Master's thesis

NTNU
Norwegian University of Science and Technology
Faculty of Natural Sciences
Department of Biotechnology and Food Science

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Host range expansion of phage cocktails

Automation and study of Appelmans protocol

Master's thesis in Biotechnology (5 year)
Supervisor: Eivind Almaas
Co-supervisor: Nikolay Martyushenko
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Preface

I would first like to express my gratitude to my supervisor Eivind Almaas, who has provided support, guidance, and occasional employment since 2017. I would also like to thank the best experimentalist I know, my co-supervisor Nikolay Martyushenko, for all his advice, and for teaching me that if you need something, just make it.

Thank you also to Thi My Linh Hoang at the Cellular and Molecular Imaging Core facility for staining my samples, and providing electron microscopy training.

Finally, thank you to Anders Nilsson and the Group Nilsson at Stockholm University for graciously sending the ECOR collection to us. I've never been more excited to receive a shipment of *E. coli*.

Abstract

As the post-antibiotic era looms, bacteriophages are receiving renewed attention as an alternative to antibiotics. This study examines two aspects of developing phages for phage therapy; isolation of bacteriophages, and an automated protocol for adaption of a phage cocktail to targeted bacterial strains.

First, five phages were isolated from municipal sewage on *Escherichia coli* strains from the standard ECOR library. Along with five previously isolated strains, the host range of each phage was tested upon the ECOR library. The structure of the resulting interaction network revealed both nestedness and modularity, and ECOR strains isolated from human hosts showed higher phage susceptibility than strains isolated from non-human hosts. Secondly, three selected phages were combined in a cocktail and subjected to an automated host range expansion protocol upon 10 ECOR strains based on Appelmans protocol. These ECOR strains were also screened for prophages. The final phage cocktail's host range expanded from 3/10 to 5/10 target strains, and observed a 44% increase in host range when tested upon all 72 ECOR strains.

Two host range mutants were isolated from the final cocktail and compared to the original cocktail phages using transmission electron microscopy. Based on these results, it seems likely the host range mutants, partially or completely, are descended from prophages resident within their ECOR hosts. Previous studies of Appelmans protocol have not reported the impact of prophages.

In addition to these results, a sterile incubation cabinet with temperature and humidity control was constructed around an Opentrons OT-1 pipetting robot programmed to carry out the automated version of Appelmans protocol. This design may have practical applications outside of phage cocktail host range expansion.

Sammendrag

Imens antibiotikaresistens sprer seg globalt, har bakteriofager fått fornyet oppmerksomhet som alternativ behandlingsmåte for bakterielle infeksjoner. Dette prosjektet tar for seg to aspekter i utviklingen av bakteriofager (også kalt fager) for fagterapi; isolering av bakteriofager, og adapsjon av fag-blandinger til nye bakterielle stammer.

Fem fager ble isolert fra et kloakkrenseanlegg ved å bruke Escherichia coli-stammer fra ECOR-samlingen som verter. Sammen med fem andre fager fra et tidligere prosjekt, ble vertsbreddene til alle fagene testet på alle de 72 stammene i ECOR. Interaksjonsnetterket ble analysert, og ECOR-stammer isolert fra ikke-menneskelige verter var i større grad immune mot fagene enn stammer isolert fra menneskelige verter. Tre fager ble så kombinert i en cocktail som ble evolvert på 10 utvalgte ECOR-stammer gjennom en automatisert protokoll basert på Appelmansprotokollen. Disse ECOR-stammene ble også sjekket for profager. Etter 30 runder av protokollen hadde vertsbredden til cocktailen økt fra 3/10 til 5/10 utvalgte stammer, og i tillegg utvidet seg 44% på hele ECOR-samlingen.

To vertsbreddemutanter ble isolert fra den evolverte cocktailen og sammenlignet med de originale cocktailfagene ved bruk av elektronmikroskop (TEM). Basert på disse resultatene er det sannsynlig at vertsbreddemutantene stammer, enten helt eller delvis, fra profager i ECOR-stammene. Tidligere studier av Appelmansprotokollen har ikke undersøkt effekten av profager.

I tillegg til disse resultatene ble et inkuberingsskap med UV-sterilisering og temperatur- og fuktighetskontroll designet og konstruert rundt en Opentrons OT-1 robot som ble programmert til å utføre protokollen. Dette designet kan være nyttig for andre prosjekter.

Table of Contents

List of Tables

List of Figures

Chapter 1

Introduction

As the current SARS-CoV-2 pandemic shows, we still live at the mercy of microbes. Once the leading cause of mortality worldwide, infectious diseases were sent into a long decline by the advent of vaccines and antibiotics, revolutionizing public health [1]. This perhaps culminated in 1980 with the global eradication of smallpox, a disease estimated to have killed 300 million people in the 20th century [2]. However, most of our diseases were not defeated, but merely suppressed into an uneasy truce. Tuberculosis, the leading cause of infectious disease mortality, kills roughly 1.3 million people annually [3]. One third of the world's population is infected with latent tuberculosis [3], making eradication virtually impossible. Given the opportunity, tuberculosis will again rise to threaten the protection afforded to us by modern medicine.

As the post-antibiotic era approaches, we have provided tuberculosis and other bacterial pathogens with this opportunity. Antibiotic resistance has been a problem for almost as long as we have had antibiotics; penicillin resistance was reported almost immediately after the widespread deployment of penicillin during World World II [4]. Through a steady stream of novel antibiotics throughout the 20th century, we managed to stay one step ahead of the microbial competition. However, the economics of antibiotics made pharmaceutical research unprofitable and the pipeline dried up [5]. At the same time, lack of governmental oversight and gross negligence in health and agricultural sectors have led to excessive antibiotic usage, fuelling the crisis [5]. An oft-cited UK governmental report warns antibiotic resistance could kill 10 million people annually by 2050 [6]. Although there is disagreement regarding the specific figure [7], antibiotic resistance is clearly a global threat against which we must take immediate action.

Bacteriophage therapy represents one such course of action [8]. Viruses that infect bacteria and archaea, bacteriophages, or phages for short, are the most plentiful and diverse biological entities on Earth, thought to outnumber every other type of organism combined [9]. First discovered by British microbiologist Frederick Twort in 1915, bacteriophages were first employed for the treatment of bacterial dysentery by Felix d'Herelle in 1919, laying the groundwork for phage therapy. Through the pre-antibiotic years of the 1920's and 30's, phage therapy became a widespread treatment for bacterial infections [10].

However, the advent of broad-spectrum antibiotics during World World II cast a shadow upon the often less than effectual phage therapy [10]. In contrast to antibiotics, phages generally have narrow host ranges, and are only effective upon a few bacterial strains [11]. Worsening matters, the most prominent proponent of phage therapy, Felix d'Herelle, had a habit of making enemies of powerful scientists, contributing to limited scientific acceptance of phage therapy. D'Herelle relocated to Tbilisi, Georgia in 1934, helping found the Eliava Institute which exists to this day. Perhaps tarnished by association with the Soviet Union, phage therapy fell completely out of fashion in the West. In the Eastern Bloc however, bacterial infections continued to be treated with phage therapy, often using commercial phage collections known as cocktails, developed at the Eliava Institute [10].

Antibiotic resistance has triggered something of a renaissance of phage therapy research in the West [8]. However, linguistic and commercial barriers have inhibited the transfer of much accumulated experience from the former Soviet Union. For example, *Appelmans protocol*, has been the main method used to develop phage cocktails in the Republic of Georgia since the 1930's, but the first mention of this protocol in modern Western journals was in 2016 [12, 13].

Appelmans protocol expands the host range of a phage cocktail evolved upon targeted bacterial strains over successive rounds of infection and reproduction. Although Appelmans protocol was shown to benefit from genetic recombination between the phages of the initial cocktail [13], there is much to be understood about how this protocol works. Appelmans protocol is also quite slow and labor-intensive; published implementations run for 30 days, requiring daily inoculation of a 96-well plate with different strains and cocktail dilutions [13].

In this thesis, I show how a modification of Appelmans protocol allows automation by the Opentrons OT-1 pipette robot, reducing run-time to less than a week with significantly less labor. I also isolate phages against *Escherichia coli* from sewage and characterize their host ranges against the ECOR library. I then test the automated Appelmans protocol using a selection of these phages, and analyze the resulting cocktail.

Chapter 2

Theory and background

In this chapter, basic phage biology will be reviewed, focusing on the aspects important to this project; phage diversity, temperate phages and lysogeny, host range determinants, and phage coinfection. This will provide a rationale for the aims of this project, and help sketch a theoretical background for the results.

2.1 Phage diversity

Phages are viruses defined by the ability to infect bacteria [14]. Beyond this, they are an incredibly diverse group, lacking any universal homologous genes and boasting a wide variety of structures, hosts, and genotypes. Even within phylogenetic groups, phage genomes often exhibit a patchy mosaicism of similarity, evidence of horizontal gene transfer vastly exceeding that of their bacterial hosts [15]. Partly because of these complications, phage taxonomy is still largely based upon morphology gleaned from electron microscopy. Like viruses of eukaryotes, phage taxonomy is determined by the International Committee on Taxonomy of Viruses (ICTV). Figure 2.1 shows schematics and electron microscopy images for all accepted phage morphologies as of 2018.

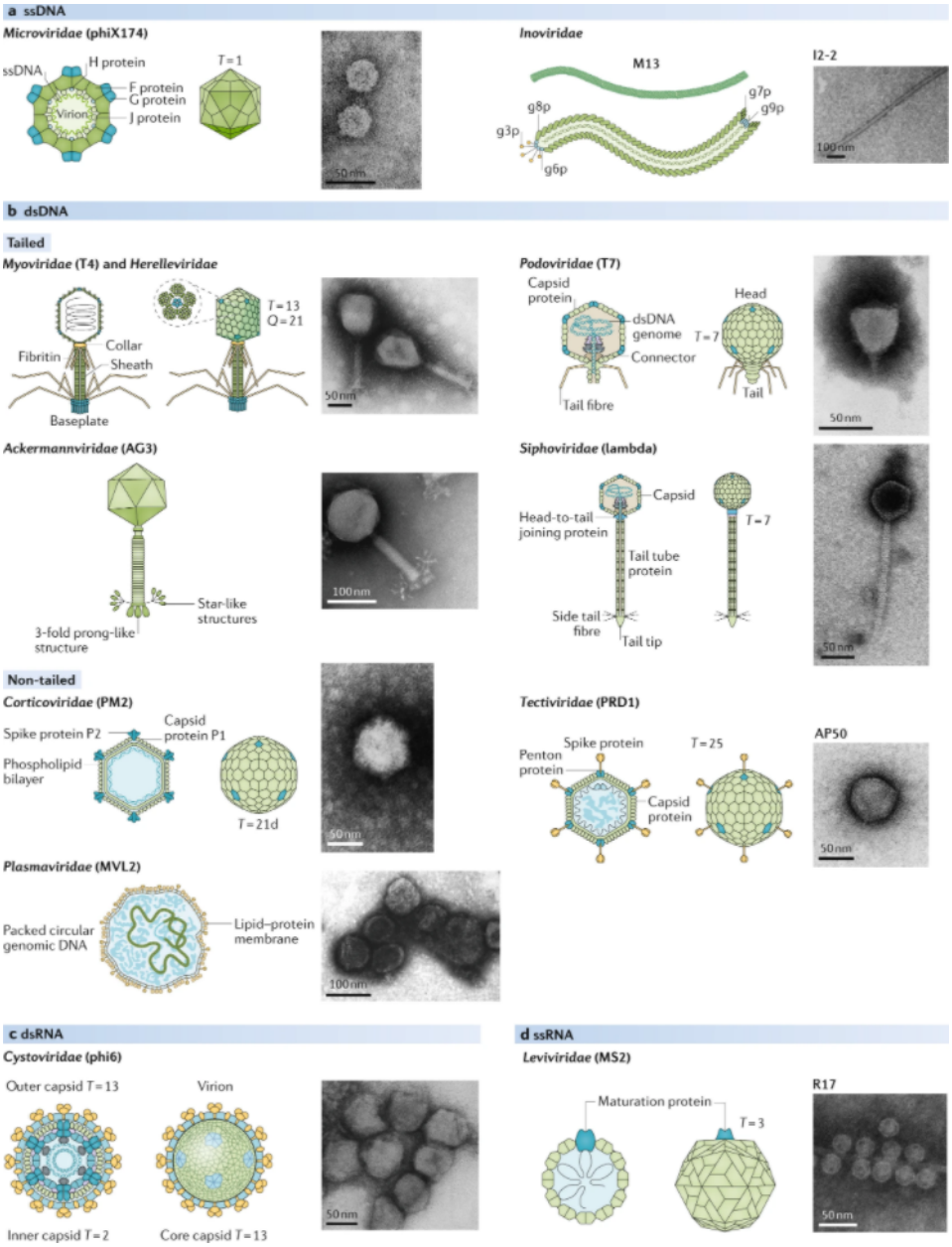


Figure 2.1: An overview of major phage families according to morphology and genome type. A schematic representation and transmission electron microscopy image are shown for each morphology. Reproduced from [16], CC-BY-4.

Most of the observed abundance and diversity of bacteriophages resides in the dsDNA tailed phages, order *Caudovirales* [17]. Tailed phages are characterized by their eponymous tails and icosahedral capsids, usually referred to as the "head". A large range of tail lengths exist, and many capsids are of irregulat length and shape. However, this order is monophyletic, indicating that this charismatic morphology is not the result of convergent evolution but instead a recapitulation of their phylogenetic heritage [16].

In addition to the head and tail structures shown in Fig. 2.1, tailed phages have a linear, dsDNA genome [14]. Their genomes however, range from 10 kbp to 500 kbp, demonstrating the tremendous amount of variety masked behind these familial traits [17]. This order currently consists of five families: *Myoviridae* with contractile tails, *Siphoviridae* with long, noncontractile tailes, *Podoviridae* with short tails, and *Ackermannviridae* and *Herelleviridae*, two recently created families on the basis of network-based genomic analyses [17, 16]. *Herelleviridae* is not shown in Fig. 2.1.

Included in the non-tailed dsDNA phages are the membranous families *Corticoviridae*, *Tectiviridae*, and *Plasmaviridae*. *Corticoviridae* and *Tectiviridae* both have an internal lipid membrane and an icosahedral capsid, but have circular and linear genomes, respectively. *Plasmaviridae* however, lacks a capsid completely and its circular genome is solely enclosed by a a lipid-protein membrane [16]. As can be seen in the transmission electron microscopy (TEM) images Fig. 2.1, these phages have far less distinct morphologies than the tailed phages.

The *Microviridae* have circular, positive sense ssDNA genomes, and are as their name suggests, small. The most well-studied species, ϕ X174, has a 5.4 kbp genome, enclosed by a 26 nm icosahedral capsid [16]. However, the *Inoviridae*, the only other family of ssDNA phages, bear little similarity. Although these the *Inoviridae* have similarly small genomes, these are enclosed by thousands of major coat protein (MCP) copies to form filamentous structures up to 2000 nm long. These phages replicate by continuous secretion of filaments from the host in a chronic infection that does not kill the host [18].

Finally, there are two families of RNA phages: the dsRNA *Cystoviridae*, and the ssRNA *Leviviridae* [19]. The *Cystoviridae* genomes are tri-segmented, and their icosahedral capsids are surrounded by lipid membranes. The *Leviviridae* however, morphologically have more in common with the small *Microviridae*, as the small icosahedral capsid solely consists of MCPs and a single maturation protein copy. The positive-sense ssRNA genomes are small (< 5 kbp), and form secondary structures vital for virion assembly [16, 19].

Thanks to the sheer diversity and ubiquity of phages in nature, it is often straight-

forward to isolate phages from natural ecosystems. Although there exist many variations of phage isolation protocols, most rely upon incubating environmental samples with a targeted bacteria. If phages capable of reproducing upon the targeted bacteria exists in the sample, amplification may occur, increasing the concentration of these phages [20].

This type of isolation protocol works well for isolating phages capable of rapid reproduction upon targeted hosts, however they do not provide a representative snapshot of phage diversity [16]. Increasingly, novel phages are discovered by metagenomics surveys where phages are not isolated but instead identified by genome assembly from metagenomic virome databases[21]. This also exemplifies the increasing degree to which phage diversity and taxonomy is investigated using genomics instead of physical isolation, and traditional morphology-based taxonomy supplemented with phylogenetic clades [16, 17, 21].

2.2 To lyse or lysogenize; phage life cycles

Traditionally, phage replication is split into the lytic and lysogenic cycles. In the lytic cycle, the phages' genetic material is replicated without integration into the host chromosome, and upon viral assembly and maturation, the cycle terminates in the destruction (lysis) of the bacterial host cell. In the lysogenic cycle however, the phage genome is integrated into the host chromosome as a prophage, and resides here indefinitely until an appropriate signal triggers transcription of the prophage and subsequent induction into the lytic cycle. These interlocking cycles are illustrated in Fig. 2.2. Phages able to undergo the lysogenic cycle are often referred to as temperate, while phages unable of undergoing this cycle are obligately lytic [14].

There are good reasons not to immediately lyse a host. Although an obligately lytic strategy allows for a potentially exponential growth, environments rarely allow for this. Such a phage may thrive in a chemostat culture with high concentrations of susceptible hosts, but struggle in a low-nutrient, stationary phase culture with few viable hosts. For some phages like T4, amplification upon a stationary phase culture seems to be impossible without the addition of nutrients. In these situations, it may be prudent for a phage to "hedge its bets" and wait for better conditions in the relative safety of a host [14, 22].

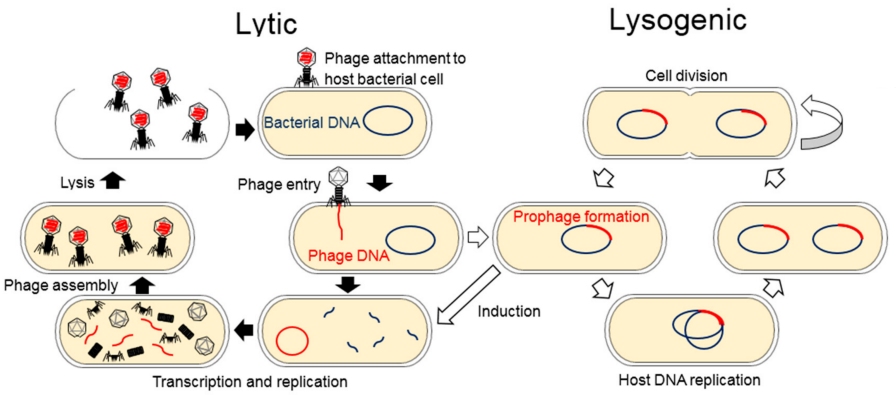


Figure 2.2: An overview of the basic lytic and lysogenic phage life cycles. Lytic phages operate strictly through the lytic cycle, where phage attachment and entry immediately leads to phage replication and assembly, followed by lysis of the bacterial host cell. Temperate phages may operate via both the lytic and the lysogenic cycle, where phage attachment and entry may lead to prophage DNA integration in the host genome, where the prophage may be stably passed through host cell division. Induction into the lytic cycle may occur via various signals, including UV radiation and infection by another phage. Reproduced and altered from [23], CC-BY-4.

Upon prophage formation shown in Fig. 2.2, phages employ several strategies for prophage maintenance. Perhaps the most familiar strategy is that employed by the λ phage: chromosomal insertion using site-specific recombinases. This appears to require circular phage genomes, so phages with linear genomes, including λ, must first convert to a circular intermediate. After circularization, the phage-encoded protein integrase mediates recombination between the λ genome and the host chromosome at a 15 bp overlap. Although this insertion site lies within intergenic DNA and hence does not interrupt host transcription, many other phages insert themselves within structural genes [14].

A second prophage strategy is that of insertion by transposition, as exhibited by Mu-1. The linear phage genome inserts itself randomly in the host chromosome, often causing phenotypic mutations. During replication in the buildup to lysis, the prophage acts as a transposon, and copies of the genome are replicated and inserted randomly throughout the host chromosome. During packaging, the prophage is excised somewhat randomly, with roughly 2 kbp of host genome included at either end [24]. In this sense, these types of phages are never truly removed from the host genome, and the virion simply acts to enable transposon activity between bacterial cells [14].

The final type of prophage are those that maintain lysogeny in plasmid form. Phage P1 is the model organism for this type of replication. Upon infection of a host cell, phages destined for lysogeny circularize their genome in similar fashion to λ. However, at this point, the circular prophage remains as a plasmid, replicating only once per host cell replication so that both daughter cells inherit the prophage [14]. This is contrary to the traditional model of prophage integration into the host genome as shown in Fig. 2.2, and represents a fundamentally different type of prophage maintenance.

For many temperate phages, lysogeny is fairly stably maintained: the rate of spontaneous lysis often lies between $10^{-4} - 10^{-5}$ per bacterial generation. However, the lytic/lysogeny decision is not usually one made at random: environmental factors such as nutrient depletion and phage densities have been shown to impact the rate of λ phage lysis [22]. Some temperate phages are also sensitive to the host SOS response to DNA damage. In E. coli, DNA damage activates protease activity by the host protein RecA. RecA cleaves the host control protein LexA, which triggers transcription of numerous genes who code for DNA repair proteins. λ phage utilizes this mechanism to trigger lysis in response to DNA damage. The constitutively expressed λ transcription repressor is sensitive to RecA proteolysis, and cleavage of the repressor protein results in transcription of λ lysis genes [22]. This type of SOS response can be triggered by UV radiation, or by incubation with the potent antibiotic mitomycin c, both of which induce bacterial DNA damage. Of the two, mitomycin c appears to more stable induce prophage activation [22, 25] However in general, the lysis/lysogeny decision is poorly understood for most temperate phages, which raises barriers to potential therapeutic applications [14, 26, 22].

The major concern temperate phages raise, is that of horizontal gene transfer. Lysogeny represents a fundamentally different host relationship than lytic bursting. Temperate phages benefit from host cell growth, and hence symbiotic host-phage interactions are advantageous. Prophages sometimes encode virulence factors, such as the gene for Shiga toxin carried by some lambdoid phages. Phages may also carry genes for antibiotic resistance, and the ability of some phages like Mu-1 to carry random sections of host genomes within its virion means beneficial novel genes and mutations may spread quickly throughout a bacterial population [27, 26].

On the other hand, temperate phages might also provide potential therapeutic benefits. In an *in vivo* environment such as the human gut where resources are scarce and competition severe, treatment with strictly lytic phages may not be optimal. Low target bacteria concentrations and starvation conditions imply lytic phage struggle to replicate, as is borne out by studies showing the gut virome is dom-

inated by temperate phages, and a lytic coliphage therapy study showing little *in vivo* amplification of the administered phage cocktail. Perhaps an engineered temperate phage whom undergoes lysis at relatively high rates may be better fit to this type of treatment [28, 22].

Although phages are often viewed through the lens of a lytic-lysogenic dichotomy, this presents several issues. Firstly, filamentous phages such as M13 reproduce in a continuous, chronic fashion without killing their hosts. This is increasingly recognized as a third, separate cycle of phage reproduction [16]. Some phages are also capable of both chronic infection, but also able of undergoing the lysogenic cycle [29, 22].

Further complicating matters is phages' predilection for recombination. Many phages, and especially temperate phages, are known to engage in frequent genomic crossovers and exchange of genes upon coinfection within the same host cell. This occurs to such an extent that recombination is thought to be the primary driver of phage evolution [13, 30]. Although recombination between obligately lytic phages and temperate phages is thought to occur less frequently than between phages with the same life-style, it is clearly possible and can drive phage life-style changes [31]. Additionally, the very definition of temperate phages implies lysogeny is optional; under certain conditions and hosts, temperate phages are capable of acting lytically. As such, it is perhaps most appropriate to consider lysogeny as a life cycle rather than a classification of phage. This is not to suggest there is no distinction between temperate phages and obligately lytic phages; phage cluster phylogenetically according to life-style [32]. However, the distinction is blurred when one considers systems more complex than that of a single phage upon a single bacterial lab strain cured of prophages.

2.3 Bacteriophage host range determinants

Like in everything else, phages clearly exhibit a wide variety of life cycles. However, they are united with all other biology in sharing a single goal: replication. As viruses, they rely on host cells to achieve this, and their life cycle can be loosely described in 5 steps: *attachment* to the host cell, *penetration* of phage nucleic acid and protein into the host cell, *replication* of phage nucleic acid and protein, *assembly* and packaging of virions, and *release* of matured virions from the host cell. As achievement of these goals is obviously not in the best interest of the phage's host, bacteria have evolved numerous methods of avoiding successful phage infection and replication.

2.3.1 Attachment and penetration

As the initial contact between phage and bacteria, adsorption to host receptors is one of the most important factors in determining host range. Phages vary greatly in their host range, with some only infecting certain strains of a host species, while others may infect bacteria from different genera [33, 34]. A broad host adsorption range may clearly be advantageous, but not without bounds. For the phage, receptor recognition is not only about attaching to as many bacteria as possible, but also identification of a host that will be susceptible throughout its life cycle. There is no point in adsorbing to a host who cannot support a complete infection cycle [34].

In general, receptor-binding proteins (RBPs, or viral adhesins) make specific, usually non-covalent contact with a host receptor presented on the cell surface. There are a wide variety of receptors, including proteins, polysaccharides, acids, and bacterial appendages. Receptor binding occurs in three steps: initial contact, reversible binding, and irreversible binding. Intercellular phage virions cannot move on their own accord, and hence initial contact relies upon Brownian motion and environmental forces. Reversible binding serves as an initial recognition step, but the virion remains uncomitted and may disassociate with viability intact. However, subsequent irreversible binding is accompanied by conformational changes in the virion to prepare for entry into the host [33, 34].

The lipopolysaccharide (LPS) layer of Gram negative bacteria is an important determinant of phage host range. The LPS consists of a lipid A membrane anchor, core polysaccharide, and a repeating O-antigen polysaccharide, and is the source of both primary and secondary receptors, as well as serving as a host defensive layer by sterically blocking phages from attaching to their receptor. The structure of LPS is shown in Fig. 2.3 Lipid A and the core polysaccharides are relatively conserved, while the O-antigens often have tremendous diversity: more than 200 E. coli O-serotypes are known [34, 35]. Modification of O-antigen is an adaption to avoid recognition by the mammalian immune system, but also aids in avoidance of phage infections. Mutants incapable of O-antigen synthesis are termed ”rough”, as they form less regular colonies than their ”smooth” O-antigen-synthesizing cousins. Most E. coli lab strains, including DH5α and BL21, are rough mutants and have long since lost the ability to synthesize O-antigen [36]. This should be taken into account when extrapolating phage interactions in lab strains to wild-type E. coli.

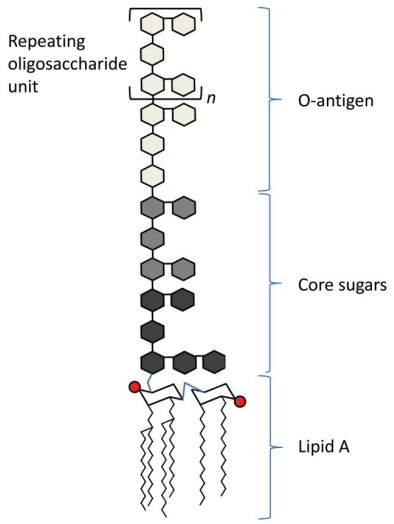


Figure 2.3: Structure of the Gram negative lipopolysaccharide (LPS) layer. The lipid A anchors the LPS to the outer membrane, while the core sugars and O-antigen polysaccharides protrude from the membrane. The Lipid A and the core sugars are relatively conserved, while the the repeating O-antigen polysaccharide is hyper-variable within species. Reproduced and altered from [37] with permission.

Unsurprisingly, phages that target elements of the inner lipid A or the core polysaccharide shown in Fig. 2.3 generally have broader host range than those that target O-antigen. As these LPS layers may be bulky and contribute to steric hindrance of phages, some phage LPS-recognizing proteins also have hydrolysis activity: upon binding their LPS receptor, they hydrolyse the polysaccharide bonds, presumably to gain access to an irreversible receptor buried beneath LPS. This appears to be especially common among podoviruses [35, 34].

Outer membrane proteins are common receptors for phages of Gram-negative bacteria. Highly-expressed proteins such as the *E. coli* porins OmpA and OmpC are over-represented as phage receptors, but rare proteins such as *E. coli* NrfA (roughly 5 copies per cell) have also been shown to function as phage receptors. Bacterial appendages such as flagella and pili may also be used as receptors. These are normally used as primary receptors, and phages have been shown to use the rotational motion of flagella to propel themselves towards their secondary receptors on the cell proper. Finally, exopolysaccharide layers such as those in biofilms may both shield hosts from phage-receptor binding, but also provide the necessary receptors for others. *E. coli* phage can attach to encapsulated hosts, and ”drill” through the layer to reach the outer membrane and latch onto its next receptor [33, 34].

In response to phage reliance on surface structures, adsorption blocks are a common class of defence against phages that physically prevent phage-receptor binding. These include capsules and biofilms, mutation of the relevant receptor [38], development of another surface structure capable of physically blocking phage adsorption [39], or even complete loss of the phage receptor [34]. Although these alterations may result in fitness defects corresponding to reduced (or loss of) function of the phage receptor, receptor mutations may in many cases block phage adsorption without significant disruption of function; [40] shows mutations to OmpA could protect the host from 15 different phages while maintaining normal function.

In addition to random mutation, many bacteria also employ more sophisticated strategies. Phase variation at select genome loci allow for fast, non-random alteration of phage receptors and other defences [41, 42]. This may be done by toggling a phage receptor on/off, as in the BvgAS system of *Bordella* spp, where phase variation alters between the phage receptor-expressing Bvg+ phenotype and the phage insensitive Bvg- phenotype. However, this phase variation entails a trade-off, as the insensitive Bvg- phenotype lacks the secretion systems of the more virulent Bvg+ phenotype [34]. Phase variation may also have less dramatic impacts upon host fitness, as for example exhibited in the LPS modification of different *Salmonella enterica* serovars [43]. There are many mechanisms by which phase variation occurs, including misalignment of repetitive sequences during DNA replication that lead to phase-variable protein expression (single strand misalignment), RecA-mediated and site-specific recombination between multiple gene alleles, and epigenetic regulation by DNA methylation [42].

Far from passive automatons in the face of escalating bacterial evolutionary innovations, phages have a host of strategies to combat bacterial immunity in an ever-escalating arms race. The simplest of these is mutation of receptor proteins to recognize a modified bacterial receptor. For example, ϕX174 is able to quickly generate variants capable of binding to a range of LPS modifications [44], while λ phage can evolve the ability to use OmpF in lieu of its normal receptor OmpC [45]. Some phages evade bacterial resistance by other strategies. The coliphage Φ92 carries multiple adsorption proteins, granting it an abnormally large host range without resorting to mutation [46]. However, most phage host range alterations do occur by genome modification, and adsorption-enabling mutations in particular usually occur in the receptor-binding phage protein [47].

Some phages also employ mechanisms for selectively mutating host-range determinants. Some phages like Mu, have shufflon systems similar to those found in their bacterial hosts [48]. By randomly switching genes at host determinant loci, these phage populations are able to better respond to corresponding phase variations in their hosts. Other phages, such as the temperate *Bordtella* phage BPP1,

use *reverse retrohoming* to generate variability in host range determinants. Certain genes are transcripted, followed by generation of a DNA template using an error-prone reverse transcriptase. This template is then substituted into the genome, thus generating host range mutants at a high rate [49].

2.3.2 Replication, assembly, and release

Upon entering the host cell proper, phages can mindlessly initiate their true "purpose": replication. Although many phages immediately start the replication of proteins and nuceleic acid, temperate phages may delay this step indefinitely by integrating their genome into the host chromosome as a prophage [22].

However, bacterial defensive tactics are not limited to preventing phage entry. Most bacteria encode restriction endonucleases that are capable of selective double-stranded cleavage of foreign DNA at specific recognition sites while sparing host DNA [41]. Distinguishing between self and non-self is often carried out by methylation of recognition sequences in the host genome, thus protecting themselves from restriction endonucleases while leaving them free to cleave non-methylated phage DNA. This strategy has been mimicked by some phages who are also capable of DNA methylation, thus protecting themselves from degradation [34]. T4 for example, as gone even further in its DNA modification, and substituted cytosine with the modified base hydroxymethylcytosine (HMC)[14]. This provides the additional benefit of allowing T4 to destroy host nucleosides and instead produce their own custom nucleosides, hence shifting translation from host proteins to phage proteins [50].

There are many variations on this theme. For example, the Phage growth limitation (Pgl) system in *Streptomyces coelicolor* modifies phage DNA instead of its own, marking out the invaders DNA during replication. After lysis, the marked phage progeny are recognized by *S. coelicolor* endonucleases upon subsequent infections [51]. This system is especially interesting as it mediates protection at the population level; the initially infected cell receives no direct benefit [41].

Systems that mediate population protection in this manner are called Abortive infection (Abi) systems and are also widespread among bacteria [41]. A common setup is that of toxin-antitoxin system, where the host encodes a toxin and antitoxin in equal concentrations. Upon infection by a recognized phage, the antitoxin is destabilized, thus releasing the toxin to kill the infected cell and prevent phage proliferation [51, 41]. Interestingly, phages have also been shown to use Abi systems to provide population-level protection. λ- infected *E. coli* lysogens encode RexAB, which upon activation by T4 infection, kills the host (and the prophage) by perforating the cell membrane [52]. It is fascinating that a genetic parasite

will commandeer its host to commit altruistic suicide in order to protect neighboring host cells which may harbor fellow prophages. This example provides an excellent illustration of the complicated, sometimes-parasitic-sometimes-mutualistic relationships between phages and their bacterial hosts.

So far, the defense mechanisms described have provided innate protection: they are always active. However, with the discovery of CRISPR-Cas immunity, we now know bacteria also have an adaptive immune system. CRISPR has been found in approximately half of sampled bacteria, and allows the host bacteria not only to degrade recognized phage DNA sequences, but also to "learn" from new phage infections and degrade them upon subsequent infection [53].

Although there are a large variety of CRISPR-Cas systems, they operate on a common principle; the integration of foreign, non-self DNA sequences at designated CRISPR loci in the host genome. These DNA sequences are of length 26-72 bp and are called called spacers. In between spacers lie palindromic repeat sequences, from which the system owes its name (Clustered Regularly InterSpersed Repeat Sequences). The proteins Cas1 and Cas2 in association with other factors, recognize foreign DNA sequences (protospacers) flanked by a recognized 2-6 bp protospacer adjacent motif (PAM). The protospacer is then excised, and upon processing, is inserted in a CRISPR locus. Since CRISPR loci exist within the host genome, all daughter cells will also inherit acquired spacers and the immunity they provide [53, 54].

If a similar phage again infects the cell, the appropriate CRISPR spacers may be transcribed, processed by Cas ribonucleases, and complex to form CRISPR ribonucleoprotein (crRNP) complex that are able to target and degrade the recognized foreign DNA. Recognition is dependent not only upon the presence of the previously-encountered spacer sequence in the foreign DNA, but also adjacency of the PAM sequence. Requiring the PAM sequence is a safeguard that protects the host's CRISPR loci from self-degradation [53, 54].

2.4 Phage therapy

The potential therapeutic applications of bacteria-killing viruses were immediately obvious to early practitioners [10]. Using phages to treat clinical bacterial infections is known as phage therapy, and became very popular in the 1920's and 1930's. However, chiefly due to the discovery of effective, broad-spectrum antibiotics in the 1940's, phage therapy fell out of popularity and was only used in the Soviet Union and Eastern Europe [10].

Although widespread antibiotic resistance has warranted a renewed interest in

phage therapy, there are many challenges to translating *in vitro* phage lysis to therapeutic treatment. The most obvious of these is that of host range. By the variety of mechanisms described above, phages generally possess very narrow host range compared to antibiotics and generally lyse only specific strains of a host species [11]. Although this specificity is sometimes described as a feature [8] rather than a bug (think targeted strikes vs a nuclear bomb), there is no doubt it mostly presents itself as a challenge for clinical practitioners.

Narrow host ranges can also cause subtler issues for phage therapy. As viruses, phages are orders of magnitude larger than chemical antibiotics. For example, a normal 500 mg dosage of the fluoroquinolone ciprofloxacin consists of $9 * 10^{20}$ molecules, whereas a Russian commercial phage cocktail measured $7 * 10^{6}$ plaque forming units/mL upon an indicator *E. coli* strain [55]. Although a direct comparison between phage and antibiotics dosages is not necessarily informative, this does illustrate a vast difference in scale.

Of course, if the phages replicate *in vivo* this not necessarily a problem; exponential growth may ensure enough phages are produced to eradicate the pathogen. However, even if the targeted pathogen is susceptible to the phage, sustained phage reproduction will not occur unless the pathogen is present at sufficient concentration. A 2016 randomised, double-blind trial of a Russian commercial oral phage cocktail against enterotoxigenic *E. coli* did not observe any treatment benefits compared to a placebo, with stool samples revealing no increase in phage titers [28]. The authors considered this most likely due to the low intestinal *E. coli* concentrations present in the patients; the susceptible host was simply not present in high enough concentrations to sustain phage reproduction.

Phagoburn [56], the only other modern, randomised double-blind phage therapy study, encountered somewhat similar problems. In this study a phage cocktail was employed against *Pseudomonas aeroginosa* burn wound infections. However, the study was halted prematurely due to insufficient efficacy of the phage cocktail. Due to manufacturing difficulties and standards for endotoxin levels, the phage cocktail was administered at very low titres (10-100 pfu/mL) which were clearly insufficient for efficient bacterial clearance.

There are perhaps three main strategies for working around narrow host ranges; isolating the pathogen and screening it against a phage library to find a viable phage [57], employing mixtures of multiple phages with complementary host range (phage cocktails) [8], and evolving phages to viability upon the pathogenic strain [12]. Of these approaches, phage cocktails are perhaps the most successful and certainly the most common approach [8, 10]. Broad(er)-range cocktails may be produced at commercial scale, used for many different patients, and subjected to

regulatory approval. However, no phage cocktail will effectively lyse every bacterial strain encountered, and the phage library and evolutionary strategies add a flexibility that pre-formulated cocktails lack. The approach presented in this thesis attempts to achieve the best of both worlds to combine phage cocktails with evolution, and cocktails with expanded host range against targeted bacterial strains.

2.5 Experimental phage evolution

2.5.1 Single phage systems

The dynamics of single phage-bacteria systems are well-studied, particularly in continuous culture chemostats [58, 59, 60]. These systems are often constructed around the same design; a lytic phage is added to a steady-state bacterial culture initially susceptible to the phage, allowing both the phage and bacterial populations to evolve. The bacterial culture is rarely completely washed out by the phage, but instead harbors resistance mutants that grow to dominance. In turn, phage populations often adapt to the new bacterial mutant, which may in turn adapt to the new phage in a coevolutionary cycle [59, 61, 62]. This coevolution is known as arms race dynamics, and is characterized by escalating resistance and counter-resistance; the current bacterial population is immune to all former phages, and the current phage can reproduce upon all previous bacterial generations [61]. Although exceptions apparently exist [62], arms race dynamics generally do not continue indefinitely [59], as within a few generations most bacterial populations will eventually produce a mutation the phage population is unable to overcome [61].

Interestingly, arms race dynamics sometimes give way to fluctuating selection dynamics, in which the new bacterial generation is immune to the current phage generation, but not a previous generation. Likewise, the next phage population will prey upon the new bacteria, but in the process lose the ability to prey upon the previous generation [61, 59]. These indefinitely fluctuating phenotypes are shown in Fig. 2.4 are characteristic of the Red Queen hypothesis, named for the eponymous Red Queen of Lewis Carrol's *Through the Looking-Glass* who runs as fast as she can just to stay in place. Similarly, the Red Queen hypothesis postulates competing species are trapped in a continuous evolutionary race even as their average fitness remains constant [63].

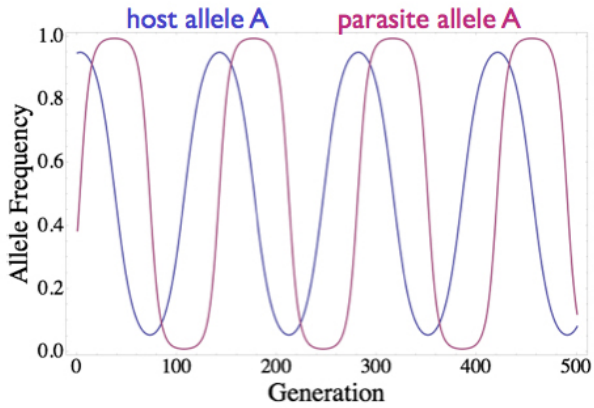


Figure 2.4: A general model of parasite-host phenotype alteration with Red Queen dynamics. Host phenotypes displaying host allele A are susceptible to parasite phenotypes displaying parasite allele A, and are hence replaced at the population level by host phenotypes displaying an alternative host allele B. However, this naturally entails a delayed parasite population replacement of parasite allele A with an allele capable of infecting the host allele B. This may again cause a shift back to host allele A, restarting the cycle. Reproduced from [64].

In Fig. 2.4, host allele A may represent a particular bacterial O-antigen, while parasite allele A may represent a particular phage tail fibre sequence. In response to phage predation, host allele A is replaced at the population level by an alternative O-antigen, host allele B. This causes a similar phage population-level shift to an alternative tail fibre sequence capable of recognizing the new O-antigen. Since there is often a limited array of potential alleles that do not entail significant fitness costs, the bacterial population may switch back to the initial host allele A which the current phage tail fibre allele is incapable of recognizing.

Interestingly, the Red Queen hypothesis is often posited as an explanation of why sex is so common in eukaryotes [63]. Sexual recombination allows for the horizontal exchange alternative alleles, thus allowing populations to more quickly adapt to shifts in the phenotypes of competing species. Similarly, recombination is thought to be the primary driver of phage evolution [65, 13], suggesting the Red Queen may have driven the evolution of widespread phage recombination as well.

In contrast to the frequency in which many phages adapt to host resistance mutations, phage adaption to novel hosts is clearly more difficult. Although it is certainly possible, phages are often unable to adapt to new hosts even of the same species [66, 67]. Many phages have large genomes, but sequencing has shown mutations in tail fibre genes are largely responsible for host range adaption [68,

66, 13]. Many phage genomes also have overlapping genes, meaning a mutation in one gene may significantly impact the function of an unrelated gene [69]. As a result, cultures of single phages often appear to have limited plasticity in terms of host range expansion.

2.5.2 Competition and cooperation in multi-phage systems

Evolutionary dynamics in multi-host/parasite systems can become rather complicated. In dense phage/bacteria populations, coinfections are common (multiplicity of infection follows a Poisson distribution) [70]. Although increased competition may result in more efficient infection of bacteria, coinfection may also select for phage-phage competition at the expense of virulence. This is analogous to the old adage about running away from an angry bear together with a friend; you don't need to outrun the bear, only your friend. In this situation, an optimal strategy might be to periodically stick your leg out in the hope of tripping your associate. However, this would be a bad strategy if running alone since it is slower than sprinting and increases your likelihood of gaining an ursine acquaintance. In other words, an optimal strategy does not exist in a vacuum, but instead depends on your competition [71, 72].

Coinfection competition manifests itself in several forms. Beyond simple competition for hosts, a phage participating in coinfection may "steal", and use structural proteins (i.e. capsid) that its competitor synthesized as its own. This type of competition may select for faster lysis times in order to secure limited reproduction at their competitors expense, resulting in sub-optimal use of the (hosts) resources in a tragedy of the commons scenario. Phages may also produce anti-phage toxins to prohibit coinfection [72, 71]. Interestingly, variable lysis time has been observed to evolve in some systems (T4, and *P. fluorescens* phage $\Phi 2$) with initially clonal phage populations and bacterial hosts. The phages evolve to respond to coinfection by premature lysis, while maintaining normal lysis time upon solitary infection [73, 70].

So far, these examples present competition as occurring between individual phages. However as previously noted, phages frequently engage in genetic recombination upon coinfection to produce mosaic offspring. There events are perhaps better understood from the context of selfish gene theory, with selection occurring upon the level of individual genes within their phage vessels. If a different permutation of genes creates a fitter phage, this new permutation will be selected for and become dominant. Viewed in this manner, a phage is simply a selection of genes assembled from within the accessible phage pangenome. The diversity present in this pangenome vastly exceeds that available from simple point mutations, and for this

reason, recombination is thought to be the primary source of phage diversity [65, 13].

Recombination clearly plays an important role in the evolution of phage host range, and attempts have been made in exploiting recombination to expand the host range of collections of phages, known as phage cocktails. One of the oldest techniques for phage host range expansion, Appelmans protocol, evolves phage cocktails upon a selection of target hosts and is thought to employ recombination [13]. Interestingly, this protocol dates back to 1922, before there was any notion of phage recombination, and has been used continuously to develop therapeutic phage cocktails by the Eliava Institute in Tbilisi, Georgia. Since phage therapy was replaced by antibiotics in the West, little-to-nothing was written about Appelmans in Western scientific journals. However, with the recent resurgence of interest in phage therapy, Appelmans protocol has resurfaced. In 2019, Burrowes, Molineux, and Fralick [13] detailed a modified version of Appelmans protocol to successfully expand the host range of a 3-phage cocktail against 10 *Pseudomonas aeruginosa* strains. Another study [12] employed the same protocol with different phages and *P. aeruginosa* strains will similar results.

Appelmans protocol is an iterative process where a phage cocktail is serially inoculated upon separate bacterial hosts, incubated, and recombined to create the next cocktail iteration. In the protocol of Burrowes, Molineux, and Fralick, each row of a 96-well plate is inoculated with a different bacterial strain. Next, a serial dilution of the previous cocktail iteration is added to the wells of each row. After incubation, the wells of each row are examined for bacterial growth. All serial dilutions that cleared bacterial growth along with the first serial dilution that did not clear bacterial growth are combined. The next cocktail iteration is assembled by combining these dilutions from each row, followed by centrifugation and filtration to remove bacteria [13]. This new cocktail is re-inoculated as before, completing the cycle. This process is continued for 30 rounds.

Although these results are very promising, the process is labor-intensive and requires 30 days to completion. The protocol presented in this thesis therefore modifies the host range expansion protocol of Burrowes, Molineux, and Fralick to facilitate automation, reducing protocol run-time to less than a week [13, 12, 57].

Chapter 3

Materials and Methods

3.1 Recipes

3.1.1 LB medium (1 L)

- 10 g Tryptone
- 5 g Yeast Extract
- 5 g NaCl

Fill with distilled water to 1 L in a 2 L flask and autoclave. Store at room temperature.

3.1.2 Agar (1 L)

- 10 g Tryptone
- 5 g Yeast Extract
- 5 g NaCl
- 7.5 g agar

Fill with distilled water to 1 L in a 2 L flask and autoclave. Store at 55 °C and dispense into agar plates.

3.1.3 Soft agar (1 L)

- 10 g Tryptone

- 5 g Yeast Extract
- 5 g NaCl
- 3.75 g agar

Fill with distilled water to 1 L in a 2 L flask and autoclave. Store at 55 °C.

3.1.4 SM buffer (1 L)

- 5.8 g NaCl
- 2 g $MgSO_4 \cdot H_2O$
- 50 mL Tris-Cl (1 M, 7.5 pH)

Fill with distilled water to 1 L in a 2 L flask and autoclave. Store at room temperature.

3.1.5 HEPES buffer (1 M, 1 L)

- 283.3 g HEPES

Fill with distilled water to 1 L, and adjust pH to 7.5 pH using 10 M NaOH.

3.2 Bacterial strains

For this project, the *E. coli* reference collection (ECOR) was used, which consists of 72 *E. coli* strains representative of the species' genetic diversity [74]. This collection was generously sent to us by the Nilsson group at Stockholm University.

The ECOR collection was assembled in the early 1980s from isolates collected from a wide range of mammalian hosts. 39 of the strains were isolated from human hosts, while the remaining 33 strains were collected from a menagerie of animals including a kangaroo rat from Nevada and sheep from New Guinea. The diversity of ECOR also spans the major *E. coli* phylogenetic groups A, B1, B2, D, E, and F [75, 76].

3.3 Basic phage protocols

3.3.1 Double agar overlay method

Many slightly different versions of the double agar overlay method exist, and the one below is the version used in our lab, which closely resembles that of [20]. First,

a liquid overnight culture of the bacteria of interest is grown under appropriate conditions. The *E. coli* strains used here were grown in LB medium at 37 °C and 200 rpm shaking. Next, a dilution series of the phage-containing stock or sample is prepared in SM buffer. The appropriate dilution series depends on the initial phage concentration, and it is generally desirable to aim for a dilution that will yield 10-100 plaque forming units (pfu)/mL upon plating. If nothing is known about the phage stock or sample, an appropriate dilution series may be [10^0, 10^{-2}, 10^{-4}, 10^{-6}, 10^{-8}]. An equal number of LB agar plates as the size of the dilution series are retrieved and labeled according to the dilution concentration. If these were stored at 4 °C, warm the plates to room temperature. 3 mL liquid soft agar is aliquoted into centrifuge tubes and placed in a heat block at 55 °C. 100 µL of the appropriate phage dilution and 300 µL of the bacterial culture are added to each tube with soft agar and mixed by vortex. The soft agar mixture is then poured onto an agar plate, evenly distributed, and placed top-up. This was repeated for each serial dilution. After the top agar had solidified (about 10 minutes), the plates are inverted and incubated overnight at 37 °C. If phages against the bacterial host were present, round plaques with reduced or no bacterial growth are visible after incubation. These can be counted to determine the pfu/mL concentration of the phage stock (number of plaques* 0.1 mL* dilution factor).

3.3.2 Plaque purification

Plaque purification is a standard technique to purify clonal phages [77]. Individual plaques produced by the double agar overlay method are excised from the surrounding agar with a sterile toothpick and suspended in an Eppendorf tube with 1 mL SM buffer by vortexing. The suspended stock is stored at 4 °C. To insure the phages are clonal, stocks should be subjected to at least 3 rounds of plaque purification and the resulting plaques should appear homogeneous.

3.3.3 Whole-plate lysis

To prepare phage stocks of concentration in excess of 10^8 pfu/mL, the method of whole-plate lysis was used. The phage stock of interest was plated with serial dilution using the double agar overlay method. After overnight incubation, the serial dilution plates which were almost completely cleared by phage lysis were chosen. These plates display a characteristic ”webbing” pattern of lysis, and are indicative of several rounds of phage infection, resulting in a very high number of phage virions. 5 mL of SM was added onto each of these plate and left to sit for 30 minutes with periodic agitation. The resulting solution was aspirated from the plates, centrifuged for 15 minutes at 5000 rpm, and passed through a 0.2 µm membrane filter. The stocks were stored in 1 mL aliquots at 4 °C.

3.3.4 Spot testing

To test host susceptibility to multiple phages on a single agar plate, a simple spot test may be used. An agar plate is marked into the number of quadrants desired (max 12 for a normal agar plate) and labeled according to the phage names. As in the double agar overlay method, 300 µL of overnight bacterial culture is mixed with 3 mL soft agar but *without* any phage dilution. The plate is allowed to solidify, and 10 µL of each phage stock is pipetted into their corresponding quadrants. The plate is then dried under a flame with the lid off until the drops are not visible (at least 20 minutes). Finally, the plate is inverted, incubated overnight at 37 °C, and examined for lysis the following day.

3.3.5 Sample preparation and TEM imaging

At least 20 mL of high titer phage stock in excess of 10^8 pfu/mL were prepared by whole-plate lysis. The stock was centrifuged at 13.000g for 24 hours at 4 °C, forming a plaque on the side of the centrifuge tubes. The supernatant was discarded, and the plaque resuspended by adding 1 mL SM buffer to the tube and incubated with shaking at 200 rpm and 4 °C.

The phage stock was then centrifuged for 24 hours under the same conditions, the supernatant was discarded, and the plaque was resuspended in ice-cold 2% paraformaldehyde in 0.1 M HEPES buffer by vortexing. 4 µL of phage stock was then pipetted onto carbon coated grids and stained with 15 µL 4% uranyl acetate. The negatively stained grids were then examined in a Tecnai 12 TEM at 100 kV. Staining was performed by Thi My Linh Hoang of the Cellular and Molecular Imaging Core Facility, and microscopy was performed by the author.

3.4 Bacteriophage isolation

To obtain a collection of phages capable of lysing a broad cross-section of ECOR, strains E10, E24, E35, E42, and E55 were used as hosts to isolate phages from environmental samples. Phage isolation is a relatively standard protocol, and this version is closely related to those reviewed by [78].

All phages were isolated from sewage samples collected at Høvringen sewage treatment plant in Trondheim, Norway. 5 mL of sewage sample was added to 20 mL SM buffer and incubated at room temperature overnight with shaking. Although many protocols call for sample incubation with added LB and a targeted bacterial culture [20], this may reduce phage diversity and bias in favor of the most virulent phage on the current host.

The sewage suspension was centrifuged for 15 minutes at 5000 rpm, and passed through a 0.2 μm membrane filter to remove any bacteria and higher organisms. Overnight cultures of each targeted ECOR host were prepared, and the sewage suspension was serially diluted and the double agar overlay protocol was run for each ECOR host to produce plates with phage plaques on each host. Several sewage suspensions and subsequent double agar overlays had to be performed for hosts E10 and E42 before any plaques were produced.

Next, individual plaques were picked according to the plaque purification protocol. Picked plaques were chosen for their perceived lytic properties (i.e. clear and large). Five phage isolates were chosen for further examination; E10p1, E24p1, E35p1, E42p1, and E55p1, isolated on ECOR strains E10, E24, E35, E42, and E55 respectively. Finally, high titre stocks in excess of 10^8 pfu/mL were then produced for each phage isolate using the method of whole plate lysis.

3.5 Determining bacteriophage host range

Each phages' host range was characterized upon the ECOR library. In addition to the five ECOR phages E10p1, E24p1, E35p1, E42p1, and E55p1, five other phages were also tested for ECOR host range: phages De1, De2, De8, and De11 previously isolated on *E. coli* lab strain DH5α, and phage Be2 previously isolated on *E. coli* lab strain BL21. All phages and their respective isolation hosts are summarized in Table 3.1.

Table 3.1: List of isolated phages tested for host range on ECOR. Isolation host indicates the strain each phage was first isolated on, along with the phylogenetic group of each host strain. The domesticated lab strains DH5α and BL21 are here considered to not belong to any phylogenetic group.

Phage	Isolation host	Host group
E10p1	E10	A
E24p1	E24	A
E35p1	E35	F
E42p1	E42	E
E55p1	E55	B2
De1	DH5α	-
De2	DH5α	-
De8	DH5α	-
De11	DH5α	-
Be2	BL21	-

Five phages of Table 3.1 were isolated upon four of the six major *E. coli* phylogenetic groups: A, F, E, and B2 [75, 76]. Although the lab strains DH5α and BL21 are both descended from group A isolates (K12 and B strain respectively), decades of domestication have altered them considerably: for example, neither are able to produce long-chain LPS that may significantly modulate phage susceptibility [36, 35]. Because of this, DH5α and BL21 are here considered to be outside the scope of wild-type phylogenetic classification.

Testing the host ranges' of 10 phages on the 72 ECOR strains generates 720 phage-bacteria interaction. In order to test this many pairs, a spot test was used for all 10 phages upon the 72 ECOR strains. The plates were examined after incubation, and each phage-bacteria interaction was recorded as either 0 (no lysis), 0.5 (partial lysis), or 1 (complete lysis).

3.6 Phage evolution strains

On the basis of their relatively broad and complementary host ranges, phages De8, E10p1, and E42p1 were chosen to create the initial phage cocktail. These phages were combined in a 1:1:1 ratio from stocks of roughly 10^9 pfu/mL. For host strains, 10 ECOR strains spanning five of the six major *E. coli* phylogenetic groups were chosen. These are shown in Table 3.2 along with their phylogenetic groupings [75].

Table 3.2: ECOR strains selected as hosts for the phage evolution protocol along with their susceptibility to the initial phage cocktail consisting of De8, E10p1, and E42p1.

Strain	Group	Initial cocktail susceptibility
E4	A	-
E13	A	De8, E42p1
E17	A	-
E21	A	-
E31	E	-
E40	F	De8, E10p1, E42p1
E53	B2	-
E57	B2	E10p1, E42p1
E64	B2	-
E70	B1	-

To ensure the cocktail does not simply die out, strains susceptible to the cocktail phages were included. As shown in Table 3.2, 3 of the 10 ECOR evolution host

strains had initial susceptibility to subsets of the initial phage cocktail, while the remaining seven strains were completely immune to the cocktail. The susceptible strains E13, E40, and E53 were also deliberately chosen their susceptibility to multiple phages, as this presumably increases the possibility of phage recombination [13].

3.7 Prophage screening

All 10 ECOR strains of Table 3.2 were screened for prophages using both bioinformatics and an experimental approach. After accessing whole genome shotgun draft sequences of the ECOR strains available on NCBI [76], each genome was run through Phaster to screen for prophage sequences. Phaster is an online tool that searches bacterial genomes for known (pro)phages by BLASTing for matches in the NCBI database combined with a custom prophage database [79]. Phaster was used to screen the ECOR genomes for intact prophages that could potentially be released.

The Table 3.2 ECOR strains were also tested experimentally for actively released prophages. In a similar approach to that of Shibata et. al. [80], each of the 10 ECOR evolution strains were cultured in LB overnight. The cultures were then centrifuged for 15 minutes at 5000 rpm, and passed through a 0.2 µm filter. The filtrates were then tested for prophages by spot-testing as before upon all 10 ECOR evolution strains in addition to DH5α. Preferably this screening would have been performed by culturing the ECOR strains with mitomycin c [81], but unfortunately it was not possible to obtain this chemical in time for the project deadline.

3.8 Incubation cabinet

In order to run the automated phage evolution platform, a sterile incubation cabinet needed to be built around the Opentrons robot. The NTNU mechanical workshop built a clear polystyrene box with a door to encase the robot with an approximately airtight seal. In order to sterilize the cabinet, all walls and contents were wiped down with 1% Virkon before each protocol run. In addition to this, a modified table lamp with a UV-C LED was duct-taped to the roof of the box which could be turned on and off as needed. To make sure bacterial cultures were not irradiated during the sterilization procedure, a system was designed to open and close plate lids. A stepper motor (SM-42BYG011-25) was mounted on a 3D-printed plate holder and super-glued to the plate lid. By rotating the motor axle 90°, the lid could be opened and shut on command. This allowed the plate containing the host ECOR strains to be placed in the incubation box prior to UV sterilization with

the lid closed, and then opened upon completion of UV sterilization. This design is shown in Fig. 3.1. The complete specifications for hardware and software are published on FighShare.

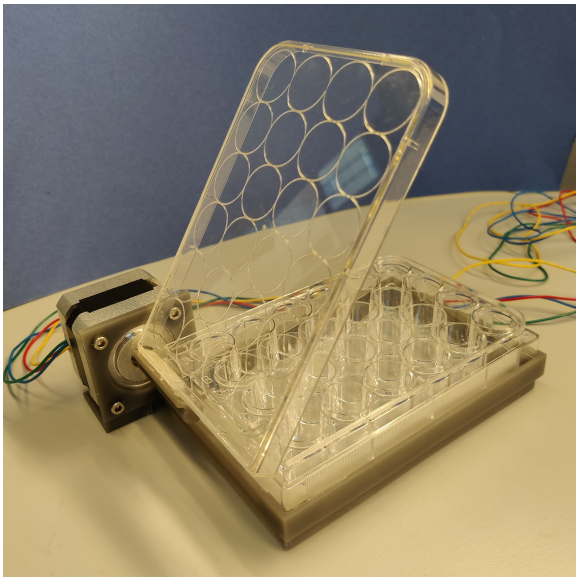


Figure 3.1: System for remote opening and closing of Corning Costar 24-well plate lid during UV sterilization protocol. The plate fits within the 3D-printed mount together with a SM-42BYG011-25 stepper motor screwed beside. An adaptor is slotted onto the stepper motor arm and super-glued to the plate lid. Using an Arduino Pro Micro, the plate lid can be raised and lowered at will.

An Arduino Pro Micro was used to control the box temperature. The temperature was measured by a LM35DZ integrated-circuit temperature sensor, and two RS Pro Silicone Heater Mats (80W, 12V) coupled with heatsinks (153AB Series, 66mm x 40mm) were used as heating elements. Using bang-bang control, the box was kept at the target temperature (37 °C). A fan (be quiet! Silent Wings 3 140mm) was mounted —next to the heat elements to circulate air within the box. All power was supplied by the Dell AC305AM-00 240V DC power supply. The circuit diagram shown in Fig 3.2 controls both temperature, airflow, and the plate lid system of Fig. 3.1.

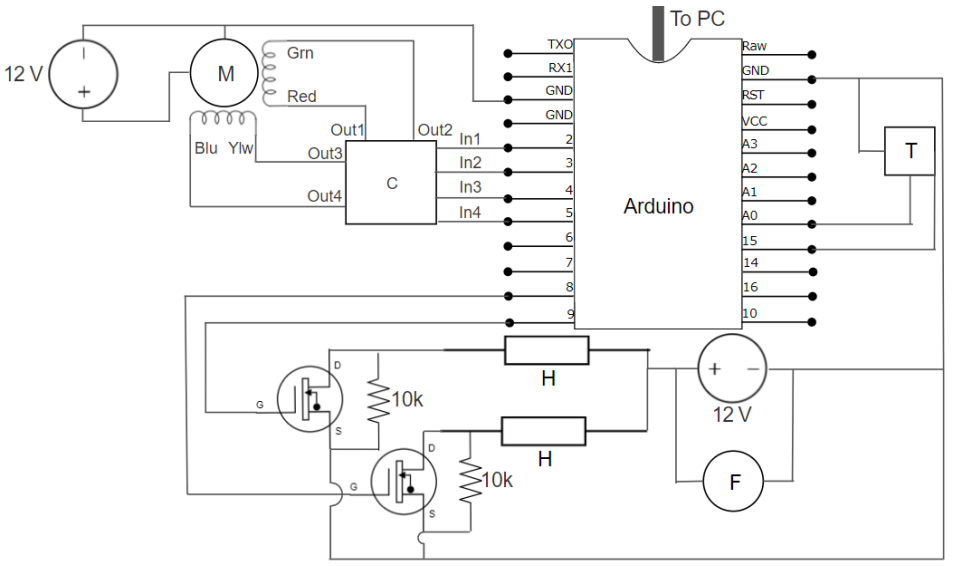


Figure 3.2: Circuit diagram of temperature control and plate lid control. Arduino Pro Micro (labelled Arduino) controlled both these functions running a script uploaded to Figshare [82]. A temperature controller (labelled T) continuously monitored the temperature. Two heat elements (labelled H) with heatsinks were turned on/off using bang-bang control mediated by NMOS transistors. A fan (labelled F) was mounted by the heat elements. The stepper motor (labelled M) which raised/lowered the plate lid was driven by a L298N H-bridge motor driver (labelled C).

The circuit of Fig. 3.2 was assembled upon a circuit board and soldered into place. In addition to the temperature control supplied by this circuit, humidity was controlled by placing a coffee filter in a small box of water. This functioned as a passive humidifier, keeping humidity at around 60% and preventing evaporation of medium from plate wells.

Finally, all these parts were assembled to complete the setup shown in Fig. 3.3. The incubation cabinet is shown encompassing the OT-1 along with the UV lamp duct-taped to the ceiling of the box, electronics, plates, and pipette tips used during the evolution protocol. Holes for wires were burnt through the box walls using a butane-powered soldering iron, and made airtight using putty.

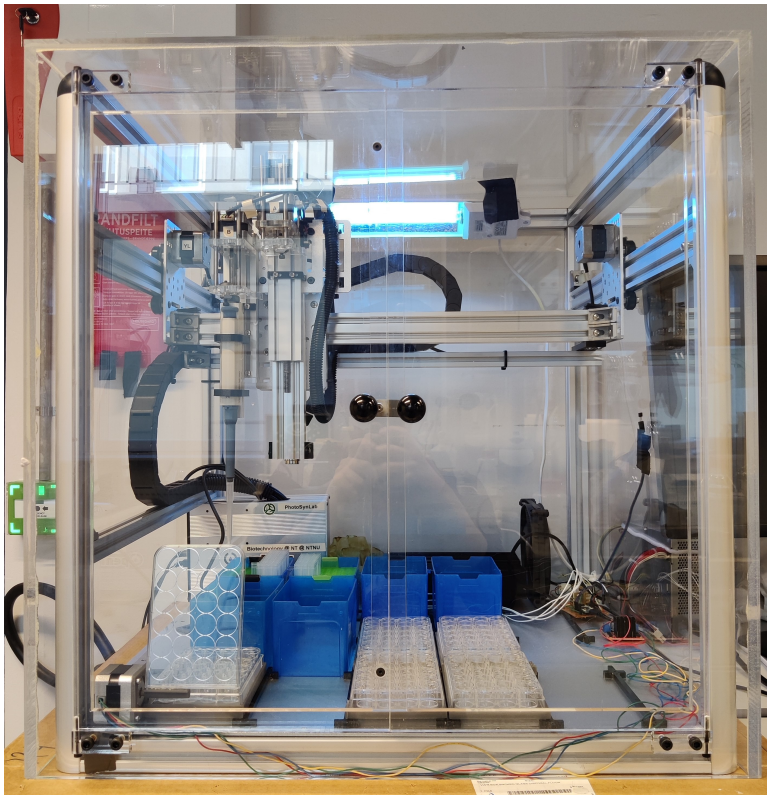


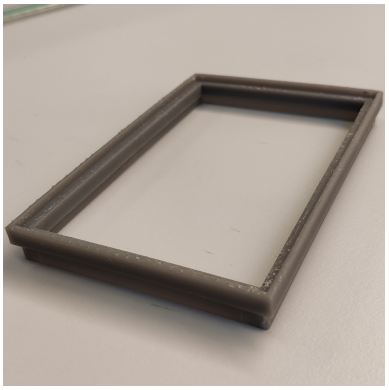
Figure 3.3: Opentrons OT-1 inside incubation cabinet along with UV-C lamp, temperature and humidity control system and deck set up for the host range expansion protocol.

Even with the two heater mats, 37 °C was at the upper limit of what the incubation cabinet of Fig. 3.3 could stably reach. To increase the effective temperature range, the OT-1 and surrounding incubation cabinet were placed upon a large double-layer of cardboard, and the cabinet doors were duct-taped during experimental runs. This had the added benefit of limiting a possible contamination source.

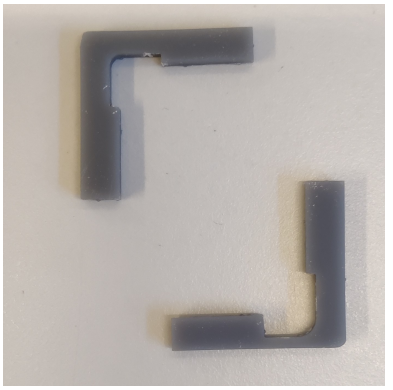
3.9 Automation and 3D printing

The host range expansion protocol was automated using an Opentrons OT-1 pro, an open-source liquid handling robot. The lab protocol was written using the Opentrons OT-1 Python API and Python 3.9.0. However, a significant amount of work was required for the robot to interface properly with the plates and pipette tips used in this protocol.

Although Opentrons is ostensibly open-source, it requires specific pipette tips and boxes that must be ordered from their website. Instead of using these, 3D parts were designed that allowed use of Fisher SureOne Micropoint graduated pipette tips and racks with the OT-1. These 3D parts were designed in FreeCAD and printed using Formlabs Form 2 3D printer with Grey Pro V1 resin. These parts are shown in Fig. 3.4, and the STL files are uploaded to FigShare [83].



(a) Plate mount



(b) Pipette box mount

Figure 3.4: 3D-printed mounts for a) the Corning Costar CellBIND 24-well plates, and b) the Fisher SureOne pipette boxes.

The 3D-printed mounts shown in Fig 3.4 allowed the Corning Costar 24-well plates and Fisher SureOne pipette boxes to sit securely in the deck slots of the OT-1. This was especially important for the pipette boxes since a high degree of accuracy was required for the OT-1 to pick up pipette tips.

The Opentrons OT-1 Python API allows users to easily register new labware for usage in protocols. However, because only the official Opentrons pipette tips and boxes are supported, the API does not accommodate custom pipette boxes. An added difficulty is that the Fisher SureOne pipette boxes and racks are slightly warped and uneven, meaning the pipette tips are not distributed in a perfect grid. The Opentrons calibration software is not good enough to account for these imperfections, and the OT-1 is unable to reliably pick up pipette tips.

The Opentrons Python API user functions do not offer access to the absolute deck coordinates, but only to features defined by the registered labware, i.e. well B3 of plate A2 instead of (x=23.455, y=52.563, z=41.692). Although this is user-friendly, it could be more accurate. The Opentrons calibration app only supports one-point calibration; the user manually maneuvers the robotic pipette-arm to the left lower plate well, and calculates the position of all other plate wells based on

the dimensions of the plate. This calibration scheme proved to be insufficient for interface with 3rd-party pipette boxes. To solve this problem, a custom two-point calibration script was written, where the user maneuvers the robotic pipette arm to both the lower-left well as well as the upper-right well, and calculates the position of all other wells by assumption of a uniform grid. Grid calibration and coordinates were wrapped in a custom container class where relative coordinates (i.e. wells) could be accessed by the same indexing scheme as the base Opentrons Python API.

Two-point calibration relieves dependence upon user-supplied lab-ware dimensions, which were difficult to measure accurately for the somewhat uneven pipette boxes. It also corrected the z-axis coordinates for the slightly sloped OT-1 deck. Two-point calibration significantly improved the robot's performance, and allowed use of the 3rd-party Fisher SureOne pipette boxes. Theo Sanderson's (one-point) OT-1 Terminal calibration script was used for the user interface [84], and modified for two-point calibration. The scripts are published on FigShare [85].

Although the two-point calibration scheme accurately picked up pipette tips, the 3rd-party tips were still problematic. During pickup, the Fisher SureOne pipette tips sometimes stuck together with a neighbor tip, and the OT-1 would lift the target tip along with its neighbor. The neighbour tip would then drop on the way up, often without falling back into its respective well and instead fall to the deck floor or to lie horizontally on top of the remaining tips. This resulted in failed runs where the offending tip was never picked up and even prevented other tips from being picked up. To avoid this, a jiggle function was written that abruptly jolted the OT-1 arm 1 cm back and forth in the four perpendicular directions of the horizontal plane. This jiggle function was run during every pipette tip pickup after the target tip had been raised 15 cm, and ensured any neighbor tips would be knocked off back into their wells before being raised too high.

3.10 Host range expansion protocol

The host range expansion protocol was written using a mix of the custom container classes and the base Opentrons API. The base API and single-point calibration were used for interactions with the 24-well plate, since high accuracy was not needed for the large wells. However, two-point calibration and the custom classes were used for the pipette tip boxes, and custom tip pick-up and drop functions were written for interfacing with these custom classes. These Python scripts are posted on [85]. Here, the host range expansion protocol is split into two parts; setup, and cocktail evolution. In Section 4.1, the entire protocol flow is illustrated and explained.

3.10.1 Protocol setup

The host range expansion protocol setup is as follows. Four sterile 24-well plates were arranged in 2x2 on the Opentrons OT-1 deck in the incubation cabinet, forming the ”evolution plates”. This creates eight columns of 12 wells representing eight ”rounds” of the evolution cycle. Each well in the lower 5 rows of each plate were filled with 2 mL sterile LB medium, while the top rows were kept empty. These 10 wells in each round were called ”strain wells”, and were serially inoculated by their respective ECOR strains and the phage cocktail. One well in each column is the ”pooling well”, where a round’s strain wells were pooled to create the next round of phage cocktail. This accounts for 11 out of the 12 wells in each column; the last well is held empty and does not participate in the protocol.

A fifth 24-well plate was also placed on the OT-1 deck, the ”source plate”. This fifth plate contained 2 mL culture of each of the 10 ECOR strains in respective wells, as well as an 11th well containing 2 mL of the initial phage cocktail.

In addition to the 24-well plates, two boxes of Fisher SureOne micropoint graduated pipette tips were placed on the deck, as well as 4 empty pipette boxes serving as trash cans. The deck layout for the host range expansion protocol including all plates, tip boxes and trash cans is shown in Fig. 3.5.

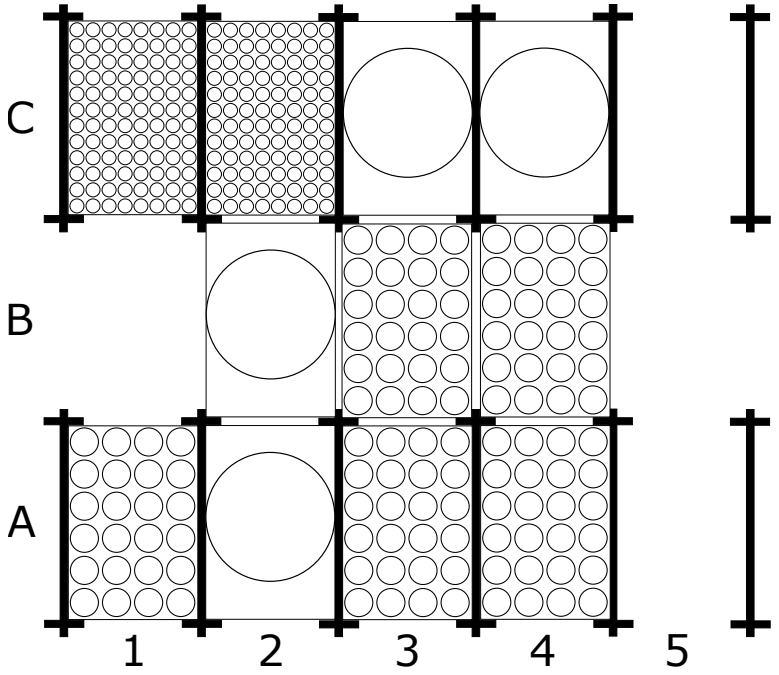


Figure 3.5: Diagram of deck layout for host range expansion protocol. Lab-ware is identified by coordinates. A1: The source plate containing target bacterial strains and initial phage cocktail. A3, A4, B3, B4: Evolution plates where all wells of the lower 5 rows of each plate are filled with LB. C1, C2: 96-tip pipette tip boxes. A2, B2, C3, C4: trash cans where tips are dropped after use.

After everything was arranged according to the setup of Fig. 3.5, the incubation cabinet temperature was set to 37 °C, and the passive humidifier was placed inside to control humidity around 60%. Next, the incubation cabinet was wiped down with a 1% Virkon solution and UV-sterilized using the modified table lamp for 30 minutes. During this time, the lids of the evolution plates were open to allow sterilization, while the lid of the source plate was closed to avoid any potential UV-induced damage. After this time, the UV light was turned off, and the source plate lid was autonomously lifted using the stepper motor to avoid opening the incubation cabinet and compromising sterility.

3.10.2 Protocol evolution

After performing protocol setup, the host range expansion protocol itself was run with all procedures automated by the Opentrons OT-1. The first round of strain wells were inoculated by transferring 100 µL of each ECOR strain from the source

plate to their respective round 1 strain wells. Each well was mixed by aspirating and dispensing a 1 mL volume twice (henceforth referred to as mixed by pipetting). Pipette tips were switched between each transfer. After incubating for 30 minutes, 100 µL phage cocktail was transferred from the source plate to each first-round strain well. Each strain well in the first round was then mixed by pipetting, with pipette tips not switched between wells.

The resulting phage-bacteria co-cultures were incubated for 3 hours, and mixed by pipetting every hour. Meanwhile, 2.5 hours into this incubation, the round 2 strain wells was inoculated from the source plate as before (with switched tips for each transfer as before). After completion of the 3 hour incubation cycle, 200 µL of all round 1 strain wells were combined in the round 1 pooling well, and mixed by pipetting. 100 µL from the round 1 pooling well was then transferred into each round 2 strain well, mixed by pipetting, and incubated for 3 hours with hourly mixing as before. This cycle of incubation and transfer continued for all 8 rounds, taking a bit more than 24 hours.

The cocktail in the final round 8 pooling well was collected, centrifuged at 5000 rpm for 15 minutes, and passed through a 0.2 µm filter. This cocktail could then be stored for several weeks at 4 °C.

Chapter 4

Results

The results of this thesis project are given in several parts. First, the performance of the incubation cabinet and the Opentrons OT-1 robot is described, particularly with respect to the issue of bacterial contamination. Next, the host range expansion protocol posited in this thesis is described and illustrated, followed by a characterization of all ECOR strains with respect to phage susceptibility, phylogeny, and isolation host. In addition, the results of the prophage screen of the 10 targeted ECOR strains are reported. Following this, the results of the host range expansion protocol are reported along with characterization of the final cocktail and host range mutants. The three initial cocktail phages along with two host range mutants are also examined with TEM imaging. Finally, the structure of the phage-bacteria interaction networks are examined.

4.1 Incubation cabinet and automation

Using the two-point calibration and jiggle function outlined in Section 3.9, the OT-1 successfully picked up the Fisher SureOne pipette tips with an accuracy significantly above 99%. This is a major improvement upon base API performance, which commonly failed 5-10 times during a run-through of a 96-tip box. Occasional fails still occurred, but they were rare enough to run the 100+ tip host range expansion protocol unsupervised with only very occasional failed runs.

Contamination proved to be a greater issue. The host range expansion protocol detailed in Section 4.2 entails that some wells contain sterile LB medium for almost over 20 hours before inoculation. Although the incubation cabinet was sterilized

with Virkon and 30 minutes UV-C irradiation prior to each experimental run, some of these late-inoculation wells were sometimes found to be turbid prior to scheduled inoculation, revealing contamination.

Several tests were conducted to isolate the source of contamination. First, plates filled with sterile LB were incubated in the cabinet with no robot action. This incubation resulted in no contamination, ruling out pre-contaminated wells, outside contamination leaking into the cabinet, and contamination source within the cabinet. Secondly, the host range expansion protocol was run as a ”dry run” where the bacterial strains and phage cocktail were replaced with sterile LB. This also did not result in contamination, again indicating there was no contamination source within the incubation cabinet itself.

Next, sterile plates of LB were incubated along with plates of LB inoculated with *E. coli* with no robot action to test whether the presence of nearby bacteria somehow resulted in cross-contamination. After incubation, there was no sign of contamination in the sterile wells, supporting the hypothesis that cross-contamination occurred by robot interaction with wells with bacterial culture.

One possibility was that the pipette tips dripped contamination upon travelling from inoculated wells to trash cans via a path above sterile wells. In response to this, the deck layout was arranged so that used pipette tips never traveled over sterile wells. This is explained in detail in Section 4.2.

However, contamination continued to occur even after this re-arrangement. By process of elimination, contamination was therefore thought to occur by bioaerosols produced upon agitation of inoculated wells by pipette action. This is further discussed in Section 5.2 along with a potential fix to this problem. For this study, contamination continued to be an occasional issue, and several protocol runs were cut short by contamination. In these cases, the run was not completely wasted, as the latest iteration of the cocktail was collected and used as the initial cocktail in subsequent protocol runs.

4.2 Phage cocktail host range expansion protocol

First, all plates, tip boxes, and trash cans were arranged as shown in Fig. 3.5. The trash can setup in particular were layed out so that used pipette tips on their way to trash cans were never routed over wells that had not yet been inoculated. This helped prevent well contamination from droplets hanging on the used pipette tips.

The host range expansion protocol then proceeded as represented schematically in Fig. 4.1, and detailed in Section 3.10.2. Each round of strain wells were succes-

sively inoculated by their respective ECOR strains, incubated for 30 minutes and inoculated with the previous cocktail generation. After 3 hours incubation, each round was pooled to create the next generation of cocktail and the cycle repeated for the next round.

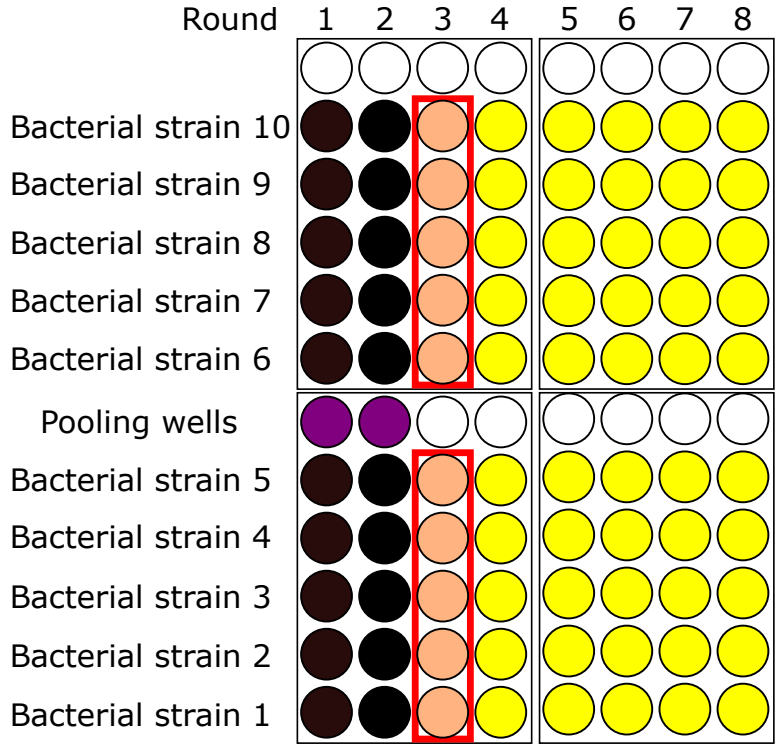


Figure 4.1: Illustration of the evolution plates of the host range expansion protocol directly before the start of round 3 (highlighted in red). All phage-bacteria co-cultures from the round 2 strain wells (colored black) have been combined in the round 2 pooling well (colored purple). All round 3 strain wells have been inoculated with their respective strains (colored peach), and are about to be inoculated with the round 2 pooling well containing the phage cocktail. Round 4-8 strain wells (colored yellow) have not yet been inoculated with anything, and consist of sterile LB medium.

After the cycle illustrated in Fig. 4.1 ran for all eight rounds on the evolution plates, the final cocktail was collected and processed as described in Section 4.2. The entire host range protocol was then run again using the new cocktail as input. In the modified Appelmans protocol described by Burrowes, Molineux, and Fralick [13], the evolutionary cycle is terminated after 30 rounds, representing 30 days of evolution. In this version of Appelmans, the protocol also runs for 30 rounds, but this takes only 5 days to run.

4.3 Determination of phage host range

As described in Section 3.5, all 10 selected phages were spot-tested against all ECOR strains for a total of 720 interactions. Each interaction was ranked as either 0 (no lysis), 0.5 (partial lysis), or 1 (complete lysis). These phage-host interactions were used as a measure of aggregated host susceptibility to the phage collection. This measure, termed *weighted susceptibility*, was defined as the sum of each ECOR strain's 10 phage interactions, with complete lysis weighted as 1, partial lysis weighted as 0.5, and no lysis weighted as 0. Fig. 4.2 shows the weighted susceptibility measure for each ECOR strain.

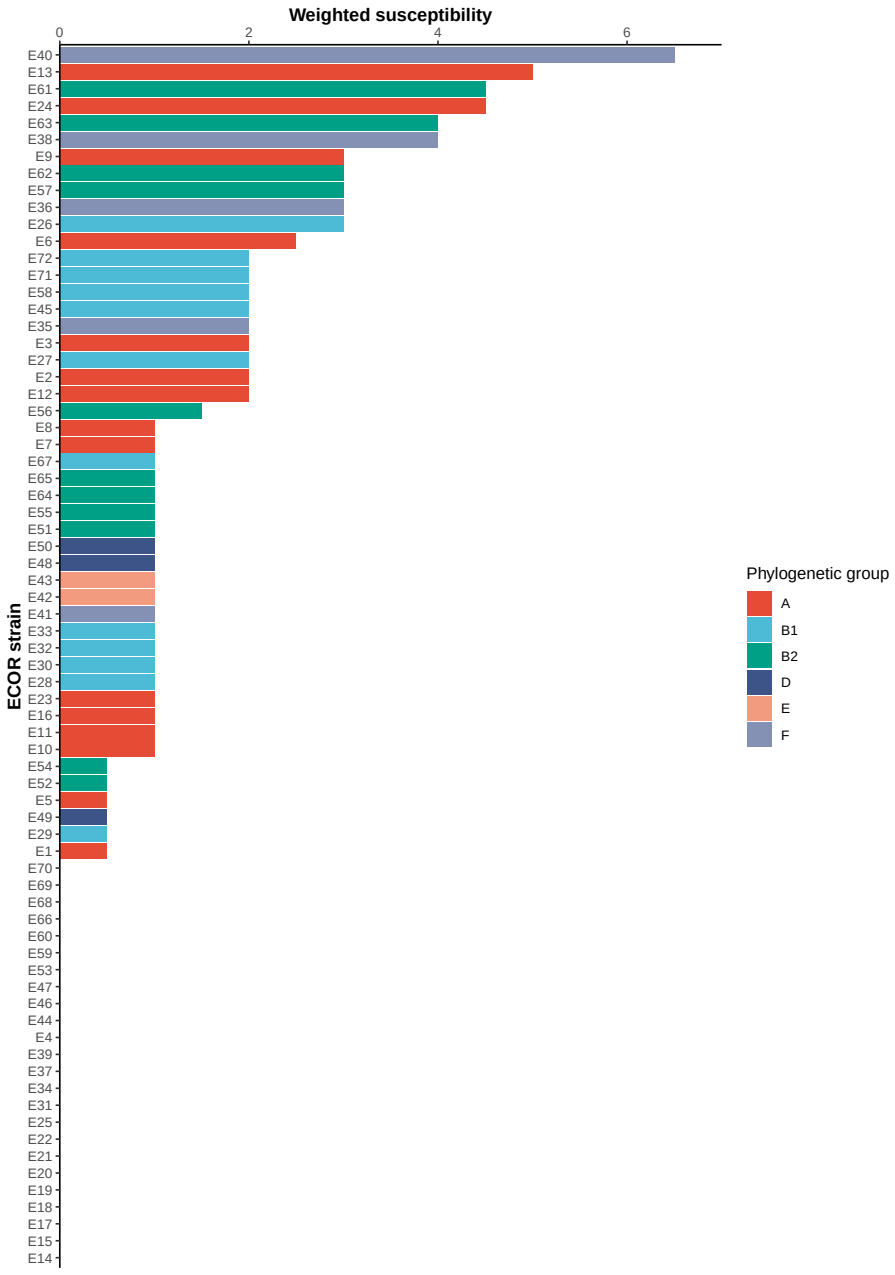


Figure 4.2: Weighted susceptibility for each ECOR strain against the isolated phages, colored according to major phylogenetic grouping. Weighted susceptibility is defined as the sum of all 10 phage interactions for each strain with complete lysis weighted as 1, partial lysis weighted as 0.5, and incomplete lysis weighted as 0. 24 ECOR strains were completely immune to all of the phages tested.

Fig. 4.2 shows that there is significant variation in phage susceptibility between the ECOR strains. Strain E40 has a weighted susceptibility of 6.5, and was at least partly susceptible to 8/10 phages tested, contrasting with the 24 ECOR strains that were completely immune to every phage tested. Fig. 4.2 also shows which of 6 major phylogenetic groups (A, B1, B2, D, E, and F) each susceptible strain lies within.

In order to better compare the weighted susceptibilities of the different phylogenetic groups, Fig. 4.3 shows a boxplot of the weighted susceptibility aggregated by phylogenetic group. Additionally, this figure shows the ECOR aggregated according to what type host they were originally isolated from (human/non-human).

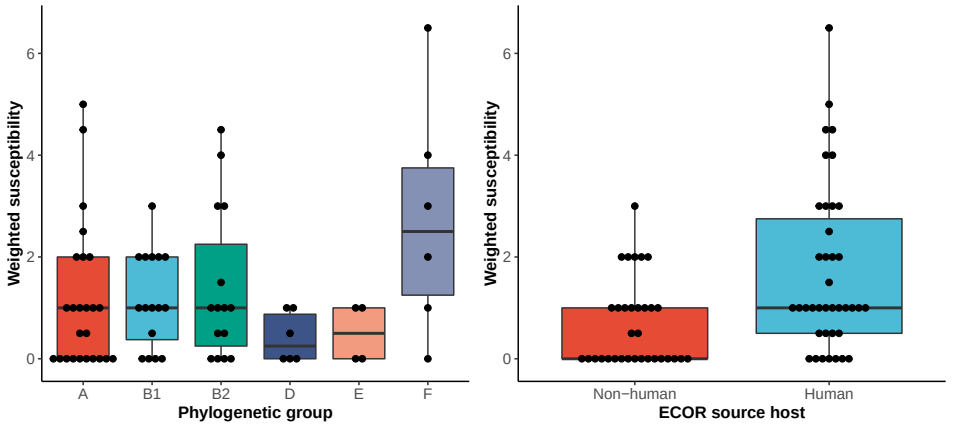


Figure 4.3: Boxplot of ECOR weighted susceptibility aggregated by a): phylogenetic group and b): whether the original ECOR source hosts were human/non-human. Weighted susceptibility is defined as the sum of all 10 phage interactions for each strain with complete lysis weighted as 1, partial lysis weighted as 0.5, and incomplete lysis weighted as 0. Each point represents an ECOR strain. The upper and lower boundaries of the boxes closest to zero indicate the group 75th (Q3) and 25th (Q1) percentile respectively, while the black line within the boxes marks the group median.

The boxplots of Fig. 4.3 show that although there are a significant variations in weighted susceptibility between the ECOR strains, these variations are not well-explained by grouping the strains according to the major *E. coli* phylogenetic groups. To test whether the groups originate from the same distribution, a Kruskal-Wallis one-way analysis of variance test was performed. This test does not assume normality of residuals, and is therefore appropriate for this dataset.

None of the phylogenetic group median weighted susceptibilities differed signif-

icantly under the Kruskal-Wallis test (P = 0.191). However, group medians *did* differ significantly between the ECOR strains isolated from human hosts and those isolated from non-human hosts (P = 0.0017).

4.4 Prophage screening

The 10 ECOR strains of Table 3.2 chosen as hosts for the host range expansion protocol were screened for prophages by searching their genomes for known prophages within the NCBI database and a custom prophage database using the bioinformatics tool Phaster [79]. These strains were also experimentally tested for prophages by spot-testing lysate from each strain against all other strains, as well as against the lab strain DH5α. These results are summarized in Table 4.1.

Table 4.1: Number of hits from Phaster genome search for intact prophages, along with results from lysate spot-test. 10 µL of lysate from overnight cultures of each ECOR strain was spot-tested for active phages upon bacterial lawns of each ECOR strain as well as the lab strain DH5α. The host range of the lysate from each strain is given.

ECOR strain	Number of intact prophages (Phaster)	Lysate host range
E4	2	-
E13	3	DH5α
E17	2	-
E21	1	-
E31	3	-
E40	5	-
E53	4	DH5α
E57	3	-
E64	3	-
E70	2	-

As shown in Table 4.1, a considerable number of intact prophages were found in the host genomes. The Phaster search for prophages revealed that all of the ECOR host strains had intact prophages within their genomes, ranging from E21 (1 intact prophage) to E40 (5 intact prophages). In addition to the 28 intact prophages found by Phaster, the 10 ECOR strains had a combined total of 16 prophage regions ranked as ”questionable” (might be intact and viable), and 32 prophage regions ranked as ”incomplete” (missing key phage genes).

The experimental spot-test for active prophages revealed that lysate from E13 and E53 successfully cleared the DH5α lab-strain (complete lysis). However, no other

lysates had any observed phage activity. Additionally, none of the ECOR strains showed sensitivity to any each others lysates, indicating none of the strains harbor active prophages capable of lysing each other.

4.5 Host range expansion from cocktail evolution

Using the evolutionary protocol detailed in Section 4.2, an initial phage cocktail consisting of phages De8, E10p1, and E42p1 was evolved upon the ECOR host strains of Table 3.2 for 30 iterations. The results of this evolution is shown in Table 4.2, where host range was determined by spot testing as before.

Table 4.2: Host range expansion from cocktail evolution experiment using an initial cocktail consisting of phages De8, E10p1, and E42p1. Host ranges determined by spot testing 10 µL of cocktail upon bacterial lawn. A value of 1 indicates the cocktail round completely lyses the strain, 0.5 indicates partial lysis, and dashes indicate no lysis.

Strain	Round 0	Round 8	Round 15	Round 22	Round 30
E4	-	-	0.5	0.5	1
E13	1	1	1	1	1
E17	-	-	-	-	-
E21	-	-	-	-	-
E31	-	-	-	-	-
E40	1	1	1	1	1
E53	-	-	-	-	-
E57	1	1	1	1	1
E64	-	1	1	1	1
E70	-	-	-	-	-

Occasional contamination of yet-to-be-inoculated strain wells (readily exposed by visible turbidity) sometimes led to early termination of an experimental run. For example, if contamination was observed in a round 8 well, the run was terminated after round 7. This lead to the slightly uneven intervals between the rounds of Table 4.2.

Over 30 rounds, Table 4.2 shows the phage cocktail's host range expanded from 3/10 ECOR strains, to 5/10 ECOR strains. In addition to the original hosts (E13, E40, and E57), the round 30 cocktail can also lyse strains E4 and E64. The cocktail's host range quickly expanded to E64 (within 8 rounds), while the expansion to E4 was more gradual: by round 15 the cocktail could partially lyse E4, and by round 30 the cocktail could completely lyse E4.

A dilution series of the round 30 cocktail was then plated upon E4 and E64 bacterial lawns. Individual plaques were picked from both series, and propagated for several rounds upon their respective hosts in the same manner as in Section 3.4 during isolation of phages from sewage samples. These new phage strains were named E4p1 and E64p1, after the respective host strains they were isolated on. Table 4.3 shows the host ranges of E4p1 and E64p1 upon the same ECOR strains.

Table 4.3: Host range as determined by spot testing of round 30 cocktail isolates E4p1 and E64p1 upon the ECOR strains they were evolved upon. A value of 1 indicates the cocktail round completely lyses the strain and dashes indicate no lysis.

Strain	E4p1	E64p1
E4	1	-
E13	-	-
E17	-	-
E21	-	-
E31	-	-
E40	-	-
E53	-	-
E57	-	-
E64	-	1
E70	-	-

As Table 4.3 shows, both E4p1 and E64p1 are only capable of lysing E4 and E64 respectively, presenting no overlap with the original round 0 cocktail host range. Finally, the host ranges' of the round 30 cocktail, E4p1, and E64p1 was tested on the entire ECOR collection. Significant changes were observed relative to the original phage cocktail's host range, and the round 30 cocktail both lost and gained certain strains from its' host range.

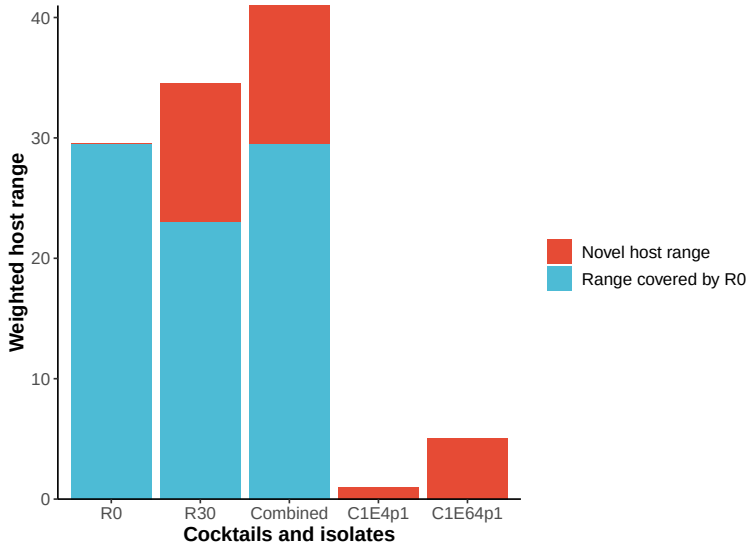


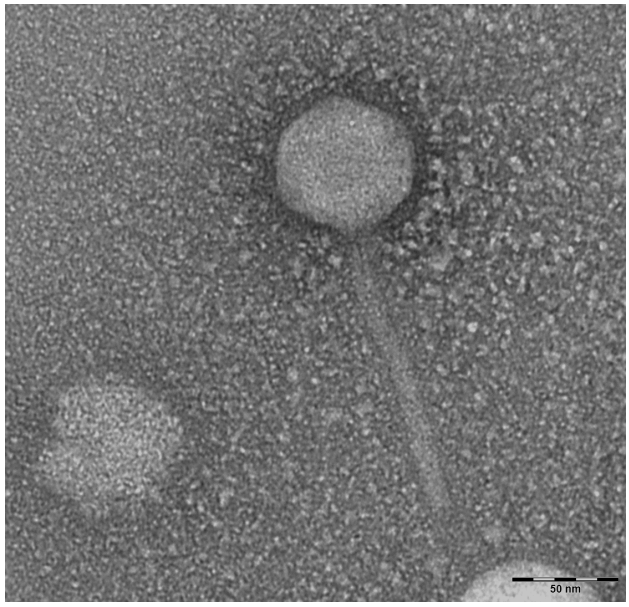
Figure 4.4: Weighted host range of cocktails and isolates. From left to right: round 0 cocktail, round 30 cocktail, round 0 cocktail and round 30 cocktail combined, cocktail isolate C1E4p1, and cocktail isolate C1E4p1. Weighted host range is defined as the sum of all ECOR interactions for each cocktail/isolate, with complete lysis weighted as 1, partial lysis weighted as 0.5, and incomplete lysis weighted as 0. Blue area represents ECOR interactions present in R0, while red represents ECOR interactions in excess of the R0 weighted host range.

Fig. 4.4 shows the weighted host ranges' of the round 0 and round 30 cocktail, a hypothetical combined cocktail of both, and the cocktail isolates C1E4p1 and C1E64p1. Weighted host range is the same measure as the weighted susceptibility of ECOR strains, but aggregated by phage/cocktail. The blue areas of the weighted host ranges' represent interactions present in the R0 cocktail, while the red areas represent interactions that were not present in the R0 cocktail. This shows that R30 has lost a considerable number of host strains from its range (-6.5 weighted host range) but also gained a considerable number (+11.5 weighted host range).

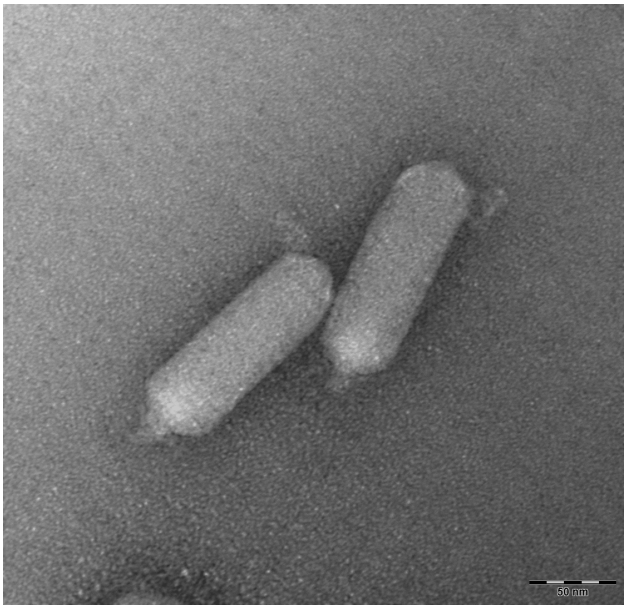
One side-effect of this dual gain and loss is that a combined cocktail of R0+R30 has an increased host range as compared to both R0 and R30, reaching a weighted host range of 41 (out of a maximum 72). It is also noteworthy that the host range of both cocktail isolates, C1E4p1 and C1E64p1, consist solely of strains not present in the host range of R0. Additionally, C1E4p1 is shown to possess the narrowest host range of all phages tested: it only lyses strain E4.

4.6 Electron microscopy

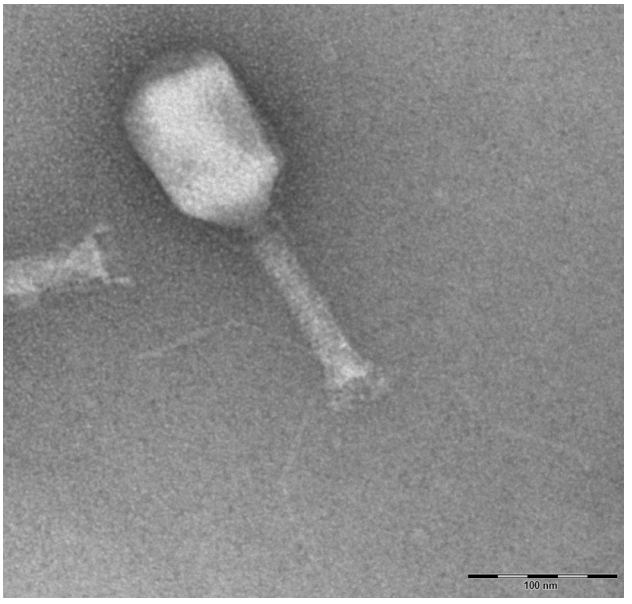
Each of the three phages included in the initial cocktail (De8, E10p1, and E42p1), as well as the cocktail isolates C1E4 and C1E64 were chosen for transmission electron microscopy. The samples were concentrated, purified, and stained with 4% uranyl acetate as described above, and examined with a Tecnai 12 TEM.



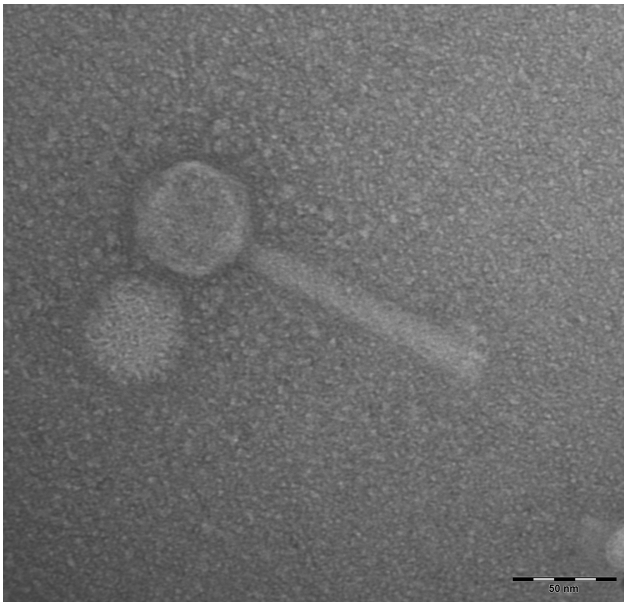
(a) Phage De8. The scale bar is 50 nm.



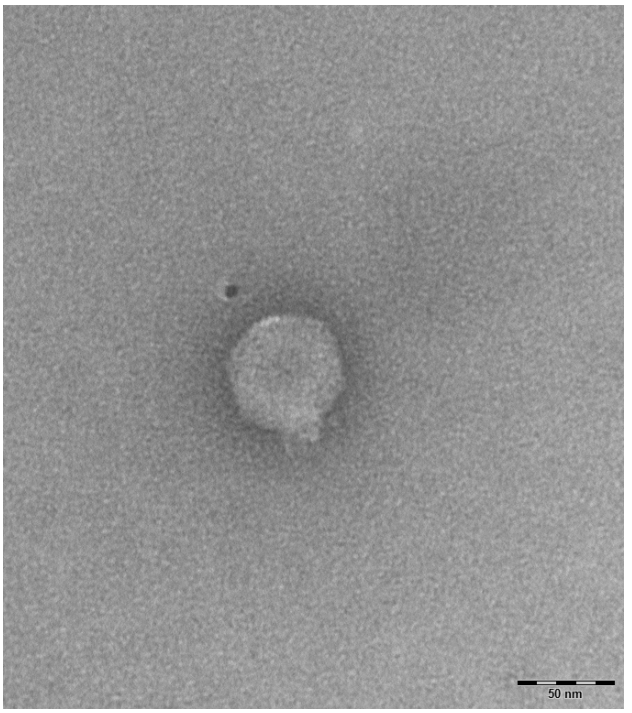
(b) Phage E10p1. The scale bar is 50nm.



(c) Phage E42p1. The scale bar is 100nm.



(d) Phage C1E4p1. The scale bar is 50 nm.



(e) Phage C1E64, The scalebar is 50 nm.

Figure 4.5: TEM images of initial cocktail phages De8, E10p1, and E42p1, and cocktail isolates C1E4p1 and C1E64p1. All images taken between 100,000x - 150,000x.

The phages shown in Fig. 4.5 demonstrate a wide variety of morphologies, with no obvious relationship between the original cocktail phages De8, E10p1, E42p1, and the round 30 cocktail isolates C1E4p1 and C1E42p1. Table 4.4 summarizes the morphological classification of each phage, as well as mean values with standard deviation for head length, head width, tail length, and tail width. All measurements were calculated based on a minimum selection of 10 separate phage virions.

Table 4.4: Morphological classifications and mean measurements with standard deviation of selected phage characteristics. All measurements are given in nanometers, and are calculated from a minimum selection of 10 phage virions.

Phage	De8	E10p1	E42p1	C1E4p1	C1E64p1
Classification	*Siphoviridae*	*Podoviridae*	*Myoviridae*	*Myoviridae*	*Podoviridae*
Head length	62.9±1.6	135.2±1.8	117.9±3.5	51.6±2.1	54.4±1.0
Head width	62.9±1.6	47.8±2.2	89.3±7.2	51.6±2.1	54.4±1.0
Tail length	142.5±3.4	14.7±1.3	115.8±4.6	122.4±5.4	13.6±0.7
Tail width	9.2±0.2	15.3±0.8	26.8±2.1	18.6±1.5	14.0±0.3

All of the five phages examined by TEM are tailed phages (*Caudovirales*), and therefore possess dsDNA genomes. Beyond this however, they are a diverse group and Table 4.4 does not shed any more light on possible relationships between them.

4.7 Phage-bacteria library analysis

Adding the isolates C1E4p1 and C1E64p1 to the ECOR-phage interaction data increases this database to a total of 864 interactions. To examine the structure of this dataset, Fig. 4.6 is a dendrogram-heatmap plot of the weighted susceptibility of each ECOR strain to each phage. The dendrogram was constructed using the Manhattan distance metric between the weighted susceptibilities of each ECOR strain, while the heatmap colors phage-bacteria interactions by their weighted susceptibility ranking.

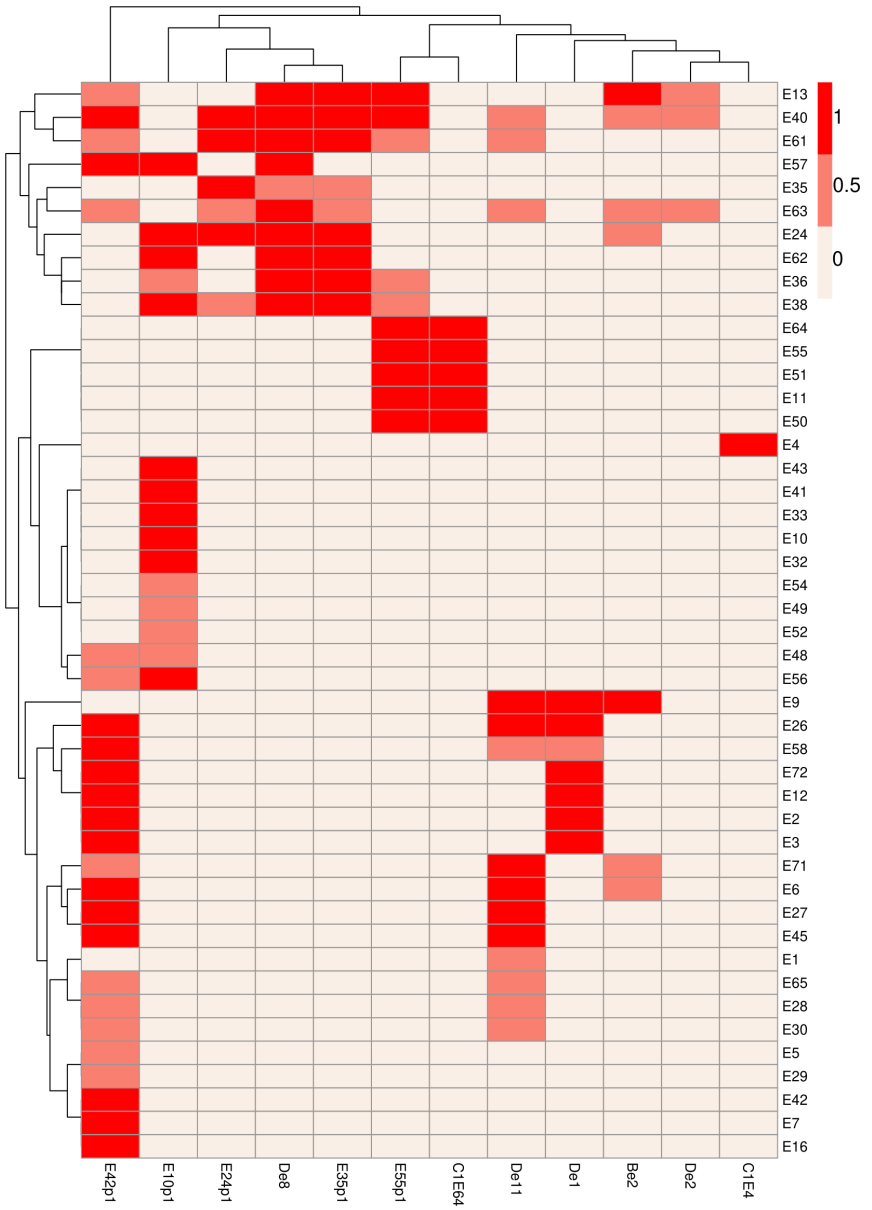


Figure 4.6: Dendrogram-heatmap of ECOR strain susceptibilities to each phage. ECOR strains immune to all phages are not included. The dendrograms use the Manhattan distance metric to hierarchically cluster the ECOR strains and phages by their vector of weighted susceptibilies and weighted host ranges. Susceptibility was determined by spot-testing, with complete lysis weighted 1, partial lysis weighted 0.5, and no lysis weighted 0.

Fig. 4.6 exposes a certain degree of clustering within the ECOR-phage interaction data. In particular, E62, E36, E38, E24, E35, E13, E63, E4, and E61 form a cluster of highly susceptible strains. Some phages also exhibit a hierarchy of host ranges. For example, E24p1's host range nests entirely within E35p1's host range, which again nests entirely within De8's host range.

To further investigate structure within the interaction data, a bipartite network was constructed with edges between phages and their bacterial hosts weighted by the same weighted susceptibility scale used in Fig. 4.6. This bipartite network was generated in Cytoscape [86] using the Edge-weighted Spring-embedded layout. This layout treats nodes as mutually repulsive objects, while edges function as attractive springs according to a force function dependent upon the edge weights. The algorithm then organizes the graph so that the sum of forces in the network is minimized [87]. The position of some overlapping nodes were slightly adjusted manually to increase readability. Each node is also colored according to its identity (phylogenetic group or phage). This network is shown in Fig. 4.7.

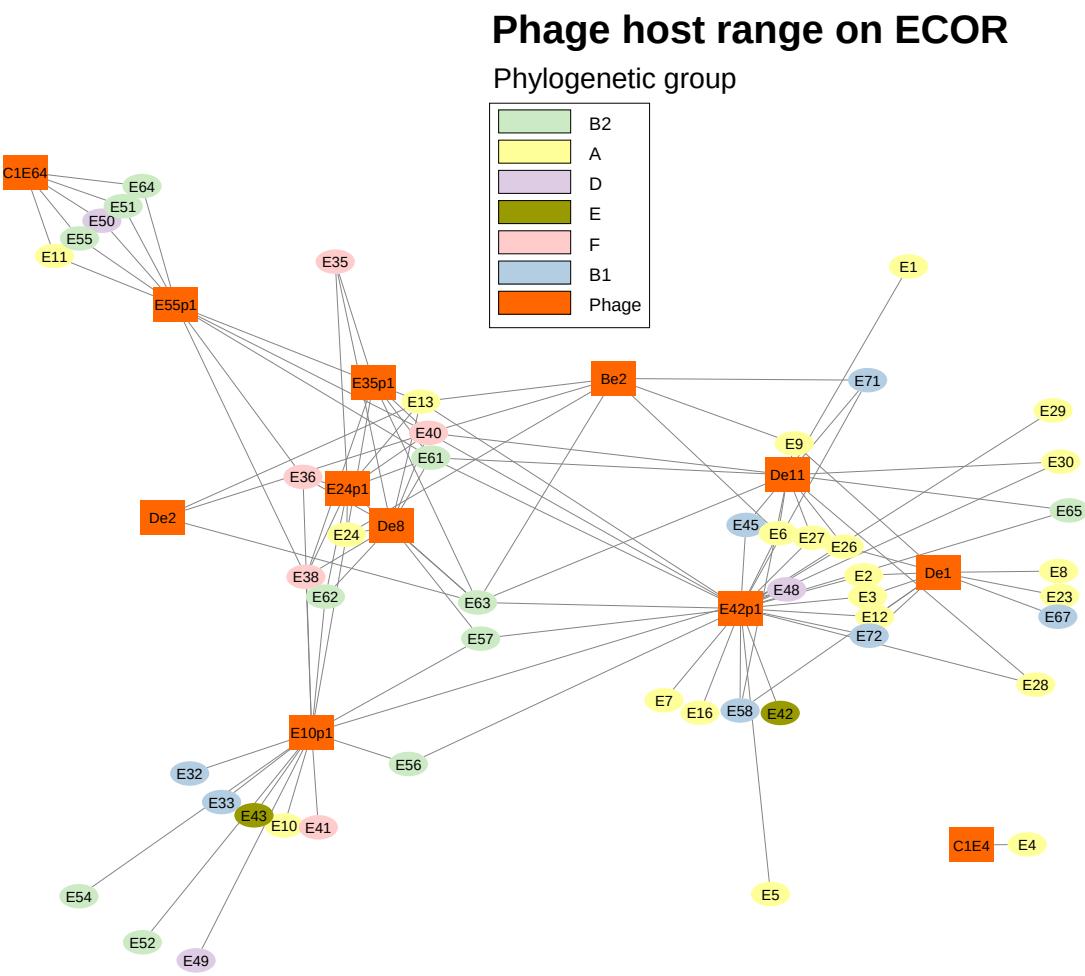


Figure 4.7: Bipartite network representing phage-bacteria interactions. The network was generating in Cytoscape using the Edge-weighted Spring-embedded layout. Oval ECOR nodes are colored according to their phylogenetic group, while phages are represented as rectangular orange blocks.

The spring-embedded bipartite network of Fig. 4.7 roughly splits the phages into two main clusters. The first cluster consists of E42p1, De11, and De1, while De8, E24p1, and E35p1 form the core of a second cluster with E10p1, De2, E55p1, and C1E64p1 loosely connected. Meanwhile, Be2 is equally split between the clusters, and the C1E4p1-E4 pairing is not connected to any other nodes.

ECOR phylogeny does not appear to be randomly distributed across the network. Excluding E4, 16/24 rightmost ECOR nodes belong to group A, compared to only

4/24 of the leftmost ECOR nodes. On the other hand, group B2 is over-represented on the left-hand side of the network (10/24) compared to the right-hand side (1/24).

Chapter 5

Discussion

In this chapter, the results of this thesis are discussed. First, limitations of the host range results along with an analysis of the phage-host interaction network are presented. Next, difficulties in automation using the OT-1 are recounted and a potential solution to the problem of bacterial contamination is proposed. Lastly, the performance of the host range expansion protocol is discussed, particularly with respect to the challenges and opportunities prophages present.

5.1 Phage-bacteria library host range and analysis

One of the commonly-touted advantages of phage therapy is the ease with which bacteriophages are isolated from (un)natural milieus. This was indeed found to be the case for this project. Phages were isolated from sewage against all five of the ECOR strains used as hosts. However, it was easier to isolate phages against some strains than others; phages against E24p1, E35p1, and E55p1 were easily isolated on the first attempt, while multiple sewage samples were required to isolate phages against E10p1 and E42p1.

In this study, phage host ranges were tested by spotting a small volume high-titer phage stock upon bacterial lawns. Although this technique is commonly used due to its high throughput (10 phages can easily be spot-tested on the same plate), spot-testing is by itself insufficient to comprehensively characterize the host range of a phage. Phage stocks may contain bacteriocidal compounds such as colicins that may present a similar spot-lysis pattern to that of phages. This being said, the ECOR collection has been shown to be broadly immune to colicins [81], so this

probably did not impact this study significantly. A better method to test phage-host interactions is by plating individual phages with hosts to test for plaque formation, but this has significantly lower throughput (only 1 phage-bacteria interaction may be tested per plate). However due to time constraints, it was not possible to plaque-test all interaction pairs individually for this project.

As the bar plot of Fig. 4.2 makes clear, there are significant variations in levels of susceptibility between ECOR strains. This pattern of varying immunity to phages is commonly observed, and phages are often considered within the framework of specialists which infect only a few bacterial strains, and generalists capable of infecting a broad range of strains [88, 89]. Statistical studies of phage-bacteria interaction networks have yielded insights beyond this observation. An analysis of 12,000 phage-bacteria interactions identified a significant level of nestedness within the network [90]. In other words, specialist phages infect commonly-infected bacteria, and generalist phages infect both commonly-infected and rarely-infected bacteria.

Although it is more difficult to observe nestedness in the relatively small network shown in the heatmap-dendrogram of Fig. 4.6, certain phages do nest neatly within the host range of others. For example, the host range of E35p1 lies within the broader host range of E24p1 which *again* lies completely within the broader host range of De8. The smallest host range observed belongs to phage De2, and fits completely within the host range of four other phages.

The nestedness of phage-bacteria interaction networks is also supported by the relative difficulty of isolating phages against E10p1 and E42p1. Both strains E10 and E42 are only susceptible to E10p1 and E42p1 respectively; they are, at least within this network, defence specialists. Perhaps not surprisingly, E42p1 and E10p1 also possess the two broadest host ranges of any phages tested. Meanwhile E24p1 and E35p1, isolated on broadly susceptible hosts, have far narrower host ranges. On this basis, the following heuristic for isolating phages with broad host ranges suggests itself; generalist phages are more likely to be isolated from bacterial strains known to be defence specialists than from broadly susceptible bacteria. Although generalist phages also prey upon broadly susceptible bacteria, it is impossible to differentiate between generalists and specialists using the traditional plaque-picking isolation technique.

Phage host ranges may be simultaneously both highly dependent upon phylogeny and completely independent of it. At the species level and above, most phages are certainly highly specific, although several studies have characterized genus-spanning phage host ranges, with one study even (rather dubiously) claiming a phage host range spanning both Gram-negative and Gram-positive bacteria. As a

general rule however, most phages are species- or genus-specific [11, 91].

This phylogeny-dependent picture changes at the intra-species level. At this level of granularity, phage host ranges are often somewhat randomly distributed [92, 93]. Because bacteria are under constant predatory pressure from phages, phage receptors and replication blocks such as LPS, and protective plasmids and prophages are highly variable even within a species [93, 90]. Interestingly, the bipartite network of Fig. 4.7 reveals a non-random distribution of phylogenetic groups within the phage-ECOR interaction network. The phages mostly form two distinct clusters with their bacterial hosts. 16/24 of the ECOR strains in the E42p1-De11-De1 cluster (cluster 1) belong to group A strains, while only 4/24 of the other major cluster (cluster 2) are group A. Meanwhile, 10/24 of cluster 2 strains are group B2, compared to 1/24 of cluster 1 strains. Group D is also over-represented in cluster 2, while groups B1 and E are evenly distributed between the clusters.

Although this is a somewhat surprising result, *E. coli* is an unusually diverse species, and ECOR is a very broad *E. coli* library. Based on the genomes available in the NCBI database, the ECOR core genome (unique genes present in all strains) consists of 1,359 genes, while the pangenome (the combined set of unique genes) is 6,768 genes. The entire *Shigella* genus has also been shown to cluster within the ECOR phylogenetic tree [75]. With this type of intra-species diversity, it is perhaps to be expected that phage host ranges would begin to take on phylogenetic patterns.

More striking is the significant difference in weighted susceptibility between ECOR strains isolated from human hosts compared to those isolated from non-human hosts. Although previous studies have collected phage libraries against ECOR [20, 94, 95], to the best of my knowledge, none have considered the importance of strain host source upon phage susceptibility.

The discrepancy in phage susceptibility between human/non-human ECOR isolates may perhaps be explained by the fact that all phages were isolated from the same source: a Norwegian municipal wastewater treatment plant. There are no giraffes in Trondheim, Norway, and it is perhaps unreasonable to expect phages collected here to efficiently lyse their resident microbiota. Despite the superficial logic, this is an interesting result; with the exception of enteroinvasive *E. coli* strains, the species is not known to exhibit significant host specificity [96]. Phylogenetic studies have shown animal and human isolates to share a common genetic background, and the molecular mechanisms of host specificity are not understood [96, 97]. This result indicates that phages are sensitive yet-unknown *E. coli* host specificity determinants, and perhaps suggests phages themselves might play a role in *E. coli* host specificity.

5.2 Experimental platform

Although the incubation cabinet and OT-1 proved capable of running the cocktail evolution platform, they were not without their problems. Perhaps the greatest issue lay in the OT-1. Even after printing 3D parts to securely lodge the pipette boxes in deck slots, it was very difficult to get the robot to reliably pick up pipette tips. Part of this difficulty was self-imposed; using the Opentrons-produced pipette tips and boxes would likely have increased reliability significantly. However, for a company that touts open-source bona fides in its very name, it is surprising there is not better support for third-party pipette tip solutions. Although it is understandable that performance cannot be guaranteed when using third-party tips, publishing 3D-models for correctly-dimensioned tip boxes that interface with common tips would have been helpful. This walled-garden approach to tips appeared to extend to software; attempting to add custom pipette box definitions in the Python API caused a frustrating variety of difficult-to-source calibration bugs.

Calibrating the OT-1 was often a rather painful process. By default, calibration is performed using the OT app. This app is a graphical user interface that allows manual control of the robotic arm. In theory this would be great, but the app appeared to contribute to calibration bugs such as huge offsets being added to each stored position. To avoid these issues, I used Theo Sanderson's excellent command-line calibration script published on GitHub. However, even correctly-recorded calibration values were not accurate enough for reliable function, and additional features were necessary. Implementing two-point calibration greatly increased performance, and it is difficult to understand the Opentrons decision not to include this feature by default. Upon adding the "jiggle function", the OT-1 performance was reliable enough to run 24-hour protocols with 100+ tip pickups, although occasionally runs were still ruined by a failed tip pickup.

Preventing well contamination in the incubation cabinet also proved difficult. The cocktail evolution protocol calls for some wells filled with sterile LB to sit exposed for almost 24 hours, and contamination was relatively frequent, necessitating early termination of cocktail evolution. To investigate the source of this contamination, several experiments were performed. Firstly, the Opentrons deck layout was designed so that the robot pipette never passed over sterile wells on its way between pipette boxes, wells, and trash cans. This ensured that contamination did not occur by drip from pipette tips. Secondly, the entire evolution protocol was tested by a run where sterile LB was placed in the source plate wells instead of bacterial cultures. During this run, no contamination was observed. This indicated that contamination did not originate in inadequate sterilization prior to the protocol run, and also excluded possible contamination from outside the incubation cabinet.

Perhaps surprisingly, this suggests that contamination occurred through aerosolization of the bacterial cultures upon agitation by pipette action. Although enteric bacteria such as *E. coli* are not generally well-known for airborne transmission, studies have shown *E. coli* to be perfectly capable of aerosolized transmission, particularly at higher temperatures and humidities such as those present in the incubation cabinet [98, 99]. Such bioaerosols have also been shown to be generated by turbulence-induced bubbles bursting at the liquid-air interface [100]. This may very well have occurred during liquid transfer and pipette mixing, resulting in well contamination. This hypothesis could be tested by incubating a plate with sterile LB wells next to a plate inoculated with bacteria periodically agitated by pipette action. If contamination of the sterile wells is observed, contamination may be assumed to occur by bioaerosol.

A possible solution to this problem might be constant UV radiation. For this study, any potential UV-induced impact upon the bacterial strains was avoided by keeping the source plate lid closed for the UV-C-cabinet sterilization prior to protocol initiation. However, this is probably not strictly necessary. UV-C does not penetrate liquid suspensions of organic molecules well [101], and bacterial growth did not appear to be impacted by running the protocol with constant UV-irradiation. However, it did appear to significantly decrease well contamination, in agreement with a previous study that showed UV radiation to greatly reduce *E. coli* bioaerosol survival rates [102].

5.3 Cocktail host range evolution

In the protocol of Burrowes *et. al*, each row of a 96-well plate is inoculated with a different bacterial strain. Next, a serial dilution of the previous cocktail iteration is added to the wells of each row. After incubation, the wells of each row are examined for bacterial growth. All serial dilutions that cleared bacterial growth along with the first serial dilution that did not clear bacterial growth are combined. The next cocktail iteration is assembled by combining these dilutions from each row, followed by centrifugation and filtration to remove bacteria.

There are several differences between the automated cocktail evolution protocol proposed in this study and that of Burrowes, Molineux, and Fralick [13]. Firstly, the latter protocol can be thought to employ a ”weight function” during the combining of serial dilutions. More weight is given to the phages of hosts that are lysed at lower cocktail dilutions, presumably providing evolutionary pressure for more efficient phages. Secondly, each cocktail iteration is purified of bacteria. This is potentially important to prevent bacterial escape mutants from hitchhiking to the next generation of evolution, and becoming dominant. Unfortunately, neither of

these features are easily automated.

The automated evolution protocol solves the problem of bacterial evolution by allowing half an hour of bacterial incubation prior to addition of the cocktail. Even though the cocktail may include bacterial escape mutants, the target strain should overwhelm any escape mutants by pure numbers, facilitating phage evolution. Meanwhile, the weight function is simply removed. Even without this feature, phage mutants that more efficiently lyse a host will be present in greater numbers, and therefore selected for in each cocktail iteration.

With these changes, it was possible to design an automated cocktail evolution protocol. In addition to the decreased workload inherent to automation, this protocol is almost significantly faster than the 30-day protocol reported by Burrowes *et. al*. Eight iterations can be performed in 24 hours, while a complete 30-iteration run takes roughly fived days, although with prep work and sterilization, 6 days is more realistic. This is an important breakthrough; the commercial cocktails designed by the Eliava Institute, as well as most research on phage cocktails, has focused on developing ready-to-use, broad-spectrum cocktails that can be given to patients without isolating the pathogen. This has several advantages; these cocktails are easy to use, and can be commercially licensed. However, in a situation where a patient presents a novel pathogenic strain immune to an existing phage library, this protocol could potentially allow for a viable cocktail to generated in time to treat the patient.

The actual cocktail evolution experiment reported here was a moderate success. The initial cocktail was effective against 3/10 target strains, while the final round 30 cocktail was effective against 5/10 target strains. Additionally, the round 30 cocktail off-target host range was significantly altered from the initial cocktail's; round 30 cocktail gained complete or partial sensitivity to 14 ECOR strains not within the initial cocktail's host range. The round 30 cocktail also lost complete or partial sensitivity to 8 ECOR strains. The combined initial + round 30 cocktail shows complete or partial sensitivity to 44% more ECOR strains than the initial cocktail alone, with a 39% increase in weighted host range as shown in Fig. 4.4.

In the host range expansion protocol detailed by Burrowes, Molineux, and Fralick, an initial three-phage cocktail was evolved upon 10 *Pseudomonas aeruginosa* strains; three lab strains, and seven clinical isolates. Two of the lab strains and none of the clinical isolates were initially susceptible to the cocktail. After 30 rounds of the protocol, all strains were lysed by the final cocktail. When tested against different *P. aeruginosa* strains, the final cocktail exhibited no host range expansion relative to the initial cocktail [13].

In a different study by Mapes *et. al* [12], the same host range expansion protocol

was used to develop an initial 4-phage cocktail against 14 *P. aeruginosa* clinical isolates and 2 lab strains. Here, the initial cocktail lysed 6/16 strains, while the final round-30 cocktail lysed 12/16 strains. When tested against 10 different *P. aeruginosa* strains, the final cocktail lysed 10/10 strains compared to the initial 2/10 strains. Combined with the results of this study, this provides a rather confused picture. I believe this is primarily due to two factors; the initial phage cocktail, and the choice of bacterial developmental strains.

Phage recombination, both homologous and non-homologous [13, 30], is thought to occur to such an extent that the concept of phage species is occasionally thrown into question [103]. However, as is pointed out by Burrowes, Molineux, and Fralick, these evolutionary protocols often occur across around 100 phage generations, which probably is not enough time for significant non-homologous recombination. This leaves homologous recombination left as the important source of phage evolution, indicating the initial phage cocktail should include closely related phages. Two of the initial cocktail phages chosen by Burrowes, Molineux, and Fralick were extremely closely related, with $>$99% DNA sequence identity. Fascinatingly, the phage isolated from their final cocktail with greatest host range (10/10 target strains), was shown to be a mosaic of these two parental phages, constructed from at least 48 crossover events. In contrast, the more modest host range expansion reported by Mapes *et. al* utilized phage cocktails consisting of phages later shown to have little homology, and the final cocktail isolates were shown to be clonal descendants of the original phages, having accumulated only point mutations. Similarly, the initial phages in this study exhibited very diverse morphologies and therefore likely little DNA homology. This is likely an important contributor to the relatively modest host range expansion.

The bacterial developmental strains also clearly determine the host range expansion. In this study, the cocktail was developed upon diverse strains picked from a sequenced collection known to span the breadth of *E. coli*, and tested upon the entire collection. Meanwhile, the two previous studies [13, 12] did not use well-characterized developmental strains, and beyond antibiotic sensitivity data [12], it is difficult to say much about diversity within their selections. As shown in Fig. 4.7, a certain degree of phage host range modularity may be expected even within a single species, implying a genetically-diverse collection of species will be more difficult for a cocktail to lyse. For this reason, I suggest host range expansion studies should sequence their bacterial strains to preclude inclusion of prohibitively similar strains.

In both previous studies, cocktail isolates were sequenced along with the initial phages to determine their ancestry. The same is being done for the phages of this study, but the sequencing data is not in yet. However, the TEM images revealed

that neither phages isolated from the final cocktail bear any particular resemblance to the initial phages. It is likely that these isolates are, partially or completely, descended from prophages resident within the bacterial genomes. As shown in Fig. 4.1, a Phaster genome search revealed that all 10 of the ECOR developmental strains used in this study had numerous intact prophages, with a total of 76 prophage sequences, intact or otherwise. Isolate C1E4p1 also bears a resemblance the widespread P2-family of temperate phages [104], which is present as a prophage in several of the ECOR developmental strains. It is more difficult to compare the morphology of C1E64p1, but the ECOR genomes possessed multiple P22-family [105] prophages that may be the ancestral source.

This menagerie of resident viruses is not the exception, but the rule; prophage sequences have been shown to make up more than 13% of the *E. coli* O157:H7 genome [106], while a survey of the *P. aeruginosa* pangenome found 8% of genes to be prophage genes. It seems inevitable this tremendous diversity could potentially release mutant or recombinant phages capable of lytically lysing target strains. Seen through this lens, it is somewhat surprising that previous studies have not observed a prophage contribution to their host range expansion; it seems inevitable that at least portions of a cocktail evolved upon wild-type bacteria will consist of prophage elements.

There is currently widespread skepticism regarding the use of temperate phages in therapeutic contexts [107, 13, 57]. This is for good reason; temperate phages are well-known contributors to bacterial virulence [107] which is obviously undesirable in a therapeutic context. However, it seems negligent to ignore the deep well of viral diversity lying within the bacterial genome, especially when temperate phages may be mutated to act in a lytic manner as presumably occurred in this study. A more prudent strategy might be to allow temperate-descended phages in cocktails, but to screen for virulence factors prior to therapeutic treatment. In any case, it is impossible to ignore lysogeny completely; even if one were to employ an obligately lytic cocktail, there is no guarantee the phages would not recombine with prophages present *in vivo*. Perhaps a phage cocktail may be thought of not as a collection of individual virions, but as the sum of pangenomic information within them: a blueprint containing genes capable of targeting bacterial strains and reshaping their surrounding microbial ecology.

Chapter 6

Conclusion

The aim of this project was threefold: to isolate and characterize phages against a cross-section of the ECOR library, to build an automated platform for phage cocktail host range expansion, and to characterize the final phage cocktail. All of these aims were accomplished.

Appelmans protocol is a protocol used to expand the host range of a phage cocktail against a collection of targeted bacterial strains. This protocol has been used in various forms for almost a century, particularly in the Republic of Georgia. Based on the version of Appelmans protocol detailed by Burrowes, Molineux, and Fralick [13], I designed and implemented an automated version of Appelmans protocol for cocktail host range expansion that both runs significantly faster and requires less labor.

The protocol was automated using an Opentrons OT-1 pipette robot placed within an incubation cabinet constructed from a polystyrene box with temperature and humidity control, and UV sterilization. This incubation cabinet may be useful for a variety of automated incubation protocols. The Opentrons calibration was also improved using a two-point calibration scheme, and 3D-printed parts were used to secure plates and pipette boxes in the deck slots. All the code and designs are posted on FigShare.

Phages against the *E. coli* ECOR strains E10, E24, E35, E42, and E55 were isolated from municipal sewage and spot-tested for host range against the entire ECOR library along with five previously isolated *E. coli* phages. Aggregating ECOR strains phage susceptibility by phylogenetic group demonstrated no significant differences in susceptibility between groups. However, aggregating the

ECOR strains by their original host (human/not human) revealed that strains isolated from human hosts were significantly more susceptible to the phage collection than were strains isolated from non-human hosts. A basic network analysis also revealed that the phage host ranges roughly separated into two clusters, and that several phage host ranges nested within that of other phages.

The automated version of Appelmans protocol was tested with an initial cocktail of phages De8, E10p1, and E42p1 upon a collection of 10 ECOR strains. After 30 rounds, the final cocktail host range had expanded fro 3/10 to 5/10 target ECOR strains, along with a total 44% increase in host range upon the entire ECOR library. Along with the initial phages, two isolates from the cocktail with novel host range were examined with transmission electron microscopy. Based on morphology and the presence of plentiful prophages within the ECOR strains' genomes, it was deemed likely that the cocktail isolates were (at least in part) descended from prophages resident within the targeted ECOR strains.

Although the cocktail host range developed in this study did not expand upon target strains to the extent that has been observed in other studies, it did observe a greatly increased host range upon non-targeted ECOR strains. This is thought to be the result of two factors. The initial phage cocktail contained highly diverse phages, making recombination unlikely and reducing the evolutionary potential of the phages. On the other hand, the targeted ECOR strains harbor plentiful prophages which may either be directly activated or evolved to act lytically and increase the cocktail host range.

The results of this study outline both a warning and an opportunity. Not only do prophages often provide their bacterial hosts with immunity to related phages, but they may also carry virulence genes which increase host pathogenicity. Hence, great care must be taken to ensure phage cocktail developed using Appelmans protocol do not contain phages which may worsen a bacterial infection. However, phages' propensity for recombination combined with the sheer diversity of prophages represents a tremendous well of genetic potential that could be harnessed to treat bacterial pathogens. Here, Appelmans protocol is shown capable of harnessing this potential to a certain extent.

Clearly there is a need for further research on phage cocktail host range expansion. Very little published research exists on the subject, particularly with respect to the effect of safety and prophages. However given the ubiquity of prophages in wild-type bacteria, the subject is unavoidable. It would also be interesting to further study the role recombination plays in expanding host range, particularly with regards to genetic exchange of prophage genes.

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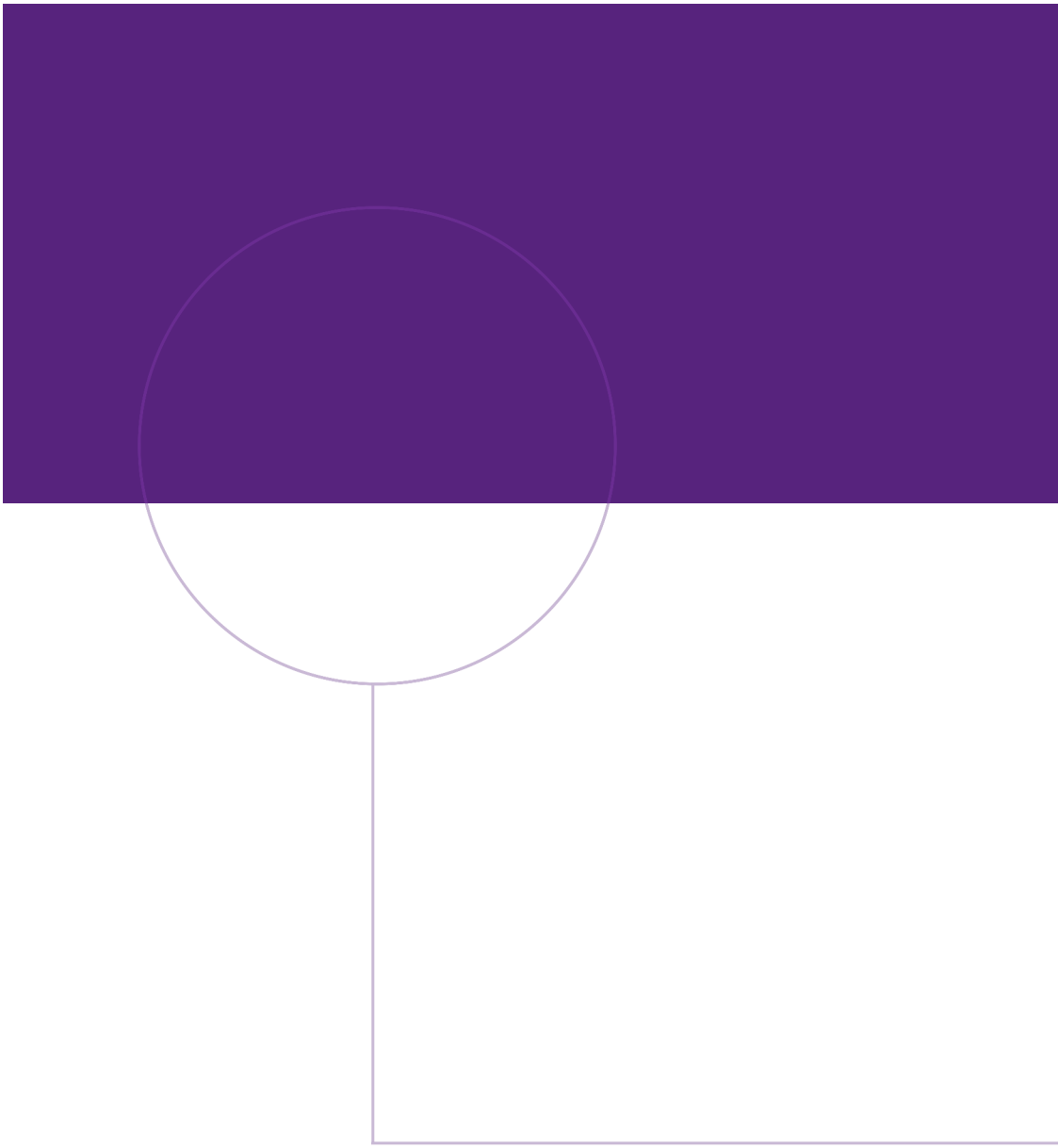

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