria within the body [14].

Unfortunately, bacteria can resist phage infection through a variety of innate immune systems and genetic mutations including blocking phage binding to cell surface receptors, enhancing extracellular defenses, preventing DNA entry, or using restriction modification systems to block the lytic cycle [15]. For phage therapy applications, the application of 'phage cocktails' or combinations of multiple phages against a bacterial strain is the most commonly used way to overcome resistance [15]. Phage cocktails have the advantage over single phage administration because the combined pressure of multiple phages often elicits a stronger adaptive response from bacterial cells. Cocktails can increase the fitness cost of acquiring phage resistance [16], decrease the frequency of genetic mutations [17], or simply expand the number of phages capable of targeting bacteria in an infection setting [18]. Phage strain type, genome size, gene content, replication rates, and phage resistance timing can vary widely between strains. Yet even subtle differences in these phage parameters appear to be correlated with inconsistent cocktail treatment outcomes [19, 20]. Although the traits desired from cocktail application are well known, the pharmacokinetics (PK) and pharmacodynamics (PD) of phages in

cocktails remain poorly understood and *in vivo* cocktail efficacy has been sporadic due to their empirical design [21, 22].

Despite their many positive attributes, phages fell out of favor as antimicrobials in the 1940s when easy to use, broad spectrum, shelf-stable antibiotics were introduced for infectious disease management [23]. A chief concern of manufacturing phage biologics is the presence of endotoxins from bacterial lipopolysaccharides (LPS) leftover from phage cultivation. LPS is a highly immunogenic component of the bacterial membrane which contaminates phage preparations during the destructive lysis of phage replication. Removing the bacterial carnage of their bacterial hosts has traditionally been laborious and time-consuming [24, 25]. Unwanted endotoxin contamination can elicit a number of inflammatory responses in humans including fever, anaphylactic shock, and even death [26]. Phages are in the same molecular weight range (~100kDa) as most endotoxins making conventional centrifugation and filtration inadequate for purification [27]. To this day, access to phage therapy remains bottlenecked by limited access to purified phage therapeutics.

Compassionate use, otherwise known as expanded access investigational new drug (eIND) in the US, treatments have been the primary means of administering phages to critically ill patients in the past 20 years. These single case studies and several clinical trials have largely focused on demonstrating that phage administration is safe [28-32]. However, phage therapy still lacks approval in the United States from the Food and Drug Administration (FDA) due to a lack of clinical efficacy [33]. Because phages are often combined with standard of care antibiotics, the question of how to validate phage treatment has remained open [34]. Several metrics of assessing phage therapy efficacy

have been suggested such as assessing whether phages reduced bacterial burden, whether phages replicated *in vivo*, and whether phages were in sufficient numbers to elicit bacterial resistance responses or host neutralization responses [34]. These phenomena would imply successful targeting of the pathogen, self-replication of phages at site, and/or sufficiently high *in situ* phage concentrations to induce pathogen and host responses. However, lack of standardization in microbiological analyses confounds comparison with clinical outcomes.

1.1 Goal and specific aims

The overarching goal of this dissertation was to develop innovative phage processes and intravenous treatment regimens to combat MDR *P. aeruginosa*. I hypothesize that deciphering the nuanced uniqueness of each phage's infectivity provides optimal calibration of therapeutic formulations, purifications, and applications.

Aim 1 is to interrogate the kinetics of individual phages in cocktails to determine biomarkers or regimens that improve formulation efficacy.

Aim 2 is to develop and improve the cultivation and purification of clinical phages.

Aim 3 is to survey clinical *Pseudomonas* targets and validate the activity of phages during clinical phage therapy.

The major objectives were:

- (i) To survey potential anti-*Pseudomonas* phages and formulate therapeutic phage cocktails.
- (ii) To study phage cocktails against *P. aeruginosa* and determine the kinetics of their interactions in microtiter plates and hollow fiber infection model.

- (iii) To develop and scale-up phage purification for clinical and immune studies utilizing cross-flow filtration technology.
- (iv) To improve removal of bacterial endotoxins by using individual growth kinetics to limit the growth of phage resistant cells during lysate production and simplify phage production.
- (v) To screen a library of phages against clinical *P. aeruginosa* isolates and determine phage suitability.
- (vi) To validate phage targeting during failed and successful therapies and determine whether phage replication *in situ* corresponded to changes in infectious load burden and genotype.

CHAPTER 2

BACKGROUND

2.1.1 The pathogen, *Pseudomonas aeruginosa*

The versatility of *Pseudomonas aeruginosa* has allowed it to maintain its spot in the World Health Organization's (WHO) list of priority pathogens since the list's inception in 2017 [1]. Originally described in 1882 by the French pharmacist Carle Gessard, the pathogen was first identified infecting bandage wounds and has since continued to be a major cause of death in humans [3]. *P. aeruginosa* is a Gram-negative opportunistic pathogen that can be isolated from most natural and human environments. The nosocomial pathogen is particularly dangerous for persons who are immunosuppressed. As a facultative aerobe, *P. aeruginosa* is equipped with five terminal oxidases that allow it to readily grow in soil, water, and host-associated environments [35]. As such, respiratory, wound, and systemic infections by *P. aeruginosa* are readily adapted into chronic infections at organ sites [36]. Paradoxically, this makes *P. aeruginosa* both a highly malleable pathogen to study *in vitro* but a nigh impossible opponent to eradicate in the clinic. Several laboratory strains of *P. aeruginosa* have become well-established in research including the PAO1 [37], PAK [38], and PA14 strains [39]. These references and the abundance of available clinical strains have made comparative genomic tools against the bacterium highly accessible, allowing researchers to improve strain identification, perform phylogenetic analyses, and standardize antimicrobial target design [40, 41].

P. aeruginosa has an array of virulence factors and defenses to resist host defenses and antimicrobial treatments [36]. The bacterium's structure protects and

contributes to its ability to cause disease. The lipopolysaccharide (LPS) structure of *P. aeruginosa* contains three domains, the lipid A, core oligosaccharide, and O-antigen [42]. This protective layer for the bacterium itself is considered an endotoxin to the human host and elicits pro-inflammatory responses, causes tissue damage, and allows bacterial attachment to cells [26]. The O-antigen of *P. aeruginosa* in particular is a potent agonist against human tissue but attempts to design vaccines against it has largely been ineffective due to the many serotypes [43]. Modification of the O-antigen also plays a role in the bacterium's transition to a chronic state, whereby the bacterium increases secretion of exopolysaccharides such as alginate, Psl, and Pel polysaccharides which allows it to form complex biofilms [44]. Complex quorum sensing pathways and stress regulators enhance the pathogen's evasion of host immunity [45, 46]. Within the body, *P. aeruginosa* can therefore acutely penetrate and transition into chronic infection in almost any organ.

P. aeruginosa also boasts innate resistance to multiple classes of antibiotics, making it often appear in the clinical as a multidrug resistant (MDR) bacterium. Molecular mechanisms such as biofilm formation [47], LPS modification [48], and possession of four multidrug efflux pumps [49] contribute to its arsenal of defenses against antibiotics. Horizontal gene transfer between *P. aeruginosa* and spontaneous mutations in the clinical setting also facilitate acquisition of antibiotic resistance genes [50]. Consequently, rates of *P. aeruginosa* multidrug resistance range from 11.5% to 24.7% in the US [2].

2.1.2 *P. aeruginosa* in human disease

P. aeruginosa is an indicator of mortality and morbidity in circulatory and respiratory infections and can chronically infect the heart and lung [8]. Heart disease is a significant

concern in the United States wherein 1 in 4 Americans will develop heart failure in their lifetime [51]. Treatment of advanced heart failure can include the use of left ventricular assist devices (LVADs) that help heart circulation while patients are awaiting transplant or serve as a permanent alternative for those compatible [52]. As with most major surgeries, a complication of LVAD usage is the risk of device-related infections which can occur in as many as one-third of LVAD recipients [53]. Although originating the device, infections by *P. aeruginosa* can extend to the driveline and even circulate the bloodstream [53]. Currently, long-term antibiotic usage is the primary way to suppress the infection but, once infected, LVAD infections are generally not curable without removal of the device [54]. In treatment of LVAD infections, typically debridement of the site of infection with oral or intravenous antibiotics is recommended [53]. Device replacement is a last resort only if the infection cannot be controlled and has spread to the bloodstream [53].

Cystic fibrosis is the result of mutations in the CF Transmembrane Conductance Regulator (CFTR) gene that encodes an ion channel expressed on the epithelial cell surfaces of many organs. In the lung, where the most severe symptoms occur, CFTR defects cause increased mucus viscosity and compromise mucociliary clearance, leading to small airways plugging, irreversible structural and functional damage, and eventually hallmark chronic bacterial infection [55]. In the upper airways, the morphology of CF disease causes structural defects such as thicker airway walls that help the sinuses form a habitat for bacterial colonization [56]. In the lower respiratory tract, thick mucus production prevents clearance of the lung cilia and changes the airway environment to allow microbes and pathogens absent in healthy lungs to colonize the lungs [57].

Cystic fibrosis sputum is composed of abnormally thick mucus, with a 5-10 fold higher mucin to water ratio [58] which enables trapping of bacteria such as *P. aeruginosa*. As CF disease pathology progresses with age, lung anatomy and sputum microstructure are altered by excessive mucin and bacterial extracellular DNA content. This creates a microaerobic or anaerobic environment in the lung that not only facilitates growth of resilient *P. aeruginosa*, but also hastens the decline of lung function [59]. This abnormal oxygen environment further alters even immune responses of the host to *P. aeruginosa* infection [60], allows the chronic infection to persist, and at times develop into acute pneumonia. *P. aeruginosa* therefore flourishes in the CF lung environment and generates a diverse multivariant infection as CF patients age [61]. Chronic infection by *P. aeruginosa* is highly associated with morbidity and mortality in CF patients [62]. Microevolution of *P. aeruginosa* in the lung environment allows colonization of a MDR mixed community consisting of typical nonmucoid *P. aeruginosa* bacterial cells and mucoid *P. aeruginosa* [63]. Mucoid growth is defined by overproduction of exopolysaccharide alginate, a mutation often associated with O-antigen modification [64]. Co-isolation of the two bacterial variants is associated with increased resistance to antibiotics, host antimicrobial response, and heightened decline in CF lung function [65].

The primary strategy to treat *P. aeruginosa* infection in CF is to delay infection for as long as possible. Once infected, chronic management of *P. aeruginosa* involves lifelong antibiotic usage, either through nebulization, oral, or even intravenous administration during hospitalization [66, 67]. CF patients will also experience acute pulmonary exacerbation events, the onset of severe lung function decline necessitates hospitalization and medical intervention [68]. To treat *P. aeruginosa* exacerbations, the

standard of care is to administer combination antibiotics to reduce the risk of selecting for resistant bacteria [69]. Antibiotics are also accompanied by other strategies to clear the CF airways including bronchodilators, recombinant DNase treatment to reduce the viscosity of sputum, and for those whose gene mutation are compatible, the use of CFTR modulator therapies to improve CFTR channel function [70-72]. These treatments in combination can help up to 75.4% of people recover up to 90% of their baseline lung function [73]. However, increased MDR strain frequencies necessitate new antimicrobials for the management of *P. aeruginosa* in CF disease.

2.2.1 Phage therapy

Bacteriophages (phages for short) are viruses that infect, replicate within, and lyse bacteria. They were independently discovered in the early 20th century by Felix d’Herelle and Frederick Twort. Both scientists were able to observe phage plaques on agar plates, but d’Herelle was the first to name and identify his as the bacteriophage [74]. Phages are the most abundant form of “life” with incredibly large biodiversity. With an estimated 10^{31} phages in the biosphere, they are the ultimate bacteria killing organisms on the planet. Their vast diversity and abundance means that these predators are readily isolated from all animals, plants, bodies of water, soils, and sewage [75].

The most abundant phages associated with the human body and human application are *Caudoviricetes* double-stranded DNA (dsDNA) phages [76]. These phages are typically composed of an icosahedral, proteinaceous capsid that protects the phage genome. *Caudoviricetes*, also commonly referred to as tailed phages, can have three different morphologies: podovirus which have a nonenveloped icosahedral head

and very short noncontractile tail, myovirus which have a nonenveloped icosahedral head and long contractile tail, and siphovirus which have a nonenveloped icosahedral head and long noncontractile tail [77]. These phage structures were originally used to taxonomically summarize the diversity of phages within groups but have since been demoted to describe only the structure of the viruses.

Like all other viruses, phages must hijack the cellular machinery of a host cell to replicate. Phages can undergo one of two lifecycles: the lytic cycle or lysogenic cycle. Lysogenic, or temperate, phages integrate into the bacterial chromosome and typically give bacteria a fitness advantage. In contrast, the process of lytic replication can be divided into the phage properties of adsorption rate (binding), eclipse period (completion of new phage virions), latent period (host lysis), and burst size (number of phage progeny) [11, 78]. Lytic phage infection results in the production of new phage particles and bacterial lysis [11]. At each step of lytic replication, phages are equipped for highly specialized bacterial killing. Phages under consideration for most human applications are those that are considered strictly lytic. This is most commonly determined by genome annotation of the phage and determining that an integrase gene is absent [79]. The presence of this gene generally disqualifies the phage as a therapeutic candidate. While most phage genomes are relatively small, typically ranging from 10-100kbp [80], other genes that are avoided are bacterial virulence factors such as antibiotic resistance genes and toxins [79].

The diversity of phage binding receptors and structures underscores the numerous mechanisms that phages use to overcome bacterial evolution [81]. Phages can bind to regions of the cell wall (e.g., lipopolysaccharide (LPS), lipoteichoic acids (LTA)) or

bacterial appendages (e.g., pili, flagella) [81]. Most phages can only infect a limited number of strains within a species due to binding receptor specificity [82, 83]. Once irreversibly bound, phages have variable replication rates. Some phages appear to produce fewer progeny quicker (short latent period, small burst) while others produce numerous virions at a slower rate (long latent period, large burst) [82]. As a potential alternative antimicrobial agent for use in human therapeutics, agriculture, or food production, phages are generally recognized as safe, as they cannot infect mammalian cells, and can rapidly translocate throughout the body to target the site of infection [84, 85]. This parasitic and enzymatic bacterium killing is different from the antibacterial mode of action by all other drugs, making phages potentially effective against MDR bacteria.

2.2.2 Dynamic *P. aeruginosa* and phage interactions

Phages specific to *P. aeruginosa* infection were first described in the late 1950s, close to four decades after the initial discovery of phages [86, 87]. Like the majority of phage-bacteria interactions in other species, anti-*Pseudomonas* phages infect their host and either perform lysis or become lysogenic and integrate with the host genome. *P. aeruginosa* boasts an array of defense systems to resist phage infection including genetic or phenotypic modification of cell surface receptors and intracellular defense systems [88]. One study found that clinical *P. aeruginosa* isolates carry at least 71% of known defense systems, making the species attractive for studying bacterial immunity against phages [89]. In comparison, the type strain PAO1 was not found to harbor CRISPR defense systems and only has a type II restriction modification system [89].

P. aeruginosa can evolve resistance to phages by modifying the structures to which phages bind, such as their LPS and pili [90]. This can occur at a per cell basis whereby phenotypic resistance can transiently prevent the adsorption of phages onto cells [91]. Resistance can be more permanent through single nucleotide polymorphisms (SNPs) in cell surface genes. Mutations generally carry a fitness cost to the bacterium, but SNPs can render entire populations of bacteria immune to all phages utilizing that receptor [92]. This ability to become “cross-resistant” to phages conveys a strong advantage to bacteria in evading phage predators using conserved LPS structures for binding [92]. In response to certain phage infections, *P. aeruginosa* can also employ a large chromosomal deletion of a gene region to acquire resistance [92, 93]. Large deletions have been associated with higher cellular stress and appear to occur in response to LPS phage pressures [92].

Other phage resistance mechanisms rely on intrinsic defense systems. These include superinfection exclusion systems, restriction-modification systems that cleave phage DNA, clustered regularly interspaced short palindromic repeats (CRISPRs), and abortive infection systems [15]. These systems have been shown to activate largely secondary to surface modifications for *P. aeruginosa* [94]. A generalized explanation for this phenomenon is that surface modifications carry a fixed cost, independent of phage type exposure while inducible defenses become costly when elicited [95]. Thus, surface modifications are more readily accessible and activated when phage exposure occurs.

2.2.3 Phage cocktail formulation to overcome bacterial defenses

One of the methods by which the emergence of resistance may possibly be prevented, or at least delayed, is the use of combination therapy. The advantage is that

the chance of bacterial offspring being resistant to a cocktail of unique phage types is exceedingly small and, provided that the resistance mechanisms are independent, it is the product of chance single mutations [96]. This feature was recognized many years ago and is one of the reasons that infections with mycobacteria are treated using multiple classes of antibiotics [17]. The same principles apply for antiretroviral therapy [97]. Current rationales for the design of combination phage therapy regimen by host range date back to the 1920s and 1930s [23], and serendipitously, was concurrent with the initial characterizations of filterable bacterial eaters (bacteriophages). The development of these regimens in the absence of any knowledge of phage type or infection mechanism was a remarkable achievement. Comparative studies indicate that emergence of resistance is less common when combination phage therapy is used [18, 83]. This is particularly true for bacteria known to develop resistance relatively quickly, such as *P. aeruginosa* [98, 99]. Although our knowledge of the genetic drivers of phage resistance has increased dramatically over the past 20 years, this information has been difficult to adapt to clinical practice [100]. As such, even commercial combination regimens often fail to cure many patients [101, 102].

Administering combination phage therapy has been suggested in two formats, either simultaneously administering two or more phages in combination or sequentially administering the phages one after another. The emergence of phage resistance signals the end of the therapeutic usefulness of phages. Unlike antibiotic resistance, which requires higher concentrations of the drug over time to achieve a therapeutic effect, phage resistance is an ‘all-or-nothing’ phenomenon [90]. Once genetic resistance blocks adsorption to the bacterium, the phage strain is no longer effective against the evolved

host. Sequential treatment has been suggested as a method to prolong the effectiveness of phage combinations. However, *in vitro* sequential resistance appears to allow a greater number of mutations to occur in comparison to simultaneous cocktails. Hall et al. found that individual phage strains could vary in effectiveness and convey cross-resistance, but that a cocktail of 4 phages was the most effective short-term treatment [103]. Furthermore, bacterial phage resistance to the multi-phage treatment resulted in greater fitness cost in the form of reduced basal growth rate in phage-resistant mutants [103]. Betts et al. similarly found that increasing the diversity of phages caused bacteria to favor more general resistance mechanisms but could not prevent eventual host resistance [16]. Expanding on Hall et al., Wright et al. found that the timing and order of phage exposure could determine the strength and fitness cost of bacterial resistance during sequential phage treatment [104].

Other cocktail studies have focused on whether large cocktail sizes can prevent resistance growth. Yang et al. found that resistance to a five-phage cocktail inhibited growth for up to 5 days before the bacterial density increased to $OD_{600} > 0.5$ [105]. Pinto et al. observed in a range of 2-5 phage cocktails that the best result was a formulation of all 5 phages, but that the number of strains did not necessarily correlate to improved cocktail activity due to competition and cross-resistance [106]. Finally, Markowitz et al. most recently found that 2 and 3-phage cocktails encouraged more bacterial mutation heterogeneity than single-phage treated bacteria [98].

Combined, these studies appear to validate that combination phage activity is preferred to delay phage resistance for as long as possible. However, the mechanisms behind phage-phage interactions in each case were not well elucidated compared to the

outcomes of mutational resistance and fitness costs. Phage selection in these studies has also varied considerably and it remains unclear which anti-*Pseudomonas* phage types are best suited for cocktail outside of receptor comparisons. Moreover, only two of these studies compared the effect of simultaneous and sequential phage treatments [103, 104].

2.3 Antimicrobial development against *P. aeruginosa*

A common *in vitro* technique to study the kinetics of antimicrobial agents is the time-kill assay (TKA). In principle, the time-kill assay is used to examine the rate at which different concentrations of an antimicrobial can kill a bacterial isolate [107]. Time-kill assays performed in 96-well microtiter plates are particularly versatile because they can capture independent stochastic events with multiple biological and technical replicates in a single experiment. The well-mixed environment of the well also ensures that the kinetics of phage-bacteria interactions begin nearly immediately upon the addition of the virus. Time-kill strategies are also easily standardized for fixed bacterial inoculums, allowing analysis between untreated controls and the antimicrobial agent as well as across multiple experiments [108]. Automated plate reader readings also allow averaging within wells and across plate samples. In this way, time-kill kinetics allow evaluation of whether the antimicrobial agent produces a concentration-dependent killing effect or a time-dependent killing effect [108]. As a result, time-kill kinetics have been used extensively to study the relationship between *P. aeruginosa* and its phages [103-106]. The majority of these experiments have focused on the study of anti-*Pseudomonas* phage combinations

and their ability to suppress bacterial growth and subsequent resistance mutation. However, *in vitro* results often fall short and translate poorly to *in vivo* systems.

The clinical pharmacokinetics and pharmacodynamics (PK/PD) of *P. aeruginosa* and phages remains one of the most difficult aspects of study for understanding phage efficacy [109]. The hollow fiber infection model (HFIM) can provide a dynamic, *in vitro* method to explore antimicrobial development in a pseudo-human circulatory system [110, 111]. The system can be set up to perform a co-culture system, as in time-kill kinetics, but also include an element of continuous flow circulation and filtration whereby phages can dynamically flow in and out of a central cartridge (Figure 2.1). This creates a two compartment model whereby phages can freely interact with bacterial cells but also selectively be filtered out akin to human circulation and kidney filtration [111].

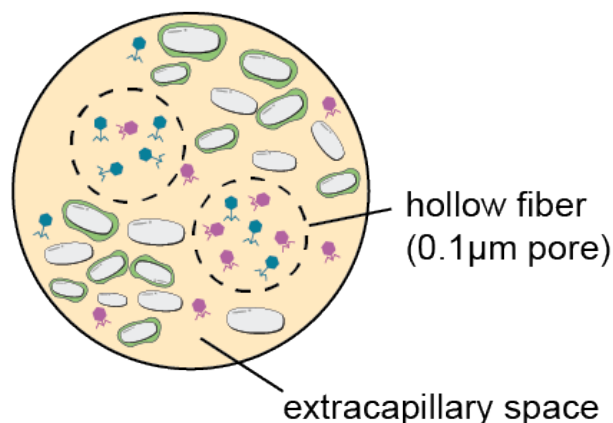


Figure 2.1 Schematic of phage-bacteria interactions in HFIM cartridge. The HFIM cartridge houses *P. aeruginosa* cells which are too large to pass through the 0.1 μm pore size of the hollow fiber membranes. Bacteria is retained in the extracapillary space while phages <0.1 μm can freely pass through the membranes along with circulatory media exchange within the cartridge.

The central cartridge of the system can then be sampled to quantify bacterial and phage samples in real-time. In practice, this system has been used more extensively in

the past decade to explore the PK/PD of *P. aeruginosa* and combination antibiotics such as ceftolozane-tazobactam [112], fosfomycin and meropenem [113], piperacillin [114], and meropenem and amikacin [115]. The model has also been used to explore the interactions between the antibiotic ciprofloxacin and phages in a more recent study and found that simultaneous phage and antibiotic administration could improve performance compared to either agent alone [116]. This would suggest that study of *P. aeruginosa* and individual or combination phages is compatible with this system. Other models of antimicrobial development include synthetic or natural drug screens and *in silico* or AI-driven drug discovery platforms [117]. Several downstream processes in these pipelines such as compound screening and hit validation would also be compatible with phage development but often fall short due to the self-replicating nature of phages.

2.4 Non-good manufacturing practice of phages for IND use

In the US, the FDA mandates that commercial drugs are produced under good manufacturing practices (GMP), a system that ensures that pharmaceutical products are made consistently and meet quality standards [118]. Because phages are currently under consideration as an investigational new drug only a limited number of phages are being produced under GMP regulations [119]. In order to proceed with compassionate phage use, non-GMP phage products are currently being used based on several ad hoc laboratory methods [120, 121]. However, to be used in US eIND treatment these products must at minimum meet United States Pharmacopeia (USP) testing standards [109]. This would include at minimum testing for product sterility (USP 71) and endotoxin (USP 85) quantification [109].

Purification of phages is largely bottlenecked by the difficulty of removing endotoxins from produced phage lysates [122, 123]. Because phages obligately require bacterial cells to replicate, the consequence of successful replication is the destruction of bacterial cells which remain in solution with freshly produced phage particles. Phages with select structures or infectivity parameters may be more difficult to separate from bacterial debris or generate a larger upstream bacterial burden. Gross bacterial impurities could trigger potentially life-threatening inflammation and septic shock in humans [124]. Endotoxins are of primary concern and the FDA limits human intravenous drugs to <5 endotoxin units (EU)/kg/h [122]. However, endotoxin removal without sacrificing phage concentration remains difficult at-scale for compassionate use. For example, phages prepared under prescription as “magistral preparations” for human treatment in Belgium [125] and for expanded access eINDs [126] were reported to yield only $\sim 10^7$ plaque-forming units (PFU)/mL at >10 EU/mL.

Current phage preparation methods are often time consuming, expensive, involve harmful chemicals, or are difficult to scale-up for generating larger batches of phage [127-129]. Low-cost methods like polyethylene glycol are popular but result in high-salinity and high-endotoxin preparations [25, 128]. Chromatographic methods are varied and more recently employed, but are costly and difficult to scale-up due to instrument limitations [130]. Organic solvents are increasingly used to reduce endotoxins in lysates [127, 131, 132], but can be toxic to users, inhibit chromogenic-based endotoxin quantification methods, and decrease phage stability in storage [132]. Sample recovery using organic solvents is also inconsistent, sometimes being as low as 15% [129]. Standardized

methods to remove endotoxin with considerations for accessibility to clinical users and small research groups are necessary.

2.5 Current state of phage therapy (published review)

This section is a reprint of a published review entitled: *Phage Therapy in the Resistance Era: Where Do We Stand and Where Are We Going?* Printed in *Clinical Therapeutics*, September 2020. Written by Tiffany Luong, Ann-Charlott Salabarria, and Dwayne R Roach. This review summarizes the past two decades (2005-2020) of phage clinical trials and patient cases and outlines the fundamentals behind phage treatment strategies under development. The remaining barriers to phage therapy development and the role of phages with standard of care antibiotics is discussed after review of these cases.

Introduction

The ability to control infectious diseases marked a revolution in human health, shifting the global burden of disease from infectious diseases to noncommunicable diseases now being the leading cause of death globally [133]. The 20th century brought an appreciation of bacteriophages (often known simply as phages) and their relationship to infectious diseases, which resulted in the implementation of “phage therapy” to decrease the incidence of several infectious diseases. From the first successful administration of phages in 1921 at Hôpital des Enfants-Malades (Paris) treating children suffering from shiga dysentery to its widespread use in humans around the world, the effectiveness of phage therapy was highly controversial [23]. In 1945, a new era began with the golden age of antibiotics, and phage products were taken off the market.

However, shortly thereafter, penicillin resistance became a substantial clinical problem. In response, a steady stream of new classes of antibiotics and modifications to old-classes, were developed and deployed [134].

However, the antibiotic pipeline has been dry for decades [134]. Today, multi-drug resistant (MDR) bacteria are becoming commonplace, making available antibiotics much less effective [135]. In 2017, the World Health Organization (WHO) released a global priority pathogens list of antibiotic-resistant bacteria (Table 2.1) [1]. Based on diverse criteria such as mortality, prevalence of resistance, treatability and current pipeline, the situation is critical for the majority of difficult-to-treat community-acquired, healthcare-associated and nosocomial infections caused by ESKAPEE (*Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, *Enterobacter spp.* and *Escherichia coli*) pathogens. We included *Mycobacterium tuberculosis* in the critical category as an additional priority global health threat. The list is an excellent guide for the prioritization of research and development aimed at discovering new antibacterial therapies, which are critically needed [136, 137].

Table 2.1 World Health Organization priority pathogens.

Table I. The World Health Organization global priority pathogens for research and development of new antimicrobials.*	
Priority	Bacterial Pathogen
Urgent	<i>Acinetobacter baumannii</i> , carbapenem-resistant
	<i>Pseudomonas aeruginosa</i> , carbapenem-resistant
	Enterobacteriaceae, [†] carbapenem-resistant, third-generation cephalosporin-resistant
	Mycobacteria, including <i>Mycobacterium tuberculosis</i>
Serious	<i>Enterococcus faecium</i> , vancomycin-resistant
	<i>Staphylococcus aureus</i> , methicillin-resistant, vancomycin-resistant
	<i>Helicobacter pylori</i> , clarithromycin-resistant
	<i>Campylobacter</i> , fluoroquinolone-resistant
	<i>Salmonella</i> species, fluoroquinolone-resistant
	<i>Neisseria gonorrhoeae</i> , third-generation cephalosporin-resistant, fluoroquinolone-resistant
* Data are from the World Health Organization. ⁵	
[†] Includes <i>Klebsiella pneumoniae</i> , <i>Escherichia coli</i> , <i>Enterobacter</i> species, <i>Serratia</i> species, <i>Proteus</i> species, <i>Providencia</i> species, and <i>Morganella</i> species.	

The inevitable demise of all available antibiotics has reignited the development of phage therapy. Phages are naturally occurring viruses that infect both Gram-positive and Gram-negative bacteria. As such, they are generally unaffected by antibiotic resistance and (unlike most antibiotics) are able to target bacteria encased in biofilms [82, 138]. A substantial volume of preclinical data, as well as a growing body of clinical evidence shows the immense therapeutic potential across a wide range of infectious diseases. For decades, the Eliava Institute in Georgia and the Ludwik Hirsfeld Institute in Poland have

provided phage therapies to hundreds of nationals and internationals. The Eliava Institute has routinely provided off-the-shelf fixed-phage products as well as personalized single-patient phage treatments as extempore medications [33]. The Ludwik Hirszfeld Institute provides phage therapy when the standard-of-care (SOC) has failed, under the Declaration of Helsinki (compassionate use) [139]. Recently, Belgium implemented single patient phage therapy regulation as magistral preparations (i.e. pharmacy compounding), with phage products prepared and deployed by the Queen Astrid military hospital [125]. France has adopted a similar exceptional single patient authorization dispensed by hospital pharmacies with a prescription [140]. In the United States (US), the Food and Drug Administration (FDA) has recently approved multiple phage therapy clinical trials, as well as several phage therapies as expanded access investigational new drug (eIND) for patients with an immediately life-threatening condition or serious disease where SOC has failed [141-143].

In this Review, we summarize the growing body of clinical data on phage therapy and discuss how phages transition from “bench to bedside” and beyond. Our aim is to highlight the importance of approaching translational and clinical research from various angles and develop innovative ways to overcome the many challenges with implementing phage therapy.

The Active Agent

Phages are bacteria-restricted viruses that exploit all natural environments, including the human body [144, 145]. With more than 10^{31} phages estimated to be in the biosphere [146], they are readily isolated from soil, bodies of water, feces and sewage [147]. Although, phages come in a variety of structural morphologies and genome types,

most discovered are non-enveloped icosahedral head and tail in structure and have dsDNA genomes. Viral tails are unique to phages and can be contractile, short non-contractile, or long non-contractile, which allow for viral classification as myoviruses, podoviruses and siphoviruses, respectively [148]. Phage genomes have enormous genetic diversity, with as much as 80% of the predicted genes having no known function [80]. In addition, phages exhibit substantial gene diversity even with phage strains that infect a common bacterial host [149]. Together with their enormous abundance, great diversity and relative ease of isolation, phages provide an unlimited source of antibacterial agents against all human bacterial pathogens.

When virulent dsDNA phages infect susceptible host bacteria *in vitro*, the number of phages in the culture supernatant does not increase until after, generally, 30 to 40 min at 37°C has passed. This period-of-time is required for a phage particle to inject its genome into the host cell, hijack host metabolism, express its viral genes, assemble phage particles (10s to 100s), and release progeny into the environment (Figure 2.2). Cell lysis is the result of viral endolysins cleaving bonds within the cell wall peptidoglycan, which causes the cell wall to destabilize and osmotic rupture to occur under the high internal pressure [150]. A successful infection by a virulent phage always leads to cell lysis.

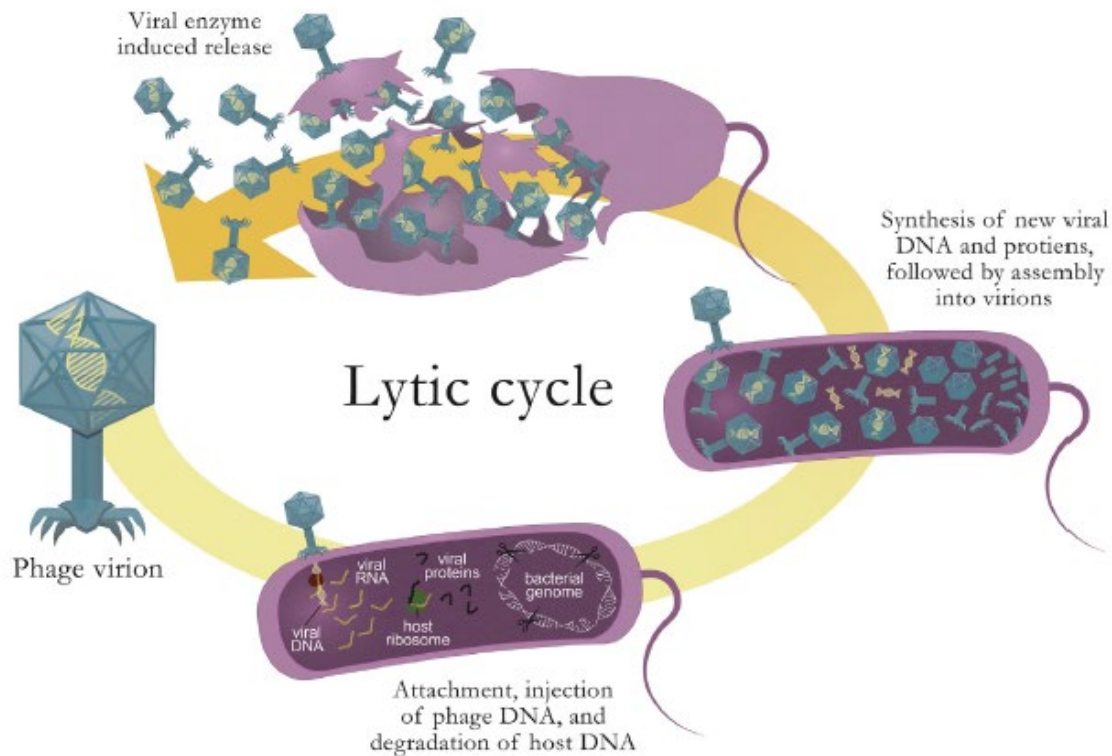


Figure 2.2 Lytic infection cycle of virulent phages. Step 1: adsorption: attachment of tail fibers onto a specific receptor on the bacterial cell wall. Step 2: injection: viral DNA penetrates the host through a hollow tube in the tail. Step 3: protein synthesis and host hijacking. Viral genes direct the synthesis of viral proteins using the machinery of the host. Steps 4 and 5: viral genome synthesis and assembly. Step 6: release: mature viral particle (virion) release occurs via viral peptidoglycan hydrolases (endolysins) that mediate host cell lysis, which liberates up to 200 infectious phages.

By contrast, infection by a temperate phage can lead to lysogeny. That is, temperate phages infect bacteria as a symbiotic prophage and replicate along with the bacterial chromosome during bacterial cell division. When exposed to environmental cues or stressors, the prophage resumes a lytic cycle to create phage particles [151]. Due to an inherent capacity of prophages to mediate the transfer of genes between bacteria by specialized transduction – an event that may increase bacterial virulence or promote antibiotic resistance — generally disqualifies temperate phages for therapeutic purposes. However, advances in synthetic biology have provided new opportunities for the use of temperate phages as a therapy against bacterial infections by removing so called

“lysogeny genes” from their genome [152, 153]. This is beneficial for infections caused by bacterial species with little to no virulent phages have been discovered, such as *Clostridium difficile* [154] and *Mycobacterium abscessus* [153].

The “parasitic” and “enzymatic” bacterial killing by phages are different from the antibacterial mode of action by all other drugs. This allows them to be effective against most MDR bacteria (Figure 2.3). In addition, self-replication in the presence of susceptible bacteria provides density-dependent dosing at the site of infection. Reducing the number of daily doses through extended-release drugs can reduce the relapse of disease, symptomatology associated with drug therapy (i.e. drug-related problems), patient's non-compliance, and costs [155]. However, the association between number of phage doses per day and relapse in infections remains uninvestigated.

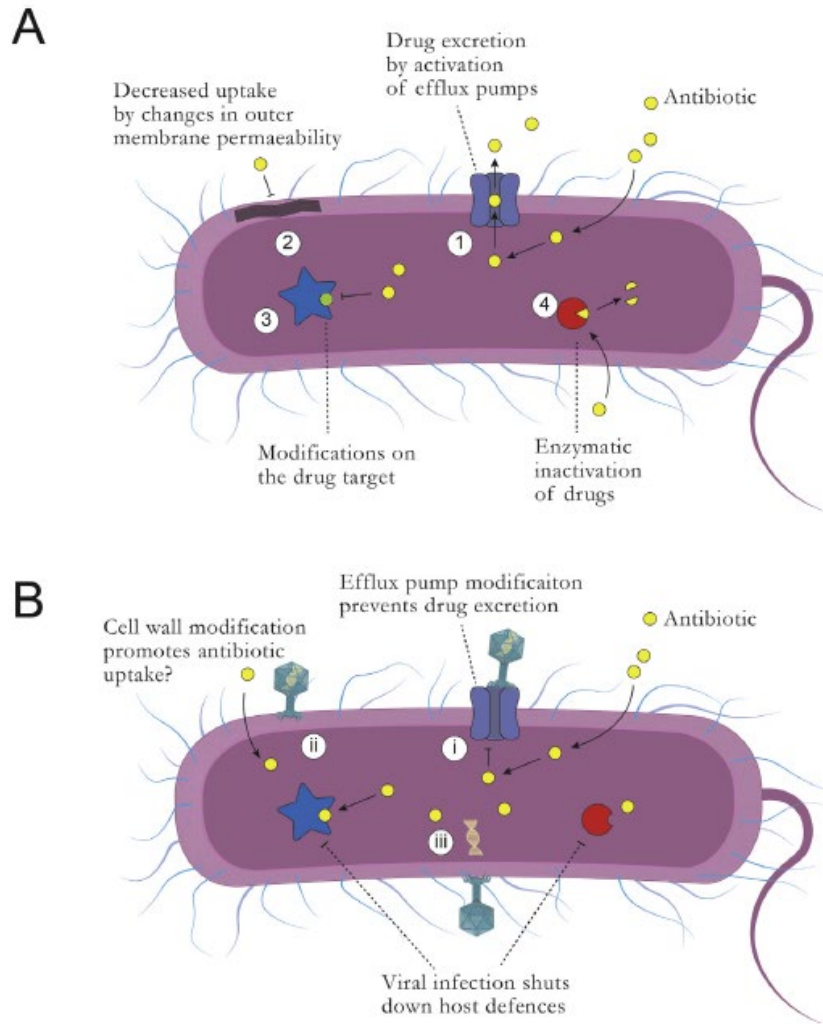


Figure 2.3 Phages as potential antibiotic adjuvants. (A) There are 4 main mechanisms of antibiotic resistance: (1) excretion by activation of efflux pumps, (2) decreased uptake by changes in the outer membrane permeability, (3) modifications on the drug target, and (4) enzymatic inactivation of the drug. (B) Phages can potentiate the antibiotics by altering the physiologic activities exhibited by phage sensitive and resistant bacteria by (1) direct blockade or mutation of drug efflux mechanism, (2) enhancement of drug uptake through cell wall mutations (untested), and (3) inhibition of antibiotic resistance elements.

A novel aspect of phage therapy is the potential for low-dose phage treatments, relying on self-replication to bring up the phage concentration at the site of infection. For self-dosing to occur, target host cell densities need to be over a “replication threshold” of 10^4 colony forming units (CFU) per mL or more [156, 157].

Phage infection can only take place after the viral tail fibers bind irreversibly to specific receptors on the bacterial cell wall, including lipopolysaccharide (LPS or endotoxin), teichoic-acid, pili, outer membrane proteins, efflux pumps, and polysaccharide. This stringent compatibility causes phages to be specific against bacterial targets and prevent tropism for mammalian cells. Nevertheless, phages can rapidly translocate throughout the body, even penetrating compartments previously considered sterile [84, 85]. However, despite the increasing number of clinical applications, there is a very limited understanding of the interactions between phages and human cells.

Clinical Data

It is well recognized that all recipients of phage therapy have so far been treated empirically, with incomplete information on, for example, the phages, adequate route of administration, dosing, duration and antibiotic compatibility. Figure 2.4 and in the subsections below briefly summarizes select phage therapy clinical trials and single patient reports between 2005-2020 grouped by route of administration. Further case data are summarized in Supplemental File 1. With limited clinical trial data, lessons learned from personalized case studies under eIND have advanced medicinal phage technologies and highlighted potential risks and challenges, which we review in subsequent sections.

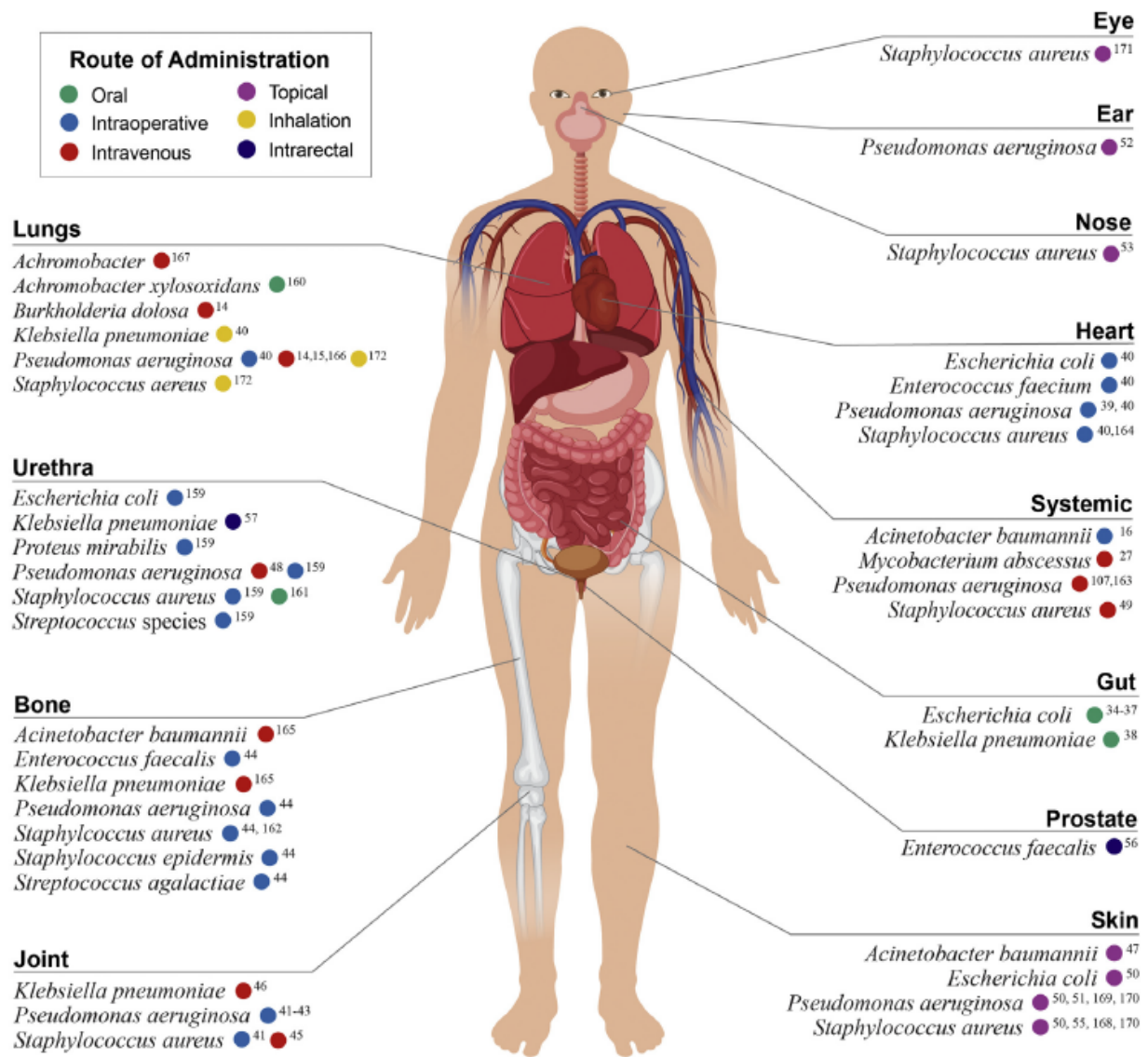


Figure 2.4 Summary of the phage therapy clinical trials and compassionate use case reports from 2005 to 2020. Case reports are categorized by the site of infection and target pathogen. The color coding represents the primary route of phage administration in each case report.

Oral route

A randomized, double-blind and placebo-controlled phase I study, oral ingestion 3x daily for 2 consecutive days of a single phage targeting *Escherichia coli* at a 'low' dose of 9.0×10^5 and 'high' dose of 9.0×10^7 plaque forming units (PFU) per dose caused no adverse effects in healthy adults [28]. In another safety study, oral ingestion did not cause serious adverse effects after oral ingestion of a 10-phage mix (cocktail) targeting *E. coli* at 1.5×10^7 to 1.5×10^8 PFU/dose in both adults and children. However, increased aspartate aminotransferase levels were observed in children, and some adults reported transient abdominal pain, dyspepsia, and toothache [29]. In a double-blind placebo-controlled crossover trial, healthy adult daily dietary supplementation with a 4-phage cocktail targeting *E. coli* at 10^6 PFU/dose had no serious side effects after 28 days [158]. Although recipients had reductions in fecal *E. coli* counts, there was no global disruption of the gut microbiota community, and both short-chain fatty acids and lipid metabolism were largely unaltered, thereby suggesting that phages can alter targeted microbiome species without inducing dysbiosis.

In a randomized controlled therapy trial, oral phage treatment targeting diarrheal *E. coli* in children did not improve acute diarrhea symptoms any more than oral rehydration therapy [159]. Children received either 1.4×10^9 PFU/dose of a Russian commercial 11-phage mix [29] or 3.6×10^8 PFU/dose of a 10-phage cocktail, 3 times daily for 4 consecutive days. No adverse events were attributable to oral application. Less than half of the recipients had *E. coli* in their stool and it accounted for less than 5% of total fecal bacteria [159]. In contrast, the Eliava Institute showed that both oral and intra-rectal phage treatments could cure a MDR *Klebsiella pneumoniae* gut infection [160].

Intraoperative route

Phages applied intraoperatively have cured a series of implant-associated MDR and recalcitrant infections. A 1-phage treatment at 1×10^8 PFU in combination with ceftazidime applied directly to an aortic fistula cleared a chronic aortic graft MDR *Pseudomonas aeruginosa* infection in a 76 year-old [161]. Similarly, a chronic aortic graft *Staphylococcus aureus* infection was cured after a 59 year-old patient received 2×10^{10} PFU/dose of a single phage applied via drainage every 12 h for 2 days [162]. A 2-1-1 phage mix targeting *P. aeruginosa*, *S. aureus*, and *Enterococcus faecium*, respectively, cleared an aortic graft multispecies infection in a 52 year-old patient after 2 local phage treatments of 2.5×10^9 PFU administration a day apart. Unfortunately, relapse of a *P. aeruginosa* infection occurred after 17 days [162].

Left ventricular assist devices (LVADs) are becoming a more frequent life-support intervention, but there has been mixed success of treating LVAD infections with phages. A 62 year-old with fulminant pleural empyema caused by *S. aureus* infection after LVAD implantation was cured after 1-phage local treatments of 2×10^{10} PFU applied via drainage every 12 h for 7 days [162]. In contrast, a 51 year-old could not be cured of chronic *S. aureus* LVAD infection after 2 weeks of daily local treatments with a 4-phage mix at 1.0×10^{10} PFU applied via drainage. The cause of treatment failure was unknown, but there were no signs of developed phage resistance or phage-neutralizing antibodies after 2 weeks [162].

Phages have also treated prosthetic joint infections (PJI). An 80 year-old with type 2 diabetes was cured of a methicillin sensitive *S. aureus* (MSSA) prosthetic hip infection after debridement and a single local administration of a 6-phage mix at 6×10^{10} PFU in

20 mL of PBS [163]. Treatment also consisted of daptomycin, amoxicillin and clindamycin to suppress *Enterococcus faecalis* and *Staphylococcus lugdunensis* (also isolated from the joint) and oral amoxicillin/clindamycin for 6 months. The patient's PJI remained infection free for 18 months [163]. Similarly, an 80 year-old with a *P. aeruginosa* PJI was cleared with a 100 mL intraoperative treatment of a 3-phage cocktail at 1×10^8 PFU followed by administration of 5×10^8 PFU/dose every 8 h via drainage for 5 days [164]. Another case showed that after removal of sacroiliac joint cement and tissue debridement, 4 local administrations of a 4-phage mix of approx. 2.0×10^{10} PFU, colistin, and a compress soaked with approx. 4.0×10^{10} PFU, combined with off-label IV ceftolozane/tazobactam, cleared a chronic MDR *P. aeruginosa* PJI [165]. Phage intervention allowed for surgical reconstruction after 2 weeks.

A patient with severe *P. aeruginosa* osteomyelitis that received intraoperative phage therapy exhibited no severe adverse events and their wound infection resolved without relapse [166]. After thorough debridement and irrigation, between 10 to 40 mL of a 10^7 PFU/mL mix of *S. aureus* and *P. aeruginosa* phages rinsed the bone/soft tissue defects. After a contact time of 10 min, a gentamicin and phage soaked collagen sponge was applied to the infected bone before closure. Postoperative administration of phage occurred for 7 to 10 days, 3 times per day, with 3 months of concomitant antibiotics [166].

Under eIND, a 68 year-old diabetic was cured of a disseminated MDR *Acinetobacter baumannii* infection from necrotizing pancreatitis with combined phage and antibiotic therapies [143]. Treatment consisted of a 4-phage 10^9 PFU/dose given intraoperatively via drainage catheters every 6-12 h. After 36 h, a second 4-phage mix at 10^9 PFU/dose was IV administered at increasing frequency to dosing every 2 h. Phage-

resistance developed after 10 days, which necessitated switching IV treatment to a third 2-phage mix, which was maintained for the remaining 2 weeks of therapy [143].

Intravenous route

Phage therapy under eIND salvaged a joint prosthesis in a 72 year-old obese adult with a chronic methicillin-resistant *S. aureus* (MRSA) infection [167]. During debridement surgery and placement of a static vancomycin spacer, two intra-articular doses of a single phage at 5.4×10^9 PFU in 10 mL of saline. Daily 2.7×10^9 PFU in 50 mL of saline IV infusions continued. However, the patient developed transaminitis after 3 days, prompting phage treatment termination. Aminotransferase and alanine aminotransferase levels returned to normal with continuation of daptomycin alone. Nonetheless, combined interventions cleared the chronic joint infection, which allowed for distal femoral cemented prosthesis implantation [167]. In another case, a 62 year-old with a potentially limb-threatening chronic *K. pneumoniae* prosthetic knee infection was treated with IV phage therapy after several failed surgical interventions and prolonged antibiotic therapy [168]. The patient received 40 daily 30 minute infusions of a single phage at 6.3×10^{10} PFU in 50 mL of normal saline. Over the course of phage therapy, the patient experienced improvement in erythema, swelling, pain, range of motion, and function of the knee. The patient reported minor and intermittent pruritus of the lower extremity about 2 weeks into the course of therapy but experienced no apparent adverse events from the infusions [168].

A 77 year-old developed a post-operative MDR *A. baumannii* infection after a craniectomy [169]. After surgical debridement, parenteral administration of 8.5×10^7 PFU in Lactated Ringer's solution every 2 hours through a central catheter line, for a total of

98 doses, cleared *A. baumannii* from the site of infection. Two hours following the first dose, the patient exhibited a hypotensive response, but the remaining doses showed no adverse effects. After finishing phage treatment the patient continued to suffer from fevers and leukocytosis, and died 9 days later [169]. LaVergne et al suggested that surgical drain phage administration might have led to a better outcome [169].

A 15 year-old patient with cystic fibrosis (CF) that developed a *M. abscessus* airway infection post lung transplant was cured after 32 weeks of treatment with 3-phage at 3×10^9 PFU/dose given every 12 h under eIND [153]. Unconventionally, treatment consisted of temperate phages with modified genomes to remove their ability to undertake lysogeny, instead of traditionally virulent phages [153]. Similarly, a 26 year-old CF patient that received a 4-phage mix at 4×10^9 PFU/dose every 6 h for 8 weeks IV, in combination with antibiotics, was cured of a MDR *P. aeruginosa* lung infection [142]. In another case, a 57 year-old was treated for a MDR *P. aeruginosa* airway infection that developed following lung transplant was cleared after a 4-week IV course of a 4-phage mix at 4×10^9 PFU/dose given every 12 h concomitantly with inhaled colistin [141]. In contrast, 10-weeks of IV phage and antibiotic therapies could not eradicate a *Burkholderia dolosa* airway infection that developed post-lung transplant [141].

A 67 year-old with a history of *P. aeruginosa* urinary tract infections (UTI) was cleared with a 6-phage mix at 2×10^7 PFU instilled directly into the bladder every 12 h for 10 days, in combination with colistin and meropenem. The patient remained free of UTI for 12 months [170]. In a single arm study, antibiotics combined with a 3-phage cocktail at 3×10^9 PFU in saline per min over 10–30 min 2x daily for 14 days showed

clinical improvement in 8 of the 13 recipients with *S. aureus* infective endocarditis and bacteremia [171].

Topical route

In a double blind, phase I study, no adverse events occurred after weekly venous leg ulcer topical washes with phages targeting *E. coli*, *P. aeruginosa*, and *S. aureus* [172]. However, wound healing following 12 weeks of weekly 3.2×10^{10} PFU in 50 mL of saline was not significantly different from saline only washing. The lack of clinical improvement was likely due to the lack of sensitivity of the infecting bacterial isolates to the treatment phages; prior phage sensitivity testing was not done [172]. Similarly, a multi-center, double-blind, randomized phase I/II study failed to find burn wound clinical improvement after a 12-phage topical treatment of *P. aeruginosa* infections when compared to silver sulfadiazine cream [102]. The study had several deficiencies, including elevated endotoxin levels and instability of phages in administered products. The inability to adjust treatment parameters of the clinical trial caused treatment to instead consist of phage counts less than 10^3 PFU per dose [102]. Phages rely entirely on chance encounters with their bacterial hosts and providing high phage doses increases the likelihood of bacterial encounters for both lysis and self-dosing via phage replication.

In a double-blind, phase I/II trial, topical phage treatment of chronic otitis media caused by *P. aeruginosa* was overall ineffective [173]. For all recipients, phages briefly reduced bacterial counts within the ear canal. Ear swabs also showed an abundance of phages, which implies phages replicated at the site of infection. However, infections relapsed in 9 of 12 recipients [173]. A rebound in bacterial counts during treatment is consistent with the emergence of phage resistance, but it was not tested. In another study,

intranasal irrigations with a 3-phage mix was shown to reduce *S. aureus* in the interim but only cleared infections in 2 of 9 recipients [30]. Patients did exhibit adverse effects, including rhinalgia after two treatments of 3×10^8 PFU, oropharyngeal pain after 22 treatments of 3×10^8 PFU, and metabolic acidosis after 28 treatments of 3×10^9 PFU [30].

In contrast, a 16 year-old with a rare congenital skin disorder called Netherton syndrome showed a significant reduction in atopic eczema after both daily topical and oral administrations of an “undefined” mix of *Streptococcus*, *Staphylococcus*, *Proteus*, *Escherichia*, *Pseudomonas*, and *Enterococcus* phages [174, 175]. The daily treatment regime consisted of 20 min application of sterile gauze soaked with the phage mix, followed by phages mixed into ointment cream. The patient also ingested 20 mL of the same phage mix [175]. Continuous daily treatments occurred for 2 weeks, with 2 week pauses, and after 3 months a second 2-phage cocktail was introduced to address the emergence of phage-resistant *S. aureus* isolates. Patient quality of life improved with decreased skin irritation and increased joint mobility [175].

Inhalation route

A *K. pneumoniae* lung infection during drug-induced immunosuppression after heart transplantation was successfully cleared in a 40 year-old after inhalation of a 2-phage cocktail at 2×10^8 PFU/dose and daily nasogastric ingestion of 1.8×10^9 PFU/dose for 4 days, under the Declaration of Helsinki. After phage therapy, *K. pneumoniae* was undetectable in bronchial lavages, but remained in the patient’s stool [162].

Intrarectal route

Three cases of *E. faecalis* chronic prostatitis were successfully treated by twice daily intrarectal (IR) phage treatments. Phages at 10^8 - 10^9 PFU/dose given for approximately 1 month. At the end of treatment all three patients' prostates were restored to normal function and physiology [176]. Similarly, a 60 year-old patient who received a kidney transplant suffered from 12 severe episodes of UTI due to *K. pneumoniae* in the 15 months following transplantation. Phage therapy was initiated after the patient's tenth UTI and continued throughout two more hospitalizations. IR phage administration had no adverse effects on the patient despite their hospitalization and in the 4 years following phage treatment the patient has not been hospitalized due to UTI [177].

Phage Selection

Not all phages are candidates for therapeutic purposes. As mentioned, virulent phages (obliquely lytic cycles) are preferred because cell lysis is indeterminate and there is a reduced risk of viral mediate horizontal gene transfer via transduction [178]. Genome sequencing and bioinformatic workflows, [179-183] permit identification of undesirable gene features to rapidly "rule out" unsuitable phage strains based on homologies to known genes on existing databases., as shown in [179-183], For example, carrying lysogeny-associated repressors and integrases, bacterial virulence, toxin, and/or antibiotic-resistance genes would disqualify a phage for human therapy [79, 184]. Although, it may be possible to knock out genes with undesirable traits when, for example, virulent phages are difficult to isolate [153, 185]. Continued efforts to improve the quality

and accuracy of archived phage genomic information will contribute to increasing the safety of phage therapy.

In general, a good phage candidate for therapy is one that infects a wide range of bacterial strains. There is evidence that myoviruses exhibit broader host range [186]. For example, a single phage lysed 17 of 28 *S. aureus* clinical isolates [187]. In contrast, narrow host range phages may present new therapeutic opportunities. For instance, targeting a prevalent and specific strain of pathogenic bacteria could avoid adverse effects associated with host microbiome dysbiosis [182].

Another feature important in phage selection is their natural ability to disrupt structured communities of bacterial cells that attach to surfaces and enmesh in biofilm [138]. Many infectious diseases in humans are the result of, or exacerbated by, biofilms. Self-made exopolysaccharide (EPS) matrix provides an essential scaffold for biofilm development, promoting bacterial adhesion to surfaces, cohesion, as well as hindering diffusion. Although, it is often assumed that biofilms confer resistance to phages, most phages readily infect bacteria within biofilms [138]. Indeed, phages have co-evolved with bacterial biofilms, and thus, their infection of encased bacteria is expected. For instance, some phage tail fibers and tail-spikes even carry depolymerases that degrade exopolysaccharides to unmask cell receptors on planktonic cells and disrupt the exopolysaccharide matrix of biofilms [188-191]. *In vitro*, phages have reduced both single-species [82, 192, 193] and dual-species [194-196] biofilms. In an artificial CF sputum, phages could reduce *P. aeruginosa* biofilm by 3-logs, indicating that phages readily penetrate and lyse bacteria encapsulated in biofilms [192]. As phages lyse distal bacteria, interior cells awaken and become more metabolically active due to increased oxygenation

and nutrient exposure, thus becoming more sensitive to phage attack. Certain phages however poorly penetrate the biofilm matrix [138], and others can promote a mucoid phenotype of host bacteria that enhances biofilm formation [197]. Although biofilm infections are common and cause clinically significant and potentially fatal infections, much work remains in the quest for effective anti-biofilm phages in clinical settings.

The extended storage of phages is another important consideration. Phages keep short-term in cold storage at 4 °C when purified in buffered solution, concentrated, and shielded from light [127, 198, 199]. Even then, phage counts generally trickle down over months. Cryopreservation can minimize the loss of phages, but phage particles are inactivated with freezing and thawing cycles [127]. Long-term storage is also feasible with phage lyophilization (freeze-drying) using stabilizing additives [200]. While lyophilization is not compatible for most clinical applications, it may be suitable for inhaled phage therapies [201, 202]. Although many phages can be stored in these conditions, specific storage conditions for each phage strain requires evaluation.

Formulation

In clinical practice, phages are typically administered as a mix (cocktail) of viral strains [102, 142, 143, 153]. Well-planned phage combinations have several benefits over using a single phage strain. These include: 1) broadening target bacterial strain spectrums; 2) targeting multiple bacterial species at once; 3) increasing dose potency by multiple phage strains attacking the same bacterial cell; and 4) limiting resistance by forcing the target bacterium to evolve resistance to multiple phages simultaneously, in order to survive [203-207]. The drawback is that individual phages generally requires a

reduction in concentration when mixed into a single dose. There is also potential that phages will interfere with one another, for example, compete for the same bacterial cell receptor and drive cross-resistance [208] [208].

Conversely, a single phage strain can precisely target a specific bacterial strain [161, 167]. The drawback of using a single phage strain is that it requires careful definition of the etiological bacterium before therapy. Thus, this approach is primarily for proof of concept/efficacy/safety *in vitro* and animal studies.

Product pipelines

There are generally three pipelines for phage drug products: off-the-shelf, pharmacy compounding, and on-demand *de novo*. Off-the-shelf, fixed phage products are designed either to target not yet identified pathogens (e.g. multiple phage strains targeting multiple bacterial species) or identified pathogens (e.g. multiple phages targeting a single bacterial species). They are manufactured under Current Good Manufacturing Practice (cGMP) production and final products undergo phased clinical safety and efficacy testing [33]. Fixed products enhance safety, quality, and uniformity. However, the clinical usefulness of fixed phage products is unclear because of, for example, high strain variability of target bacterial pathogens, rapidity of phage resistance, encountering multi-species infections, and varied phage storage stability.

Compounding by a licensed pharmacist with a physician's prescription can create phage medications that are tailored to the individual needs of a single patient [125]. This may be required, when the patient's infection isolates are not sensitive to phages in the

fixed product, dosage is unavailable, or the fixed phage product is not well tolerated (see “Drug-related problems” below). Compounding also allows the insertion of phages into topical creams, transdermal gels, or other dosage forms.

To implement phage compounding, large and diverse collections of purified phages (phage banks) are a prerequisite [125, 209]. Indeed, bacterial susceptibility and phage strain diversity have been the driving force in library construction. For instance, the Queen Astrid military hospital has an extensive phage collection. Yet only 15 of 260 patient isolates were sensitive to phages in the collection [210]. Likewise, in a bank with more than 10,000 mycobacteria phages, only 6 phages lysed *M. abscessus* isolated from a CF patient [153]. Adaptive Phage Therapeutics’ diverse and well-characterized PhageBank™ collection also did not contain a phage strain suitable against a patient’s *K. pneumoniae* isolate [168]. Thus, proper phage selection criteria and library size have yet to be identified. A potential improvement may be to ensure a diverse collection of phages that use different bacterial receptors [209].

On-demand, *de novo* personalized phage products have so far provided better overall efficacy [142, 143, 153, 168]. This pipeline allows phages to be isolated specifically against bacterial isolates directly from the patient [143, 168]. Thus, phage products can be rapidly and continually modified to respond to, for example, phage resistance [143, 211, 212]. The problem remains that on-demand phage products can take weeks to months to manufacture [213].

Pharmacokinetics/pharmacodynamics

The pharmacokinetics (PK) and pharmacodynamics (PD) of phages *in vivo* are complicated and largely unknown [214, 215]. Phage PK are unique due to their relatively large size; approximately 100 times larger than antibiotics and 10 times larger than antibodies. In addition, phages consist of several different proteins, which can increase their rate of clearance by the mononuclear phagocyte system (MPS) and neutralization by anti-phage antibodies [216, 217]. Phage efficacy in entering animal or human bodies strongly depends on the route of administration. Oral administration effectively delivers phages to the gastrointestinal tract, and efficiency of gut transit is dose dependent. Fecal phage counts peaked at 3.0×10^4 PFU/g after oral consumption 1.5×10^7 PFU in mineral water 3x daily [28]. Whereas patients orally treated with phages for acute cholera at a dose of 10^{13} PFU, together with phage propagation at sites of infection, resulted in feces concentrations of more than 10^{11} PFU/g [218]. This suggests that phage passage through the stomach can be efficient. Absorption of phages into the blood, however, was inefficient, reaching only 10^2 PFU/mL [218]. Phages can easily penetrate most organs, though they readily accumulate in the spleen and liver [219-221].

For phages to be effective, they must reach the site of infection in sufficient numbers and infect as many target bacteria as possible, without causing unwanted side effects. Conversely, removal of phages from the site of infection is an important factor in terminating action. Phages delivered systemically can penetrate the lumen of murine lungs [222]. Phages in the plasma and tissues (spleen, kidney, liver, and lung) of rats following a single 1 mL intravenous bolus of 10^{10} PFU/mL exhibited a half-life of 2.3 h and 9 h, respectively [223]. Continuous infusion of 0.1 mL/h for 24 h of the same phage

cocktail and titer resulted, however, in plasma progressively increased to reach a plateau of 10^7 PFU/mL after 6 h, which is comparable with the concentration achieved after bolus injection [223]. In humans, after a dose of 8.5×10^7 PFU in 4 mL of Lactated Ringer's solution, administered through a peripherally inserted central catheter, phage levels were detectable after 5 min in blood samples but were undetectable after 50 min [169].

Studies examining the PD of phages, defining synergy, additivity, indifference, antagonism, and host immune system interaction are scarce. The monotherapy versus cocktail versus phage-antibiotic combination therapy debates have not yet considered the potential additional value of the patient immune system. It is still unclear if the initial phage dose or phage replication most influences outcomes, as well as what is a sufficient level of target bacteria to allow phage replication to overtake bacterial replication [208]. Thus, the effectiveness of phage therapy likely increases as the infection worsens, inflating the likelihood that phages will encounter a bacterial host. Inefficient outcomes however, may arise from the tendency for phages to bind to bacterial debris [224].

With the uniqueness of phage therapy, studying PK/PD is challenging. Most proof-of-principle demonstrations of phage therapeutic applications use very high titers of phage to achieve efficacy, which is unlikely to be achieved in humans. But, given the low toxicity of phages, phage therapy should be successful provided that phages reach the site of infection in sufficient numbers and are able to replicate within target bacteria [208].

Drug-related problems

Drug-related problems (DRP) actually or potentially interfere with desired health outcomes, including adverse reactions and emerged resistance. They are relatively common in hospitalized patients and can result in morbidity and mortality, as well as increased costs. The range of adverse reactions during phage therapy varies widely. A 2 year-old experienced anaphylaxis after IV administered phages [225] and a 15 year-old CF patient became flushed after initial IV phage treatments [153]. Topical application of *S. aureus* phages has caused mild adverse reactions such rhinalgia, oropharyngeal pain, and metabolic acidosis [30]. Multiple patients experienced hypoxemia and hypothermia during topical *Pseudomonas* phage treatment of burn wounds [102]. Oral administration of *E. coli* phages has caused transient abdominal pain, dyspepsia and toothache in adults [29].

We speculate that adverse reactions can arise due to bacterial lysis by the phages, and/or direct phage-mammalian host immune system interactions and/or residual bacterial toxins in phage products [213, 226]. Phage-induced rapid release of bacterial cell wall components may have immediate adverse effects for the patient. Animal studies have shown that phage-induced endotoxin release is significantly lower in lysis-deficient phages compared to lytic phages, which leads to decreased proinflammatory cytokines (e.g., TNF α and IL-6) [227, 228]. However, the amount of endotoxin released during antibiotic action is also clinically important. For instance, some classes of β -lactam antibiotics lead to markedly increased levels of free endotoxins, while treatment with carbapenems and aminoglycosides produces relatively low amounts of endotoxins.

Dufour et al. found that phage-mediated bacterial lysis released endotoxins were similar to β -lactam antibiotics *in vitro* [229].

Direct interactions between phages and the host immune system can induce signaling cascades that can result in elevated cytokines, increased immune cell infiltration, increased phagocytosis and adaptive immunity activation (T-cell response and antibody production by B-cells). Phages have been shown to stimulate anti-viral interferons (IFN) through activation of Toll-like receptor 9 (TLR9), which play crucial roles in the innate immune system by recognizing microbial DNA as a pathogen-associated molecular pattern (PAMP) [230]. Treating mice with phages led to immune cell expansion in the gut and increased IFN- γ production, which worsened murine colitis [230]. Human lung epithelial cells increased IL-6 and TNF α production in response to exposure to certain *Pseudomonas* phages [231]. Similarly, certain staphylococcal and pseudomonal phages induce proinflammatory cytokines like IL-6 and IL-1 β , in murine dendritic cells [231], while other staphylococcal phages did not elicit human dendritic cell cytokine expression [232]. Together, data suggests immune responses are phage strain dependent.

Moreover, some phages can bind to cell surface polysialic acids as a receptor for cell entry [233], while other phages will internalize through epithelia cell pinocytosis [234]. However, the physiological and immunological consequences of harboring internalized phage particles is unclear.

Certain structural proteins of phages can elicit weak humoral responses after prolonged exposure, which can lead to the antibody neutralization of phage particles [235, 236]. Anti-phage antibodies have been recovered from patients that received phage

therapy [217] as well as those that have not; presumably due to natural contact with phages during infections with associated bacteria [237]. A phage safety trial in humans showed no anti-phage IgG, IgM, or IgA antibodies detected in the blood of recipients after oral ingestion of phages for two consecutive days [28]. In animal models, however, continuous oral consumption of phages can induce weak anti-phage antibodies (IgG after 36 days and IgA after 79 days) [235]. A different phage type, induced IgG and IgM antibodies after prolonged murine oral ingestion [238], and *Staphylococcal* phages induced IgM, IgG, and IgA antibodies in blood [236]. Notably, phage numbers in murine models are significantly higher in comparison to human therapeutic doses. Although phages appear to induce only weak antibody responses, anti-phage antibody responses may lead to the activation of the complement system [239]. Human blood from recipients of phage therapy was found to contain anti-phage IgG, IgM, and IgA antibodies *in vitro*. However, there was no correlation between the induction of phage-neutralizing antibodies and the outcome of phage therapy [240]. Anti-phage antibodies, while likely not harmful for the patients, could limit the effectiveness of phages given therapeutically. To date, this problem has not been reported.

Identifying immunological interactions with phages that contribute to the development of adverse reactions may aid in the prevention of problems related to phage therapy.

Table 2.2 Adverse events reported in 12 of 59 phage therapy cases.*

Primary Route of Administration	Clinical Trial	Infection Site	Target	Clinical Improvement	Adverse Events	References
Oral	Yes	gut	<i>Escherichia coli</i>	N/A	Stomach pain, nausea, increased peristalsis, and/or sore throat.	Bruttin et al., 2005
Oral	Yes	gut	<i>E. coli</i>	N/A	Adult: transient abdominal pain, dyspepsia, and toothache Children: ear pain and loose stool	McCallin et al., 2013
IO	No	pelvis	<i>Pseudomonas aeruginosa</i> and <i>Staphylococcus epidermis</i>	Y	Local redness and pain	Onsea et al., 2019
IO	Yes	urethra	<i>E. coli</i> , <i>Proteus mirabilis</i> , <i>P. aeruginosa</i> , <i>Staphylococcus aureus</i> , and <i>Streptococcus</i> species	6 of 9	Fever and chills	Ujmajuridze et al., 2018
IV	No	lungs	<i>Mycobacterium abscessus</i>	Y	Sweatiness and flushing without fever	Dedrick et al., 2019
IV	No	skin (scalp)	<i>Acinetobacter baumannii</i>	N	Brief hypotension	LaVergne et al., 2018
IV	No	joint	<i>K. pneumoniae</i>	Y	Intermittent pruritus but experienced no apparent adverse events from the infusions	Cano et al., 2020
IV	No	blood	<i>P. aeruginosa</i>	N	Anaphylaxis	Duplessis et al., 2018
Topical	Yes	leg	<i>E. coli</i> , <i>P. aeruginosa</i> , <i>S. aureus</i>	N	Increased pain at target ulcer and wound area size	Rhoads et al., 2009
Topical	Yes	skin	<i>P. aeruginosa</i>	N	Hypothermia	Jault et al., 2019
Topical	Yes	ear	<i>P. aeruginosa</i>	N	Headache/dizziness, abdominal pain, ear pain, thirst, insomnia, pharyngolaryngeal pain, rash	Wright et al., 2009
Topical	Yes	nose	<i>S. aureus</i>	2 of 9	Diarrhea, epistaxis, upper respiratory tract infection, rhinalgia, oropharyngeal pain, metabolic acidosis	Ooi et al., 2019

IO (intraoperative), IV (intravenous), Y/N (yes/no), N/A (not applicable)

*All case data are in the Supplemental File 1.

Phage Resistance

Another complication of phage therapy is that bacteria can thwart phage attack through an arsenal of anti-viral mechanisms, targeting almost every step of the phage replication cycle [15]. These include spontaneous chromosomal mutations, the ability to block the entry of phage genetic material (e.g., superinfection exclusion), DNA restriction-modification enzymes, abortive infection, and CRISPR-Cas adaptive immunity. A major mechanism driving phage resistance is spontaneous chromosomal mutations governed by Darwinian dynamics. While treating an infection, a small number of mutations will spontaneously arise (rate of ca. 10^{-8}), followed by phage-resistant sub-population regrowth [241-243]. Animal studies have shown bacteria can rapidly shrug off phage attack leading to uncontrolled proliferation of phage-resistant mutants and treatment failure [12, 244, 245]. However, target bacterial populations are likely to experience large, cyclic swings (predator-prey dynamics) in population size before resistance dominates [243, 246].

Among 12 phage therapy clinical studies, 7 cases confirmed the emergence of phage resistance during treatment [247]. For example, *A. baumannii* isolates from blood cultures exhibited resistance to all 8 phages after just 1 week of therapy [143]. Similarly, resistance developed during topical treatment but after nearly continuous dosing for 3 months [175]. Most studies have not adequately tested for the emergence of phage resistance, likely due to successful outcomes. However, cases continue to show that resistance emerges during phage therapy [141], which might attribute to treatment failures not reported.

Despite the near certainty that phage resistance will arise, multiple scenarios occur with which phage resistance does not impede a positive therapeutic outcome, for clinical examples see [143, 247]. First, the patient immune system, given an adequate response, can eradicate emerged resistant bacterial mutants [12]. If resistance to phage treatment dose arise, switching to new phages with different binding sites or order of exposure can be therapeutic strategies to improve efficacy [104]. The maintenance of bacterial anti-viral defense systems comes with a cost, such as DNA restriction-modification and CRISPR-Cas enzymes [248, 249]. Evolution of resistance can lead to attenuation of bacterial virulence [188, 250, 251] or promote collateral sensitivity to antibiotics [252, 253]. Lastly, phages also harbor their own defenses to counteract bacteria immunity, such as anti-restriction and anti-CRISPR enzymes [254, 255] as well as counter-adapt to re-infect resistant bacteria [246, 256].

Phage adjuvants and *vice versa*

One approach that has received little exploration is the use of phage adjuvants, also referred to as “phage potentiators” and “resistance breakers”. These are active compounds with little or no effect on bacterial growth in isolation and, when co-administered with phages, act to enhance phage activity or block phage resistance. The combination of synergistic antimicrobials are also adjuvants. Chemical antibiotics can act as phage adjuvants by boosting phage particle production. For instance, a subinhibitory concentration of cephalosporin increased an *E. coli* cell's production of the phage particles by 7-fold [257]. Phage infected *Burkholderia cenocepacia* cells produced higher phage counts in the presence of subinhibitory concentrations of meropenem,

ciprofloxacin, and tetracycline [258]. Antibiotics that act by inhibiting cell division (β -lactam, quinolones, nalidixic acid) or cause an elongated morphology (ciprofloxacin, meropenem) can permit higher production of phage particles [257, 258]. Likewise, subinhibitory concentration of tetracycline causes cell clustering, which may promote increased phage infections by minimizing lateral travel between adjoined cells, thereby enhancing contact with phage receptors on uninfected cells. [258]. Importantly, elevated phage production was unaltered when bacterial cells became antibiotic resistant [258]. Moreover, combining phages with sub-inhibitory concentrations of ciprofloxacin or meropenem can inhibit regrowth of phage-resistant mutants in a murine endocarditis model and thus improve the phage therapy outcome [223].

Bacterial biofilms represent a major obstacle in the fight against bacterial infections, as they are inherently refractory to various antibiotics. A potential adjuvant to phage therapy include DNases that can degrade extracellular DNA, which plays important roles in both the aggregation of bacteria and in the interaction of the resulting biofilm with polymorphonuclear leukocytes during an inflammatory response [259]. Sugar alcohols such as xylitol, sorbitol and erythritol can also be phage adjuvants [260]. They inhibit bacterial growth by diffusing into the biofilm and accumulate as a toxic, non-metabolizable sugar alcohol phosphate [260]. Together, this suggests that phage adjuvants may be a way to improve the efficacy of bacterial killing during treatment.

Conversely, phages in combination with antibiotics offer an orthogonal approach to newer antibiotic discovery [261, 262]. For example, some phages infect Gram-negative bacteria by binding to TolC or its homologues, a common component of a wide variety of multidrug efflux pumps [252, 263, 264]. Phage infection of MDR bacteria can lead to

phage resistance (e.g. *tolC* mutants), which can induce a collaterally sensitive response to antibiotics [252]. Similarly, the phage OMKO1 infects *P. aeruginosa* by binding to the efflux pump MexAB/MexXY [253]. Mutations in the gene encoding of the outer membrane porin M, which is part of the efflux pump complex, impaired phage infection, but restored the sensitivity to ciprofloxacin [253]. *E. faecalis* is a human intestinal pathobiont with intrinsic and acquired resistance to many antibiotics, including vancomycin. Despite gaining phage resistance, mutant strains exhibited a loss of resistance to cell wall-targeting antibiotics *in vitro* [265]. Identifying evolutionary trade-offs is an emerging strategy for combating antibiotic resistance. For instance, prescribing sequences of synergistic antimicrobials wherein the evolution of resistance to the first induces susceptibility to the second [266, 267].

Treating biofilms with phage has shown promise in a handful of experimental and case studies. The unique action that phages exert on bacteria offers the potential for the two agents to act synergistically; their total efficacy is much greater than each individual action. Most antibiotics alone were ineffective at disrupting *S. aureus* biofilms low concentrations *in vitro*. However, the addition of phages led to substantial improvements in efficacy [268]. Using phage with rifampin also proved effective at reducing the outgrowth of resistant strains during the course of treatment *in vitro* [268]. Likewise, the addition of *E. coli* phages decreased in the minimum biofilm eradication concentration of cefotaxime [269]. Thus, combining phage therapy with traditional antibiotics could help better manage antibiotic-resistant bacterial biofilms. In some cases, even though no synergism in antimicrobial activity is observed, the combined use of phages and

antibiotics significantly reduces or even prevents the development of antibiotic- and phage-resistant bacteria [268, 270].

However, there may be antagonism whereby the molecular action of phages somehow interferes with the action of antibiotics [258, 271]. Quinolones can be synergistic with phages against *P. aeruginosa* in one study while antagonistic in another [253, 271]. High antibiotic concentrations can also cause a reduction in phage propagation [268]. This is particularly evident when using antibiotics that target cell protein synthesis [272].

From a clinical standpoint, phage-antibiotic combination therapy can improve or prolong the lifespan of the available antibiotic arsenal, and the growing pressure to develop phage therapy can be somewhat alleviated. The principal disadvantage of their use is that more complex studies are required to establish effective co-dosing regimens since compatible pharmacokinetic and pharmacodynamic properties between the antibiotic and phages are required. Unfortunately, in most cases, the mechanism underlying potential phage-antibiotic interactions is unknown. In addition, although *in vitro* assessments of synergy justify combination therapy, these studies are rarely further pursued within animal models.

Conclusions

Antibiotic resistant bacteria threaten the extraordinary health benefits achieved with antibiotics. As we face the current antibiotic crisis, phage therapy has the potential to alleviate the ever-growing problem of infectious diseases, either as an alternative to

antibiotics, or in combination with antibiotic therapies. While clinical successes suggest the safety and, in some cases, efficacy of phage therapy, the clinical functionality still requires careful definition. The lack of well-controlled clinical trial data and complex regulatory frameworks have driven most recent human data generation on a single patient compassionate use basis. Most include the concomitant use of antibiotics, which makes it difficult to draw solid conclusions as to the effectiveness of phages alone. However, compassionate use human data supports exploiting the combination of phages and antibiotics and is a promising avenue for near-term clinical development.

Recent improvements in phage genomics, purification and formulation have greatly contributed to improving the efficacy and reliability of phage therapy. Current knowledge gaps exist on the appropriate routes of administration, phage selection, frequency of administration, dosage, phage resistance, and PK/PD of phage. In addition, phages require thorough investigation into the immunological response they may elicit. Advancement of other technologies supporting phage therapy will have to follow suit and evolve. This includes (i) rapid and accurate phage assessment methods and criteria; (ii) standardization of phage manufacturing; (iii) phage banking; (iv) phage product stability during storage and transport; and (v) new quantitative methods that allow for precise monitoring of pharmacological parameters of phages.

Some may decide, of course, that the transition to phage therapy cannot be done, perhaps due to the lack of necessary capabilities or financial resources, or because the belief that chemical antibiotics are superior and can defeat the resistance problem. The redoubling efforts to enhance ineffective antibiotics has in many cases succeeded in improving their performance [273]. However, this may only be postponing the day of

reckoning for a post-antibiotic era. Thus, development of alternative antimicrobial therapies is critical. Although more translational research is needed before the clinical implementation of phage therapy is feasible, phages may be pivotal in safeguarding the overall health of humans in the near future.

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Author Contributions Chapter 2.5

Conceptualization, TL, ACS, and DR; methodology, TL and DR; validation, TL and ACS; investigation, TL and ACS; formal analysis, TL, ACS, and DR; resources, DR; data curation, TL and ACS; writing—original draft, TL, ACS, and DR; writing—review & editing, TL, ACS, and DR; visualization, TL and DR; supervision, DR; funding acquisition, DR.

CHAPTER 3

KINETICS OF PHAGE COCKTAILS

INTRODUCTION

Phages are vicious predators and are a major determinant in shaping bacterial lives and communities. Despite the abnormally high predator-prey conversion factor however, stable coexistence of phages and bacteria occur in the environment and it would seem, even within the human body [274]. Other attributes include enhanced biofilm degradation properties and their potential application for refined species targeting which lowers impact on beneficial microbes in host microbiomes [275]. Whenever phages are foreseen for clinical application, they generally undergo a careful pre-selection after intensive application-oriented biological investigation. These include genome and infection characteristics, spectrum of activity, effectiveness against biofilms, ability to overcome anti-phage systems, and storage stability [100]. Therapeutic phages should also have high clearance rates of target strains and maintain their killing ability during treatment for as long a period as possible. Nevertheless, while it is easy to find potential therapeutic phage strains, it is still arduous to down select those truly worthy of clinical development.

While phage therapies have proven highly effective in lab-scale models, successes have been mixed in the healthcare setting [21, 276, 277]. Two commonly identified knowledge gaps with regard to clinical therapy are how to effectively provide a broad-spectrum therapeutic product and how to effectively limit phage resistance [18, 204]. In practice, treatment consisting of multiple phages strains known as phage ‘cocktail’

therapy could address both of these. For the first, multiple phage species together can infect a greater number of hosts strains or host species depending on the phage types. For the second, phage types binding to different receptors on the same susceptible hosts can better limit resistance [204]. In the last two decades, cocktails between two to twelve phages were given in 44/57 cases [277]. However, because cocktail formulations have largely been chosen empirically, based on availability or host-range, there is limited information about the lysis activities of phages within so-called ‘successful’ cocktails. In order to leverage complex cocktails as therapeutics it is important to understand the lysis activities, or kinetics, of combination phage killing. Many of these determinants will be case-specific, but there may also be general phage-bacteria and phage-phage principles that can be incorporated into the rational selection of viruses that comprise cocktails.

Attempts have been made to quantify the selection pressures of phages in cocktail [83, 96, 104, 278]. However, the mechanistic information about how phages in cocktails interact to promote combinatorial effects and anticipated resistance evolution patterns, remain elusive, in part because these interactions depend on each phage species, the bacterial species, and spatial patterns of microbial distribution. Whether the bacteriolytic activity, or potency, of individual phage strains combined can function independently, synergistically, or antagonistically remains underexplored. Because phages obligately hijack bacterial cells for replication, at the exclusion of other phage particles, I hypothesize that competition for available bacterial cells likely constrains initial phage replication and activities in cocktail.

Here, the kinetics of *P. aeruginosa* and its phages in two *in vitro* models are described. Although the current diversity of 32 anti-*Pseudomonas* phage genera initially

suggests many potential patterns of phage activity, only six genera of phages are frequently selected for therapy, suggesting that phage cocktail kinetics may not be as complex as previously believed. Two phage strains were selected from these genera, LPS-binding *Pbunavirus* E215 and type IV pilus-binding *Phikmvvirus* LUZ19. In microwell time-kill assays, although phage resistance mutation against a two-phage cocktail is inevitable, the duration of resistant bacterial suppression can be extended through a combination of phage selection and phage dose. That is, that two phages with high lytic activities appear to be more effective in combination than phages with unequal activities.

Although sequential phage administration was thought to reduce phage competition, only one of the regimens recapitulated cocktail activity suggesting directionality of receptor mutations. Alternating treatment order produced a novel lysis pattern that appeared superior to cocktail administration for select host strains. In comparison with the hollow fiber infection model (HFIM), competition between phages constrained cocktail phage potencies, that is, that phages were slower to infect and reduce bacterial population numbers in cocktail compared to their respective single phage activity. Although cocktail performance still suppresses bacterial resistance for far longer, this initial competition affects the frequency at which exponential resistance growth can occur. These results suggest that phage kinetics can be predicted by study of phage replication kinetics and that combining phages from the perspective of phage-phage competition can inform the design of multi-phage combinations for clinical consideration.

MATERIALS & METHODS

Survey of phage types

Survey of known phages was conducted by filtering the *INPHARED* [279] database (accessed April 2024) for dsDNA phages classified under the host '*Pseudomonas aeruginosa*'. The collection of therapeutic bacteriophages was compiled by searching NCBI for *P. aeruginosa* clinical case reports between 2005 and June 2024. A list of the phages identified and their associated reports are listed in Table 3.1.

Strains & culturing

P. aeruginosa PAO1 (NC_002516.2) and PAK (LR657304.1) were grown under normal atmospheric conditions at 37°C in Luria broth (LB) Lennox with shaking (180 rpm). Bacteriophages E215, LUZ19, and PYO2 are *Caudoviricetes* originally isolated on *P. aeruginosa* strain PAO1 and previously described [82, 280]. Phages were propagated on PAO1 as previously described [281]. Briefly, purified phages were added to mid-log growing PAO1 at the multiplicity of infection (MOI) 0.1 and cultured overnight at 37°C with shaking. Lysates were centrifuged at 8000 ×g to remove bacterial debris and collected supernatant containing phages was 0.22 µm dead end filter sterilized and stored at 4°C. Phage quantification was performed by serial dilution followed by spot titering.

Spot⁺ titration

Bacterial cultures and phage stocks were quantified by double aliquot 48-spot serial titration. Briefly, bacteria culture lawned over agar was air-dried. In a 96-well microplate, two identical stock samples were taken and each serially diluted at least 8 times before spotting 4 µL in triplicate on the dried bacterial lawn. Titrations were

incubated at 37°C until colony forming units (CFU) or plaque forming units (PFU) were visible.

Time-kill assays

P. aeruginosa inoculum was prepared freshly for each experiment by growing a single CFU overnight (18-24 h) in LB broth. The overnight culture was then diluted into fresh medium the morning of each plate experiment (1:10) to obtain bacteria in the early logarithmic phase of growth. Microtiter plate wells were filled with 170 μ L of fresh LB, inoculated with $\sim 2 \times 10^6$ CFU, and dosed with phosphate buffered saline (PBS, control) or phages, before being sealed with a BreathEasy™ membrane (Diversified Biotech). A microplate reader (Clariostar; BMG) incubated the microplates at 37°C under shaking conditions (120 rpm), while measuring OD₆₀₀ at 6 min intervals between shaking steps. After 8 h, microtiter plates were aseptically unsealed, and a second phage dose (or control) was added to wells and a fresh membrane was applied before incubation and OD₆₀₀ monitoring continued.

In order to observe and compare phage lysis trends, the growth of PAO1 was standardized for the plate reader. The goal was to calculate a minimum starting density for which *P. aeruginosa* would be above the plate reader limit of detection, at optical density reading 600nm (OD₆₀₀) 0.18. A standard curve was created to compare plated bacterial counts against OD readings on the ClarioStar plate reader and fit a semi-log line (based on bacterial exponential growth [282]) to create a formula for estimating the CFU/mL density of the plate reader (Figure S3.1). Thereafter, a density of $\sim 2 \times 10^6$ CFU was added per well for each experiment and verified by plating on solid agar and

estimation > OD₆₀₀ 0.18 in plate reader. The theoretical limit of detection (LOD) limit was OD₆₀₀ 0.18, corresponding to 7.84×10^5 CFU/mL.

Next, a pipeline for the normalization and analysis of time-kill outcomes was developed (Appendix, RMarkdown). In order to compare the outcomes of replicate experiments, raw data for each plate experiment was corrected using its internal blank control. Plates were then normalized to the same starting density to allow comparison across different plate experiments. Each treatment condition could then be subset for comparison across concentrations and combinations.

The normalized experimental data derived from time-kill assays were analyzed using GraphPad Prism 5.03 (GraphPad Inc., San Diego, CA, USA). Normalized optical density values were plotted against time for each phage singly or in combination. The timing of resistance was calculated as the point which OD₆₀₀ exceeded 0.3. The percentage of turbid wells was similarly calculated as the number of wells with OD₆₀₀ > 0.3 at T28.

Hollow fiber infection model setup

PAO1 was studied over 7 days using polyvinylidene fibers (C2025, FiberCell, New Market, MD) with 0.1 µm pore size. The system half-life was fixed to 2 h based on assessment of limited available literature concerning *in vivo* PK [109, 283, 284]. The total system volume of distribution was 125 mL with a clearance rate of 0.7 mL/min. For bolus strategies with a target C_{max} = 7 log₁₀ (PFU/mL), a stock concentration of 11.6 log₁₀ PFU/mL was used. For continuous infusion strategies with a target C_{ss,avg} = 2 log₁₀ PFU/mL, a stock concentration of 6.58 log₁₀ (PFU/mL) was used. Serial samples were

collected for enumeration of bacteria and phages during treatment. Bacteria were quantified from samples obtained from the extracapillary space of the HFIM cartridge. Bacteria were quantified by serially diluting samples and plating 50 μ L on Mueller Hinton Agar (MHA) then incubating for 24 h prior to automated enumeration (Protos, Synbiosis). By comparison, phages were quantified in both the central reservoir and the extracapillary space of the HFIM cartridge to fully account for full disposition of phage in the system. Individual phage strains were distinguished through quantification on PAO1-resistant mutant lawns.

Phages were quantified by double aliquot 48-spot serial titration, as previously described. Briefly, phage-resistant PAO1 mutant cultured at OD₆₀₀ 0.2 was lawned and dried over agar medium before spotting two identical 8-well columns of tenfold serial diluted phage 4 μ L samples. Agar plates were incubated overnight at 37°C before plaque enumeration. Data were graphed using *R* (version 4.3.0) with no observable counts (i.e., 0 PFU) plotted as 0 log₁₀ PFU/mL for visualization purposes.

True counts of bacteria and phages from hollow fiber infection model

Serial samples were collected for enumeration of bacteria and phages during treatment. Centrifugation at 8000xg for 5 min separated the bacterial pellet from the phage supernatant. Bacteria were quantified from samples obtained from the extracellular space of the HFIM cartridge. Bacteria were quantified by serially diluting samples and plating 50 μ L on MHA then incubating for 24 h prior to automatic enumeration (Protos, Synbiosis). By comparison, phages were quantified in both the central reservoir and the extracapillary space of the HFIM cartridge to fully account for full disposition of phage in

the system. Individual phage strains were distinguished through quantification on *in vitro* evolved phage resistant PAO1 mutant lawns. Phages were quantified using the Spot⁺ titer method as described above.

DNA extraction, genome sequencing, and bioinformatics

Microtiter wells from 28 h time kill assays were individually streaked out and individual CFUs were subcultured and archived with 25% glycerol at -80°C. Genomic DNA was extracted by Macherey-Nagel NucleoSpin[™] Microbial DNA kit (MN). Sequencing libraries were prepared by the Microbial Genome Sequencing Center (MiGS) using the Illumina Nextera Kit and then sequenced on the Illumina NextSeq 550 platform (Illumina, San Diego, CA, United States) with paired-end 300 nucleotide reads. Raw reads were trimmed by *fastp* v0.20.1 [285] and *de novo* assembled using *SPAdes* v3.15.1 [286]. Reference guided scaffolding was conducted using *RAGTAG* v2.1.0 using the PAO1 NCBI reference genome (NC_002516.2). Annotation of specific function of open reading frames (ORFs) was conducted using Rapid Annotations of Subsystems Technology (*RAST*) v2.0 [287]. Identification of tRNAs was conducted using [288] and *ARAGORN* v1.2.41 [289]. Mutational analyses relative to control *P. aeruginosa* PAO1 (grown under microplate conditions for 28h) was conducted using the *BRESEQ* v0.35.5 pipeline [290].

Metagenome DNA Extraction

Metagenomic DNA was extracted using phenol-chloroform extraction method. In brief, samples were treated with detergent sodium dodecyl sulfate (SDS) and protease and bulk bacterial debris/viral capsids were removed with centrifugation. The supernatant was transferred to a Phase Lock Gel (QuantaBio) and phenol-chloroform extracted. The

supernatant was removed, and the remaining phenol was removed after mixing in a second Phase Lock Gel with chloroform extraction. DNA was then treated with sodium acetate and precipitated using ice cold absolute ethanol. After precipitation, DNA was washed with 70% ethanol, the DNA pellet was spun down, ethanol was evaporated, and the DNA was resuspended in tris-EDTA buffer. MiGS prepared a library for Illumina NextSeq 550 paired-end sequencing using the Nextera DNA Flex sample preparation kit (Illumina, San Diego, CA, USA). The paired-end reads were processed with *fastp* (version 0.23.2) [285] with default settings. Reads were then taxonomically classified using *Kraken2* [291] (v2.1.2) with default settings. Relative abundance was determined as a ratio of taxon reads/total classified reads.

Efficiency of plating experiments

Microtiter wells from time kill assays were individually streaked out and single CFUs were resuspended in 5mL LB and tested for phage resistance. Resistant isolates were used as the bacterial lawn culture and *pot*⁺ titration was performed as described above for comparison of E215 and LUZ19 activities on PAO1 and E215- or LUZ19-phage resistant lawns.

Statistics

All data are shown as means $\pm$ standard errors of the means (SEMs). Area under the curve (AUC) calculations were taken from time 0 h to 8 h to quantify the decline of sensitive biomass and from 8 h to 28 h to quantify the growth of phage resistant biomass. AUC calculations and statistical analyses were performed using GraphPad PRISM v9. A student's two-way t-test or two-way analysis of variance (ANOVA) with Tukey's multiple-

comparison test was used to determine differences between two or multiple groups, respectively. A p-value <0.05 was considered significant.

RESULTS

3.1: Only a limited number of phage genera are therapeutic candidates

With over 830 characterized anti-*Pseudomonas Caudoviricetes* (tailed dsDNA phages) I sought to subgroup tailed phages by their properties that provide unique therapeutic potentials. A survey of the phage types used in investigational new drug (IND) patient cases over the last two decades revealed that of the 32 tailed phage genera that infect clinical *P. aeruginosa*, only *Pbunavirus*, *Pakpunavirus*, *Phikmvvirus*, *Litunavirus*, *Bruynoghevirus*, and *Phikzvirus* have been the main groups of phages used in eIND cases (Table 3.1). As expected, phages in these genera are virulent with strictly lytic lifecycles. Moreover, phages within each genus are grouped by high intergenomic similarities, virion morphology, and genetic content [292]. Comparison of between genera uniqueness therefore suggests that their phages should have different therapeutic properties.

For instance, the aforementioned genera are delineated by two types of viral tail structures, differently sized genomes, diverse genetic content, and replication kinetics (Appendix, Tables S3.1-S3.6). These same characteristics have formed the basis of phage selection in recent clinical trials and case series [100, 293] which suggests that phages from multiple genera are typically selected for therapy. Moreover, a case series reporting treatment of a whopping 49 *P. aeruginosa* infections selected treatment from only 9 phages singly or in cocktail [21]. Of the phages, 4 used were from the *Pbunavirus*,

3 from *Litunavirus*, 1 from *Pakpunavirus*, and 1 from the *Phikmvvirus* genus [21]. In total, 63% of the cocktails surveyed combined phages from these genera (Table 3.1). A caveat of this however is that this number is expected to be an underestimation because several surveyed cases do not include detailed phage taxonomic information.

Table 3.1 Phage selection for *P. aeruginosa* treatments from 2009-2024.

Case Number	Author	Year	Treatment	Genus	Phage
1	Wright et al.	2009	Biophage-PA Cocktail	nd	BC-BP-01
				nd	BC-BP-02
				nd	BC-BP-03
				nd	BC-BP-04
				nd	BC-BP-05
				nd	BC-BP-06
2	Khawaldeh et al.	2011	PYOPhage Cocktail ¹	<i>Bruynoghevirus</i>	TL
				<i>Litunavirus</i>	PEV2
3	Kvachadze et al.	2011	PYOPhage Cocktail ¹	<i>Bruynoghevirus</i>	TL
				<i>Litunavirus</i>	PEV2
4	Rose et al.	2014	BFC1 Cocktail	<i>Pbunavirus</i>	14-1
				<i>Phikmvvirus</i>	PNM
5	Jennes et al.	2017	BFC1 Cocktail	<i>Pbunavirus</i>	14-1
				<i>Phikmvvirus</i>	PNM
			AB-PAO1 m1 Cocktail (+Pa193, Pa204, Pa222, Pa223)	nd	Pa176
				<i>Pbunavirus</i>	Pa193
			AB-PAO1 Cocktail	<i>Pbunavirus</i>	Pa204
				<i>Bruynoghevirus</i>	Pa222
6	Aslam et al.	2018		<i>Bruynoghevirus</i>	Pa223
				nd	Paφ1
			PA Navy Phage Cocktail 1	nd	PaSKWφ17
				nd	PaSKWφ22
			PA Navy Phage Cocktail 2	nd	PaATFφ1
				nd	PaATFφ3
7	Chan et al.	2018	Single	<i>Phikzvirus</i>	OMKO1
8	Duplessis et al.	2018	Cocktail	nd	nd
			2 unspecified phages		
9	Ferry et al.	2018	Single	nd	PP1450
			Single	nd	PP1777
			Single	<i>Bruynoghevirus</i>	PP1792
			Single	nd	PP1797
10	Ujmajuridze et al.	2018	PYOPhage Cocktail ¹	<i>Bruynoghevirus</i>	TL
				<i>Litunavirus</i>	PEV2
11	Jault et al.	2019	PP1131 Cocktail 12 unspecified phages	nd	nd
				<i>Pbunavirus</i>	Pa193
12	Law et al.	2019	AB-PAO1 Cocktail	<i>Pbunavirus</i>	Pa204
				<i>Bruynoghevirus</i>	Pa222
				<i>Bruynoghevirus</i>	Pa223

Table 3.1 Phage selection for *P. aeruginosa* treatments from 2009-2024. (continued)

Case Number	Author	Year	Treatment	Genus	Phage
13	Maddocks et al.	2019	AB-PAO1 Cocktail	<i>Pbunavirus</i>	Pa193
				<i>Pbunavirus</i>	Pa204
				<i>Bruynoghevirus</i>	Pa222
				<i>Bruynoghevirus</i>	Pa223
14	Onsea et al.	2019	BFC1 Cocktail (+ <i>S. aureus</i> phage ISP)	<i>Pbunavirus</i>	14-1
				<i>Phikmvvirus</i>	PNM
15	Rubalskii et al.	2020	Cocktail	<i>Pbunavirus</i>	PA5
				<i>Pakpunavirus</i>	PA10
16	Tkhilaishvili et al.	2020	PYOPhage Cocktail ¹	<i>Bruynoghevirus</i>	TL
				<i>Litunavirus</i>	PEV2
				<i>Pbunavirus</i>	PP1450
17	Ferry et al.	2021	Cocktail	<i>Pbunavirus</i>	PP1777
				<i>Bruynoghevirus</i>	PP1792
				<i>Pakpunavirus</i>	PT07
18	Tkhilaishvili et al.	2021	Cocktail (with PNM and 14-1)	<i>Pbunavirus</i>	14-1
				<i>Phikmvvirus</i>	PNM
				<i>Litunavirus</i>	vB_PaeP_4029
19	Ferry et al.	2022	Cocktail	<i>Litunavirus</i>	vB_PaeP_4032
				<i>Litunavirus</i>	vB_PaeP_4034
				<i>Pbunavirus</i>	14-1
20	Nieuwenhuysen et al.	2022	BFC1 Cocktail	<i>Phikmvvirus</i>	PNM
				<i>Pbunavirus</i>	PaWRA01Phi11
				<i>Pbunavirus</i>	PaWRA01Phi39
21	Tamma et al.	2022	WRAIR-PAM_CF1 Cocktail	<i>Bruynoghevirus</i>	PAWRA02Phi83
				<i>Bruynoghevirus</i>	PAWRA02Phi87
				<i>Bruynoghevirus</i>	Pa53
22	Cesta et al.	2023	Single	<i>Pbunavirus</i>	6959
				<i>Pbunavirus</i>	6917
23	Green et al.	2023	Cocktail	<i>Pbunavirus</i>	6917
24	Köhler et al.	2023	Single	<i>Pakpunavirus</i>	vFB297
25	Onallah et al.	2023	Single	<i>Pbunavirus</i>	PASA16
				<i>Pakpunavirus</i>	PAK_P1
				<i>Pbunavirus</i>	E217
26	Aslam et al.	2024	Cocktails PAK_P1+E217 PAK_P1+PAK_P5	<i>Nankokuvirus</i>	PAK_P5
				nd	PaBAP5φ3
				nd	PaMTAE8φ1
				nd	PaMTAE8φ3
				nd	PaMTAE8φ3

Table 3.1 Phage selection for *P. aeruginosa* treatments from 2009-2024. (continued)

Case Number	Author	Year	Treatment	Genus	Phage
27	Pirnay et al.	2024	BFC1 (2 <i>PA</i> phages) BFC2 (2 <i>PA</i> phages) IntestiPhage (23 phages) PYOPhage Cocktail ¹ Cocktail (Custom)	<i>Pbunavirus</i>	4P
				<i>Pbunavirus</i>	14-1
				<i>Phikmvvirus</i>	PNM
				<i>Pakpunavirus</i>	PT07
				<i>Litunavirus</i>	4029
				<i>Litunavirus</i>	4032
				<i>Litunavirus</i>	4034
				<i>Pbunavirus</i>	Phage C
				<i>Pbunavirus</i>	DP1

¹ The commercial product "PYOPhage" is expected to be different across batches but metagenomic analysis suggests presence of at least these two phage strains (Villarroel et al., 2017).

A well-designed phage cocktail should accomplish three major goals: 1) broaden the host range, 2) increase bacteriolytic potency by having multiple phage strains attack the bacterial population, and 3) extend treatment longevity by forcing the target bacterium to evolve resistances to multiple phage strains simultaneously [103, 104, 294]. Mechanistic information about how phages in cocktails interact to promote synergistic/antagonistic effects and anticipated resistance evolution patterns remain elusive, in part because these interactions depend on each phage species, the effect of two phages on one host, and spatial patterns of microbial distribution. Deep phage characterization and generalized selection parameters are needed to improve the selection of phages for therapeutic formulation.

Given the prominence of these phage genera, I designed for the study an anti-*Pseudomonas* cocktail by selecting *Pbunavirus* E215 whose related phages were included in 56% of single or cocktail treatments developed for eIND (Table 3.1). E215 infects host cells by binding to bacterial lipopolysaccharide (LPS) and has been shown to have antibiofilm activity and broad host range on a panel of clinical isolates [82]. It has

been suggested that pairing phages binding to different cell surface receptors provides an ideal cocktail to increase the cost of resistance while limiting the strength of evolved resistances [16, 104]. Therefore, to pair with E215 in cocktail I added *Phikmvvirus* LUZ19 which binds to host cell type IV pilus. LUZ19 was previously characterized and found to complete its infection cycle in under 25 min with potentially accelerated transcription due to its encoded RNA polymerase [295]. Both phages were found to be free of integrase or lysogeny associated genes and considered obligately lytic [82, 295].

3.2: Single time-kills allow resistant escape independent of phage concentration

To determine baseline activity patterns of each individual phage strain, time-kill curves were used to measure the kinetics of bacterial decline, suppression, and exponential resistance regrowth in the presence of phages. PAO1 was exposed to phages at multiplicities of infection (MOI) where virions outnumber host cells 10:1 (MOI 10), equal ratio (MOI 1), and where cells outnumber virions (MOIs 0.1 and 0.01). Both E215 and LUZ19 could produce similar lysis kinetic by suppressing all bacterial population growth for approximately 10 hours longer than no treatment (Figure 3.1A). Thereafter, canonical resistance regrowth to near carrying capacity was observed. In total, once exponential growth began 98% (417/423) of the single phage time-kills reached carrying capacity OD₆₀₀ 0.7-1.0 (Figure 3.1B).

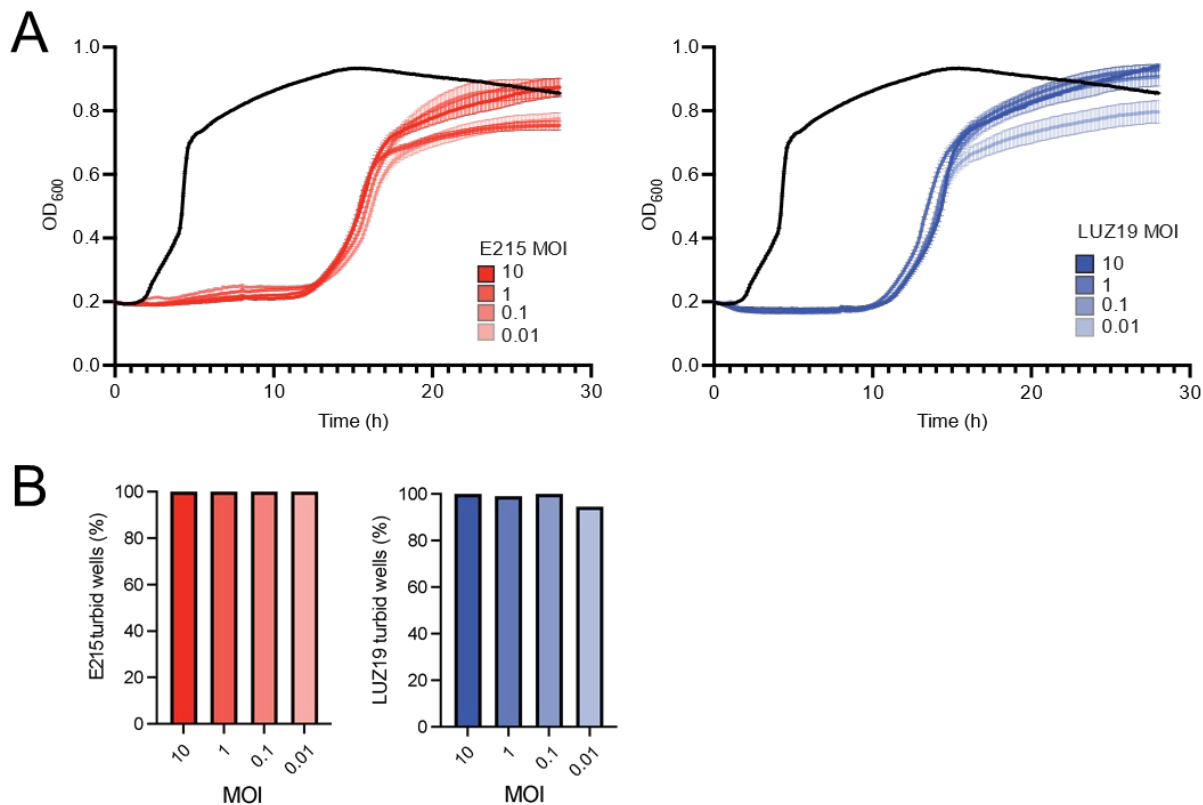


Figure 3.1 Single phage time-kill kinetics. (A) Averaged time-kill kinetics of E215 (red) and LUZ19 (blue) at MOIs 10-0.01. Phage treatment was given at T0. Error bars represent standard error of the mean (SEM). (B) Percentage of wells grown turbid, above OD₆₀₀ 0.3 after 28 h phage treatments.

Because bacterial regrowth in phage lysates were most likely due to evolution of mutational resistance resistant colonies were isolated, short-read sequenced, mapped to the untreated reference genome, and the accumulated single nucleotide polymorphisms (SNPs) were analyzed to identify putative phage-induced bacterial modifications. Consistent with host cells modifying their cell surface to block phage binding there were abundant phage receptor mutations. Host cells became resistant to LUZ19 by modifying its type IV pili (T4P) or resistance to E215 by modifying their lipopolysaccharide (LPS) gene *wzy* (Appendix, Table S3.7). However, known surface LPS modifications accounted for only 40% of E215 resistant mutants. Therefore, non-receptor gene modifications may

also be promoting resistance to E215, such as transporter genes *OppB* and *PotB*, transcriptional regulators or tRNAs in the AcrR family, or others in an array of unidentified genes (Appendix, Table S3.7).

3.3: Inequal phage activities increases frequency of cocktail resistance escape

It has been suggested that pairing phages binding to different cell surface receptors provides an ideal cocktail to increase the cost of resistance while limiting the strength of evolved resistances [104]. Cocktail of E215 and LUZ19, which was composed by mixing equal phage ratios, suppressed bacterial growth for longer in liquid time-kill culture compared to each phage individually (Figure 3.2A). The phage cocktail prevented bacterial growth for 28 hours in 75% of the 118 cultures (Figure 3.2B). While individual phages could prevent bacterial growth for ~12 hours the cocktail was suppressive for over 24 hours, more than doubling the effective duration of phage treatment (Figure 3.2A). Phage resistant cocktail mutants also appeared to plateau at a lower carrying capacity than mutants of the individual phages, suggesting increased fitness cost from two phage resistance (Figure 3.2A). Nonetheless, resistant mutants still arose ~22h after addition with timing independent of dose (Figure 3.2C). It appeared that the cocktail mutants were most likely resistant to both phages through surface modifications against each phage receptor gene [241]. Indeed, in streak-purified cocktail resistant mutants the frequency of a pilus mutation being accompanied by an LPS mutation was 100% (Appendix, Table S3.8).

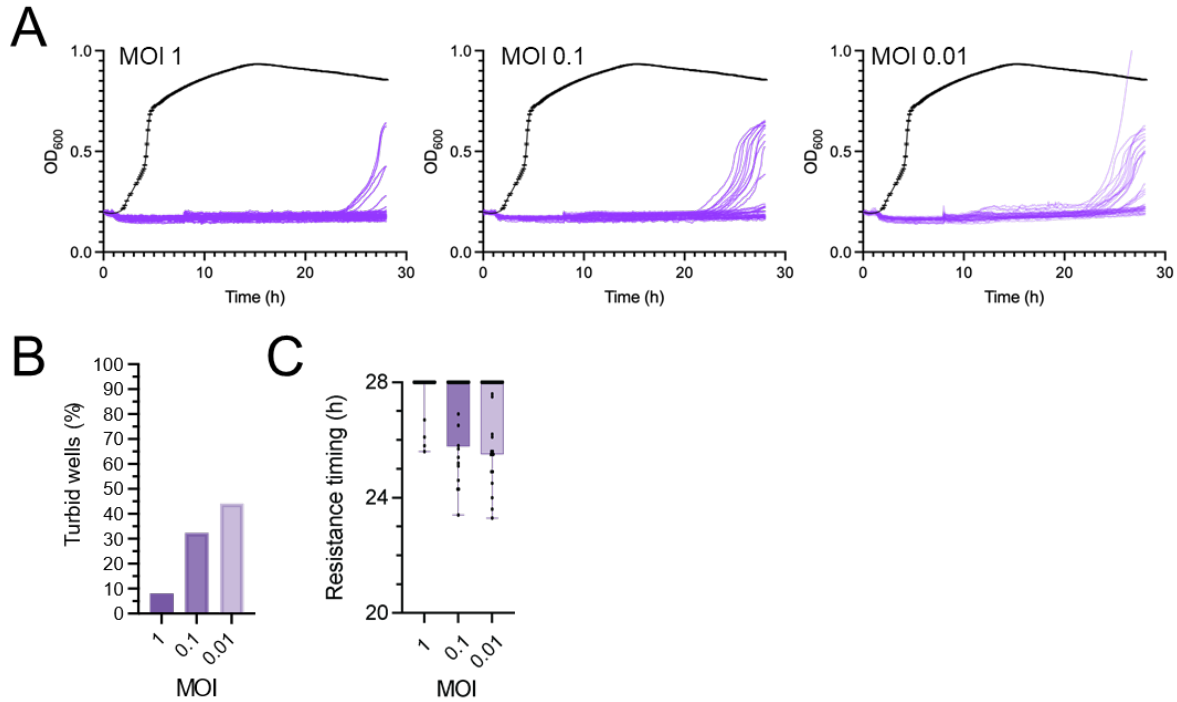


Figure 3.2 Cocktail E215+LUZ19 time-kill kinetics. (A) Individual time-kill replicates for MOI 1-0.01 E215+LUZ19 cocktail treatment are shown. Cocktails were composed of equal phage densities to achieve the total MOI, e.g. MOI 0.5 E215/MOI 0.5 LUZ19 for MOI 1 cocktail. Cocktail phage treatment was given at T0. Error bars represent standard error of mean. (B) Percentage of wells grown above OD₆₀₀ 0.3 after 28 h cocktail treatment. (C) Timing of bacterial growth that surpassed >OD₆₀₀ 0.3 during cocktail treatment.

Although the combination of E215+LUZ19 complemented to delay the onset of resistance, the frequency of resistance in culture appeared to be dose-dependent. Cocktail E215+LUZ19 was most effective at MOI 1 (MOI 0.5/phage) where as few as 8% (4/50) of cultures escaped phages to become turbid (Figure 3.2B). Tenfold reductions in phage concentration were subsequently associated with increased bacterial escape as often as 44% (15/34) (Figure 3.2B). The dose-dependent pattern of resistance frequency suggests that cocktail failure may be a consequence of insufficient treatment inoculum as seen during resistance development in antibiotics [296] and/or a consequence of insufficient activity by one or both phages [104].

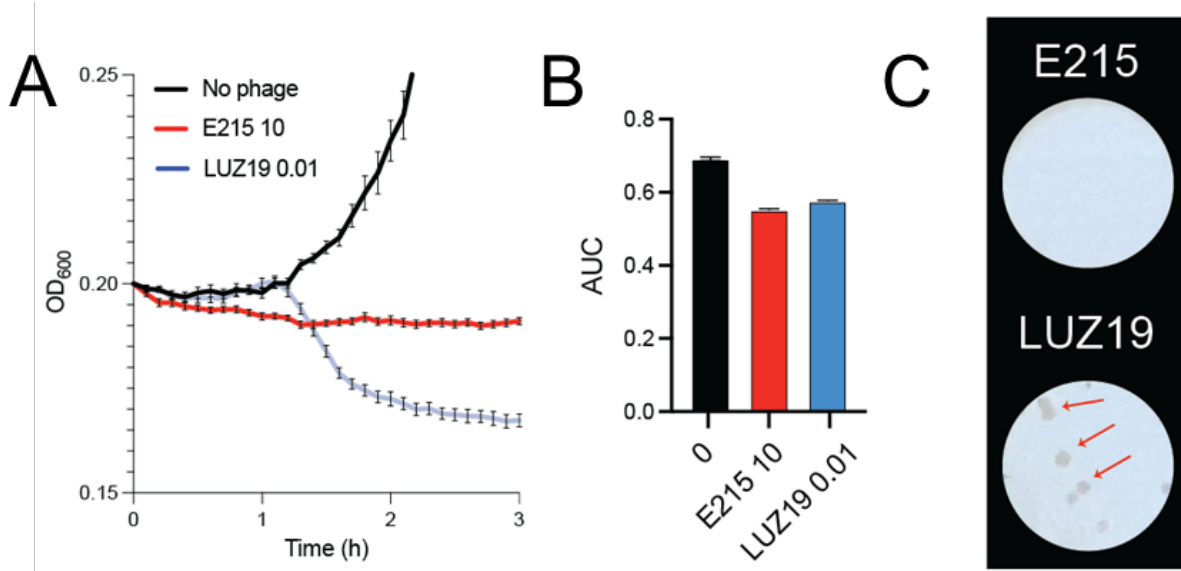


Figure 3.3 Comparison of E215 and LUZ19 lysis potencies. (A) Early time-kill kinetics (T0-T3) of single phage treatments. Error bars represent SEM. (B) Area under the curve (AUC) comparison as a proxy to determine bacterial reduction by phages E215 and LUZ19 from T0-T3. Treatments at MOI 10 E215 and MOI 0.01 LUZ19 were selected to normalize lysis comparison. Error bars represent SEM. (C) Representative image of LUZ19 and E215 clearance of PAO1 after 18 h incubation of 4μL phage spots. Phage concentrations were standardized to 10^8 PFU/mL for each stock. Red arrows indicate resistant CFUs.

Therefore, next the initial time-kill kinetics of each phage were compared to determine whether the activity, or potencies, of the phages were similar. During the first 3 h of treatment, in order for E215 to achieve similar lysis kinetics it had to be added at 1000× the concentration of LUZ19 (Figure 3.3A). Moreover, MOI 10 E215 lysis activity reduced bacterial growth but could not decrease the load to the limit of detection (OD₆₀₀ 0.17), which LUZ19 was capable of at all concentrations (Figure 3.1A). Nonetheless, during initial infection both phages were able to maintain a lower bacterial burden than the untreated control (Figure 3.3B), albeit following different infection patterns whereby E215 appeared to infect and prevent bacterial growth more quickly with slower lysis and LUZ19 infecting more slowly but lysing more rapidly (Figure 3.3A). This appears to

correspond to the individual replication kinetics of each phage. Accordingly, E215 binds to or adsorbs to host cells more rapidly than LUZ19 (Appendix, Tables S3.1 and S3.3; 1.40×10^{-9} vs $1.20 \times 10^{-7} \text{ mL}^{-1} \text{ min}^{-1}$). Moreover, LUZ19 had an infection latent period 13 min shorter than that by E215 (Appendix, Tables S3.1 and S3.3; 17 min vs. 30 min), although longer E215 latent period translated to a burst of 250 phage progeny, compared to LUZ19 burst of 122 (Appendix, Tables S3.1 and S3.3).

Together, this would suggest that analysis of phage replication characteristics can predict initial time-kill patterns in *in vitro* co-culture. Comparison between LUZ19 and E215 also suggests that latent period, more so than burst size, predicts how rapidly bacterial burden is reduced when host infection is maximized ($\text{MOI} > 1$), that is that a shorter latent period allows reduction of bacterial cells more efficiently than a large burst size. This suggests that LUZ19 activity is much more potent than E215, hence lowered concentrations of LUZ19 compounded by the deficiency in E215 potency could allow more frequent cocktail resistance at low cocktail MOIs.

On the other hand, increased bacterial resistance was also correlated with higher phage potency. On solid agar both E215 and LUZ19 formed clear plaques on PAO1 lawns (Figure 3.3C). However, LUZ19 allowed nearly twice (193%) as many highly pronounced resistant colony-forming units (CFU) to emerge per μL of phage spotted (10^8 PFU/mL) compared to E215 (Figure 3.3C). This suggests an alternative scenario wherein the high potency of LUZ19 pressures an increased rate of bacterial escape in cocktail. Moreover, among the various LUZ19 resistant mutants sequenced SNPs occurred at 13 genes distributed across the PAO1 genome and spanning all four of the sub-complexes [297] that make up the type IV pilus assembly system (Appendix, Tables S3.7-S3.9). In contrast

only a single LPS associated gene *wzy* was consistently mutated in 30/52 E215 resistant mutants sequenced (Appendix, Tables S3.7-S3.9). Resistance rate analysis states that the overall mutability of resistance is the sum of independent mutability values from the number of possible resistance producing genes [298]. This would mean that the sum resistance rate against LUZ19 is higher than the resistance rate against E215. Because individual phage activities and resistance rates are unequal in cocktails, this suggests that uneven competitiveness may be reducing the overall cocktail efficacy. That is, either E215 is not competitive enough and fails to reduce bacterial burden or that LUZ19 is too competitive and drives more rapid, higher rates of resistance.

3.4: Substitution with higher potency phages decreases cocktail escape frequencies

A cocktail composed of an LPS binding phage and pili binding phage has been by far the most rigorously studied among the potential anti-*Pseudomonas* combination treatments [299]. However, there has been little direct pairwise phage comparison in cocktail. Because cocktail efficacy of E215+LUZ19 appeared to be dampened only in the absence of high LUZ19 starting inoculum, E215 was substituted for an alternate LPS binding phage to see if efficacy could be improved in cocktail. The list of other prevalent anti-*Pseudomonas* phage genera was used to identify an alternate LPS phage (Appendix, Table S3.5). *Litonavirus* PYO2 was isolated alongside E215 and found to likewise bind to *P. aeruginosa* LPS [82]. Following single phage time-kills, PYO2 resistant isolates were found to harbor SNPs in the same *wzy* gene as E215 in 60% (3/5) of sequenced mutants (Appendix, Tables S3.7). Despite binding to the same receptor, cross resistance between

PYO2 and E215 was directional. That is, E215 was unable to plaque on a PYO2 resistant *wzy* mutant, while PYO2 could still plaque on the E215 resistant *wzy* mutant but at a lower efficiency (Figure 3.4). This implies each phage binds to different O-antigen molecules. In contrast, there was no cross-resistance between LUZ19 and either PYO2 or E215.

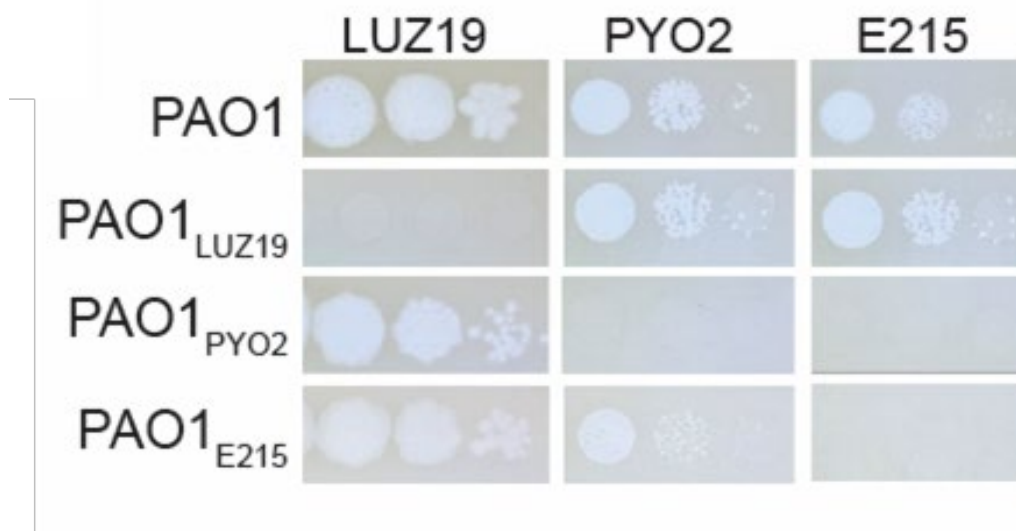


Figure 3.4 Cross resistance analysis of PAO1 phages. Phage stocks were diluted to 10^8 PFU/mL and titered on bacterial lawns of wild-type and phage resistant mutants.

The PYO2+LUZ19 combination was much more effective at preventing bacterial escape than E215+LUZ19 (Figure 3.5A). Out of the 118 cocktail treatments, only a single well at the lowest concentration allowed bacterial escape before the 28-hour time point (Figure 3.5A). As expected, this exponentially growing mutant harbored both *pil* and *wzy* mutations in its genome (Appendix, Tables S3.8). This demonstrates that an effective treatment was possible using an LPS+pili phage cocktail.

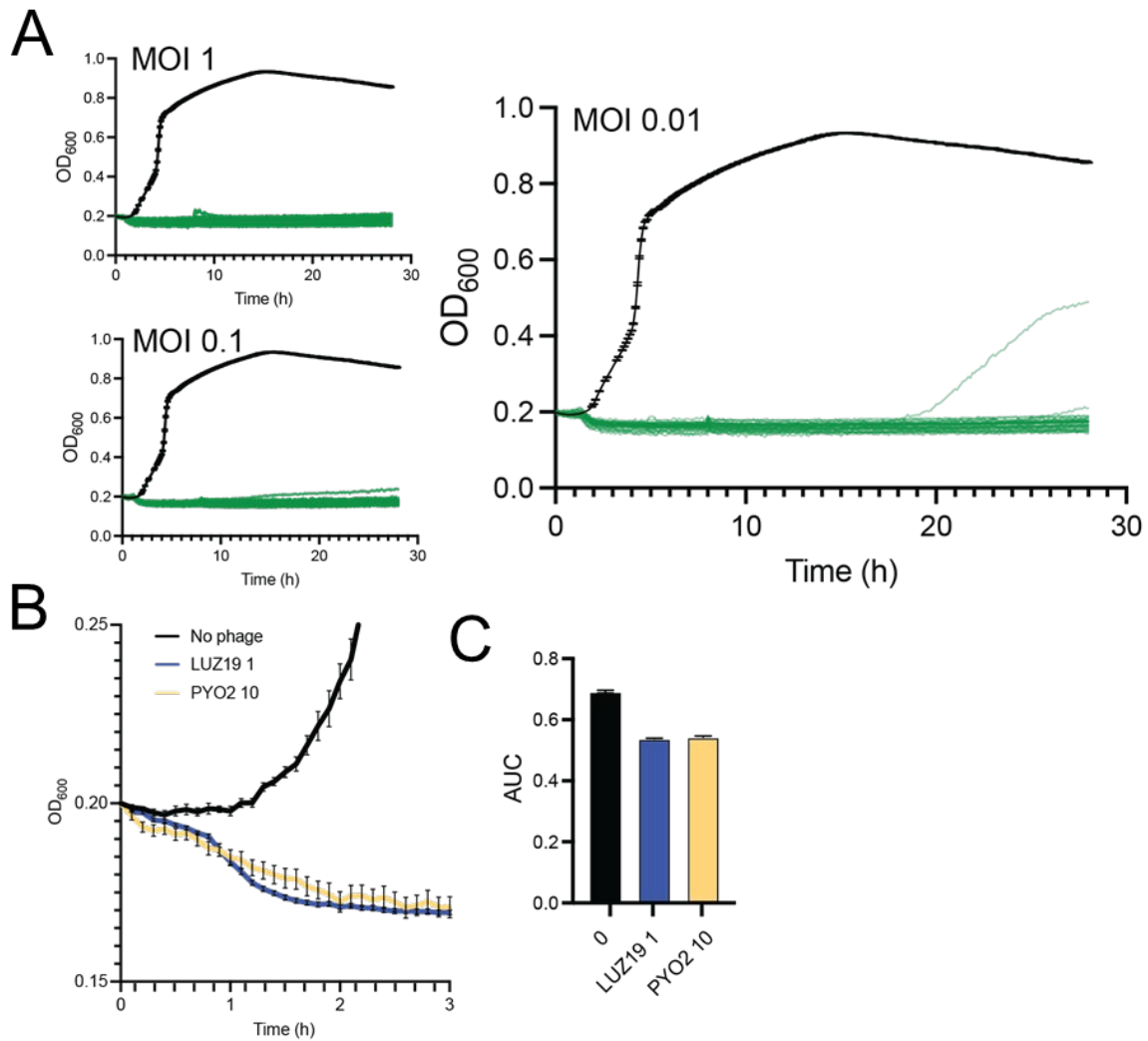


Figure 3.5 Cocktail LUZ19+PYO2 time-kill kinetics. (A) Individual time-kill replicates for MOI 1-0.01 LUZ19+PYO2 cocktail treatment are shown. Cocktail phage treatment was given at T0. Error bars represent standard error of mean. (B) Comparison of LUZ19 and PYO2 lysis potencies from T0-T3. Error bars represent SEM. (C) AUC comparison of initial bacterial reductions by MOI 1 LUZ19 and MOI 10 PYO2. Error bars represent SEM.

Although this suggests that PYO2 is the more potent phage compared to E215, it is unclear whether its morphology, genome, and/or lytic cycle confers its advantage. The two phages also have different morphologies, with PYO2 having a podovirus, short noncontractile tail structure while E215 is a myovirus with a long contractile tail structure. The genomes of the two phages appeared similar in size and gene number but PYO2

encodes its own RNA polymerase (Appendix, Table S3.5). Compared with E215, PYO2 was also found to replicate 1.15x faster but produces ~20% fewer phages per lytic cycle (Appendix, Table S3.1 and S3.5). It would appear that the longer latent period of E215 is compensated by its larger burst size but, again, the correlation between lytic cycle and potency remains unclear (Appendix, Table S3.1 and S3.5). Comparison of initial single phage activities confirmed however that the initial potency of PYO2 was still 10x lower than LUZ19 (Figure 3.5B). This, in turn, emphasizes that PYO2 is approximately 100x more potent than E215 singly administered. Combined, this suggests that their difference in initial phage activities may account for their divergent cocktail efficacies when mixed with LUZ19 (Figure 3.2A vs 3.5A).

In contrast to assumptions of phage synergism in cocktail, the potency of LUZ19 alone was greater than that of PYO2+LUZ19 together (Figure 3.6A). This would suggest that cocktail potency is driven by the most potent phage in cocktail. Moreover, because the kinetics of cocktail treatment are slower in the presence of PYO2 it appears that the presence of a second, less potent phage strain hampers treatment efficacy. Because there is no collateral resistance between either LPS and pili binding phages, this would suggest that the reduced potency is a limitation of susceptible bacterial cells. That is, if cells LUZ19 could have otherwise monopolized were occupied by PYO2 binding, fewer cells would be available for LUZ19 replication.

Indeed, when the number of available host cells was increased 100x by decreasing phage numbers, the reduction of cocktail potency relative to single phages was reversed (Figure 3.6B). There was also a modest improvement in cocktail wherein lysis kinetics mirrored the patterns of PYO2 followed by LUZ19 lytic activities (Figure 3.6B). That

cocktail kinetics now hybridize the activities of both phages instead of an 'average' of the two curves suggests improved co-existence of both phage strains compared to the MOI 1 cocktail (Figure 3.6 A vs B). On the other hand, potency over time remained constrained by the availability of LUZ19 phages and did not appear further improved (Figure 3.5A). Together, this suggests that when two phages are combined in cocktail, the activity of the individual phages are reflected in their kinetics even when the receptor basis of the combination remains the same, i.e., LPS-binding phage and pili-binding phage.

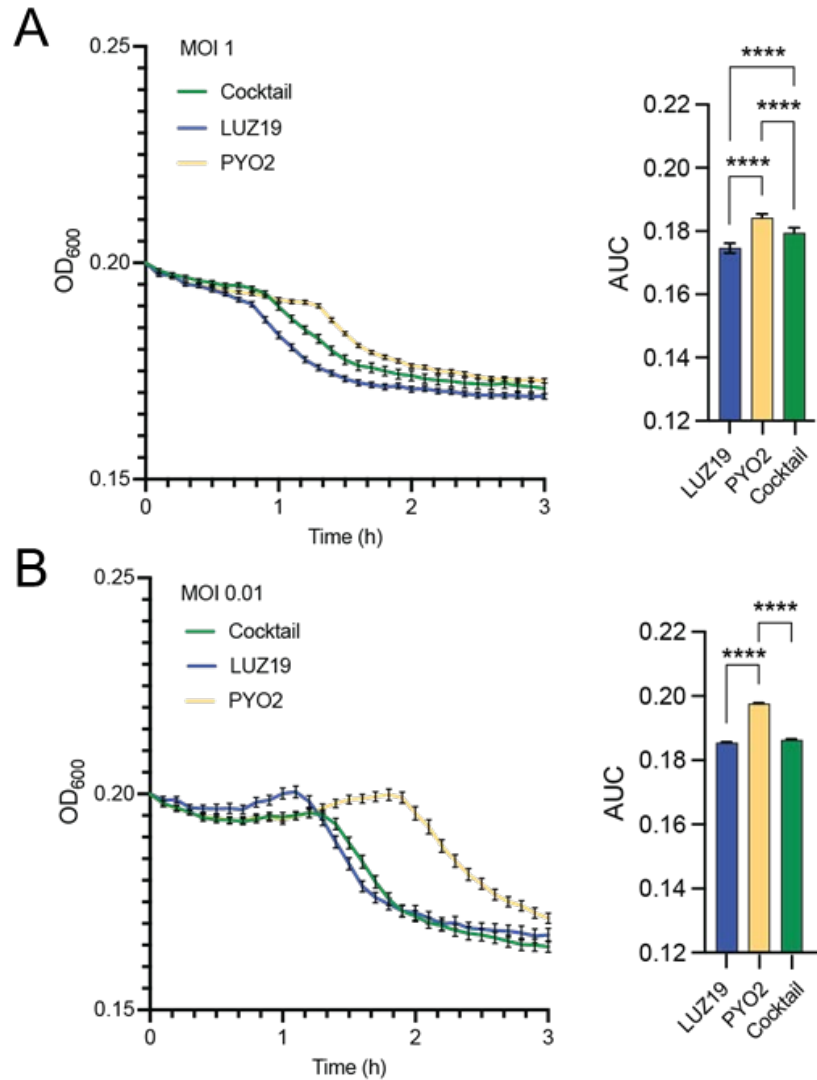


Figure 3.6 Comparison of LUZ19+PYO2 cocktail and single phage potencies. Comparison of individual phages and cocktail activity and bacterial reduction (AUC) when (A) bacterial numbers are equal to phage (MOI 1) and (B) higher than phages (MOI 0.01). Error bars represent SEM. * $p < 0.05$

3.5: Asymmetric phage concentrations in cocktail dampens cocktail potency but not efficacy

To further decipher whether lower PYO2 potency and/or high LUZ19 potency contribute to bacterial escape, different compensatory cocktail doses were compared.

When the dose of PYO2 was increased 10x and 100x relative to LUZ19 the reduction of sensitive cells from T0-T3 was reduced 3.3% and 4% compared to MOI 1 LUZ19 treatment, respectively (Figure 3.7A). Increasing LUZ19 concentration to MOI 100 also slightly dampened potency by increasing AUC by 1% (Figure 3.7A). This would suggest that phage potencies are adversely affected by high phage doses > MOI 100. Treatment of LUZ19 together with filtered PYO2 lysate did not evoke similar decreases in lysis potency, further suggesting that the direct effects of two phages versus a limited number of bacterial hosts was the cause of slowed initial bacterial lysis (Figure 3.7A). This suggests that initial phage efficacy cannot be compensated for or improved by addition of higher phage starting inoculums.

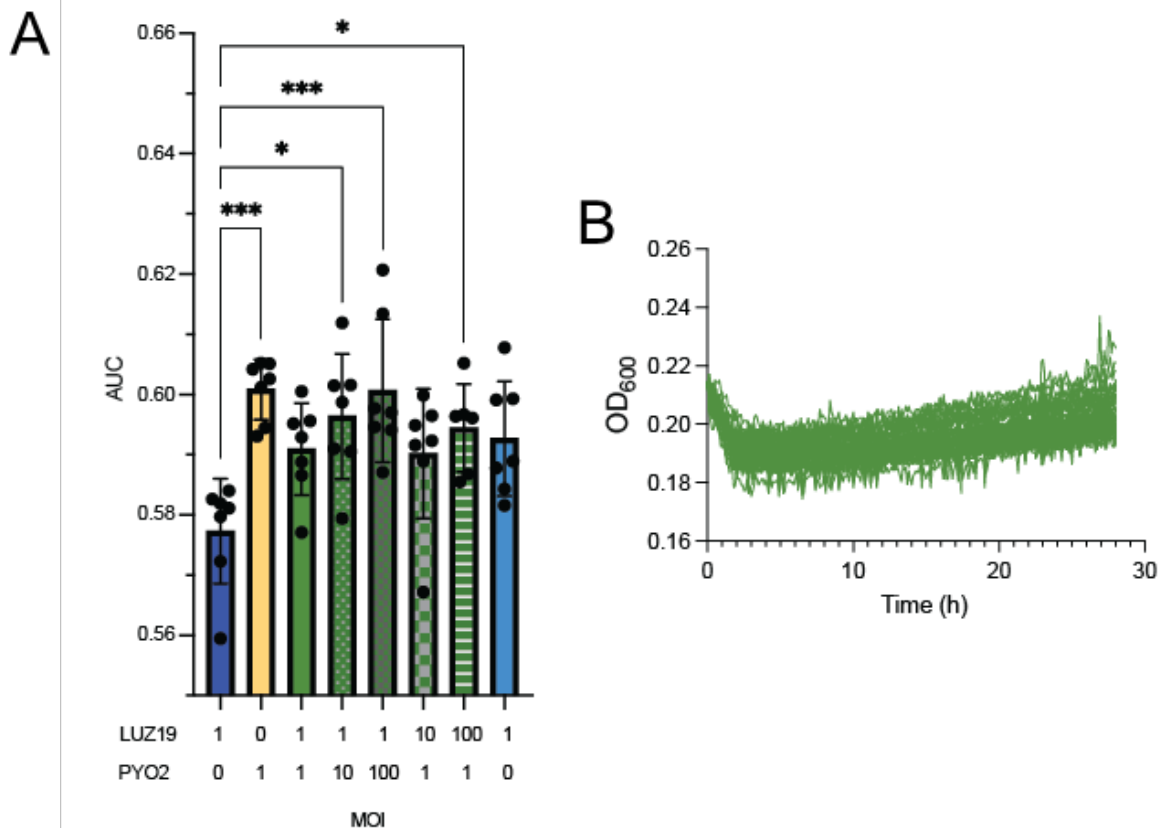


Figure 3.7 Asymmetric cocktail potency versus single phage potency. (A) Comparative potency from T0-T3 of asymmetric cocktail concentrations combining LUZ19 and PYO2. Cocktails were composed of phages to create total MOIs of 2, 11, or 101. A filtered PYO2 lysate fraction (<100kDa) free of phages was used for comparison (light blue). Error bars represent SEM. * $p < 0.05$ (B) Time-kill kinetics of asymmetric cocktails. No exponential growth was observed in any of the cocktail treatment wells, $n=35$.

Despite the slight decrease in potencies however, no cocktail escape mutants were observed (Figure 3.7B). This would further suggest, as seen with previous escape frequency, that cocktails combining LUZ19 and PYO2 with total MOI 1 or higher appear sufficient to prevent bacterial growth for 28 h (Figure 3.5A vs 3.7B). This would be in line with the high predator-prey conversion rate of phages whereby additional phage starting inoculum would be expected to have diminishing returns in light of the rapid host cell turnover rate [300]. Combined, although increased concentrations of phages slightly

dampened initial phage lysis activities, these results suggest that a MOI equal to, but not in large excess of, bacterial density best optimize cocktail performance when both phages are potent. Although comparison of these activities in E215 cocktail has yet to be explored, this suggests that phage selection should play a larger role relative to dose for increasing cocktail efficacy during treatment design.

3.6: Reducing phage competition through sequential addition worsens individual phage activities

Lytic phages and bacteria create a unique predator-prey dynamic wherein the death of a bacterial cell is typically accompanied by the release of hundreds of new phages [11]. Because this strategy constrains phage co-existence in nature [300], it would follow that competition between phages can similarly be a problem during phage cocktail design. Because the cocktail of PYO2+LUZ19 outperformed that of E215+LUZ19, this suggests that the increased initial potency of PYO2 enabled it to better compete with LUZ19 in co-culture. Thus, I next hypothesized that our original E215 and LUZ19 cocktail could be improved by limiting the competitive pressure exerted by LUZ19 onto E215. Two sequential treatment regimens were devised to give E215 a competitive advantage: 1) either by allowing E215 to monopolize cells by having 8 hours of uncontested replication or 2) by allowing E215 to prey upon a subpopulation of LUZ19-resistant cells (Figure 3.8A). We selected an 8-hour time-point to capture a period of time when bacterial cells resistant to the first phage in sequence could begin approaching the limit of detection $\sim 6.5 \times 10^3$ CFU (LUZ19) or before they could outcompete sensitive cells $\sim 2.8 \times 10^6$ CFU (E215) and grow unencumbered (Figure 3.8B).

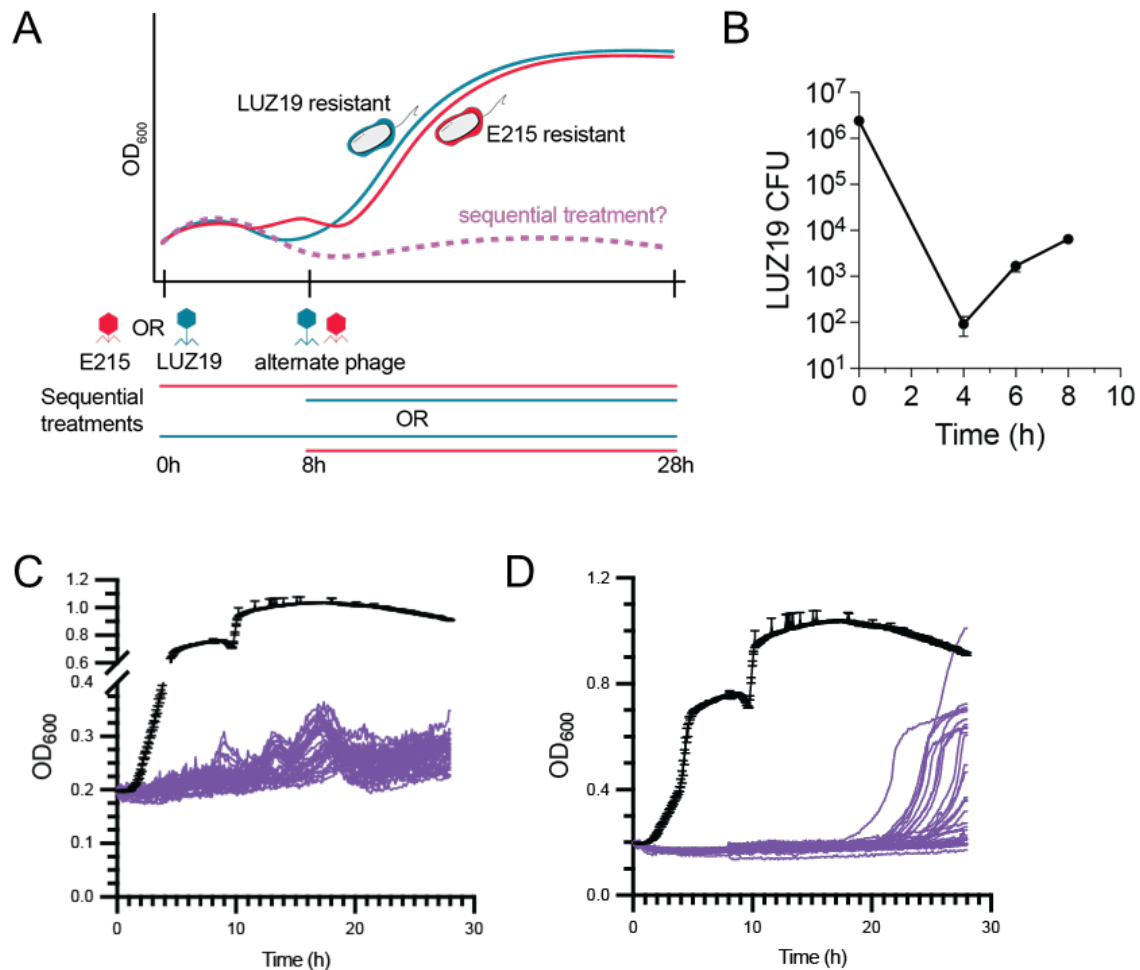


Figure 3.8 Sequential treatment schematic and time-kill results. (A) Schematic depicting the resistance outcomes of single and cocktail phage treatments. The design of sequential treatment using single phages (MOI 1) at T0 and T8 is below. (B) Titer of bacterial colony forming units sampled from LUZ19 treated microtiter wells. Error bars represent SEM. Sequential treatment time-kill kinetics of (C) E215 (T0) followed by LUZ19 (T8) and (D) LUZ19 (T0) followed by E215 (T8). Error bars represent SEM for no phage control and individual replicates shown for phage treated wells.

In the first scenario, the treatment initiated with E215 followed by LUZ19 appeared to worsen the activity of the more potent phage (Figure 3.8C). Although the second phage was added when bacterial cells were at what appeared to be a sufficiently high density (OD₆₀₀ >0.2) for phage reduction, no decrease in density was apparent until T18, approximately 10 hours after addition (Figure 3.8C). Furthermore, bacterial cultures

appeared to steadily increase in density from T8-T18 despite the abundance of both phage strains (Figure 3.8C). This suggests that the activity of LUZ19 was dampened as compared to cocktail, even though plenty of *wzy* mutants were expected to be present and indeed were confirmed after 28 h treatment (Appendix, Table S3.9). Although the density of the wells stayed close to $\sim OD_{600}$ 0.3, 100% of these wells were turbid in comparison to cocktail. We had previously verified that there was no collateral resistance between E215-resistant mutants and LUZ19 binding by calculating the efficacy of plating (EOP) between phage sensitive and phage resistant variants of the phages (Figure 3.4). This would suggest that a new phenomenon caused the delay in LUZ19 activity nonspecific to *wzy* mutation.

In the second scenario, administering the sequential treatment in reverse with LUZ19 then E215, we observed that the kinetics of treatment more closely resembled that of cocktail (Figure 3.8D). In sequential treatment however, the frequency of turbid wells increased 6.5x (52% vs 8%) compared to cocktail treatment at the same concentration (Figure 3.8D vs Figure 3.2A). Again, phage activity appeared worsened despite E215 having a plethora of *pil* mutants for replication (Appendix, Table S3.9). Although we set out to improve cocktail efficacy by reducing competition between the two phages, the activities of both sequences appeared to worsen the activity of the second phage added. However, there was strong dependence by bacterial resistance dynamics based on the sequence of phage addition. Moreover, although the addition of E215 to LUZ19 did not perform as expected the lack of exponentially growing resistance mutants was intriguing.

3.7: Sequential phage treatment performance is strain-dependent

If reduced phage potencies were correlated to lower lytic activity in cocktail, we reasoned that testing the phages on non-isolation host strains would show similar lysis dynamics or infer host-related differences that could be used to compare an 'optimized' cocktail against other susceptible strains. For comparison, another well-characterized *P. aeruginosa* strain PAK [38] was selected and challenged with single, cocktail, and sequential phage treatments, focusing now on MOI 1 concentration. Plating on PAK bacterial lawns found that E215 and LUZ19 had EOPs of 56.2% and 100% respectively. This would suggest reduced potency of phage E215 on the new host. In contrast to this expectation, only LUZ19 kinetics were divergent from its activity on PAO1 (Figure 3.9A). Despite its efficacy being halved, E215 kinetics nonetheless showed a similar, consistent low level of bacterial control near the limit of detection followed by growth of resistant mutants at ~16 h, two hours longer than on PAO1 host. Approximately 17.6% (3/17) of LUZ19 treated wells remained clear for 28h (Figure 3.9A). This conversely suggests that activity of both phages in liquid co-culture is improved relative to PAO1 kinetics despite lowered E215 EOP on solid agar.

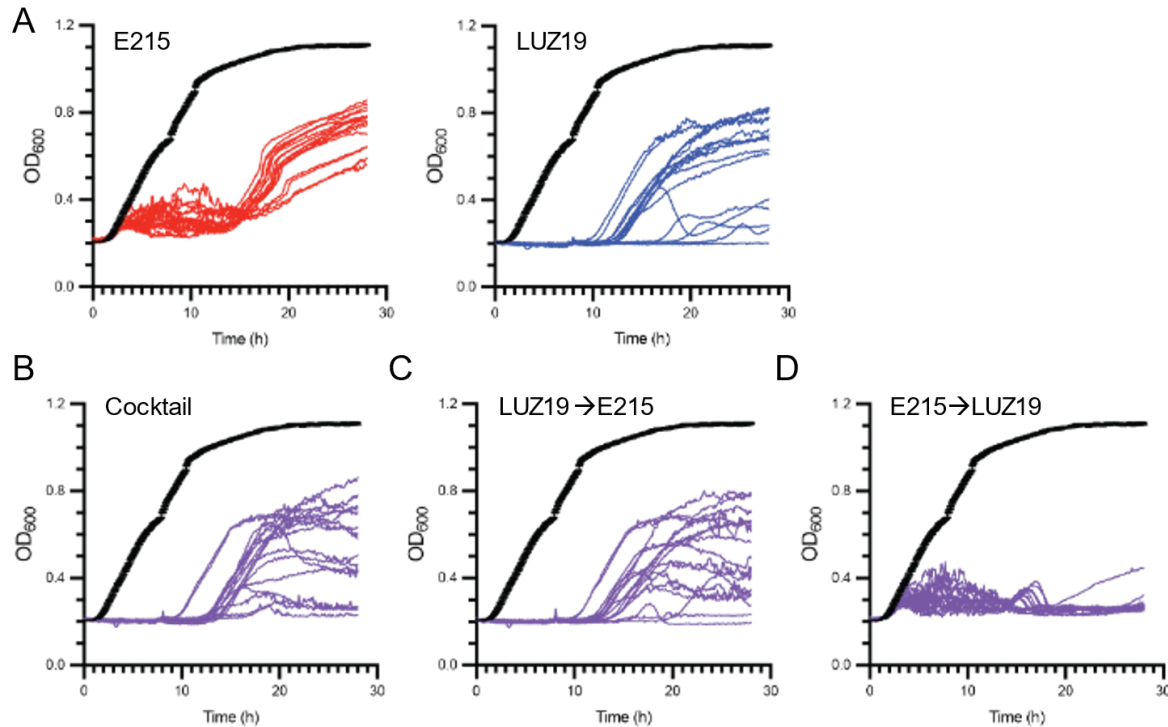


Figure 3.9 Time-kill kinetics of PAO1-phages on non-host PAK strain. Single phage time-kill kinetics of MOI 1 (A) E215 and (B) LUZ19 on PAK. Combination phage performance as cocktail, sequential E215 (T0) to LUZ19 (T8) treatment, and sequential LUZ19 (T0) to E215 (T8) treatment.

When cultured in cocktail however, the majority of E215+LUZ19 treatments 76.5% (13/17) allowed resistance escape, unlike only 8% in PAO1 (Figure 3.9B vs 3.2A). With this decrease in cocktail efficacy, sequential treatments were expected to worsen as well. Treatment patterns of LUZ19 followed by E215 appeared no different compared to cocktail (Figure 3.9C). On the other hand, reversed treatment of E215 followed by LUZ19 improved kinetics compared to cocktail both in suppression timing and frequency of resistant escape (Figure 3.9D). There was a similar delay in phage activity of the second phage added as on PAO1, where bacterial growth appeared to briefly grow exponentially before being reduced to the limit of detection (Figure 3.9D vs 3.8C). All but one well then remained clear for 28h (Figure 3.9D). Reduction to the limit of detection implies either that

the phages were more potent against the PAK strain or that the fitness cost of resistance against the phages was higher compared to PAO1.

Regardless, the combination of E215 and LUZ19 appears to potentially reduce *P. aeruginosa* growth in the absence of competition for host cells despite seemingly reduced potency on alternate strains. This suggests not only a broad applicability of the cocktail, but that co-administration method and order may play roles depending on specific isolate susceptibility and/or fitness. Further screening on patient isolates and non-host strains will be necessary to determine these effects.

3.8: HFIM recapitulates cocktail competition patterns

Although phage competition limits the initial potency of phages in well-mixed *in vitro* culture, a more dynamic environment would be required to decipher the more complex phage kinetics that occur *in vivo*. The Hollow Fiber Infection Model (HFIM) is the gold standard for quantifying small molecule antimicrobial pharmacokinetics (PK) and pharmacodynamics (PD) and resistance [301, 302]. The HFIM was adapted for this study based on several key features of the model: 1) continuous fluid-flow exchange of phage and media, 2) heterogenous distribution of bacteria and phages, 3) quantification of bacterial growth dynamics, and 4) protracted experimental length and timepoint sampling. The HFIM consists of a media-filled cartridge with multiple fine fibers porous to phage, but not to bacteria (Figure 2.1). Consequently, this allows phages to be injected in the HFIM *after* bacteria has been established in the extracapillary space (ECS) of the cartridge. In contrast to well-mixed micro-well cultures, infusion of phages through selective fibers allows phages to diffuse into an area rich with bacteria without disruption of the ECS.

Similar to its performance in the time-kill assay, single treatment of phage LUZ19 was effective at rapidly reducing sensitive bacterial burden within the HFIM (Figure 3.10A). Bacterial counts increased by 1.5-log during the first 6 h of treatment (compared to 2-log increase untreated) before being reduced from 10^7 to 10^3 CFU/mL by 8 h. Sampling at 12 h revealed that bacterial counts rebounded to 10^9 CFU/mL, suggesting that bacteria within the HFIM acquired resistance to LUZ19. Phage counts corresponded to the changes in bacterial density, with titers increased by 4-logs after only 2 h following treatment, up to 10^9 PFU/mL, and continued to increase until it plateaued around 12 h at 10^{11} PFU/mL. This is consistent with the large decrease in bacterial count at 6 h and subsequent bacterial regrowth at 12 h, where it appears phage no longer replicated efficiently within the HFIM. Both phage and bacterial counts reached a plateau of around 10^{10} to 10^{11} CFU or PFU/mL after 36 h with final phage titers surpassing bacterial counts. The central reservoir, through which fresh phages and media are exchanged and waste is filtered out, consistently contained 10^5 to 10^7 PFU/mL of LUZ19 suggesting efficient phage replication occurred to exceed the expected steady state concentration (C_{ss}) infusion of 10^5 PFU/mL (Figure 3.10A).

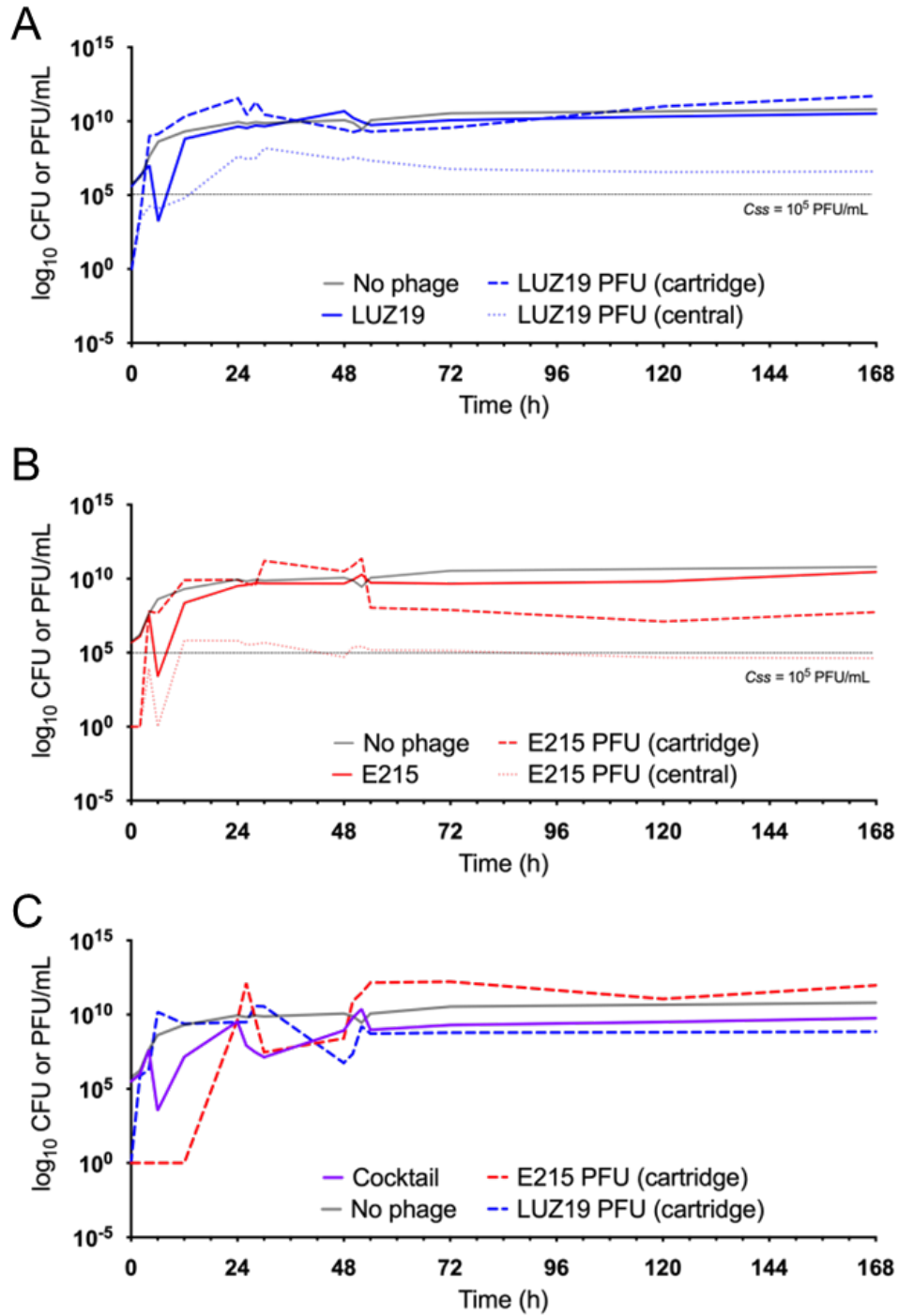


Figure 3.10 HFIM time-kill kinetics. Phage and PAO1 kinetics in HFIM central cartridge for (A) LUZ19 treatment at MOI 0.1, continuous infusion of 10^5 PFU/mL, (B) E215 treatment at MOI 0.1, continuous infusion of 10^5 PFU/mL, and (C) cocktail E215 and LUZ19 treatment at MOI 0.1, continuous infusion of 10^5 PFU/mL.

Single E215 treatment was similarly effective in HFIM and allowed bacterial counts to increase by 2-logs (from 10^5 to 10^7 CFU/mL) 2 h after phage treatment before effectively reducing cells to 10^3 CFU/mL (Figure 3.10B). Again, sampling at 12 h showed that bacterial counts rebounded to 10^8 CFU/mL and peaked at 10^{10} CFU/mL after 24 h. As to be expected, phage titers did not immediately increase at the first 2 h timepoint (given its longer latent period compared to LUZ19), but dramatically increased to 10^8 PFU/mL at the 4 h timepoint. Phage titer increased to a peak of 10^{11} PFU/mL 30 h after phage treatment. Although the titer remained high for a further 22 h (up to the 52 h timepoint) the counts decreased by 3-logs at 54 h, decreasing lower than bacterial counts.

In contrast to LUZ19, the central reservoir contained fluctuating counts of E215. E215 was only briefly detected in the central reservoir after 6 h, despite phage counts increasing in the cartridge, before shortly dropping below the limit of detection. At 12 h phage counts rebounded to 10^6 PFU/mL and stayed at C_{ss} of 10^5 PFU/mL shortly thereafter. Thus, although E215 was able to replicate within the cartridge, it did not appear to replicate as efficiently or in excess of the C_{ss} as LUZ19 did (Figure 3.10B vs 3.10A). Altogether, single phage treatment in the HFIM was comparable to our observations from time-kill curves. The effect of phage replication on bacterial counts was evident six hours after treatment when they sharply declined while phage titers increased. Unlike microwell time-kill curves where resistance to LUZ19 occurred sooner, bacterial resistance to each phage strain seemed to emerge at similar amplitudes between 8-12 h for both phage strains. In the HFIM, LUZ19 seemed to stay at stable high counts compared to E215 whose titer dropped several logs at 54 h.

Next, bacterial colonies were plated to assess the performance of cocktail treatment within the HFIM (Figure 3.10C). Bacterial counts from combination treatment increased 2-logs to 10^7 CFU/mL from 0-4 h, similar to single phage treatment, before dropping 10^3 CFU/mL at 8 h. Unexpectedly, instead of stronger or prolonged suppression of bacterial growth, bacterial counts rebounded to 10^7 CFU/mL after only 12 h of phage treatment, and up to 10^9 CFU/mL before a second drop in bacterial titer occurred at 30 h. However, a second surge in bacterial growth occurred between 48-52 h where bacterial counts increased to 10^{10} CFU/mL. Surprisingly a third decline in bacterial count occurred at 54 h before the bacterial population plateaued and remained at 10^9 CFU/mL, only 1-log lower than single phage treatments.

To examine the dynamics of the individual phage strains, phage-resistant strains to each cocktail phage (sub-cultured from single HFIM experiments) were used to determine the titers of the mixed supernatant from the cartridge. Between 0-8 h, LUZ19 phage titers increased to 10^{10} PFU/mL before plateauing until 30 h. Phage titers decreased between 30-48 h from 10^{10} PFU/mL to 10^7 PFU/mL before increasing from 48-52 h back to 10^9 PFU/mL. On the other hand, phage E215 did not appear to replicate efficiently for the first 12 h following addition. One day (24 h) after combination phage treatment was initiated, E215 counts increased to 10^9 PFU/mL and further to 10^{12} PFU/mL after 26 h. Strangely, E215 titers declined from 26-48 h before increasing back to 10^{12} PFU/mL at 52 h and plateauing afterwards. Within the central reservoir, LUZ19 titers reached and maintained 10^5 PFU/mL from 2-24 h before increasing and plateauing at 10^7 PFU/mL, suggesting similar replication efficiency as seen in single LUZ19 treatment. In contrast, although E215 titers still fluctuated for the first 8 h, phage titers

approached 10^5 PFU/mL by 12 h and increased 2-logs at 24 h. Shortly after, E215 titers were maintained at a much higher titer than during single treatment and stayed at 10^8 PFU/mL, exceeding that of even LUZ19 (Figure 3.10C).

Based on the phage and bacterial counts, the HFIM suggests that 1) double-phage resistance occurs even in a continuous flow system, 2) double-phage resistance appears stepwise, and 3) resistance likely occurs first to the pili binding phage (LUZ19) before the LPS binding phage (E215). Correlating the bacterial counts to the changes in phage titer, this suggests that the first reduction in bacterial count largely to the activity of phage LUZ19 and the second bacterial reduction to phage E215. Although both phages should be capable of replication at the initiation of dual-phage combination treatment, LUZ19 replicated much more efficiently than E215. This suggests that LUZ19 dominates the early bacteriolytic dynamics within the HFIM despite there being an equal number of each phage strain present. That E215 only begins replicating after LUZ19 titers plateau suggests that E215 is predominantly replicating on the LUZ19-resistant bacterial population present at 12 h and no longer competing with LUZ19 over phage-sensitive ancestral bacteria. This appears consistent with our observations in microwell time-kill assays wherein cocktail potencies seem to preferentially favor LUZ19 (Figure 3.6B). This also resembles dynamics in microwell sequential potencies where application of LUZ19 followed by E215 more closely resembles cocktail dynamics, and whereby resistance to type IV pili appears more fixed than *wzy* (Figure 3.8D, Appendix, Table S3.9).

It is unclear whether this is due to a difference in phage potency, the fitness cost of phage resistance, or to ease with which *pil* genes can be mutated compared to *wzy* (12 genes vs. 1 gene, Appendix, Table S3.7). Finally, although this was not previously

observed in the time-kill curves, the third smaller reduction in bacterial counts could hypothetically be due to co-evolution between PAO1 and LUZ19 since phage counts increased by 2-logs following bacterial reduction (Figure 3.10C). After 48 h, there were no apparent changes in phage or bacterial titers in either the cartridge or central reservoir, suggesting that bacteria are largely phage-resistant either to single or both phages (Figure 3.10C).

3.9: HFIM metagenome captures true microbial abundances

Although a large number of samples and isolates could be harvested from HFIM timepoints, it was difficult to ascertain whether bacterial cells remained phage sensitive or resistant during intermediate timepoints (before 8 h regrowth) using experimental methods. Developing a method to extract and quantify bacterial and phage abundances bioinformatically could potentially in one step quantify abundances of both sample types. For sample extraction, the traditional phenol-chloroform method was used based on its relative ease, speed, and compatibility with both bacterial and viral samples [303].

Three samples were analyzed after DNA extraction and short-read Illumina sequencing which showed only a $\pm 2\%$ difference between taxonomically classified reads and plated counts of either PAO1, E215, and LUZ19 (Figure 3.11A vs 3.11B). It appeared that viral abundances were only slightly underestimated by the bioinformatic analyses which captured largely bacterial reads (>90% reads mapped) (Figure 3.11B). Although we have yet to find bioinformatic markers to delineate between phage sensitive and insensitive bacterial variants, this method appears to bridge the gap between high sample

count HFIM analysis and capturing subtle changes in bacterial populations, and perhaps even of phage co-evolution, for PK/PD analysis.

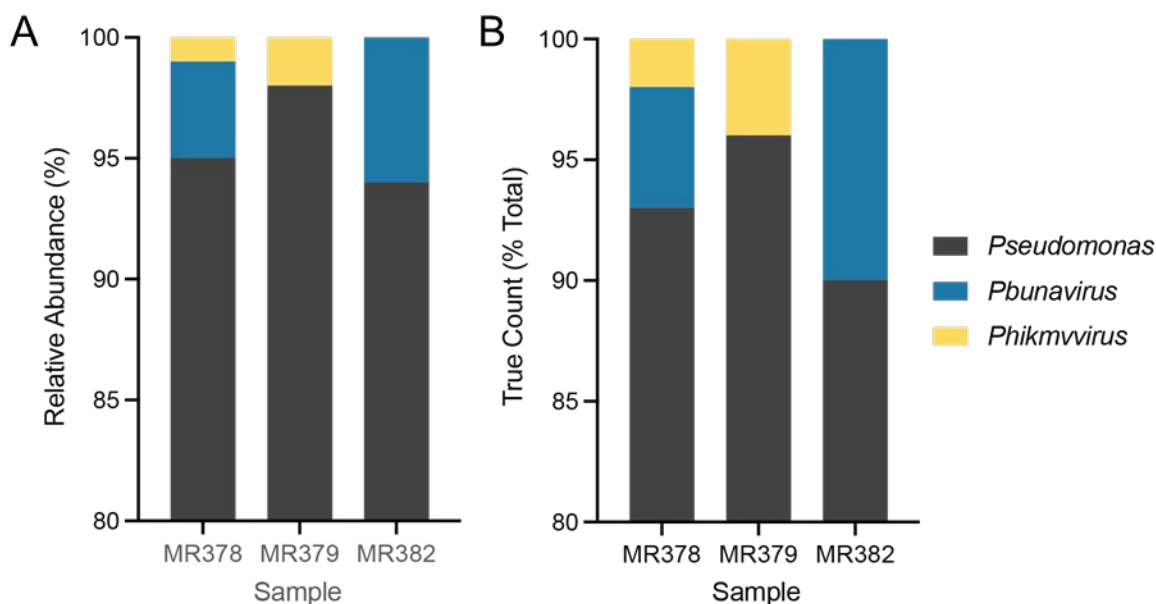


Figure 3.11 Metagenome analyses successfully recapitulates plated counts of bacterial and viral abundances. (A) Bioinformatic classification and comparison of bacteria (*Pseudomonas*, black) and phage (*Pseudovirus* E215, blue and *Phikmvvirus* LUZ19, yellow) abundances from samples extracted at 120h. DNA reads captured the (B) *in vitro* abundances of the samples counted from the HFIM cartridge at 120h. True counts below 2×10^6 PFU/mL were not classified by Kraken2 as containing >1% of total reads.

DISCUSSION

In this study, we evaluated the interactions between two phages on a single bacterial host and the efficacy of phage combinations in the context of short-term phage proliferation and long-term treatment sustainability. Understanding the competition kinetics and dynamics between phage strains will allow us to identify biomarkers for improving phage therapy cocktail design. Our work strives to understand the gladiatorial stage of phage competition: whereby phages must compete not only for the finite

resource of host cells, but also be restricted in their ability to bind to select host strains and types.

With over 100 years of experimental phage therapy, we were surprised to find that only a small subset of anti-*Pseudomonas* phages has been used therapeutically. Nonetheless, the six genera we identified appear to be promising therapeutic candidates, or failing that, could be used as exclusion references to screen for novel phage types. Although only two representative phage strains *Pbunavirus* E215 and *Phikmvvirus* LUZ19 were extensively studied, we found that individual phage activities or potencies were secondary to receptor mixing in cocktail, but that individual phage characteristics such as shorter latent periods could be leveraged to improve already efficient cocktails. For instance, the ability to substitute E215 with *Litunavirus* PYO2 suggests that phage cocktail formulations should be focused on harnessing the individual early lytic activities of phages to improve long-term resistance prevention.

Despite their seeming simplicity, time-kill kinetics were highly manipulable for concise comparison of phage bacterial killing and for comparison of single versus combination phage activities at early time points. Although the initial potency of the cocktail can be manipulated to enhance lysis kinetics, we found that activity between two phages did not seem to exceed the maximum potency of the ‘strongest’ phage. Moreover, contrary to the often perceived ‘synergism’ of phages in cocktail, we found instead that phage competition for susceptible hosts limited the potency of two-phage cocktails at administration. When hosts became abundant, this drawback of phage potency was lost but the frequency of mutational escape was found to increase. This would suggest that

both high phage potencies and abundance are necessary at phage administration to optimize phage activities in cocktail.

When we attempted to reduce competition between phage species, we found that sequential treatments worsened phage activity for the secondary phage in each scenario tested irrespective of receptor. Together, this suggests that cocktail phage formulation requires not only the selection of phages with unique receptors but also of phages with similar lytic activities on host to maximize both potency and efficacy. Comparison of these dynamics on an alternative strain PAK however suggests that sequential dynamics may be beneficial in the right context, although the mechanism of this interaction remains unknown.

Combined, examination of bacterial and phage counts in the HFIM suggests that combination phage therapy at MOI 1 had short-lived and limited success. While microwells time-kills remained clear for upwards of 20 h, bacterial resistance was detectable as early as 12 h (to a single phage) in HFIM and to both phages at 48 h. We estimated that bacteria populations in the HFIM were well over 10^7 CFU/mL after a mere 12 h. Thus, it is clear that combinatory treatment appeared more successful during microwell time-kills than in the HFIM. We hypothesize that this is due to a difference in the spatial distribution of phage and bacterial subpopulations in each of these different culture scenarios. Future work will require microbial or metagenomic analysis of HFIM populations contrasted to microwell populations to decipher the differences in phage activities, resistance emergence, competition between bacterial populations, and competition between phage strains in context of continuous flow circulation (e.g., in human blood). Although we were able to assess the relative potency of two different LPS

phages in this work, another complement to this work would be to examine an alternate pili-binding phage, whether more or less potent than LUZ19, to assess its relative contribution in cocktail.

ACKNOWLEDGMENTS

Chapter 3 is currently being prepared for submission for publication of the material. Authors: Tiffany Luong, Lindsey Camara, Nicholas Smith, and Dwayne Roach. The dissertation author was the primary researcher and first author of this material.

Author Contributions Chapter 3

Conceptualization, TL and DR; methodology, TL, NS, and DR; validation, TL, LC, and NS; investigation, TL, LC, and NS; formal analysis, TL, LC, NS, and DR; resources, NS and DR; data curation, TL, LC, and NS; writing—original draft, TL and DR; writing—review & editing, TL and DR; visualization, TL; supervision, DR; funding acquisition, NS and DR.

CHAPTER 4

CLINICAL PHAGE CULTIVATION AND PURIFICATION

INTRODUCTION

Production and purification of clinically safe phages is bottlenecking access to phage therapy. Phages must be produced in a culture-dependent manner. When bacterial cells are lysed by phages, their cell membranes rupture and endotoxins are released into the solution. Endotoxins in solution range from molecular weights of 10 to 1,000 kilodaltons (kDa) [304]. However, since phages are in the same molecular weight range as endotoxins, centrifugation and filtration cannot be used to adequately separate the endotoxins from the target phages. A harmonized standard for recommended maximum endotoxin exposure is 5.0 EU per kg of body weight (interpreted as within 1 hour) for most drugs based on an average patient weight of 70 kg [122].

The bottleneck for producing phages for human administration therefore is separating phage particles from bacterial cell debris. Gross bacterial impurities could trigger potentially life-threatening inflammation and septic shock in humans [124]. Endotoxins are of primary concern and the FDA limits human intravenous drugs to $<5 \text{ EU} \cdot \text{kg}^{-1} \text{ h}^{-1}$ [122]. However, endotoxin removal without sacrificing phage concentration remains difficult at-scale for compassionate use. For example, phages prepared under prescription as “magistral preparations” for human treatment in Belgium [125] and for expanded access eINDs by Chan et al [126] were reported to yield $\sim 10^7 \text{ PFU} \cdot \text{ml}^{-1}$ at $>10 \text{ EU} \cdot \text{ml}^{-1}$. Standardized and streamlined methods of high-quality phage

preparations are necessary to improve studies of phage safety and increase access for compassionate use and phased clinical trials.

Here, we hypothesize that phage purification can be improved by introducing cross-flow filtration (CFF) to phage production. Following traditional overnight cultivation and sterile filtration, phage lysates can be concentrated, and phages will be 'washed' with PBS using a 100kDa CFF membrane. During CFF, any material smaller than the cross-flow membrane pore of 100 kDa passes through the membrane, while larger suspended phage particles are retained. Therefore, this process both filters spent media solutes (e.g., yeast, tryptone, salts) out of the phage solution, while simultaneously reducing the liquid volume of the preparation by ~100-fold, improving downstream handling of the preparation. A membrane pore size of 100 kDa provides an equivalent to a spherical particle with a 3 nm diameter and is therefore sufficient to retain all known phages [305].

Moreover, we further hypothesize that reducing the initial bioburden of phage resistant cells during phage amplification can allow us to simplify phage purification and reduce the time needed for clinical phage preparations. Processing of the phage lysate prior to resistant bacterial growth by harvesting the lysate or reducing growth temperatures will limit the number of resistant bacterial cells present in the final phage lysate. Combined with the concentration of phages ~100-fold, this suggests that further time-consuming downstream purification can be eliminated, e.g., cesium chloride density ultracentrifugation and/or organic solvent extraction. Optimization of conventional chromatographic endotoxin removal kits are therefore sufficient for residual endotoxin removal. Clinical phage purification can therefore take as few as 48 hours to complete and with the use of only conventional microbiological equipment.

MATERIALS AND METHODS

Phage stability assay

PAK_P1 (GCA_000891855.1) phages were produced according to the method described in detail in Chapter 4.1.1. Purified phages were produced for final storage in phosphate buffered saline (PBS). The final stock solution of the phages was 0.2 μ m sterile-filtered into a 50-mL plastic conical. Further sterile dilutions into glass or plastic Eppendorf tubes were performed in a biosafety cabinet using Gibco Dulbecco's PBS (dPBS) without calcium or magnesium (14190144) to produce 1mL diluted stocks. At indicated timepoints, 2 μ L of phages were removed and quantified on lawns of *P. aeruginosa* PAK (LR657304) by double aliquot 48-spot serial titration. Briefly, bacteria culture lawned over agar was air-dried. In a 96-well microplate, two identical stock samples were taken and each serially diluted at least 8 times before spotting 4 μ L in triplicate on the dried bacterial lawn. Titrations were incubated at 37°C until colony forming units (CFU) or plaque forming units (PFU) were visible.

RESULTS

4.1.1: Cross-flow filtration improves the concentration and purification of phages (published protocol)

This section is a reprint of a published protocol entitled: *Standardized Bacteriophage Purification for Personalized Phage Therapy*. Printed in *Nature Protocols*, September 2020. Written by Tiffany Luong, Ann-Charlott Salabarria, Robert A Edwards, and Dwayne R Roach. This protocol details a 14 to 21 day procedure to produce phages using a combination of modified classical techniques, cross-flow filtration (CFF)

processes, and no organic solvents to produce phages against Gram-negative pathogens. The protocol described aims to standardize how phages are produced in order to bridge the need for clinically-safe phages where phages produced under good manufacturing practices (GMP) are not yet available for emergency IND cases.

Abstract

The world is on the cusp of a post-antibiotic era but researchers and medical doctors have found a way forward – by looking back at how infections were treated before the advent of antibiotics, namely using phage therapy. Although bacteriophages (phages) continue to lack drug approval in Western medicine, an increasing number of patients are being treated on an expanded access emergency investigational new drug (eIND) basis. In order to streamline the production of high-quality and clinically safe phage preparations, we developed a systematic procedure for medicinal phage isolation, liter-scale cultivation, concentration and purification. The 14-21 day procedure described in this Protocol utilizes a combination of modified classical techniques, modern membrane filtration processes, and no organic solvents, to yield on average 23 mL of 10^{11} PFU·mL⁻¹ for *Pseudomonas*, *Klebsiella* and *Serratia* phages tested. Thus, a single production run can produce up to 64,000 treatment doses at 10^9 PFU, a commonly prescribed IV dose, which would be sufficient for most expanded access phage therapy and potentially for clinical phase I/II applications. The protocol focuses on removing endotoxins early by conducting multiple low-speed centrifugations, microfiltration and cross-flow ultrafiltration, which reduced endotoxins 10^6 -fold in phage preparations. Implementation of a standardized phage cultivation and purification across research laboratories participating

in phage production for expanded access phage therapy may be pivotal to re-introduce phage therapy to Western medicine.

Introduction

Academic and military research institutions are being called to immediate action to produce bacteriophages —viruses that kill bacteria— for the treatment of antibiotic resistant infections prior to drug approval [153, 306-308]. Sometimes called compassionate use, expanded access provides a patient with an immediately life-threatening condition or serious disease rapid access to an investigational new drug (IND) when no satisfactory alternative therapy options are available [309]. While demand for bacteriophages (phages for short) is increasing in several fields including human and veterinary drugs, biological products, food supply, and cosmetics [262, 308, 310], only a limited number of phage products are produced under the regulations covering good manufacturing practices (GMP) [119, 120]. However, an argument can be made that an effective, consistent, and controllable process for phage production, which also meets safety and efficacy demands for human and animal clinical use, has yet to be achieved [121, 311]. That is, medicinal phage products have generally been of low purity and titer [306, 308, 312-314].

Deaths from antibiotic drug resistant bacteria are at historic highs according to the Center for Disease Control (CDC) and World Health Organization (WHO) [315, 316]. In the United States more than 2.8 million multi-drug resistant (MDR) infections occur each year, and more than 35,000 people die as a result [315]. As the usefulness of antibiotics is waning, there is an urgent need to develop new ways to treat infections or MDR will be the leading cause of human death worldwide by 2050 [316]. Now, researchers and

medical doctors have found a way forward – by looking back at how infections were treated before the advent of antibiotics [153, 306]. Phage therapy started with its first clinical application in 1919 [317] and has seen continuous improvement to this day. Although phages continue to lack drug approval in Western medicine, an increasing number of patients have been treated on an emergency IND (eIND) basis under U.S. Food and Drug Administration (FDA) or the European Medicines Agency approval [153, 306-308, 314]. With IND clinical approvals taking an average of eight years from entry into clinical trials, patients experiencing antibiotic failure and their family members are themselves advocating for expanded access phage therapy. Thus, as the antibiotic resistance crisis continues to rise globally, expanded access phage therapy is bridging the gap between current antibiotic failures and future clinical trials.

As a type of virus, phages can only reproduce within a bacterial cell [318, 319]. Therefore, a major hurdle to creating medicinal phages is the separation of phages from bacterial cell debris like endotoxin (i.e. lipopolysaccharide or LPS), peptidoglycan, exotoxins, flagella, nucleic acids and other compounds. If not adequately removed, these gross impurities could trigger potentially life-threatening inflammation, sepsis and septic shock in humans [27, 124, 320]. To date, the major safety concern with phage products for human use has been endotoxin content [306, 311, 314]. Adequately removing endotoxins from phage products for human eIND phage therapy has been problematic, often requiring significant product dilution, which concurrently reduces the active agent of phages [306-308]. In addition, GMP pharmaceutical phage products have reported endotoxin quantity that would not be actionable for low dose or topical applications and could be indicative of adverse effects if administered in certain routes of administration,

like intravenously (IV); a common route of phage administration recommended by the Center for Innovative Phage Applications and Therapeutics (IPATH) [153, 306, 308]. Moreover, phage production methods have largely disregarded other unknown gross impurities that may risk human health. Indeed, phage preparations can be safe in animal models [12, 82, 321, 322]. However, experimental phage therapy studies generally cultivate phages with laboratory-adapted reference bacterial strains. For expanded access eIND use, large quantities of a pathogen — often a clinical MDR bacterial isolate — are required to produce a high number of phages.

Several ad-hoc laboratory approaches are currently employed for phage cultivation and purification for human use, which have been largely developed for small experimental studies [24, 127, 128, 153, 226, 306-308, 314, 318, 323-326]. However, several pitfalls have materialized, including low phage recovery, high gross impurities, inadequate endotoxin removal, and addition of toxic chemicals (see, Comparison with other practiced protocols). Large-scale GMP pharmaceutical production of large phage libraries will likely be needed to meet the demands for personalized phage therapy. However, this approach is currently not time and cost-effective, thus creating an unmet need for systematic small batch phage production to excel current efforts for expanded access phage therapy.

In this protocol, we outline a systematic, comprehensive, and practicable procedure for phage isolation, selection, liter-scale cultivation, concentration and purification (for schematic see Figure 4.1). Our protocol utilizes aspects to demonstrate product identity, purity, and quality while protecting scientific data integrity for batch therapeutics. This production process utilizes typical microbiology laboratory equipment

and no organic solvents. The method yields sufficient phages for most expanded access eIND and potentially for clinical phase I/II applications. We show phage preparations obtained using this protocol have high phage purity measured by *Limulus* amoebocyte lysate (LAL) chromogenic assays, protein analysis, and cell viability analyses (see Anticipated Results). In our hands, all executed runs of the procedure were successful, vouching reproducibility of this Protocol.

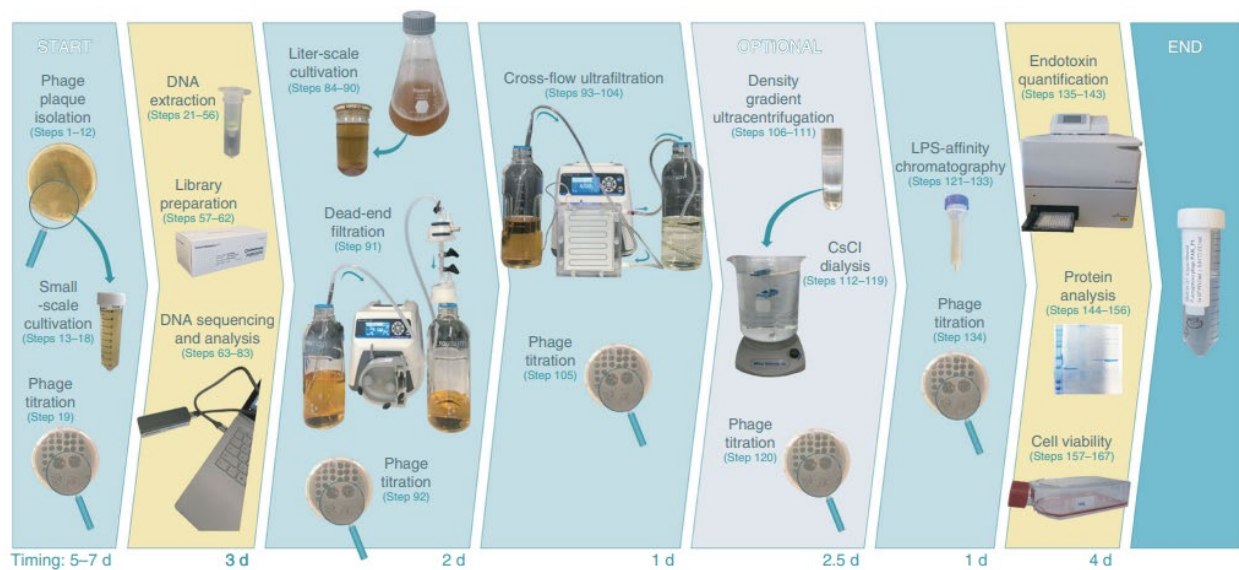


Figure 4.1 Overview of bacteriophage cultivation and purification. The procedure starts with sourcing and isolating phages with a target bacterial strain. After multiple rounds of agar plaque isolation, a single plaque is small-scale cultivated overnight. Next, the newly isolated phage genome is sequenced, annotated and screened for lysogenic and harmful genes. Phages deemed potentially safe for human use are then liter-scale cultivated. After overnight culturing, phage lysate is sterilized by pressure-driven double dead-end filtration and cross-flow ultrafiltration (see Figure 4.2 for filtration scheme). Cross-flow ultrafiltration also diafiltrates to remove growth medium and concentrates phage particles in buffer. As an option, CsCl density gradient ultracentrifugation and dialysis can be used to further confirm phage stock homogeneity. LPS-affinity chromatography is used to remove residual endotoxins. Lastly, the final phage preparation purity and safety is tested by quantifying endotoxin level, protein abundance and cell viability after phage sample exposure.

Application of purified phage preparations

In recent years, the demand for small quantities of phages for expanded access eIND and clinical phase I/II trials has been growing. Small aliquots of phages are useful for animal experiments and pilot studies in which high-titer and high-purity are required. For this purpose, we developed a liter-scale phage production process. The production method is a scaled-up version of previously described preparation processes [12, 82] and is able to deliver high-titer phages (10^9 - 10^{12} plaque forming units [PFU]) with low endotoxin (4.3-24.1 units) per milliliter (Table 4.1). We are currently in the process of using final patient formulations in expanded access eIND phage therapy. Our production method has three major advantages: 1) It does not require specialized production equipment, like a bioreactor or chromatography system, 2) By using a cross flow filtration (CFF) method to concentrate phages (Steps 93-105) and exchange growth medium, no organic solvents are required, 3) Filtration materials are disposable, avoiding any cleaning validation, which saves additional time and costs. Therefore, this protocol provides a standard-of-production for medicinal phages, allowing expanded access phage therapy, to reach more of those in need, starting by increasing the global production of safe personalized phage products.

Table 4.1 Bacteriophage purities from completed protocol.

Table 1 Bacteriophage product final titer (Step 134), endotoxin level (Step 143) and estimated number of doses produced						
Phage strain ^a	Bacterial host	PFUs (ml ⁻¹) ^b	Endotoxin units (ml ⁻¹) ^c	Final volume (ml)	Estimated doses at 10 ⁹ PFUs	EU 10 ⁹ PFUs ⁻¹
PAK_P1	<i>P. aeruginosa</i>	5.78 × 10 ¹⁰	4.49	24	1,387	0.0173
PAK_P5	<i>P. aeruginosa</i>	4.00 × 10 ¹¹	4.30	30	12,000	0.00250
E217	<i>P. aeruginosa</i>	1.89 × 10 ¹¹	5.26	21	3,970	0.00529
PYO2	<i>P. aeruginosa</i>	4.00 × 10 ¹²	5.05	16	64,000	0.000250
JG265	<i>K. oxytoca</i>	1.30 × 10 ¹⁰	24.10	28	364	0.07
JG266	<i>K. oxytoca</i>	2.00 × 10 ⁹	17.30	29	58	0.05
SM219	<i>S. marcescens</i>	2.90 × 10 ¹⁰	18.85	19	552	0.0345

^aGenBank accession nos.: PAK_P1, KC862297.1; PAK_P5, KC862301.1; E217, MF490240; PYO2, MF490236. ^bPFUs determined in Step 20B. ^cEUs determined by the Pierce LAL Chromogenic Endotoxin Quantitation Kit, Steps 135–143.

Recently, phages have been shown to play important roles in health through unexpected host immune interactions [12, 230, 320, 327]. However, immunological response studies have used varying degrees of purified phages, ranging from sterile filtered lysates to low endotoxin preparations in saline buffer [12, 328, 329]. Although, there is no evidence of direct toxicity induced by phage particles, study of mammalian cell-phage interaction requires phage preparations to be free of bacterial cells, toxins and other compounds to avoid skewing host responses. Due to the high-titer and purity of phage production, it is suitable for cell culture and animal studies.

We have used this Protocol for the production of four *Pseudomonas* phages PAK_P1, PAK_P5, E217 and PYO2, two *Klebsiella* phages JG265 and JG266, and a *Serratia* phage SM219 (Table 4.1 and Appendix, Table S4.1). Although the selection criteria of therapeutic phages is still under investigation [12, 120, 262], and not covered

here, the presented production method can be generally used for other Gram-negative phage and final formulation designs.

Comparison with other practiced protocols

This phage production protocol offers several distinct advantages over currently practiced methods conducted at laboratory scales. Table 4.1 shows that a six-liter production run can produce between 6.0×10^{10} - 6.4×10^{13} phage virions in total, providing between 58 to 64,000 doses at 10^9 PFU; a commonly prescribed intravenous treatment dose [126, 153, 306]. In addition, endotoxin measured by LAL chromogenic analysis ranged between 0.0003-0.03 EU (endotoxin units) per 10^9 PFU dose (Table 4.1). In comparison, phages prepared under prescription as “magistral preparations” for human treatment in Belgium [125] and for expanded access eINDs by Chan et al [126] were reported to yield $\sim 10^7$ PFU·ml⁻¹ at >10 EU·ml⁻¹. Because our phage preparations are of higher titer and purity than those reported as being administered to patients for expanded access phage therapy, implementation of our protocol would conceivably allow for administration of higher phage titer per treatment dose and maintain a FDA human intravenous limit of <5 EU·kg⁻¹ h⁻¹ [330].

In contrast to the polyethylene glycol (PEG) precipitation method conducted in the majority of competing protocols (for example [120, 128]), the CFF method employed here (Steps 93-105) is a pressure-driven scalable membrane filtration process that markedly decreases labor and improves purification reproducibility (Figure 4.2). PEG precipitation methods can produce comparably high phage yields [153, 320]. However, high yields are phage strain dependent [128, 226, 326] and PEG precipitation does not effectively

remove endotoxin [320]. Therefore, PEG precipitated phages typically require substantial dilution or implementation of other endotoxin removal techniques.

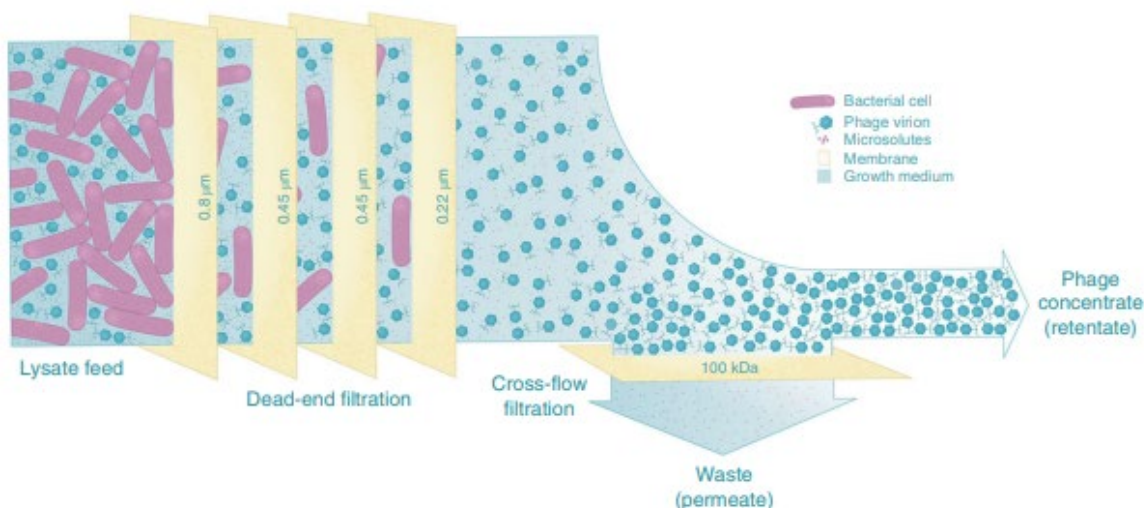


Figure 4.2 Schematic of phage lysate dead-end filtration (Step 91) and cross-flow filtration (Steps 93–104) removal of impurities. Phage lysates are sterilized by inline 0.8-, 0.45-, 0.45-, and 0.22- μ m membrane filtration to remove whole bacterial cells and cellular debris. Then, CFF is used to remove growth medium and microsolute <100 kDa (e.g., endotoxins, peptidoglycans, exotoxins, flagella and nucleic acids), while concentrating the phages in phosphate buffer.

CFF is able to reliably fractionate phages from gross bacterial impurities (e.g. <40 EU·ml⁻¹) and diafilter and concentrate phage particles 4-100 fold (Figure 4.3). Importantly, this all occurs in a cost-effective, programmed and semi-automated single-step (Figure 4.1). CFF was shown to be highly effective at concentrating phages [128]. The use of a CFF molecular weight cut-off (MWCO) of 100 kDa in combination with several sterile washes appear to be important for the significant removal of endotoxin from phage lysates because CFF using MWCO <30 kDa has been shown to concentrate endotoxin along with phages [128]. In addition, CFF with MWCO of 100 kDa would potentially remove exotoxins, which all have been found to be smaller than 100 kDa [331]. Together, the inclusion of CFF is a cost-efficient way to concentrate and purify phages. It is also scalable

with no barrier to running several apparatuses with additional hardware (not described in this protocol).

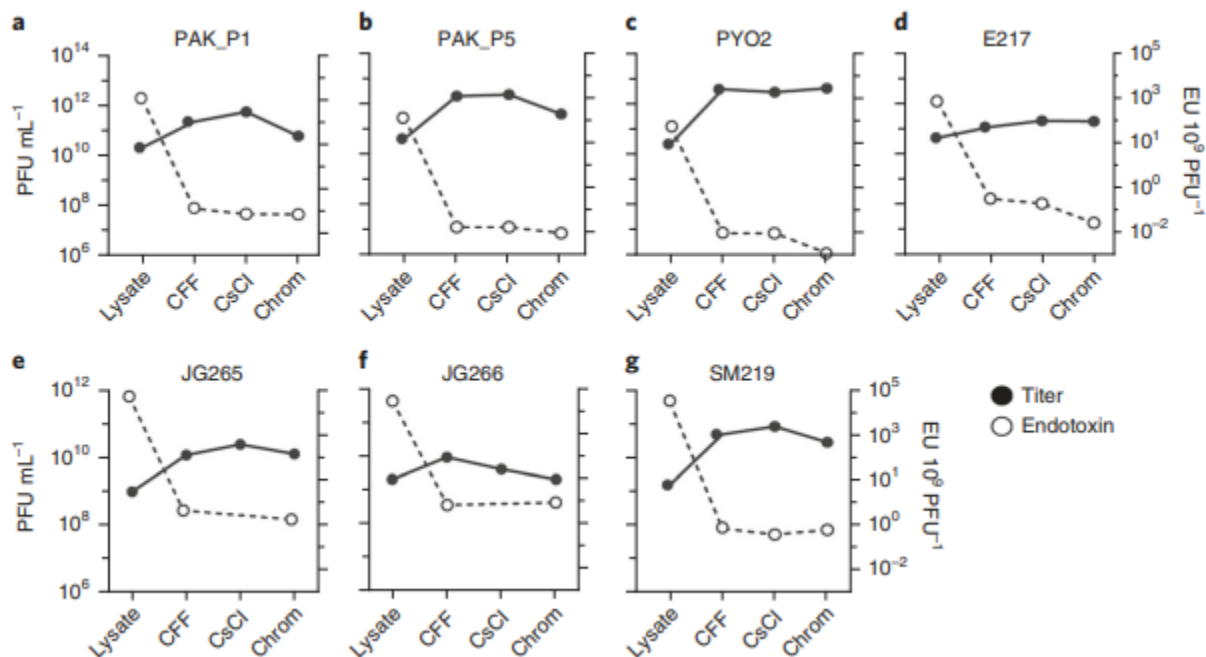


Figure 4.3 Process stepwise phage titer and endotoxin concentration throughout processing. PFUs per mL (right y axis; closed circles) and endotoxin units (EUs) normalized to 10^9 PFUs (left x axis, open circles) after phage lysate sterilization (Lysate, Step 92), CFF (Step 105), density gradient ultracentrifugation and dialysis (CsCl Step 120) and LPS-affinity chromatography (Chrom, Step 134). (A–D) *P. aeruginosa* phages PAK_P1, PAK_P5, PYO2 and E217. (E,F) *K. oxytoca* phages JG265 and JG266. (G) *S. marcescens* phage SM219. See Step 20B and Steps 121–134 for phage titration and endotoxin quantification procedures, respectively.

The downstream cesium chloride (CsCl) density gradient ultracentrifugation (Steps 106-111), dialysis (Steps 112-120), and affinity chromatography (Steps 123-134) processes may not be required to meet FDA drug product endotoxin safety limits [330]. Without including these steps, our approach yields ~30 mL of between 2.7×10^{11} to 1.1×10^{14} phage virions in total per six-liter batch post CFF (Appendix, Table S4.1). This provides an estimated 270-110,000 doses at 10^9 PFU with <0.22 EU; the allowable endotoxin exposure for a 70 kg person would be 350 EU h^{-1} . Omission of lengthy and

costly density gradient ultracentrifugation, dialysis, and affinity chromatography steps would reduce phage preparation time to approximately five days. However, if time permits, CsCl density gradient ultracentrifugation, dialysis, and affinity chromatography offer further separation of potential gross impurities and provide a strategy to avoid unwanted phage contaminants [121, 226]. Density gradient ultracentrifugation can offer visual confirmation that preparations are homogeneous containing a single phage strain because unwanted contaminating phages appear as a second band due to their differing density (Figure 4.1).

Our protocol avoids the use of chloroform, denaturing solvents and detergents. Competing protocols often recommend using chloroform to disrupt the bacterial cell wall to release internal phage particles in lysates or as standard procedure to remove PEG after precipitating phages [332]. There are risks with adding chloroform in the purification process such as denaturing certain phages [318, 324]. Long-term exposure to chloroform by inhalation in humans has resulted in hepatitis, jaundice, and central nervous system effects and it has been shown to be carcinogenic in animals after oral exposure, resulting in kidney and liver tumors [333]. In our experience, chloroform also increases the amount of bacterial cell debris by lysing phage resistant cells—which harbor no phage particles—that can emerge with extended bacterial culturing. Organic solvents and detergents can be an effective method to reduce endotoxins in phage lysates [127, 131, 226]. However, they can be toxic to users, inhibit chromogenic-based endotoxin quantification methods and decrease phage stability in storage [226].

Lastly, we use Oxford Nanopore MinION sequencing over other sequencing technologies due to several added benefits. The MinION offers rapid generation of

sequence information, with the reads being generated and available for study within minutes after initiation of the run. Importantly, the latest generation of the MinION sequencer (cat. no. SQK-RAD004), in our recent experience with phage genomics, allows a few reads to cover the whole genome. Furthermore, the assembly of the sequences generated are nearly identical to the Illumina assembled sequences, in our case by only a single base. Finally, the Oxford Nanopore sequencing is significantly cheaper than other sequencing technologies, with a Flongle generating over 1 GB of sequence data, more than sufficient to accurately assemble a phage genome.

Limitations of this Protocol

We have used the production method described in this protocol for the cultivation and purification of phages that infect Gram-negative bacteria including *Pseudomonas aeruginosa*, *Klebsiella oxytoca* and *Serratia marcescens* grown aerobically at 37 °C. Pathogenic strains of *Pseudomonas aeruginosa* and *Klebsiella*, along with Gram-negative *Escherichia coli* and *Acinetobacter*, according to the CDC, are becoming increasingly resistant to most available antibiotics [315]. Therefore, the case studies presented here are relevant for current expanded access eIND phage therapy global needs. Notwithstanding the individual culture conditions for each bacterium-phage pair, there should be minor optimization needed to use this protocol for the production of a variety of phages. For instance, larger volumes of low-titer phage lysates can be processed with CFF to obtain a sufficiently high titer sample for downstream procedures. Nonetheless, further validation of this protocol with other phages such as those that infect Gram-positives or anaerobes is needed.

Another drawback, as with most purification techniques, is the loss of phages with an increased number of manipulation techniques. For instance, we found phage yield decreases significantly after LPS-affinity chromatography, which appear to be phage strain dependent. For example, *Pseudomonas* phage PAK_P1 and PAK_P5 use LPS as its bacterial cell wall binding site. We found PAK_P1 and PAK_P5 titers each decreased from 5.19×10^{11} to 5.78×10^{10} and 2.30×10^{12} to 4.00×10^{11} PFU·mL⁻¹, respectively (Appendix, Table S4.1). In contrast, the titers pre- and post-affinity chromatography remained stable for the other phages tested. We propose that the losses for some phage strains are dependent on how strongly phages associate with the remaining LPS in samples prior to affinity chromatography.

There might be a health risk of using CsCl. Our protocol includes phages retrieved in 1500 mg·mL⁻¹ CsCl, followed by 1:1000 dialysis against phosphate buffer saline (PBS) and high-titer aliquots diluted in sterile saline to the required therapeutic phage dose. This suggests that treatments would contain trace amounts of cesium (<80 ng·mL⁻¹). Although, radioactive cesium can cause seizure, syncope, hypokalemia, and chronic diarrhea [334], non-radioactive cesium is readily found in the human body, mostly derived from consumption of plant and animal products [335].

The scalability of the presented phage production process is unclear. The principles of our protocol are compatible with other existing bioprocess methods used in the manufacture of biotherapeutics. For example, cultivation of phages in bioreactors would offer scalability over the use of shake-flasks [336], while still being compatible with presented downstream filtration steps that are easily scalable with larger filters.

Centrifugation steps however can have scalability limitations and affinity chromatography is not ideal for high throughput processes.

EXPERIMENTAL DESIGN

The Protocol outlines a combination of classical and modern viral cultivation and purification techniques, which are experimentally validated for Gram-negative phages (See 'Anticipated Results' below). The protocol design is modular and adaptable, allowing each laboratory to decide how to best implement the necessary controls, by using scientifically sound design, and testing procedures to achieve higher quality through continual improvement. Implementing this formal system will ensure the identity, titer, quality, and purity of human and animal phage products and prevent instances of contamination, mix-ups, deviations, and errors.

Plaque assay phage isolation

Plaque assays are routinely used to discover new phage strains from a wide range of sources such as sewage, bodies of water, liquefied soil, and bodily fluids. We use a modified agarose overlay technique, described previously [318], where a filtered natural sample is serially diluted and poured over a lawn of exponentially growing compatible bacteria (Steps 1-12). As the bacterial lawn grows, small zones of lysis become visible. Each PFU is derived from an initial infection with a single virion followed by phage-induced lysis of neighboring cells [318]. PFUs displaying different morphologies are selected to inoculate small-scale liquid cultivation on the compatible bacteria. A critical factor in the isolation of highly functional phages is the preparation of a pure solution of

identical phages. Therefore, the PFU selection process is repeated several times until all plaques exhibit a similar morphology, suggesting a single phage strain has been isolated.

Phage titration

Agar Petri plating techniques are routinely used to quantify phage particle numbers in preparations. One option is to use the agarose overlay technique as previously described [318], which consists of mixing serially diluted phage samples with susceptible bacteria in molten agar and pouring over solid agar in a Petri plate (Step 20 Option A). A more rapid option is to perform several tenfold serial dilutions of a phage stock in a microplate and spot these dilutions on a bacterial seeded agar Petri plate (Step 20 Option B; Figure 4.1). Conventional plaque titering can also be substituted or supplemented by the use of real-time quantitative PCR (qPCR) if primers for the phage strain are available [337, 338].

Annotation and bioinformatic analysis of phage genomes

The primary goals of genome sequencing and analysis (Steps 57-83) are different when the phages are required for human therapeutic applications than when they are used for research purposes. For phages that will be used for expanded access phage therapy, the primary goal of sequencing and annotation is to ensure that no harm is likely to be introduced by the phage preparation, and the primary concern is the transfer of genetic material between bacteria by transduction. Prophages directly influence the microbes where they reside: they often express genes that are beneficial to the bacteria (called lysogenic conversion factors) [339]. There are three elements that are the primary concern for transfer: 1) toxins, 2) antibiotic resistance genes, and 3) virulence factors.

The toxins associated with cholera [340], diphtheria [341], scarlet fever [342], shigella [343-345], and botulinum [346] are all encoded by phages integrated into their host bacterial genome [347, 348]. Were one of those toxins to be transferred by a phage used for expanded access phage therapy, the expression of the toxin may lead to increased morbidity and mortality [345]. Similarly, the introduction of antibiotic resistance genes either already on the phage genomes [349] or transferred between bacterial hosts by specialized or generalized transduction [350] may render traditional antibiotic therapies ineffective. Although the precise role of phages in the spread of antibiotic resistance genes between bacteria is somewhat controversial [351], it has been demonstrated previously [352, 353]. Moreover, the relative ease of antibiotic resistance gene(s) identification warrants analysis. In addition to antibiotic resistance genes and toxins, phages are also implicated in the horizontal transfer of virulence genes [354].

Computational analysis of toxins, antibiotic resistance, and bacterial virulence factors present their own challenges. Novel toxins and mechanisms of virulence are continually being discovered [355]. This limits understanding of the mechanisms of disease and our ability to predict the detrimental effect of horizontal gene transfer. The identification of genes that provide direct antibiotic resistance mechanisms is straightforward, however the identification of resistant alleles of housekeeping genes is more complex. For example, it is trivial to identify a β -lactamase encoding gene, but associating specific point mutations with resistance is more computationally challenging without thousands of genomes [356]. Multiple databases can be used to identify toxins, antibiotic resistance alleles, and virulence factors. For example, *Abricate* (<https://github.com/tseemann/abrigate>) uses ensemble methods to compare sequences

to the most up-to-date databases including the Comprehensive Antibiotic Resistance Database (CARD) [357], ResFinder [358], NCBI's AMRFinder [359], Antibiotic Resistance Gene-ANNOtation (ARG-ANNOT) [360], the virulence factor database [361], and PlasmidFinder [362].

In expanded access phage therapy, care must be taken to avoid temperate phages that are able to lysogenize their bacterial host. While there are genetic approaches to inactivate the lysogenic lifecycle [153], it is preferable to begin with phages that are unable to lysogenize their host. Phage genome sequencing allows rapid confirmation of whether a phage is likely to be temperate or virulent. Whole-genome sequencing may also provide clues to other biological contaminants in the preparation, depending on the abundance of the contaminants relative to the phage. However, in each of these considerations, phage genome sequencing will only identify known features. Computational identification of a toxin, antibiotic resistance gene, or virulence factor should not infer that these elements are not present, rather than known elements are not present.

The Oxford Nanopore MinION sequencer is the most convenient “long-read” sequencer currently on the market, and sequencing phages with this device often results in a single read representing the entire phage [363]. After sequence assembly, the genomes are explored for antibiotic resistance genes and virulence factors (e.g. with the aforementioned Abricate) and by annotating with PATRIC [364]. PHACTS is used to determine whether the phages are likely to be virulent or temperate [179]. Phages without genes of concern and that are predicted to be virulent (i.e. strictly lytic lifecycle) are selected for downstream liter-scale cultivation.

Finally, the approach described in this protocol is appropriate for the rapid analysis of phage genomes for expanded access therapy. As we focus on those characteristics of the phage however, further techniques should be applied to the phage and its genome for full characterization [180, 365, 366].

Liter-scaled shake flask cultivation

Test tube and smaller shake flask cultivation in complex medium on a rotary shaker are convenient for initial phage cultivation for bacterial susceptibility testing and DNA isolation. Large shake batch cultures in complex medium on a rotary shaker are required for downstream process sterilization of cultivated phages (Figure 4.1). Phage enrichment by shaking flask cultivation can be scaled to produce six liters per batch, limited here by centrifuge rotor capacity. Seeding cultures with a multiplicity of infection (MOI) of 0.1 can produce phage lysates with about 10^9 to 10^{10} PFU·mL⁻¹. Although, mid-log stage of bacterial growth is the most amenable to infection by most phages [367], basic parameters for bacteria-phage interaction should be optimized. For example, selected complex medium and temperature for the bacterium are factors that influence the infectivity of phages and burst size.

Membrane applications in phage production

We use two flow configurations for membrane processes, namely dead-end microfiltration (Steps 91-92) and cross-flow ultrafiltration (Steps 93-105; illustrated in Figure 4.2). Dead-end microfiltration is used to retain both bacterial cells and particulates that are collected on the membrane surface forming a filtration cake. This layer then makes additional filtration effects, improving the separation efficiency. Dead-

end filtration is usually a batch-type process, with extensive membrane fouling being its main disadvantage.

We employ several methods to minimize membrane fouling while filtering phage lysates. First, we avoid the use of chloroform to sterilize lysates to help reduce bacterial debris that would otherwise be derived from the final lysis of phage-resistant bacterial cells, which typically develop with prolonged culturing. Moreover, in our experience, chloroform lysis does not significantly improve phage yield and it can harm certain phages [324]. Second, we subject phage lysates to two rounds of centrifugation with a sterile bottle change in between. Third, we use a pleated cartridge filter design, which provides a higher surface area. Lastly, two pleated cartridge filters are used for two-step membrane pore sizing.

Next, we use the VivaflowTM CFF cassette as an efficient way to ultrafilter lysates with a high concentration of phages (Steps 93-105). CFF works by introducing dead-end filtered lysate under pressure across the membrane surface, instead of directly onto the micro-filter. During filtration, any material smaller than the cross-flow membrane pore of 100 kDa passes through the membrane, while larger suspended phage particles remain in the retentate stream. A membrane pore size of 100 kDa provides an equivalent to a spherical particle with a 3 nm diameter and is therefore sufficient to retain all known phages [305]. We employ diafiltration with freshly autoclaved buffer to increase the purity and improve the separation of phages from bacterial debris and the complex growth medium. Simultaneously, permeate is withdrawn at the same rate that the buffer is added, to flush out microsolute from the phage solution. For instance, CFF with a 100 kDa membrane pore size can remove both endotoxins, which are approximately 10 kDa in

size, and all known exotoxins, which are typically smaller than 30 kDa [331]. Thus, CFF is used to “wash” the phage particles while concentrating up to 2 L by a factor of 30.

Upscaling the purification process using density gradient ultracentrifugation

Density gradient ultracentrifugation developed by Brakke [368] is a common technique used to isolate and purify a wide range of phages purely on the basis of their density [24, 226]. We perform a self-forming density gradient isopycnic ultracentrifugal separation technique making use of differences in density between gross impurities and viruses of a CFF phage preparation (Steps 106-111; Figure 4.2). In self-forming gradients, the phage solution is layered on top of the gradient medium, centrifuged, and as the solute molecules sediment to form the gradient identical phages band at their isopycnic points. Following ultracentrifugation, virus bands may be visualized as a result of their light scattering. We employ this technique for accomplishing both removal of other unwanted phage strains and gross impurities. That is, different phages will be separated by their different densities during ultracentrifugation [121]. In addition, large endotoxin aggregates will remain near the top of the gradient, while microsolutes will pass to the bottom of the gradient.

We then use dialysis to facilitate the removal of small, unwanted CsCl from phage particles in solution by selective and passive diffusion through a semi-permeable membrane (Steps 112-120; Figure 4.2). Phage particles that are larger than the 100 kDa membrane-pores are retained, but small molecules and buffer salts pass freely through the membrane, reducing the concentration of those molecules in the phage preparation. Dialysis also accomplishes both removal of bacterial small molecules (e.g. 2.5 to

7000 kDa LPS molecules) and storage buffer exchange for phage products but dilutes the phage preparation.

Chromatographic removal of endotoxins

Endotoxin removal is critical when producing therapeutic phages in bacterial systems. We further remove this hydrophobic molecule through commercial purification affinity chromatography (Steps 121-134), which is likely the most reliable and widely applied method used to remove endotoxin. We then use the *Limulus* amoebocyte lysate (LAL) assay (Steps 135-143) because of its sensitivity, reproducibility, and simplicity for endotoxin detection; which uses the blood coagulation system of the horseshoe crab, and clots upon exposure to endotoxin [369].

Phage preparation protein analysis

Bacterial protein contamination is an immunogenic component of phage preparations. We use semi-quantitative sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and Coomassie staining to visualize potential bacterial protein contamination in the final phage preparations (Steps 144-156; Figure 4.4A). For example, the presence of protein smears across a variety of sizes indicates high bacterial protein contaminants as seen in the *Klebsiella* phage JG265 lysate in Figure 4.4. Phage structural proteins will dominate clean preparations.

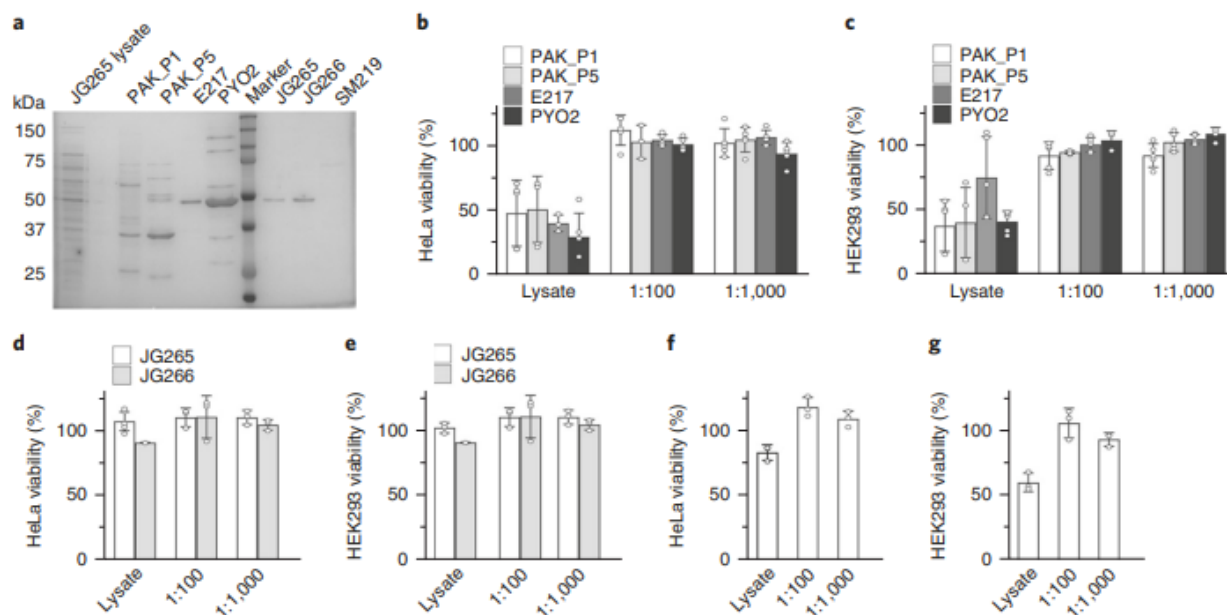


Figure 4.4 Purity and safety analyses of final phage preparations. (A) SDS–PAGE analysis of protein content in final phage samples (Step 133) at 10^9 PFUs compared to an exemplified sterilized phage lysate (produced by Steps 84–92) and Precision Plus Protein ladder (Bio-Rad). (B–G) Human HeLa cell line (B,D,F) and HEK293 cell line (C,E,G) viability after 24 h of co-incubation with final preparations of *P. aeruginosa* phages (B,C), *K. oxytoca* phages (D,E) and *S. marcescens* phages (F,G) at cell:phages ratios of 1:100 and 1:1,000 quantified by the CellTiter-Glo assay (Steps 157–167). Viability was normalized to untreated cells. *Pseudomonas* and *Serratia* sterilized phage lysates were significantly lower than corresponding final phage preparations ($P < 0.006$), whereas there was no significant difference in cell viability between *Klebsiella* sterilized lysate and phage treated. One-way ANOVA; error bars represent s.e.m.; $n = 3–6$.

Phage preparation effects on human cell viability

Phages cannot infect human cells. However, phages can indirectly influence mammalian cell activities [230, 327, 370]. In addition, bacterial contaminants in phage preparations may elicit cell inflammatory responses [329, 371]. Cell-based assays are often used for screening medicinal products to determine if the test molecules have effects on cell proliferation or show direct cytotoxic effects that eventually lead to cell death [372]. In this protocol, we describe a method to test the effects of phage preparations on human cells that is based on a multi-well format where adenosine triphosphate (ATP) production is recorded using a plate reader (Steps 157–167). ATP has

been widely accepted as a valid marker of viable cells [372]. When cells lose membrane integrity, they lose the ability to synthesize ATP and endogenous ATPases rapidly deplete any remaining ATP from the cytoplasm. The measurement of ATP using luciferase is the most commonly applied method for estimating the number of viable cells in high throughput screening applications. The ATP assay has a high sensitivity and is less prone to artifacts. The ATP detection reagent contains a cell lysing detergent, ATPase inhibitors to stabilize released ATP, luciferin as a substrate, and luciferase to catalyze the reaction that generates luminescence [372].

MATERIALS

Biological materials

Strains

- PAK_P1 Phage (GenBank accession no. KC862297.1 | RRID:NCBITaxon_743813)
- PAK_P5 Phage (GenBank accession no. KC862301.1 | RRID:NCBITaxon_1327964)
- PYO2 Phage (GenBank accession no. MF490236 | RRID:NCBITaxon_2034342)
- E217_A65 Phage (GenBank accession no. MF490240 | RRID:NCBITaxon_2034346)
- SM219 Phage (this study, available from the corresponding author upon request)
- JG265 Phage (this study, available from the corresponding author upon request)
- JG266 Phage (this study, available from the corresponding author upon request)
- *P. aeruginosa* strain PAO1 (ATCC no. 47085 | RRID:NCBITaxon_208964)
- *P. aeruginosa* strain PAK [373] (RRID: NCBITaxon_1009714)
- *S. marcescens* strain (this study, available from the corresponding author upon request)
- *K. oxytoca* strain (this study, available from the corresponding author upon request)

Cell lines

CAUTION: The cell lines used in your research should be regularly checked to ensure they are authentic and not infected with mycoplasma.

- HeLa Cells (ATCC no. CRM-CCL-2 | RRID:CVCL_0030)
- HEK293 Cells (ATCC no. CRL-157 | RRID:CVCL_0045)

Reagents

- Tryptone (Fisher, cat. no. BP1421-2)
- Yeast Extract (Fisher, cat. no. BP1422-2)
- Sodium chloride (NaCl; Fisher, cat. no. 7647-14-5)
- Agar (CulGenes, cat. no. C6002)
- Phosphate buffer saline (BioPioneer, cat. no. MB1001)
- Tris base (Fisher, cat. no. 77-86-1)
- Glycine (Millipore Sigma, cat. no. G4392)
- SDS (sodium dodecyl sulfate; Fisher, cat. no. BP166-500)
- Ethanol, Absolute (Millipore Sigma, cat. no. E7023-500ml)
- Nanobind CBB Big DNA Kit (Circulomics, cat. no. NB-900-001-01)
- DNase I (Life Technologies, cat. no. 90083)
- RNase A (Life Technologies, cat. no. EN0531)
- PEG-8000 (Fisher Scientific, cat. no. BP233-100)
- Magnesium Sulfate Heptahydrate (MgSO₄; Fisher Scientific cat. no. M63-500)
- Acetic acid (Fisher Scientific, cat. no. A38S 500)

Equipment

- Biosafety cabinet (ESCO, cat. no. AC2-4S9-NS)
- Microtiter plate reader (BMG, model: Clariostar, cat no. 0430-100)
- Vortex mixer (VWR, cat. no. 12620-838)
- Spectrophotometer (Denovix, model. no. DS-11 FX+)
- High-speed Centrifuge (Beckman Coulter, model: Avanti JXN-26 IVD, cat. no. B38623)
- Fixed-angle centrifuge rotor for 1 L (Beckman Coulter, model: JLA-8.1000, cat. no. 969328)
- Ultracentrifuge (Beckmann Coulter, Optima-L-90K, cat. no. 365672)
- SW41 rotor (Beckman Coulter, cat. no. 331336)
- Microcentrifuge (Sartorius, cat. no. A-14C)
- Mini-PROTEAN™ Tetra Vertical Electrophoresis System (Bio-Rad, cat. no. 1658028FC)
- Gel imager ChemiDoc™ XRS+ System (Bio-Rad, cat. no. 1708265)
- Thermal cycler (Eppendorf, cat. no. 6311000010)
- Bunsen burner (VWR, model no. 89038-530)
- Pipette (1000, 200, 20, and 10 µl; Sartorius, cat. no. UX-24505-33, -34,-35,-36, respectively)
- Multichannel pipette (8x 10 µl; Sartorius, cat. no. UX-24505-44)
- Electronic pipette controller (Argos, model: OmegaZen, cat. No. 25300-96)
- 10 L water bath (Cole Parmer, cat no. WE-14576-08)
- Peristaltic pump (Cole Parmer, model: Masterflex, cat. no. EW-07522-20)

- Pump head (Cole Parmer, cat. No. EW-77253-00)
- Tubing (Thermo Scientific, cat. no. 8060-3015; Cole-Parmer, cat. no. ZX-06422-01)
- Microbiological incubator/shaker (Eppendorf, model: S44i, cat. no. S44I200005)
- Cell incubator (Eppendorf, model: C170i, cat. no. 6731010015)
- Incubator (VWR, model: 5420, cat. no. 97005-252)
- Thermomixer (Eppendorf, cat. no. 2231000574)
- Stir plate (Heathrow, cat. no. HP8885794)
- Hemocytometer (Hauser Scientific, cat no. 3110V)
- Water Purification System (Millipore, cat. no. ZRXQ010T0)
- Magnetic Tube Rack (ThermoFisher, model: DynaMag-2, cat. no. 12321D)
- DNA sequencer (Oxford Nanopore, model: MinION, cat. no. SQK-RAD004)
- Linux or Apple Macintosh computer

Consumables

CRITICAL: Autoclave glassware and tubing to degrade phage particles before use.

- 10 cm Petri plate (Fisher Scientific, cat. no. 08-757-100D)
- 1.7 mL microcentrifuge tubes (Sorenson, cat. no. 11500)
- 2.0 mL microcentrifuge tubes (Sorenson, cat. no. 12030)
- Glass test tubes (VWR, cat. no. 10545-922)
- Glass 250 mL Erlenmeyer flasks with GL45 screw-cap (Kimble-Chase, cat. no. 26720-250)
- Glass 2 L Erlenmeyer flasks with GL-45 screw-cap (Kimble-Chase, cat. no 26720-2000)
- GL45 0.22 μm PTFE membrane vented cap (Corning cat. no. 1395-45LTMC)

- Pipette tips (10 – 1000 μ L; Sartorius, cat. no. 37001-162, 53503-294, 83007-372)
- Pasteur pipette (VWR, cat. no. 16001-182)
- 1 L centrifuge bottles (Beckman Coulter, cat. no. A98812)
- 0.8/0.45 μ m and 0.45/0.22 μ m capsule filters (Sartorius, model: Sartopore 2, cat. no. 5441306G5, 5441307H5)
- 100kDa Cross-flow ultrafiltration cassette (Sartorius, model: Vivaflow 50R, cat. no. VF20P4)
- 100,000 MWCO PES spin tubes (Sartorius, model: Vivaspin 500, cat. no. VS0141)
- Ultraclear Tubes (14x89mm) (Beckman Coulter, cat. no. 344059)
- Luer-Lok syringe (5 ml; BD, cat. no. 309628)
- 0.45 and 0.22 μ m syringe filters (Sartorius, cat. no. 16537, 16541)
- 0.22 μ m GL45 filter caps (Sartorius, cat. no. 16541-k)
- 26 G needle (BD, cat. no. 305111)
- 15 and 50 mL conical centrifuge tubes (VWR, cat. no. 430790 and 430828)
- 2 L glass beaker (VWR, cat. no. 10754-760)
- Dialysis membrane tubing, 100 kDa MWCO (Spectra/Por, cat. no. 131408)
- 96-well clear microplate (Corning, cat no. 35177)
- 96-well white microplate (Greiner bio-one, cat no. 655088)
- PCR eight-tube strips and caps (Sarstedt, cat. no. 772.991.1002)
- T75 culture flask (Corning, cat. no. 430639)
- MinION flow cell (Oxford Nanopore, cat. no. R9.4.1)
- Kimtech wipes (Fisher Scientific, cat. no. 06-666)
- Gloves (Xceed cat no. XC-310-S, M, L)

Software

- MinKNOW MinION Release 19.12.5 <https://community.nanoporetech.com/downloads> (registration required)
- *canu* v2.0 <https://github.com/marbl/canu>
- *abricate* v1.0 <https://github.com/tseemann/abricate>
- PHACTS website <https://edwards.sdsu.edu/PHACTS/>
- PATRIC website <https://patricbrc.org>

Reagent set-up

CRITICAL: Prepare all solutions with sterile double distilled water (ddH₂O) and store at room temperature (RT; 20 to 25 °C) for up to 3 months, unless otherwise indicated.

Liquid Growth Medium Mix 10 g tryptone, 5 g yeast extract, and 5 g NaCl in 1 L of ultrapure ddH₂O. Autoclave.

Solid Growth Medium Add 14 g of agar to growth medium and autoclave. Then, cool to 55 °C in a water bath for 30 min to 1 h. Sterilely pour solid growth medium into sterile Petri plates. Thickness should be approximately 5-10 mm thick. Let cool at room temperature and store at 4 °C for up to 2 weeks.

Molten soft-agar Mix 7 g of agar to growth medium and autoclave. Then, cool to 55 °C in a water bath to prevent agar solidification before plating. Make fresh.

Tris-sodium chloride (TN) buffer Mix 10 mM Tris (pH 7.0) and 150mM NaCl. Adjust to pH 7.0. Autoclave and store at 4 °C for up to 3 months.

Tris-EDTA (TE) Buffer Mix 10mM Tris (pH 7.0) and 1 mM EDTA (pH 8.0). Adjust to pH 7.5.

10x SDS-PAGE Running Buffer Mix 30.0g of Tris base, 144.0g of glycine, and 10.0g of sodium dodecyl sulfate (SDS) in 1 L of ddH₂O. Make fresh.

Cesium Chloride (CsCl) Solutions Mix 41.2 g (d=1.6), 34.13g (d=1.5) or 20.49g (d=1.3) in 50 mL of TN buffer. Sterile filter.

Nuclease solution Mix 20 mg·mL⁻¹ DNase I and 20 mg·mL⁻¹ RNase A. Store at -20 °C for up to 30 months.

Precipitant solution (30% w/v PEG-8000 and 3 M NaCl). In a sterile bottle add 110 mL ddH₂O and 35 g NaCl, dissolve completely. Add 60 g PEG-8000, cap bottle and shake. Incubate bottle in a 50 – 60 °C water bath for about 2 h, shaking occasionally (at this point, it is normal for the solution to be turbid and separated into two phases). Remove and let cool to RT, shaking occasionally. The solution should be clear or slightly turbid. Dilute to 200 mL with ddH₂O.

Resuspension buffer (5 mM MgSO₄) Add 0.0123 g of MgSO₄ heptahydrate per 10 mL ddH₂O.

Sourcing of Phages We outline sourcing phages without enrichment cultures by plating environmental samples directly onto an isolation host and sample from formed plaques (i.e. zones of lysis). For rarer phage sourcing, an enrichment step may be required. For details see [374].

CRITICAL. Solid source material like soil or feces should be suspended in buffer or liquid growth medium to generate an aqueous sample that can be plated directly on an isolation host.

PROCEDURE

Phage plaque isolation TIMING 3-5 d, depending on repetitions needed to obtain a single plaque morphology for each isolated phage.

CAUTION. Phage isolation (Steps 1-12) should be completed in a Class II Biosafety cabinet.

- 1| To isolate phage strains *de novo*, centrifuge aqueous environmental viral sample at 8,000 ×g for 30 min at 4 °C to remove bulk debris. **CRITICAL STEP.** Volume of aqueous viral sample will be dependent on separation of liquid from solid debris after centrifugation.
- 2| Decant the supernatant into a clean tube without disturbing debris pellet.
- 3| 0.2 µm syringe filter the environmental viral sample.
- 4| In a sterile test tube, mix 100 µL of bacterial host at OD₆₀₀ 0.2 and 100 µL of the filtered viral sample.
- 5| Add 3 mL of molten soft-agar, mix gently, and pour over solidified agar Petri plate. **CRITICAL STEP.** If the bacterial lawn is clear after incubation, dilute the environmental sample to obtain single plaques.
- 6| Incubate under appropriate temperature and atmospheric growth conditions for 12-18 h or until plaques form on a confluent lawn of bacteria.
- 7| Using a Pasteur pipette, select one PFU and re-suspend in 100 µL of PBS in a microcentrifuge tube.
- 8| Prepare serial dilutions (e.g. tenfold) in PBS.
- 9| In sterile test tubes, mix 10 µL of the 10⁻⁵, 10⁻⁶ or 10⁻⁷ dilutions with 500 µL of bacteria grown to OD₆₀₀ 0.2.

10| Add 3 mL of molten soft-agar to each, mix gently, and pour over solidified agar Petri plate.

11| Incubate under appropriate temperature and atmospheric growth conditions for 12-18 h or until plaques are visible on a confluent lawn of bacteria.

12| Repeat Steps 7 through 11. **CRITICAL STEP.** Repeat Step 12 three times, or until all PFUs exhibit the same observed plaque morphology.

Small-scale cultivation TIMING 15-21 h

13| Using a Pasteur pipette, select one PFU and re-suspend in 100 μ L of PBS in a microcentrifuge tube.

14| Warm 50 mL of growth medium in a sterile 250 mL GL45 screw-top flask with a GL45 0.22 μ m PTFE membrane vented cap.

15| Add 500 μ L of bacteria grown to OD₆₀₀ 0.2 and incubate at appropriate temperature and atmospheric growth conditions for 20 min.

16| Add 50 μ L phage obtained in Step 13 and incubate under appropriate bacterial growth conditions for 12-18 h.

17| To remove bulk bacterial debris, transfer lysates to 50 mL conical centrifuge tubes and centrifuge at 6,000 $\times$ g for 30 min at 4 °C.

18| Filter sterilize the supernatant with a 0.2 μ m syringe filter into a sterile 50 mL conical tube. **CRITICAL STEP.** Do not disturb the pellet during decanting.

19| Titer the sample as described in Step 20.

PAUSE POINT. Store phage lysate at 4 °C for up to 12 months.

Phage titration

20| For routine phage titering, we describe two techniques (see Experimental Design – Phage titration): Follow ‘Option A’ for agar overlay titering and ‘Option B’ for spot plaque titering. Alternatively, phages can be quantified via their genome copy number by qPCR (For details see [337, 338]).

OPTION A| Agar overlay titering TIMING 20 h

- i. Add 100 μL of phage sample into a microcentrifuge tube containing 900 μL of PBS, mix well.
- ii. Pipette 100 μL from Step i dilution into a 2nd a microcentrifuge tube containing 900 μL of PBS, mix well.
- iii. Repeat step ii for the remaining microcentrifuge tubes to create a dilution series of 10^{-1} to 10^{-8} .
- iv. In sterile test tubes, mix 100 μL of each serial dilution with 100 μL of bacteria grown to OD_{600} 0.2.
- v. Add 3 mL of molten soft-agar, mix gently, and pour the mixture over a solidified agar Petri plate.
- vi. Incubate under appropriate temperature and atmospheric growth conditions for 12-18 h or until plaques form on a confluent lawn of bacteria.
- vii. Determine phage stock concentration as $\text{PFU} \cdot \text{mL}^{-1}$.

OPTION B| Spot plaque titering TIMING 19 h

- i. Dry a growth agar Petri plate for 30 min in a biosafety cabinet.
- ii. To seed a lawn of bacteria, pour 3 mL of bacteria grown to OD_{600} 0.2 onto the dry growth agar Petri plate and quickly remove excess culture.

- iii. Dry the Petri plate in a biosafety cabinet for 15 min.
- iv. While drying, add 90 μL of PBS to 8 wells row-by-row in a 96-well microtiter plate.
- v. Add 10 μL of phage sample to the first well, mix well.
- vi. Pipette 10 μL from the 1st well into the 2nd well, mix well.
- vii. Repeat step vi for the remaining wells to create a dilution series (10^{-1} to 10^{-8}).
- viii. Using an 8-channel pipette, spot 4 μL from each well onto a dried seeded lawn of bacteria prepared in (i-iii). **CRITICAL STEP.** Completely dry spots before moving the plate to incubator.
- ix. Incubate plate at appropriate temperature and atmospheric growth conditions for 12-18 h or until plaques form on a confluent lawn of bacteria.
- x. Determine phage stock concentration as PFU $\cdot\text{mL}^{-1}$.

DNA extraction TIMING 2 h

CRITICAL Steps 21-56 describe how to extract genomic DNA from small scale phage lysates following a modified version of a previously published protocol [375]. There are many DNA extraction kits, including the Promega Wizard, Qiagen PowerViral Kit, and Norgen Biotek. This protocol uses the Ciculomics Nanobind Tissue Big DNA Kit to generate long DNA fragments suitable for the Oxford Nanopore MinION sequencer.

- 21| Place the lysate from Step 18 into a clean 50 mL centrifuge tube. Add 0.5 μL of nuclease solution per mL of lysate ($10\text{ }\mu\text{g}\cdot\text{mL}^{-1}$ DNase & RNase final). Incubate the lysates at 37 °C for 30 min, or at RT for 2 h.
- 22| Add $\frac{1}{2}$ volume of the precipitant solution compared to the lysate (10% (wt/vol) PEG-8000, 1 M NaCl final concentration). Mix gently by inversion. Incubate on ice for at

least 60 min; precipitation works best when incubated at 4 °C overnight. Most phages are stable in this state for several days.

- 23| Centrifuge the precipitated phage lysate at 10,000 ×g, 4 °C, 10 min.
- 24| Carefully remove the supernatant by either aspiration or carefully pouring into a separate tube and retain the transparent or slightly opaque pellet in the original tube.
- 25| Gently, resuspend the pellet in 0.5 mL of 5 mM MgSO₄ and briefly (5-10 s) centrifuge at 10,000 x g at RT to pellet any remaining insoluble particles.
- 26| Transfer the supernatant to a sterile microcentrifuge tube for the remaining steps.
- 27| Before proceeding, dilute the supplied CW1 and CW2 buffers with 96%-100% ethanol, as described by the manufacturer.
- 28| Add 20 µL of proteinase K solution.
- 29| Add 20 µL of the supplied CLE3 buffer.
- 30| Pulse vortex 10 times for 1 s each.
- 31| Incubate in the ThermoMixer at 56 °C and 900 rpm for 30 min.
- 32| Add 200 µL of the supplied BL3 buffer.
- 33| Pulse vortex 10 times for 1 s each.
- 34| Incubate microcentrifuge tube in the ThermoMixer at 56 °C and 900 rpm for 30 min.
- 35| Add the Nanobind disks to the lysate.
- 36| Add 300 µL of isopropanol.
- 37| Mix microcentrifuge tube by inversion 5 times.
- 38| Mix on a rotator at 10 rpm for 10 min.
- 39| Place microcentrifuge tube in the Magnetic Tube Rack.

- 40| Carefully remove the supernatant taking care not to disturb the Nanobind disks.
- 41| Add 700 μ L of the supplied CW1 buffer.
- 42| Mix microcentrifuge tube by vigorous inversion 4 times.
- 43| Place microcentrifuge tube in the Magnetic Tube Rack
- 44| Carefully remove the supernatant taking care not to disturb the Nanobind disks.
- 45| Add 500 μ L of the supplied CW2 buffer.
- 46| Repeat Steps 42-45.
- 47| Mix by vigorous inversion 4 times.
- 48| Place microcentrifuge tube in the Magnetic Tube Rack.
- 49| Carefully remove the supernatant taking care not to disturb the Nanobind disks.
- 50| Spin the microcentrifuge tube for 5 s.
- 51| Remove any remaining liquid without disturbing the Nanobind disks.
- 52| Repeat Steps 50-51.
- 53| Add 100 μ L of the supplied elution buffer (EB) and incubate at RT for 10 min.
- 54| Transfer the DNA eluate to a new, sterile, microcentrifuge tube.
- 55| Spin the microcentrifuge tube containing the Nanobind disks for 5 s. Remove additional eluate and pipette into the same microcentrifuge tube in Step 54.
- 56| Leave at RT for ~1 h to let the DNA re-dissolve.

PAUSE POINT. Phage DNA can be stored at 4 °C for <2 weeks and -20 °C <6 months.

Sequencing library preparation TIMING 10 min

CRITICAL: Steps 57-62 use Oxford Nanopore MinION for rapid DNA sequencing of phage genomes. Thaw all components on ice, and store on ice until needed. Centrifuge all components briefly before use.

- 57| In a 0.2 mL PCR tube, mix 7.5 µL of phage DNA (>400 ng) preparation from Step 56 and 2.5 µL of fragmentation mix (solution FRA).
- 58| Mix by gently tapping the tube, and then centrifuge briefly to bring all the contents to the bottom of the tube.
- 59| Incubate the tube at 30 °C for 1 min and then at 80 °C for 1 min.
- 60| Add 1 µL of rapid adapter (solution RAP)
- 61| Mix by gently tapping the tube, and then centrifuge briefly to bring all the contents to the bottom of the tube.
- 62| Incubate the tube at RT for 5 min.

PAUSE POINT. The sequencing library can be stored briefly on ice until loaded into the DNA sequencer.

DNA sequencing TIMING 1-48 h, depending on desired reads coverage, with first reads recovered in an hour. More reads will increase confidence.

- 63| Open MinKNOW software on the computer
- 64| Plugin the MinION device and insert a flow cell (Figure 4.1)
- 65| Enter the sample ID and flow cell ID on the computer
- 66| Run the Platform QC script and confirm active pores available
- 67| Open the cover of the flow cell, and drawback a few microliters of fluid to remove bubbles, taking care not to remove the buffer from the pores.
- 68| To prepare the flow cell priming mix, add 30 µL of Flush Tether (FLT) directly to the tube of mixed flush buffer (FLB), and mix by pipetting up and down. Centrifuge briefly.
- 69| Load 800 µL of the priming mix into the flow cell via the priming port. Be careful to avoid introducing air bubbles into the flow cell. Incubate at RT for 5 min.

- 70| Mix the sequencing reaction in a new tube:
- 34 μ L Sequencing Buffer (SQB)
 - 25.5 μ L Loading Beads (LB)
 - 4.5 μ L Nuclease-free water
 - 11 μ L DNA library from Step 62
- 71| Mix by pipetting and centrifuge briefly
- 72| Access the SpotON sample port by lifting the cover.
- 73| Load 200 μ L of the priming mix into the flow cell via the priming port used in Step 69. Be careful to avoid introducing air bubbles into the flow cell.
- 74| Carefully add the 75 μ L of sequencing reaction from Step 71, in a dropwise fashion. Be careful to ensure that each drop enters the flow cell.
- 75| Carefully replace the SpotON sample port cover ensuring the port is filled by the bung, close the priming port cover and close the MinION lid.
- 76| On the MinKNOW software, click Start Run.
- 77| Upon completion of the sequencing, flow cells can be washed and retained for further use. DNA sequences will be available almost immediately and will continue to accumulate while there are active pores.

Bioinformatics analysis TIMING 12 h

- 78| Upload the fastq files from MinKNOW to a Linux server
- 79| Assemble the long reads with *canu* [376] by running the following code:
- a. `canu -p phage -d assembly genomeSize=40k -nanopore-raw phage.fastq`

The primary options are -p for the name, -d for the location to write the output, and the fastq output from MinKNOW.

80| Compare the phage genomes to antimicrobial and virulence gene databases using *Abricate* (<https://github.com/tseemann/abricate>) by running the following code:

a. for DB in argannot card megares ncbi plasmidfinder resfinder vfdb; do abricate -- threads 10 --db \$DB assembly/ done > abricate.out

81| Upload the genome to PHACTS to predict whether it is virulent or temperate virus [179].

82| Upload the genome to PATRIC (<https://patricbrc.org/>) to annotate other genes in the genome.

83| Select phages for large-scale production based on the lack of predicted antibiotic and virulence genes, and the likelihood that the isolated phage is virulent (i.e. strictly lytic lifecycle).

Liter-scale shake flask cultivation TIMING 18-24 h

CRITICAL: Large phage production batches are limited by maximum centrifuge rotor capacity. Steps 84-90 describe how to process 1 L cultures; thus 6 flask cultures are performed in parallel.

84| In a sterile 2 L GL45 screw-top flask with a GL45 0.22 μm PTFE membrane vented cap, warm 1 L of complex growth medium (Figure 4.1).

85| Add 5 mL of bacteria grown to OD₆₀₀ 0.2 and incubate appropriate temperature and atmospheric growth conditions for 20 min.

86| Add phage from Step 18 at a Multiplicity of Infection (MOI) of 0.1 and incubate under appropriate bacterial growth conditions for 12-18 h.

87| To remove bulk bacterial debris, transfer lysates to 1 L centrifuge bottles and centrifuge at 8,000 $\times g$ for 45 min at 4 °C (Figure 4.1).

88| Decant the supernatants into fresh, sterile 1 L centrifuge bottles without disturbing the bacterial pellet.

89| Centrifuge again at 8,000 ×g for 45 min at 4 °C.

90| Decant supernatant into sterile 1 L glass bottle.

Dead-end filtration TIMING 1 h

91| Assemble 0.8/0.45 µm and 0.45/0.2 µm capsule filters inline as shown in Figure 4.1. Use the peristaltic pump to filter sterilized supernatant into sterile glass bottles.

CRITICAL STEP. Capsule filters can process up to 2 L before clogging but can be cleaned and reused following manufacturer's protocol.

92| Titer the sample as described in Step 20. **CRITICAL:** The phage lysate titer should be $>10^9$ PFU·mL⁻¹.

PAUSE POINT. Phage preparation can be stored at 4 °C for <6 months.

Ultrafiltration, diafiltration, and concentration TIMING 8 h (2 h per 1.5 L)

93| Assemble the peristaltic pump and CFF cassette as shown in Figure 4.1.

94| Circulate 500 mL of sterile ddH₂O through the cassette and discard.

95| Place the intake and retentate hoses into the 0.2 µm sterile phage supernatant solution from Step 92.

96| Place the filtrate hose into a sink or waste container.

97| Recirculate (up to 1.5 L per batch) supernatant until ~200 mL remains.

98| Add 400 mL of sterile ddH₂O.

99| Recirculate supernatant until ~200 mL remains.

100| Add 400 mL of sterile TN buffer.

101| Recirculate supernatant until ~200 mL remains.

102| Repeat steps 100 and 101, until supernatant becomes clear and colorless.

103| Pause pump, place intake and retentate hoses in a sterile 50 mL conical tube and recirculate while continuously adding remaining supernatant until 40 mL of concentrate remains (1st fraction).

104| Pause pump, place intake hose with retentate hose into a sterile 50 mL conical tube with 30 mL of TN buffer. Circulate briefly and remove intake hose. Collect 30 mL of concentrate (2nd fraction). **CRITICAL STEP.** Subsequent fractions can be collected if lower concentration phage stocks are desired.

105| Titer the sample as described in Step 20. The phage concentrate should be ≥10-fold higher in titer than that in Step 92.

PAUSE POINT. Phage preparation can be stored at 4 °C for <2 weeks.

Density gradient ultrapurification TIMING 6 h

CRITICAL: Density gradient ultracentrifugation (Steps 106-111) and dialysis (Steps 112-120) may not be required to meet regulatory endotoxin safety limits of phage products [330]. Optionally, proceed to Step 135 for endotoxin quantification.

106| In an open top ultraclear (14×89 mm) round bottom tube, prepare a CsCl step density gradient by layering 2 mL of d=1.6, 3 mL of d=1.5, and 3 mL of d=1.3, from the bottom up. **CRITICAL STEP.** Avoid disturbing the previous density layer during preparation.

107| Fill the remaining tube volume with phage concentrate (~4 mL) from Step 104.

108| Place tubes inside buckets and balance on a SW41 rotor. **CRITICAL STEP.** Cool rotor to 4 °C and centrifuge with all buckets, even if unused.

- 109| Ultracentrifuge at 28,000 ×g for 4 h at 4 °C.
- 110| Carefully extract tubes from buckets using tweezers.
- 111| With a concentrated light source, extract the visible band containing the phage particles by puncturing the thin-walled ultraclear tube with a 26G needle and syringe (Figure 4.1).

CsCl concentrate dialysis TIMING 1 d

- 112| Chill up to 3 L of sterile ddH₂O (4 °C) in a large beaker placed on a stir plate.
- 113| Place a magnetic stir bar in the beaker.
- 114| Clamp one end of the 100k MWCO dialysis tubing and add the CsCl phage concentrate from Step 111.
- 115| Clamp the other end and secure the clamped dialysis tubing to a float.
- 116| Dialyze CsCl phage concentrate in pre-chilled sterile ddH₂O, stir 30 min at 4 °C.
- 117| Exchange ddH₂O with up to 3 L of pre-chilled sterile storage buffer (e.g. PBS) and dialyze for 1 h.
- 118| Repeat step 117 but prolong dialysis to 24 h.
- 119| Recover phage concentrate.
- 120| Titer the sample as described in Step 20. The phage titer should be between 10⁹–10¹² PFU·mL⁻¹.

PAUSE POINT. Phage preparation can be stored at 4 °C for <2 weeks.

LPS-affinity chromatography TIMING 2 h

CRITICAL: Steps 121-134 are specific to the Pierce™ High Capacity Endotoxin Removal Spin Column.

- 121| Equilibrate and regenerate the spin column as per the manufacturer's instructions.
- 122| Place the spin column into a collection tube and centrifuge at 500 ×g for 1 min at RT to discard the regeneration solution.
- 123| Remove the cap and insert the bottom plug. Add 8 mL of 2M NaCl, replace the cap and invert the column several times.
- 124| Loosen the cap and remove the bottom plug. Place the column in a collection tube and centrifuge at 500 ×g for 1 min at RT to discard the solution.
- 125| Remove the cap and insert the bottom plug. Add 8 mL of supplied endotoxin-free H₂O. Replace the cap and invert the column several times.
- 126| Loosen the cap and remove the bottom plug. Place the column in a collection tube and centrifuge at 500 ×g for 1 min at RT to discard the water.
- 127| Remove the cap and insert the bottom plug. Add 8 mL of endotoxin-free PBS, replace the cap and invert the column several times.
- 128| Loosen the cap and remove the bottom plug. Place the column in a collection tube and centrifuge at 500 ×g for 1 min at RT to discard the PBS.
- 129| Repeat steps 127 and 128 two additional times.
- 130| Remove the cap and insert the bottom plug. Add 10 mL dialyzed phage concentrate from Step 119 to the resin, replace the cap and invert the column several times.
- 131| Incubate the column with gentle end-over-end mixing at 4°C for 45 min.
- 132| Loosen the cap and remove the bottom plug. Place column in a sterile collection tube and centrifuge at 500 ×g for 1 min at RT to collect the sample.
- 133| Repeat steps 121-132 until all phage concentrate is processed.

134| Titer the sample as described in Step 20. The phage titer should be between 10^9 – 10^{12} PFU·mL⁻¹.

PAUSE POINT. Phage preparation can be stored at 4 °C for <6 months.

Phage preparation endotoxin quantification TIMING 2 h

CRITICAL: Steps 135-143 are specific to the Pierce™ LAL Chromogenic Endotoxin Quantitation Kit.

135| Equilibrate solutions to room temperature, as per the manufacturer recommendation.

136| Prepare LPS standards provided in the kit using “High Standards” option.

137| Add prepared standards, the blank, and samples from Step 133 to a 96 well microtiter plate. **CRITICAL STEP.** Appropriately dilute phage samples to be within the linear range of the high standard.

138| Warm plate to 37 °C and add 50 µL of LAL to each well. Tap plate lightly 10 times to mix.

139| Reconstitute the chromogenic substrate solution and warm it at 37 °C, 5 min before use. **CRITICAL STEP.** Work quickly to prevent inactivation of solutions after reconstitution.

140| Add 100 µL of the chromogenic solution to each well.

141| At 6 min, add 50 µL of 25% (vol/vol) acetic acid to stop the reaction.

142| Immediately, measure OD_{405nm} in a microplate reader.

143| Extrapolate the endotoxin level from the standard curve. Refer to Appendix, Table S4.1 for anticipated endotoxin concentration.

Phage preparation protein analysis TIMING 4 h

- 144| Determine the absorbance of phage preparations from Step 133 at 280 nm to measure protein level. This will vary between phage strains but should be between 1-3mg·mL⁻¹.
- 145| Dilute phages to 15 µg in 20 µL of 1x Laemmli sample buffer.
- 146| Incubate samples at 90 °C for 5 min.
- 147| Centrifuge samples at 13,000 rpm for 60 sec at RT.
- 148| Prepare a 10% (wt/vol) acrylamide gel for SDS-PAGE electrophoresis.
- 149| Mount the gel into the tank, remove combs, and completely fill the inner chamber of the tank and ¾ of the outer chamber with 1x SDS-PAGE running buffer.
- 150| Pipette 3 µL of the standard and 20 µL of sample into the subsequent wells.
- 151| Run electrophoresis at 100V for ~60 min.
- 152| Wash the gel with ddH₂O 3 times for 15 min each.
- 153| Incubate the gel with 50 mL Coomassie blue staining solution at RT for 1 h.
- 154| Decant solution and add 50mL ddH₂O. Incubate at RT for 30 min on a rocker.
- 155| Decant and repeat Step 154 three times.
- 156| Image the gel using a gel dock station or conventional camera.

Phage preparation effects on human cell viability TIMING 3 d

CRITICAL: In Steps 157-167, we describe how to use the CellTiter-Glo® luminescent cell viability assay (Promega) to determine the number of viable cells in culture after phage preparation exposure based on quantitation of the ATP present, an indicator of cell proliferation and cytotoxicity.

157| In a 96-well tissue culture plate, seed wells with 100 μL of media containing ~20,000 of HeLa or HEK293 cells. **CRITICAL STEP.** Use a clear bottom white walled microplate to minimize well luminescence cross talk.

158| Incubate at 37 °C, 5% CO_2 , for 24 h.

159| The next day, check for a confluent monolayer of cell culture.

160| From Step 133, dilute phage stock to $10^8 \text{ PFU} \cdot \text{mL}^{-1}$ and $10^9 \text{ PFU} \cdot \text{mL}^{-1}$.

161| Add 10 μL of $10^8 \text{ PFU} \cdot \text{mL}^{-1}$ stock for an approximate concentration of 1 cell: 100 phages, 10 μL of the $10^9 \text{ PFU} \cdot \text{mL}^{-1}$ stock for a concentration of 1:1000 phages.

162| For positive control add 10 μL 1% (wt/vol) SDS and for negative control add 10 μL PBS.

CRITICAL STEP. Ensure that two wells are filled with medium only.

163| Incubate at 37 °C, 5% CO_2 , for 24 h.

164| Equilibrate the 96-well plate to room temperature for 30 min before adding 100 μL CellTiter-Glo™ to all wells.

165| Mix plate on an orbital shaker for 2 min to induce cell lysis.

166| Incubate at 22 °C for 10 min.

167| Measure luminescence on a microplate reader (see ‘Anticipated results’ below for example data).

TROUBLESHOOTING

Troubleshooting advice can be found in Table 4.2.

Table 4.2 Troubleshooting advice for purification protocol.

Table 2 Troubleshooting table			
Step	Problem	Possible reason	Solution
7	No visible clear zones (i.e., plaques)	Bacterial cells poorly lysed	Select alternative bacterial host strain
		Low viral count in environmental sample	Concentrate environmental sample by, for example, CFF (Steps 93–105) or PEG/NaCl precipitation ^{9,32}
20	Not quantifiable owing to the presence of pinpoint plaques	Actual plaque morphology on target bacterial host strain	Use a lighted magnifying glass over a dark backdrop to count plaques or switch to alternative bacterial host strain ⁹⁵
91	Filter clogging	Large amounts of cell debris in supernatant after centrifugation	Centrifuge phage lysate a third time (Steps 88 and 89) or process 1 L of lysate per batch
92	Phage titer $<10^9$ PFUs·mL ⁻¹	Poor bacteria growth	Optimize bacterial growth conditions and/or reduce phage MOI for Step 86
		Poor phage replication or low phage burst size	Produce a larger volume of phage lysate (set up more flasks at Step 84) before proceeding to Step 71
111	Fuzzy band observed at top of gradient	Gross bacterial debris present in phage concentrate	0.2-μm syringe filter the CFF fraction before proceeding to Step 106
	Multiple bands observed	Unwanted phage contaminant present in the phage concentrate	Repeat Steps 106–109 but with 7 mL of $d = 1.5$ in Step 106 and ultracentrifugation at 38,000g for 18 h at 4 °C in Step 109
120	Phage titer drops by >1 log	Phages might be damaged by osmotic shock	Use a 50/50 mix of water and 0.5 M PBS for the first dialysis in Step 112
134	Phage titer drops by >1 log	Phages bound to column resin	Increase NaCl concentration to 40 mM in the sample and equilibration buffer before adding phages to the column in Step 130
143	Endotoxin signal beyond recommended standard range	Endotoxin >1 EUs·mL ⁻¹	Dilute phage sample 10- to 1,000-fold in sterile PBS before adding to the microtiter plate in Step 137

TIMING:

The timing information is an estimate for phages for aerobic Gram-negative bacteria.

Steps 1–12, phage plaque isolation: 3–5 d, depending on repetitions needed to obtain a single plaque morphology.

Steps 13–19, small-scale cultivation: 15–21 h

Step 20 Option A, phage titration using agar overlay titering: 20 h

Step 20 Option B, phage titration using spot plaque titering: 19 h

Steps 21–56, DNA extraction: 2 h

Steps 57–62, sequencing library preparation: 10 min

Steps 63–77, DNA sequencing: 1–48 h, depending on desired reads coverage.

Steps 78–83, bioinformatics analysis: 12 h

Steps 84-90, large-scale cultivation: 24-30 h

Steps 91-92, dead-end filtration: 1h

Steps 93-105, ultrafiltration, diafiltration, and concentration: 8 h (2 h per 1.5 L)

Steps 106-111, density gradient ultrapurification: 4 h

Steps 112-120, CsCl concentrate dialysis: 24 h

Steps 121-134, LPS-affinity chromatography: 2 h

Steps 135-143, phage preparation endotoxin quantification: 2 h

Steps 144-156, phage preparation protein analysis: 4 h

Steps 157-167, phage preparation effects on human cell viability: 3 d

ANTICIPATED RESULTS

Process optimization at liter-scale production

This protocol for purifying phages employs a combination of modified classical techniques, modern membrane filtration processes, and omission of certain common practices (for overview see Figure 4.1). A typical six-liter shake-flask cultivation yields between 16-30 mL of final product, providing up to 64,000 treatment doses at 10^9 PFU — a commonly prescribed IV dose ¹—, depending on the phage-bacteria pair (Table 4.1 and Appendix, Table S4.1). Because final phage products contain very low endotoxin levels, theoretically would be possible that treatments of up to 10^{12} PFU could be prescribed to humans, and be within regulatory limits of $5 \text{ EU} \cdot \text{kg}^{-1} \text{ h}^{-1}$ [330]. In comparison, Belgium is the only western country that routinely produces phages in a laboratory under prescription, as a “magistral” preparation (i.e. drug compounding) [125]. To the best of our knowledge, magistral phage products are generally purified by high-speed lysate

centrifugation and subsequent affinity chromatography endotoxin removal [125]. In practice, this approach yields $\sim 10^7$ PFU·mL⁻¹ and 12.5 EU·mL⁻¹ [126]. A recent extended access phage therapy reported PEG/NaCl, density gradient ultracentrifugation and dialysis purification of *Mycobacterium* phages yielded a comparably high 10^{11} PFU·mL⁻¹ and undetectable endotoxin [153]. *Mycobacteria*, however, do not produce LPS. PEG-CsCl-dialysis can effectively reduce LPS to <0.05 EU·mL⁻¹ experimentally, it does so by adding organic solvents and generally at the expense of phage yield [320].

In Figure 4.3, we show that a relatively high phage titer is maintained during downstream processes, while endotoxin is being reduced to a safe level for human intravenous use. In addition, we show that high titer and low endotoxin phage preparation is independent of phage strain and viral structure. For example, myophages PAK_P1 and PAK_P5 were isolated from French wastewater with the *P. aeruginosa* strain PAK [377], while myophage E217 and podophage PYO2 were isolated from Italian wastewater with the *P. aeruginosa* strain PAO1 [82]. Final production runs produced titers of 6×10^{10} , 4×10^{11} , 2×10^{11} and 4×10^{12} PFU·mL⁻¹, respectively (Table 4.1). High-titer final stocks are dependent on the initial phage cultivation performance. Six-liter lysates of *Pseudomonas* phages produced titers of 10^{10} PFU·mL⁻¹ that could be concentrated up to 10^{12} PFU·mL⁻¹, whereas lysates of *K. oxytoca* and *S. marcescens* phages produced lower titers of 10^9 PFU·mL⁻¹ that could be concentrated to 10^{10} PFU·mL⁻¹ (Figure 4.3 and Appendix, Table S4.1). Because CFF was found to be the main phage concentrating technique, a larger volume of lysate from phages that do not produce high titers would be required to achieve higher final product concentrations.

Other practiced phage cultivation and purification approaches, such as polyethylene glycol precipitation, centrifugal ultrafiltration, organic solvent extraction, enzymatic inactivation, and anion-exchange chromatography were previously shown to produce equally high phage concentrations, but can retain high amounts of endotoxins [24, 121, 127, 128, 131, 226, 318, 323-326]. Our protocol focuses on early removal of endotoxins by conducting multiple low-speed centrifugation steps, microfiltration and cross-flow ultrafiltration with phage particle washing steps, which reduce the majority of LPS in phage samples (Figure 4.3). By contrast, we found subsequent CsCl density gradient ultracentrifugation increases, in some preparations, LPS quantity by up to 30% (Appendix, Table S4.1). Our results conflict with the recent study by Van Belleghem et al. that compared several endotoxin removal methods and concluded that CsCl density gradient ultracentrifugation was the most effective at removing endotoxin [226]. The study also showed effective endotoxin removal came at the expense of a phage yield reduction. In this study, we observe that the phage titer generally increases after CsCl density gradient ultracentrifugation and dialysis, except for phage JG266 (Figure 4.3).

Our results suggest that CFF with a MWCO 100 kDa pore size, in combination with several buffer washes, produces a phage product with endotoxin quantities within FDA regulatory limits for human intravenous use. Importantly, most exotoxins are smaller than 100 kDa [331]. The semi-automated CFF used in our approach is able to concentrate phages between 10- to 100-fold, while removing endotoxin by >4000-fold and rich bacterial growth medium in a single-step (Figure 4.3). CFF is an effective phage concentration technique [127, 128]. However, using lower MWCO pore sizes appears to trap endotoxins along with the phage particles [128]. The recent “Phage On Tap”

purification protocol showed that centrifugal ultrafiltration was more rapid technique to concentrate phage particles, but also trapped endotoxins [127]. Nevertheless, we show that endotoxins in phage samples can be further reduced by commercially available LPS-affinity chromatography (Figure 4.3 and Appendix, Table S4.1).

Phage product safety testing

Throughout the phage cultivation and purification process we maintain phage strain homogeneity by monitoring for changes in plaque morphology while phage titering. After each processing step, phage stocks should maintain a distinct single plaque phenotype. Homogeneity is further confirmed by obtaining a distinct single band after density gradient as would be expected with a sample containing phage particles with the same density and shape [368].

As mentioned above, at a therapeutic dose of 10^9 PFU, endotoxin in phage lysates are reduced at least a 10^6 -fold for all seven phages tested, without the use of organic solvents (Figure 4.3 and Table 4.1). Simultaneously, <100 kDa microsoluble permeate is withdrawn from phage preparations during CFF, suggesting that small molecules, such as exotoxins that are typically smaller than 30 kDa [331], are also removed from final phage products. However, none of our phages or bacterial strains contained a known exotoxin to validate this claim.

Figure 4.4A shows that our example phage preparations are free of gross bacterial proteins with SDS-PAGE staining not showing significant protein smearing. This becomes especially clear when comparing the $0.2\ \mu\text{m}$ filtered lysate of *Klebsiella* phage JG265 with its purified preparation (Figure 4.4A). The lysate displays many bands, while the purified

phage preparation displays a few distinct bands unique for each denatured phage, indicating differences in their structural proteins. PAK_P1 and PAK_P5 share a major protein band at ~50kDa, and E217, PYO2, JG265 and JG266 at 75 kDa. Between phage strains, other proteins are not conserved with a variety of bands ranging from 20 to 150kDa. To identify these specific phage proteins further mass spectrometry analysis would need to be conducted [378].

Because SDS-PAGE is not sufficient to guarantee absence of harmful toxins, we further conducted cell viability testing [379]. *In vitro* models to test for phage preparation effects on human cells and exclude potentially harmful products is an important consideration, but there is no standard model of phage pharmaceutical standard available. As a rapid test for eIND request, quantitation of intracellular adenosine triphosphate can be used as a measurement of the metabolic activity of human cells. With two human cell lines, HeLa and HEK293, we show that cell viability is not significantly different from untreated cell controls after exposure to each of the phage strains purified after cultivation with *Pseudomonas*, *Klebsiella* or *Serratia* (Figure 4.4B-G). By contrast, centrifuged phage lysate causes a significant decrease in cell metabolic activity. Therefore, our results suggest that downstream purification of phage lysates can produce a product free of harmful bacterial toxins and other components.

Together, these results suggest that our protocol for phage purification is reliable and reproducible, helping to ensure the safety and efficacy of phage products for human use.

4.1.2: Improved rapid preparation and sterile handling of clinical phages (published protocol)

This section is a reprint of a published protocol entitled: *Rapid Bench to Bedside Therapeutic Bacteriophage Production*. Printed in *Methods in Molecular Biology*, December 2023. Written by Tiffany Luong, Andrew D Sue, and Dwayne R Roach. This protocol is an updated version of the process described in Chapter 4.1.1. The main improvements to the work include enhancing the clarity of the procedures, leveraging the resistance kinetics of each phage of interest to decrease the upstream burden of bacterial cells during cultivation, and improvements in CFF concentration to 1) improve the removal of endotoxins and 2) reduce the steps and time necessary for purification. Additional steps to ensure the safety and sterility of the phage preparation are also included to gear this protocol for increased accessibility and clinical application compared to the original protocol.

Abstract

It has been over 100 years since bacteriophages (phages) were used as a human therapeutic. Since then, phage production has dramatically evolved. Current phage preparations have fewer adverse effects due to their low bacterial toxin content. As a result, therapeutic phages have become a predominant class of new antimicrobials and are being widely used for compassionate treatment of multidrug-resistant (MDR) infections. We describe herein a protocol for the production and ultrapurification of phages. By this technique, it is possible for a lab experienced with the process to produce $>10^9$ plaque forming units (PFU) per mL of Gram-negative phages that meet FDA

endotoxins limits for intravenous infusions in as little as 48 hours. We provide illustrations of the process and tips on how to safely remove bacterial toxins from phage lysates. Although dependent on the phage strain, the approach described herein to rapidly generate and purify phages is extremely useful for a variety of applications.

1. Introduction

Bacteriophage, also known as phage, is a virus that attacks and destroys bacteria. Phages are found anywhere and everywhere bacteria can be found: water, air, soil, and in the body. Their activities control the levels of all bacteria in nature. In 1919, Félix d'Hérelle, a French-Canadian microbiologist, who at the time was at the Pasteur Institute in Paris, used phages to treat dysentery bacillus. d'Hérelle's work set the basis for phage therapy. Currently, only the Eliava Institute in Tbilisi, Georgia, has maintained a mainstream use of phage therapy since its discovery. For the past several years, the practice of using phages for patients has been under redevelopment and modernization [277, 380, 381]. Antibiotic resistance has become a leading public health threat, and the discovery of new classes of antibiotics has stagnated [9]. Phages are not affected by antibiotic resistance mechanisms in general [277, 380], and virions themselves are generally considered safe for human and animal use [28, 29, 159].

Virulent phages exclusively use the lytic cycle for replication in bacterial host cells [11]. A phage seeks a host and, when found, binds to the cell surface and injects its DNA genome inside the host. In a matter of seconds, the host cell is hijacked for intensive virion replication that results in the bacterium bursting open, releasing newly produced phages to repeat the process. In this manner, the phage lysate can expand to $>10^{10}$ plaque forming units per milliliter (PFU/mL) of growth medium under optimal host

conditions. Many phage strains are easy to produce with standard microbiology equipment.

However, the production bottleneck arises during phage purification. Phage replication accumulates a high amount of toxins in lysates, such as endotoxins, exotoxins, soluble bacterial proteins, peptidoglycans, nucleic acids, and induced prophages [382, 383]. Their presence in the bloodstream may cause septic reactions with a variety of symptoms such as fever, hypotension, nausea, shivering, and in extreme cases, fatal shock. Endotoxins are lipopolysaccharide (LPS) molecules associated with the outer membranes of Gram-negative bacteria and make up the most common toxins that must be removed from pharmaceutical phage products. When these bacterial cells are lysed by phages, their cell membranes rupture and endotoxins are released into the solution. Endotoxins in solution range from molecular weights of 10,000 to 1,000,000 [304]. However, since phages are in the same molecular weight range as endotoxins, filtration cannot be used to adequately separate the endotoxins from the target phages. A harmonized standard for recommended maximum endotoxin exposure is 5.0 endotoxin units (EU) per kg of body weight (interpreted as within 1 hour) for most drugs based on an average patient weight of 70 kg [122]. Troubleshooting endotoxin-contaminated phage suspension may become necessary during phage production. Moreover, cold-stored phage suspensions may see the number of viable phages decrease over time, while the titer of endotoxins detected may not [314].

Double polyethylene glycol (PEG) precipitation is a traditional approach for purifying phages [25, 128]. As phage particles are made up mostly of coat proteins around their genetic material, solutions containing PEG and high salt force phage particles to

aggregate into clusters. This low-cost method needs minimal equipment, and the precipitation is performed by general centrifugation. PEG precipitation, however, faces several disadvantages. The precipitation method requires the addition of large amounts of PEG and NaCl, some of which remain as a residue in the phage pellet and are difficult to eliminate. Certain other contaminants, such as LPS and exotoxins, are generally co-precipitated with phages [129, 130]. For these reasons, depending on the purpose, PEG-precipitated phages usually require further methods of purification, such as density gradient ultracentrifugation (DGU), to remove co-precipitated impurities [25, 128]. DGU is a highly versatile method that has been widely used for the concentration and purification of phages [128, 281, 306]. This approach allows the separation of phages and contaminants based on buoyant density differences independent of size and shape. In this method, the sample is passed through a steep density gradient that contains a very high concentration of CsCl and ultracentrifugation. Multiple bands may be visible after ultracentrifugation; top band(s) corresponds to debris, LPS, and unassembled phages (e.g., genome-free capsids). While the lower band contains the target phage that can be collected by puncturing specialized tubes [281, 384]. Drawbacks associated with CsCl DGU are that some phages cannot withstand long periods of ultracentrifugation at more than 100,000×g force, and osmotic shock or interaction with CsCl and/or extensive dialysis may lead to loss of phage infectivity [332]. Last, not all laboratories can access the required equipment for this method.

Diverse chromatography-based methods enable the separation and purification of phages. The target phage can be purified based on its interaction types as well as its characteristics, such as shape, size and total charge, hydrophobicity, and binding

capacity. Accordingly, the separation functionality is broadly categorized into 1) size, 2) charge, 3) hydrophobicity, and 4) affinity, etc. Size exclusion chromatography (SEC) separates molecules based on their size by filtration through a gel. Given the large size of phages relative to most bacterial components as well as other small compounds such as LPS, exotoxins, ions, and salts, SEC can be applied as a complementary step for phage purification [130]. The main advantages of SEC are the low costs of resins and simplicity in operation as samples are eluted isocratically, so there is no need to use different buffers during the separation. However, this technique lacks selectivity, suffers from low productivity, and scaling up is restricted because the column can get saturated with host cell debris, consequently preventing the separation of large phages from endotoxins. Another nonspecific technique is ion exchange chromatography (IEC), which is one of the most efficient methods for separating charged particles. Phages are separated according to the strength of their overall ionic interaction with a solid phase material [128, 129, 132]. There are two types of IEC: anion- and cation-exchange chromatography (AEC and CEC, respectively). A disadvantage of this method is the risk of eluting contaminant proteins and endotoxins with similar electrostatic interactions as the target phage. Phages have a pH-dependent negative surface charge in most solutions, as do LPS [385]. The other drawback is the need to connect the chromatography column to a costly FPLC or HPLC instrument. By contrast, affinity chromatography, also called affinity purification, makes use of specific binding interactions between molecules [128, 129, 132, 281]. This method uses synthetic ligands for specific elution and involves the impregnation of poly(ϵ -lysine) into cellulose beads, which provide high endotoxin selectivity [386]. A combination of electrostatic,

hydrophobic, and hydrogen bond interactions are present in the affinity chromatography. The current drawbacks of affinity chromatography are low yield and high salt concentration requirement for substance elution.

More recent methods have been applied to phage purification. Extraction with organic compounds can efficiently remove endotoxins from phage samples, such as 1-octanol [131, 387]. The removal of octanol is achieved by multiple centrifugation steps and use of a vacuum to remove the residual organic solvent [129, 132, 387]. The main drawback is inconsistency in sample phage recovery [129, 131, 387], sometimes being as low as 15% [129].

With several options at each step of the production process, this protocol selects appropriate methods for the production and purification of phages using different criteria, including cost, equipment, ease, time, yield, and purity of the product. The steps intrinsic to our process to rapidly generate highly pure small-batch phage stocks that meet regulatory guidelines for intravenous (IV) administration are timed process execution to match phage-bacteria kinetics and modifications that have been used to optimize ultrafiltration (UF) and diafiltration (DF) by both us [281] and others [128, 129, 384]. Our process first minimizes the quantity of bacterial toxins that accumulate during phage replication by using several techniques to limit bacterial overgrowth and reduce bacterial debris in sterile lysates. Next, we use a semi-permeable membrane with active force applied to drive UF and DF to get target phages ready for the next processing step by enabling sample cleanup, purification, concentration, buffer exchange, and desalinization. Last, we use affinity chromatography resin to selectively capture the conserved region of the inner core of LPS molecules and thereby remove all kinds of

endotoxins from Gram-negative bacteria. We also include our aseptic/sterile fill-finish methods which are ideal for small-batch biomedical applications. Together, this streamlined process efficiently produces many phages in as little as 48 hours and economically.

2. Materials

2.1 Equipment

1. Biological safety cabinet (BSC)
2. Microbiological incubators: static and refrigerated incubator shaker (see **Note 1**)
3. Single and multichannel pipettes: various volumes
4. Serological pipette controller
5. Spectrophotometer
6. High-speed centrifuge with fixed angle rotors (e.g., Beckman Coulter J-Lite JLA-8.1000 fixed angle rotor)
7. Thermocycler
8. Electrophoresis chamber and gel imager
9. Double-pan balance scale
10. Peristaltic pump and size 16 pump head (see **Note 2**)
11. Tube rotator
12. Multimodal microplate reader

2.2 Bacteriophage and Bacterial Host Strains

We recommend before phage production that both phage and host bacterial genomes have been sequenced and annotated. Both phage and host bacteria genomes

require screening for virulence factors, antibiotic resistance and toxin genes, and prophages [348, 366, 388]. Prophages integrated into the host genome may not only harbor additional virulence factors, but also introduce an added risk of horizontal gene transfer (transduction) or may interfere with the infectivity of the target phage [347, 348]. If any undesirable elements are identified in either the phage or host genome, we recommend using alternative strains.

1. Bacteriophage stock (recommend sequenced and 0.2 μm filtered)
2. Bacterial strain used to propagate bacteriophage strain (recommend sequenced)

2.3 Reagents

Reagents should be prepared using sterile Milli-Q water, autoclaved (45 min at 121°C), and stored at room temperature unless otherwise indicated.

1. Bacterial broth and solid (agar) media
2. Phosphate buffered saline (PBS) (10X): weigh 17.8 g of Na_2HPO_4 , 2.4 g of KH_2PO_4 , 80 g of NaCl, 2 g of KCl, and dissolve in 800 mL of sterile water. Add water to 1L. Dilute 10:1 in sterile water to make a 1X working solution (10 mM Na_2HPO_4 , 1.8 mM KH_2PO_4 , 137 mM NaCl, 2.7 mM KCl)
3. PCR primers (10 μM concentration)
4. Tris-acetate-EDTA (TAE) buffer (10X): weigh 48.5 g Tris and dissolve in 800 mL of sterile water. Add 11.4 mL glacial acetic acid and 20 mL 0.5 M EDTA (pH 8.0). Add water to 1 L. Dilute 10:1 in sterile water to make 1X working solution (40 mM Tris, 20 mM acetic acid, 1 mM EDTA)
5. 2% agarose gel: 0.8 g agarose, 40 mL 1X TAE
6. 0.5 N NaOH

7. Endotoxin-free water
8. 95% Ethanol and 0.2 N NaOH solution
9. 2 M NaCl
10. 10 mM sodium phosphate buffer (PBS): Dilute 10X PBS 10:1 in endotoxin-free water and filter sterilize
11. DNase I
12. RNase A
13. 100% Ethanol

2.4 Consumables

When appropriate (i.e., if plastics are not sterilely packaged), non-sterile glassware and plastics should be autoclave-sterilized prior to use.

1. Disposable inoculating loops: 1 μ L
2. Disposable plastic cuvettes
3. Test tubes and caps
4. Petri plates: 100 x 15 mm
5. Pipette tips: various volumes
6. Serological pipets: various volumes
7. Polypropylene conical centrifuge tubes: 15 mL and 50 mL
8. DNase/RNase-free strip PCR tubes and caps
9. Microcentrifuge tubes: 1.5 and 1.7 mL
10. Graduated glass media bottles: various volumes
11. Glass Erlenmeyer flasks with GL45 screw cap: 250 mL and 2 L

12. GL45 screw cap with 0.22 µm polytetrafluoroethylene (PTFE) hydrophobic membrane (Corning)
13. Polycarbonate centrifuge bottles with cap assemblies (recommend 1 L)
14. Luer Lock tip syringes: 10 mL and 50 mL
15. Syringe filters: 0.2 µm
16. Syringe needles: 23G x 1-1/2"
17. Rx-Vent™ Filtered Venting Needles
18. 0.8 | 0.45 µm polyethersulfone (PES) double layer dead-end filter capsule
19. 0.2 | 0.1 µm PES double layer dead-end filter capsule
20. Vivaflow® 200 Crossflow Cassettes: 100 KDa MWCO PES membrane
21. ¼" flexible rubber tubing and clamps
22. Depyrogenated glass vials (pre-assembled with stopper and aluminum crimp seal)
23. 96-well microplates: clear U-bottom and black flat-bottom

2.5 Recommended Kits

1. Endotoxin Removal: EndoTrap® HD (Lionex) or Pierce™ High-Capacity Endotoxin Removal Resin assay (Thermo Scientific)
2. Endotoxin Quantification: ENDONEXT™ EndoZyme® II – Recombinant Factor C (rFC) Endotoxin Detection assay (BioVendor) or Pierce™ Chromogenic Endotoxin Quant assay (Thermo Scientific)

3. Methods

All procedures must be performed aseptically/sterilely in a biological safety cabinet.

3.1 Pre-Processing

3.1.1 *Bacterial host standard curve*

1. Streak bacteria on an agar plate from a glycerol stock using a sterile loop. Incubate overnight at the temperature that is optimal for bacterial growth.
2. Pick a colony forming unit (CFU) and culture it in the appropriate nutrient-rich broth, overnight at the optimal temperature for growth.
3. In a BSC, air dry 5 agar plates for 1 h.
4. Subculture 50 μL in 5 mL of fresh liquid broth in a sterile test tube. Incubate until an OD_{600} 0.8 is reached. Place the test tube on ice.
5. Aliquot culture into a sterile cuvette and measure absorbance using a spectrophotometer. Dilute the culture to OD_{600} 0.8 with sterile bacterial broth in a sterile cuvette. Continue dilutions in twofold increments to obtain cuvettes with OD_{600} 0.8, 0.4, 0.2, 0.1, and 0.05.
6. Prepare two identical 8-well columns of PBS diluent in a 96-well microtiter microplate. Add 198 μL of PBS to the top 2 wells and 90 μL in the remaining 14 wells.
7. Sample 2 μL from the OD_{600} 0.8 bacterial culture and add to the first microwell containing 198 μL of PBS and mix by repetitive pipetting (repeatedly dispensing and withdrawing solution from the microwell). Sample 2 μL of bacterial culture again and add to the second microwell containing 198 μL .

8. Use a multichannel pipette to serially dilute in tenfold increments by aliquoting 10 μ L from the first row of wells to the second and mix by repetitive pipetting. Change pipette tips and repeat for the remaining six wells.
9. Withdraw 4 μ L from the first column of 8 microwells using a multichannel pipette. Spot $8 \times 4 \mu$ L aliquots to the leftmost edge of the agar plate. Repeat $8 \times 4 \mu$ L sampling from the first column and spot on the agar plate next to the first column of spots. Repeat $8 \times 4 \mu$ L sampling from the first column a third time, spotting on the agar plate next to the second column of spots (Figure 4.5A).
10. Repeat 4 μ L spotting in triplicate, withdrawing from the second column of 8 microwells. Dry spots completely before closing the Petri plate lid and moving the plate (see **Note 3**).
11. Repeat dilution and titration steps 6-10 for the remaining diluted cuvettes.
12. Incubate all agar plates at the appropriate bacterial growth conditions until colonies are countable (Figure 4.5B).
13. Calculate bacterial CFU/mL by averaging the number of CFUs visible in all 6 spots from both dilution series. Selected spots should contain between 10-40 countable CFUs. Divide the average CFUs by 4 (volume spotted), times the dilution factor (column position), and times by 1000 (convert μ L to mL).
14. Plot the CFU/mL (y-axis) against OD₆₀₀ reading (x-axis). Generate a trendline and check that the R^2 of the line is >0.9 . The equation of the trendline will allow you to approximate the host OD₆₀₀ value necessary to achieve the desired concentration CFU/mL.

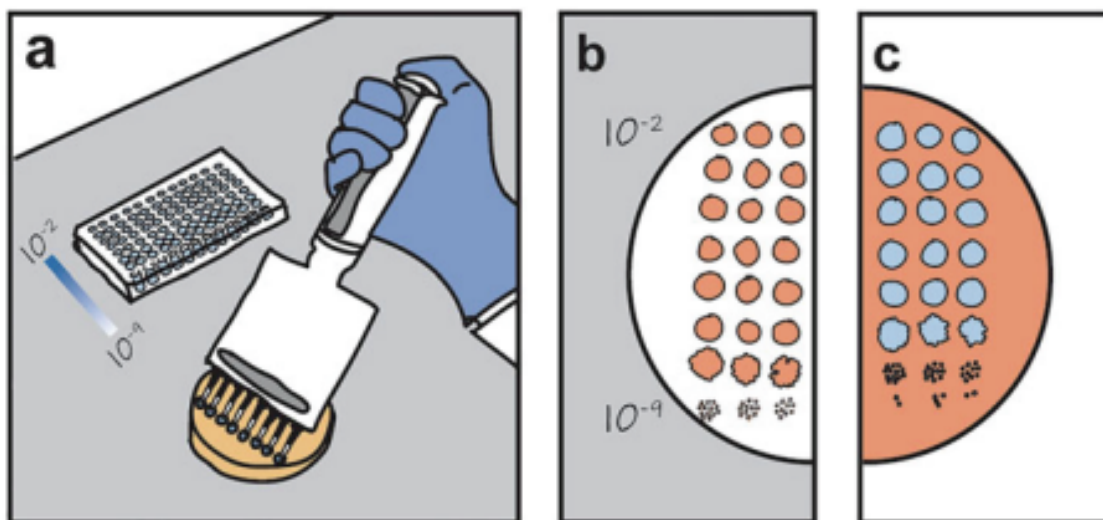


Figure 4.5 Illustration of bacteria titration or Spot⁺ phage titration. (A) Example setup using a multichannel pipette and 96-well plate for serial dilutions. (B) Desired 48-spot plating for bacterial quantification. Counting should proceed from the row containing ten or more distinct CFUs. (C) Desired 48-spot plating for Spot⁺ phage titration. Counting should proceed from the row containing ten or more distinct PFUs.

3.1.2 Spot⁺ phage titration

Traditionally, phages are quantified using the top agar overlay method [11]. We recommend a double aliquot 48-spot serial titration per sample of interest. This quantification method is quick and easily performed with general laboratory equipment. The Spot⁺ method increases sample coverage, depth, and technical repetition to improve accuracy over a typical spot titration method.

1. In a BSC, air-dry agar plates for 1 h. Dry one plate for each sample to be titrated.
2. Pour 2 mL of bacterial culture at OD₆₀₀ 0.2 onto a dried agar plate to seed. Gently swirl to spread the liquid evenly over the entire surface. Tilt the Petri plate and immediately collect excess liquid for disposal. Repeat seeding for each dried agar plate needed.
3. Air dry seeded plates for 30 min.

4. Prepare two identical 8-well columns of PBS diluent in a 96-well microtiter microplate. Add 198 μL of PBS to the top 2 wells and 90 μL in the remaining 14 wells.
5. Sample 2 μL from the phage preparation and add to the first microwell containing 198 μL of PBS and mix by repetitive pipetting (repeatedly dispensing and withdrawing solution from the microwell). Sample 2 μL of phage again and add to the second microwell containing 198 μL .
6. Use a multichannel pipette to serially dilute in tenfold increments by aliquoting 10 μL from the first row of wells to the second and mix by repetitive pipetting. Change tips and repeat for the remaining six wells.
7. Withdraw 4 μL from the first column of 8 microwells using a multichannel pipette. Spot the 8 aliquots in the leftmost area of the seeded agar plate. Repeat $8 \times 4 \mu\text{L}$ sampling from the first microwell column and spot onto the agar plate next to the first column of spots. Repeat $8 \times 4 \mu\text{L}$ sampling from the first microwell column a third time, spotting on the agar plate next to the second column of spots (Figure 4.5A-5.5B).
8. Repeat step 7 and perform 4 μL spotting in triplicate, withdrawing from the second column of 8 microwells.
9. Dry spots completely before closing Petri plate lid and moving. Incubate at the appropriate bacterial growth conditions until plaques are countable (Figure 4.5B).
10. Calculate phage PFU/mL by averaging the number of PFUs visible in all 6 spots from both dilution series. Selected rows should contain 10-40 PFUs. Divide the average PFUs by 4 (volume spotted), times the dilution factor (column position), and times by 1000 (convert μL to mL).

3.1.3 Pilot phage amplification

1. Pick a fresh CFU from the host strain plate and grow to $\sim 10^8$ CFU in 5 mL of broth medium.
2. Add phages at $\sim 10^7$ PFU to achieve a multiplicity of infection (MOI) of 0.1.
3. Incubate the culture for 10-12 h, then harvest or cool to 10°C for the remainder of the time (see **Note 4**).
4. Determine the window for cooling the phage preparation by titering and checking the OD₆₀₀ of the lysate the following day. (see **Note 5**).
5. Harvest the phage lysate in a sterile 15 mL conical and centrifuge for 10 min at 8000×g.
6. Decant the supernatant into a sterile syringe with an attached filter unit. Filter into a fresh 15 mL conical.
7. Aliquot 100 µL of the filtered lysate into a PCR tube.
8. Store at 4°C.

3.1.4 Lysate prophage detection

Even if a putative prophage is detected bioinformatically, it may not necessarily be liberated during lysate production. Use the pilot phage lysate as a PCR template for verification.

1. Identify prophages from a fully assembled host strain genome using PHASTER or other software of choice. (see **Note 6**).
2. Obtain the FASTA file for the putative prophage(s) from the tool of choice.
3. Design primers for the prophage(s) using NCBI Primer-BLAST or other tools of choice.

4. Make a master mix for each potential prophage or phage strain tested. (see **Note 7**)
5. Vortex briefly to mix. Centrifuge briefly to settle.
6. Distribute 22.5 μ L of master mix into freshly labeled PCR tubes.
7. Add 2.5 μ L of the pilot phage lysate as template into PCR tubes.
8. Set the PCR temperatures according to the polymerase/kit chemistry. Use an annealing temperature specific for the phage primers designed in **step 3**.
9. While the PCR reaction is running, prepare a 2% agarose gel and add DNA gel stain.
10. Load the PCR reaction after the gel has set for 20 min. Run gel electrophoresis using an appropriately sized DNA ladder and samples.
11. Analyze and image gel using a gel documentation system. (see **Note 8**).

3.2 Batch Phage Amplification

Phage production typically begins with long culture incubations (e.g., overnight/18-24 h) followed by lysate centrifugation and 0.2 μ m filter sterilization. As mentioned, bacterial toxins accumulate during phage amplification, and a large quantity of free endotoxin results from violent cell lysis. It is also common for target bacterial cells to evolve resistance to phage infection during these long incubations. These evolved mutants thrive in optimal culture conditions and overgrow in the lysate. We recommend switching to cold growth incubation, 1-2 h prior to the visible outgrowth of phage-resistant bacteria. Time-kill kinetics assays help understand interactions that exist between host bacteria and phages, including time to resistance. For example, we recommend when using *Pseudomonas aeruginosa* phages to reduce the incubation temperature after ten hours to suppress the outgrowth of evolved phage resistant mutant cells while maintaining phage production. Similarly, we recommend for *Staphylococcus aureus* phages to reduce

the incubation temperature after 22 hours to achieve the same suppression. This temperature drop prevents resistant bacterial growth, which causes a clearer lysate while maintaining long incubation times for higher phage yields. The addition of two centrifugation steps significantly enhances the removal of intact bacterial cells and bacterial debris, including large endotoxin aggregates. Figure 4.6 shows a flow diagram briefly describing this process.

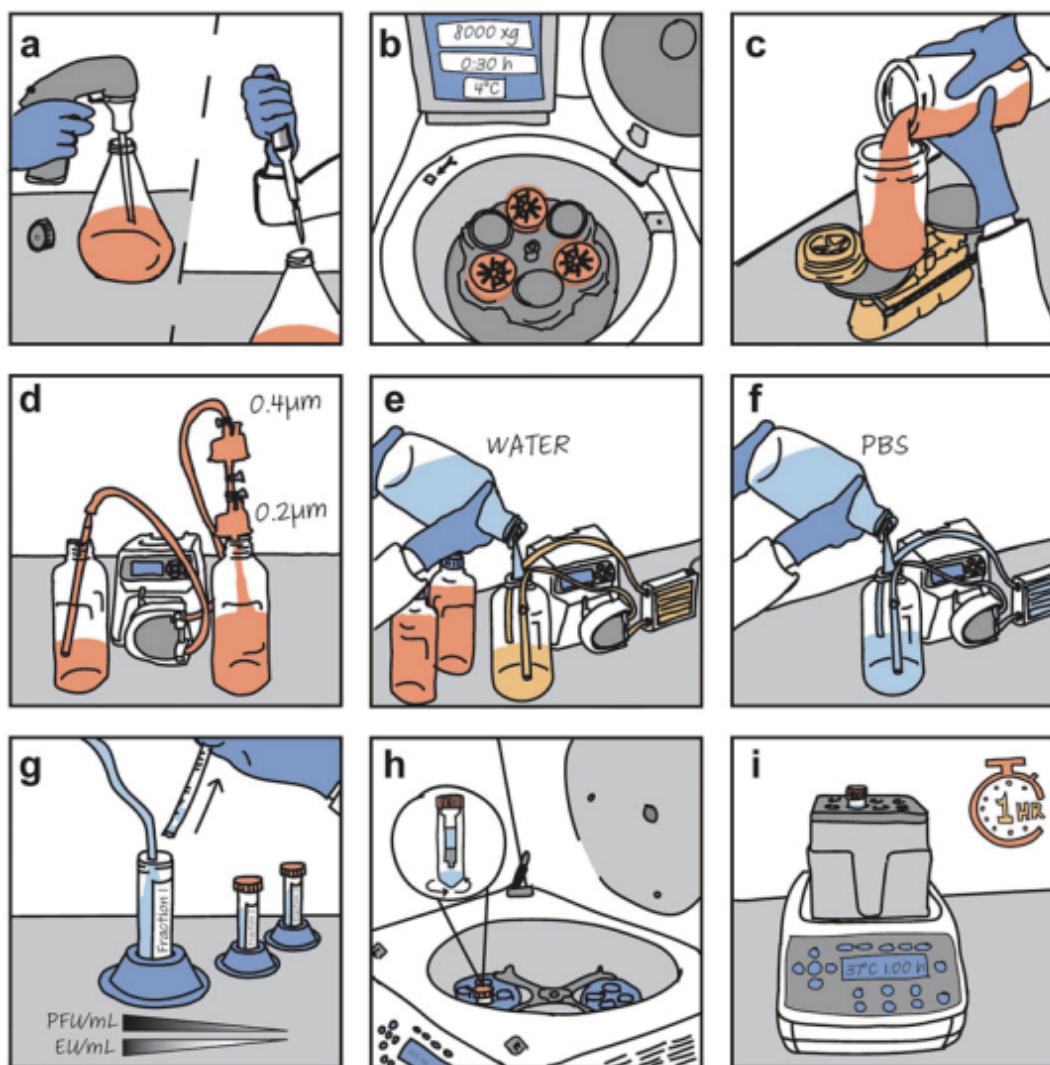


Figure 4.6 Illustrated schematic of phage production and purification process. (A) Phage amplification. Bacteria should be added to the sterile growth medium and grown for one generation with a vented screw cap to allow gas exchange (left). Phages should be added to achieve a MOI of 0.1 (right). (B) First centrifugation. Centrifuge produced phage lysates at $8000 \times g$ for 30 min at 4°C . (C) Second centrifugation. Avoid the bacterial pellet and carefully decant the supernatant into new centrifuge bottles. Re-balance bottles and repeat centrifugation at $8000 \times g$ for 30 min at 4°C . (D) Dead-end filtration sterilization. Sterilize the lysate by connecting in tandem a $0.8 \mu\text{m}$ | $0.45 \mu\text{m}$ capsule filter to a $0.45 \mu\text{m}$ | $0.2 \mu\text{m}$ filter capsule. (E) Diafiltration. Remove storage buffer from the CFF cassette, flush with water, and set up the CFF cassette as pictured. The intake and retentate lines should recirculate from the bottle containing lysate. Flow all phage lysate through the CFF. Concentrate to 50% volume and dilute with cold ultrapure water. (F) Washing. Exchange spent lysate for cold ultrapure storage buffer. Stepwise concentrate and dilute phage with storage buffer. (G) Concentrating. When the phage concentrate appears visibly clear, purge the CFF and collect phage particles in three or more fractions. (H) Endotoxin removal. Sterilely prepare reagents for endotoxin removal. Perform endotoxin removal per manufacturer's instructions with suggested modifications to prevent phage loss. (I) Removal of nucleic acids. Remove free DNA/RNA by 1 h enzyme treatment.

1. Pick a CFU isolated using the streak plate method on solid growth medium and culture in the appropriate nutrient-rich broth medium overnight to bulk up a pure culture. (see **Note 9**).
2. Subculture 50 μ L in fresh liquid broth to get the bacterial population growing exponentially (i.e., OD₆₀₀ 0.1-0.8).
3. Add 1 L of broth medium to each of the 3 sterile autoclaved glass Erlenmeyer flasks with GL45 screw cap and pre-warm to bacterial growth temperature.
4. Add exponentially growing bacteria at an optical density that corresponds to a count of $\sim 10^{10}$ CFU per flask and exchange the flask's cap for an autoclaved GL45 vented screw cap with 0.22 μ m PTFE hydrophobic membrane. Incubate the flasks for one generation at growth temperature with gentle agitation (Figure 4.6A).
5. After bacteria have acclimated and grown for one generation, add phages at $\sim 10^9$ PFU to achieve a multiplicity of infection (MOI) of 0.1 (Figure 4.6A).
6. Incubate the flasks for 10-12 h, then cool to 10°C for the remainder of the time until 18 h. (see **Note 4**).
7. Harvest amplified phages by decanting lysates into sterile centrifuge bottles (e.g., 1 L bottles) and balance the three bottles against one another using a double pan balance scale (Figure 4.6B).
8. Centrifuge at 8000 $\times g$ for 30 min at 4°C.
9. Decant supernatant containing phage particles without disturbing the solid pellet into new sterile centrifuge bottles (Figure 4.6C). Repeat centrifugation at 8000 $\times g$ for 30 min at 4°C (see **Note 10**).

10. Collect phage particles without disturbing the tiny pellet into sterile glass storage bottles.

3.3 Phage Purification

3.3.1 Dead-end filtration sterilization

1. In a BSC, connect a 0.8 | 0.45 μm heterogeneous PES double layer filter capsule to a 0.2 | 0.1 μm heterogeneous PES double layer filter capsule with autoclaved ¼" flexible tubing and clamps as shown in Figure 4.6D.
2. Prepare a disposable 10 mL serological pipette by removing the cotton or filter from its end. Feed the tubing through the peristaltic pump head, insert the serological pipette into the end of the tubing, and place it into sterile water.
3. Precondition the filter membrane by pumping 500 mL of sterile water through the capsule filters. Discard the flow through.
4. Switch the serological pipette and tubing to the bottle containing phage lysate and pump it into sterile glass bottles (see **Note 11**).
5. Store all filtered lysates at 4°C. This is a potential pause step; depending on the phage strain, the lysate can be stored at 4°C for up to several months without significant loss in titer.

3.3.2 Ultrafiltration and diafiltration

This process uses crossflow filtration (CFF) for combined UF and DF of up to 5 L batch volumes. UF and DF are commonly used for the development and manufacturing of biological therapeutics, such as proteins and antibodies, as well as therapies that rely on viral nanoparticle delivery [389, 390]. The Vivaflow 200 CFF cassette uses a

polyethersulfone membrane (PES), which is popular for phage applications given its hydrophilic properties and low protein binding. The semi-permeable membrane retains larger target phages while allowing smaller molecules to filter out. A 100 kDa MWCO is adequate to retain most phages. DF enables medium to buffer exchange by adding new cold buffer to the phage retentate. By returning the sample to the original volume, UF/DF can be repeated until the sample reaches a targeted level of clarity with the new buffer. The Vivaflow's unique switchback channels parallel to the filtration membrane improves phage dissociation from endotoxins and require less pressure to drive filtration. The cassette design is easy to set up, gentle on phage particles, and its transparency allows for monitoring flow.

1. In a BSC, connect the CFF cassette with autoclaved 6.4 mm OD tubing as shown in Figure 4.6E. Connect the filtrate line to the port on top of the cassette, the retentate line to the upper side port, and the intake line to the lower side port.
2. Feed the intake line through the peristaltic pump head, insert a disposable 10 mL serological pipette into the end of the tubing, and place the pipette into a bottle with sterilized chilled (4°C) ultrapure water. Keep the cassette in an open circuit by placing both the retentate and filtrate lines in a waste container.
3. Pump 500 mL of autoclaved cold ultrapure water through the cassette to remove storage EtOH and to condition the membrane with pure water.
4. Transition the cassette into a continuous circuit. Insert the intake and retentate lines into the glass bottle containing sterilized cold phage lysate, as shown in Figure 4.6E. Only the filtrate line should remain in the waste container.

5. Recirculate the phage lysate through the cassette until 50% volume remains in the bottle and add cold ultrapure water to double volume (see **Note 12**).
6. Concentrate to 50% again and repeat the ultrapure water dilution by doubling the volume (see **Note 13**).
7. Concentrate to 50% again and dilute with cold sterile PBS to double the volume (Figure 4.6F).
8. Concentrate to 30% and dilute with cold sterile PBS to double the volume.
9. Concentrate to 20% and dilute with cold sterile PBS to double the volume.
10. Concentrate to 10% and dilute with cold sterile PBS to double the volume, repeat until the lysate is visibly clear (see **Note 14**).
11. To collect phage particles, set up and label 3 sterile conical centrifuge tubes as “1st, 2nd, 3rd Fractions”. Add 30 mL of sterile cold PBS to tubes 2 and 3.
12. Concentrate to 30 mL and pause the peristaltic pump. Pour the 30 mL of phage solution into the empty conical tube and carefully insert the intake and retentate lines into the conical. Restart pumping until ~5 mL remains in the conical tube. Pause the pump. See Figure 4.6G.
13. Collect the 1st fraction by draining the cassette, this will allow you to collect the remaining ~30 mL of dead volume of the cassette and tubing. Remove the intake line from the conical and restart the pump to completely flush the cassette into the 50 mL conical. Pause the pump (see **Note 15**).
14. Immediately insert the intake and retentate lines into the 2nd conical tube containing 30 mL sterile cold PBS and start the pump to recirculate buffer to recover the phage remaining in the cassette. When the volume reaches ~5 mL, pause the pump,

remove the intake line, and restart the pump to completely flush the cassette again.

Pause the pump after all the liquid is collected.

15. Repeat the recirculation and draining from step 14 using the 3rd conical tube containing sterile cold PBS. Pause the pump after all liquid is collected. Immediately refill the cassette with buffer for short-term storage until cleaning using manufacturer's instructions (see **Note 16**).
16. Store fractions at 4°C (see **Note 17**).

3.3.3 Chromatographic removal of endotoxin

Although all prior processing steps reduce and remove endotoxins from phage samples, CFF fractions generally contain endotoxins above regulatory limits for intravenous applications. For example, up to 10^5 - 10^6 EU/mL. Therefore, chromatographic endotoxin removal may be required depending on phage concentration and intended application. For instance, a 10^{11} PFU/mL phage sample with an endotoxin level of 10^4 EU/mL would not need additional endotoxin removal steps if dilution in pyrogen-free saline was done to achieve a therapeutic dose of 10^9 PFU/mL. The commercially available Pierce High-Capacity Endotoxin Removal Resin and EndoTrap HD assays have a binding capacity of 2×10^6 and 5×10^6 EU/mL, respectively. Below is described the specific use of a 1 mL Pierce Chromogenic Endotoxin Removal Spin Column with a sample capacity of 10 mL with modifications to the manufacturer's instructions. Samples should be prepared using endotoxin-free water, and the column should only be uncapped in a BSC.

1. Prepare the 1 mL column according to the manufacturer's instructions. Each time you spin out a solution, loosen the column cap and remove the column's bottom

plug. When adding a solution to the resin slurry, secure the column cap and replace the bottom plug (see **Note 18**).

2. Prepare buffers for use according to the manufacturer's instructions. Kit modification: Spike the endotoxin-free equilibration buffer with sterile-filtered NaCl solution to achieve a final solution of 10 mM PBS 0.4 M NaCl.
3. Following the removal of the ethanol storage solution, NaOH regeneration buffer, salting with NaCl buffer, and equilibration with water and sodium phosphate buffer, the column is ready for sample addition. Kit modification: Increase the phage concentrate's NaCl concentration to 0.4 M NaCl by spiking in sterile NaCl solution (Figure 4.6H) (see **Note 19**).
4. Mix end-over-end at 4°C for 1 h.
5. Collect the sample in a sterile conical tube (see **Note 20**).
6. Once endotoxin column removal is completed, filter (0.2 µm syringe filter) the final phage preparation into a sterile conical tube (see **Note 21**).
7. Store at 4°C and maintain sterility for qualification/quantification.

3.3.4 Free nucleic acids digestion

Phage products can contain residual DNA from host cell substrates. It is, therefore, possible that such residual DNA could encode or harbor harmful molecules or elicit an innate immune response in the patient. It is not clear what health risk the DNA can pose to the product recipients, but often manufacturing can be designed to minimize the risk by reducing the levels of DNA. The World Health Organization and U.S. Food and Drug Administration guidelines recommend that 10 ng/dose and 200 base pairs be the limits of content and size of residual DNA in the final product dose [391]. Enzymatic digestion of

phage samples removes free DNA and RNA while intact virions are unharmed and protected by their protein capsid.

1. Pre-heat a heat block to 37°C.
2. Dilute DNase I stock to a concentration of 100 U/mL. Place on ice.
3. Dilute RNase A stock to a concentration of 100 U/mL. Place on ice.
4. Determine how many mLs of phage preparation were produced. If you are not using the entire stock, sterilely aliquot the fraction that will be treated.
5. Add 2.5 µL DNase I and 2.5 µL RNase A per 100 µL of phage preparation being treated.
6. Treat phage preparation for 1 h at 37°C, with the lid heating off (Figure 4.6I).
7. Store the phage preparation at 4°C.

3.4 Final Preparation Validation

3.4.1 Sterilization and titration

1. Prepare a fresh exponentially growing culture of host bacteria.
2. Air-dry an agar plate for 1 h in a BSC.
3. Pre-wet a 0.22 µm PES syringe filter using sterile phage storage buffer (i.e., PBS).
4. Decant the nucleic acid-free phage preparation into the syringe. Filter sterilize into a fresh sterile 15 mL conical tube (Figure 4.7A).
5. Aliquot 100 µL of the phage preparation into a microcentrifuge tube.
6. Store 0.22 µm filtered phage preparation at 4°C and protect from UV for longer-term storage.
7. Perform spot titration using the phage aliquot as outlined in subheading 3.1.2.

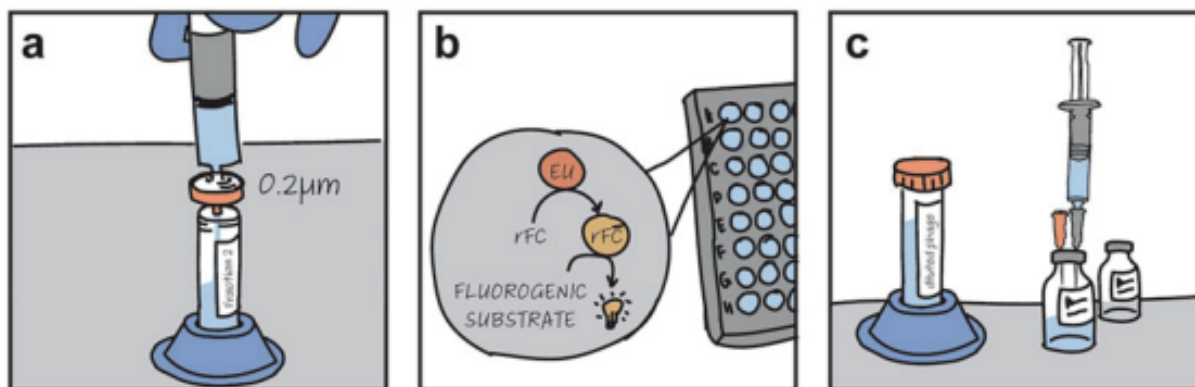


Figure 4.7 Illustrated schematic of phage sterilization, validation, and fill-finish. (A) Syringe sterilization. Syringe filter the final phage preparation before obtaining a final phage titer, validation, and/or long-term storage. (B) Endotoxin quantification. Perform endotoxin quantification using a kit with an appropriate detection range. (C) Vial filling. UV sterilize all supplies and vials before use. Filter sterilize the diluted phage preparation before sterile distribution into vials.

3.4.2 Endotoxin quantification

We recommend choosing either the EndoZyme recombinant factor C or Pierce chromogenic LAL-based assays. Traditional endotoxin quantification uses the Limulus amoebocyte lysate (LAL) test, which contains specialized blue blood cells from the wild Atlantic horseshoe crab, *Limulus polyphemus*, as a component because they react to the presence of endotoxins in a way that can be measured and quantified. The EndoZyme assay is a sustainable test, using a recombinant version of the first enzyme in the LAL clotting cascade. The EndoZyme assay also has a larger detection range from 0.005 to 50 EU/mL.

1. Select an assay and prepare the assay reagent mixture and set the microplate reader to the settings as per the manufacturer's instructions.
2. Dilute the phage sample in endotoxin-free water to achieve a dilution series of 10^{-4} , 10^{-6} , and 10^{-8} PFU/mL to ensure at least one measurement is within the linear dynamic range of the assay.

3. Add reagents and run the microplate reader protocol as per the manufacturer's instructions (Figure 4.7B).

3.5 Sterile fill-finish

The term “sterile fill-finish” is used for drug products that are sterile injectables (e.g., liquids filled in vials or syringes). Because there is no process to sterilize the phage product in its final container, it is critical that containers be filled and sealed in an extremely controlled environment [392]. Ensuring sterility is not a trivial task, and failure can have catastrophic—even life-threatening—consequences for a patient. The success of aseptic processing and sterile fill-finish operations relies on mitigating contamination from each of these sources: (i) personnel, (ii) drug product components and containers, (iii) cleanroom facilities, and (iv) equipment and processes.

1. Clean a BSC with HEPA filtration using 70% EtOH and UV sterilize for 20 min.
2. Place all containers and consumables in the cabinet and UV treat for 20 min (see **Note 22**).
3. Dilute the phage stock with the desired sterile and endotoxin-free chilled solution in a sterile 50 mL conical tube. Gently vortex (see **Note 23**).
4. Pre-wet a 0.22 μ M PES syringe filter with phage diluent (i.e., PBS), and filter sterilize the diluted phage preparation into a fresh sterile 50 mL conical tube.
5. Insert a filtered sterile vented needle into the vial's rubber stopper (see **Note 24**).
6. Draw 4.5 mL in a 5 mL Luer-Lock tip syringe with a 23G x 1-1/2" needle. Pierce the rubber stopper and aliquot 1 mL into the vial (Figure 4.7C).
7. Carefully remove both the syringe needle and the vented needle (see **Note 25**).
8. Repeat until all vials are filled.

9. When finished, protect vials from UV light and store at 4°C.

4. Notes

1. If your incubator is not equipped with refrigeration, phage production will need to be manually paused during pilot production (see Subheading 3.1.3).
2. A pump with a convex-roller head design is gentler on phages compared to a flip-type head design.
3. A 115 mm Petri plate will support a 48-spot grid of 6 columns of 8 spots each (see Figure 4.5A). We recommended using this layout to titer CFU/PFU in two sets of triplicate spots.
4. Modern microbiological shaking incubators can be refrigerated and programmed to support temperature cycles. Alternatively, cultures can be moved to 4-14°C environment. We find that harvesting phage production after 10 hours achieves titers upwards of 10^8 PFU/mL while minimizing bacterial overgrowth during additional incubation. This timing may change depending on the growth rate of the host bacteria, so perform pilot phage production (see Subheading 3.1.3).
5. Once bacterial density reaches OD₆₀₀ 0.1-0.2, it should be immediately harvested or transferred for cooling. Additional bacterial growth will result in difficulty during dead-end filtration.
6. At the time of this writing, PHASTER is accessible using a web browser and accepts FASTA files or GenBank accession numbers [393]. Alternate tools include PhiSpy and Prophage Hunter [394, 395].

7. A standard PCR protocol (such as [396]) should be used. Individual reagent volumes will depend on the polymerase/PCR kit being used. Each reaction will require 22.5 μ L of the master mix.
8. Bacterial strains can also be cured of prophages using mitomycin C induction or UV light treatment [397]. However, prophage curing can cause changes in phage susceptibility/bacterial fitness. We recommend repeating pilot phage lysate production if prophage curing is performed.
9. Culture in liquid broth medium gives bacteria easy access to the available nutrients compared to static bacterial growth on solid medium, also known as an agar culture. Agitation to keep the bacteria dispersed through the medium during incubation can aid this access further. Liquid media will also dilute out waste products as they are formed, distributing them through the culture. Consequently, a greater mass of bacteria may be obtained for an equivalent volume of liquid as opposed to solid media.
10. When pouring to a new container, the pellet may not be easily observed. Nonetheless, carefully handle the centrifuge bottles to avoid disturbing the pellet. The supernatant may appear clear; however, it is critical to perform a second spin to enhance the removal of gross bacterial debris and prevent clogging of capsule filtration units.
11. By centrifuging lysates twice, 3 L can generally be filtered with the capsule filters without the need for cleaning in between. If the 0.2 | 0.1 μ m filter becomes clogged, follow the manufacturer's instructions to clean the filter (e.g., using 0.5 M NaOH), rinse with sterile water, and resume filtration.

12. If phage lysates are in multiple storage bottles, continue to add all phage lysate to the CFF before switching to the ultrapure water dilution step.
13. The CFF will wash proteins and salts through the membrane while leaving phage particles in the retentate and exchange spent growth media for the desired storage buffer. This buffer exchange will occur simultaneously with concentration, where liters of lysate are concentrated up to 100-fold.
14. Repeat this step until the phage solution has visible clarity, denoting the remaining broth and small particles have been washed away (i.e., diafiltration).
15. This will briefly dry the CFF membrane and proceed through fraction collection quickly to ensure the membrane does not stay dry for long.
16. Phage may be collected with further repeats. However, as you collect fractions, phage concentration decreases.
17. Typically, CFF concentrates phage lysates by 1-2 orders of magnitude and concentrates endotoxin to $\sim 10^4$ - 10^5 EU/mL of buffer. We recommend proceeding with endotoxin removal using Fraction 2, which has lower endotoxin content than Fraction 1, while the phage concentration typically stays within the same order of magnitude. If time permits, titer the phage concentrate before proceeding.
18. For improved endotoxin removal, we recommend column regeneration using 0.2N NaOH overnight (16-18 h) incubation at room temperature. Regenerate the column during phage overnight production if you plan to remove endotoxin immediately after CFF concentration.
19. When using the Pierce endotoxin removal resin, an insufficient salt concentration significantly decreases final phage sample recovery. Spiking the equilibration buffer

and phage concentration to ~0.4 M NaCl, as recommended in the manufacturer's troubleshooting guide, has significantly improved the recovery of *P. aeruginosa* phage particles.

20. If desired, the column can be immediately regenerated, and the endotoxin removal process can be repeated. A second removal step may further reduce endotoxin concentration by up to twofold. However, we do not recommend going beyond two column passages as the final phage recovery is reduced in each passage.
21. Pre-wet the syringe membrane with endotoxin-free buffer to prevent loss of phages during final sterile filtration.
22. UV does not penetrate into materials, particularly plastics, very well. Although UV can disinfect an empty BSC, it will only disinfect the outer surface of any material in a BSC.
23. Filtration typically causes a loss in phage concentration. Calculate and add at least 10% more phage stock to compensate for diluted phage losses.
24. For sterile glass vials, it is necessary to vent the gas from the vial and allow the solution in.
25. At minimum, replace the needles each time a new syringe is used.

4.2: Purified phages are shelf-stable at high concentrations

When purified phage preparations are created, one concern is the loss of phage particles over time in the absence of bacterial hosts [398]. We found that PAK_P1 phages produced using the method described in Chapter 4.1.1 remained stable in IV safe, dPBS storage buffer for more than 1 year when stored undiluted in either glass or plastic containers (Figure 4.8). However, higher phage dilutions experienced a similar pattern of

decline as soon as one-week after dilution when reduced below 10^9 PFU/mL. This would suggest that phages have improved shelf-stability when stored at higher concentrations, regardless of glass or plastic container material.

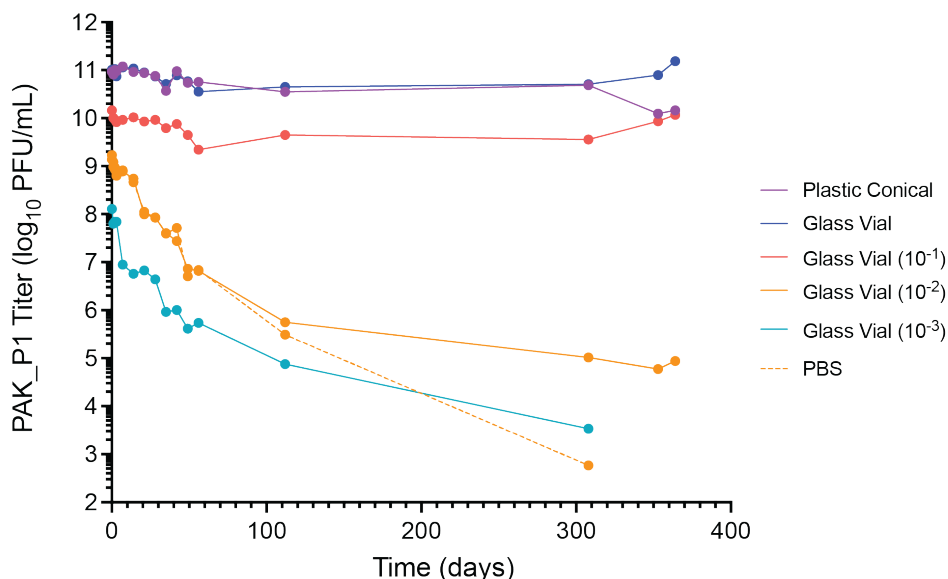


Figure 4.8 Stability of PAK_P1 phages over one year. Phages were purified using the protocol described in Chapter 5.1 (purple) and stored at 4°C, away from light or transferred into a glass vial (dark blue). Dilutions were prepared in glass vials in dPBS at 10^{-1} dilution (red), in glass vial in dPBS at 10^{-2} dilution (orange), in glass vial in PBS at 10^{-2} dilution (dashed, orange), and in glass vial in dPBS at 10^{-3} dilution (light blue).

DISCUSSION

Improved phage purification protocols have the potential to improve not only access to clinical phage therapeutics but also to help improve study of phage immunology. Because phage host interactions can be confounded by residual endotoxins, microsolute, or other components of phage lysates the production of pure phage preparations can also be used for immunological studies. How phages have the potential to interact with the human host during administration of trillions of phages directly into the body is a critical gap in our knowledge and application of phage therapeutics [76].

The method and improvement thereof described in this chapter have been used safely for human application described in Chapter 4, as well as in studies and trials elsewhere [399-401]. There remains room for future improvement however, in the initial production of phages. The kinetics of phage strains suggests that there are diminishing returns when phage resistance emerges *in vitro*, therefore strain-optimized timing during phage production is a further optimization to the production process that can reduce the bioburden of the initial phage lysate. This would also prevent induction of bacterial SOS response, including DNA repair, elongation of bacterial cells, cell surface alteration, increased mutation rates, induction of latent bacteriophages (prophages), secretion of exotoxins, and inhibition of cell division [402]. These processes could be unfavorable stressors to phage production. Additionally, because we have observed that phage activity can vary between bacterial hosts, optimizing production hosts by elimination of prophages or other virulence factors could be an additional step to limiting immunogenic byproducts. Finally, although the FDA currently limits phage preparations based on sterility and endotoxin quantification, other material may remain immunogenic to human cells. Produced phage lysates can contain a high level of free DNA (fDNA) and RNA from lysed bacterial cells. Although we recommend DNase and RNase treatments in our modified protocol, additional screening to quantify residual fDNA could be considered for batch quality assurance.

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Author Contributions Chapter 4.1.1

Conceptualization, TL and DR; methodology, TL, ACS, RE, and DR; software/code, RE; validation, TL, ACS, RE, and DR; investigation, TL, ACS, and DC; formal analysis, TL, ACS, and DR; resources, DR; data curation, TL and ACS; writing—original draft, TL, ACS, and DR; writing—review & editing, TL, ACS, RE, and DR; visualization, TL and DR; supervision, DR; funding acquisition, DR.

Author Contributions Chapter 4.1.2

Conceptualization, TL and DR; methodology, TL and DR; software/code, TL; validation, TL and DR; investigation, TL and AS; formal analysis, TL, AS, and DR; resources, DR; data curation, TL; writing—original draft, TL and DR; writing—review & editing, TL, AS, and DR; visualization, TL; supervision, DR; funding acquisition, DR.

CHAPTER 5

VALIDATION OF PHAGE THERAPY EFFICACY

INTRODUCTION

Currently phage therapy is not an FDA-approved treatment in the United States and needs to undergo phased clinical trials, largely for efficacy, to enter mainstream medicine. Traditional drug development often fails at this point due to inadequate pre-clinical target validation techniques [403]. For clinical phage use, it is therefore essential to profile phage-target interactions to maximize therapeutic action while minimizing undesirable effects. Target validation is also crucial to ensure, particularly in the complex infection environment, that phages were capable of reaching the site of infection, replicated using microbial target(s), and reduced bacterial load.

P. aeruginosa is an opportunistic bacterium with high incidences of multi-drug resistance. As a result, *P. aeruginosa* infections can be difficult to treat and can often develop into life-threatening acute or debilitating chronic infections in people with weakened immune systems, such as persons with cystic fibrosis [61, 404]. Although phages are often sought to target a variety of acute and chronic *P. aeruginosa* infections, it remains unclear whether phages actively replicate once they reach the site of infection [34]. Moreover, the suitability of *P. aeruginosa* host targets remains an open question because phages obligately require susceptible host cells to remain lytically active. Clinical *P. aeruginosa* strains thrive in different host environments and can vary widely despite the ease of their transmissibility in sensitive populations [405, 406]. In principle then, a

desirable *P. aeruginosa* target is therefore one that phages can broadly access, such as LPS, which is conserved across infection types [407].

Although phages can be isolated for nearly any bacterial target, it remains difficult to equally target multivariant infections using narrow-spectrum phages. The notion of antimicrobial ‘synergism’ using phages as adjuvants to antibiotics is therefore attractive due to targeting of a bacterium with multiple mechanisms of action [408, 409]. However, this would also suggest that phages need to compete with antibiotic killing for replication hosts. Here, we hypothesize that phages can target and replicate most effectively in clinical settings in the absence of antibiotic competition. That is, when treatment phages and standard of care antibiotics do not overlap in host range to provide phages with high initial bacterial densities for *in vivo* replication. We evaluated the ability of phages to infect chronic and acute *P. aeruginosa* isolates and found high compatibility between select anti-*Pseudomonas* phages and MDR isolates. We also perform retrospective analyses of two *P. aeruginosa* emergency investigational new drug (eIND) cases to decipher whether phages were effective against their intended targets and replicated within the human host. Finally, we screened phages against a library of clinical isolates to identify the breadth of infection types for phage targeting.

MATERIALS & METHODS

Clinical strain time-kill assays under atmospheric conditions

A hypoxic incubator chamber (Coy Laboratory Products, Grass Lake, MI, United States) was used to grow overnight cultures of each clinical strain and to precondition LB media at 2% O₂/5% CO₂. The overnight culture was then diluted into fresh medium the

morning of each plate experiment (1:10) to obtain bacteria in the early logarithmic phase of growth. Microtiter plate wells were filled with 170 μ L of fresh LB, inoculated with $\sim 2 \times 10^6$ CFU, and dosed with phosphate buffered saline (PBS, control) or phages, before being sealed with a BreathEasy™ membrane (Diversified Biotech). A microplate reader (Clariostar; BMG) incubated the microplates at 37°C under shaking conditions (120 rpm) while measuring OD₆₀₀ at 6 min intervals between shaking steps. To mimic the *in vivo* growth conditions of those isolates (1.1-2.0% O₂ in the human heart), the time-kill protocol as previously described was modified for hypoxic (2% O₂/5% CO₂) time-kill kinetics. The experimental data derived from time-kill assays were analyzed using GraphPad Prism 5.03 (GraphPad Inc., San Diego, CA, USA). Data were normalized in R Studio by blank correction and normalization to the same starting optical density (see Appendix, R Markdown).

Clinical host range analysis

Clinical isolate identities were verified through Matrix-Assisted Laser Desorption/Ionization Time-of-Flight (MALDI-TOF) or PCR and confirmed as *P. aeruginosa*. Each clinical isolates was grown from fresh CFUs inoculated into fresh LB medium and grown for 18-48 h, until an optical density of OD₆₀₀ 0.2 was reached. Bacterial culture was lawned over a pre-dried agar plate and air-dried. Plaques were measured after phages were spotted on bacterial lawns and incubated for 18h at 37°C. Phage strains tested had the following accession numbers: PAK_P1 (NC_015294), PYO2 (MF490236), LUZ19 (NC_010326), E215 (NC_042080.1), and PaCe (ON376263).

RESULTS

5.1.1: Phage suitability against chronic *P. aeruginosa* lung infiltrate (published)

This section is a reprint of a published protocol entitled: *Draft Genome Sequence of the Multidrug-Resistant Pseudomonas aeruginosa Strain PA291, Isolated from Cystic Fibrosis Sputum*. Printed in *Microbiology Resource Announcements*, September 2021. Written by Tiffany Luong, Ashley Schumann, Douglas Conrad, and Dwayne Roach. This publication describes the processes required to characterize, test antimicrobial susceptibilities of, and annotate the genome for a novel clinical isolate. Although this patient was not treated for their infection, the processes and tools described in this publication were adapted for work in Chapter 5.2.2.

ABSTRACT

Here we report the genome sequence of PA291, a non-mucoid, multidrug-resistant strain of *Pseudomonas aeruginosa* isolated from cystic fibrosis sputum. Short reads were *de novo* assembled into 190 contigs and scaffold assembled to a length of 6.26 Mbp. PhiSpy predicts that PA291 is free of prophages. Given its characteristics, PA291 may be a suitable candidate for phage therapy.

RESULTS

Pseudomonas aeruginosa causes difficult-to-treat and often multidrug resistant (MDR) infections, which severely impacts individuals with Cystic Fibrosis (CF) [410, 411]. CF patients are often required to take daily antibiotics, which promotes the development of chronic MDR infections [412]. Under investigation is phage therapy to combat MDR

infections [82, 277], which in part requires both phenotypic and genomic characterization of the patient's infectious isolate(s).

In this study, a bacterial colony from CF sputum (under UC San Diego institutional-review board approved protocol #160078) was cultured overnight on blood agar at 37°C and matrix-assisted laser desorption ionization-time of flight (MALDI-TOF) mass spectrometry identified that it belonged to the species *P. aeruginosa*. The strain was named PA291 and found to have a non-mucoid phenotype and a broth microdilution assay (performed by UCSD Clinical Molecular Microbiology Laboratory following FDA/CLSI guidelines) revealed strong antibiotic resistance to ceftazidime (minimum inhibitory concentration (MIC) > 16 mcg/mL) and intermediate resistance to aztreonam (MIC 4 mcg/mL), gentamicin (MIC 8 mcg/mL), and meropenem (MIC 4mcg/mL). Moreover, PA291 was lysed by phages PAK_P1, E215, and E217, which are currently under investigation to treat *P. aeruginosa* infections [12, 82, 281].

PA291 DNA extraction and purification was performed after overnight growth in LB Lennox broth at 37°C using the phenol-chloroform method [413]. DNA was sent to the Microbial Genome Sequencing Center (Pittsburg, PA), which prepared indexed paired end library with the Illumina Nextera DNA Flex Library Prep Kit and sequenced using the NextSeq 550 sequencer. A total of 4,939,438 reads were obtained with a mean length of 134 bases. QC of FASTQ files was conducted with *fastp* v0.21.0 [285] and short reads were *de novo* assembled using *SPAdes* v3.14.1 [414] into 190 contigs. Contigs were assembled together using the scaffolder *MeDuSa* v1.6 [415] using reference genomes: PAO1 (AE004091.2), W16407 (CP008869.2), VA-134 (CP013245.1), DVT779 (CP050330.1), SE5357 (CP054844.1), H47921 (CP008861.1), SE5443 (CP046405.1),

PAO1161 (CP032126.1), PABL048 (CP039293.1), and PAC6 (CP053705.1). Quality and metrics were analyzed using *QUAST* v5.02 [416]. All software was performed using default settings. PA291 was found to have a length of 6.26 Mbp over 3 contigs and a N₅₀ of 6.16 Mbp. Its GC content is 66.55%. *RAST* (Rapid Annotation using Subsystems Technology) [417] predicted that PA291 encodes 6114 genes including motility genes (*PilE/Tfp*, *PilB*) and alginate biosynthesis genes (*Alg*, *KinB*) [418]. In addition, *RAST*, *RGI* v5.1.1, and *CARD* v3.1.1 [419] identified 47 antibiotic resistant genes, including efflux pumps (*MATE* family, *Opr*), polymyxin resistance (*ArnC*), and fosfomycin resistance (*FosA*). PhiSpy v4.2.6 [420] did not predict PA291 carried complete viral genomes (prophages).

In summary, we report the near-complete genome of a non-mucoid MDR *P. aeruginosa* strain isolated from CF sputum. PA291's size is consistent with other *P. aeruginosa* strain genomes size of between 5.5 to 7 Mbp [421]. This strain carries several antibiotic resistant, motility, and alginate biosynthesis genes commonly associated with strains isolated from chronic CF infections [422]. Extensive antibiotic usage has negative health impacts and further promotes drug resistance. Given PA291 MDR and lack of prophages, phage therapy may be a suitable alternative treatment.

Data Availability

Data for *P. aeruginosa* PA291 can be found in the NCBI database under BioProject accession number PRJNA727612 and BioSample accession number SAMN19026471. Raw reads can be found in the Sequence Read Archive (SRA) under accession number SRR14436913. The *RAST* annotation of the genome is available on Zenodo

(<https://zenodo.org/record/5114517#.YPXH VehKguV>). The genome sequence of *P. aeruginosa* PA291 was deposited in GenBank under accession number JAGYWJ000000000, where PGAP annotation is also available.

5.1.2: Target validation of phages against *P. aeruginosa* clinical collection

One obstacle in employing phages clinically is the specificity to which phages bind their bacterial hosts. Unlike antibiotics which are shelf-stable and target a broad host-range, phages must be matched one to one with clinical isolates and often produced only after a match is confirmed [83]. To that end, major efforts have been made globally to establish ‘phage libraries’ to improve availability of phages for emergency cases [423]. Here, we found in a collection of 83 isolates from 16 patients that a collection of only five phage types were able to target 96.4% (80/83) of strains (Table 5.1).

For each clinical isolate, we grew a single, isolated CFU to exponential density ($OD_{600} \sim 0.2$) and lawned bacteria on solid agar. After allowing the lawn to dry, spots of 10^9 PFU/mL and 10^7 PFU/mL phage stocks were spotted on top. Susceptibility was scored based on whether no plaques appeared (insensitive), plaquing occurred only at the highest density (weakly susceptible), or phages cleared at both densities (strongly susceptible). The phages screened were among the six highly represented phage genera described in Chapter 3.1. This included LPS-binding phages *Pakpunavirus* PAK_P1 (NC_015294), *Litunavirus* PYO2 (MF490236), *Pbunavirus* E215 (NC_042080.1), *Bruynoghevirus* PaCe (ON376263), and type IV pili-binding *Phikmvvirus* LUZ19 (NC_010326). Unfortunately, our collection did not extend to the final genus in the list, that of *Phikzvirus* which groups anti-*Pseudomonas* jumbo viruses.

Table 5.1 Host range of 83 clinical *P. aeruginosa* isolates from 16 patients.

Reference	Site	MDR? ¹	Mucoid? ¹	Phage strain susceptibility ²				
				PAK_P1	PYO2	E215	PaCe	LUZ19
PAK	NA							
PAO1	NA							
Patient	Isolate #							
1	1	blood						
2	1	bone						
2	2	bone						
3	1	lung						
3	2	lung						
4	1	blood						
5	1	lung						
5	2	lung						
6	1	lung						
7	1	blood						
8	1	lung						
9	1	bone						
10	1	lung						
10	2	lung						
10	3	lung						
10	4	lung						
10	5	lung						
10	6	lung						
10	7	lung						
10	8	lung						
10	9	lung						
10	10	lung						
10	11	lung						
10	12	lung						
10	13	lung						
10	14	lung						
10	15	lung						
10	16	lung						
10	17	lung						
10	18	lung						
10	19	lung						
10	20	lung						
10	21	lung						
10	22	lung						

Table 5.1 Host range of 83 clinical *P. aeruginosa* isolates from 16 patients. (continued)

Patient	Isolate #	Site	MDR? ¹	Mucoid? ¹	Phage strain susceptibility ²				
					PAK_P1	PYO2	E215	PaCe	LUZ19
10	23	lung							
10	24	lung							
10	25	lung							
10	26	lung							
10	27	lung							
10	28	lung							
10	29	lung							
10	30	lung							
10	31	lung							
10	32	lung							
10	33	lung							
10	34	lung							
10	35	lung							
10	36	lung							
10	37	lung							
10	38	lung							
10	39	lung							
10	40	lung							
10	41	lung							
10	42	lung							
10	43	lung							
10	44	lung							
10	45	lung							
10	46	lung							
10	47	lung							
11	1	blood							
11	2	blood							
12	1	lung							
12	2	lung							
12	3	lung							
12	4	lung							
13	1	heart							
13	2	heart							
13	3	heart							
13	4	heart							
13	5	heart							
13	6	heart							

Table 5.1 Host range of 83 clinical *P. aeruginosa* isolates from 16 patients. (continued)

Patient	Isolate #	Site	MDR? ¹	Mucoid? ¹	Phage strain susceptibility ²				
					PAK_P1	PYO2	E215	PaCe	LUZ19
13	6	heart							
13	7	heart							
13	8	heart							
13	9	heart							
14	1	lung							
14	2	lung							
14	3	lung							
15	1	blood							
15	2	blood							
15	3	blood							
15	4	blood							
15	5	blood							
16	1	lung							

¹ Grey boxes indicate a positive result for multidrug resistance (MDR) or mucoid phenotype.

² Phage susceptibility is indicated for insensitivity in orange, weak susceptibility in light blue, and strong susceptibility in dark blue.

We first studied susceptibility patterns from the perspective of infection type. The majority of the isolates in the collection came from lung infections 73.4% (61/83), 12% (10/83) from blood, 10.8% (9/83) from heart, and 3.6% (3/83) from bone (Table 5.1). Therefore, the types of infection ranged from systemic to localized infections, but infection type did not appear correlated to susceptibility to a particular phage strain. Isolates from all 16 patients were susceptible to at least 1 phage in the collection, with 81.9% (68/83) of strains susceptible to at least 2 phages (Table 5.1).

Across the collection, we noted two consistent phenotypes which were that strains plated on solid agar displayed nonmucoid or mucoid colonies. These variants are frequently co-cultured in the lungs of chronically infected patients, with antimicrobial resistant advantages conveyed by the mixed variant infection [65]. In our collection mucoid variants were present in both lung (19/20) and heart infections (1/20) (Table 5.1).

We found that the phage collection was less effective across the mucoid isolates, with only phages PYO2 and E215 able to infect 30% and 75% of strains, respectively. This appears consistent with PYO2 and E215's ability to target biofilms, as previously described by in [82].

Because only two phages appeared poised to target mucoid isolates, we next surveyed whether phage binding receptors were related to host range. Indeed, we found that LPS-binding PAK_P1, PYO2, and E215 infected 67%, 63%, and 72% of strains, respectively (Table 5.1). Both PYO2 and E215, with their broadest host ranges, bind to the O-antigen region, located on the outermost region of LPS. This would suggest that these clinical isolates, despite several being from chronic infections or having mucoid phenotypes, conserve their O-antigen structure for immune evasion [424]. The prevalence of PYO2 and E215 in other therapeutic cases therefore seems apt given their ability to bind to what appears to be a conserved LPS molecule.

On the other hand, although PaCe like PAK_P1 also binds to the LPS core, its host range was only 31%. This would suggest variation in the binding region of these two phages, unlike PYO2 and E215 which we previously observed caused the same resistance mutation. PAK_P1, a phage which we have employed clinically twice now, appeared to demonstrate the most consistent strong plaque clearance across 59% of the library. Finally, phage LUZ19 had the poorest host range, infecting only a quarter of the isolates in the collection (20/83) (Table 5.1). This would be consistent with the loss of or change in piliation during bacterial transition into chronic infections [297]. Together, these results validate microbial targeting of these five therapeutically relevant phage genera against clinical strains.

5.2.1: Phage validation after failed *P. aeruginosa* LVAD infection treatment

Ventricular assist devices are increasingly being employed for heart disease patients awaiting organ transplant or to provide support while cardiac recovery occurs [425]. However, device-related infection occurs in 18-59% of cases and is associated with increased morbidity and mortality [425]. An 82-year-old male with a left ventricular assist device (LVAD) placed two years prior was diagnosed with recurrent *P. aeruginosa* bacteremia, driveline infection, and admitted to hospital [399]. Despite an intraoperative (IO) dose of phages at the infection site and a combined 86 days of intravenous (IV) phage treatment, the intervention ultimately failed and the patient passed away [399]. During the course of treatment, the patient was first given a cocktail combining *Pakpunavirus* PAK_P1 and *Pbunavirus* E217. After a lack of plaque clearance was observed for phage E217, the cocktail was substituted using a combination of PAK_P1 and *Nankokuvirus* PAK_P5. Here we retrospectively continue our analysis of phage activity on patient isolates to determine whether a lack of sufficient phage activity was a plausible reason for therapeutic failure.

Under atmospheric conditions emulating the heart environment (2% O₂), we retroactively tested patient isolates PAD1, PAD3, and PAD4 which were obtained before phage therapy (Figure 5.1A). In liquid co-culture single phages PAK_P1 and PAK_P5 were both effective against pre-treatment isolates, suppressing bacterial growth for upwards of 10 h. On the other hand, phage E217 activity was no different from untreated control. This would suggest that although E217 produced plaques solid agar, its activity in liquid culture was insufficient to prevent bacterial growth. However, when combined in cocktail with PAK_P1, lack of E217 activity did not appear to interfere with overall

suppression of the bacterial growth and activities were at least equal to PAK_P1 activity alone (Figure 5.1A). On the other hand, compared to expected cocktail performance, the combination of PAK_P1 and PAK_P5 did not improve suppression of bacterial growth in comparison to single phage activities (Figure 5.1A). This would suggest that combined phage pressures were insufficient to kill sensitive bacterial cells or alter resistance trajectory of the clinical isolates. Alternatively, because the phage receptor for PAK_P5 is unknown, this could imply that phages were cross-resistant, and therefore activity was no better together than separately.

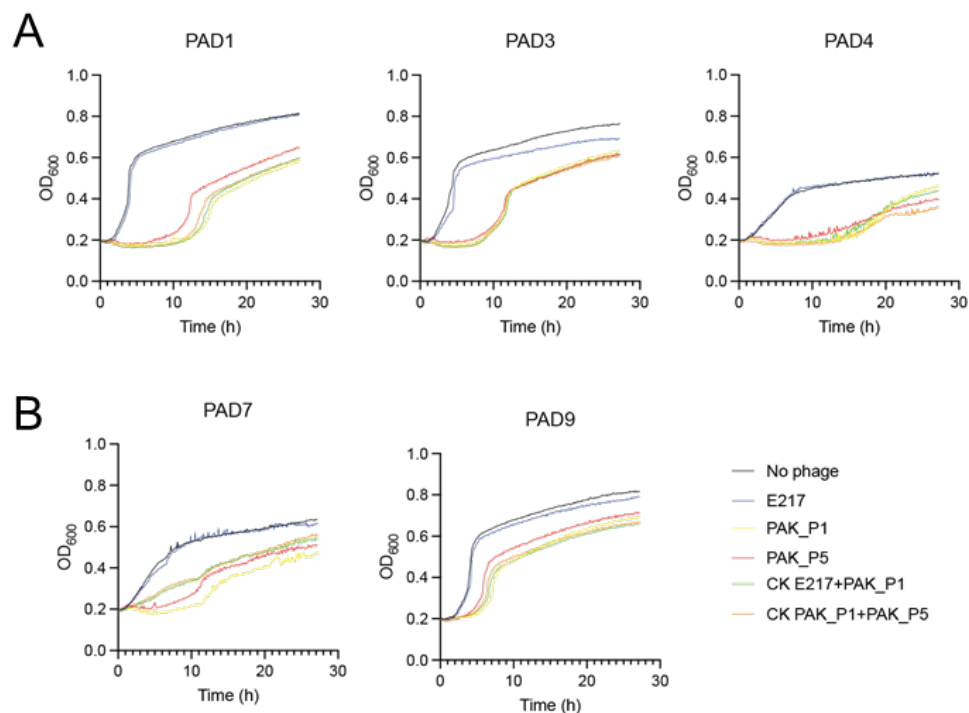


Figure 5.1 Time-kill kinetics of LVAD patient isolates. (A) Phage-naïve isolates. PAD1 was isolated from driveline 52 days prior, PAD3 from driveline 22 days prior, and PAD4 from blood 2 days prior.

When post-treatment isolates PAD7 and PAD9 obtained 56 and 92 days after initiating phage treatment respectively, were tested we saw similar trends (Figure 5.1B).

Isolates obtained post-treatment remained insensitive to phage E217, confirming that the phage was not effective for any of the variants present in the patient's infection. Moreover, we observed that phages even appeared to antagonize one another when combined in cocktail against PAD7. Both the PAK_P1+E217 and PAK_P1+PAK_P5 cocktails appeared to perform worse in the presence of both phages compared to single PAK_P1 activity (Figure 5.1B). This would suggest that competition or another form of incompatibility between phage strains limited PAK_P1 lytic activity against PAD7. PAD9 appeared to be less sensitive to both PAK_P1 and PAK_P5 with the phages suppressing growth for only half as long ~4 h compared to pre-treatment isolates. This would suggest that although phages did not appear very effective on individual isolates, their combined pressure *in vivo* may have eventually evolutionarily selected for a less phage sensitive variant to emerge/become dominant at the infection site (Figure 5.1B).

Together this would suggest that phages would have had a challenging time managing the multivariant nature of the patient's infection. Only one of the three pre-treatment variants PAD4 was potently suppressed by phages *in vitro* for >12 h. Moreover, the lack of phage activity observed during cocktail treatment would suggest that phage-phage competition may have contributed to poor *in vivo* results as well. The lack of phage E217 activity throughout treatment would suggest that phage plaquing was insufficient to select phages against a multivariant infection. No change in E217 activity was detected post-treatment, but reduced PAK_P1 and PAK_P5 activities on PAD9 would suggest that those phages may have had enough *in vivo* replication or lysis activity to select for this clinical variant. It remains unclear why phage therapy failed in this patient, but limited

phage activity on target host, phage competition, and/or developed phage insensitivity appear to have been contributing factors.

5.2.2: Phage validation after successful treatment of an acute *P. aeruginosa* exacerbation in cystic fibrosis (in preparation)

This section is a reprint of work being prepared for submission entitled: *Deep dive into second line anti-pseudomonal phage therapy to reduce the severity of resistant pulmonary exacerbations in cystic fibrosis*. Written by Tiffany Luong, Lukeman Kharrat, Kevin Champagne-Jorgensen, Wai Hoe Chin, Jennifer Melendez, David Pride, Douglas Conrad, and Dwayne Roach. This paper describes phage therapy given intravenously to an adult male with CF to treat an acute pulmonary exacerbation caused by *P. aeruginosa*. The results herein compare the clinical and microbiological outcomes of phage-antibiotic treatment to analyze phage efficacy and replication during patient recovery. This work is also in part the culmination of work performed in Chapters 3 and 4 of this dissertation to aid in the selection and purification of phages for therapy.

ABSTRACT

For many people with cystic fibrosis (pwCF) the treatments of recurrent pulmonary infections, frequently antimicrobial resistant (AMR), remain for the foreseeable future a core component of cystic fibrosis care. Bacteriophages (phages) are a quickly advancing therapeutic for human health worldwide and could offer more effective ways of addressing antibiotic resistance and fight infections prophylactically and therapeutically. But phage variability, narrow range, mutational resistance, and immunogenicity challenge efficacy.

Here we show that anti-pseudomonal phage cocktail therapy can complement standard antibiotic in reducing the severity of acute pulmonary exacerbation by nonredundantly reducing multiple infection subpopulations simultaneously. Symptom improvement and substantial recovery of lung function were associated with sufficient intravenous delivery of phages and a strong self-dosing effect that nearly eradicated the relative abundance of the narrow-targeted infection subpopulation. Nonetheless, phage cocktail efficacy was restricted almost immediately by unwanted *in vivo* immune responses and over time by the development of mutational resistance. Phage effects persisted after interventions end with treatment phages joining the pwCF lung microbiome.

INTRODUCTION

Pseudomonas aeruginosa is the most prevalent and significant pulmonary pathogen in people with the genetic disease cystic fibrosis (pwCF) [426]. Once chronic infection is established, the highly antibiotic-refractory bacterium is nearly impossible to eradicate and is associated with increased mortality and morbidity (including a more rapid decline in lung function and more hospitalizations) [426, 427]. A crucial obstacle in antimicrobial therapy is the ability of *P. aeruginosa* to rapidly adapt to the ever-changing physiology within the CF airways [65, 428] as well as current antibiotics becoming less effective due to rise in antimicrobial resistance (AMR). In the past decade, no new antibiotics have been developed and there have only been minor improvements in anti-pseudomonal antibiotic treatments [429]. Due to these highly adaptive resistance mechanisms and intraspecies competition between strains, *P. aeruginosa* is capable of persisting in the CF airways irrespective of intensive antibiotic regimens [65, 428]. There

are new therapeutic options that enhance or restore the basic defect of CF, the transmembrane conductance regulator (CFTR)-modulating protein. Modulators can improve the flow of water and chloride into and out of lung epithelial cells. However, modulator therapies are effective only in some pwCF, depending on their age and specific mutations [430]. As such, the treatment of pulmonary infections remains a core component of cystic fibrosis and new antibacterial therapies are in continuous demand [426, 430].

Bacteriophages, also known as phages, are obligatory parasites of bacteria and phage predation is a principal force controlling bacteria numbers in all habitats on Earth. There has long been interest in using phages as antimicrobial therapeutics, with more than 6,300 patients having received phage therapy around the globe since 1922 [10]. However, in the U.S. and most other countries, phage therapy has not gained regulatory approval by health authorities. Modern clinical trials have been limited, and many were primarily concerned with the safety of phages [10, 277]. Randomized clinical trials have tested the effectiveness of phage therapy compared to standard of care and so far, have demonstrated varying efficacy of phage as medicinal products [10, 31]. Most recently, a study cohort of 100 phage treatments has reinforced the safety of personalized phage therapeutics while also suggesting that clinical improvement can be achieved in as many as 77.2% of patients [21]. Despite this high overall success rate *P. aeruginosa* respiratory infections remained difficult to treat, with higher instances of resistance arising and 35% of patients showing no clinical improvement [21].

An established treatment approach is combining two or more phages, mainly aimed at decreasing the risk of phage resistance, but also to treat polymicrobial infections,

and to promote viral or antibiotic synergism [18]. Phage therapy is deemed generally safe with few reported adverse events [32]. Phages also appear compatible with most common routes of antimicrobial administration including intravenously. Beyond that, many questions remain. These include selection of patients, adequate dosing and delivery, formulation, pharmacokinetics and pharmacodynamics, and interactions with antibiotics [431-433]. Further analyses of factors associated with success and failure are necessary to improve understanding of the role of bacterial resistance and neutralizing antibody responses during phage therapy. Lastly, there are several challenges with the production of Gram-negative phages when attempting to meet current regulatory criteria [432].

In the present study, we evaluated the efficacy of anti-pseudomonas phage therapy in reducing the severity of acute pulmonary exacerbations in cystic fibrosis (Figure 5.2A). We hypothesize that reduction of a target strain within a multi-strain infection with corresponding phage replication *in situ* are markers of intravenous phage treatment efficacy. These biomarkers can be identified through assessment of expectorated sputum metagenomes and direct enumeration of strain specific bacterial densities. Furthermore, we used meta-analysis of health metrics to identify the effectiveness of phages in tandem with second-line antibiotics to maintain improved lung function after a pulmonary exacerbation and colistin-induced acute kidney injury. Our work also strives to understand the effect of both preexisting and induced phage neutralizing antibody responses. The present findings highlight phages as promising candidates for development as intravenous antimicrobials for problematic and multi-resistant *P. aeruginosa* CF lung infections.

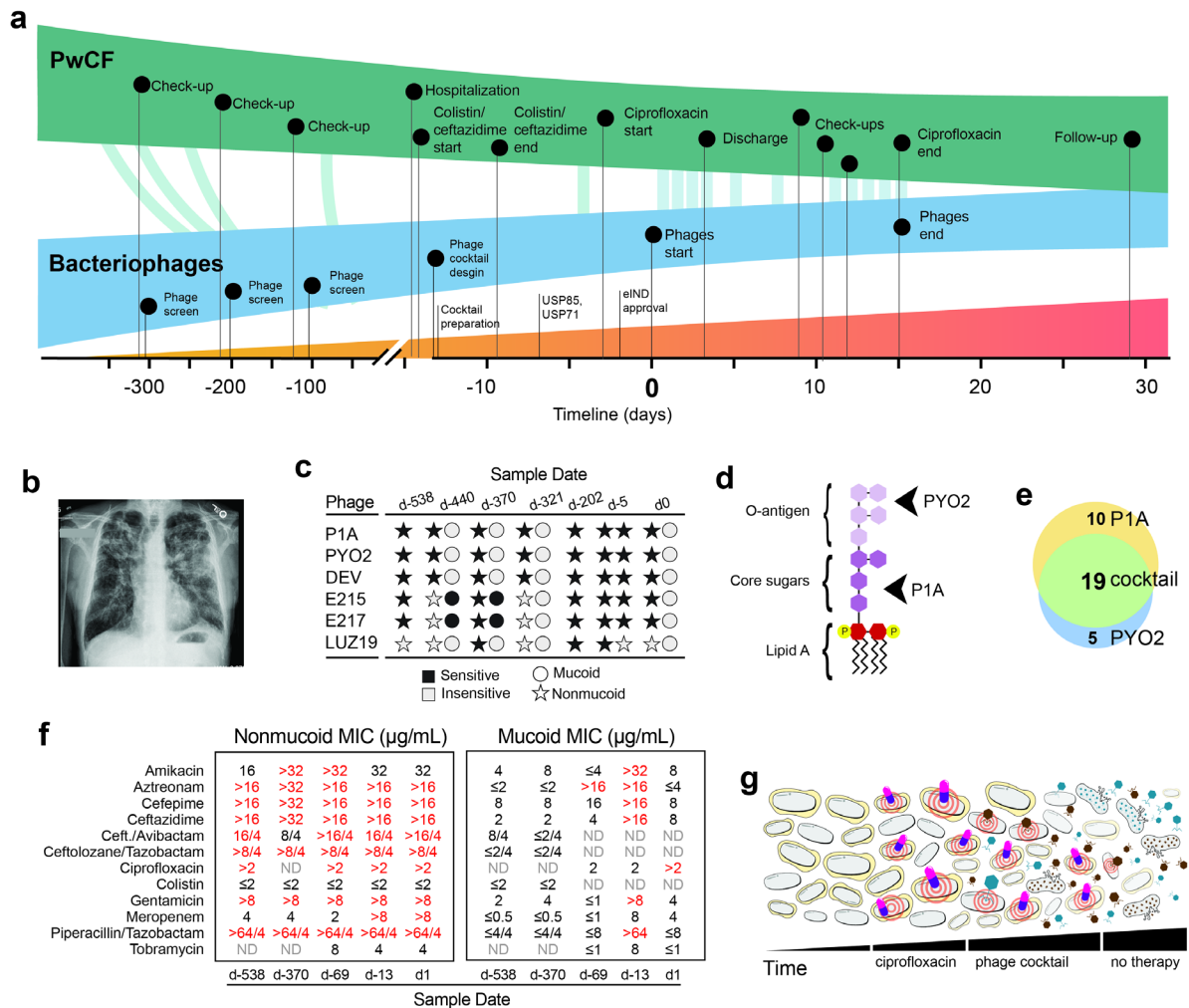


Figure 5.2 Antimicrobial treatments for acute multistrain *P. aeruginosa* pulmonary exacerbation in person with cystic fibrosis (pwCF). (A) Treatment timeline of an acute pulmonary exacerbation relative to initiating IV phages. Patient monitoring and interventions occurred at labeled circles. Twice daily IV ciprofloxacin and phages were given 8 h apart, with antibiotics and phages delivery separated 2 h. (B) Chest radiograph taken d-2 indicating ongoing bacterial lung obstruction. (C) Phage strain host range on sputa bacterial isolates. Phage sensitivity determined by plaque formation on isolate lawns after 24 h incubation. Isolates further defined by colony morphotype. (D) Schematic of phage strain receptors on *P. aeruginosa* lipopolysaccharide. (E) Venn diagram of 29/45 P1A and PYO2 susceptibility and host overlap of *P. aeruginosa* strains isolated from 13 other phage therapy candidate patients. (F) Minimum inhibitory concentration (MIC) of patient sputa bacterial isolates. Red numbers are above the resistance cut-off, black numbers are below the resistance cut-off, and values not determined (ND) are in gray. (G) Schematic of hypothetical ciprofloxacin and phage co-treatment effect on multistrain *P. aeruginosa* infection. Mucoïd cells are depicted with an alginate layer (light yellow) and nonmucoïd cells without. Ciprofloxacin is represented as a pill. Phages P1A (blue) and PYO2 (brown) are depicted corresponding to myovirus and podovirus structures, respectively.

METHODS

Patient and study design

The patient suffered an acute pulmonary exacerbation and was hospitalized 13 days prior to the start of the phage therapy. Standard of care antibiotics were given consistent with the patient's prior hospitalizations and contemporary antibiograms. The study protocol and subsequent amendments were approved by the Food and Drug Administration with local approval established by the Institutional Review Board of UC San Diego IRB#802083. Written informed consent was obtained prior to study-specific administration and consent was re-obtained when applicable. Treatments were delivered inpatient IV twice daily through a peripherally inserted central catheter (PICC) line. After discharge, the patient continued self-administration of antibiotics and phages through the PICC line, with high adherence to therapy.

Bacterial and bacteriophage strains

Pseudomonas aeruginosa PAK (NZ_CP020659.1) and PAO1 (NC_002516.2) were the laboratory strains used to propagate bacteriophage stocks (being the isolation host for phage P1A and PYO2, respectively) and perform *in vitro* screenings. Additional *P. aeruginosa* clinical strains were isolated from patient sputum using selective ceftrimide agar. Laboratory strains of *P. aeruginosa* were grown in Luria broth (LB) Lennox medium overnight (16-18 h) at 37°C with shaking. Patient strains, with a doubling time of approximately 1-2 h, were grown in LB Lennox at 37°C with shaking for 24-36 h to achieve exponential growth phase. Bacteriophages P1A (PAK_P1) and PYO2 were previously described [82, 434] and purified aliquots of each phage were used to independently cultivate stocks for clinical use and *in vitro* assays. Additional phage strains used for

susceptibility screening included DEV, E215, E217, and LUZ19 which were previously described [82, 295].

Phage quantification and efficacy testing

Phage preparation concentrations were quantified throughout the purification process and for *in vitro* experiments using the spot⁺ titering method [435]. For each sample, we performed tenfold serial dilutions of a phage stock in a microplate and spotted dilutions on a bacterial seeded agar Petri plate. For efficacy of plating (EOP) experiments, the same assay was used with standardization on phage isolation strains. Phages were serially diluted and spotted on a bacterial lawn (OD₆₀₀ = 0.2) of the patient strain. To calculate efficacy, the formula $EOP = \frac{\text{Patient Isolate PFU} \cdot \text{mL}}{\text{Isolation Host PFU} \cdot \text{mL}}$ was used.

Phage susceptibility screening

For each sample, 4μL of phage concentrate (10⁹ PFU/mL) was spotted on a bacterial seeded agar Petri plate. Spots were dried for ~15 mins and plates were incubated for 24 h at 37°C. The presence or absence of plaques was recorded for each bacterial isolate.

Antibiogram

Full panel bacterial antibiotic susceptibility testing was carried out at UCSD Clinical Microbiology laboratory using VITEK® 2 (BioMérieux, Marcy-l'Étoile, France) for most antibiotics and the disk diffusion method for ceftazidime-tazobactam. Minimum inhibitory concentration (MIC) cutoffs were determined using Clinical and Laboratory Standards Institute breakpoints [436]. For *in vitro* testing of ciprofloxacin sensitivity, representative nonmucoid (d0) and mucoid (d0) isolates were grown overnight in LB media at 37°C.

Each replicate was grown from an independent CFU, and cultures were pelleted and resuspended in sterile Milli-Q water and each well of a 96-well plate was seeded with 2×10^6 CFU. Ciprofloxacin was 2-fold serially diluted using a microtiter plate from 16 µg/mL down to 0.03 µg/mL and added to corresponding wells. Isolates (n=3) were grown at 37°C for 24 h with shaking in LB. MIC endpoints were determined from wells with no bacterial growth for 24 h. Wells with an $OD_{600} < 0.3$ were visibly clear at endpoint.

Phage cocktail preparation

Phages were prepared for clinical use as previously described [281]. In brief, liter-scale lysates of phages P1A and PYO2 were produced on laboratory strains PAK and PAO1, respectively. Amplified phages were sterilized by double centrifugation and 0.8/0.45/0.22 µm filtration. Phages were then cold cross-flow diafiltered and washed with sterile PBS before concentration. Phage concentrates were cesium chloride (CsCl) density-gradient ultracentrifuged, and a single band collected by piercing a hole in the side of the tube. Final LPS removal was done by affinity chromatography Pierce™ High Capacity Endotoxin Removal Spin Columns (Thermo Scientific, Waltham, MA, USA). Final preparations of phages P1A and PYO2 were quantified using the spot plaque titering method as described above on their respective hosts. Aliquots from each phage concentrate stock were diluted in sterile Dulbecco's phosphate buffered saline (dPBS) without Calcium, Magnesium, or Phenol Red (Gibco, Waltham, MA, USA) and mixed to create a two-phage cocktail at a final concentration of 2×10^8 PFU/mL (1×10^8 PFU/mL per strain). This solution was 0.22 µm filtered and sterile fill-finished into glass vials and stored protected from UV at 4°C. FOCUS Laboratories (Allentown, PA, USA) determined the

phage batch was sterile (USP 85) and contained <1 endotoxin unit (EU) per mL (USP 71).

Patient health metrics and pulmonary function tests

The patient was seen at UCSD Medical Hospital for all pulmonary function and hematology tests. Clinical microbiology, hematology, and x rays were performed during normal care and clinical follow ups of the patient. At each visit height, weight, BMI, and spirometry were collected. Spirometry measurements were conducted according to American Thoracic Society (ATS) guidelines and normalized using the Hankinson equations [437]. Forced expiratory volume in 1 second (FEV₁), forced vital capacity (FVC), and maximum mid-expiratory flow (MMEF) values were collected. FEV₁ and FVC tests values were then used to calculate the percentage of predicted (pp) to obtain ppFEV₁ and ppFVC as previously described [438]. Quantitative computed tomography was likewise performed at UCSD Medical Hospital, and an automated CT algorithm was used for CT quantification of % emphysema, % air trapping, inspiratory volume, and expiratory volume as previously described [439].

Principal component analysis (PCA) and hierarchical cluster analysis

Data standardization was conducted on the following variables: FEV₁, FVC, FEV₁/FVC, creatinine, and MMEF to achieve zero mean and unit variance, preparing them for principal component analysis (PCA). PCA was executed using the *prcomp* function, with centering and scaling, to simplify the dataset into principal components that capture the most significant variance and patterns. All functions used for PCA and dendrogram generation were sourced from the R *stats* package (version

3.6.2). For visualization, the *ggplot2* package (v. 3.4.3) was used to graph the first two principal components. PCA components were then hierarchically clustered using the *hclust* function, where a distance matrix derived from PCA scores was analyzed using Ward's method (*ward.D2*), which squares the dissimilarities before cluster updating to minimize the within-cluster variance [440] . The distance matrix was then converted into a dendrogram using the *as.dendrogram* function. Clusters were identified by segmenting the hierarchical tree into four groups with the *cutree* function. All statistical analyses were performed in accordance with SAS standards [441].

Sputum metagenomics

Spontaneously expectorated sputum was collected in a sterile container and briefly stored at 4°C until metagenomic DNA extraction using the freeze-boil method. In brief, homogenized sputum was stored in a temperature resistant cryovial and subjected to four 5 min cycles of freezing and boiling. Once liquified, the sample was spun down to remove debris and the supernatant was passed through the NucleoSpin Microbial DNA Isolation kit (Machery-Nagel, Düren, Germany). DNA was processed following the manufacturer's protocol for binding and elution. SeqCenter (Pittsburgh, PA, USA) prepared a library for Illumina NextSeq 550 paired-end sequencing using the Nextera DNA Flex sample preparation kit (Illumina, San Diego, CA, USA). For taxonomic classification we used the protocol outlined by Lu et al., [442]. In brief, the paired-end reads were processed with *fastp* (version 0.19.4) [285] with default settings. For removal of contaminating human reads, raw reads were masked using an 85% cutoff using *BBMap* [443] with the human reference hg19 (NCBI RefSeq: GCF_000001405.40). Reads were then taxonomically classified using *Kraken2* [291] (v2.1.3) with default settings. *Pavian* [444] was used to

visualize the results from *Kracken2* reports and obtain the number of classified and unclassified reads for each taxon. Relative abundance was determined as a ratio of taxon reads/total classified reads.

Quantification of total microbial load

Sputum samples were archived with 50% glycerol and stored at -80°C until colony quantification. Archival sputum glycerol stocks were scraped to obtain 50µL samples which were further serially diluted in sterile dPBS. After dilution, 650µL of each dilution series was spread onto Luria broth (LB) agar plates and incubated at 37°C for 24 h. Total CFU's were counted and were categorized into nonmucoid and mucoid phenotypes. Plating was repeated in triplicate for each sputum sample date (n=3).

Patient isolate growth curves and phage-antibiotic killing in microtiter plate

Representative phage-naïve (d0) nonmucoid and mucoid isolates were grown overnight in LB media at 37°C. Each replicate was grown from an independent CFU (n=3), and cultures were pelleted and resuspended in sterile Milli-Q water which were diluted with water. In a 96-well microtiter plate, 2x LB was added to each well followed by bacteria (2×10^6 CFU), phage (MOI 1), and ciprofloxacin (2 µg/mL). Sterile water was used to replace either bacteria, ciprofloxacin, or phages for growth controls or wells without treatment. The plate was incubated in a microplate reader (BMG LABTECH, Ortenberg, Germany) at 37°C with orbital shaking for 24 h where optical density readings at 600 nm wavelength (OD₆₀₀) were taken every 6 min. Phage and bacterial densities were quantified by the spot⁺ method as described with phages titers quantified on isolation host strains lawns. For ciprofloxacin-phage efficacy assays, fold-change in phage densities

was calculated by dividing viral titers at $t = 24$ h with the initial phage concentration in the well at $t = 0$ h, while fold-change in bacterial densities was determined by dividing bacterial concentration between treatment and growth control wells at $t = 24$ h.

Genome sequencing

Chromosomal DNA was extracted from overnight culture from a single colony for each isolate on Luria Broth (LB) agar at 37°C using the NucleoSpin Microbial DNA Isolation kit (Machery-Nagel, Düren, Germany). For phage DNA, phenol-chloroform extraction using gel lock tubes was performed from phage stocks purified as described above. In brief, 2mL samples were pre-treated with 10mM MgCl₂, DNase (40U), and RNase (80µg) and incubated at 37°C for 30 min until 20mM EDTA was added to stop DNase/RNase activity. Proteolysis of the phage capsid was performed by adding 200µg of proteinase K and 0.5% SDS and incubation at 55°C for 30 min. Samples were mixed with equal volume phenol:chloroform:isoamyl alcohol and centrifuged using phase lock gels (QuantaBio, Beverly, MA, USA). The aqueous phase was removed and the process was repeated with fresh phase lock gels. A final separation was performed by mixing with equal volume chloroform and the aqueous phase was collected in a fresh conical. The final solution was treated with sodium acetate (300mM) and ice-cold absolute ethanol, the solution was homogenized and DNA was allowed to precipitate. The DNA pellet was collected by centrifugation and washed twice with ice cold 70% ethanol. After one more round of centrifugation ethanol was removed and the DNA pellet was resuspended with sterile water. SeqCenter (Pittsburg, PA, USA) prepared a library for Illumina NextSeq 550 paired-end sequencing using the Nextera DNA Flex sample preparation kit (Illumina, San Diego, CA, USA). The paired-end reads were processed with *fastp* (version 0.19.4) [285]

with default settings. *REALPHY* [445] (version 1.13) was used to generate a phylogenetic tree using processed short reads mapped against reference PAO1. Phages P1A and PYO2 were previously sequenced [82, 434] and accessed from NCBI under accession numbers NC_015294.2 and MF490236.1 respectively.

Phage quantification in patient samples

Samples were collected to measure phage counts as follows: sputum was spontaneously expectorated into sterile containers, blood was extracted by during hospital and clinical visits a syringe, and urine was collected into sterile containers. Sputum was manually homogenized in 1mL of sodium magnesium (SM) buffer, weighed, and aliquoted for DNA extraction, microbial counts, phage quantification, and glycerol storage. The portion of the sputum sample for phage enumeration was 0.2µm filtered and plated onto lawns of PAK and PAO1. No viable phage plaques were found.

An aliquot of whole blood was diluted 1:100 in SM buffer and plated onto lawns of PAK and PAO1. No viable phage plaques were found, save for one sample taken 30 min after phage administration. The remainder of the whole blood was allowed to clot in untreated (no anti-coagulant) tubes for 30-60 min at room temperature (20°C) to produce serum samples. Samples were then stored at -80°C until further processing. Collected urine samples were stored on ice or at 4°C for processing. Urine was concentrated 100x using Vivaspin 20 Centrifugal Concentrator 100kDa (Sartorius, Göttingen, Germany). Concentrated samples were then spotted onto lawns of PAK and PAO1. Again, no viable phage plaques were found.

Patient antibody responses

Endotoxin-free phage preparations of P1A and PYO2 were diluted to 1×10^6 PFU/mL using dPBS. Collected serum was diluted in sterile dPBS to a final concentration of 10% total volume (accounting for the volume of the added phage). Phages were added to serum for a final concentration of 1×10^5 PFU/mL and incubated at room temperature for 2 h and 24 h. Using the spot plaque titer assay, phages were serially diluted and plated onto their isolation host strains. After overnight incubation (24 h), plaques were counted.

Cell culture preparation

Lung epithelial cell line A549 was obtained from ATCC (Manassas, VA, USA) and cultured in Dulbecco Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (Gibco, Waltham, PA, USA) at 37°C in 5% CO₂. A549 cells were seeded at 9×10^5 cells/well and grown for 24 h to a density of 1×10^6 cells/well in a 6-well tissue culture plate. The cells were then incubated in serum-free DMEM for 2 or 6 h with 10 µg/mL of PAK or P1A DNA isolated as described above. Untreated A549 cells were cultured under the same medium conditions and incubated with sterile dPBS for 2 h.

RNA-Seq

After 2 or 6 h, the culture supernatant was removed, and cells were washed in triplicate with sterile dPBS. Total RNA was prepared from treated and untreated A549 cells using the RNeasy Kit (Qiagen, Hilden, Germany). The samples were stored at -80°C. RNA sequencing was performed by SeqCenter (Pittsburgh, PA, USA). Samples were processed using the Illumina Stranded Total RNA Prep kit and were run on the Illumina NovaSeq platform. Quality control and adapter trimming was performed with

Illumina's *bcl2fastq2* Conversion Software (v. 2.20). Read quantification was performed with *RSEM* [446]. Read counts were normalized using *edgeR*'s [447] Trimmed Mean of M values (TMM) algorithm. Subsequent values were then converted to counts per million (cpm). Differential expression analysis was performed using *edgeR*'s exact test (*exactTest*) functionality against treatment groups. Because there were no replicates in each group, dispersion was estimated as .04 as per the *edgeR* user manual. Genes exhibiting an absolute log fold change (logFC) greater than 1 and a p-value < 0.05 are identified as differentially expressed. Finally, pathway analysis was performed using *limma*'s [448] “*goana*” and “*kegga*” functionality. The genes that were considered Up/Down in this analysis were at FDR < 0.1.

Differential gene expression analysis

Differentially expressed genes were used to investigate gene expression changes across different samples and time points (2h PAK, 2h P1A, 6h PAK, 6h P1A). All differentially expressed genes were combined from each sample and time point to generate a list of the top 871 differentially expressed genes. Significant genes were considered with a p-value < 0.05 and a false discovery rate (FDR) < 0.1. LogFC values of genes that did not meet the significance threshold were calculated by subtracting the treatment from the control from all quantified genes for each sample to generate a heatmap of upregulated and downregulated genes. Data for the heatmap was first converted into a matrix in preparation for heatmap generation to visually distinguish between upregulated and downregulated genes. The heatmap was created using the *ComplexHeatmap* function from the *Bioconductor* package (v. 3.18) in R. Genes with a logFC greater than 0 were categorized as upregulated, whereas those with a logFC less

than 0 were categorized as downregulated. The *ggplot2* package (v. 3.4.3) was then used to create a bar plot of the significantly upregulated or downregulated gene counts. To determine which genes were considered immunologically related, the Immunology Database and Analysis Portal (ImmPort) [449] was compared against the list of differentially expressed genes for each sample and time point.

Gene ontology and KEGG analysis

Gene ontology (GO) enrichment analysis was done using differentially expressed up and downregulated genes for each sample and time point using the *ShinyGO* enrichment tool (v. 0.80) with an FDR cut-off of 0.1 [450]. The top pathways for each GO category—Biological Process (BP), Cellular Component (CC), and Molecular Function (MF)—was curated using the *Bioconductor* (v. 3.18) package *rrvgo* (v. 1.14.1) in R to reduce the redundancy of the GO lists [451]. Significant pathways had an FDR-adjusted p-value < 0.05. The results of the top enriched pathways were visualized with a bar plot using the *ggplot2* package (v. 3.4.3). Additionally, this package was used to produce an enrichment plot that highlights the top pathways identified within the Kyoto Encyclopedia of Genes and Genomes (KEGG) via the *ShinyGO* (v. 0.80) enrichment tool using the same settings as GO [452].

RESULTS

Last resort antibiotic replaced with a combination of antibiotic and phage cocktail

A 76-year-old male with CF (genotype c.3849+10kBC>T and c.2012delT) and multiple co-morbidities experienced an acute pulmonary exacerbation diagnosed from a number of symptoms including dyspnea, increased sputum production, weight loss, an

infiltrate in his left upper lobe, and decreased lung function (for patient health summary see Supplementary Table 1). The patient's recurrent sputum cultures were positive for *P. aeruginosa* with two colony morphotypes: mucoid and nonmucoid. The multi-strain *P. aeruginosa* infection was consistent with typical adult CF exacerbations [65, 427]. The first-line treatment of the airway infiltration included the last resort antibiotic colistin adjusted for nephrotoxicity risk (1mg/kg/q12h), antibiotic ceftazidime (25mg/kg/q8h), inhaled hypertonic saline, recombinant human DNase, and bronchodilators. The first-line treatment however caused serious acute kidney injury after 7 days and had to be stopped. After premature stoppage of last resort colistin, persistent airway obstruction remained in the patient's chest x-ray and CT scan (Figure 5.2B), thus we pursued phages in the second-line therapy.

Because the patient's lungs were found to continuously harbor multiple variants of *P. aeruginosa*, we used 5 of the patient's historical isolates and 2 post-colistin contemporary isolates to screen six different phages and guide empirical phage cocktail design. We found that all the patient's nonmucoid isolates tested were sensitive to at least three of the phage strains (Figure 5.2C). In contrast, mucoid isolates were largely insensitive to the panel of phages and those active phage strains formed hazy plaques suggesting they were not that potent. We therefore hypothesized that phages would only be effective at managing the nonmucoid population. In addition, genetically and morphologically different P1A and PYO2 in cocktail would be the most potent against the nonmucoid variants. Combining P1A and PYO2 also limits collateral-resistance as these phages bind to different host lipopolysaccharide molecules (Figure 5.2D) and broadened host range by ~20% compared to either phage alone (Figure 5.2E and Supplementary

Table 2). Because the selected phage strains had poor performance on the mucoid variants, a complementary antibiotic would be necessary. Antibigrams indicated that the mucoid isolates were susceptible to most antibiotics and the nonmucoid were largely pan-resistant (Figure 5.2F and Supplementary Table 3). Therefore, we opted that the optimal second-line treatment regimen would be a combination of intravenous (IV) phage P1A and PYO2 cocktail and ciprofloxacin (400mg/kg/q18h) (For treatment schematic see Figure 5.2G). To evaluate the effects of each intervention type and delineate any adverse reactions, we staggered treatment starts (Figure 5.2A). Phage and antibiotic were given ~2 hours apart in an attempt to limit potential antimicrobial antagonism [453]. With no adverse reaction to a phage test dose at (2×10^7 PFU), a regimen of twice daily phages at 2×10^8 PFU was initiated.

Lung function recovery pattern relationship with antibiotic and phage treatments

The detrimental outcomes associated with acute pulmonary exacerbations highlight the need to understand the time course of treatment and level of recovery. Therefore, we sought to describe the patient's symptom recovery, lung function and physiology to demonstrate we provided a timely and effective second-line treatment with phages. We show that percent predicted forced expiratory volume in 1 s (ppFEV₁) and forced vital capacity (ppFVC) based on the patient's age, height, and sex had improved the most during colistin treatment before nephrotoxicity (Figure 5.3A). Adding the phage cocktail after 3 days of ciprofloxacin provided another 4 and 6% increase in ppFEV₁ and ppFVC, respectively. Overall improvements in lung function allowed the patient to be transitioned to outpatient care after 3 days of phage therapy. An elevated lung function appeared stable that first phage treatment week, before gradually receding the second

week. Despite co-therapy efficacy appearing to wane, ppFEV₁ stabilized 4% below and ppFVC at the patient's previous pulmonary function levels before his exacerbation (Figure 5.3A and Supplementary Table 1). Because exacerbations and inflammation can restrict airflow, we used thoracic quantitative computed tomographic (QCT) to compare air trapping before and after co-treatment [439]. After therapy, lung density expiratory/inspiratory ratios decreased in each of the patient's lung lobes, implying less air trapping and improvements in lung physiology (Figure 5.3B and Supplementary Table 4). Overall, regaining more than 90% pulmonary function and improving anatomical and physiological CF lung structure after an exacerbation constitutes a successful recovery [439, 454].

which we interpreted to reflect different dimensions of pulmonary function. The loadings onto PC1 and PC2 explained 90% of the total variance in the health dataset (Supplementary Table 5). The majority of sample variance is explained by FEV₁ (loading = 0.52) and FVC (loading = 0.65) in PC1 and PC2, respectively. This suggests that FVC may be associated with phage clinical outcomes. Next, we built a hierarchy of health metric variance (Figure 5.3D). Hierarchical clustering analysis revealed that the patient's early pulmonary exacerbation health status (d-13) is distinct from samples taken during therapy. Health metrics shortly after the first-line treatment (i.e., antibiotic holiday) clustered with those at the patient's baseline, albeit somewhat distantly. Health metrics clustered even more distantly after an additional three days of antibiotic holiday. These observations together suggest that the variance captured by PC1 is influenced by the efficacy of first-line therapies (Figure 5.3C). Subsequently, the formation of a new cluster comprising ciprofloxacin only (d-1) and the first ten days of ciprofloxacin-phage therapy (d1-d10) may indicate that the variance associated with PC2 is influenced by the efficacy of second-line therapies (Figure 5.3C), consistent with findings in ppFVC (Figure 5.3A). This newly formed cluster represents the patient's highest relative health state by the end of second-line treatment (d15) before health sample metrics in PC1 retreated to align with the patient's baseline samples (Figure 5.3D).

About as far back as a year prior to hospitalization, metagenomes suggesting that the patient's lung sputum carried relatively low microbial load (11-13%) compared to during his pulmonary exacerbation (Figure 5.3E and Supplementary Table 6). We found that 8 weeks prior to hospitalization, the patient's sputum microbial abundance had already increased 6-fold, which foreshadowed his exacerbation. Lacking metagenomes

at admission and early therapy, we were unable to determine microbial abundance or the initial effects of treatment on sputum metagenomes. Nonetheless, microbial abundance 3 days into phage co-treatment was at similarly elevated levels as 8 weeks prior to hospitalization (d-69). This seemingly suggests the patient's lung microbial load had declined from his max load during first-line treatment. This microbial reduction continued during antibiotic-phage treatment receding a further 50% by day 7 and 97% by day 12 (Figure 5.3E). Reducing microbial load 74% less than that carried during non-exacerbation states implies antibiotic-phage co-therapy efficacy, prescription of adequate dose and duration, and the presence of adequate tissue concentrations at the target site.

Phages target a specific bacterial morphotype in the lungs

As expected, the patient's CF lung metagenome had an extremely low bacterial diversity, virtually being only *P. aeruginosa* (Figure 5.4A). Although relative bacterial abundance declined during therapy (Figure 5.3E), *P. aeruginosa* did not lose its dominance (Figure 5.4A). We therefore cultured *P. aeruginosa* from sputa to quantify each colony morphotype's abundance. We found mucoid colonies were dominant in sputa throughout the treatment period and thereafter. This would be consistent with the notion that mixed strain infections are dominantly mucoid in chronic CF with pathoadaptations to enhance survival of both mucoid and nonmucoid variants [65, 455]. However, mucoid counts steadily declined by 1.5 logs during treatment (Figure 5.4B), which correlates with reductions in the metagenome relative abundance (Figure 5.3E). Because mucoid isolates were non-hosts to the phages (Figure 5.4C), the reduction of mucoid population was likely due to the antibiotic alone. On the other hand, phage treatment was catastrophic to the nonmucoid population reducing burden by nearly 4-logs within the first

3 days; majority of the decline was in the first 24 hours. However, as phage treatment went on it became less effective allowing the nonmucoid population to increase by a log before plateauing by the end of treatment (d15) and thereafter (Figure 5.4B). Altogether, combining antibiotics and phages to nonredundantly target different infection populations led to clinical improvement beyond expectations with either agent alone.

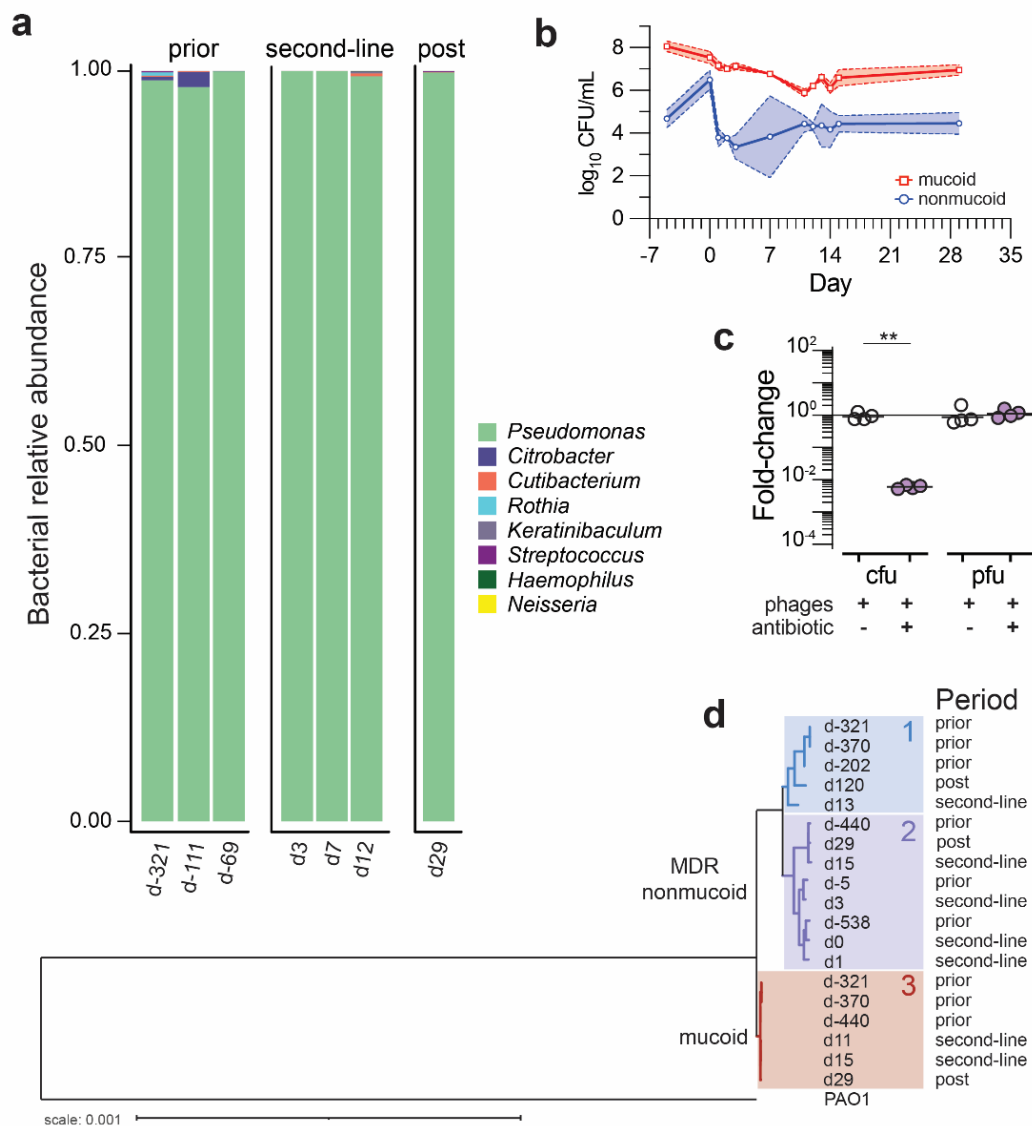


Figure 5.4 *P. aeruginosa* morphotypes are reduced in the CF lung by nonredundant antimicrobial action. (A) Relative abundance of bacterial reads in extracted patient sputum metagenome. (B) Direct quantification of colony-forming units (CFU) in patient sputa. Diluted archival sputa directly cultured on solid growth media and colonies quantified by morphotype. Error bars are 95% CI. (C) Effect of phage cocktail and ciprofloxacin-phage treatments on liquid mucoid culture. Phage-naïve mucoid cells co-cultured with phages at equal ratio and/or ciprofloxacin (2µg/mL). Bacteria and phages were quantified after 24 h by plating on solid growth media and bacterial lawns, respectively. Error bars are SEM; $p < 0.05$. (D) Maximum likelihood phylogenetic tree of patient *P. aeruginosa* isolates rooted by outgroup PAO1. Branch insets are arranged by morphotype and colored according to the numbered descendant isolate subclade.

We next retrospectively surveyed the accumulated isolates of *P. aeruginosa* to determine whether there were changes in the chronic infection landscape post treatment. Although we initially thought there were two major strain populations, isolate phylogeny uncovered that the patient's airways harbored *three* main phylogenetic groups (Figure 5.4D). Nonmucoid isolates fell into two clades, while the mucoid variants fell into a single clade. Nonetheless, maintenance of three main lineages after treatment implies that phage therapy did not open a niche for new strain populations, *P. aeruginosa* or otherwise (Figure 5.4A). Historical nonmucoid isolates from ~1 year prior to the exacerbation episode and three months post treatment grouped in clade 1, while most nonmucoid isolates during treatment grouped together in clade 2. Within clade 2 was also the sole historical isolate (d-440) collected 6 weeks before the patient's last exacerbation episode (d-393).

Together, this suggests that clade 2 variants were the secondary dominant population during acute pulmonary exacerbation along with the predominant mucoid population (Figure 5.4B). As such, clade 1 variant dominance appears to have a modest association with periods of the patient's stable chronic infection state. On the other hand, historical and contemporary antibiotic sensitive mucoid isolates were highly similar, with minimal genome mutations occurring after treatment. The simple structural properties of the phylogenetic tree, combined with culture findings, highlights the heterogeneous populations during the exacerbation and relative stability of the variant lineages despite aggressive antimicrobial treatment targeting all three clades.

Intravenous phages are delivered to the airways and self-replicate *in situ*

IV antimicrobials are held to be the most effective form of drug delivery and as such, was considered the preferred phage delivery method. Recently, however, the assumption that IV phages are optimal for treatment of pulmonary infections in pwCF has been questioned [32]. To establish if IV phages were sufficiently transited to the lungs, we first attempted to isolate treatment phage from expectorated sputa. After numerous failed attempts to observe phage plaquing (data not shown), we instead used metagenomic quantification of phages in patient sputa. Perhaps not surprisingly after years of chronic *P. aeruginosa* infection, the patient's CF virome consisted of phages in the same genera *Pakpunavirus* and *Litunavirus* as P1A and PYO2, respectively (Figure 5.5A and Supplementary Table 5). Interestingly, both genera along with *Pbunavirus* dominated total viral abundance. Within 3 days of IV phages, *Pakpunavirus* relative abundance increased to 99% of viral reads for at least the next 5 days. By the end of the second treatment week, *Pakpunavirus* relative abundance declined by 50%. Although pulmonary delivery of IV drug is complex in CF due to airway mucus plugging and heightened defense mechanisms, this indicated sufficient delivery of P1A into the CF airways. In addition, the higher relative abundance P1A during early dosing versus a lower relative abundance during later doses provides evidence that P1A replicated *in situ* (Figure 5.5A). Because the phages possess the ability to self-replicate in the course of their antibacterial action, a reduction in bacterial abundance was the reciprocal effect (Figure 5.3E and 5.4B).

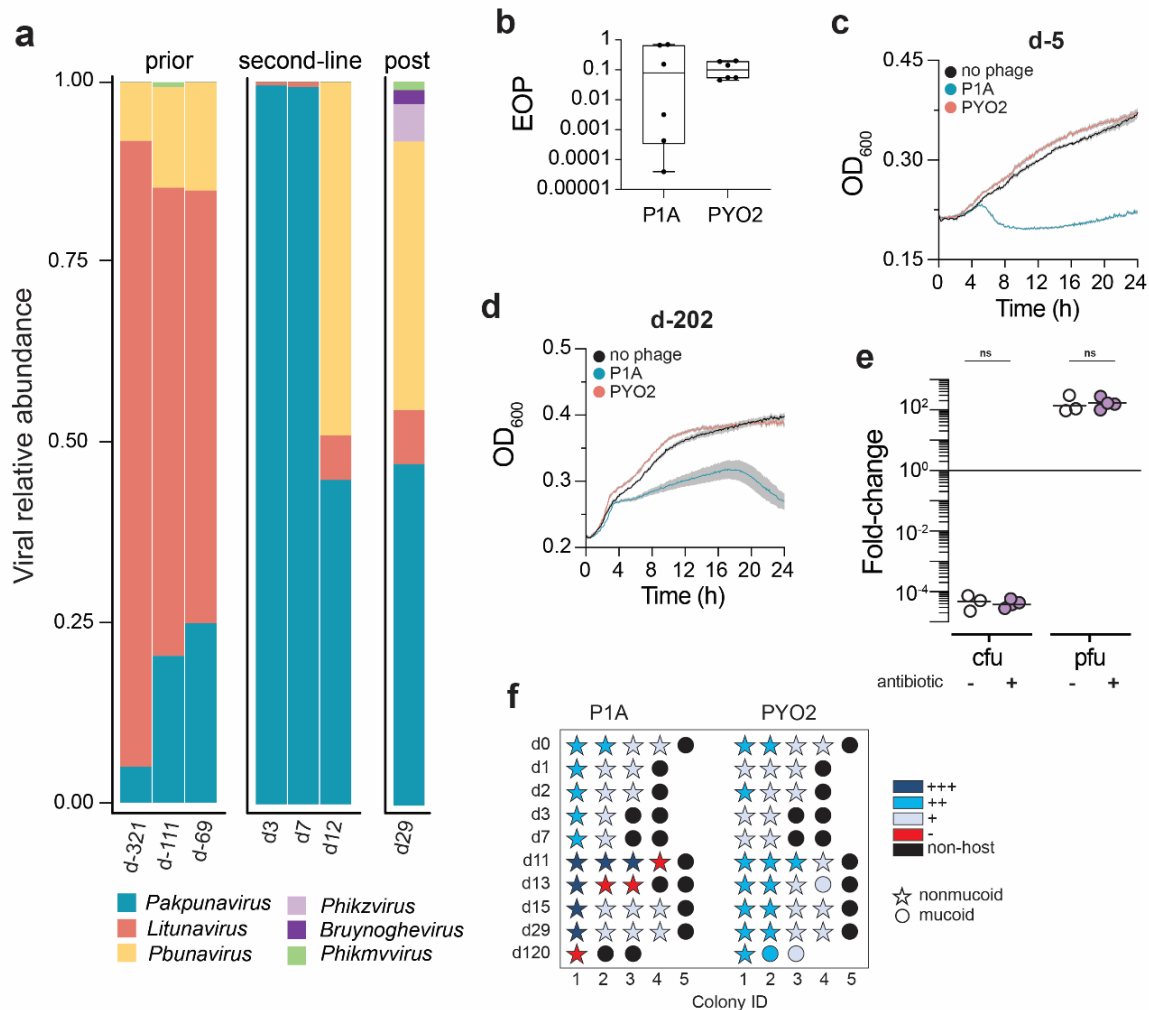


Figure 5.5 P1A demonstrated robust in situ replication following intravenous administration. (A) Relative abundance of bacterial reads in extracted patient sputum metagenome. (B) Efficiency of plating (EOP) of phages on 9 historical (phage-naïve) *P. aeruginosa* nonmucoid isolates. EOP is the ratio of isolate lawn PFU/mL divided by isolation host PFU/mL. Error bars are SEM. (C-D) Time-kill kinetic curve of phages co-cultured at equal ratio (MOI 1) with phage-naïve nonmucoid isolates from (C) d-5 (clade 2) and (D) d-202 (clade 1) in liquid growth media. Error bars (shaded) are SEM. (E) Effect of phage cocktail and ciprofloxacin-phage treatments on liquid nonmucoid culture. Phage-naïve nonmucoid cells co-cultured with phages at equal ratio (MOI 1) and/or ciprofloxacin (2µg/mL). Bacteria and phages were quantified after 24 h by plating on solid growth media and bacterial lawns, respectively. Error bars are SEM; $p < 0.05$. (F) Phage strain host range on sputa bacterial isolates. Colonies collected during and post second-line treatment were further defined by morphotype. Phage activity was scored by plaque clarity on isolate lawns after 24 h incubation.

However, adequate IV delivery and/or *in situ* replication may be phage strain dependent. PYO2 had declined in relative abundance during phage treatment (Figure 5.5A). Thus, PYO2 delivery may have been blocked or the phage was delivered but

replicated poorly. To test the latter, we compared efficiency of plating (EOP) between PYO2 and P1A (Figure 5.5B). There was no apparent mean EOP difference on solid growth medium. We then compared time kill kinetics between PYO2 and P1A (Figure 5.5C and 5.5D). On a clade 2 isolate associated with the patient's exacerbation variants (Figure 5.4D), P1A was significantly better at rapidly reducing the bacterial population compared to an apparent lack of activity by PYO2 (Figure 5.5C). Although PYO2 was then expected to outperform on clade 1 isolates, having been at high relative abundance in the virome when this variant was assumed to be in high abundance (Figure 5.5A), we observed a similar lack of activity in liquid culture in comparison to P1A (Figure 5.5D). This showed that PYO2 was significantly inferior at controlling nonmucoid isolates in liquid culture compared to P1A. Moreover, Figure 5.5E shows that MIC ciprofloxacin neither inhibited nor enhanced phage activity in the same time kill assay. Together, we cannot determine whether PYO2 was sufficiently IV delivered, but it does appear PYO2 was outcompeted by P1A in the CF airways.

As mentioned, prolonged treatment showed that P1A lost its dominance in sputa relative abundance (Figure 5.5A: d12). This timeframe coincided with the isolation of phage P1A resistant colonies (Figure 5.5F). It was unclear whether the resistant subpopulation had a detrimental effect on *Pakpunavirus* abundance since the phages remained >40% long after IV phage treatment ended (Figure 5.5A: d29). With these retrospective insights, P1A was appropriate for the given pulmonary infection with both the route of administration and duration of treatment being sufficient. In addition, dose did not appear to be a major influence on phage abundance relative to self-replication. The same cannot be concluded for PYO2.

Mapping the effects of phages on the immune system, including neutralization

Phages of all types have the likelihood to influence the human immune system [76]. Because the mechanisms of these perturbations are poorly understood, we set out to understand the development, specificity, and neutralizing potency of the patient's humoral immune response against the treatment phages. Indeed, phage treatment led to a sharp increase of sera neutralizing activity against both PYO2 and P1A (Figure 5.6A). Several key points regarding the kinetics of neutralization can be made. First, significant neutralization of P1A appeared after 15 days of treatment. This is consistent with the time needed to develop neutralizing antibodies in detectably large amounts after first exposure to phage antigens [456]. Second, a strong neutralization of PYO2 occurred as early as 11 days after treatment onset (Figure 5.6A). This would have been too early to develop humoral immunity against a *newly* detected viral antigen, suggesting the patient had previously been exposed to PYO2-like phages and had developed immunity then. This would be consistent with a high relative abundance of *Litunavirus* phages related to PYO2 in the patient's airway virome before therapy (Figure 5.5A). CF is a classic example wherein dysfunctional defenses lead to a chronic pulmonary infection [426, 455], which also promotes the overabundance of phages that infect these same chronic pathogens. Taken together, these findings illustrate that both phage-specific and neutralizing antibody responses occur rapidly after initiating phage therapy.

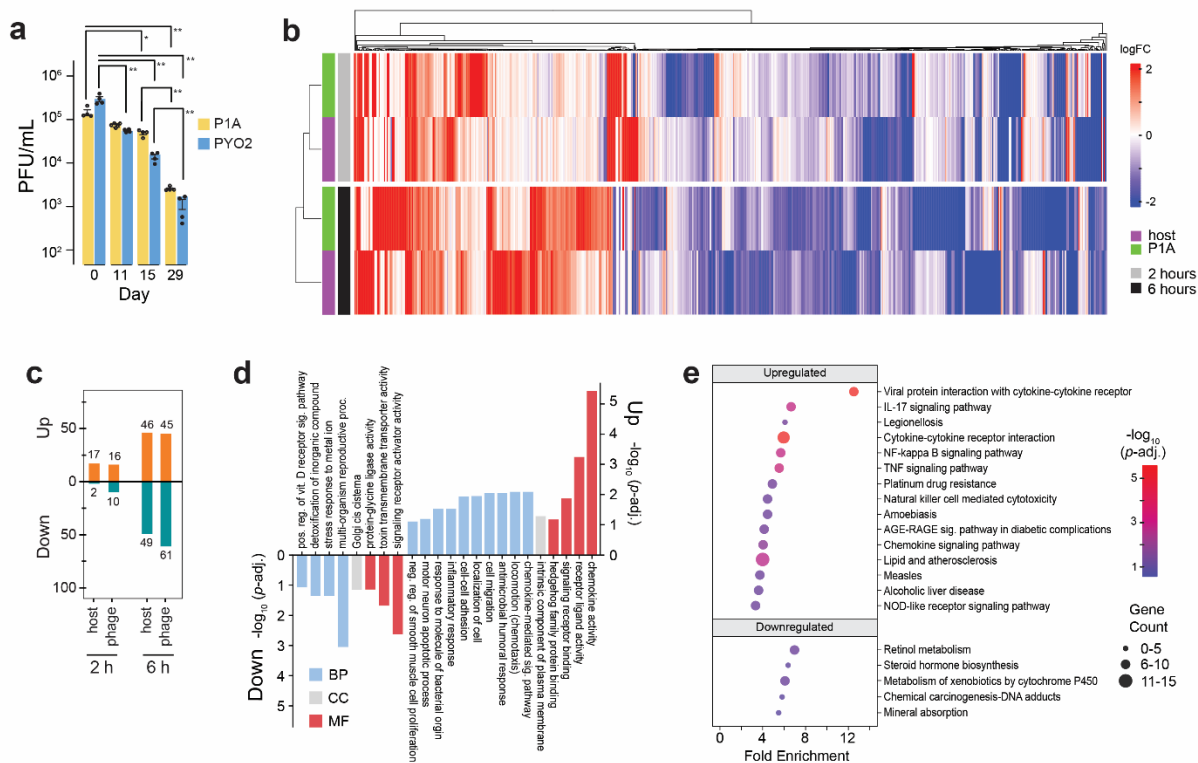


Figure 5.6 Significant neutralization and recognition of phage P1A potentially limited phage efficacy. (A) Anti-phage neutralization activity of treatment phages in patient sera. Phages (10^5 PFU/mL) were co-cultured with 10% diluted archival sera and plaque formation was quantified after 24 h incubation. (B) RNA-seq heatmap of differentially expressed genes (DEGs) in A549 lung epithelial cells following DNA stimulation. DEGs were determined as $|\log_{2}FC| > 1$. (C) Significantly regulated immunological DEGs in A549 cells after DNA stimulation. (D) Gene ontology (GO) enrichment analysis for DEGs after 2 h phage DNA exposure. GO terms were grouped as biological processes (BP), cellular components (CC), or molecular functions (MF). p-values were adjusted for false discovery rate (FDR) < 0.1 . (E) Kyoto encyclopedia of genes and genomes (KEGG) pathway enrichment after 2 h of P1A exposure. p-values were adjusted for FDR < 0.1 .

To further understanding of how P1A could affect the immune system and its consequences for treating pulmonary disease, we catalogued the response of lung epithelia after exposure to the phages' DNA. Viral DNA, which is easily exposed during infection, is a typical PAMP that is sensed by host cells [76]. Differential expression analysis identified 871 differentially expressed genes (DEGs) (p -adjusted < 0.05), spanning 179 upregulated DEGs and 352 downregulated DEGs (Figure 5.6B,

Supplementary Figure 2, and Supplemental Data File 2). P1A-exposed cells also exhibited increased transcription levels of many inflammatory cytokines/chemokines, including CXCL1, CXCL2, CXCL3, CXCL5, interleukin-8 (IL-8), IL1RAP, IL-27RA, IL32, LIF, and MST1P2. The upregulation of type I interferon (IFN)-stimulated genes (ISGs) was also detected, including IFI44, IFIT2, BISPR, and IFN-induced oligoadenylate synthetase (OAS) antiviral genes. There was also a marked upregulation of nucleic acid sensing genes including DHX58 and notably, several DEGs revealed a change in the expression of factors mediating humoral immunity, including IL-11, ICAM1, ILDR2, CCL2, CD40, PILRA, PILRB, RAET1G, RAET1E, and EBI3.

Significantly enriched Gene Ontology (GO) terms (p -adjusted < 0.05) among top up-regulated DEGs included antimicrobial humoral response, chemokine-mediated signaling pathway, cell-cell adhesion, and signaling receptor binding, while down-regulated DEGs were involved in tubulin glycine ligase activity and growth factor activity (Figure 5.6D and Supplemental Data File 2). Downregulated genes associated with microtubules that make up extracellular structures such as cilia, likely reflect host epithelial stress. Notable annotations from the Kyoto Encyclopedia of Genes and Genomes (KEGG) include viral protein interaction with cytokine-cytokine receptor (hsa04061), TNF signaling (hsa04668), and NF-Kappa B signaling (hsa04064) and IL-17 signaling pathways (hsa04657) (Figure 5.6E and Supplemental Data File 2). The highly enriched “viral protein interaction with cytokine-cytokine receptor” pathway suggests viral engagement of toll-like receptors [457] and enrichment of both TNF and IL-17 signaling indicates initiation of cell defense and repair responses that would include the production of chemokines, in particular neutrophil chemoattractant [458]. This is largely consistent in

our previous study that found phage mildly stimulated peripheral blood neutrophils to secrete the chemoattractant interleukin-8 (IL-8) [459]. Overall, these results obtained in epithelial culture suggest a key role of phage DNA in the induction of both the interferon and inflammatory pathways that can drive host humoral responses.

DISCUSSION

Currently, we are facing a growing crisis as resistance to antibiotics continues to spread [429]. Thus, it is more important than ever to use alternative antimicrobials as effectively and appropriately as possible. We found it is possible, and indeed likely, that phage therapy can be used as a first-line treatment with antibiotics to prevent deep-seated bacterial growth in a complex infection environment. Although herein was an assessment of anti-pseudomonal phages given as second-line treatment for a pulmonary exacerbation, we show how effective phages were at reducing growth of an existing *P. aeruginosa* population in the harsh CF lungs. We conducted a first-of-its-kind adaptive treatment strategy to replace failed colistin with a nonredundant ciprofloxacin–phage regimen. The efficacy of the strategy, as compared to standard treatment, was superior in that it combined potencies of each antimicrobial singly to broadly manage growth of a mixed strain infection. This treatment strategy was also associated with a shorter total duration of therapy than would have been possible with standard treatments and with no evident safety concerns. In total, expansion of chronic lung pathogens is associated with the onset of acute exacerbation where a quarter of pwCF fail to recover to baseline lung function despite extensive antibiotic usage [454, 460]. Against extremely poor recovery odds, the patient described herein successfully recovered his baseline lung function.

As pwCF age, treatment of *P. aeruginosa* is often complicated by chronic, mixed-strain infections that have gained several selective advantages to withstand antimicrobials [65, 455]. As a result, combinations of antimicrobials have become the common approach to treat pulmonary exacerbations [426]. The main strength of this study was the pragmatic design that relied on co-operation of antimicrobials with different mechanisms of action and target strains to eradicate the patient's pulmonary infection. In addition, we conducted a difference-in-difference analysis with staggered treatment initiation to assess the impact of each change on patient outcome. We found that antimicrobial co-operation was neither synergistic nor antagonistic, wherein ciprofloxacin inhibited the growth of a portion of the population (i.e. mucoid) and the phages then eliminated the survivors (nonmucoid). Although IV phage treatment provided a more immediate effect with greater potency compared to ciprofloxacin (Figure 5.4B), neither antimicrobial alone would have been adequate for patient recovery. Indeed, phages were self-dosing (i.e. amplified *in situ*) during the bacterial-killing process, though with some limitations such as dependence on relatively high nonmucoid densities (Figures 5.3E vs 5.5A) and continued host cell sensitivity (Figure 5.5F). Decline post-treatment corresponded to continued high abundance of phage P1A in sputum, implying active phage replication in the airways and continued therapeutic effect long after active administration. Due to their implications in chronic disease, the persistence of phages in the microbiome has recently become the topic of much exploration [461, 462]. Together, our findings establish nonredundant functions for phages in the presence of antibiotics during infection treatment and argue for multilayered effects on the basis of a division of labor.

Likewise to several studies [20, 463], we found that traditional phage susceptibility testing failed to account for extrinsic determinants of phage activity present in the complex infection environment and was therefore a poor predictor of phage treatment outcome. Beyond genetically encoded mechanisms of phage resistance, the determinants of phages activity during infection remain poorly understood, and treatment failure is common [399, 464]. We had planned to implement duplicative phages (i.e. cocktail) in such a way that if one phage failed, the alternative phage would continue to provide treatment efficacy [465]. However, we essentially provided monophage therapy with P1A as PYO2, the second component of the cocktail, failed to achieve a substantial presence in the lungs (Figure 5.5A). We found several factors that likely contributed to the therapeutic inhibition of PYO2. First, PYO2 exhibited a low potency in inhibiting nonmucoid population growth in liquid culture compared to P1A, despite having a similar EOP on solid culture medium (Figure 5.5B vs 5.5C). This suggests that even if PYO2 was sufficiently delivered to the airways, the phage would replicate poorly and thus be outcompeted by P1A (Figure 5.5A). Second, we found a strong PYO2 neutralization *preceding* the time required for the patient to generate a humoral response, which would have hampered the phage's delivery to the airways. The hyperresponsive microenvironment of the CF lungs is known to continuously recruit and sequester high numbers of immune cells [466]. During chronic bacterial infection plgR expression and IgA production are selectively upregulated in CF lung, sputum, and sera [467]. Although primarily targeting *P. aeruginosa*, excessive inflammation and antibody sequestration in the CF lungs suggests that extracellular antigens such as phage capsids inadvertently become IgA targets [76, 456]. Together, as observed in other phage treatments of chronic

pulmonary infection [468, 469], cross-reactivity with prior phage exposure may have limited the effect of PYO2 despite active administration into a host rich environment.

It is unclear if bacterial debris from high cell lysis compounded as an adjuvant to stimulate the immune response against the phage particles. However, activity by other bacteriolytic agents such as lysozymes is correlated to localized increases in inflammatory cytokine production due to the enhanced release of immunomodulatory bacterial products such as peptidoglycan [470]. In this context, elevated levels of bacterial products released from violent phage P1A replication may have likewise provoked increased localized innate immune activation in the CF airways. Therefore, treatment was a double edge sword. Moreover, highly efficient phages also appear to trigger strong adaptive immune responses that in time clear the therapeutic agent [20, 463]. We surmise that extremely high abundances of P1A from both sufficient delivery and robust viral replication provoked an antiviral immune response, leading to the development of a P1A neutralization response. This is also in line with anecdotal evidence that direct (i.e., IV) phage administration appears to elicit stronger antibody responses in comparison to indirect routes such as oral ingestion [76, 235]. In addition to host responses, the lack of phage redundancy reduced the activity depth [18] of phages on the nonmucoid population, thereby indirectly limiting long-term P1A potency. Although treatment was designed to constrain the evolution of bacterial phage resistance through the use of multiple phage receptors (Figure 5.2D), resistance to P1A emerged by d11. Fortunately as we previously studied [12], sufficient host immune responses appear to have prevented a P1A-resistant population from overtaking the CF environment as resistant isolates did not allow nonmucoid numbers to increase or form a fourth isolate lineage

(Figure 5.4B, 5.4D). Against compounding human and bacterial host factors, P1A remained relatively high post-treatment. Indeed, a continual presence maintained a small therapeutic effect post treatment and kept the nonmucoid population at bay.

These retrospective analyses together support the selected phages, antibiotics, and provide a modest framework for future treatment design. Combined, immune responses to the treatment phages were asymmetrical but suggest that a phage abundance threshold determines tolerance or elimination of phages in context of the CF microenvironment. When considering future phage candidates, i.e. E215, the risk of neutralization therefore appears to primarily depend on their pre-treatment abundances in the lung virome. This suggests an understudied role for studying the pre-treatment infection CF virome in context of phage selection. We also designed co-treatment for improved breadth using antibiotics as opposed to depth. Alginate overproducing mucoid isolates are implicated in persistent CF lung biofilm formation and the clogging of CF airways [428]. Reduction of the mucoid cell numbers therefore appears consistent with observed FEV₁ improvements in first-line and early second-line treatment (Figure 5.3A, 5.3C, 5.4B). On the other hand, pulmonary infection studies have also suggested that FVC improvements independent of FEV₁ change is related to reductions in airway inflammation [471, 472]. Bronchodilators for COPD patients or high-dose ibuprofen (non-steroidal anti-inflammatory drug) significantly improved or prevented FVC decline with decreased inflammatory cytokine markers [471, 472]. Similarly, our meta-analysis of lung health suggested that the addition of phages had little bearing on FEV₁ change but was correlated to improved FVC (Figure 5.3C). Phage targeting of nonmucoid cells, therefore,

may have instead reduced the inflammatory milieu of the airways to improve patient FVC over their two-week course.

To conclude, while phages are potent bacterial killers it is unclear whether they are effective in concert with standard of care antibiotic, precisely identify and attack certain types of bacterial cells, replicate sufficiently in situ to be self-dosing, or have direct effects related to immune cell function in a clinical setting. Individualized phage therapy cases have substantially improved understanding and implementation over the past several decades, but sophisticated interrogation of efficacy in context of the specific infection environments is needed. In this study, the designed division of antibiotic and phage activity provided a clear framework to study the outcomes of phage treatment. Although the patient's mixed strain infection and specific susceptibilities lent itself to the design, future development of phage studies and clinical efficacy trials will require such context to unravel the complexity of antibiotics, phages, bacteria, and extrinsic host interactions.

SUPPLEMENTARY INFORMATION

Supplementary information includes one .pdf file (two figures, five tables) and one .xlsx file.

Extended figures, tables and data files:

Supplemental Data File 2 (LUONG_CH5_CF_SUPPLEMENTARY1.pdf) containing:

- Supplementary Figure 1 (related to Figure 5.2F)
- Supplementary Figure 2 (related to Figure 5.6B)
- Supplementary Table 1 (related to Figures 5.2A, 5.3A, and 5.3C)
- Supplementary Table 2 (related to Figure 5.2E)

- Supplementary Table 3 (related to Figure 5.2F)
- Supplementary Table 4 (related to Figure 5.3B)
- Supplementary Table 5 (related to Figure 5.3C)
- Supplementary Table 6 (related to Figures 5.3E, 5.4A and 5.5A)

Supplemental Data File 3 (LUONG_CH5_CF_SUPPLEMENTARY2.xlsx) containing:

- Tab 1 DEG (related to Fig 5.6C)
- Tab 2 GO (related to Fig 5.6D)
- Tab 3 KEGG (related to Fig 5.6E)

Code availability

Data were analyzed using the standard R studio pipeline, or with the stated variations. User scripts will be shared upon request.

DISCUSSION

Many of the techniques and approaches classically applied to studying the pharmacokinetics of other small drugs or molecules are incompatible with phages due to their size, active replication, and phage-bacterium-host interactions [215]. Indeed, attempts to track phage abundance using PCR or qPCR have been largely unsuccessful or inconsistent [469, 473]. Microbiological and bioinformatic approaches are therefore necessary to steer the course of phage therapy and improve outcomes [34]. Though real-time analyses may not always be possible, assays that quantitatively assess phage replication, changes in bacterial abundance or variation, and clinical biomarkers of phage activity are necessary to understand phage pharmacokinetics and treatment outcomes.

Failed phage therapy to treat an LVAD infection appeared to have multiple components working against phage treatment. Phage activity appeared to be limited by the patient's multivariant infection. Additionally, although two of the phages did have activities on the patient's variants, loss of sensitivity to phages PAK_P1 and PAK_P5 appears to have limited phage activity *in vivo*. Moreover, we were surprised to find that the two-phage cocktail combining PAK_P1 and PAK_P5 did not have improved activities against any of the patient strains, despite both phages having different single phage activities. This would suggest that the phages conferred collateral resistance during resistance mutation and/or incompatibility between the phages for another reason. Although we were unable to confirm the bacterial burden at the start of this patient's infection, limited phage activity combined with the patient's consistent intake of antibiotics would suggest that phages would have replicated poorly *in vivo*. However, this could not be confirmed given the lack of direct sampling for phages in the blood or at the site of infection.

In contrast, our analyses of the outcomes of phage treatment in a CF patient implies that phages can replicate and dominate the lung virome given sufficient host cells. Because the design of the treatment was non-redundant such that phages could target antibiotic resistant variants within the patient's multivariant infection, we were able to quantify from plated sputa the change in bacterial variant abundance as they corresponded to clinical phage abundances obtained in sputa metagenome. Together, these results suggested that phages were able to target specific populations of the patient's infection independent of antibiotic activity and replicate. Even in the presence of ciprofloxacin, a DNA gyrase inhibiting antibiotic that could potentially block phage

replication [474], the phages were able to replicate on their target cells. This validation of phage targeting and replication is among the first to suggest successful *in vivo* replication of phages following therapy. Alongside other clinical markers of the patient's recovery, this analysis of phage therapy clinical and microbiological outcomes can serve as a guide for validation of phage success in future clinical trials.

Although the goal of these studies was to decipher whether phages sufficiently targeted clinical hosts, we also found that pili and LPS phage cocktails were less efficient than we previously believed. The limited host range of LUZ19 on our clinical library suggests that an alternative to pili binding phages or isolation of broader range pili binding phages will be necessary to develop effective phage cocktails. Moreover, although we had then employed a cocktail of two different LPS binding phages to treat a second patient, the almost immediate neutralization of treatment phage PYO2 also appeared as an unexpected hurdle in cocktail formulation. Thus, although large phage libraries do not seem necessary to target at least some of the clinical variation present in infection, identifying new phage genera to avoid neutralization may be a more pragmatic approach to expand phage availability in the future.

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Melendez, David Pride, Douglas Conrad, and Dwayne Roach. The dissertation author was the primary researcher and first author of both papers.

Author Contributions 5.1.1

Conceptualization, TL and DR; methodology, TL, ARS, DC, and DR; software/code, TL; validation, TL and ARS; investigation, TL and ARS; formal analysis, TL and DR; resources, DC and DR; data curation, TL; writing—original draft, TL and DR; writing—review & editing, TL, ARS, DC, and DR; supervision, DC and DR; funding acquisition, DR.

Author Contributions 5.2.2

Conceptualization, TL, DC, and DR; methodology, TL, LK, KC-J, WC, DC, and DR; software/code, TL and LK; validation, TL and DR; investigation, TL, LK, KC-J, WC, JAM, and DC; formal analysis, TL, LK, KC-J, WC, DC, and DR; resources, DP, DC, and DR; data curation, TL and LK; writing—original draft, TL, LK, DC, and DR; writing—review & editing, TL, DC, and DR; visualization, TL, LK, and DR; supervision, DC and DR; funding acquisition, DC and DR.

CHAPTER 6

CONCLUSIONS

After a century of scrutiny and now several decades of renewed interest, the efficacy of phage therapy has yet to be proven and phages still lack formal approval by regulatory authorities [10]. Although phage treatment safety has now been better established through a series of modern trials [475], therapeutic efficacy remains stalled due to insufficient methods to produce clinically safe phages, empirical selection of phage cocktail formulae, and uncertainty as to how phages compare to standard of care antibiotics [34]. The overarching goal of these studies was to develop innovative phage processes to formulate, purify, and validate phage treatment against *P. aeruginosa*. The focus of these studies was on understanding individual phage kinetics in order to leverage them to improve phage production and track phage PK and PD *in situ*. A small group of well-characterized dsDNA *Caudoviricetes* phages E215, PYO2, LUZ19, and PAK_P1 were studied in this dissertation. Limiting the diversity and deepening understanding of select phages allowed comparison of how phage replication characteristics corresponded to phage activities in two *in vitro* models, in purification and stability studies, in targeting of clinical isolates, and even within patient metagenomes.

In early cocktail kinetics, individual phage potencies were limited by competition for bacterial cells, but long-term efficacy appeared enhanced when phages with shorter latent periods were combined. This would suggest that competition between phages may contribute to failed or asymmetric cocktail replication *in vivo*. This is emphasized by competition kinetics in HFIM which suggest that single bolus phage cocktail administration or low dose cocktails *in vivo* would adversely affect survival or persistence

of at least one phage strain during treatment. More screening of two-phage combinations and administration regimens against clinical *P. aeruginosa* variants will be necessary to correlate how short-term phage potency can influence the long-term suppression of resistant isolates. It will be important to investigate the specific effects of these findings on clinical strains and settings. Although these results suggest that two phages are sufficient for *in vitro* resistance prevention for upwards of 28 hours, it remains unclear whether more phage strains will be necessary *in vivo* and the effect of phage competition between more species.

The purification of phage biologics was enhanced by implementing patterns derived from phage-bacteria kinetics. For instance, because we found that phage resistant *P. aeruginosa* grew exponentially after ~12 hours of co-culture with single phage, we implemented a strain-timed cooling or stop cultivation of phages in the improved protocol to limit bacterial bioburden. This additional step not only allows shortening of the purification process but reduces downstream large endotoxins and bacterial debris. Improving ease of purification not only makes clinical phage production more rapid but also more accessible in developing countries where interest and advocacy in phage applications are increasing [476]. For future process development, refinement to phage cultivation should include engineering or selecting production strains with altered endotoxin structures that could be readily eliminated. Further improvements to phage purification should focus on removing non-endotoxic immunogenic elements such as residual DNases or RNases and preventing potential bacterial SOS responses that cause confounding factors and/or stressors. Testing of these protocols on other bacterial

species, such as Gram-positive bacteria, and slow growing clinical isolates will also be necessary for amplifying rare phages with hard-to-culture hosts.

Finally, validation of *in vivo* phage PK and PD is a critical gap in designing and implementing efficacy trials. During failed phage therapy, conventional EOP plating was a poor indicator of phage-bacteria efficacy under mimicked oxygen conditions. In retrospect, this could have been determined 50 days prior to the patient's treatment with what was only one effective phage in cocktail. Because standardized regimens do not currently exist, future screening of phage biologics will require a combination of sensitivity assays to better determine suitability of the biologic against the infection. During successful therapy, we attempted to perform unsupervised machine learning (principal component analysis) to decipher phage efficacy patterns but had some difficulty in determining the types of clinical outcomes pertinent to the analyses and limited sampling during treatment. Fortunately, the combined quantification of a phage-specific bacterial variant and measuring phage abundances in the lung virome appear to be a highly-sensitive pair of assays to avoid challenges with plating clinical samples such as blood, urine, or sputum which may block phage activity when plated [463, 477]. In the future, controlled clinical trials that safely separate phage and antibiotic timelines will be necessary to delineate and verify the activities of each antimicrobial. Together, these analyses suggest that better guidance of phage selection is required for efficacy trials prior to treatment and both microbial and metagenomic outcomes will be needed to demonstrate phage PK/PD.

APPENDIX

Supplementary tables, figures, and materials.

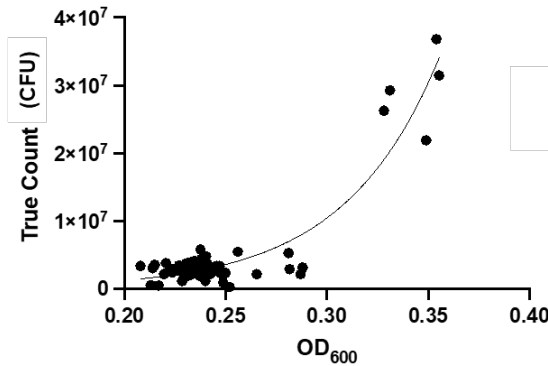


Figure S3.1 Semi-log curve fit of PAO1 growth in plate reader versus plated counts. The formula for calculating CFU/mL is $Y=10^{(4.2155+9.3295 \times X)}$ where x is the OD₆₀₀ reading above the detection limit of OD₆₀₀ 0.18.

Table S3.1 Characteristics of *Pbunavirus* phages.

Genus	<i>Pbunavirus</i>										
	PA5*	PASA16*	PA14-1*	EPA11*	EPA39*	V523	V524	DRL-P1	vB_PaeM_SCU T-S1	JG024	E215
Genome											
Morphotype	Myovirus	Myovirus	Myovirus	Myovirus	Myovirus	Myovirus	Myovirus	Myovirus	Myovirus	Myovirus	Myovirus
NCBI accession number	NC_041902.1	MT933737.1	NC_011703	MT108727	MT118303	MW595220	MW595221	MN564818	MK340760	GU815091	MF490241.1
Genome size (bp)	66182	66127	66235	66800	66708	66301	65447	66243	66046	66275	66789
GC%	55.49	55.6	nd	55.7	nd			54.9	55.43	55.62	55.6
tRNAs	0	nd	nd	nd	nd			0	0	0	0
RNA polymerases	0	nd	nd	nd	nd			0	0	0	0
Open reading frames	nd	93	nd	nd	nd			93	93	94	95
Growth											
Bacterial isolation host	PAO1	PA14	PAO1	PaWRA01	PaWRA01	PAO1	PAO1	MTCC 1688	PAO1	PAO1	PAO1
Binding receptor	nd	nd	LPS	nd	nd			nd	nd	LPS	O-antigen
Adsorption rate (mL/min)	nd	nd	nd	nd	nd			nd	nd	nd	1.40E-09
Eclipse period (min)	nd	nd	nd	nd	nd			nd	nd	nd	26
Latent period (min)	nd	nd	nd	nd	nd	55	50	30	40	50	30
Burst size (PFU)	nd	nd	nd	nd	nd	124	134	100	134	180	210
Plaque size (mm)	nd	nd	2	nd	nd			2	nd	nd	0.5

*Phages used therapeutically.

Table S3.2 Characteristics of *Bruynoghevirus* phages.

Genus	<i>Bruynoghevirus</i>				
Genome	PA222*	PA223*	Delta*	PACE	vB_PseuP-SA22
Morphotype	Podovirus	Podovirus	Podovirus	Podovirus	Podovirus
NCBI accession number	MK837011.1	MK837012.1	MG432151.1	ON376263	OP793496.1
Genome size (bp)	45770	45703	45970	45365	45458
GC%	nd	nd	52.2	52.5	52.5
tRNAs	nd	nd	3	4	3
RNA polymerases	nd	nd	nd	nd	0
Open reading frames	nd	nd	nd	73	58
Growth					
Bacterial isolation host	nd	nd	PA-4U	PAO1Δ PW	clinical strain
Binding receptor	nd	nd	nd	nd	nd
Adsorption rate (mL/min)	nd	nd	nd	nd	nd
Eclipse period (min)	nd	nd	nd	nd	nd
Latent period (min)	nd	nd	nd	nd	30
Burst size (PFU)	nd	nd	nd	nd	300
Plaque size (mm)	nd	nd	2.5-5.0	nd	1.5±2.5

*Phages used therapeutically.

Table S3.3 Characteristics of *Phikmvvirus* phages.

Genus	<i>Phikmvvirus</i>									
Genome	PNM*	PT5	PT2	φKF77	LKA1	LKD16	vB_PaeP_AS P23	PAXYB1	φKMV	LUZ19 ^a
Morphotype	Podovirus	Podovirus	Podovirus	Podovirus	Podovirus	Podovirus	Podovirus	Podovirus	Podovirus	Podovirus
NCBI accession number	OP29228	NC_0111	NC_0111	NC_0124	NC_00993	NC_0099	MN602045	KY6188	NC_0050	NC_01032
	8.1	05	07	18	6.1	35		19	45	6.1
Genome size (bp)	42721	42954	42961	43152	41593	43200	42735	43337	42519	43548
GC%	62.3	62	62	62.8	60.9	62.6	62.15	62.29	62.3	62.3
tRNAs	nd	nd	nd	nd	nd	nd	0	0	0	0
RNA polymerases	nd	nd	nd	nd	nd	nd	1	1	1	1
Open reading frames	51	52	54	53	56	54	54	60	52	54
Growth										
Bacterial isolation host	573 (clinical)	573 (clinical)	573 (clinical)	nd	PAO1	PAO1	L64	PAO1	PAO1	PAO1
Binding receptor	nd	nd	nd	nd	nd	nd	nd	nd	type IV pili	type IV pili
Adsorption rate (mL/min)	nd	7.30E-09	9.20E-09	1.50E-08	3.90E-09	nd	nd	3.70E-09	1.60E-08	1.20E-07
Eclipse period (min)	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
Latent period (min)	nd	21	21	23	41	27	10	30	15	22
Burst size (PFU)	nd	134	116	129	225	120	140	141	30	122
Plaque size (mm)	nd	4	4	nd	1	nd	nd	0.8	nd	1.3

*Phages used therapeutically.

Table S3.4 Characteristics of *Pakpunavirus* phages.

Genus	<i>Pakpunavirus</i>					
Genome	PAK_P1*	PAK_P2	PAK_P4	JG004	C2-10_Ab1	PaP1
Morphotype	Myovirus	Myovirus	Myovirus	Myovirus	Myovirus	Myovirus
NCBI accession number	NC_015294.2	KC86229.8	KC862300	NC_019450	NC_019918.1	NC_019913.1
Genome size (bp)	93398	92495	93147	93017	92777	91715
GC%	49.5	49.3	49.3	49.26	49.28	49.4
tRNAs	13	11	13 + 1 pseudo	12	12	13
RNA polymerases	1	nd	nd	nd	nd	nd
Open reading frames	181	176	174	108	158	157
Growth						
Bacterial isolation host	PAK	PAK	PAK	WT PAO1	C2-10	PA1
Binding receptor	LPS	nd	nd	LPS	nd	LPS
Adsorption rate (mL/min)	nd	nd	nd	nd	nd	nd
Eclipse period (min)	nd	nd	nd	nd	nd	nd
Latent period (min)	14	nd	nd	31	nd	nd
Burst size (PFU)	100	nd	nd	13	nd	nd
Plaque size (mm)	nd	nd	nd	nd	nd	nd

Table S3.5 Characteristics of *Litunavirus* phages.

Genus	<i>Litunavirus</i>								
Genome	YH6	vB_PaeP_CM S1	LIT1	vB_PaeS_V L1	vB_PaeP_PS 28	PYO2	4029*	4032*	4034*
Morphotype	Podovirus	Podovirus	Podovirus	Podovirus	Podovirus	Podovirus	Podovirus	Podovirus	Podovirus
NCBI accession number	KM974184.1	OM937766.1	NC_013692	OK665488	OQ134474.1	MF490236.1	ON815901.1	ON815902.1	ON815903.1
Genome size (bp)	73050	72673	74901	73308	72283	72697	72063	72063	72063
GC%	54.88	54.9	53.4	54.7	54.75	55	nd	nd	nd
tRNAs	0	0	0	0	0	0	nd	nd	nd
RNA polymerases	1	1	1	1	1	0	nd	nd	nd
Open reading frames	90	92	92	92	94	91	nd	nd	nd
Growth									
Bacterial isolation host	PA	PAO1Δ pilA	US449	ATCC27853	PS28	PAO1	PAO1	ATC15442	PAO1
Binding receptor	nd	nd	nd			nd	nd	nd	nd
Adsorption rate (mL/min)	nd	nd	nd	nd	nd	1.80E-10	nd	nd	nd
Eclipse period (min)	nd	nd	20	nd	nd	24±2	nd	nd	nd
Latent period (min)	30	nd	25±5	30	15	26±2	nd	nd	nd
Burst size (PFU)	100	nd	100±50	404±10	210	183±20	nd	nd	nd
Plaque size (mm)	1-2	nd	1-2	4	2	0.75	nd	nd	nd

*Phages used therapeutically.

Table S3.6 Characteristics of *Phikzvirus* phages.

Genus		<i>Phikzvirus</i>					
Genome		phiKZ	OMKO1*	SL2	Koomba boorn-mokiny kep-wari Wadjak 1	KTN4	φPA3
Morphotype		Myovirus	Myovirus	Myovirus	Myovirus	Myovirus	Myovirus
NCBI accession number		NC_004629.1	ON631220	NC_042081.1	OP310983.1	KU521356.1	NC_028999.1
Genome size (bp)		280334	281755	279696	278796	279593	309208
GC%		36.8	nd	36.9	36.91	36.9	48
tRNAs		6	nd	5	6	6	5
RNA polymerases		1	nd	nd	nd	nd	6
Open reading frames		306	nd	355	nd	368	378
Growth							
Bacterial isolation host		clinical	PAO1	MDR-PA2	PAO1	nd	nd
Binding receptor		flagella/efflux pump	outer membrane porin/efflux	nd	nd	type IV pili	nd
Adsorption rate (mL/min)		nd	nd	nd	nd	nd	nd
Eclipse period (min)		210	nd	nd	nd	nd	nd
Latent period (min)		nd	nd	30	nd	40	50-60
Burst size (PFU)		100	nd	22±6	nd	6-8	100
Plaque size (mm)		nd	nd	nd	nd	1.7	2.5

*Phages used therapeutically.

RMarkdown

The following is the RMarkdown code used to normalize time-kill kinetic data to blank controls and to normalize the starting density reading for comparison across different plate experiments.

```
library(readxl)
library(plyr)
library(tibble)
library(tidyr)
library(ggplot2)
library(ggh4x)
library(viridis)
library(csv)
library(rstudioapi)
library(growthcurver)
library(pracma)
setwd(dirname(getActiveDocumentContext())$path))

#input raw data file and organize

sheet_names <- excel_sheets("20231213_R_Master_TKA_TL.xlsx")

list_all <- lapply(sheet_names, function(x) {
  as.data.frame(read_excel("20231213_R_Master_TKA_TL.xlsx", sheet = x)) } )
names(list_all) <- sheet_names

raw <- rbind.fill(list_all)
detach(package:plyr)

data <- raw
num_info_cols <- 6
data_long <- pivot_longer(data = data, cols = (num_info_cols+1):ncol(data), names_to = "HOURS", values_to = "OD")
data_long$DATE <- as.factor(data_long$DATE)
data_long$PLATE <- as.numeric(data_long$PLATE)
data_long$INTERRUPTED <- as.factor(data_long$INTERRUPTED)
data_long$TREATMENT <- as.factor(data_long$TREATMENT)
data_long$MOI <- as.factor(data_long$MOI)
data_long$HOURS <- as.numeric(data_long$HOURS)
data_long$OD <- as.numeric(data_long$OD)
data_long$HOURS <- as.numeric(data_long$HOURS)
data_long$OD <- as.numeric(data_long$OD)

#blank correction
data_long_blanks <- data_long %>% subset(TREATMENT %in% "BLANK")

data_long_blanks_avg <- data_long_blanks %>% group_by(PLATE, TREATMENT) %>% summarise(blank_avg = mean(OD))
```



```

data_long_blanks_avg$correction <- (data_long_blanks_avg$blank_avg - 0.2)
data_long$blankcorrected_OD <- data_long$OD - data_long_blanks_avg$correction
[match(data_long$PLATE, data_long_blanks_avg$PLATE)]

#Normalize time=0 to the same starting OD600, LOD 0.18
# Calculate the OD difference between 0.18 and OD for each PLATE and WELL combination at HOUR 0.0

od_difference <- data_long %>%
  filter(HOURS == 0.0) %>%
  group_by(PLATE, WELL) %>%
  summarize(od_diff = 0.18 - blankcorrected_OD)

# Merge the OD difference with the original data
data_with_od_diff <- merge(data_long, od_difference, by = c("PLATE", "WELL"))

# Normalize OD values for every other timepoint
normalized_data <- data_with_od_diff %>%
  mutate(OD_normalized = blankcorrected_OD + od_diff)

# Now 'normalized_data' contains a new column 'OD_normalized' with normalized OD values

# Subset data for analysis

subsetting_data <- normalized_data[, c("TREATMENT", "INTERRUPTED", "PLATE", "WELL", "MOI", "HOURS", "OD_normalized")]

subset_tkadata <- subsetting_data %>% filter(TREATMENT == "CFU", INTERRUPTED == "0", MOI == "0")

#process data to output for Prism graphing

subset_tkadata_prism <- pivot_wider(
  data = subset_tkadata,
  names_from = c("PLATE", "WELL"),
  values_from = "OD_normalized",
  names_glue = "{PLATE}_{WELL}")

subset_tkadata_prism<- apply(subset_tkadata_prism, 2, as.character)
write.csv(subset_tkadata_prism, "CFU_1_prism.csv")

#call for resistance timing
timing <- read.csv("CK_LE_10_prism.csv")
timing_long <- pivot_longer(data = timing, cols = 5:ncol(timing), names_to = "WELLS", values_to = "OD")
timing_unique <- timing_long %>% group_by(WELLS) %>% filter(OD >= 0.3) %>% slice(1)
View(timing_unique)

```

Table S3.7 Mutational analysis of single phage resistant mutants from TKA. Five resistant isolates were sequenced per phage treatment.

Phage Treatment					E215	PYO2	LUZ19
Protein Function	Genome Position	Annotation	Gene	Putative gene function			
Type IV Pilus/Fimbral Assembly							
	2913857	coding (109/357 nt)	<i>PilZ</i>	pilus biogenesis protein			1
	1970808	S141* (T C G→T A G)	<i>PilF</i>	pilus biogenesis protein			1
	1203710	T230P (A C C→ C CC)	<i>PilR</i>	fimbriae expression regulatory protein			1
	1228607	D388A (G A C→G C C)	<i>PilB</i>	fimbral assembly, ATPase			1
	1203506	coding (892/1338 nt)	<i>PilR</i>	fimbriae expression regulatory protein			1
O-antigen							
	2694486	coding (136/1317 nt)	<i>Wzy</i>	Oligosaccharide repeat unit polymerase		2	
	2694486	coding (136/1317 nt)	<i>Wzy</i>	Oligosaccharide repeat unit polymerase	1	1	
	2694975	coding (625/1317 nt)	<i>Wzy</i>	Oligosaccharide repeat unit polymerase	1		
Transporter							
	4174863	coding (203 302/312 nt)	<i>OppB</i>	Oligopeptide transport system permease protein	5	5	5
	3686613	S361R (A G C→ C GC)		Organosulfonate ABC transporter substrate binding protein	1		
	5063859	L201R (C T G→ C GG)		Permease of the drug/metabolite transporter (DMT) superfamily	1		
Regulator							
	844803	intergenic (-339/+118)		MarC family integral membrane protein/probable sensor/response regulator hybrid	4	5	5
	2718830	intergenic (+2/ 158)		tRNA Glu TTC/Transcriptional regulator, AcrR family	4	5	5
	5220913	intergenic (+100/+2)		Transcriptional regulator/tRNA Arg ACG	5	5	5
	305077	intergenic (+35/+10)		Transcriptional regulator, AraC family/N acetylglutamate kinase (EC 2.7.2.8)		1	
	4786916	A150G (G C G→G G G)		Transcriptional regulator, AcrR family	1		
Enzymes							
	677087	H116R (C A C→ C GC)		Glycosyl transferase in large core OS assembly cluster	1		
	2107228	E649A (G A G→ C CG)		Phosphoenolpyruvate carboxylase	1		
	3281129	R308G (C G C→ G GC)		Glutathione S transferase, omega			
	3281131	A307G (G C G→ G GG)		Glutathione S transferase, omega	1		
	3743705	L25R (C T G→ C GG)		Succinate dehydrogenase/fumarate reductase, flavoprotein subunit	1		
	4509471	intergenic (220/ 107)		23S rRNA (cytidine(2498) 2' O) methyltransferase, /Aconitate hydratase @ 2 methylisocitrate dehydratase		1	
	4540353	R711M (A G G→ A TG)		DNA polymerase III subunits gamma and tau	1		
	4540357	A710S (G C G→ I CG)		DNA polymerase III subunits gamma and tau	1		1
	4849887	intergenic (+36/ 57)		1 pyrroline 4 hydroxy 2 carboxylate deaminase, 2,5 dioxovalerate dehydrogenase	1		

Table S3.8 Mutational analysis of phage resistant mutants from cocktail TKA. Five resistant isolates were sequenced per phage treatment.

Phage Treatment				E215+PYO2 E215+LUZ19 PYO2+LUZ19		
Protein Function	Genome Position	Annotation	Gene	Putative gene function		
Type IV Pilus/Fimbral Assembly						
	623834	coding (2078 2096/2145 nt)	<i>PilQ</i>	pilus biogenesis protein		1
	1203459	coding (910 939/1338 nt)	<i>PilR</i>	fimbriae expression regulatory protein		2
	619280	E127* (GAG→TAG)	<i>PilM</i>	pilus biogenesis protein		1
		*199R (TGA→AGA)	<i>PilN</i>			
	620560	M1K (ATG→AAG) †	<i>PilO</i>	pilus biogenesis protein		1
	623491	E579* (GAA→TAA)	<i>PilQ</i>	pilus biogenesis protein		1
				fimbriae expression regulatory protein		
	1203161	A413P (GCG→CCG)	<i>PilR</i>			1
	1228272	G500S (GGT→AGT)	<i>PilB</i>	fimbral assembly, ATPase		1
	1228607	D388A (GAC→GCC)	<i>PilB</i>	fimbral assembly, ATPase		2
	619443	coding (542/1065 nt)	<i>PilM</i>	pilus biogenesis protein		1
O-antigen						
	2695229	coding (879/1317 nt)	<i>Wzy</i>	Oligosaccharide repeat unit polymerase	1	
	2695221	R291C (CGT→TGT)	<i>Wzy</i>	Oligosaccharide repeat unit polymerase		1
	2694486	coding (136/1317 nt)	<i>Wzy</i>	Oligosaccharide repeat unit polymerase	3	
	2694486	coding (136/1317 nt)	<i>Wzy</i>	Oligosaccharide repeat unit polymerase		5
	2694975	coding (625/1317 nt)	<i>Wzy</i>	Oligosaccharide repeat unit polymerase		1
	2695254	coding (904/1317 nt)	<i>Wzy</i>	Oligosaccharide repeat unit polymerase		1
Transporter						
	3644812	intergenic (+199/ 447)		ABC transporter, permease protein (cluster 10, nitrate/sulfonate/bicarbonate)/ Coenzyme F420 dependent N5,N10 methylene tetrahydromethanopterin reductase and related flavin dependent oxidoreductases		4
	4174863	coding (203 302/312 nt)	<i>OppB</i>	Oligopeptide transport system permease protein	5	5
	1417186	D32A (GAC→GCC)		Uncharacterized MFS type transporter	1	
	2304981	intergenic (+49/ 464)		SAM dependent methyltransferase/ABC transporter, ATP binding protein (cluster 1, maltose/g3p/polyamine/iron); ABC transporter, ATP binding protein (cluster 10, nitrate/sulfonate/bicarbonate)	1	
	4432149	intergenic (55/+53)		Osmosensitive K ⁺ channel histidine kinase	1	
	4587167	L215V (CTG→GTG)		KdpD/Potassium transporting ATPase C chain (EC 3.6.3.12) (TC 3.A.3.7.1)		1
	4587194	A224P (GCC→CCC)		Eukaryotic type low affinity urea transporter	1	
	5947991	intergenic (+377/+65)		Eukaryotic type low affinity urea transporter		
				4 hydroxybenzoate transporter/Nucleoside binding outer membrane protein	1	
Regulator						
	844803	intergenic (-339/+118)		MarC family integral membrane protein/probable sensor/response regulator hybrid	4	5
	1843223	coding (30 129/657 nt)		Transcriptional regulator, LuxR family		3
	2718830	intergenic (+2/ 158)		IRNA Glu TTC/Transcriptional regulator, AcrR family	5	5
	5220913	intergenic (+100/+2)		Transcriptional regulator/IRNA Arg ACG	5	5
	268037	P175T (CCG→ACG)	<i>PhoB (SphR)</i>	Phosphate regulon transcriptional regulatory protein		3
Enzymes						
	16749	intergenic (+79/+13)		Quinone oxidoreductase /Threonylcarbamoyl AMP synthase	1	1
	16925	A163G (GCG→GGG)		Threonylcarbamoyl AMP synthase	1	
	3281131	A307G (GCG→GGG)		Glutathione S transferase, omega	1	
	3902594	C39R (TGC→CCG)		Putative short chain dehydrogenase	2	2
	4378449	P136A (CCG→GCG)		1,2 dihydroxy 3 keto 5 methyl hiopentene dioxygenase	1	
	4540357	A710S (GCG→TCG)		DNA polymerase III subunits gamma and tau		1

Table S3.9 Mutational analysis of phage resistant mutants from sequential TKA. Five resistant isolates were sequenced per phage treatment.

Phage Treatment					LUZ19→E215	E215→LUZ19
Protein Function	Gene	Genome Position	Annotation	Putative gene function		
Type IV Pilus/Fimbral Assembly						
	<i>PilQ</i>	621795	coding (39-65/2145 nt)	pilus biogenesis protein		1
	<i>PilR</i>	1203459	coding (910-939/1338 nt)	fimbriae expression regulatory protein	1	
	<i>PilM</i>	619730	Q277* (CAG→TAG)	pilus biogenesis protein	1	
	<i>PilN</i>	620425	T154P (ACC→CCC)	pilus biogenesis protein	1	
	<i>PilE</i>	1194711	R53P (CGC→CCC)	pilus biogenesis protein	1	
	<i>PilA</i>	1230305	G102D (GGT→GAT)	pilin		1
	<i>PilY1</i>	1198030	Q224* (CAA→TAA)	fimbrial biogenesis protein		1
	<i>PilB</i>	1228607	D388A (GAC→GCC)	fimbral assembly, ATPase	1	
	<i>PilC</i>	1227721	coding (345/1221 nt)	fimbrial assembly protein		1
O-antigen						
	<i>Wzy</i>	2694486	coding (136/1317 nt)	Oligosaccharide repeat unit polymerase	1	
	<i>Wzy</i>	2694486	coding (136/1317 nt)	Oligosaccharide repeat unit polymerase	5	1
	<i>Wzy</i>	2694975	coding (625/1317 nt)	Oligosaccharide repeat unit polymerase	1	
	<i>Wzy</i>	2694975	coding (625/1317 nt)	Oligosaccharide repeat unit polymerase	1	
Transporter						
	<i>OppB</i>	4174863	coding (203-302/312 nt)	Oligopeptide transport system permease protein	5	5
Regulator						
		844803	intergenic (-339/+118)	MarC family integral membrane protein/probable sensor/response regulator hybrid	5	5
		1843223	coding (30-129/657 nt)	Transcriptional regulator, LuxR family	1	
		2718830	intergenic (+2/-158)	tRNA-Glu-TTC/Transcriptional regulator, AcrR family	5	5
		5220913	intergenic (+100/+2)	Transcriptional regulator/tRNA-Arg-ACG	5	5
	<i>PhoB (SphR)</i>	268037	P175T (CCG→ACG)	Phosphate regulon transcriptional regulatory protein	5	2
Enzymes						
		3996382	coding (803-813/840 nt)	UTP--glucose-1-phosphate uridylyltransferase		1
		3732283	intergenic (-63/+37)	L-asparaginase I, cytoplasmic/Sodium:alanine symporter family protein		1
		3902594	C39R (TGC→CGC)	Putative short-chain dehydrogenase	2	1
		3996579	R206C (CGC→TGC)	UTP--glucose-1-phosphate uridylyltransferase		1
		4540357	A710S (GCG→TCG)	DNA polymerase III subunits gamma and tau	2	

Table S4.1 Bacteriophage cultivation and purification titers and endotoxin units after each production process.

Phage Strain*	Bacterial Host	Sterile Phage Lysate (Steps 84-92; mL ⁻¹)		Cross-Flow Filtration (Steps 93-105; mL ⁻¹)		Density Gradient Ultracentrifugation & Dialysis (Steps 106-120; mL ⁻¹)		LPS-affinity Chromatography (Steps 121-134; mL ⁻¹)	
		PFU [†]	EU [‡]	PFU	EU	PFU	EU	PFU	EU
PAK_P1	<i>P. aeruginosa</i>	2.04 × 10 ¹⁰	2.09 × 10 ⁵	2.13 × 10 ¹¹	29.14	5.19 × 10 ¹¹	42.77	5.78 × 10 ¹⁰	4.49
PAK_P5	<i>P. aeruginosa</i>	4.33 × 10 ¹⁰	5.84 × 10 ⁴	2.00 × 10 ¹²	39.48	2.30 × 10 ¹²	39.91	4.00 × 10 ¹¹	4.30
E217	<i>P. aeruginosa</i>	3.05 × 10 ¹⁰	1.94 × 10 ⁵	1.14 × 10 ¹¹	37.08	2.00 × 10 ¹¹	40.61	1.89 × 10 ¹¹	5.26
PYO2	<i>P. aeruginosa</i>	2.50 × 10 ¹⁰	1.35 × 10 ⁴	3.67 × 10 ¹²	39.38	2.81 × 10 ¹²	35.83	4.00 × 10 ¹²	5.05
JG265	<i>K. oxytoca</i>	1.00 × 10 ⁹	4.98 × 10 ⁵	1.20 × 10 ¹⁰	55.67	2.50 × 10 ¹⁰	ND [†]	1.30 × 10 ¹⁰	24.10
JG266	<i>K. oxytoca</i>	2.00 × 10 ⁹	5.43 × 10 ⁵	9.00 × 10 ⁹	60.58	4.00 × 10 ⁹	ND	2.00 × 10 ⁹	17.30
SM219	<i>S. marcescens</i>	1.60 × 10 ⁹	5.16 × 10 ⁵	5.00 × 10 ¹⁰	37.22	8.50 × 10 ¹⁰	33.84	2.90 × 10 ¹⁰	18.85

* GenBank Accession #: PAK_P1, KC862297.1; PAK_P5, KC862301.1; E217, MF490240; PYO2, MF490236.

[†] PFU (plaque forming units) determined by Step 20 option B; ND (not determined)

[‡] EU (endotoxin units) determined by the Pierce™ LAL chromogenic endotoxin quantitation Steps 135-143.

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