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Polyvalent Bacteriophages: Isolation, Modification, and Applications in Environmental Systems

by

Pingfeng Yu

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APPROVED, THESIS COMMITTEE

Dr. Pedro J. Alvarez, Chair,
George R. Brown Professor,
Civil and Environmental Engineering.

Dr. Qilin Li,
Professor,
Civil and Environmental Engineering.

Dr. Yizhi Jane Tao,
Associate Professor,
BioSciences at Rice,

Dr. Jacques Mathieu,
Research Scientist,
Civil and Environmental Engineering

HOUSTON, TEXAS
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ABSTRACT

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Bacteriophages are garnering significant interest for microbial control predominantly driven by the widespread and increasing onset of antibiotic-resistant bacteria (ARB). However, the scope and efficacy of phage applications in environmental systems have been hurdled due to narrow infective spectrum, development of phage resistance, inefficient biofilm penetration and infectivity loss during phage delivery. This dissertation contributes to advance our understanding of polyvalent (broad-host-range) phages and provides proof-of-concept on polyvalent phage-based antimicrobial approaches.

To enhance research on polyvalent phages, we developed two sequential multi-host isolation methods and tested both culture-dependent and culture-independent phage libraries for broad infectivity. In contrast to the conventional single host enrichment method, which biased for fast-replicating narrow host-range phages, sequential multiple-host approaches enriched generalist phages in each step while left the specialist phages behind. Lytic polyvalent phages capable of interspecies or even inter-order infectivity can be readily isolated from common environmental samples without significant reduction in efficiency of infection. Due to their stable and broad host range, these phages can be
enriched with a nonpathogenic host (i.e., *Pseudomonas putida* F1 and *Escherichia coli* K-12) and subsequently used to infect model problematic bacteria (*Pseudomonas aeruginosa, Pseudomonas syringae*).

Microcosm studies were conducted to validate the importance of phage polyvalence on phage propagation and bacterial suppression in biofilm-associated environments. The microbial community structure in biofilm had a significant impact on phage propagation and migration, and therefore the suppression of the target bacteria within the biofilm. Polyvalent phages could outcompete narrow host-range phages in the suppression of target bacteria in biofilm-associated environments, although narrow host-range phages were more effective than polyvalent phages in monoculture or simple mixed-species culture. Combining bacterial competitors with phages resulted in meaningfully greater inhibition of target bacteria than separate treatments in planktonic conditions, suggesting that phage-based biocontrol would be more effective in combination with compatible microbial control strategies.

The infectivity of commonly isolated narrow-host-range phages decreases quickly because of environmental stresses. Therefore, polyvalent phage-based biocontrol approaches were proposed for safe phage production and improved ARB inhibition in environment. Polyvalent phages can proliferate and thrive in activated sludge microcosms, especially when added along with their nonpathogenic production hosts. Due to the presence of alternative hosts, polyvalent phages reached greater densities, which offset phage loss during delivery and increased the probability of ARB infection. The fraction of surviving *E. coli* containing the *bla*NDM-1 resistance gene was also
significantly lower for the polyvalent phage cocktail treatment, mainly because the
development of phage resistance increased the fitness costs of ARB.

Biofilms may shelter pathogenic or problematic microorganisms that are difficult to
eradicate due to hindered penetration of antimicrobial chemicals. Here, we demonstrated
the potential for efficient bacterial suppression using polyvalent phages attached to
magnetic nanoparticles that facilitate biofilm penetration under a relatively small
magnetic field. The *Podoviridae* polyvalent phage PEL1 was immobilized onto Fe$_3$O$_4$-
based magnetic CNCs that had been coated with chitosan (and thus functionalized with
amino groups). This facilitated conjugation with phages via covalent bonding and
enabled phage loading with optimal orientation. The phage-nano complex significantly
improved the efficacy of phage biocontrol in biofilm compared with free phages because
of high local concentration and optimized phage orientation. The conjugation also
extended the application of phages for microbial control by enhancing their delivery to
relatively inaccessible locations within biofilms.

Overall, polyvalent phages can be preferentially isolated by sequential multiple hosts
and hold the potential for ARB control and ARG mitigation. Polyvalent phages exhibit
more multifaced propagation dynamics, which increase phage fitness and boost target
bacterial infection in biofilm-associated systems and complex bacterial communities.
Polyvalent phages are compatible with engineered functional nanomaterial and their
conjugation can not only further enhance the efficacy of phage treatment, but also extend
the scope of phage application in environmental systems.
I would like to express my sincere gratitude to my advisor, Dr. Pedro Alvarez, for his unconditional trust, unwavering support, and meticulous care during my time at Rice. I could not have imagined having a better advisor and mentor for my Ph.D. study. My special thanks go to Dr. Jacques Mathieu, Dr. Qilin Li and Dr. Jane Tao, who have been always generous with sharing their expertise, experience, and wisdom in research and beyond. Their insightful comments and encouragement incented me to widen my research from various perspectives.

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Ultimately this is for my family, I thank my parents, my wife Jialu Xu and my newborn daughter Esther Yu, who have always supported and loved me. Thank you for making me laugh during the hard times and for sharing in my excitement during the good times.

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Based on this research, the following papers are published:


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## Nomenclature

<table>
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<tr>
<th>Acronym</th>
<th>Description</th>
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<tbody>
<tr>
<td>ARB</td>
<td>antibiotic-resistant bacteria</td>
</tr>
<tr>
<td>ARG</td>
<td>antibiotic resistant gene</td>
</tr>
<tr>
<td>AS</td>
<td>activated sludge</td>
</tr>
<tr>
<td>BIM</td>
<td>bacteriophage insensitive mutant</td>
</tr>
<tr>
<td>CFU</td>
<td>colony forming unit</td>
</tr>
<tr>
<td>CI</td>
<td>combination index</td>
</tr>
<tr>
<td>CNC</td>
<td>colloidal nanoparticle cluster</td>
</tr>
<tr>
<td>CRISPR</td>
<td>clustered regularly interspaced short palindromic repeats</td>
</tr>
<tr>
<td>EOP</td>
<td>efficiency of plating</td>
</tr>
<tr>
<td>ICTV</td>
<td>International Committee on Taxonomy of Virus</td>
</tr>
<tr>
<td>MDR</td>
<td>multi-drug resistant</td>
</tr>
<tr>
<td>MOI</td>
<td>multiplicity of infection</td>
</tr>
<tr>
<td>ORF</td>
<td>open reading frame</td>
</tr>
<tr>
<td>PEG</td>
<td>polyethylene glycol</td>
</tr>
<tr>
<td>PFU</td>
<td>plaque forming unit</td>
</tr>
<tr>
<td>QPCR</td>
<td>quantitative polymerase chain reaction</td>
</tr>
<tr>
<td>SEM</td>
<td>scanning electron microscope</td>
</tr>
<tr>
<td>TEM</td>
<td>transmission electron microscope</td>
</tr>
<tr>
<td>TSB</td>
<td>tryptic soy broth</td>
</tr>
<tr>
<td>VLP</td>
<td>viral-like particle</td>
</tr>
<tr>
<td>WWTP</td>
<td>wastewater treatment plant</td>
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Chapter 1

Introduction and Objectives

1.1. Problem statement

The overuse and misuse of antibiotics over the last decades have resulted in unforeseen consequence of growing urgency due to the rise of antibiotic-resistant bacteria (1). Whereas antibiotic-resistance compromises the effectiveness of pathogenic bacterial prevention and treatment, it has been observed to nearly all antibiotics that have been developed and saved millions of lives (2). Notably, the antibiotic resistance crisis is in broad scope as illustrated by the mounting research publications covering the thrive of antibiotic-resistant bacteria in natural and medical settings of numerous countries all around the world (3, 4). A recent report estimated cumulative economic losses of $100 trillion and 300 million premature deaths by 2050 due to substantial rises in rates of antibiotic resistance (5). Therefore, there is an enormous impetus to both reduce antibiotic use (to alleviate selective pressure for ARB proliferation) and find alternative means of microbial control.
Figure 1.1 Timeline of antibiotics discovery and resistance development. The widespread and rapid emergence of antibiotic resistance, as well as reduced rate of novel antibiotics discovery, has led to the antibiotic resistance crisis. Edited from Blair JMA (2015).

Predominantly stimulated by the antibiotic resistance crisis, there has been a resurgence of interest in the utilization of phages for microbial control (6). Phages are viruses infecting and replicating within bacterial hosts and recognized as the most abundant and diverse biological entities in the biosphere (7). Having co-existed and co-evolved with their bacterial hosts for billions of years, phages possess the capability of infecting their hosts specifically and efficiently, making phages potent antibacterial agents (8). The most important advantage of phages over antibiotics or other chemical disinfectants is their specificity against the bacterial targets, which allows controlling the problematic bacteria without significant impact on the functional bacterial communities.

However, there are several factors limit the scope and efficacy of phage-based microbial control in environmental systems because of heterogeneity and diversity of these microbial communities (9). The major hurdles include phage narrow host ranges, development of phage resistance, poor phage penetration into biofilm and loss of infectivity during phage delivery (10). Therefore, there is an urgent need to advance our
understanding of phage-host interactions and to explore novel strategies for enhanced microbial control in environmental systems.

Polyvalent (broad host range) phages can recognize and replicate in multiple bacterial hosts (i.e., more than one bacterial species) (11). Their extended host ranges mainly result from that they can recognize receptors of different hosts and circumvent various extracellular and intracellular antiviral systems (12). While there is strong indication that polyvalent phages are common in certain ecosystems with low bacterial density or high bacterial diversity (13), we currently lack targeted methods for their reliable isolation. Their broad host range may foster new opportunities to overcome the conventional hurdles facing phage-based biocontrol and enhance biological control of ARB and other problematic bacteria in environmental systems.
1.2. Objectives, hypothesis and significance

The general goal of this research is to develop novel selection strategies for preferential isolation of polyvalent phages with subsequent assessment of their potential for enhanced microbial control in environmental systems. The research contributes to overcoming conventional application challenges such as narrow host ranges, poor biofilm penetration, and phage decay during delivery, which would facilitate the application of phages in environmental engineering. Specifically, we seek to:

- Modify conventional phage isolation methods for preferential isolation of polyvalent phages from common environmental samples and characterize the isolated phages in terms of host range and infectivity.

**Hypothesis:** Environmental samples with high bacterial abundance and diversity are good phage reservoir since phages co-exist with their bacterial hosts. In contrast to the standard use of a single host, high-density planktonic conditions, which are strongly biased for the enrichment of fast-growing narrow-host-range phages, the sequential multi-host isolation approaches will provide optimal conditions for the replication of polyvalent phages and facilitate their isolation from these environmental samples. Polyvalent phages may display reduced growth rate and infectivity toward individual host relative to narrow host-range phages as costs of extended host ranges. A comprehensive genomic analysis of polyvalent phages would advance our understanding of phage host range determinants.

- Investigate the effect of phage host range on phage propagation, interspecies competition and target bacterial suppression under different bacterial compositions and growth conditions (suspended in liquid vs embedded in biofilm).
Hypothesis: Both phage infection and interspecies competition can suppress the growth of target bacteria in microbial communities. Polyvalent phages propagation in multiple bacterial hosts would enhance phage infection but attenuate interspecies competition. Therefore, the overall effect of phage host range on bacterial target suppression may depend on the composition and structure of microbial communities. Biofilms show a heterogeneous spatial structure of microbial populations and a complex matrix that is difficult for free phages to penetrate, which may compromise the efficacy of phage-based microbial control.

• Develop polyvalent phage biocontrol approaches to simplify phage preparation process and enhance target bacterial control in environmental systems (e.g., activated sludge).

Hypothesis: The enrichment process of polyvalent phages could be achieved by safer (non-pathogenic) and more effective (faster growing) hosts if the polyvalent phages have stable host range and high efficiency of infection among multiple hosts. Using polyvalent phages in conjunction with their production host, phage polyvalence would enhance their proliferation in the production hosts and other possible non-target bacteria, which would offset the phage loss during phage delivery and increase the probability of infecting target bacteria.

• Conjugate polyvalent phage with magnetic colloidal nanoparticle clusters (CNCs) for enhanced biofilm penetration and selective microbial control.

Hypothesis: Phage-based biofilm control is limited by inefficient phage penetration into the biofilm matrix. Polyvalent phages immobilized on CNCs would be easier to penetrate the established biofilm under the magnetic field as well as maintain higher in situ phage abundance to facilitate control of target bacteria in the mixed-species biofilm. CNCs
surface properties (functional group and surface charge) could greatly affect phage conjugation efficiency and the corresponding infectious activity since directional immobilization of phage particles via their heads is needed to ensure that tail fibers are exposed to the host.

**Significance:** Polyvalent phages with broad and stable host range would enable safer and more effective phage production compared with conventional phage production for narrow host-range phages. Since the target antibiotic-resistant bacteria could be substituted with benign bacteria during phage production, there may be no need to purify them from the host and other metabolites prior to utilization, circumventing the complex purification process. Polyvalent phages propagation in multiple hosts could offset phage inactivation by environmental stresses and facilitate phage spreading within the mixed-species biofilm, holding the promise for enhanced microbial control in environmental systems. The phage-CNCs conjugation approach could further extend the application of phages for microbial control by enhancing their delivery to relatively inaccessible locations within biofilms. With synthetic biology advancing exponentially, a better understanding of genes related to phage polyvalence can help modify phages for enhanced microbial control and gene delivery.
1.3. Dissertation Organization

This dissertation is organized into eight chapters in total. Chapter 1 provides the background knowledge leading to the formation of research objectives, hypothesis, and environmental significance. Chapter 2 reviews the recent studies and present understandings on phage host range, phage-based antimicrobial agents, opportunities and challenges of phage application in environmental engineering. Potential strategies to overcome these challenges are also delineated in this chapter. Chapter 3, entitled “Isolation of Polyvalent Bacteriophages by Sequential Multiple-Host Approaches”, introduces two sequential multiple-host approaches to preferentially isolation of phages with broad host ranges, which has been published in Applied and Environmental Microbiology. Chapter 4 presents the effect of phage polyvalence on phage propagation, interspecies competition and bacterial suppression in environments harboring enteric pathogens and soil bacteria, which is edited from an article published in Environmental Science and Technology. Chapter 5 validates polyvalent phages safely produced by non-pathogenic hosts could control problematic ARB in activated sludge microcosms and mitigate the propagation and discharge of associated resistance genes to the environment, this novel polyvalent-phage based ARB control approach has been published in Environmental Science and Technology Letter. Chapter 6 reports findings on a novel biofilm mitigation strategy, which immobilizes polyvalent phages onto CNCs and manipulates their transfer and penetration under a magnetic field. This chapter is edited form a published coauthored article in Environmental Science: Nano. Chapter 7 summarizes the key findings, their implications and their environmental significance of this dissertation and outlines several suggestions and directions for future research.
Chapter 2

Literature Review

2.1. Antibiotics and antibiotic resistance

Antibiotics have heralded a new age of small molecule treatments for bacterial infections and served as a cornerstone of modern medicine due to their effectiveness and ease of use. However, the selective pressure exerted by the persistent use of broad-spectrum antibiotics prompted the proliferation and horizontal transfer of resistance genes (14). The widespread and rapid emergence of antibiotic resistance, as well as reduced rate of novel antibiotics discovery, has led to the antibiotic resistance crisis. According to the Centers for Disease Control and Prevention, each year in the United States, at least 2 million people become infected with bacteria that are resistant to antibiotics and at least 23,000 people die each year as a direct result of these infections (15).

2.1.1. Antibiotics and their action modes

Antibiotics are generally broad-spectrum antimicrobial agents with diversified structures and specific target sites. There are five major modes of action against bacteria based on their target sites (Fig. 2.1): (1) inhibition of cell wall synthesis (e.g., beta-
lactam), (2) disruption of cell membrane function (e.g., polymyxin), (3) interference of nucleic acid transcription (e.g., quinolones), (4) inhibition of protein synthesis (e.g., tetracycline), and (5) interruption of metabolic pathways (e.g., sulfonamides). (16)

**Figure 2.1 Common antibiotics and their target sites.**
The major modes of action against bacteria include inhibition of cell wall synthesis, disruption of cell membrane function, interference of nucleic acid transcription, inhibition of protein synthesis, and interruption of metabolic pathways. Edited from Kohanski MA (2010).

Beta-lactam antibiotics (e.g., penicillin) inhibit the synthesis of the peptidoglycan layer and thus suppress the formation of cell wall structure (17), dramatically reducing bacterial tolerance to fluctuating environmental stresses such as pH, ion strength, and temperature. (18) Cell membrane, which separates extracellular and intracellular environments, is responsible for important cellular processes such as cell signal sensing and ion conductivity. Polymyxin and gramicidin bind to lipopolysaccharide in the outer
membrane and disrupt both the outer and inner membranes of gram-negative bacteria, increasing the permeability of the bacterial cell membrane (19). Quinolones trap DNA gyrase and DNA topoisomerase to form complex and the genomic fragmentation stimulates excessive accumulation of highly reactive oxygen species (20). By binding to the 30S ribosomal subunit, tetracycline suppresses the binding of tRNA to the mRNA-ribosome complex, constraining the protein synthesis processes (21). Sulfonamides compete with dihydropteroate synthase, an enzyme associated with folate synthesis, hinder the growth and replication of cells (22).

2.1.2. Antibiotic resistance development, maintenance, and dissemination

Antibiotic resistance can be achieved through a variety of mechanisms (Fig. 2.2), which include decreased permeability (23), increased active efflux (24), enzymatic inactivation or modification (25), alteration in target sites (26), and bypass of metabolic pathways (27). To minimize intracellular antibiotics, bacteria can limit their entry by reducing (or altering) porin channels or enhance their active efflux through the multidrug-resistant (MDR) efflux pumps. Large-scale metagenomic studies revealed that MDR efflux pumps are the most prevalent resistance mechanism found in the environment (28). Besides physical exclusion of antibiotics, enzymatic reactions also contribute to resistance by hydrolysis, group transfer, and redox. As the best-known antibiotic-resistance mechanism, production of β-lactamases is conductive to the degradation of the β-lactam antibiotics, such as penicillin, cephalosporin, clavams, carbapenems and monobactams (29). Since most antibiotics bind to their target sites with high specificity and affinity, alterations in the target structure that interfere their binding can also confer resistance to bacteria. For example, the chloramphenicol–florfenicol resistance (cfr)
methyltransferase methylates 23S rRNA and prevents the binding of a wide range of antibiotics (30). In terms of pathway alternation, resistance to β-lactam can also be achieved through the bypassing of penicillin-binding proteins by the β-lactam-insensitive L, D-transpeptidase (Ldtfm), which leads to the high-level resistance of Enterococcus faecium to glycopeptides and β-lactams (27).

**Figure 2.2 Antibiotic resistance mechanisms in bacteria.**
Bacteria develop antibiotic resistance through decreased permeability, increased active efflux, enzymatic inactivation or modification, alteration in target sites, and bypass of metabolic pathways. Edited from E. Gullberg.

Once antibiotic resistance genes (ARGs) are acquired in chromosome or plasmid, the resistant mutants must compete with the wild-type ancestors and other bacteria in the community (31). Although ARGs generally reduce bacterial fitness due to the associated metabolic burdens, they can also provide fitness advantages in the presence of antibiotics selective pressures (32). ARGs can still be maintained in bacterial populations under environmental conditions in the absence of antibiotic selective pressure (33). The genomic basis for their durability includes compensatory evolution and genetic linkage. Compensatory evolution, which refers to secondary mutations compensating for the
fitness costs of resistance mutations, can potentially restore fitness and avoid the loss of ARGs (34). Genetic linkage between resistance mutations and other selected genetic markers (e.g. virulence factors) may also promote the persistence of resistant genes (35).

Moreover, horizontal gene transfer further promotes the spread of ARGs among the bacterial community in the forms of transformation, conjugation, and transduction (36). In natural bacterial transformation, exogenous DNA released from donor bacteria is directly taken up from the environment by competent bacteria across their cell wall (37). During conjugation, a donor cell extends one pore and attach to a recipient cell, then one strand of plasmid DNA passes into the recipient bacterium (38). Specifically, conjugation can be achieved not only in the form of plasmids but also in the forms of transposons, integrons and gene islands. Transduction of ARGs between bacterial cells occurs via gene transfer agents (e.g., phages), which consists of generalized transduction and specialized transduction (39). Generalized transduction occurs during the phage lytic life cycle, in which bacterial genome is hydrolyzed into small fragments, packed into phage capsids randomly and then transferred into the recipient bacteria (40). Specialized transduction happens under lysogenic life cycle, where prophages are incorrectly excised from the host chromosome and the transferred genes are adjacent to the prophages (41).

One 2014 World Health Organization (WHO) report stated that the antibiotic resistance crisis is an emerging global health threat that is happening right now in every region of the world and will require large-scale, concerted efforts to mitigate. Besides the prudent utilization of antibiotics in human and animals, the exploration of antibiotics alternatives and supplements also contribute to resolving the crisis of antibiotic resistance (42). Tackling ARGs and ARB in environment cannot be overlooked since environmental
systems (e.g., wastewater treatment plants and agricultural fields) may also serve as breeding grounds and point sources for environmental dissemination of ARGs (43, 44).
2.2. Bacteriophage in nature

Bacteriophages (phages) are viruses that specifically infect bacteria. As with all viruses, phages are obligate intracellular parasites without intrinsic metabolism, which require the metabolic machinery of the host cell to support their reproduction. The complete structure of a phage commonly consists of a nucleic acid core (single or double-stranded RNA or DNA), an outer shell of protein capsid and in some cases a lipid envelope (45). Most phages are semi-microscopic with a size distribution between 20 and 200 nm. Therefore, phages cannot be visualized under the normal optical microscope but can be observed through transmission electron microscopy after capsid staining (46).

Electronic microscopic images show phages are diverse in terms of morphology including 6 morphological types (Groups A - F). The majority of characterized phages (around 96%) are nonenveloped tailed phages with icosahedral protein capsids and dsDNA genomes (Groups A, B and C) (47). According to the International Committee on Taxonomy of Virus (ICTV), these phages are mainly members of order Caudovirales, which includes the families of Myoviridae (Group A, contractile tail), Siphoviridae (Group B, long noncontractile tail) and Podoviridae (Group C, short noncontractile tail). (48) Phages in Groups D and F (Filamentous phages) contain single-stranded DNA while Group E phages contain single-stranded RNA.

2.2.1. Phage ecology

Viral metagenomic studies have rapidly provided illumination on phage abundance, diversity and ecological importance (8, 49). Phages are now well recognized as the most abundant and diverse biological entities in the biosphere, with an estimated global phage
population of about $10^{31}$ (13, 50). Phages have co-existed and co-evolved with their host for billions of years and widely distributed in almost all the ecosystems. There is a dictum that phages exist where bacteria thrive, not only in marine, terrestrial, and underground ecosystems but also on human skins and in human guts. (51) With the high concentration of phages, even rare phage-induced events manifest with high frequency in microbial communities regardless of whether they are autotrophs or heterotrophs.

Lytic phages are responsible for the majority of bacterial mortality or morbidity in the natural microbial communities depending on the sensitivity of their hosts (52), which accelerates the release of cellular materials and essential nutrients back to the environment. As the host population become more abundant, so do the population of their phages, which prevents mono-specific dominance and increases microbial diversity (53). The contributions of lysogenic phages to their hosts not only include host immunity against another phage, but also the horizontal transfer of functional genes (54, 55). Lysogenic phages serve as the most dominant gene transfer agents, covering genes associated with antibiotic resistance, toxins and biofilm formation (56). Considering their abundance and contribution in shaping microbial communities, the importance of phages in global ecosystems and biogeochemical cycles can never be overestimated.

Fluctuation in phage composition is a commonly observed phenomenon in natural and artificial environmental systems (57), demonstrating a dynamic relationship between phages and their bacterial hosts. In fact, the phage composition is the net result of two opposing processes: phages replication due to productive infection and phage inactivation caused by surrounding stresses (58). In general, more new phages are produced when conditions are favorable for bacterial growth. The half-life of free phages in ecosystems
is generally less than 48 hours, and the actual extracellular durability depends on the environmental stress such as solar radiation, temperature, salinity and other physical-chemical conditions. (59) Individual phages can display major difference in environmental tolerance. For example, phage PRD1 has high structural stability over a wide range of temperature and ionic strength and weak affinity towards organics and sediments, which makes phage PRD1 durable in soil and groundwater environments (60). Other phages like MS2, can only survive in a narrow spectrum of salinity and pH (61), and find it challenging to maintain structural integrity and infectious activity in unfavorable environments.

Spatial heterogeneity in phage-host interactions and temporal changes in phage susceptibility define bacterial escape strategies from phage lysis and thus ensure the dynamic equilibrium between bacteria and phage (62). Adsorption resistance, restriction-modification (R-M) systems, CRISPR-Cas systems and abortive infection systems are the most commonly antiviral strategies developed by the bacteria during the coevolution with phages. There are three categories of mechanisms involved in adsorption resistance: blocking of phage receptors, production of capsule or slime layers and presence of competitive inhibitors. (63) Prevention of phage recognition can be achieved through surface protein shield, surface protein alteration or additional production of extracellular matrix. Restriction-modification systems represent a barrier against foreign genomes and reduce the efficiency of unmodified phages by $10^4$ to $10^5$-fold. (64) CRISPR-Cas systems serve as adaptive immune systems by acquiring short phage sequences in the CRISPR locus, which allow the host to recognize and destroy the previously encountered phage genome. (65) Abortive infection systems are altruistic cell death systems to limit phage
replication, which composite the wedge to phage infection through the bacterial community. (66)

2.2.2. Phage life cycles

A typical phage life cycle includes (1) phage adsorption (phage attachment to host), (2) genome uptake (phage nucleic acid injection into host cytoplasm), (3) host takeover (synthesis of proteins and nucleic acid), (4) virion assembly (phage maturation) and (5) progeny release. In terms of life cycle, phages can be divided into two categories: lytic (virulent) phages and lysogenic (temperate) phages (Fig. 2.3). (67) The phages that only use the lytic life cycle are classified as lytic phages, among which coliphage T4 is the most studied (68). The lysogenic phages can either enter the lysogenic cycle by integration into the host genome or enter a lytic life cycle. *E. coli* phage λ is the prototypical representative of lysogenic phages (69), whose life cycle can be either lysogenic or lytic. The life cycle of lysogenic phages largely depends on the states of the phage and bacterial host as well as the surrounding environmental stresses (67).
Figure 2.3 Bacteriophage lytic and lysogenic life cycles.
Phage lytic life cycle leads to lysis of the host while lysogenic life cycle can co-exist with the host. Edited from Salmond GP (2014).

Lytic life cycle. Lytic phages inject their nucleic acid into the host upon attachment to the cell surface. Early expressed genes tend to encode proteins that further take over the host cell, while later expressed genes allow production of phage genomes and capsid proteins, which are subsequently assembled into phage progeny. The phage progeny are released by fatal cell lysis and free to infect new cells (70). This unique life cycle makes lytic phages promising antimicrobial agents against both gram-positive and gram-negative pathogens. Soon after the discovery of phages, the phages have been introduced to control bacterial infections from both gram-negative pathogens (e.g., *Shigella*, *Salmonella*, and *Vibrio*) and gram-positive pathogens (e.g., *Listeria*, *Clostridium*, *Streptococcus*, and *Staphylococcus*).(71)

A series of pathogen detection approaches have been developed based on phage lytic life cycles. Phages with high specificity and short latent time are preferred for phage-based pathogen detection. The underlying mechanisms include phage amplification,
phage-induced cell lysis, and phage-encoded reporter gene expression. In phage amplification assays, samples are mixed with phages specific for the target bacteria to allow infection occur, then extracellular phages are inactivated by viricides and new helper host are introduced to enrich phages. (72) Plate assay or qPCR are used to detect the abundance of phages, which is directly related to the abundance of targeted bacteria in the samples. On cell lysis by infected phages, adenosine triphosphate (ATP), adenylate kinase (AK) and other cytoplasmic markers (e.g., β-D-galactosidase, α- and β-glucosidase) can be harnessed for bacterial detection. Enzymatic conversion of these released compounds into chromogenic substrates provides a signal for bacterial detection. (73) Engineered phages modified with luciferase gene (e.g., luxAB) or green fluorescent protein (GRP) genes allow the expression of bioluminescence or fluorescence on phage infection (74) (75). Due to their sensitivity and accuracy, reporter phages are widely used for pathogen detection in the form of ready-to-use kits.

\[ r = k \times Pt \left( B \times e^{-rL} - 1 \right) \]

**Equation 2-1 Intrinsic growth rate of phage.**

where \( P_t \) is phage abundance at time \( t \), \( k \) is adsorption rate constant, \( L \) is latent time and \( B \) is burst size.

As shown in Equation 2.1, adsorption rate constant, latent time and burst size are three most important parameters to define the propagation of lytic phages. (76, 77) Adsorption rate constant describes the affinity of a phage towards bacterial hosts, latent time is the period between phage attachment and progeny release, and burst size refers to the number of progeny released by each infected cell. Higher phage propagation rate, which is beneficial for phages survival under high host densities, is generally associated with larger adsorption rate constant and shorter latent time.
Lysogenic life cycle. Infection by lysogenic phages may not result in immediate lysis of the host cell. Instead, these phages can stably integrate their genome into the host genome forming a dormant prophage. The phage genome is replicated in concert with the division of the host bacterium. Now it is widely recognized that the coexistence of lysogenic phages confers fitness advantage to their hosts. The contributions of prophages to their hosts include protecting the host against other phages, increasing antibiotic resistance, and coding for toxins and adhesion factors. (54, 55) For example, the inserted phage λ genome changes external membrane of *E. coli*, making the host more resistant to the invasion of some other coliphages. (55) As in the case of *E. coli* O157:H7, eighteen prophages constitute 16% of its genome. Increased bacterial virulence (e.g., shiga toxin), which offered *E. coli* O157:H7 a competitive advantage, results from the infection by the prophages VT1 and VT2 (78). Another study demonstrated that the *bla*TEM and *bla*CTX-M ARGs from phage DNA were transferred to susceptible *E. coli* strains and render resistance to β-lactam antibiotics (39).

Because of these features, lysogenic phages can serve as gene transfer agents to modify the undesirable genes (e.g., virulent genes and resistant genes) (79), switch off host response genes (e.g., suppress SOS genes), and deliver lethal genes into the bacteria. For example, CRISPR-Cas systems were incorporated in phage λ to specifically delete the antibiotic-resistant genes and thus resensitize antibiotic-resistant bacteria. (79) Suppressing the SOS network in *E. coli* with engineered phage enhances killing by quinolones (antibiotics damaging bacterial DNA) by several orders of magnitude. (80) Delivery of lethal genes into target bacteria provides green and specific microbial control
method, which selectively kill the unwanted bacteria without the release of intracellular toxins and virulence genes (81).

It is noted that lysogenic phages possess both lytic and lysogenic life cycles. The lytic-lysogenic decision is dependent upon environmental signals, the number of infecting phages per cell and the health state of the bacterial hosts. (82) When multiplicities of infection are high, lysogenic life cycle would be ecologically favored due to the limited abundance of unaffected bacterial hosts. In contrast, lytic life cycle with productive infections is an advantageous strategy where there are sufficient bacterial hosts. Lysogeny is stable in healthy cells, however, when certain stresses (e.g., ultraviolet light or mitomycin C) cause damage to host genome, the prophage would be induced to re-enter the lytic cycle (83). When the microbial community faces destructive challenges, the phage-to-host ratio becomes significantly higher compared with that in the normal microbial community, because of prophage excision and re-entering lytic life cycle. (84) (85)

Pseudolysogeny. In addition to this classical bifurcation into either lytic or lysogenic propagation, some phages can enter into a carrier state, known as pseudolysogeny (86). The phage genome is incorporated into the cell as free DNA, but neither lyses the host cell nor integrates its DNA into the chromosome. The cessation of the normal life cycle results from lack of adenosine triphosphate (ATP) in host under extreme conditions such as starvation. A good example of pseudolysogeny is an infection of sporulating cells of Bacillus subtilis by phages PBS1 and SP10 (87). The phage genome is incorporated into the developing endospore as free DNA. Upon germination of the spore under favorable conditions, the pseudolysogenic state is resolved, resulting in production of progeny
virions and lysis of the actively growing bacterial cells. Pseudolysogeny increases the possibility of phage survival under unfavorable conditions, which may provide useful information for phage applications in harsh environments.

2.2.3. Phage host range

Host range, which is the breadth of hosts a parasite can infect, is generally determined by parasite, host and environmental factors. Specifically, phage host range describes an assortment of susceptible bacterial types. From host’s perspective, phage host range is mainly limited by bacterial antiviral mechanisms, which include adsorption resistance, uptake blocks, restriction systems, abortive infection, reduced infection vigor and interference with dissemination. To achieve broad host-range, phages need to recognize receptors on each of the different hosts, be able to carry out replication using the divergent machinery available in each, and circumvent any antiviral systems present in each host. Therefore, it is not surprising that for the past 100 years the accepted dogma has been that phages possess stringent host specificity, and examples of polyvalence are considered rare.

Most phages are considered as narrow host-range, which can infect only a few strains of bacteria within the same species. Host specialization is highly correlated with higher propagation rate, so that its progeny can take advantage of the local host abundance. Optimal foraging model implies that these specialist phages would generally outcompete generalist phages when host density is high or host diversity is low. These characteristics are ecologically beneficial in planktonic states without restriction on phage spreading or in nutrient-rich conditions with stable host abundance.
Nevertheless, recent studies have suggested that polyvalent phages may be more widespread than previously recognized (89-91), and may have been overlooked due to the use of biased isolation methods that preferentially select for narrow host-range phages (92, 93). The most popular methods for host range determination are spot tests and plaque assay, which are both culture-dependent. Considering the relatively low ratio of culturable bacteria to total environmental bacteria, these methods may underestimate the breadth of one phage. Moreover, phages propagation remained active and phage abundance was maintained higher than expected in environments where bacterial communities show low host densities but high diversity (94), how can phages survive and propagate in such conditions are poorly understood. Although studies have shed light on the biofilm-phage interactions, there is still knowledge gaps on how phage migration and proliferation in mixed-species biofilms with heterogeneous microbial communities (95, 96).

<table>
<thead>
<tr>
<th>Table 2.1 Phage lifestyle tradeoffs based on optimal forging model</th>
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<tbody>
<tr>
<td><strong>Narrow host-range phages</strong></td>
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<tr>
<td>Specialists (r-strategists)</td>
</tr>
<tr>
<td>Faster replication and decay</td>
</tr>
<tr>
<td>Higher adsorption (lower diffusion)</td>
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<tr>
<td>Permissive hosts</td>
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Recently, the prevalence of polyvalent phages has been more widely recognized due to the continuing isolation of novel phages with broad host range as well as introduction of additional hosts to well-characterized phages. For example, phage T4, originally isolated against *Escherichia*, has been shown to infect certain strains of *Erwnia* and
Shigella (97). Due to a dual set of tail fiber genes, the host range of temperate phage Mu is across different genera, including E.coli, Shigella dysenteriae, and Citrobacter freundii (98). Phage SN-T has been reported to acquire 16S rRNA genes from both Sphaerotilus natans and Pseudomonas aeruginosa, indicating its capability of intergeneric transduction (99). Polyvalent phages with host range spanning both Gram-negative and Gram-positive bacteria have been isolated from activated sludge systems (100) and nutrient-limited lakes (101). Their broad infectivity may result from host-phage coevolution in environments with low host density and/or high host diversity.

Besides natural polyvalent phages, genomic engineering can also extend host range and enhance the efficiency of infection. Since tail fibers are responsible for host recognition, engineered phages with chimeric tail fibers exhibit a broader host range due to recognizing additional bacterial receptors (102). When the vulnerable sites of phage genome are modified to circumvent the restriction-modification systems, the efficiency of phage infection toward nonproduction hosts improves significantly.

In principle, Overall, polyvalent phages may be common in certain environments and their broad host range hold the opportunity of advancing phage applications, especially in environmental systems.
2.3. Bacteriophage-inspired antimicrobial approaches

In the pre-antibiotic era, few treatments for bacterial infections were available and thus the exploration of phages as potential antimicrobial agents continued to flourish until the discovery of antibiotics. With increasing occurrences of antibiotic resistance among pathogenic bacteria, as well as better understanding of phage-bacteria interactions, the utilization of phages for microbial control get revived (103). Phages and their derivatives offer various strategies to tackle the antibiotic resistance such as selective control of target bacteria, dispersal of biofilms, and resensitize of antibiotic resistance (79). Their applications in medical areas have been nearly one century and now have been extended into the fields of agriculture, wastewater treatment, aquaculture and food industry (104-106).

Phages have several beneficial properties over antibiotics in microbial control. Firstly, phages are environment-friendly and naturally existing antimicrobial agents, so isolation of novel phages can usually be achieved more easily and cheaply than the development of new antibiotics. Evolution drives the rapid emergence of new phages that can destroy bacteria that have become resistant to previous phages [24]. Also, phages are relatively more specific than antibiotics and inhibit problematic bacteria with little direct effect on beneficial microbial flora (9).
Figure 2.4 Phage-inspired microbial control approaches.
(A) Phages as self-replicating antimicrobial agents, (B) Phages as lethal genes delivering vehicles, (C) Phages for selective gene modification, (D) Phage-derived antimicrobial proteins.

2.3.1. Phages as self-replicating antimicrobial agents

The ability to multiply in the presence of host makes phages self-regulating tools (107). Phages continue to multiply when problematic bacteria are present, while they decrease as soon as the target bacteria are eliminated. The applications of phages in environmental systems have been tested in terms of biological foaming control, sludge volume reduction, biofilm formation inhibition, and pathogenic population suppression (104-106). The overgrowth of filamentous bacteria results in excess foaming and bulking of the activated sludge, disrupting the wastewater treatment process. Phages infecting *Gordonia* were isolated from activated sludge and the phage cocktails decreased the abundance of filamentous bacteria and controlled the foaming and bulking of activated sludge in microcosms. Membrane fouling caused by biofilm formation on membrane
surface compromises membrane bioreactor process. Phage isolated from raw sewage inhibit the formation of biofilm by *P. aeruginosa*, *A. johnsonii* and *B. subtilis* on the ultra-filtration membrane, reducing biofouling and increasing membrane permeability (108).

Applications of natural and engineered phages for foodborne pathogens (e.g., *Escherichia*, *Salmonella*, *Listeria* and *staphylococcus*) are showing encouraging results “from farm to fork” throughout the entire food chain (109). Phages have been successful in (1) preventing or reducing colonization of pathogens in livestock, (2) disinfecting equipment, containers and other contact surfaces, (3) decontaminating raw products (e.g., meat, fruit and vegetables) and (4) extending the lifetime of final products. Because phages are harmless to mammalian or plant cells and specifically control the pathogenic bacteria, US Environmental Protection Agency (EPA) has approved the use of certain phage preparations against plant pathogens and US Food and Drug Administration (FDA) has approved the use of one phage formula against *Listeria* in meat and poultry products.

**2.3.2. Phages for targeted gene-delivery**

Instead of taking advantage of the natural ability to lyse a target cell, some studies have focused on using virus particles for their capacity to deliver nucleic acids to target cells. The delivered nucleic acids can either encode fatal proteins or disrupt the cell resistant systems. For example, Westwater *et al* utilized the non-lytic, filamentous phage M13 to deliver specialized phagemid DNA, which encoded the addiction toxins Gef and ChpBK, into target cells (110). Hagens and Bläsi used a similar principle to deliver genes encoding the restriction enzyme BglIII and genes associated with λ S holing. The former
enzyme could introduce double-stranded breaks in the chromosome, while the latter could create cytoplasmic membrane lesions to inhibit *E. coli*. (81) These genetically engineered phages exerted a high killing efficiency while leaving the cells structurally intact thus minimizes endotoxin release.

Temperate phages can also deliver functional genes to resensitize ARB to antibiotics. Phage λ was modified to deliver clustered regularly interspaced short palindromic repeats (CRISPR)–CRISPR-associated (Cas) system. The CRISPR-Cas system can specifically recognize and destroy the antibiotic genes, which reverse antibiotic resistance and eliminate the transfer of resistance between strains (79). Other researchers use lysogenic phages to delivery excess antibiotic sensitive genes (i.e., genes *rpsL* and *gyrA*) into the ARB (111). The genes *rpsL* and *gyrA* were integrated with the chromosomal genome of the infected ARB during lysogenic pathway, thus the resistant bacteria were found sensitive to antibiotics (e.g., streptomycin and nalidixic acid).

2.3.3. **Phage-derived antimicrobial proteins**

Phage endolysins are peptidoglycan hydrolases produced at the later stage of phage infection (112). Since peptidoglycan provides structural integrity and rigidity to the cell, degrading the peptidoglycan layer leads to cell wall instability. Endolysins are typically the product of a single gene in a single open reading frame, which can be modified to improve bacteriolytic activity or expand lytic spectrums. For example, exchanging the cell wall binding domain (CBD) of phage endolysin Ply187 with that from Src Homology 3b drastically improved the lytic activity of the chimeric enzyme (113). Similarly, the combination of Cpl-7 CBD from streptococcal prophage LambdaSa2 endolysin and the
SH3b CBD from staphylococcal endolysin LysK make the chimeric enzyme effective to both *streptococcal* and *staphylococcal*. (114)

Phage-encoded polysaccharide depolymerases can degrade macromolecule carbohydrates within extracellular polysaccharides (EPS) and liposaccharides (LPS). Polysaccharides are important for bacterial survival since they are involved in cell integrity, migration, virulence and biofilm formation. (115) Therefore, treatment of bacterial EPS and LPS with phage depolymerases can prevent the colonization of pathogenic bacteria or reduce their virulence and resistance. For example, phage depolymerase DpoEa1h removed the EPS layer of plant pathogen *Erwinia amylovora*, making the amylovora deficient mutant harmless to its host plant. Degrading capsular EPS with phage depolymerase resensitize *K. pneumoniae* to gentamicin. As a result, the combination of purified depolymerase and gentamicin synergistically suppressed the infection caused by *K. pneumoniae*.

Overall, there has been a renewed interest in the application of phages as green antimicrobial agents because of their selectivity and self-replication. The ease of their manipulation and production provides a diverse range of applications in modern biotechnology. Recent studies have emphasized the importance of the microbiome on gut health (116), soil fertility (117) and activated sludge capability (118). The concepts of selective microbial control and targeted drug delivery become more and more popular (119, 120). Phage-inspired microbial control approaches will play more important roles in combating global antimicrobial resistance.
2.4. Challenges of phage applications in environmental systems

To achieve successful microbial control, a sufficient number of phages with infectious capability should be delivered where target bacteria thrive. In environmental systems, the problematic bacteria could exist in sludge flocs, microcolonies, and biofilms, which is not easily accessible. In contrast medically-relevant pathogens, the problematic bacteria in environmental systems are more genotypically and phenotypically diverse, making it difficult to prepare phage formulations. Current major challenges facing phage-based microbial control include narrow host ranges, development of phage resistance, loss of activity during phage delivery and inefficient phage penetration into biofilm (Fig. 2.5). Due to the better understanding of phage-host interactions, a series of strategies have been proposed to help overcome these hurdles and facilitate phage applications (10), although they were previously designed for medical utilization.

![Diagram of challenges facing phage-based microbial control](image)

Figure 2.5 Major challenges facing phage-based microbial control. (A) narrow infective spectrum, (B) development of phage resistance, (C) loss of activity during delivery and (D) inefficient biofilm penetration. Edited from
Narrow infective spectrum. It is well recognized that phage specificity enables suppression of the target bacteria without significant impact on the functional microbial communities. However, high specificity may be a disadvantage for phage applications in environmental systems, where problematic bacteria include distantly related species. For example, sulfate-reducing bacteria and iron oxidizing bacteria are classified based on their metabolic pathways and thus their members can be distributed among different genus or even different phylum (121, 122), which make narrow host range phages ineffective in solving this problems. Given the heterogeneity and diversity of these microbial communities (122), it is challenging for a phage with narrow host range to thrive. Therefore, the success of phage treatment depends largely on the accurate characterization of the microbial community of interest.

There are two major opportunities to overcome the intrinsic drawbacks due to narrow host range: preparation of narrow host-range phage cocktails and application of broad-host-range phages. Previous studies have focused on the former strategy mainly because of the ready availability of narrow host-range phages. The introduction of phages with overlapping host range not only extend the infectious spectrum but also reduce the frequency of bacterial phage-resistance. The introduction of narrow host-range phage cocktail has been proven effective for treating pathogenic infections caused by a certain species of bacteria.

Development of phage resistance. During the arms race, bacteria have evolved to develop a series of defense strategies against phages invasion, which include preventing phage adsorption, destroying phage genomes, and aborting phage life cycles. (88) The frequency of bacteriophage insensitive mutants (BIMs) and the fitness costs associated
with phage resistance mainly depend on the characteristics of bacterial hosts, phages and the surrounding environments.

There are already some strategies proposed to address the challenge of bacterial phage resistance, which include the adoption of phage cocktails, phage training, and the combination of phage with antibiotics. It’s reported that phage cocktails can significantly delay the evolution of phage-resistance (123) or even prevent the instances of resistance by rational design of phage cocktails (124). The previous study also showed one evolved phage cocktail, trained by the wild-type isolates and consecutively phage-resistant variants as host, not only displayed broader host range but also ensured that bacteria resistant to one phage remain susceptible to others. (125) The production frequency of BIMs following phage cocktail treatment ($7.5 \times 10^{-7}$ cfu/ml) was significantly less compared with ancestor phage treatment ($9.5 \times 10^{-4}$-$3.0 \times 10^{-5}$ cfu/ml). The application of natural or engineered phage as antibiotic adjuvants reduced the minimal inhibition concentration of antibiotics and significantly enhanced the efficacy of bacterial suppression by several orders of magnitude (80).

However, all these methods share one common drawback, which is high dependence on the culture of the problematic bacteria. The introduction of problematic bacteria make the purification process complicated because of the existence of cell debris, toxins and virulence genes (126). In some cases, the problematic bacteria grow so slow (i.e., the doubling time is too long) (127) that it’s unsuitable to adopt these culture-dependent approaches to produce or train phages.

**Loss of activity during phage delivery.** Inactivation during phage delivery is another major hurdle for phage-based microbial control in environmental systems. since
phages may be damaged or adsorbed before reaching the target bacteria (128). Phage inactivation mainly results from either structural protein damage, core nucleic acid destruction or in some cases envelope loss. (129) The damage of phage capsid restricts phage attachment onto the host receptor or disables delivery of phage genome after receptor recognition. Due to the destruction of its genome, the phage cannot complete a normal life cycle after genome entering the cell. Phage with envelopes utilize membrane fusion to enter host bacterium, so the loss of envelope blocks phage initial infection.

Environmental factors contributing to phage inactivation include, but are not limited to, physical and chemical conditions (e.g., sunlight UV, temperature, acidity, salinity, and ions) (130), biological predation and enzymatic digestion (131), and molecular compounds binding with tail fibers (132). Removal of viruses during phage delivery occurs by non-specific adsorption to sludge flocs, suspended particles, sediment and nontarget bacteria (133).

There has been some researches on phage encapsulation to attenuate phage loss for phage therapy in the medical field (134) (135). However, these methods are expensive or technically challenging, making them unsuitable for environmental application. Considering the rarity of studies concerning phage protection during delivery, there is still much space for developing better phage delivery strategies in natural and artificial environments.

**Physical barriers and heterogeneity within biofilms.** Another important factor that cannot be neglected is that microorganisms tend to form biofilms with heterogenous bacterial communities, in which extracellular matrix acts as a physical barriers to phage attack. (136) Bacteria in biofilm also display reduced metabolic activity and thus show
increased resistance to phage infection. Especially in the mixed-culture biofilms, aside from EPS protection, the susceptible organism can gain protection by surrounding resistant organisms, which makes the biofilm notoriously difficult to treat with traditional antimicrobial agents. Poor phage penetration into biofilm may therefore limit phage treatment efficacy as phages have difficulty in reaching the target host cells.

To overcome the physical barriers of biofilm, the gene encoding an enzyme that degrades a polysaccharide adhesin implicated in biofilm formation, was incorporated into phage T7. This modified phage effectively cleared *E. coli* biofilms through phage-mediated cell lysis and release of the recombinant dispersin enzyme. The specificity of depolymerase may compromise the efficacy of this approach in treating mixed-species biofilms, which are common under environmental conditions. Therefore, there is an urgent need to develop additional strategies to enhance phage penetration into biofilm and manipulate their migration within biofilm.

Given the global abundance of phages and limited number of inventory phages, there must be many discoveries to be derived from basic phage work. Based on the experience of previous phage research, the versatility of phages and their derivatives will undoubtedly create novel antimicrobial strategies and overcome the challenges facing current phage-based biocontrol approaches (137).
Chapter 3

Isolation of Polyvalent Phages by Sequential Multiple-Host Approaches

This chapter is edited from a published article in Appl Environ Microbiol.
3.1. INTRODUCTION

The total phage population on Earth is estimated at $10^{31}$ or more, making them by far the most abundant biological entities on the planet (138). As such, phages exert a major influence over global biogeochemical cycles (7, 139) and are important drivers of bacterial diversity (140). Considering their ecological importance and value as a potential genetic resource, increasing our fundamental understanding of phage biology may facilitate the development of novel applications in medical, environmental, agricultural and industrial biotechnologies.

However, research on phage diversity and ecology may be inadvertently limited by the use of biased isolation techniques that preferentially select for narrow host-range phages (141), while broad host-range phages are consistently overlooked. The classic approach to isolate and study phages is typically performed with high density, nutrient-rich batch monocultures grown under planktonic conditions, and most often results in the isolation of narrow host-range phages (76, 77, 142).

The occurrence of polyvalent phages has been more widely recognized as more polyvalent phages are isolated from natural systems (e.g., sewage, activated sludge and lake water). (98, 143) However, their broad host-range properties were discovered accidently, and few studies have explored methods for isolating and enriching polyvalent phages. Jensen et al. developed a multiple-host enrichment method to identify phages capable of interclass infectivity (144), and Bielke et al. used a sequential isolation method to isolate phages with intergenus infectivity (145). However, these phage isolation methods have yielded variable results, with a later experiment showing no
significant difference in phage host-range (146). Furthermore, multiple-host enrichment
tend to dramatically reduce phage infectivity, as measured by the efficiency of plating
(EOP) on non-production hosts (144). Nevertheless, these studies demonstrate that
modification of current isolation method based on host-phage interactions may help
isolation of broad host-range phage from common environmental samples.

To facilitate research on the role of polyvalent phages in microbial ecology and
evaluate their potential applications for microbial control or gene transfer, this chapter
compared three different phage isolation and enrichment methods. These included
simultaneous multi-host enrichment method and sequential multi-host isolation
approaches (linear and circular, the latter involving return to original host). Polyvalent
phages were enriched by a benign host (for safer production) and then tested for their
inter-genus infectivity and capacity to suppress growth of model problematic bacteria.
The utility of phage libraries created using either host enrichment or culture-independent
concentration was also compared.
3.2. MATERIALS AND METHODS

**Bacterial strains and culture conditions.** The bacterial strains in this study included *P. aeruginosa* PA01 (ATCC 15692), *P. aeruginosa* HER1018 (ATCC BAA-47), *P. aeruginosa* Migula (ATCC 700829), *Pseudomonas* sp. CF600 (147), *P. putida* F1 (ATCC 700007), *P. syringae* van Hall (ATCC 19310), *E. coli* K-12 (ATCC 10798), and *E. coli* C3000 (ATCC 15597). Culturing was performed using Tryptic soy broth (TSB) medium. The double agar layer method used Tryptone Base-Layer Agar (TBA) as base agar and Tryptone Soft Agar (TSA) as soft agar (148). All bacteria were cultured at 37°C. Bacteriophages were stored at 4°C in SM buffer (50 mmol/L Tris-HCl pH 7.5, 0.1 mol/L NaCl, 8 mmol/L MgSO4, 0.01% gelatin) (149).

**Preparation of phage stock.** An activated sludge sample was taken from the 69th Street Wastewater Treatment Plant in Houston, TX. A portion of this sample was initially mixed with an equal volume of TSB medium and incubated overnight at 37°C. Phages were detached from sediment particles using the sodium pyrophosphate method (150). Centrifugation and filtration were used to remove particles larger than 0.2 µm as previous described (149). The filtrate was treated as phage stock A. Another portion of the original sample was used to obtain phage stock B. Phages were similarly detached from sediment particles. Centrifugation and filtration were used to remove particles larger than 0.2 µm. The phage particles in the filtrate were further concentrated by polyethylene glycol 8000 (PEG) precipitation (150) and resuspended in SM buffer to obtain the phage stock. The
Phage stocks were stored at 4°C and used within one week.

**Figure 3.1 Sequential multi-host isolation method A.**

In each step one host was adopted to enrich and collect phages that can infect this host. The phage that can form plaques on the lawn of the last host is theoretically the one can infect all the hosts.

**Bacteriophage isolation.** Two modified sequential multi-host isolation methods were tested to isolate phages of interest. For sequential multi-host isolation method A (Fig. 3.1), in step 1, phage stock and Host 1 were added to the upper layer of a double-layer agar plate and the plate was incubated until plaques formed on the lawn of Host 1. In step 2, all the plaques from step 1 were collected and cultured in batch with Host 2 for 4 h. The phages and Host 2 were subject to double-layer plates and the plates were incubated until plaques formed on the lawn of Host 2. Similarly, in step 3, the plaques from step 2 were collected and cultured with Host 3. The phages and Host 3 were subject to double-layer plates and the plates were incubated until plaques formed on the lawn of Host 3, so on and so forth.
**Figure 3.2 Sequential multi-host isolation method B.**

1. Phage stock was added to Host 1 of exponential phase. 2. Phages infecting Host 1 were adsorbed by the Host 1. 3. Free phages and adsorbed phages are separated by centrifugation. Phages infecting Host 1 are enriched afterwards. 4-6. Enriched phages are added to Host 2 of exponential phase and repeated the previous procedures with Host 2. 7. Phages enriched by the last host were subject to double-layer plate assay.

For sequential multi-host isolation method B (Figure 3.2), phage stock was added to Host 1 at exponential phase to allow phages infecting Host 1 to be adsorbed for 10 min. Free phages and adsorbed phages were then separated by centrifugation at 10,000 g for 5 min. The supernatant was added to Host 1 for another 10 min to allow the adsorption of phages with low adsorption rate. Phages infecting Host 1 were enriched with Host 1 afterwards for 4 h. The enriched phages were added to Host 2 at exponential phase and the previous adsorption, separation and enrichment procedures were repeated. The enriched phages were added to Host 3 at exponential phase and the same procedures were repeated again. Phages enriched by the last host were subject to double-layer plate assay and the plates were incubated until plaques formed on the lawn of the last host.

**Bacteriophage purification.** A single phage plaque from the lawn of last host was harvested and diluted in SM buffer. The phages were further purified three times using
standard procedures to ensure removal of any contaminant phages (151). The phage titer was expressed as Plaque Forming Units (PFU)/ml using a double-layer plaque assay in triplicate. For morphological analysis, phage particles were further purified by ultracentrifugation using cesium chloride gradients (152), and then dialyzed in pure water to remove ions.

**Transmission electron microscopy.** The purified and dialyzed phage (about 10⁸ PFU/ml) was loaded onto carbon film copper grids and then negatively stained with 2% uranyl acetate (pH 4.5) (46). The excess stain was removed immediately and the stained specimens were air-dried for 30 min. The specimens were observed with JEOL 2010 transmission electron microscopy at 80 kV. Based on their morphology, phage identification and classification was conducted according to the International Committee on Taxonomy of Viruses (153).

**Bacteriophage host range and EOP.** The phage host range was initially determined by spot test assay on potential host lawn (154). In the spot test, 10 µl of phage suspension (about 10⁸ PFU/ml) was added to the potential host lawn and then incubated at 37 °C overnight. The results were further confirmed by measuring the optical density (OD₆₀₀) of the liquid medium in 96-well plates. Each well was inoculated with bacteria of exponential phase to a final concentration of 10⁶ CFU/ml. The initial multiplicity of infection (MOI) was 10, which is a common MOI used in bacterial challenge test (155). The plate reader (Molecular Devices, MA) was set as follows: 600 nm, 37°C, 12 h measurement with an interval of 30 min, and shaking for 5 s before and after each measurement. As shown in Equation 3.1, EOP was quantified by calculating the ratio of
phage plaque titers obtained with indication hosts (PFUi) to those obtained with the production host (PFUp).

\[ EOP = \frac{PFU_i}{PFU_p} \]

**Equation 3-1 Efficiency of plating (EOP) calculation**

PFUi represents phage plaque titers obtained with indication hosts and PFUp represents phage plaque titers obtained with production host.

**Bacteriophage adsorption rate constant, latent time and burst size.** Adsorption rate constants for phages PX01 and PEf1 attaching to host cells were determined as proposed by Kropinski (156), based on the assumption that the adsorption of phage particles to bacterial cells followed first-order kinetics. One-step growth curve experiments were conducted to determine the latent time and burst size of these phages in multiple hosts as previous described (157). All parameters were measured in TSB medium shaking at 120 rpm at 30°C. Briefly, phages were added at an MOI of 0.01 to 1 ml of mid-log phase bacterial culture (diluted to OD_{600} 0.1) and allowed to adsorb for 5 min. Free phages were removed by centrifugation (7000 g, 2 min, 4°C ) and cell pellets were resuspended with same volume of medium. 50 µl of the resuspended culture was transferred to 50 ml medium in a 100 ml flask. Samples were collected at an interval of 5 or 10 min and immediately subjected to plate assays for phage titration. Plaque counts were averaged and plotted to generate the one-step growth curve.

**Bacteriophage host range stability and bacterial challenge tests.** To assess the stability of host range, the purified phage stock (1st generation) was diluted until it formed several clear plaques on double-layer agar plates. A single plaque was selected and then cultured with *P. putida* F1 as host in TSB medium at 37°C for 12 h (Figure 3.3). The enriched phage in tube 2 was considered 2nd generation. Similarly, we enriched
phages with the same hosts for the 3rd and 4th generations. Spot tests were used to test host range. Optical density at 600 nm (OD<sub>600</sub>, measured during bacterial batch growth in liquid culture) was used to assess the inactivation of multiple hosts by 1<sup>st</sup> and 4<sup>th</sup> generation phages.

**Figure 3.3 Host range stability test.**
In each step one single plaque formed on the lawn of Host 1 was collected and then enriched in the liquid culture with Host 1 as host overnight. Phages of all generations were subject to spot test to determine their host range.

To avoid the interference caused by cell debris, the viable bacterial density was measured when the uninfected (control) bacteria reached stationary phase, using plate assays and expressed as Colony Forming Units (CFU). Specifically, the culture in the well was collected and rinsed (centrifugation at 4 °C and resuspension) three times with 200 μl of 0°C PBS, serial dilution was made by 0°C PBS and plated at 37°C overnight. The lethality was calculated by the following equation (first or fourth generation, P1 or P4 respectively).
\[ Lethality = \frac{CFUc - CFUp}{CFUc} \times 100\% \]

Equation 3-2 Host lethality caused by phage infection
CFUc is the CFU from the control culture without phage and CFUp is the CFU from the culture amended with a phage.
3.3. RESULTS

3.3.1. Simultaneous multi-host enrichment methods are not sufficient to isolate broadly polyvalent phages.

Initially, the isolation of polyvalent phages was attempted using a previously reported multiple-host enrichment method (144). In this method, phage enrichment is first conducted using environmental water samples amended with growth medium containing multiple hosts, with bacteriophages subsequently detected using plaque assays. This method was duplicated with the exception of using activated sludge as the phage source. Using this method, we isolated multiple phages capable of infecting different strains of either *E. coli* or *P. aeruginosa*, but no phages capable of infecting more than one of the four *Pseudomonas* species tested (Table 3.1).

![Electron microscopic images of isolated polyvalent phages.](image)

Panels depict phage (A) PX01 (Podoviridae), (B) PPJ2 (Podoviridae), (C) PEa1 (Siphoviridae), and (D) PEf1 (Siphoviridae).

This method was then modified to use a purified and concentrated phage stock from the same sludge sample (phage stock B). This resulted in the isolation of phage PPJ1, which is capable of infecting both *P. putida* F1 as well as *Pseudomonas sp. CF600*. However, further phage characterization yielded an EOP of $3.08 \times 10^{-4}$ for *Pseudomonas sp. CF600* relative to *P. putida* F1 (Table 3.2). No other phages were isolated that were capable of inter-species infectivity using these particular conditions and hosts.
Table 3.1 Influence of phage stock and isolation method on phage host range

<table>
<thead>
<tr>
<th>Phage Stock</th>
<th>Isolation Method</th>
<th>Phages</th>
<th>Host range a</th>
</tr>
</thead>
<tbody>
<tr>
<td>Stock A</td>
<td>Multi-host</td>
<td>Group1 E. coli K-12, E. coli C3000</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Enrichment</td>
<td>Group2 P. a. PA01, P. a. HER1018, P. a. ATCC 700829</td>
<td></td>
</tr>
<tr>
<td>Stock B</td>
<td>Multi-host</td>
<td>PPJ1 P. putida F1, P. CF600</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Enrichment</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Stock A</td>
<td>Method A</td>
<td>PX01 - PX4 P. a. PA01, P. a. HER1018, P. putida F1, P. CF600, P. s. van Hall</td>
<td></td>
</tr>
<tr>
<td>Stock B</td>
<td>Method A</td>
<td>PEa1 - PEa3 E. coli K-12 , E. coli C3000, P. a. PA01, P. a. HER1018</td>
<td></td>
</tr>
<tr>
<td>Stock A</td>
<td>Method B</td>
<td>PPJ2 P. a. PA01, P. a. HER1018, P. putida F1, P. CF600, P. s. van Hall</td>
<td></td>
</tr>
<tr>
<td>Stock A</td>
<td>Method B</td>
<td>PEf3 E. coli K-12 , E. coli C3000, P. a. PA01, P. a. HER1018, P. putida F1</td>
<td></td>
</tr>
<tr>
<td>Stock B</td>
<td>Method B</td>
<td>PEf1, PEf2 E. coli K-12 , E. coli C3000, P. a. PA01, P. a. HER1018, P. putida F1</td>
<td></td>
</tr>
</tbody>
</table>

a P. a. represents P. aeruginosa, and P. s. represents P. syringae.

3.3.2. Sequential multi-host isolation methods can successfully isolate polyvalent phages with inter-order infectivity.

Both sequential multi-host isolation methods enabled the isolation of phages capable of infecting all four species of Pseudomonas tested from phage stock A (Table 3.1). Phage PX01, PX2, PX3 and PX4 were isolated with method A using the same hosts (P. aeruginosa PA01, P. sp. CF600, P. putida F1 and P. syringae van Hall), but with different infection sequences. Phage PPJ2 was isolated with method B according to the sequence P. aeruginosa PA01 > P. sp. CF600 > P. putida F1 > P. syringae van Hall > P. putida F1 > P. sp. CF600 > P. aeruginosa PA01.
Both method A and method B allowed the isolation of phages capable of infecting both *E. coli* and *Pseudomonas* from phage stock B. Phage PEa1, PEa2 and PEa3 were isolated with method A according to the sequence *E. coli* K-12 > *P. aeruginosa* PA01 > *P. putida* F1 or *P. aeruginosa* PA01 > *E. coli* K-12 > *P. putida* F1. However, the plaques collected from the second host could not infect *P. putida* F1 in step 3. In other words, PEa1, PEa2 and PEa3 could only infect *E. coli* K-12 and *P. aeruginosa* PA01. Phage PEf1, PEf2 and PEf3 were isolated with method B according to the sequence *E. coli* K-12 > *P. aeruginosa* PA01 > *P. putida* F1 > *P. aeruginosa* PA01 > *E. coli* K-12. PX01, PPJ2, PEa1 and PEf1 showed almost no differences in EOP on the hosts used during their isolation (Table 3.2). Figure 3.4 shows electron microscopic images of these four phages.

Based on their morphology, PX01 and PPJ2 belong to the *Podoviridae* family, while PEa1 and PEf1 belong to the *Siphoviridae* family.

**Table 3.2 Efficiency of infection of selected phages on their hosts**

<table>
<thead>
<tr>
<th>Phage</th>
<th>Production</th>
<th>Indicator Host</th>
<th>EOP</th>
</tr>
</thead>
<tbody>
<tr>
<td>PPJ1</td>
<td><em>P. putida</em> F1</td>
<td><em>P. putida</em> F1</td>
<td>1.00</td>
</tr>
<tr>
<td></td>
<td></td>
<td><em>P. CF600</em></td>
<td>3.08×10^{-4}</td>
</tr>
<tr>
<td>PX01</td>
<td><em>P. putida</em> F1</td>
<td><em>P. putida</em> F1</td>
<td>1.00</td>
</tr>
<tr>
<td></td>
<td></td>
<td><em>P. a. PA01</em></td>
<td>0.80</td>
</tr>
<tr>
<td></td>
<td></td>
<td><em>P. CF600</em></td>
<td>0.84</td>
</tr>
<tr>
<td></td>
<td></td>
<td><em>P. s. van Hall</em></td>
<td>1.15</td>
</tr>
<tr>
<td>PPJ2</td>
<td><em>P. putida</em> F1</td>
<td><em>P. putida</em> F1</td>
<td>1.00</td>
</tr>
<tr>
<td></td>
<td></td>
<td><em>P. a. PA01</em></td>
<td>0.45</td>
</tr>
<tr>
<td></td>
<td></td>
<td><em>P. CF600</em></td>
<td>0.61</td>
</tr>
<tr>
<td></td>
<td></td>
<td><em>P. s. van Hall</em></td>
<td>0.65</td>
</tr>
<tr>
<td>PEa1</td>
<td><em>E. coli</em> K-12</td>
<td><em>E. coli</em> K-12</td>
<td>1.00</td>
</tr>
<tr>
<td></td>
<td></td>
<td><em>E. coli</em> C3000</td>
<td>0.82</td>
</tr>
<tr>
<td></td>
<td></td>
<td><em>P. a. PA01</em></td>
<td>0.61</td>
</tr>
<tr>
<td>PEf1</td>
<td><em>E. coli</em> K-12</td>
<td><em>E. coli</em> K-12</td>
<td>1.00</td>
</tr>
<tr>
<td></td>
<td></td>
<td><em>E. coli</em> C3000</td>
<td>1.06</td>
</tr>
<tr>
<td></td>
<td></td>
<td><em>P. a. PA01</em></td>
<td>0.60</td>
</tr>
<tr>
<td></td>
<td></td>
<td><em>P. putida</em> F1</td>
<td>0.75</td>
</tr>
</tbody>
</table>

Data shown are the means of triplicate independent experiments.
3.3.3. Phage growth parameters indicate broader host range is associated with lower propagation rate.

Both phage PX01 and PEf1 had variable (host-dependent) adsorption rate constant, latent time and burst size (Table 3.3 and Table 3.4). The adsorption rate constant of PX01 ranged from $(3.54$ to $5.88) \times 10^{-10}$ ml/min, and the adsorption rate constant of PEf1 ranged from $(5.96$ to $8.53) \times 10^{-10}$ ml/min. These values are lower than those of prototypical narrow-host-range phage T4 $(2.4 \times 10^{-9}$ ml/min), T7 $(2.0-4.0 \times 10^{-9}$ ml/min) or λ $(1.3-9.9 \times 10^{-9}$ ml/min) (158, 159). Figure 3.4 and Figure 3.5 show the one-step growth curve of PX01 and PEf1, respectively, with different hosts. Phage latent time ranged from 40 to 55 min and burst size ranged from 45 to 99 PFU/cell. For each phage, the latent time and burst size in different hosts were positively correlated ($R^2 = 0.906$ for PX01, $R^2 = 0.948$ for PEf1), which is consistent with previously observed results (77). The adsorption rate constants were negatively correlated with latent time ($R^2 = 0.805$ for PX01, $R^2 = 0.920$ for PEf1).

<table>
<thead>
<tr>
<th>Phage</th>
<th>Host</th>
<th>Adsorption rate constant</th>
<th>Latent time</th>
<th>Burst size</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>$10^{-10}$ ml/min</td>
<td>min</td>
<td>PFU/Cell</td>
</tr>
<tr>
<td>PX01</td>
<td><em>P. a. PA01</em></td>
<td>3.63</td>
<td>50</td>
<td>60</td>
</tr>
<tr>
<td></td>
<td><em>P. CF600</em></td>
<td>3.54</td>
<td>55</td>
<td>84</td>
</tr>
<tr>
<td></td>
<td><em>P. putida F1</em></td>
<td>5.88</td>
<td>45</td>
<td>54</td>
</tr>
<tr>
<td></td>
<td><em>P. s. van Hall</em></td>
<td>5.83</td>
<td>40</td>
<td>45</td>
</tr>
<tr>
<td>PEf1</td>
<td><em>P. a. PA01</em></td>
<td>8.53</td>
<td>40</td>
<td>72</td>
</tr>
<tr>
<td></td>
<td><em>P. putida F1</em></td>
<td>6.59</td>
<td>45</td>
<td>80</td>
</tr>
<tr>
<td></td>
<td><em>E. coli K-12</em></td>
<td>5.96</td>
<td>50</td>
<td>99</td>
</tr>
</tbody>
</table>

Table 3.3 Growth parameters of phage PX01 and corresponding host lethality.
### 3.3.4. Polyvalent phages isolated by sequential multi-host isolation have stable host ranges and significant infectivity.

Spot tests revealed that phage PX01, isolated with phage stock A, formed clear lytic zones on *P. aeruginosa* PA01, *P. sp.* CF600, *P. putida* F1 and *P. syringae* van Hall, but had no effect on *E. coli* K-12 or *B. subtilis* 168. Phage PEf1 formed clear lytic zones on *P. aeruginosa* PA01, *P. putida* F1, *E. coli* K-12 and *E. coli* C3000. Halos were formed around the lytic zone of *E. coli* K-12 and *E. coli* C3000. However, PEf1 showed no infectivity towards *P. sp.* CF600, *P. syringae* van Hall, *Enterobacter cloacea*, *Klebsiella oxytoca* or *B. subtilis* 168. Phages PX01 and PEf1 were enriched by *P. putida* F1 individually and the following generations were subjected to spot tests. The spot test results for subsequent generations corresponded to those of the 1st generation. This indicates that PX01 (isolated with method A) and PEf1 (isolated with method B) retained their broad host range when enriched with a single host.

Liquid culture experiments were used for bacterial challenge tests and to confirm phage host range. The first generation (P1) and fourth generation (P4) of PX01 and PEf1 were tested for their impact on growth with an MOI of 10. The host growth in the test groups (either P1 or P4) was significantly lower than that in control group (*p* < 0.01). There was little difference in host growth inhibition between the P1 and P4 phages (Figs. 3.7 and 3.8). Using either P1 or P4 of PX01 similarly delayed the onset of exponential growth for each host by about 3 h and reduced the maximum viable bacterial density (CFUs reaching the stationary phase) by more than 50% except for *P. syringae* van Hall (Fig. 3.7 and Table 3.3). PEf1 significantly decreased both the growth rate and final bacteria concentration for all hosts (Fig. 3.8 and Table 3.4). The viable bacterial counts of
*E. coli* K-12 decreased by 90% and those of *P. aeruginosa* PA01 by 65% over the course of the experiment. The liquid culture experiments indicated the isolated polyvalent phages alone could not prevent the emergence of phage resistant bacteria in monocultures.

Figure 3.5 One-step growth curves of phage PX01 in multiple hosts. Panel A is *P. aeruginosa* PA01, Panel B is *P. CF600*, Panel C is *P. putida* F1, and Panel D is *P. syringae* van Hall. Error bars in all the figures indicate standard deviations from the mean of triplicate independent experiments.
Figure 3.6. One-step growth curves of phage PEf1 in different hosts. Panel A is *E. coli* K12, Panel B is *P. aeruginosa* PA01, and Panel C is *P. putida* F1.
3.4. DISCUSSION

This chapter contributes to the study of viral ecology and the advancement of biological control of bacteria by developing methods to isolate and enrich broad host-range phages. We show that the use of different sequential hosts selects for polyvalent phages and is apparently biased against narrow host-range phages, likely due to continual dilution after exposure to the initial host. Spatial separation of the target hosts, which differentiates our approach from previous attempts to isolate broad host-range phages (144, 145), is critical for the selection of polyvalence. Our data also demonstrate that these polyvalent phages can be enriched by benign hosts and potentially be used for microbial control of pathogenic bacteria (Figs. 3.5 – 3.8).

In order to isolate polyvalent phages, we first attempted to replicate previously reported methods that utilized simultaneous addition of multiple hosts for enrichment prior to isolation by plaque assay. However, this method was not sufficient to isolate phages with broad infectivity. The EOP of the isolated polyvalent phage (PPJ1) was greatly reduced on the second host. We hypothesized that the separation of hosts would help prevent such large decreases in EOP, and devised two sequential multi-host isolation methods that utilize an optimized incubation time followed by isolation via plaque assays.

Sequential multi-host isolation method A eliminates the initial host enrichment step to maintain phage library diversity. The isolation of phages by sequential plaque assay ensures that specialist phages are eliminated and that the isolated generalist phages have comparable EOPs to the previous host. Sequential multi-host isolation method B was
designed to separate specialist phages from generalists using multiple adsorption and centrifugation steps. In each step, the phages are exposed to a single host and the adsorbed phages are separated by centrifugation, along with the host. These adsorbed phages are then enriched by this host and introduced to a new host. We postulate that specialist phages that associate with the initial host are separated during centrifugation of subsequent hosts, which increases the relative abundance of polyvalent phages.

**Figure 3.7** Host range tests of polyvalent phage PX01 using batch growth experiments.
Bacteria were cultured in TSB medium with MOI of 10 at 30°C.
It is unclear why the sequence of host exposure was a critical factor in determining phage EOP among its different hosts. Specifically, polyvalent phages isolated using a circular host sequence (the initial and final host being the same, and isolation occurs from this strain) did not suffer significant losses in EOP. In contrast, polyvalent phages isolated with linear host sequence (no duplicate hosts, and isolation occurs from the last strain) exhibited dramatically lower cross-infectivity, with EOPs at least $10^{-4}$ or lower on other bacteria. Regardless of the underlying mechanisms responsible for this interesting phenomenon, this observation suggests the importance of circular host isolation approaches when using pooled phage libraries, to enhance both recovery and infectivity of polyvalent phages.

To assess the effect of phage stock preparation on the isolation of polyvalent phages, two different phage stocks were compared. Phage stock A used an enrichment step intended to increase phage concentration, and was considered culture-dependent. Phage stock B was only filtered and concentrated, which represents a culture-independent method. We were not able to isolate phages capable of infecting both *E. coli* and *Pseudomonas* with method A from phage stock A, but succeeded at this using stock B. It is generally recognized that during the enrichment process, the bacterial community structure changes, likely becoming much less diverse, and the viral community structure evolves accordingly (70). Optimal foraging theory posits that host discrimination (narrowing of host range) is beneficial under conditions of high phage growth rate and host abundance (159). For phages with a very narrow host-range living in planktonic conditions, it is important to bind with the greatest affinity when coming into contact with a potential host, and to be able to replicate quickly. Importantly, manipulation of
phage adsorption rate constants through tail fiber mutagenesis also affects optimal lysis time; phages with higher adsorption constants have lower optimal lysis times and vice versa (76). Lower lysis times are directly related to a smaller burst size as well (77). However, high adsorption rates may be detrimental to phage fitness in biofilm-like environments due to lower phage emigration after lysis (160). An implication of these results is that phages with high adsorption constants for a particular host will generally outcompete those with low absorption constants under planktonic conditions and high host densities. While each individual phage will have fewer progeny, the lower lysis time will allow the progeny to take advantage of the high host abundance and propagate more quickly. Consequently, the bias caused by the enrichment processes should be considered when isolating polyvalent phages, although it may be unavoidable at times.
Figure 3.8 Host range tests of polyvalent phage PEf1 using batch growth experiments.
Bacteria were cultured in TSB medium with MOI of 10 at 30°C.

Interestingly, the infectivity of phages PEa1, PEa2 and PEa3 (isolated using method A) and phages PEf1, PEf2 and PEf3 (isolated using method B) was limited to the species used during their isolation. Though the number of hosts tested in this study was limited, the data suggest that it is possible to bias selection of host-range through careful design of sequential isolation hosts.
Although polyvalent phages infecting both Gram-positive and Gram-negative bacteria have been isolated from activated sludge samples (100), we failed to isolate such polyvalent phages using the bacteria hosts reported. Further method optimization may be needed to achieve this goal. While this work has been performed mainly with *Escherichia* and *Pseudomonas* species, further work may include testing the efficacy of the proposed isolation approaches for isolation of polyvalent phages infecting other genera.
3.5. CONCLUSIONS

Research on phage ecological importance and biotechnological applications has been inadvertently limited by the use of biased isolation techniques that preferentially select for narrow host-range phages, while polyvalent phages are consistently overlooked. In contrast to the standard use of single host, high-density planktonic conditions, which are strongly biased for the enrichment of narrow host-range phages, we developed two novel methods for the rapid isolation and safer enrichment of broadly polyvalent phages, and demonstrated that the isolation of phages capable of inter-order infectivity can be easily achieved with the use of different sequential hosts. This corroborates the emerging perception that polyvalent phages are more widespread than previously perceived (144), and may lead to increased understanding of phage host range and ecology. This work may also incentivize research to harness the extremely broad targeting capability of polyvalent phages for microbial control (161) or gene delivery in uncharacterized environments, which may greatly expand and enhance many biomedical and environmental engineering applications.
Chapter 4

Effect of Phage Polyvalence on Interspecific Competition and Bacterial Suppression

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4.1. INTRODUCTION

As antibacterial agents and important vectors for horizontal gene transfer (162), phages are significant drivers of bacterial evolution and community structure. Consequently, phages can also significantly influence biogeochemical cycles and energy flows in various ecosystems (163). Given their ecological importance and potential applications for selective microbial control (164), there is a need to advance fundamental understanding of phage-host interactions in complex microbial communities. This includes discerning how phage host range affects their propagation dynamics and microbial community structure in both suspended and biofilm systems.

Phages alter their hosts’ metabolic repertoire, fitness and competitive capability, or directly eliminate the most susceptible bacteria (165, 166). Interspecific bacterial competition in the presence of phages has been studied theoretically (166, 167) and empirically (168, 169). Depending on the nature of phage-host associations and environmental conditions, phages can facilitate competitive exclusion (167, 170) or enhance the bacterial hosts’ coexistence (169, 171). However, most these studies have only considered phages that typically infect only one species and cannot reproduce by using hosts from other genera in the microbial community. No previous publications have considered how polyvalent phages affect bacterial suppression and interspecific competition. This is a critical knowledge gap given the recent recognition of the prevalence of polyvalent phages in the environment (89, 100, 172) and their potential to exhibit more complex propagation dynamics. Specifically, whereas polyvalent phages use a wider variety of bacteria to reproduce, they generally experience lower efficiency of
infection and slower growth (100, 173), which may offset their ability to influence microbial community structure.

This chapter addresses how phage host range affects interspecific bacterial competition and enteric bacteria suppression. We compare the effect of coliphage T4 versus polyvalent phage PEf1 on three competing bacteria: (1) *Escherichia coli*, representing an enteric bacterium commonly associated with fecal pollution and (for some *E. coli* strains) infectious disease (174), (2) *Pseudomonas putida* (alternative host to PEf1 but not to T4) and (3) non-host *Bacillus subtilis*, which cannot be infected by these phages. The latter two represent indigenous soil bacteria commonly associated with biodegradation of pollutants (175). *E. coli* suppression is quantified under both planktonic conditions and model biofilms, which offer potential resistance to phage diffusion and to propagation of phage-resistant mutants. We discern the contributions to *E. coli* suppression from phages versus competition by soil bacteria (demonstrating synergism), and highlight the importance of polyvalence in enhancing phage propagation in alternative hosts (*e.g.*, soil bacteria) and significantly boosting suppression of potential enteric pathogens.
4.2. MATERIALS AND METHODS

**Bacteria, bacteriophage and culture conditions.** The bacterial strains in this study included *E. coli* K-12 (ATCC 700926), *P. putida* F1 (ATCC 700007), and *B. subtilis* 168 (ATCC 23857). Culturing was conducted in M63 glucose medium at 30°C [12 g of KH$_2$PO$_4$, 28 g of K$_2$HPO$_4$, 8 g of (NH$_4$)$_2$SO$_4$ per liter water supplemented with 1 mM of MgSO$_4$, 0.2% glucose and 0.5% Casamino Acids] (176). Coliphage T4 (Carolina Biological 12-4330), which was reported to infect only *E. coli* and closely related *Shigella* species (177), infected *E. coli* K-12 but none of other tested bacterial strains. The polyvalent phage PEf1, which can infect both *E. coli* and *P. putida* but not *B. subtilis*, was previously isolated by a sequential multi-host approach. Bacteria in exponential phase were used for phage treatment, phage enumeration and phage characterization. Double-layer plaque assays used tryptone agar plates containing 0.7% agar for the soft agar and 1.1% for the base layer. Phage titration was performed using *E. coli* K-12 as the host and expressed as PFU per ml. The samples were centrifuged to obtain supernatant for extracellular phage titration. Phages were enumerated after direct dilution using the double-layer plaque assay. Quantitative phage abundance analysis was performed by observation of virus-like-particles (VLP) via fluorescence microscopy after staining with SYBR Green I according to the standard protocol (178).

**Bacteriophage growth parameters characterization.** The fraction of free phages was measured as function of time in adsorption tests, and phage adsorption rate constants were determined based on the first-order kinetics:
\[ kB = \frac{(\ln(P_0) - \ln(P_t))}{t} \]

**Equation 4-1 Phage adsorption kinetics**

k is the adsorption rate constant (mL/min), B is bacterial density (CFU/mL), \( P_0 \) is number of free phages at time 0 (PFU/mL), and \( P_t \) is number of free phages at time t (PFU/mL).

One-step growth curve experiments were conducted to measure the burst size and latent time of each phage (179). Efficiency of plating (EOP) was quantified by calculating the ratio of phage plaque titers obtained with a given host to the phage plaque titers obtained with a reference strain (180) (i.e., *E. coli* K-12 in this case). All parameters were determined with bacteria in exponential phase in M63 medium at 30 °C (Table 4.1).

**Table 4.1 Growth parameters of T4 and PEf1 measured with *E. coli* K-12**

<table>
<thead>
<tr>
<th>Phage</th>
<th>Host</th>
<th>Affinity</th>
<th>Latent time</th>
<th>Burst size</th>
<th>EOP</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>10⁹ ml/min</td>
<td>min</td>
<td>PFU/Cell</td>
<td></td>
</tr>
<tr>
<td>T4</td>
<td><em>E. coli</em> K-12</td>
<td>2.31</td>
<td>35</td>
<td>117±8</td>
<td>1.00±0.08</td>
</tr>
<tr>
<td>T4</td>
<td><em>P. putida</em> F1</td>
<td>0.11</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>PEf1</td>
<td><em>E. coli</em> K-12</td>
<td>0.60</td>
<td>50</td>
<td>99±5</td>
<td>1.00±0.06</td>
</tr>
<tr>
<td>PEf1</td>
<td><em>P. putida</em> F1</td>
<td>0.66</td>
<td>45</td>
<td>80±5</td>
<td>0.72±0.04</td>
</tr>
</tbody>
</table>

**Bacterial challenge test in planktonic state.** To compare the inhibitory efficiency of phage T4 and PEf1 in planktonic states, 10 ml 10⁵ CFU/ml *E. coli* K-12 were subjected to the following treatments: (i) 10⁶ PFU/ml phage T4, (ii) 10⁶ PFU/ml phage PEf1, (iii) soil bacteria (1 *P. putida* and 1 *B. subtilis* per *E. coli*), (iv) soil bacteria phage mixture I (1 *P. putida*, 1 *B. subtilis* and 10 T4 per *E. coli*), and (v) soil bacteria phage mixture II (1 *P. putida*, 1 *B. subtilis* and 10 PEf1 per *E. coli*). During the 72-h experiment, to avoid nutrient depletion and the accumulation of phage particles (which confounds analysis), samples were collected at 12h intervals and 0.1 ml culture were transferred into 9.9 ml fresh medium. The samples were centrifuged to obtain supernatant for extracellular phage...
titration and cell pellets for bacterial DNA extraction. Since cultures were diluted 100-fold each step, bacteria were considered completely eliminated when densities were below 100 CFU/ml.

To avoid interference by dead cells, ethidium bromide monoazide (EMA) was used to bind the DNA from dead cells (174). Briefly, cell suspensions were stained with 20 μg/ml EMA (Sigma-Aldrich) in the dark for 5 min, placed on ice for 1 min, and exposed to bright visible light for 10 min prior to DNA extraction. Bacterial genomic DNA was then extracted using an UltraClean Microbial DNA Isolation Kit (MoBio, Carlsbad, CA) according to the manufacturer’s instructions.

**Challenge tests with bacteria embedded in soft agar.** The two-species mixture (10^5 CFU E. coli K-12 and B. subtilis 168, or 10^5 CFU E. coli K-12 and P. putida F1) and three-species mixture (10^5 CFU E. coli K-12, B. subtilis 168 and P. putida F1) were inoculated with coliphage T4 or polyvalent phage PEf1 (10^5 PFU) in the soft agar of double-layer plates. The plates were incubated for 12h at 30°C and then three disk-shaped samples (2cm in diameter) were randomly taken from the soft agar of each plate. The samples were physically ground and bacterial DNA was extracted using a PowerSoil DNA Isolation Kit (MoBio, Carlsbad, CA). Phage λ DNA was used as an internal standard to calculate the recovery rate of all DNA extractions (181).

**Isolation and characterization of BIMs.** Bacteriophage-insensitive mutants (BIMs) resistant to phage PEf1 and T4 were isolated as follows (182). The soft agar of double-layer plates was seeded with 10^7 CFU hosts and 10^9 PFU phage (multiplicity of infection, MOI = 100) and then incubated for 24 h at 30°C. Surviving colonies were picked from the confluent lysis assay plate and streaked onto a nutrient agar. Bacterial contamination
was excluded by using selective agar for plating (EMB agar for *E. coli* and *Pseudomonas* selective agar for *P. putida*), and verifying purity by colony PCR with specific biomarkers (*E. coli*-specific universal stress protein gene A (183) and *P. putida*-specific toluene dioxygenase gene *todC1* (184)). The ratio between the number of surviving colonies and the number of initially inoculated bacteria (10⁷ CFU) was calculated as the BIM frequency.

BIMs were streaked once to avoid re-sensitizing and remove potentially co-evolving phages (185). Spot tests were conducted to confirm the phage resistance of new isolated BIMs (180). To determine potential fitness costs associated with phage resistance development, growth kinetics and biofilm formation were measured. Bacterial specific growth rates were determined based on OD₆₀₀ measurements during the 12-h exponential growth phase (all tested bacteria reached stationary phase after 12 h). The biofilm formed on the surface of 96-well tissue culture plates after a 24h incubation was measured according to a microtiter dish biofilm formation assay (186).

**Inhibition of *E. coli* K-12 in newly-established biofilm.** *E. coli* K-12, *P. putida* F1 and *B. subtilis* 168 (10⁵ CFU/ml for each bacterium) were incubated in glass vials (Kimble™ 20 mL Disposable Scintillation Vials) containing 5 ml of medium and 1.0 g of quartz sand (Fig. 1). The microcosms were shaken at 240 rpm and 30 °C using a Multi-Therm™ shaker (Benchmark Scientific Inc. Edison, NJ). After 24 h incubation, the bulk solution was replaced with 5 ml fresh medium inoculated with bacteria (10⁵ CFU/ml for each strain) and phages (T4 or PEf1 at 10⁶ PFU/ml) to adjust the initial MOI in the bulk solution to the same value used in the prior liquid culture experiments in order to facilitate comparison. The time point when phages were added was considered as time
zero. No phages were added in the control group. During the 5 day experiment, 4.9 ml of liquid culture was replaced with an equal volume of fresh medium at 24 h intervals.

![Image of SEM images](image.png)

**Figure 4.1 SEM images of sand particle surface with and without biofilm.** Sand particles in microcosms(A), sand surface without bacterial colonization (B), and sand surface with newly established biofilm(C).

The bulk solution was collected and centrifuged to obtain supernatant for phage titration and cell pellets for bacteria enumeration. The sand was gently rinsed three times with 0.2 ml of PBS. Sand sampled were suspended in 0.2 ml of PBS supplemented with 0.05% (v/v) Tween-20, and gently sonicated at 40 kHz for 20 min in a 4°C bath sonicator (Branson, Danbury, CT) to disrupt the biofilm matrix and disperse the bacterial cells and entrapped phages (187). The 0.2 ml suspension was centrifuged to obtain the supernatant for phage titration and the cell pellet for bacteria quantification.

**Primer design and real-time quantitative PCR analysis.** The *E. coli*-specific universal stress protein gene A (*uspA*) and toluene dioxygenase gene *todC1* were chosen as specific biomarkers to assess the abundance of *E.coli* K-12 and *P. putida* F1, respectively. Both genes were quantified by qPCR using SYBR Green. The bacterial 16S rRNA gene, quantified by Taqman qPCR, was used for the total bacteria enumeration (188). The extracted genomic DNA from *E. coli* K-12 and *P. putida* F1 with different bacterial densities were used to establish the standard curve between threshold cycle (Ct) value and log_{10} CFU for each bacteria individually. Detailed information of SYBR Green
qPCR and Taqman qPCR is included in Table 4.2. The temperature program for Taqman:

50 °C for 2 min, followed by 95 for 10 min, 40 cycles of 95°C for 15 sec and annealing temperature for 1 min. The temperature program for SYBR Green: 95 °C for 10 min, 45 cycles of 95°C for 15 sec and annealing temperature for 1 min.

Table 4.2 Primers and probes for qPCR analysis

<table>
<thead>
<tr>
<th>Target gene</th>
<th>Reagent</th>
<th>A.T.</th>
<th>Primers/probes sequences</th>
</tr>
</thead>
<tbody>
<tr>
<td>16S rRNA</td>
<td>TaqMan</td>
<td>55°C</td>
<td>5'-ATG GCT GTC GTC AGC T-3'</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>5'-ACG GGC GGT GTG TAC-3'</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>5'-FAM-CAA CGC GAA CCA CCC-TAMRA-3'</td>
</tr>
<tr>
<td>Phage λ</td>
<td>TaqMan</td>
<td>60°C</td>
<td>5'-ACG CCA CGC GGG ATG-3'</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>5'-AGA GAC ACA GGA CTG TC-3'</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>5'-TET-ACC TGT GGC ATT TGT GCT GCCG-TAMRA-3'</td>
</tr>
<tr>
<td>uspA</td>
<td>SYBR</td>
<td>60°C</td>
<td>5'-CTG ACC GAG CTT TCC ACT AAT-3'</td>
</tr>
<tr>
<td></td>
<td>Green</td>
<td></td>
<td>5'-GCG GAA CAA TCA GCA TAT CAA C-3'</td>
</tr>
<tr>
<td>todC1</td>
<td>SYBR</td>
<td>62°C</td>
<td>5'-GCC ATT ACA TCA CGA CCT ACA T-3'</td>
</tr>
<tr>
<td></td>
<td>Green</td>
<td></td>
<td>5'-GGG CTC CAT TCC TTC TTG TT-3'</td>
</tr>
</tbody>
</table>

Scanning electron microscopy of biofilm on sand surface. The washed sand samples were fixed in 4% glutaraldehyde 0.1M phosphate buffer solution (PBS, pH=7.4) overnight at 4°C. The samples were dehydrated in a series of ethanol-water solutions (50, 60, 70, 80, 90, 95, 100%, vol/vol) for 30 min at 4°C. The sand was placed on the carbon tape pasted stub and then sputter-coated with ~7 nm gold film under vacuum using Denton Desk V Sputter System. The microstructure of biofilm on the sand surface was observed using JEOL 6500 scanning electron microscopy. Duplicates were performed for samples from both control and phage treatment microcosms and more than 10 representative images were captured for each sample.
Synergy quantification of combination treatments. Synergy quantification of combination treatments was performed using the Bliss independence model (189). The Bliss model was selected because the modes of action for bacterial inhibition by interspecies competition and phage infection are independent. (190) Each suppression factor was calculated as the ratio of \( E. coli \) density in the presence of a specific stressor (phages or competitors) to the density of unexposed \( E. coli \). Combination indices (CI) were calculated as follows:

\[
CI = \frac{(E_B + E_P - E_B \times E_P)}{E_{B+P}}
\]

**Equation 4-2 Combination indices (CI) calculation**

\( E_B, E_P \) and \( E_{B+P} \) represent suppression factors for \( E. coli \) caused by interspecific competition, phage infection and combination of interspecific competition and phage infection, respectively.
4.3. RESULTS

4.3.1. Coliphage T4 suppressed *E. coli* monocultures more effectively than polyvalent phage PEf1.

*E. coli* growth in suspended pure culture was inhibited to a greater extent by T4 than by PEf1 (Fig. 4.2A). Relative to uninfected controls, *E. coli* viability after 12-h incubation in the presence of T4 at MOI = 1 was 12±4%, which is significantly lower (*p* < 0.05) than 28±4% in the presence of PEf1 at the same MOI. Similar results were observed for biofilm formation, T4 was more effective than PEf1 (10±3% versus 18±4% *E. coli* viability after 24 h exposure, *n* = 3, *p* < 0.05) at inhibiting *E. coli* growth in microtiter dish biofilm formation assays (Fig. 4.2B). These results are consistent with the higher adsorption rate constant (2.3×10^-9 vs. 0.6×10^-9 ml/min), shorter latent time (35 vs. 50 min), and larger burst size (117±8 vs. 99±5 PFU/cell) for T4 than PEf1 (Table 4.1), which results in faster propagation and higher probability of infecting *E. coli* in monoculture.
Figure 4.2 *E. coli* K-12 growth curve and biofilm formation in presence of phages. The initial MOI was 1 for both tests. The biofilm formation was measured after 24h incubation. Coliphage T4 was more effective than polyvalent phage PEf1 at suppressing *E. coli* in pure culture. Error bars indicate ± one standard deviation from the mean of triplicate independent experiments.

4.3.2. *E. coli* resistance to T4 results in higher fitness costs than resistance to PEf1.

Development of resistance is a common bacterial response to phage infection, but a given host may experience different susceptibility and fitness costs for different phages. We assessed the BIM frequency of *E. coli* exposed to T4 or PEf1 (MOI = 100) and compared the resulting fitness costs associated with phage resistance. The BIM frequency of *E. coli* with T4 was 6.2±1.1×10⁻⁷, which is significantly lower (p < 0.05) than that of *E. coli* toward PEf1 (3.8±0.5×10⁻⁶). Resistance to T4 decreased the (exponential phase) specific growth rate of *E. coli* by 28.8±3.6% (from 0.52±0.03 to 0.37±0.02 h⁻¹), compared to a 19.3±1.6% decrease (from 0.52±0.03 to 0.42±0.02 h⁻¹) associated with resistance to PEf1 (Fig. 4.3A). Similarly, the 24 h biofilm formation of *E. coli* resistant to T4 decreased by 22.4±2.1% (from 0.69±0.04 to 0.53±0.04 OD₅₉₅), while that of *E. coli* resistant to PEf1 decreased by only 14.3±2.9% (from 0.69±0.04 to 0.60±0.05 OD₅₉₅) (Fig. 4.3B). The higher fitness cost associated with resistance to T4 than to PEf1 is consistent with the more effective suppression of *E. coli* pure cultures by T4.
4.3.3. Phage PEf1 proliferates better than T4 in mixed-culture sandy slurry microcosms.

Although PEf1 was less effective than T4 in suppressing *E. coli* in mono-culture, it was significantly more effective than T4 under more realistic mixed cultures in both sandy slurry microcosms (Table 1, Fig. 4.4 and Fig. 4.5A) and in soft agar microtiter formation assays (Fig. 4.5B). In these mixed cultures, polyvalence enhanced PEf1 propagation in other hosts (*i.e.*, *P. putida* F1) and reached significantly higher concentrations than T4 in the bulk solution (7.88±0.16 vs 6.83±0.17 log$_{10}$PFU/ml, *p* < 0.05), which enhanced *E. coli* suppression.
Figure 4.4 Bacterial and phage densities in the bulk solution of mixed-species sand slurry microcosms.
Coliphage T4 (A) or polyvalent phage PEf1 (B) was introduced to the mixed-species sand slurry microcosms. PEf1 was able to reproduce in both *E. coli* and *P. putida*, reaching higher densities than T4. Error bars indicate ± one standard deviation from the mean of triplicate independent experiments.

In sandy slurry microcosms, PEf1 could reproduce in both *E. coli* and *P. putida* and reached higher (10-fold) concentrations than T4, which could reproduce only in *E. coli* (Figs. 4.4 - 4.5). Higher PEf1 concentrations in mixed cultures facilitated *E. coli* infection and suppression. *E. coli* concentrations in the bulk liquid stabilized after 3 days at 4.7±0.1 log$_{10}$CFU/ml in the presence of PEf1, which is 1.3 orders of magnitude lower than in microcosms with T4 (Fig. 4.4).
Figure 4.5 Abundance of viral-like-particles in the bulk solution of microcosm under fluorescence microscopy. Panel A is in presence of T4 and Panel B is in presence of PEf1. The samples taken on day 5 were stained with SYBR Green I. Scale bar represents 10 µm.

4.3.4. PEf1 is more effective than T4 at suppressing *E. coli* in mixed cultures under simulated biofilm conditions.

PEf1 also suppressed *E. coli* to a greater extent and for a longer duration than T4 in the mixed-culture biofilm attached on the sand surface (Fig. 4.6A). Prior to phage amendment, the initial densities of *E. coli* in newly formed mixed-species biofilm were 5.64±0.15 log$_{10}$ CFU/mg sand (Fig. 4.6A). After PEf1 amendment, the attached 5-day *E. coli* density decreased by 93±2% to 4.51±0.21 log$_{10}$ CFU/mg sand, while that in microcosms amended with T4 it increased by 44±26% to 5.80±0.23 log$_{10}$ CFU/mg sand (Fig. 4.6A). Compared to controls without phage, PEf1 reduced attached *E. coli* viability by 2.4 orders of magnitude, while T4 achieved only 1.1 order of magnitude suppression. Furthermore, scanning electron microscopy of the sand biofilm revealed significantly greater disruption in microcosms treated with PEf1 than those treated with T4 (Fig. 4.7).
Figure 4.6 Comparison of PEf1 and T4 efficacy to suppress *E. coli* in mixed-species communities.

Panel A is sand slurry microcosms and Panel B is in soft agar. *E. coli* K-12 densities in soft agar were quantified after 12h incubation. B, E, and P represent *B. subtilis* 168, *E. coli* K-12, *P. putida* F1 respectively. Error bars indicate ± one standard deviation from the mean of triplicate independent experiments.

Additional experiments were conducted with bacteria embedded in soft agar plates to assess the effect of phages under biofilm-like conditions without confounding effects such as phage reproduction in suspended hosts or recolonization from the bulk solution. Whereas soft agar tests do not represent the complexity of biofilms commonly encountered in natural and engineered systems, they offer a simple system to investigate factors that influence bacterial attachment and biofilm formation in reductionist experiments that exclude such confounding factors.(191)
**Figure 4.7 Mixed-species (E. coli, P. putida and B. subtilis) biofilms under electronic microscope.**

Intact biofilm without phage treatment (A) and damaged biofilm treated with T4 (B) and PEf1 (C). Sand samples were taken at the end of the 5-day microcosms tests. The biofilm treated with T4 was partially damaged (red circles), while that treated with PEf1 was damaged to a greater extent. The scale bar represents 10 µm.

When *E. coli* and *B. subtilis* were co-cultured without phages, the *E. coli* density reached $8.57 \pm 0.17 \log_{10} \text{CFU/cm}^2$ after 12 h (Fig. 4.6B). *E. coli* densities decreased dramatically when the bacteria were challenged with either PEf1 ($6.60 \pm 0.18 \log_{10} \text{CFU/cm}^2$) or T4 ($6.77 \pm 0.18 \log_{10} \text{CFU/cm}^2$) (Fig. 4.6B, $p < 0.05$). Notably, when *P. putida* was also present, a lower *E. coli* density ($6.06 \pm 0.20 \log_{10} \text{CFU/cm}^2$) was observed for the community challenged with PEf1, while the *E. coli* density increased to $7.15 \pm 0.21 \log_{10} \text{CFU/cm}^2$ in the community challenged with T4 (Fig. 4.6B). Hypothetically, *P. putida* served as a physical barrier for T4 diffusion through static hindrance and off-target adsorption (T4 adsorption constant to *P. putida* was $0.1 \times 10^{-9} \text{ml/min}$), protecting *E. coli* from phage infection. In contrast, *P. putida* enhanced PEf1 proliferation, facilitating its propagation through the biofilm.
Table 4.3 Mean bacterial and viral densities in the microcosms

<table>
<thead>
<tr>
<th>Phase</th>
<th>Component</th>
<th>Treated with T4</th>
<th>Treated with PEf1</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bulk solution</td>
<td>Phage (T4 or PEf1)</td>
<td>6.8±0.3</td>
<td>7.9±0.3</td>
</tr>
<tr>
<td>E. coli K-12</td>
<td></td>
<td>5.7±0.3</td>
<td>4.8±0.2</td>
</tr>
<tr>
<td>P. putida F1</td>
<td></td>
<td>8.3±0.1</td>
<td>7.5±0.3</td>
</tr>
<tr>
<td>Sand surface</td>
<td>E. coli K-12</td>
<td>5.4±0.3</td>
<td>4.5±0.2</td>
</tr>
<tr>
<td>P. putida F1</td>
<td></td>
<td>6.9±0.4</td>
<td>5.9±0.4</td>
</tr>
</tbody>
</table>

Units are log_{10} CFU/ml for bacteria in bulk solution, log_{10} CFU/mg for bacteria on sand surface and log_{10} PFU/ml for phages.

4.3.5. Interspecies competition synergistically contributes to E. coli suppression

Additional bacterial challenge tests were conducted under planktonic conditions to discern how interspecies competition contributes to E. coli suppression without the confounding effects of restricted phage diffusion. In the absence of phages, the presence of bacterial competitors (1 P. putida and 1 B. subtilis per E. coli) decreased E. coli density by 0.55±0.03 log_{10} CFU/mL after three days (Fig. 4.7B1). Thus, interspecies competition alone suppressed E. coli by 72.1±3.0%. Significantly higher suppression of E. coli was observed by the combined effects of phages plus competing soil bacteria (Figs. 4.7A1 and 4.7B1). For example, after 3 days we observed 99.990±0.002% E. coli suppression for the combined stress of T4 plus P. putida and B. subtilis versus 50.3±3.4% individually for T4 alone. The corresponding numbers for PEf1 were 99.974±0.003% for combined versus 43.8±2.8% for phage alone. The Bliss independence model was used to determine whether this combined effect of competing bacteria and phages was synergistic. The combination indices were below 1.0 for all time points (Table 4.4), indicating significant synergistic interactions.
Figure 4.8 Batch growth experiment with *E. coli* K-12 exposed to phages in different bacterial mixtures.

Panels A1 and A2 depict the densities of *E. coli* and phages in monoculture. Panels B1 and B2 depict densities in mixed culture with *B. subtilis* 168 (B), *E. coli* K-12 (E) and PEf1 host *P. putida* F1 (P). Panels C1 and C2 depict densities in more complex culture; M represents a mixture of soil bacteria. All strains were added at equal initial concentrations as *E. coli*. Error bars indicate ± one standard deviation from the mean of six independent experimental replicates.

Individual competition by *P. putida* or *B. subtilis* in two-species mixed cultures decreased the 12-h *E. coli* abundance by 51±6% and 32±4%, respectively. Thus, *P. putida* was a more effective competitor than *B. subtilis* under the tested conditions. When both *P. putida* and *B. subtilis* were used as competitors in the planktonic bacterial challenge tests, PEf1 reached significantly higher concentrations (3- to 45-fold higher) than T4 (Fig. 4.8B2) due to its ability to use *P. putida* as an alternative host. Surprisingly, the less abundant T4 exerted higher *E. coli* suppression (Fig. 4.8B1). Specifically, the mean *E. coli* density treated with T4 (4.53±0.45 log_{10} CFU/ml) was significantly lower (*p* < 0.05) than that treated with PEf1 (5.28±0.35 log_{10} CFU/ml). Apparently, higher *E. coli*
due to enhanced PEf1 propagation was offset by *P. putida* lysis (i.e., as shown in Fig. 4.9, its concentration decreased from 8.37±0.09 log<sub>10</sub> CFU/mL for cultures with T4 to 7.61±0.14 CFU/mL for cultures with PEf1), which attenuated interspecies competition.

**Figure 4.9** Batch growth experiment with *P. putida* F1 in the multispecies culture. Panels A depict densities in mixed culture with *B. subtilis* 168 (B), *E. coli* K-12 (E) and PEf1 host *P. putida* F1 (P). Panel B depict densities in more complex culture; M represents a mixture of soil bacteria. All strains were added at equal initial concentrations as *E. coli*. Error bars indicate ± one standard deviation from the mean of six independent experimental replicates.

As the number of competing soil species increased (equal initial concentrations of *B. subtilis* 168, *P. putida* F1, *B. subtilis* subsp. *subtilis*, *Pseudomonas* sp. CF600, *Pseudomonas nitroreducens*, *Shewanella oneidensis*, *Serratia marcescens*, and *E. coli*), suppression of *E. coli* was more pronounced for both T4 and PEf1, resulting in complete elimination of *E. coli* (Fig. 4.8C1). After 12 h, both T4- and PEf1-treated cultures displayed continually decreasing *E. coli* concentrations in tandem with decreasing phage titers, a pattern indicative of successful bacterial control. In such more complex cultures, competition is exacerbated and suppression of a single competitor (e.g., *P. putida*) is less critical in mitigating interspecies competition. Accordingly, in contrast to
the simpler three-species culture, *E. coli* suppression was faster in the presence of PEf1 than T4, with complete elimination occurring in 36 vs. 42 h (Fig. 4.8C1 and 4.8C2).
4.4. DISCUSSION

Phages have been described as the “dark matter of the biosphere” because, though they are the most dominant biological entities in terms of numbers, diversity and ubiquity, we still know relatively little about them (163, 193). Viral metagenomic studies have begun to shed light on bacteriophage diversity in various environments (8, 194, 195). However, how phages behave in and influence such complex communities is still largely unknown. Historically, most phages have been considered to have relatively narrow host ranges, with polyvalent phages being the exception (89, 196). Nevertheless, recent studies have shown that polyvalent phages may be more widespread than previously assumed (91, 172, 197, 198); a finding that has important implications for their potential impact on horizontal gene transfer and nutrient cycling, and as drivers of microbial diversity. A major goal of this study was to determine how phage dynamics are influenced by polyvalence in both liquid culture and biofilms, and whether this information could be applied to enhance suppression of problematic enteric bacteria in microbial communities.

Consistent with previous studies (170), combining bacterial competitors with phages (PEf1 or T4) resulted in significantly greater inhibition of *E. coli* growth than separate treatments. The resulting Bliss combination indices were below 1.0 for all time points (increasing with incubation time), which indicates significant synergistic interactions (Table 4.4). One possible explanation for the observed synergy is that BIMs acquired resistance by losing or modifying genes coding for proteins that phages might use as receptors (199), and this could incur fitness costs such as reduced motility, suppressed growth rate or decreased biofilm formation capability, which would make BIMs more
susceptible to interspecies competition. Another possibility is that interspecies competition decreases nutrient and substrate availability to the target bacteria, thus delaying the development of phage resistance (200). This would make the target bacteria more vulnerable to phage infection. Whereas further research is needed to elucidate the underlying mechanisms for the observed suppression synergism, these results suggest that phage therapy or biocontrol would be more effective in combination with compatible microbial control strategies.

Table 4.4 Synergistic suppression of *E. coli* by interspecific competition and phage infection

<table>
<thead>
<tr>
<th>Time(day)</th>
<th>Competitorsa + T4 (CP)*</th>
<th>Competitors + PEf1 (CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.5</td>
<td>0.98±0.01*</td>
<td>0.96±0.02*</td>
</tr>
<tr>
<td>1.0</td>
<td>0.97±0.01*</td>
<td>0.92±0.01*</td>
</tr>
<tr>
<td>1.5</td>
<td>0.92±0.02*</td>
<td>0.87±0.04*</td>
</tr>
<tr>
<td>2.0</td>
<td>0.90±0.02*</td>
<td>0.85±0.02*</td>
</tr>
<tr>
<td>2.5</td>
<td>0.86±0.03*</td>
<td>0.86±0.03*</td>
</tr>
<tr>
<td>3.0</td>
<td>0.85±0.02*</td>
<td>0.85±0.02*</td>
</tr>
</tbody>
</table>

a *B. subtilis* 168 and *P. putida* F1 were used as competitors.
b The asterisks (*) represent Combination Index (CI) significantly smaller than 1.0.

In surveys of the natural environment, the most dominant species in any given community generally represents a mere 2–5% of the population (201). Since most phages do not sustainably reproduce below a bacterial density of about $10^4$ CFU/ml (202), and many ecosystems contain greater than 100 different species of bacteria, a monovalent phage would only be expected to reproduce sustainably in well-mixed, planktonic environments (representative of most lab-scale studies) containing greater than $10^6$ CFU/ml. However, the vast majority of bacteria in the environment exist embedded in a biofilm or in micro-colonies (203, 204), where there is little mixing to increase the likelihood of a collision between a phage and its host. Since diffusion
controls phage dispersion within a biofilm, phage properties (e.g., adsorption affinity and host range) as well as the attached microbial community structure might have a significant impact on phage propagation, and therefore the response of the bacterial host within the biofilm (205). Indeed, high adsorption rates have been found to be detrimental to phage fitness in biofilm-like environments due to lower phage emigration after lysis (160).

Our results demonstrate that phage efficacy to suppress target bacteria in a biofilm is also greatly affected by the composition of the microbial community. In two-species biofilms of \textit{E. coli} and \textit{B. subtilis}, we observed little difference between T4 and PEf1. However, addition of \textit{P. putida} (a PEf1 host) caused a decrease in T4 efficacy while it improved the ability of PEf1 to suppress \textit{E. coli} (Fig. 4.6B). This is intuitive because \textit{P. putida} not only facilitated PEf1 propagation, but also likely hindered T4 diffusion. Interestingly, PEf1 also suppressed \textit{E. coli} more effectively than T4 in the bulk solution of microcosms containing the same three-species mixture (Fig. 4.4), but not in liquid batch culture without biofilm (Fig. 4.7B1). Our data suggest that this occurred because the biofilm in the microcosm may have partly served as a protective bacterial refuge. Specifically, concurrently high phage and bacterial densities generally indicate poor bacterial suppression due to the presence of bacterial refuges (e.g., biofilms and micro-colonies). (168, 192) This was observed for microcosms with T4, where phage densities were over 100-fold higher in the bulk solution (Fig. 4.4A) than in liquid batch cultures (Fig. 4.7B2) at comparable time points. Apparently, biofilm-associated \textit{E. coli} was not easily accessible to T4. In contrast, biofilm-associated \textit{E. coli} was more accessible to PEf1 despite its slower proliferation than T4 in \textit{E. coli} (Table 1), likely due to enhanced
proliferation and propagation by the presence of additional hosts, and the resulting higher suppression is conducive to fewer \textit{E. coli} cells repopulating the bulk solution (Fig. 4.4). Importantly, PEf1 genome sequencing did not reveal the presence of depolymerases that may have contributed to its ability to suppress biofilm growth, thus the enhanced efficacy of PEf1 is putatively due to its expanded host range and lower adsorption rate constant.

The literature includes some contradictory results on the efficacy of phages to exert long term suppression of target bacteria, possibly confounded by development of phage resistance that facilitates co-existence. Interspecies competition is also an influential factor, often enhancing phage efficacy to suppress target bacteria. For example, some phages have been shown to exert long term suppression of a target bacterial population in a natural soil community, but not in monocultures (206). Both phage host range and bacterial growth conditions (planktonic vs. biofilm) can also affect suppression of target bacteria. For instance, a study with narrow host-range phages reported long-term suppression of a planktonic, two-species bacterial community (170), while similar tests did not result in significant suppression in either single or mixed-culture biofilms (168), which agrees with our finding of more difficult suppression of \textit{E. coli} in biofilms.
4.5. CONCLUSIONS

An inability to exert long-term suppression of biofilm-associated target bacteria would pose a significant challenge to the use of phages for microbial control (207), since most bacteria live within mixed-species biofilms (204). Previous publications did not consider polyvalent phages in mixed-cultures (208-211), where both intra- and interspecies competition and complex phage propagation dynamics, may substantially affect suppression efficacy. Although narrow host-range phages may be more effective than polyvalent phages in bacterial inhibition in pure cultures, polyvalent phages can be more effective in suppressing target bacteria under multispecies biofilm conditions because polyvalence enhances their propagation in other hosts. In contrast, resistant bacteria do not propagate narrow host-range phages and hinder their diffusion through the biofilm.

Under planktonic conditions significantly higher suppression of targeted bacteria was observed by the combined synergistic effects of phages plus competing bacteria. Polyvalence enhanced phage infection while attenuated interspecies competition. In more complex cultures, competition is exacerbated and suppression of a single competitor is less critical in mitigating interspecies competition. Accordingly, bacterial suppression was faster in the presence of polyvalent phages than narrow host-range phages. Overall, polyvalent phages may provide a new approach for long-term suppression of problematic bacteria in both planktonic and biofilm mixed-species systems.
Chapter 5

Control of Antibiotic Resistant Bacteria by Production Host-Phage Mixture

This chapter is edited form a published article in Environ Sci Technol Lett.
5.1. INTRODUCTION

Phage biocontrol is receiving increasing interest to mitigate the propagation of ARB (212). Having co-evolved with bacteria for billions of years, phages possess the capability to infect their hosts specifically and efficiently, which enables control of problematic bacteria with little impact on the rest of the microbial community (9). Unlike antibiotics or biocides, whose concentration decreases with time after dosage, phages may continue to replicate and infect the target bacteria (213). Recently, improved understanding of phage-host interactions has extended phage-based microbial control applications from the medical field to water and wastewater treatment (104-106).

As with any antimicrobial agent, sufficient phage concentrations (i.e., adequate phage to host ratio) must be attained to ensure efficient microbial control (214, 215). However, given the complexity of activated sludge, phages can be removed by adsorption to sludge flocs, suspended particles and commensal bacteria before infecting their hosts (216). Furthermore, environmental stresses, such as solar radiation and extreme temperature, salinity and pH, may also decrease phage numbers and hinder infection (217). Overall, the phage titer is the result from two opposing processes: phage replication after productive infection and phage decay caused by various stressors (214). This underscores an opportunity to enhance phage proliferation and ARB suppression in WWTPs, which is important to mitigate ARB discharge to the environment (218).

In this chapter, a non-pathogenic production host (P. putida) was used to grow polyvalent phages (PER01 and PER02), and the host-phage mixture was tested for its ability to suppress multidrug- (β-lactam-) resistant E. coli NDM-1 in activated sludge microcosms. Narrow-host-range coliphage cocktails (MER01 and MER02) were also
tested to discern the benefits of phage polyvalence. We propose a novel approach to mitigate ARB proliferation in WWTPs, based on adding polyvalent phage cocktails with their benign production host. This strategy would simplify phage production and improve phage survival and ARB control efficacy.
5.2. MATERIALS AND METHODS

Bacterial strains and culture conditions. *E. coli* NDM-1 carrying the plasmid-encoded *bla*\textsubscript{NDM-1} gene is resistant to β-lactam antibiotics (219). M9 medium was used for bacterial and viral growth parameters measurement. Activated sludge for phage isolation and microcosm studies was obtained from the aeration tank of 69th Street WWTP, Houston, TX. The temperature, pH and dissolved oxygen (DO) of AS in aeration tank were provided by the WWPT. The total suspended solids (TSS), volatile suspended solids (VSS) and settled sludge volume (SSV) were determined using standard methods after the samples were brought back to the lab (220). The sludge volume index (SVI) (mL/g) was calculated as follows: \( \text{SVI} = \frac{\text{SSV (mL/L)}}{\text{TSS (g/L)}} \). All the physical chemical parameters were summarized in Table 5.1.

### Table 5.1 Physical and chemical characteristics of activated sludge

<table>
<thead>
<tr>
<th></th>
<th>Bioreactor</th>
<th>Microcosm (Day 5)</th>
</tr>
</thead>
<tbody>
<tr>
<td>pH</td>
<td>6.9±0.2</td>
<td>6.8±0.1</td>
</tr>
<tr>
<td>Temperature (°C)</td>
<td>27.8±0.3</td>
<td>25.0±0.5</td>
</tr>
<tr>
<td>DO (mg/L)</td>
<td>4.28±0.51</td>
<td>4.23±0.24</td>
</tr>
<tr>
<td>TSS (g/L)</td>
<td>2.14±0.06</td>
<td>2.32±0.08</td>
</tr>
<tr>
<td>VSS (g/L)</td>
<td>1.28±0.06</td>
<td>1.31±0.07</td>
</tr>
<tr>
<td>SVI (mL/g)</td>
<td>80.4±0.1</td>
<td>87.4±0.2</td>
</tr>
</tbody>
</table>

Values are (Means ±SD) of triplicate independent experiments in all tables in this chapter

Isolation of bacteriophages against *E. coli* NDM-1. A culture-independent phage stock was obtained from activated sludge as previous described (221). Polyvalent phages (PER01 and PER02), which can infect both *E. coli* strains and *P. putida* F1, was isolated by sequential multi-host method A (221), according to the sequence of *E. coli* NDM-1 > *P. putida* F1 > *E. coli* K-12 > *P. putida* F1. Coliphages (MER01 and MER02) were isolated by enrichment in *E. coli* NDM-1 liquid monoculture (222). Isolated phages were
purified by standard procedure and stored in SM buffer (222). The phages were characterized in terms of morphology, adsorption rate constant, latent time and burst size.

**Activated sludge microcosms.** The microcosms studies were conducted with 1.0 L amber glass bottle (Photographers' Formulary). The microcosms were put in the room temperature (25 °C) and aeration with Top Fin air pump. The pH and DO of AS in the microcosms were measured using VWR sympathony™ Multisensor. Initial concentrations of total bacteria, natural *E. coli* and natural *P. putida* in the sludge were 7.8±0.7×10¹¹ CFU/mL, 1.3±0.4 ×10³ CFU/mL, and 2.8±0.4 ×10³ CFU/mL, respectively. During the 5-day experiment, 20 ml sludge samples were collected at 24-hour intervals. The bacterial DNA was extracted using a PowerSoil DNA Isolation Kit (MoBio, Carlsbad, CA) according to the supplier’s instructions. Phage λ DNA was used as an internal standard to calculate the recovery rate of DNA extraction (223). Phages were detached from sediment particles using the sodium pyrophosphate method (150). Centrifugation (8000g, 10min, twice) and filtration (Whatman Syringe Filter) were used to remove bacteria and other particles larger than 0.2 μm. The filtrate was serial diluted for phage enumeration by double-layer plaque assay using *E. coli* NDM-1 as host. To test the biological activity of AS treated with phage cocktails, AS oxygen consumption was monitored with a PF-8000 respirometer (RSA, Inc., Springdale, AR).

**Bacterial challenge tests in activated sludge microcosms.** Microcosm studies were conducted using 1.0 L bottles containing 400 ml activated sludge with 0.2% glucose at 25°C. Group 1 microcosms were antibiotic-free and Group 2 microcosms were supplemented with 50 μg/L (each) ampicillin and kanamycin. *E. coli* NDM-1 was introduced at 10⁶ CFU/mL and incubated for 6 h before treatment with phages.
Microcosm treatments were (a) MER01 and MER02 (5×10^6 PFU/mL each); (b) PER01 and PER02 (5×10^6 PFU/mL each); and (c) SM buffer as control. *P. putida* F1 (10^6 CFU/mL) was also added to all microcosms. During the 5-day experiment, 20 ml sludge samples were collected at 24-h intervals, and 20 mL M9 medium with 4% glucose was added to sustain volume and nutrient levels.

**Isolation, verification and characterization of BIMs.** The isolation of bacteriophage-insensitive mutants (BIMs) was performed as previously reported (224). Briefly, double-layer agar plates were seeded with 10^9 PFU/mL phage and a series of 10^8, 10^7, or 10^6 CFU/mL *E. coli* NDM-1 as the host. The colonies formed on the confluent lysis assay plate were streaked on EMB selective agar. Colony PCR with *E. coli*-specific universal stress protein gene A was used to verify the surviving colonies were BIMs of *E. coli* NDM-1 instead of bacterial contamination. The colonies of BIMs were picked and further purified by co-culture with phages in the soft agar overnight. Spot tests were conducted to confirm the phage-resistance of new isolated BIMs before fitness costs tests. After 24 h at 30°C, the ratio of surviving colonies on the confluent lysis assay plate and the initially inoculated bacteria was calculated as the BIM frequency (224). Surviving colonies were picked and further purified by co-culture with phages in the soft agar overnight. Specific growth rate and biofilm formation of BIMs was measured to assess the fitness costs of phage resistance (224). Phage-resistant *E. coli* NDM-1 were challenged with *P. putida* F1 to test the stability of *bla*_{NDM-1} in various phenotypes of *E. coli* under interspecies competition.

**PCR and qPCR of selected biomarkers.** The *E. coli*-specific universal stress protein gene A (*uspA*) (183), *bla*_{NDM-1} gene (225), toluene degradation gene (*todC1*) (184) and
bacterial 16S rRNA gene (188) were chosen as biomarkers to assess the abundance of total *E. coli*, β-lactam antibiotic-resistance gene, *P. putida* and total bacteria, respectively (Table S2). Standard curves were prepared using bacterial CFUs (plate assay) and Ct values (qPCR). Normal PCR and RT qPCR were performed on T100™ Thermal Cycler and CFX96™ Real-Time System, respectively. For SYBR Green qPCR reactions, the reaction mixture contained 1 μL of DNA, 500 nM primers, 7.5 μL of 2× SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA), and DNA-free water, yielding a total volume of 15 μL. For Taqman qPCR reactions, the reaction mixture contained 1 μL of DNA, 500 nM primers, 200 nM probe, 7.5 μL of 2× Taqman PCR Master Mix (Applied Biosystems, Foster City, CA), and DNA-free water, yielding a total volume of 15 μL. The temperature program for Taqman qPCR: 50 °C for 2 min, followed by 95 for 10 min, 40 cycles of 95°C for 15 sec and annealing temperature for 1 min. The temperature program for SYBR Green qPCR: 95 °C for 10 min, 45 cycles of 95°C for 15 sec and annealing temperature for 1 min. Melting curve analysis was conducted after PCR completion to ensure nonspecific PCR products were not generated. The standard curves were prepared using bacterial CFUs (plate assay) and Ct values (qPCR). As shown in Equation 5-1.

\[
ARB \text{ fraction} = \frac{bla_{NDM-1}}{uspA} = \frac{\text{Resistant CFU}}{\text{Total CFU}}
\]

**Equation 5-1 Fraction of *E. coli* with *bla*<sub>NDM-1</sub>**

The fraction of *E. coli* with *bla*<sub>NDM-1</sub> was calculated as the ratio of resistance gene *bla*<sub>NDM-1</sub> to the *E. coli*-specific gene *uspA*. The initial ratio of *bla*<sub>NDM-1</sub> to *uspA* at the time point of inoculation was set as 1.0.
5.3. RESULTS AND DISCUSSION

5.3.1. Isolation and characterization of phages infecting *E. coli* NDM-1.

Consistent with previous studies (226, 227), enrichments using high-density, nutrient-rich liquid monocultures of *E. coli* resulted in the isolation of narrow host-range coliphages (MER01 and MER02), while a sequential multiple-host approach preferentially selected for phages with broader host range (PER01 and PER02) (Table 5.2). Specifically, optimal foraging theory posits that host discrimination (narrowing of host range) is beneficial under conditions of high host abundance (228). Phages with rapid propagation rates in the dominant host are more likely to outcompete those with slow propagation rates under planktonic conditions with high host densities. Our sequential multiple-host approach ensures spatial separation of multiple hosts, which dilutes specialist phages and enriches generalist phages in each step (221).

![Image of isolated phages](image)

**Figure 5.1 Morphology of isolated phages infecting *E. coli* NDM-1.**

Polyvalent phage PER01 (A), polyvalent phage PER02 (B), coliphage MER01 (C) and coliphage MER02 (D) belonged to the families of *Myoviridae, Podoviridae, Myoviridae* and *Siphoviridae*. Scale bar represents 100 nm.

Based on electron microscopy, phage PER01 belongs to the *Myoviridae* family, PER02 to the *Podoviridae* family, MER01 to the *Myoviridae* family, and MER02 to the
Siphoviridae family (Fig. 5.1). Phages PER01 and PER02 were capable of infecting all isolation hosts (E. coli NDM-1, E. coli K-12, and P. putida F1) as well as E. coli C3000 and E. coli J53 without a dramatic reduction in the efficiency of plating (0.43 - 1.00), while coliphages MER01 and MER02 showed narrower host range, infecting only two of the four tested E. coli strains (Table 5.2).

Table 5.2 Host range of isolated phages against E. coli NDM-1

<table>
<thead>
<tr>
<th>Bacteria</th>
<th>ATCC #</th>
<th>Infectivity (EOP)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>PER01</td>
<td>PER02</td>
</tr>
<tr>
<td>E. coli NDM-1</td>
<td>BAA-2452</td>
<td>0.89±0.10</td>
</tr>
<tr>
<td>E. coli J53</td>
<td>BAA-196</td>
<td>0.46±0.09</td>
</tr>
<tr>
<td>E. coli K-12</td>
<td>10798</td>
<td>0.82±0.12</td>
</tr>
<tr>
<td>E. coli C3000</td>
<td>15597</td>
<td>0.71±0.08</td>
</tr>
<tr>
<td>P. putida F1</td>
<td>700007</td>
<td>1.00</td>
</tr>
</tbody>
</table>
**Figure 5.2 Inhibitory effect of coliphage versus polyvalent phage cocktails.**
The phage cocktails of MER series exerted a more significant inhibitory effect than the cocktails of PER series on the growth of *E. coli* NDM-1 (MOI = 10). Error bars in all figures indicate ± one standard deviation from the mean of triplicate independent experiments.

The coliphages (MER series) displayed larger adsorption rate constants and shorter latent times compared to the polyvalent phages (PER series) (Table 5.3). The estimated growth parameters indicate faster propagation of the coliphages in *E. coli* NDM-1, which is consistent with previous reports that narrow-host-range (specialist) phages generally outcompete polyvalent (generalist) phages in liquid monoculture. (229) (230, 231) However, polyvalent phages may more effectively suppress target bacteria in mixed cultures with alternative hosts that facilitate their proliferation. Both phage cocktails (MER and PER series) exerted significant inhibitory effect on the growth of *E. coli* NDM-1 in monoculture (Fig. 5.2), indicating their potential application as antimicrobial agents.

**5.3.2. Phage polyvalence and presence of production host facilitated control of antibiotic resistant bacteria.**

When added with production host *P. putida* F1, polyvalent phage cocktails (PER01 and PER02) were more effective at suppressing *E. coli* NDM-1 than coliphage cocktails (MER01 and MER02, which were also added with *P. putida* F1 to facilitate comparison). For example, the viable concentration of *E. coli* NDM-1 decreased by 2.4±0.2 orders of magnitude after 48 h in microcosms treated with the polyvalent phage cocktails, which is 1.2±0.1 orders of magnitude lower than that for coliphage cocktails treatment at a similar time, and represents over 99% inhibition (Fig. 5.3A). Treatment with polyvalent phage
cocktails was significantly enhanced by co-amendment with the production host, since viable *E. coli* NDM-1 concentrations were 1.1±0.2 orders of magnitude higher in treatments with phages alone after 48 h (Fig. 5.4A). This reflects enhanced phage proliferation in the presence of the production host, which increases the probability of productive infection.

**Figure 5.3 Abundance of target host, production host, **bla**NDM-1** gene and phage (infecting *E. coli* NDM-1) in the microcosms spiked with antibiotics.

*E. coli* (A), and *P. putida* (D) densities were enumerated based on selected biomarkers. Phage density was measured with plaque assay using *E. coli* NDM-1 as host (B). Fraction of *E. coli* with **bla**NDM-1 gene (C) was calculated as the ratio of resistant gene **bla**NDM-1 to *E. coli*-specific gene **uspA**.
Accordingly, the polyvalent phage titer was significantly higher (i.e., by 1.1±0.3 orders of magnitude) than that of the coliphages (Fig. 5.3B) or polyvalent phages in control microcosms without *P. putida* F1 within the first 48 h (Fig. 5.4B). Eventually, as is commonly observed after successful phage treatment (232), an unsustainable high phage-to-host ratio was reached, followed by a rapid decrease in polyvalent phage titer after 72 h (Fig. 5.3B and 5.3D).

Figure 5.4 Comparison of bacterial suppression efficacy between phage-only treatment and host-phage mixture treatment.

No *P. putida* F1 was inoculated in the microcosms. Phage treatment used 7.0 log$_{10}$PFU/mL PER series phage cocktails. Host-phage mixture contained 6.0 log$_{10}$CFU/mL *P. putida* F1 and 7.0 log$_{10}$PFU/mL PER series phage cocktails.

Enhanced polyvalent phage proliferation in non-target hosts (e.g., *P. putida* F1) can offset the relatively fast phage decay in the activated sludge. In the absence of hosts, PER01 and MER01 decay rates followed first order kinetics (Fig. 5.5). Decay rate constants were 1.03±0.06 h$^{-1}$ for PER01 and 1.12±0.08 h$^{-1}$ for MER01, which are similar to reported values for laboratory-scale activated sludge systems.(105) When *P. putida* F1
(production host for PER01 but not MER01) was co-amended (MOI = 1.0), the phage
density of PER01 increased within the first 2 hours and then fluctuated around 6.2±0.2
log_{10} PFU/mL, while that of MER01 continued to decay in the same pattern (Fig. 5.5).

![Figure 5.5](image_url)

**Figure 5.5 Survival of polyvalent phage and coliphage in activated sludge**
No *E. coli* NDM-1 was inoculated in these activated sludge microcosms. Introduction of
production host and phage mixture (MOI=1.0) offset polyvalent phage (PER01) decay.

### 5.3.3. Phage cocktails accelerated the loss of plasmid-encoded *bla*<sub>NDM-1</sub> gene.

Antibiotic-resistant genes (ARGs) enhanced multidrug-resistant *E. coli* NDM-1
survival in the presence of ampicillin and kanamycin (50µg/L each). In antibiotic-spiked
microcosms, *E. coli* densities were significantly higher than those in antibiotic-free
microcosms at each comparable exposure time (Fig. 5.3A and Fig. 5.6A). At the end of
the 5-day experiment, the *E. coli* density was 5.5±0.2 log_{10} CFU/mL and the fraction of *E.
coli* with *bla*<sub>NDM-1</sub> was 0.86±0.09 in antibiotic-spiked microcosms, while the
corresponding *E. coli* density in antibiotic-free microcosms was 4.0±0.2 log_{10} CFU/mL
and the fraction of *E. coli* with *bla*<sub>NDM-1</sub> was 0.61±0.07 (Table 2).
Figure 5.6 Abundance of *E. coli* and *bla*<sub>NDM-1</sub> gene in the microcosms free of antibiotics.

*E. coli* (A) density was enumerated based on selected biomarker *uspA*. Fraction of *E. coli* with *bla*<sub>NDM-1</sub> gene (B) was calculated as the ratio of resistant gene *bla*<sub>NDM-1</sub> to *E. coli*-specific gene *uspA*.

ARGs and plasmids exert a metabolic burden associated with their energy requirements for expression, maintenance and replication, (233) and can be prone to segregational instability under growth-limiting conditions or phage infection.(234, 235) Accordingly, the plasmid-borne ARG *bla*<sub>NDM-1</sub> was lost to a significantly greater extent ($p < 0.05$) when *E. coli* NDM-1 was treated with the polyvalent phage cocktails (Fig. 1C). In these antibiotic-spiked microcosms, the fraction of *E. coli* containing *bla*<sub>NDM-1</sub> was significantly lower when treated with polyvalent phage cocktails (0.57±0.07) than the control group without phages (0.86±0.09) (Table 5.4). Overall, the phage cocktails inhibited the growth of ARB (*E. coli* NDM-1) and decreased the fraction of target
bacteria harboring bla<sub>NDM-1</sub>, resulting in a significant reduction in the overall abundance of this ARG.

**Table 5.3 E. coli NDM-1 abundance after 5-day treatment**

<table>
<thead>
<tr>
<th>Microcosm</th>
<th>Treatment</th>
<th>E. coli density&lt;sup&gt;a&lt;/sup&gt;</th>
<th>bla&lt;sub&gt;NDM-1&lt;/sub&gt; gene concentration&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Fraction of E. coli with bla&lt;sub&gt;NDM-1&lt;/sub&gt;&lt;sup&gt;b&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Antibiotics-spiked</td>
<td>Control</td>
<td>5.54±0.24</td>
<td>5.47±0.24</td>
<td>0.86±0.09</td>
</tr>
<tr>
<td></td>
<td>MER series</td>
<td>4.72±0.31</td>
<td>4.59±0.29</td>
<td>0.74±0.11</td>
</tr>
<tr>
<td></td>
<td>PER series</td>
<td>3.75±0.21</td>
<td>3.50±0.24</td>
<td>0.57±0.07</td>
</tr>
<tr>
<td>Antibiotics-free</td>
<td>Control</td>
<td>3.97±0.23</td>
<td>3.76±0.21</td>
<td>0.61±0.07</td>
</tr>
<tr>
<td></td>
<td>MER series</td>
<td>3.26±0.28</td>
<td>3.01±0.25</td>
<td>0.56±0.07</td>
</tr>
<tr>
<td></td>
<td>PER series</td>
<td>2.72±0.27</td>
<td>2.37±0.25</td>
<td>0.45±0.06</td>
</tr>
</tbody>
</table>

Units are log<sub>10</sub>CFU/mL for E. coli density and log<sub>10</sub>(copy number/mL) for bla<sub>NDM-1</sub> gene concentration.

**5.3.4. Development of phage resistance incurred fitness costs and promoted plasmid curing.**

The frequency of E. coli NDM-1 resistant to both MER01 and MER02 was 1.2±0.4×10<sup>-7</sup>, while the corresponding frequency resistant to both PER01 and PER02 was 6.2±0.5×10<sup>-7</sup>. All (n=50) of the E. coli NDM-1 BIMs were bla<sub>NDM-1</sub> positive as determined by colony PCR, and survived exposure to 10 μg/mL kanamycin or ampicillin, suggesting that development of phage resistance alone did not result in resistance plasmid loss. However, development of phage resistance was associated with fitness costs, as observed in previous studies (236, 237). The specific growth rate of E. coli NDM-1 during exponential growth decreased by 28.5±1.5% due to MER series-resistance, and by 19.2±2.3% due to PER series-resistance. MER series-BIMs exhibited decreased biofilm formation capability by 25.7±1.7%, while PER series-BIMs showed an 18.6±1.9% decrease (Fig. 5.7A).
Figure 5.7 *E. coli* fitness costs associated with phage resistance.
Panel (A) shows decreased specific growth rates and biofilm formation capacity of *E. coli* NDM-1 due to development of phage resistance. Panel (B) shows faster loss of plasmid-borne antibiotic resistant gene *bla*NDM-1 (in the absence of antibiotics) under interspecies competition with *P. putida* F1; Error bars represent ± one standard deviation from the mean of 10 replicates. The asterisks (*) represent significant decrease compared with wild type *E. coli* NDM-1.

ARGs may be retained by bacteria long after antibiotic exposure stops (238). In the absence of antibiotics, ARG maintenance and reproduction exerts a metabolic burden that can be exacerbated by interspecies competition and the fitness cost imposed by the development of phage resistance (233). For example, under interspecies competition with *P. putida* F1, the plasmid-borne ARG *bla*NDM-1 was lost to a significantly greater extent (*p* < 0.05) from phage-resistant *E. coli* NDM-1 compared to wild type *E. coli* NDM-1 (Fig. 5.7B). This suggests that phage biocontrol may accelerate resistance plasmid curing in activated sludge.
Figure 5.8 Activated sludge cumulative oxygen consumption under different treatment conditions. The phage dosage was $7.0 \log_{10} \text{PFU/mL}$, 10 times the concentration of target bacteria ($6.0 \log_{10} \text{CFU/mL} E. \text{coli DNM-1}$). Antibiotic dosage was 50 µg/L (each) ampicillin and kanamycin.

5.3.5. Proposed modified phage biocontrol approach.

Our results demonstrate that polyvalent phages may enable the specific control of target bacteria, while having little impact on activated sludge activity measured as oxygen consumption (Fig. 5.8). Importantly, polyvalent phages can be produced using multiple hosts, and therefore may circumvent the need to utilize pathogenic or difficult-to-culture hosts during production (239). Conventional phage biocontrol relies on narrow-host-range bacteriophages that must be produced using the bacterial target, which may create major safety or logistical concerns. Additionally, because polyvalent phages can be produced using benign hosts, there may be no need to purify them from the host
prior to use (Fig. 5.9), leading to significant savings in capital and operating costs. and may also hold promise for microbiome engineering (240).

Figure 5.9 Comparison of conventional and modified phage biocontrol approaches. Conventional phage biocontrol approach based on narrow-host-range phages (A), and modified phage biocontrol approach based on polyvalent phages (B). Host T represents target host, which is used for phage production in conventional phage biocontrol. In the modified approach, the phage production process is surrogated by benign production hosts (Host P), and the host-phage mixture is used for microbial control.

Whereas polyvalent phages may target multiple resistance carriers and utilize alternative hosts to increase phage titer, not all ARBs present in activated sludge are necessarily susceptible to a specific polyvalent phage. This challenge might be addressed by using trained polyvalent phage cocktails (241) or additional microbial control methods such as effluent disinfection (242, 243) or liming of biosolids (244).
5.4. CONCLUSIONS

Bacteriophage-based microbial control could help address a growing need to attenuate the proliferation of ARB in WWTPs. However, the infectivity of commonly isolated narrow-host-range phages decreases quickly when added to activated sludge. Here, we showed that polyvalent phages proliferate and thrive in activated sludge microcosms, especially when added along with their production hosts. Polyvalent phages can be safely grown and introduced with non-pathogenic hosts to enhance their proliferation in activated sludge, thus enhancing suppression of target bacteria. This approach offers the potential to serve as a supplement or alternative to biocides and disinfectants for microbial controlling problematic bacteria in activated sludge and mitigate the propagation and discharge of associated resistance genes to the environment.
Chapter 6

Biofilm Dispersal by Polyvalent Phages Conjugated with Magnetic Nanomaterial

This chapter is edited form a published coauthored article in *Environ Sci Nano.*
6.1. INTRODUCTION

Bacterial biofilms are most often composed of multi-species communities embedded in heterogeneous, extracellular polymeric substances (EPS). Whereas biofilms have important applications in wastewater treatment and industrial fermentation owning to their enhanced reaction rates and resistance to exogenous stresses, they also may shelter pathogenic or problematic microorganisms and pose public health concerns. Additionally, biofilms harboring bacteria involved in metal deterioration can accelerate microbially influenced corrosion, causing billions of dollars in damage annually. Therefore, there is growing interest in novel microbial control approaches that preferentially suppress problematic bacteria without significantly hindering the beneficial functions of biofilms.

The self-replicating properties, coupled with their specificity, make phages promising anti-microbial agents for targeted control of problematic bacteria (including biocide- and multidrug-resistant strains). Phages can subsequently disappear together with the host, thus avoiding the problem of residual disinfectants. Recent studies have shown that polyvalent (broad host-range) phages have the potential for simultaneous targeting of multiple bacterial hosts without impairing total microbial heterotrophic activity. Polyvalent phages, due to their lower adsorption rate constants and broader host range, exhibit higher diffusion within biofilms relative to narrow host-range phages. These traits could be useful for targeting problematic bacteria in complex biofilms, where the presence of multiple species can result in enhanced resistance or virulence - exemplified by the coexistence of *P. aeruginosa* and *Burkholderia cepacia* in biofilms associated with cystic fibrosis patients.
Despite these potential advantages, phage-based biofilm control is limited by two factors: (1) phage dilution due to dispersion in the bulk solution (256), and (2) limited phage penetration into the biofilm matrix (257) (258). To address these challenges (i.e., both increase phage concentration locally and enhance biofilm penetration), we conjugated polyvalent phages with magnetic CNCs. Previous studies with CNCs have shown their potential for controlled drug delivery (259). CNCs have also been conjugated with phages to bind and facilitate the magnetic separation of bacteria for rapid detection of waterborne pathogens (260, 261).

In this chapter, we evaluate the efficiency of polyvalent phage-CNC complexes to treat a well-defined two-species biofilm, using *E. coli* C3000 and *P. aeruginosa* PA01 as model target organisms. *E. coli* represents an enteric bacterium commonly associated with fecal pollution and infectious disease (262), whereas *P. aeruginosa* exhibits multiple mechanisms of antibiotic resistance and is highly active in biofilm formation (263). This model biofilm facilitates visualization of how magnetic field manipulation can control the migration of the phage-CNC conjugate. Polyvalent phage PEL1 (isolated from soil) was conjugated with various Fe₃O₄-based CNCs to advance understanding of how the morphology and surface charge of the CNCs affect phage attachment and infectivity. We show that biofilm penetration by the PEL1-CS-Fe₃O₄ conjugate can be controlled through magnetic field manipulation, and that the infection efficiency of PEL1-CNC complexes is influenced by the surface charge and amino density of the CNCs.
6.2. MATERIALS AND METHODS

Bacterial strains, bacteriophage, and cultural conditions. *E. coli* C3000 (ATCC 15597) and *P. aeruginosa* PA01 (ATCC 15692) were grown in tryptic soy broth (TSB) medium. Polyvalent phage PEL1, which infects both *E. coli* C3000 and *P. aeruginosa* PA01 (Fig. 6.1), was isolated using a sequential multi-host isolation method and characterized in terms of growth parameters and host range as previously described. All bacterial incubations and viral assays were performed at 30°C. Bacteriophages were stored at 4°C in SM buffer. The phage titer was expressed as plaque forming units (PFU) per milliliter by using a double-layer plaque assay (252) (tryptone base layer agar as a base layer and tryptone soft agar as a soft agar) in triplicate.

![Figure 6.1 Polyvalent phage PEL1 infected both *E. coli* and *P. aeruginosa*.](image)

Phage PEL1 inhibited the growth of each bacterium during batch growth experiments at MOI of 10 (A), and formed clear plaques in the soft agar inoculated with both bacteria (B). Error bars in all figures indicate ± one standard deviation from the mean of triplicate independent experiments.
Microscopic analysis of phage particles. Fluorescence microscopy (Olympus IX71) was used to examine polyvalent phages stained with SYBR Gold (Invitrogen) (264). Briefly, 1 ml isolated phage was digested with OmniPur DNase I (Calbiochem, Gibbstown, NJ) at 37°C for 1 h and then stained with 2.5× SYBR Gold for 10 min in the dark. The phage stock was filtered through a 0.02-μm-pore-size Al2O3 Anodisc membrane filter (Whatman, Clifton, N.J.) at approximately 20 kPa vacuum. The stained Anodisc filter was mounted on a glass slide with a drop of ProLong Gold Antifade reagent (Invitrogen) and a coverslip.

Synthesis and characterization of Fe3O4-based magnetic CNCs. Fe3O4 CNCs (Fig. 6.2A), core-shell Fe3O4@SiO2 CNCs (Fig. 6.2B), amino group modified Fe3O4@SiO2 CNCs (Fe3O4@SiO2-NH2, Fig. 6.2B), carboxyl groups modified Fe3O4@SiO2 CNCs (Fe3O4@SiO2-COOH, Fig. 6.2B), and chitosan-coated Fe3O4 CNCs (CS-Fe3O4, Fig. 6.2C) were used for phage conjugation and to investigate the effect of material properties on conjugation efficiency. Briefly, Fe3O4 CNCs were synthesized using a solvothermal reaction with sodium acetate and FeCl3•6H2O (265). Fe3O4@SiO2 CNCs were prepared by a versatile solution sol-gel method (266). Amino groups were introduced onto the surface of Fe3O4@SiO2 CNCs by conventional sol-gel reaction with (3-aminopropyl)triethoxysilane (APTES) as a modifying agent (267). Carboxyl groups were then functionalized by chemical reaction between the amino groups and succinic anhydride. Chitosan-coated Fe3O4 (CS-Fe3O4) CNCs were synthesized using FeCl3•6H2O, NaOAc, chitosan, and 1-ethenylpyrrolidin-2-one (PVP) via a versatile solvothermal reaction to obtain magnetic CNCs with porosity and high protonation of amino group (268).
Figure 6.2 Morphology and size distribution of magnetic nanomaterials. Fe$_3$O$_4$ CNCs (A), Fe$_3$O$_4$@SiO$_2$-NH$_2$ CNCs (B), and Chitosan-coated Fe$_3$O$_4$ (CS-Fe$_3$O$_4$) CNCs (C). Fe$_3$O$_4$@SiO$_2$ CNCs and Fe$_3$O$_4$@SiO$_2$-COOH CNCs showed similar morphology and size distribution with Fe$_3$O$_4$@SiO$_2$-NH$_2$ CNCs. Size distributions of CNCs were obtained from 100 clusters observed under TEM.

Material samples were dispersed in DI water and dried onto lacy carbon copper grids for TEM analysis. Specimens were observed with a JEOL 2010 transmission electron microscope at 200 kV and size distributions of CNCs were estimated based on 100 particles under TEM. The surface zeta potentials of all CNCs were determined in phosphate buffer (PBS, pH=7.2) at 20°C using the Nanosized Zetasizer instrument (Malvern Instruments Co., UK). The crystalline properties and phase identification were characterized by XRD (Fig. 6.3), using a Japan Rigaku DMax-γA rotation anode X-ray diffractometer equipped with graphite monochromatized Cu K$_\alpha$ radiation. The attenuated total reflectance Fourier transform infrared (ATR-FTIR) spectra were recorded on a
Vertex 70 FTIR spectrometer (Bruker Co., Germany) equipped with a deuterated triglycine sulfate detector.

Figure 6.3 XRD patterns of synthesized magnetic materials. JCPDS card NO. 65-3107 (black, Fe$_3$O$_4$, magnetite).

**Polyvalent phage conjugation with magnetic CNCs.** Polyvalent phage PEL1-MM complexes were prepared by reaction between carboxylic and amino groups under activation by N-hydroxysuccinimide (NHS) and 1-ethyl-3-(3-(dimethylamino)propyl)carbodiimide hydrochloride (EDC) (260, 269). Accordingly, CNCs (1 mg) were dispersed in 1 mL DI water, mixed with EDC (200 μL, 20 mg/mL) and NHS (100 μL, 20 mg/mL) by ultrasonication for 30 min at 25 °C. Then, PEL1 phage stock (200 μL, $6 \times 10^9$ PFU/mL) was mixed with the activated CNCs and rotated at 30 rpm overnight at 4 °C. Next, PEL1-CNCs were separated, washed to remove excess PEL1 phage, and dispersed in 1 mL PBS buffer with 0.05 % BSA for 2 h at 4 °C to block residual reactive sites.
The number of phages immobilized onto CNCs (LE, PFU/mg) was quantified by double-layer plaque assay as the difference between the initial phage amount ($P_0$, PFU/mL) and the total number of free phages remaining ($P_{re}$, PFU/mL) in the conjugation plus washing solutions.

During the washing step (repeated three times), PEL1-CNC complexes were vortexed at 240 rpm for 10 seconds and precipitated under magnetic field to detach loosely bound phages. Phage stock was also cultivated with EDC and NHS (but no CNCs) as a control to estimate the antiviral effect of these chemicals ($P_m$, PFU/mL). Phage stock cultivated with CNCs alone (i.e., no EDC or NHS to avoid stimulating conjugation) was used as additional control to assess the effect of various CNCs on phage adsorption and viability. The amount of phages adsorbed to the CNCs phages were similarly calculated as the difference between the initial phage amount and the total number of remaining free phages in the conjugation plus washing solutions. Following the separation and washing steps, the PEL1-CNCs were stored in 1 mL PBS buffer at 4 °C. The PEL1-CNCs complexes were further confirmed by JEOL 2010 TEM and fluorescence microscopy (using SYBR Gold stained phage).

**Infecive activity of PEL1-CNCs complexes.** The plaque formation capability of PEL1-CNCs complexes were quantified in triplicate by double-layer plaque assay using mixed bacteria ($OD_{600} = 0.5$, equal ratio of *P. aeruginosa* and *E. coli*). The plates were incubated at 30 °C for 12 hours. MATLAB was used to estimate the fractional area of plaque formed on the bacterial lawn. The infectivity of PEL1-CNCs towards bacterial hosts were further confirmed by scanning electron microscopy (270) using a JEOL 6500 instrument. The samples were fixed with 3% glutaraldehyde (wt/vol) in PBS buffer followed by gradient ethanol dehydration (271). The samples were placed on the carbon
tape pasted stub and then putter-coated with ~5 nm gold film under vacuum (Denton Desk V Sputter System).

**Bacterial challenge tests in biofilm conditions.** 96-well special optical plates were used to cultivate the mixed biofilm which contained 120 μL M63 buffer, inoculated with overnight cultures of *P. aeruginosa* and *E. coli* at a final cell density of OD$_{600}$ = 0.1 each. After 24 h cultivation by horizontally shaking at 100 rpm and 30 °C, the wells which contained biofilm growth were gently washed 3 times with PBS buffer to remove the unattached cells, and 160 μL PBS buffer with 10 mM MgSO$_4$ was then added. Phage PEL1 only, a mixture of PEL1 and CS-Fe$_3$O$_4$, or PEL1-CS-Fe$_3$O$_4$ complex was added to a final concentration of 4×10$^5$ PFU/mL. CS-Fe$_3$O$_4$ was also added to assess the effect of mechanical disruption on biofilm integrity. After 6 h treatment in a static state, the CNCs, suspended cells and cell debris were removed and the residual biofilms were stained with propidium iodide (PI) and SYTO9 from LIVE/DEAD BacLight kit (Invitrogen AG, Basel, Switzerland) according to the manufacturer’s instructions. The stained live and dead bacteria were examined under fluorescence microscopy.

**Transport of PEL1-CNCs complexes with magnetic orientation control.**
Transport of conjugated phage PEL1 was conducted in a double-layer plate with *P. aeruginosa* and *E. coli* embedded in the soft layer. A thin film of SM buffer was added on top of the soft layer and the PEL1-CNCs complexes were loaded in the center of the plate. A magnetic cylinder (K&J Magnetics, 660 gauss) was used to control the movement of the PEL1-CNCs. After air-drying in the hood for 10 min, the plates were incubated at 30 °C overnight to allow the conjugated phages to form clear spots on the bacterial lawn. Penetration of conjugated phages through 0.1 % agarose was performed
on microscope slides with magnetic orientation control (660 gauss). The SYBR Gold stained phages were conjugated with CNCs to render fluorescent signal on PEL1-CNCs complexes. The slides were observed with a fluorescent microscope after the gel was air-dried in the dark.
6.3. RESULTS AND DISCUSSION

6.3.1. Polyvalent phages were covalently immobilized on nanomaterial with high efficiency.

Using a sequential multiple-host isolation approach (252), phage PEL1 was isolated with the ability to infect *E. coli* C3000 and *P. aeruginosa* PA01 and significantly suppress their growth (Fig. 6.1). Host range tests showed that phage PEL1 can infect additional (but not all tested) *E. coli* and *P. aeruginosa* strains. Based on its short tail and non-enveloped morphology observed under electronic microscopy (Fig. 6.4A), phage PEL1 belongs to the family of *Podoviridae*.

![Figure 6.4 Microscopic characterization of phage conjugation with magnetic CNCs.](image)

Transmission electron microscopic images of (A) uranyl acetate negatively stained polyvalent phage PEL1, (B) newly synthesized CS-coated Fe₃O₄, and (C) polyvalent phage PEL1-conjugated CS-Fe₃O₄; Effective phage conjugation is corroborated by fluorescence images, which depict (D) SYBR Gold stained polyvalent phage PEL1, (F)
polyvalent phage PEL1 conjugated to CS-Fe₃O₄, and (E) the corresponding light micrograph depicting the CS-Fe₃O₄.

Spherical CS-Fe₃O₄ CNCs with a rough surface and mesoporous structure (Fig. 6.4B) were used to immobilize phage PEL1 following an EDC/NHS covalent coupling procedure. After conjugation with PEL1, the CNCs appeared wrapped, suggesting successful surface coating (Fig. 6.4C). Phage conjugation was confirmed by fluorescence images (Figs. 6.4D to 6.4F). CS-Fe₃O₄ displayed strong fluorescence (Fig. 6.4F) only after the conjugation with phage PEL1, which were pre-stained with SYBR Gold (Fig. 6.4D). Given the initial phage number of \(1.2 \times 10^9\) PFU, the residual \((9.0 \pm 0.2) \times 10^8\) PFU free phage after conjugation, and the \((1.4 \pm 0.1) \times 10^8\) PFU phage inactivated by EDC and NHS, the total phage immobilized onto 1 mg CS-Fe₃O₄ was \((1.6 \pm 0.2) \times 10^8\) PFU (n=3). Control tests showed that non-covalent associations of phages with CNCs were relatively small compared to conjugation (Fig. 6.5) since adsorbed phages could be easily recovered by CNC washing. Furthermore, CNCs did not exert a significant adverse effect on phage viability (Fig. 6.6A).
Figure 6.5 Phage PEL1 immobilized on CNCs due to conjugation and non-specific attachment.

The number of phages immobilized onto CNCs was quantified by double-layer plaque assay as the difference between the initial phage amount and the total number of free phages remaining in the conjugation plus washing solutions. During the washing step (repeated three times), PEL1-CNC complexes were vortexed at 240 rpm for 10 seconds and precipitated under magnetic field to detach loosely bound phages.
Figure 6.6 Lack of phage inactivation or bacterial inhibition by different CNCs.
Panel A showed these CNCs displayed little toxicity towards phage PEL1 since the sum of PFUs from free phages in bulk solution and attached phage on CNCs has no statistical difference with the PFUs in initial phage solution. Panel B showed CS-Fe$_3$O$_4$ (CS-only), Fe$_3$O$_4$@SiO$_2$ core-shell CNCs (FS-only), Fe$_3$O$_4$@SiO$_2$-NH$_2$ CNCs (FN-only), Fe$_3$O$_4$ CNCs (F-only) and Fe$_3$O$_4$@SiO$_2$-COOH CNCs (FC-only) caused no obvious inhibitory effect on bacterial hosts.

The PEL1-CS-Fe$_3$O$_4$ complexes retained broad infectious activity as demonstrated by plaque assays (Fig. 6.7A2) and electron microscopic analysis (Fig. 6.7C). Specifically, the two-species bacterial lawn was lysed by PEL1-CS-Fe$_3$O$_4$, which confirmed its capability to infect both bacterial hosts. Each PEL1-CS-Fe$_3$O$_4$ complex served as a center of infection (COI) and would form a single plaque on the bacterial lawn. Serial dilution
and plating assays demonstrated that the plaque formation capability of 1.0 mg PEL1-CS-Fe₃O₄ was about \((5.2 \pm 0.7) \times 10^6\) COI on the two-species bacterial lawn. This corresponds to an average of 30 ± 8 active phages loaded onto a CNC, and therefore one phage-CNC complex formed a relatively larger plaque compared with one free phage \((5.7 \pm 0.9\) vs \(2.6 \pm 0.6\) mm, n=50) (Fig. 6.7B). For the following biofilm treatment, the equivalent titer of immobilized PEL1 was used as the control group.

![Plaque formation capability of PEL1-CS-Fe₃O₄.](image)

**Figure 6.7 Plaque formation capability of PEL1-CS-Fe₃O₄.**
Panel A1, A2 and A3 showed the plaque formation capability of \(10^3\) PFU of free phage, 1.0 µg PEL1-CS-Fe₃O₄, and \(10^4\) PFU of free phage, respectively. Panel B1 and B2 showed the plaque morphology from free phages and PEL1-CS-Fe₃O₄ complexes, respectively. The plaque formation capability of 1.0 µg PEL1-CS-Fe₃O₄ lay between \(10^3\)-\(10^4\) PFU free phage. Scale bar represents 10 mm. Panel C showed SEM image of mixed bacteria infected by PEL1-CS-Fe₃O₄.

Covalent immobilization of phages on magnetic particles is stable and irreversible (Fig. 6.5), and previous studies have shown that direct covalent coupling with EDC-NHS
produces the highest coverage of phage on the superparamagnetic particles, even compared with antigen-specific interactions. (272) Thus, CNC-phage complexes hold great promise for achieving the targeted contact (273) while enhancing biofilm penetration.

6.3.2. Nanomaterial surface amination contributed to efficient conjugation and microbial control.

Several magnetic CNCs (Fig. 6.2) were tested for their ability to graft a high density of active phages and facilitate bacterial infection after conjugation. TEM images show that the synthesized naked Fe$_3$O$_4$ particles (50-100 nm) tended to agglomerate (Fig. 6.2A). Fe$_3$O$_4$@SiO$_2$-NH$_2$, which also underwent some agglomeration prior to coating with a 20- to 30-nm silica shell, exhibited a broader size distribution (100-220 nm) (Fig. 6.2B). In contrast, CS-Fe$_3$O$_4$ formed stable, dispersed particles with a typical smaller size of 80 to 140 nm (Fig. 6.2C), which is conducive to a larger specific surface area and more COIs after conjugation with phages.

Conjugated CNCs with amino modification (PEL1-CS-Fe$_3$O$_4$ and Fe$_3$O$_4$@SiO$_2$-NH$_2$) had a higher phage loading and displayed significantly ($p < 0.05$) higher infection ability compared with those conjugated with carboxyl modified CNCs (PEL1-Fe$_3$O$_4$@SiO$_2$-COOH) (Fig. 6.5). Specifically, PEL1-CS-Fe$_3$O$_4$ loaded $1.6 \pm 0.2 \times 10^8$ PFU/mg, compared to $1.1 \pm 0.2 \times 10^8$ PFU/mg for PEL1-Fe$_3$O$_4$@SiO$_2$-NH$_2$, $8.6 \pm 0.8 \times 10^7$ PFU/mg for PEL1-Fe$_3$O$_4$, and $5.7 \pm 0.6 \times 10^7$ PFU/mg for PEL1-Fe$_3$O$_4$@SiO$_2$-COOH (n=3). Correspondingly, PEL1-CS-Fe$_3$O$_4$ had the highest infection ability with a plaque area fraction of 99.1±0.6 %, while less
plaque formed in plates infected by PEL1-Fe₃O₄@SiO₂-NH₂ (50.4±2.7 %), PEL1-Fe₃O₄@SiO₂ (30.4±1.9 %), PEL1-Fe₃O₄ (12.1±1.4 %), and PEL1-Fe₃O₄@SiO₂-COOH (3.2±0.4 %) (Fig. 6.8). The CNCs themselves (without phages) were not bactericidal and did not contribute to plaque formation (Fig. 6.6B).

Directional immobilization of phage particles via their heads is needed to ensure that tail fibers (which are responsible for host recognition) are exposed to the host (274). It has been reported that the net charge on most viruses is negative, and the capsids acquire a negative overall charge above pH 4 (275), while another earlier study suggested that the head of T7 phage (Podoviridae family) was responsible for the overall negative charge and the tail fibers could be positively charged (276). Therefore, increased amino group density on the particle surface may not only provide more covalent binding sites for the carboxylic groups on the phage head (increasing phage density) (269), but also orient the tail fibers outwards to facilitate host recognition and infection efficiency.
Figure 6.8 Plaque formation capabilities of PEL1 immobilized on different CNCs. Phage plaque formation area after 12 h treatment with 10 μg of PEL1-conjugated CS-Fe₃O₄ (CS-PEL1), PEL1-conjugated Fe₃O₄ CNCs (F-PEL1), PEL1-conjugated Fe₃O₄@SiO₂ core-shell CNCs (FS-PEL1), PEL1-conjugated Fe₃O₄@SiO₂-NH₂ CNCs (FN-PEL1), PEL1-conjugated Fe₃O₄@SiO₂-COOH CNCs (FC-PEL1), and without treatment (Control). Percentages (means of triplicates) represent the fraction of plaque formation area.

Coating the Fe₃O₄ CNCs with SiO₂ shells increased the zeta potential from -34.6 to -24.4 mV. The zeta potential further increased to +4.0 mV when the CNCs were coated with APTES (Fe₃O₄@SiO₂-NH₂), and decreased to -45.3 mV after coating with carboxyl groups (Fe₃O₄@SiO₂-COOH) that were conjugated to the amino group, which neutralized the positive charges. Plaque formation tests indicate a high correlation between zeta potential and phage immobilization, with higher infection efficiency corresponding to the higher zeta potential (Fig. 6.9A, $R^2 = 0.966$). Nevertheless, PEL1-CS-Fe₃O₄ resulted in a much higher infectivity than predicted by this positive correlation.
(Fig. 6.9A). Apparently, the amino groups on PEL1-CS-Fe₃O₄ facilitated phage conjugation with proper orientation (i.e., with tail fibers exposed to the host), which enhanced infectivity. For example, the number of phages immobilized onto PEL1-CS-Fe₃O₄ was about 2.8-fold higher than that immobilized onto PEL1-Fe₃O₄@SiO₂-COOH, while its plaque area formed was about 31-fold higher (Fig. 6.8). This reflects the importance of proper orientation when phages are conjugated with CNCs.

Polyvalent phage PEL1 conjugated with CS-Fe₃O₄ showed significantly higher infection efficiency than that with PEL1-Fe₃O₄@SiO₂-NH₂ \( (p < 0.05) \), which had a higher positive charge \(+4.0 \text{ mV} \) vs \(-13.2 \text{ mV} \) and larger specific surface area \( (86 \text{ m}^2/\text{g} \) vs \( 71 \text{ m}^2/\text{g} \). One possible explanation is that CS-Fe₃O₄ contained a higher density of amino groups than Fe₃O₄@SiO₂-NH₂, and thus could load more properly-oriented phages on its surface. Therefore, FTIR spectroscopy was used to compare the amino group density between CS-Fe₃O₄ and Fe₃O₄@SiO₂-NH₂ (Fig. 6.9B). Due to its SiO₂ shell, Fe₃O₄@SiO₂-NH₂ exhibited the characteristic vibration peaks of SiO₂ \( (793 \text{ cm}^{-1}) \), Si-OH \( (954 \text{ cm}^{-1}) \) and Si-O-Si \( (1096 \text{ cm}^{-1}) \). \( (268) \) The absorption bands at 2972 and 2928 cm\(^{-1}\) assigned to the stretching vibration of the C-H bond of the propyl amine group, which proves the successful grafting of APTES on silica coated magnetic CNCs \( \text{(Fe₃O₄@SiO₂-NH₂)} \),\( (277) \) and of chitosan on magnetic CNCs \( \text{(CS-Fe₃O₄)} \).\( (278) \) Compared with Fe₃O₄@SiO₂-NH₂, CS-Fe₃O₄ exhibited much stronger characteristic absorption peaks of the primary amine (-NH₂); one overlaps with the \(-\text{OH} \) band at 3410 cm\(^{-1}\), and the second is visible at 1640 cm\(^{-1}\).\( (268) \) Based on the above results, PEL1-CS-Fe₃O₄ was chosen for further experiments.
Figure 6.9 Effect of CNC zeta potential on plaque formation capability. Panel (A) shows that the Zeta potential of CNCs is positively correlated with antimicrobial effect after phage conjugation ($R^2=0.966$). CS-Fe$_3$O$_4$ was an outlier possibly due to its high amino group density that facilitates higher phage loading with proper orientation for enhanced infectivity. Panel (B) compares the FT-IR spectra of Fe$_3$O$_4$@SiO$_2$-NH$_2$ and CS-Fe$_3$O$_4$, highlighting the higher amino group density of the latter.
6.3.3. Nano-phage complexes exhibited higher biofilm suppression than free phages.

For bacterial control, the conventional phage therapy approach is to apply lytic virulent phages directly on the targeted bacteria (249). Approaches to increase microbial control efficacy include (1) applying a cocktail of different phages with overlapping host ranges or polyvalent phages to deal with single or dual species biofilms (279); (2) combining phages with chemical disinfection methods to enhance biofilm control (280); and (3) combining antibiotic agents (i.e., rifampicin) with phage treatment to remove pathogenic (Staphylococcus aureus) biofilms (281). Nevertheless, insufficient phage penetration into deeper layers of the biofilm is still a major limiting factor (257, 282). This limitation can be significantly ameliorated by magnetic control.

Figure 6.10 Fluorescence microscopic analysis of mixed biofilms disruption. (A) Comparison of the remaining biofilm determined by a live (green)/dead (red) assay without any treatment (Control), with free phage treatment only (PEL1-only) and materials treatment only (CS-Fe₃O₄-only), with both free phage and materials added (No Immobilization), and with PEL1-CNCs complexes in the presence (PEL1-CS-Fe₃O₄) or absence (No Magnetic field) of a magnetic field; (B) Histograms showing the fraction of
remaining biofilm (area of both live and dead bacteria), and the coverage of Control was defined as 100 %.

For example, in the free phage-only treatment, the biofilm coverage area decreased by 35.5±6.6 %, and the Dead/Live ratio (45.9±12.1 %) increased relative to the control group (11.8±2.1 %) (Fig. 6.10B). However, since dead cells may hinder PEL1 diffusion in biofilms and protect otherwise vulnerable bacteria (283), there was a need for enhanced phage penetration and treatment efficacy. Due to mainly physical disruption, the CS-Fe₃O₄-only treatment achieved total biofilm coverage removal of (10.2±3.3 %) with Dead/Live ratio of (10.2±0.5 %) in the remaining biofilm. The diffusion of CNCs through the biofilm disrupted the biofilm structure and facilitated phage infection. Accordingly, the combination of free phage and CS-Fe₃O₄ resulted in 70.8±4.2 % biofilm removal and a 36.4±5.2 % Dead/Live ratio in the remaining biofilms. PEL1-CS-Fe₃O₄ complexes showed an even higher efficacy of biofilm coverage removal (88.7±2.8 %), suggesting that phage immobilization can enhance infection due to higher local phage concentrations and effective penetration and directional adsorption. In contrast, free phages are dispersed in the solution and do not effectively penetrate the biofilm by diffusion alone.

Whereas the magnetic properties of the phage-CNC complex clearly enhanced biofilm penetration and physical disruption under a magnetic field, immobilization of multiple phages on a single CNC is also conducive to higher localized phage concentrations reaching the biofilm. This increases the likelihood of an effective phage-host collision, as does the exposed-tails orientation of the immobilized phages.
The emergence of phage-resistance is a challenge for the application of phages in disease control. Conjugation with CNCs and the resulting higher local phage concentrations may not prevent phage resistance, but this approach would disrupt the biofilm matrix faster and incur fitness costs to the surviving bacteriophage insensitive mutants, which facilitates further microbial control (e.g., reduced growth rate, decreased antibiotic resistance, or less virulence). Possible strategies to avoid or attenuate phage-resistance include immobilizing phage cocktails onto CNCs and combining phages with antibiotics.

6.3.4. Enhanced biofilm penetration and directional control of the nano-phage complex was achieved by magnetic-field-controlled migration.

Well-established biofilms show a fractal and spatial structure of populations and a complex matrix that is difficult for free phages to penetrate, mainly due to static hindrance and non-specific adsorption. This may compromise the efficacy of phage-based microbial control. However, CNCs-conjugated polyvalent phages could help overcome these limitations by enhancing penetration and physical disruption of biofilms and facilitating directional control, as illustrated by the induced horizontal migration (Fig. 6.12). Specifically, using a relatively weak (660-gauss) magnetic field, we manipulated the horizontal transport of PEL1-CNCs conjugates within a bacterial lawn containing both *E. coli* and *P. aeruginosa*. The plates without magnetic field formed round plaques on the bacterial lawn, while the plates with oriented magnetic field formed arrowhead-shaped plaques (Fig. 6.11A). Agarose gel can to some extent simulate the biofilm conditions, and the conjugated phages penetrated through the agarose gel with
magnetic field (Figs. 6.11B), consistent with the significantly enhanced biofilm removal efficacy of the PEL1-CS-Fe₃O₄ complex after magnetic orientation control (Fig. 6.10).

![Figure 6.11](image)

**Figure 6.11 Horizontal transport and vertical penetration of PEL1-CS-Fe₃O₄ complexes under a magnetic field.**
Horizontal transport of conjugated phage PEL1 was conducted on the soft layer containing *P. aeruginosa* and *E. coli* (A). Red circle or rectangle shows host lysis. The penetration of conjugated PEL1 was performed in 0.1 % agarose gel and stained by SYBR Gold (B). The yellow line shows the initial location of conjugated phages, and the blue arrow represents the direction of the magnetic field.

### 6.4. CONCLUSIONS

Diffusion of free phages through biofilm is often limited by the presence of EPS matrix (257), bacterial lysis debris (283) and phage-resistant bacteria (288). Here we demonstrate that magnetic CNCs can be used for phage immobilization to enhance their penetration and microbial control in biofilms that are generally resistant to chemical disinfection. The phage-CNC complexes physically disrupt biofilm matrices as they penetrate under a magnetic field, and enhance phage infiltration and delivery to otherwise inaccessible host cells. Compared with phage-only treatments, immobilization mitigates
phage dilution by the medium to maintain high phage concentrations locally, and ensures that phage tail fibers are exposed to the hosts for easier infection.

This work suggests that the scope and efficacy of phage applications can be enhanced by magnetic-field-controlled migration with paramagnetic CNCs, offering opportunities for more accurate targeting of problematic bacteria in complex biofilms. Nevertheless, further research is needed with well established, complex biofilms to enhance practical applications, including quantitative characterization of dose-response patterns for various phage-CNC complexes, treatment time and frequency optimization (including potential rotation of phage cocktails to minimize resistance development), and effects of environmental factors on treatment efficiency.
Chapter 7

Summary and Conclusions

7.1. Summary and Conclusions

Bacteriophages are garnering renewed interest for microbial control predominantly driven by the widespread and increasing onset of ARB. Conventional phage-based antimicrobial methods are limited in environmental systems where target bacteria are diversified, scattered distributed and often embed within biofilms. Environmental stresses detrimental to phage activity compromise the efficiency of phage treatment. This dissertation contributes to developing safe, precise and efficient strategies for ARB control and ARG mitigation using phages with broad host range. This study advances our understanding of broad host-range phages and provides proof-of-concept on polyvalent phage-based antimicrobial approaches.

Established sequential multiple-host approaches for polyvalent phage isolation.

Many studies on phage biology are based on isolation methods that may inadvertently select for narrow host-range phages. Consequently, broad host-range phages, whose
130 ecological significance is largely unexplored, are consistently overlooked. To enhance research on such polyvalent phages, Chapter 3 introduced two sequential multi-host isolation methods and tested both culture-dependent and culture-independent phage libraries for broad infectivity. Lytic phages isolated from activated sludge were diverse in morphology and capable of interspecies or even inter-order infectivity without significant reduction in efficiency of infection. Two polyvalent phages (PX01 of the Podoviridae family and PEf1 of the Siphoviridae family) were characterized in terms of adsorption rate, latent time and burst size using different hosts. These phages were enriched with a nonpathogenic host and subsequently used to infect model problematic bacteria. Using an MOI of 10 in bacterial challenge tests, significant lethality was observed for both *P. aeruginosa* and *Pseudomonas syringae* relative to uninfected controls. This work suggests that polyvalent phages are common in certain ecosystems and may be enriched from common environments (by using different sequential hosts) to enable their study and potential broad targeting capabilities.

**Investigated the effects of phage host range on phage fitness and bacterial suppression.** Bacteriophages are widely recognized for their importance in microbial ecology and bacterial control. Chapter 4 explored how phage polyvalence affects phage propagation, bacterial suppression and interspecies competition in both planktonic and biofilm environments. Phage properties (*e.g.*, adsorption affinity and host range) as well as the attached microbial community structure had a significant impact on phage propagation, and therefore the suppression of the target bacteria within the biofilm. The results highlighted the importance of polyvalence in enhancing phage propagation in alternative hosts and spreading within mixed-species biofilms. As a result, polyvalent
phages could outcompete narrow host-range phages in the suppression of target bacteria in biofilm-associated environments, although narrow host-range phages were more effective than polyvalent phages in monoculture or simple mixed-species culture. Combining bacterial competitors with phages resulted in meaningfully greater inhibition of target bacteria than separate treatments in planktonic conditions, suggesting that phage-based biocontrol would be more effective in combination with compatible microbial control strategies.

**Developed polyvalent phage-based biocontrol approaches.** The loss of phage infectivity during phage delivery limited phage applications in environmental systems (e.g., activated sludge). Chapter 5 develops polyvalent phage-based biocontrol approaches to provide safe and long-term ARB inhibition. Polyvalent phages can proliferate and thrive in activated sludge microcosms, especially when added along with their production hosts. Such polyvalent phage cocktails were significantly more effective than narrow-host-range coliphage cocktails in suppressing a model enteric ARB (β-lactam-resistant *E. coli* NDM-1). Due to the presence of alternative hosts, polyvalent phages reached greater densities, which increased the probability of ARB infection. The fraction of surviving *E. coli* containing the *bla*<sub>NDM-1</sub> resistance gene was also significantly lower for the polyvalent phage cocktail treatment, mainly because of the fitness costs caused by development of phage resistance. Therefore, polyvalent phages safely produced by non-pathogenic hosts could offer a novel potential approach to control problematic ARB in sewage treatment plants and mitigate the propagation and discharge of associated resistance genes to the environment.
Examined polyvalent phage-nanomaterial complex for biofilm dispersal.

Biofilms may shelter pathogenic or other problematic microorganisms that are difficult to eradicate due to hindered penetration of antimicrobial chemicals. Chapter 6 demonstrated the potential for efficient bacterial suppression using polyvalent phages attached to magnetic nanoparticles (e.g., CNCs) that facilitate biofilm penetration under a relatively small magnetic field. The polyvalent phage PEL1 (Podoviridae family) was immobilized onto Fe$_3$O$_4$-based magnetic CNCs that had been coated with chitosan (and thus functionalized with amino groups). This facilitated conjugation with phages via covalent bonding and enabled phage loading with optimal orientation. The plaque formation capability of PEL1-CS-Fe$_3$O$_4$ on bacterial lawns was significantly higher than that of phages conjugated with similar CNCs that had been functionalized with carboxyl. This conjugation approach could improve the efficacy of phage biocontrol and extend the application of phages for microbial control by enhancing their delivery to relatively inaccessible locations within biofilms.

Explored the genomic basis for phage broad host range. Using a polyvalent N4-related phage isolated with a sequential multiple-host isolation approach, Chapter 7 explored the genomic basis for phage NA01 which can efficiently and productively infect multiple gram-positive and gram-negative hosts from different taxa. The types of bacterial receptors used by the phage appear to be carbohydrate in nature. The genomic analysis of NA01 revealed that both genes related with host recognition (i.e., tail structure) and those related with phage genome transcription (i.e., virion-borne RNA polymerase) and protection (i.e., DNA methylase) may be important to extend phage host range.
In conclusion, polyvalent phages are more widespread in certain environments than previous expected and can be preferentially isolated by sequential multiple hosts. Whereas polyvalent phages use a wider variety of bacteria to reproduce, they may experience lower efficiency of infection and slower growth compared with narrow host-range phages. Polyvalent phages exhibit more complex propagation dynamics, which facilitates their spread and bacterial suppression in mixed-species biofilm. Due to the presence of alternative hosts, polyvalent phages can reach greater densities and thus increasing the probability of infection, although it may reduce interspecies competition. As natural existing nanoparticles, phages are compatible with engineered functional nanomaterial. The conjugation of polyvalent phages with nanomaterial may not only further enhance the efficacy of phage treatment, but also extend the scope of phage application in environmental systems.

7.2. Significance and Implications

The impact that a phage may have on a microbial community is dictated by its host-range. Historically, most phages have been considered to be narrow host-range, infecting only certain bacterial strains within one species (9). However, there have been numerous reports of broad host-range (polyvalent) phages capable of inter-generic infectivity (239, 289). Much more rarely have phages been shown to possess the capacity for inter-family infectivity (93, 173). Narrowness of host range is also a major, oft-cited challenge of phage therapy and biocontrol (10, 290, 291). As obligate parasites, survival of phages in microbial communities depends on the availability of their bacterial hosts, and propagation on multiple hosts could maximize opportunities for reproduction in complex
natural communities, increasing the environmental fitness of phages. Therefore, the existence of polyvalent phages would have substantial biotechnological implications, particularly in regards to their use for microbial control (e.g., antibiotic resistant bacteria) and environmental gene delivery (164, 292).

From a biotechnological perspective, reliable polyvalent phage isolation approaches serve as the foundation for polyvalent phage-inspired biotechnology (e.g., microbial control, gene delivery and pathogen detection), which holds the potential for overcoming the challenges due to phage narrow infective spectrum. Polyvalent phages have several advantages over narrow host-range phages in environmental systems. First, a small library of polyvalent phages could potentially treat a wide range of bacterial targets, simplifying the process of phage formulation. Moreover, when it comes to gene delivery, polyvalent phages may serve as more powerful gene transferring agents spanning different genera. In terms of pathogen detection, polyvalent phages can also empower phage-based detectors with the capability of detect multiple microbial targets by a single reporter strain (293).

The polyvalent phage biocontrol can be an effective and environment-friendly ARB control approach in environmental systems (e.g., livestock, agriculture and WWTP). Phage polyvalence circumvents the complex phage purification process by enrichment within benign production hosts. The avoidance of pathogenic bacteria in phage production eliminates the possibility of phage contamination by problematic bacteria, toxins or virulence genes. Polyvalent phages propagation in co-amended production hosts can offset phage inactivation by environmental stresses during phage delivery, enhancing microbial control in environmental systems. The selective pressures from phage cocktails
increase the fitness costs of the surviving ARB and promote plasmid curing (thus mitigating the overall ARGs).

The phages can be considered as self-replicating natural nanomaterial with high selectivity. The advances of nanotechnology have provided numerous versatile engineered nanomaterial. The conjugation of polyvalent phages with magnetic nanomaterials can extend the application of phages by enhancing their delivery to relatively inaccessible locations. Migration of nano-phage complex under magnetic field ensures higher local phage concentration and more effective phage infection relative to free phages. The efficacy of phage-based biotechnology can be further improved due to the synergistic effect of phages and nanomaterial.

Figure 7.1 Strategies to overcome challenges facing conventional phage biocontrol
(A) Isolation of polyvalent phages to broaden phage infective spectrum. (B) Formulation of phage cocktails to reduce resistant frequency and increase fitness costs. (C) Adoption of the host-phage mixture to offset phage loss during delivery. (D) Conjugation of phages with nanomaterial to enhance biofilm penetration.
The major discoveries in this dissertation have significant ecological implications, ranging from bacterial evolution and biogeochemical cycling to antibiotic resistance gene propagation and microbiome engineering. Perhaps foremost is that a phage that can productively infect bacterial hosts from distantly related taxa raises the possibility that phages facilitate transduction across different orders, and consequently may have a larger role in bacterial genome evolution than previously appreciated. Considering the global dissemination of antibiotic resistance, the role of phages in the horizontal transfer of resistance genes may need further evaluation. Additionally, it is highly likely that phages with even broader host-ranges exist. The polyvalent phage PRD1 is known to infect many different Gram negative bacteria that harbor P-, N-, or W-type conjugative plasmids (294). Studies of cross-infection within marine bacteria have revealed the existence of generalist phages capable of infecting 31 out of 286 (10.8%) bacterial isolates (295). Gene transfer agents (GTAs), which are host-encoded, phage-like particles that package random fragments of their host chromosome, have been shown to transduce as high as 47% of the culturable fraction of a natural community (296). Recently, an RNA virus capable of infecting both plants and honey bees was reported, providing evidence for inter-kingdom infectivity (297). Thus, there is likely much left to learn regarding the role that polyvalent phages play in horizontal gene transfer, microbial evolution and diversity, and biogeochemical cycles. Polyvalent phages might serve as rich sources of biotechnologically-important enzymes, antimicrobial proteins (e.g., lysins), and broad host-range regulatory elements for use in synthetic biology applications.
7.3. Suggestions for Future Research

7.3.1. Reversion of sequential multiple-host isolation approach.

The current version of sequential multiple-host approaches preferentially isolate phages with broad host range. The resilience of isolated phages to environmental stresses (e.g., UV radiation, temperature and heavy metals), which is crucial for phage application in environmental systems, cannot be guaranteed. The frequency of bacterial phage resistance and the corresponding fitness costs are also important factors when considering phage candidate for phage formulation. Therefore, modified sequential multiple-host approaches will be proposed for selective isolation phages with high environmental resilience or high lethality.

7.3.2. Development of novel phage protection and delivery strategy.

Bacterial spores are the most resilient biological entities in the biosphere, which can survive various extreme environmental stresses. Since phages are susceptible to environmental factors, the bacterial spores would protect phages from the harsh environment during phage delivery, extending their range of application in natural and artificial environments. (298, 299) The challenge of this study will be the isolation of polyvalent phages infecting both spore-forming bacteria and problematic bacteria. The DNA of polyvalent phage can be incorporated into bacterial spores, which will protect the phage DNA from the harsh environment during phage delivery. (300) Upon arrival to the site where the problematic bacteria flourish, germination of the spore and outgrowth of vegetative cells would activate the normal virulent life cycle and release more virions.
7.3.3. Optimization of the phage-nanomaterial complex for biofilm removal.

Well-established biofilms show a fractal and spatial structure of populations and a complex matrix that is difficult for phages to penetrate, mainly due to static hindrance and non-specific adsorption. In theory, the performance of phage-nano complexes could be further improved by using genetically modified phages that disrupt bacterial biofilms by expressing EPS depolymerase (187) and/or quorum-quenching enzymes (301). Further research that includes consideration of engineered phages and versatile nanomaterial would be needed to assess their feasibility.

7.3.4. The implication of polyvalent phage on horizontal ARG transferring.

Historically, most phages have been considered as narrow host-range, recent studies (including this one) have shown that polyvalent phages may be more widespread than previously recognized. Therefore, previous research may underestimate the impact of phage on ARGs horizontal transfer through both generalized transduction and specialized transduction. Recent studies indicate lytic phages can also boost ARGs spread due to incompletely digest of plasmids. Therefore, it is necessary to estimate the contribution of polyvalent phages on horizontal ARG transferring in different settings.
References


