THE ISOLATION OF *STREPTOMYCES* BACTERIOPHAGE HOST RANGE MUTANTS

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Abstract

Bacteriophages are viruses that infect bacteria. They are ubiquitous in nature; found in every environmental niche where there are bacteria. Generally, phages infect a narrow range of hosts due to the specificity of their receptor binding proteins for substrates on the bacterial cell surface. Specificity is also determined by the defense mechanisms of the host and the phage's ability to bypass them, as well as compatibility with the host replication machinery. The mechanisms behind phage-host specificity are poorly characterized, especially for the individual virus. It has been shown that mutations gained through natural evolution to the receptor binding proteins results in an expanded host range, compared to their wild-type counterparts. *Streptomyces* is the most extensively studied genus of mycelial *Actinobacteria*. These bacteria are filamentous, spore forming, and produce a chemically diverse variety of secondary metabolites. There are few phages found to infect this genus, with a bias towards those infecting *S. griseus*. The relationship between phages and *Streptomyces* is influenced by the unique characteristics of this genus. The various morphologies of and metabolites released by this host have been shown to combat phage infection. To expand the reservoir of *Streptomyces* phages, 50 novel phages were isolated on either *S. avermitilis, S. coelicolor,* or *S. venezuelae* using direct isolations. Five phages, EnochSoames, Celery, Superstar, Rideau and Wilkos were sequenced and annotated, shown to belong to the BD1, BD2, BD3, BF, and BN clusters, respectively. Sequencing revealed high homology amongst the BD cluster phages, and none with the other clusters. The host range of 24 phages were tested across a variety of *Streptomyces* species. One phage, EnochSoames, demonstrates a broad host range, infecting all three species tested. All other phages demonstrate a narrow host range and/or a reduction in infection on alternative hosts. This reduction was characterized through isolation of phage Rosita host range mutants, which are representative of individual phages which had mutated

during amplification on the native host. Sequencing efforts of Rosita revealed that this phage may have DNA modifications. Overall, this data suggests phages can naturally mutate to infect a broader range of hosts and remain viable. In addition, *Streptomyces* phages are prevalent in nature and genetically diverse.

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Chapter 3 Results

3.1 Establish and optimize phage protocols using the host *Streptomyces*

List of Abbreviations

A
Acr – anti-CRISPR
AH – alternative host
ATP – adenosine triphosphate
attB – bacterial attachment
attP – phage attachment

B
Bp – base pair

C
CFU – colony forming units
CRISPR-Cas – clustered regularly interspaced palindromic repeats-CRISPR-associated regions
crRNA – CRISPR RNA

D
DNA – deoxyribonucleic acid
DNB – Difco Nutrient Broth

E
EDTA – ethylenediamine tetraacetic acid
EM – extracellular matrix
EOP – efficiency of plating

H
HRM – host range mutant

I
IP – immunoprecipitation

K
Kbp – kilo-base pair

L
LPS – lipopolysaccharide

M
MOI – multiplicity of infection
MYM – Maltose-Yeast-Malt

N
NCBI – National Center for Biotechnology Information
NH – native host

O
OCR - overcome classical restriction

P
PEG – polyethylene glycol
Pfu – plaque forming unit
pgl – phage growth limitation

R
RBP – receptor binding protein
RM – restriction modification
RNA – ribonucleic acid

S
SDS – sodium dodecyl sulfate

T
TEM – transmission electron microscopy

W
WT – wild-type

Chapter 1

Introduction

1.1 General information

Bacteriophages, commonly referred to as phages, are viruses that exclusively infect bacteria. With a global population of 10^{31}, phages are the most abundant biological entity on the planet, capable of killing 15-40% of the ocean's bacteria everyday (1, 2). Phages are ubiquitous in nature and have been found in every explorable environment on Earth, ranging from forests, oceans, deserts, swamps, and the human gastrointestinal tract (1, 3). The relationship between virus and host involves the exertion of major evolutionary pressures, with both partners attempting to thwart the others' mechanisms of or against infection (4–7). Phages have been found to influence a variety of biological systems through lysogeny and bacterial cell lysis, these including modulating the gut microbiome and biogeochemical cycling of carbon (2, 3).

1.2 Phage Genomics

Phages contain genetic material in the form of either RNA or DNA which may be single- or double-stranded (ds) (1). As of 2024, the NCBI Nucleotide database reports 32303 phage genomes with a sequence length greater than 5kbp (8). This number is more than double the 13132 entries reported in 2020 (9). However, discrepancies and errors found within the 2020 database reduced the number to 8245 unique complete phage genome entries, accounting for elimination of a quarter of provided sequences. Following this trend, the number of complete phage sequences available in 2024 is likely closer to 20000.

The size of bacteriophage genomes varies widely; of the sequences currently available, phages gravitate towards three major size distributions (9). On the smaller end of the spectrum (<25kbp), phages are found between 10 and 15kbp. In the middle (25≤100kbp), the most populated category, phages are found between 40 and 45kbp. And finally, at the large end of the spectrum (>100kbp), phages are found between 150 and 175kbp. It should be noted that there are some megaphages reaching 500kbp and beyond, and others barely 5kbp, demonstrating the breadth of phage diversity (10, 11). Phage genome structure is organized into functional clusters that are generally conserved. These clusters include infection, virion structure, DNA replication, bacterial cell lysis, and lysogen formation; each of these clusters can have distinct evolutionary histories (12–14). Additional genes commonly responsible for defense mechanisms or non-essential functions that can support infection or survival in niche environments are typically located at the end of the genome and can vary widely between phages (12). Active participation in horizontal gene transfer with other phages and bacteria explains the mosaicism of bacteriophage genomes and the unmatched diversity of the entire population. Coinfection with other phages may result in recombination events, highlighting that phages with similar host ranges and those within ecological proximity are more likely to share a common gene pool. During infection, the bacterial chromosome is degraded and may undergo viral uptake which would explain how phage genomes frequently contain homologs of bacterial genes (15, 16). Phages are grouped into clusters and further subclusters based on nucleotide similarity. Members of a cluster commonly share high nucleotide and amino acid sequence homology, which translates into sharing protein functions (17).

1.3 Phage Structure / Genes

Phages have a variety of morphologies. Their capsids, where the genetic material is stored and whose size is dependent on the length of the genome, may be pleomorphic, polyhedral, filamentous, or connected to a tail (1). As mentioned previously, NCBI reported ~8000 complete phage genomes sequences in 2020. Of those, 93.6% are tailed and encapsidate dsDNA, classifying them as *Caudovirales* that contains the morphotypes *Siphoviridae, Myoviridae*, *Podoviridae*, *Ackermannviridae* and *Herelleviridae.* Within this group, more than half are *Siphoviridae* (9). *Siphoviridae* are characterized by an icosahedral capsid and a long non-contractile tail; *Myoviridae* are characterized by an icosahedral capsid and contractile tail; *Podoviridae* are characterized by an icosahedral capsid and a short, non-contractile tail; *Ackermannviridae* encode multiple tail-spike proteins and infect gram negative bacteria; *Herelleviridae* specifically infect the genus *Firmicutes* (1, 18, 19).

Phages have specific structural components that make up the viral particle depending on their morphology. For *Caudovirales*, there is high homology among structural genes. The major capsid protein, portal protein, endolysins, and the terminase are conserved proteins among all tailed phages (20). These proteins are essential to phage structure and infection. For example, the portal protein is part of a collection of proteins that connect the capsid to the tail. It is essential for proper capsid formation, DNA packaging and ejection, and is structurally and biochemically conserved amongst *Siphoviridae* (21). Additional protein functions include the major and minor tail proteins, tape measure protein, tail tube protein, tail assembly chaperone, immunity repressor, and scaffolding protein which may or may not be found within the genome depending on the phage and its morphology.

The minor tail proteins can be further classified as tail spike and tail fiber proteins (collectively referred to as receptor binding proteins (RBPs)). They are involved in host recognition through structures on the cell surface, which mediate adsorption. The structure(s) a phage recognizes likely evolved based on how abundant it is, whether it is essential to the host, such as LPS on gram negative bacteria and teichoic acid on gram positive bacteria, and if that structure is conserved across bacterial genotypes (22). The host range of a phage is defined as the "taxonomic diversity of the hosts it can successfully infect" (23). If the RBP binds to a common structure found amongst a wide variety of bacteria, the phage is predicted to have a broad host range and vice versa.

RBPs have been shown to be exchanged between phages and to undergo reversible mutations, demonstrating the ability for a phage to expand and/or alter its host range (23–25). Several studies demonstrated host range expansion through natural evolution as the phages acquired random mutations. Interestingly, each study found that the mutations occurred in the tail spike, tail fiber, and/or baseplate, indicating how critical these proteins are for dictating host range (26–29). Phages may encode for multiple RBPs that can recognize different bacterial structures within the same and/or different species or possess genetic strategies to differentially express RBPs depending on the environment, known as phase variation (30). These structural proteins are encoded by genes that are typically found earlier in the genome as part of the conserved functional clusters (12). The specificity of RBPs for their receptor means identification through homology-based methods is not effective within novel phage genomes. Thus, their biology remains elusive (25).

1.4 Phage Life Cycles

A phage has two life cycles. The lytic cycle is characterized by lysis of the bacterial cell at the end of infection. Phage adsorption is initiated through recognition of specific structures on the bacterial surface by the receptor binding proteins (31). These can be any major or minor structure such as peptides, fimbriae, carbohydrates, lipopolysaccharides, or larger protruding structures like pili or flagella. Generally, infection begins with a reversible binding of the phage to the cell; this interaction is stochastic, and similar to the way an enzyme interacts with its substrate. Phage adsorption is influenced by the concentration of phage (ie. enzyme) and bacteria (ie. substrate) that are present as well as the affinity of the receptor for the structure. Once a phage has adsorbed to the cell surface, irreversible binding to the same or secondary receptor may occur that mediates injection of the phage DNA through the portal protein into the cell cytoplasm (32). Some phages require the presence of specific molecules, such as calcium or magnesium ions, to facilitate binding (33).

Once the linear phage DNA has been injected into the bacterial cell, it will circularize to evade degradation by host nucleases (6, 7, 34). The phage DNA will either hijack the host machinery or transcribe its own RNA polymerase to begin transcription (35). Transcription of phage genes is regulated through promoters controlling operons that may be temporally repressed and/or activated. Structural proteins are translated using host translation machinery in conjunction with replication of the phage genome using phage-encoded DNA polymerases (36, 37). The phage must have a certain level of compatibility with the host machinery to transcribe its genome and be maintained within the cytoplasm. In some cases, phages can encode additional machinery to improve this compatibility. For example, phages with large genomes often encode additional

tRNAs that increase translation of rare codons and increases burst size to improve overall fitness (38).

As the components of the phage particle accumulate in the host, they assemble into new virions with the phage DNA being packaged into the capsid. Concurrently, viral gene transcription and translation continues to produce proteins that will mediate the release of these newly formed viruses from the cell (37). Holins form pores in the bacterial membrane to provide endolysins with access to the cell wall, resulting in lysis of the cell (23). If these enzymes are blocked by or not able to degrade the cell, the phage progeny will remain trapped, highlighting another determinant of host range. The infection of a bacterial cell by one phage can result in the release of anywhere from 50 to 200 phages, referred to as a phage's burst size (37).

Unlike the lytic cycle, the lysogenic cycle does not produce new viral particles. Once inside the cell, the *attP* site within the phage genome aligns with an *attb* site within the bacterial chromosome and recombines into the genome, forming a prophage with the help of a phage encoded serine or tyrosine integrase. This integration is accomplished through site-specific recombination or through insert-directed integration using the host recombination machinery (39). Most phages will utilize this integrase system to form a prophage. Alternatively, some phages encode a *parABS* partitioning system for phage DNA that exists as an extrachromosomal plasmid instead of a prophage.

In addition to integration of their genome or maintenance of the extrachromosomal plasmid, all lysogenic phages are thought to have a mechanism that represses lytic gene expression and promotes genes needed for the maintenance of lysogeny. The most common mechanism employed by phages utilizes a transcription factor that is capable of both functions, known as the immunity repressor. The prophage is stable through bacterial replication cycles and is present in

daughter cells until certain factors such as changes in bacterial cell physiology triggers prophage induction, when the phage excises from the genome and enters the lytic cycle. In phages that utilize the *parABS* partitioning system, the replicated phage plasmid is segregated into daughter cells by the par proteins and is stably maintained until triggered into the lytic cycle (40, 41).

In many phages (Lambda being the most studied) the repressor protein is used to protect the cell from subsequent phage infections (42). Superinfection immunity occurs when the repressor protein binds to the DNA of highly similar phages and represses the lytic cycle, thereby preventing a successful infection (7, 43, 44).

Phage infection of a bacteria has further impact on the host than just lysis. During the lytic cycle, the bacterial genome is degraded. Fragments of that genome may be incorporated into the viral capsid, meaning that upon subsequent infections, the phage will inject the bacterial DNA it has acquired into a different cell. This process is called transduction and is one of the main sources of bacterial diversity and the major driver of the spread of antibiotic resistance.

Prophages are ubiquitous. Metagenomic studies investigating phage and bacterial sequences across a variety of ecosystems have revealed that most bacterial species contain a prophage, and clinical isolates are often polylysogens (contain multiple prophages) or have remnants of prophages within their genome (45–47). The incorporation of phage DNA can alter bacterial gene expression, as well as provide new genes that gives rise to strain variation within a species. For example, *Vibrio cholerae* was a non-virulent strain until it acquired a prophage that encodes the cholera toxin, transforming it into a human pathogen (48).

1.5 Phage and host encoded defense mechanisms

As mentioned previously, a major evolutionary pressure between host and phage is their attempts to thwart each other's mechanisms of or against infection. These adapted mechanisms are

also determinants of host range. A common defense the host will employ is blocking the phage receptors. This can be mediated through phase variation, production of extracellular matrix (EM) to physically block the phage, production of competitive inhibitors that bind to the receptors, and superinfection exclusion which is mediated specifically by the prophages that alter gene expression to change the cell surface. In response, the phage may adapt to overcome the absence of one of its receptors in the case of phase variation; some phages have developed receptors for the polymers of the EM or have acquired lyases that can degrade the EM so they can access their ligand (49).

Two very well studied mechanisms employ cutting of the phage DNA to halt infection, these being restriction modification (RM) systems and CRISPR. RM systems are found in ~90% of bacterial genomes and employ nucleases that upon recognition of specific sites, cut the DNA (34). There are several types of restriction nucleases, ranging from Type I-IV. To combat these mechanisms, phages can evolve to select against restriction sites by acquiring point mutations, so they are no longer targeted. Phages may have proteins, such as the OCR (overcome classical restriction) protein that acts as a DNA mimic to inhibit the nuclease; phages T3 and T7 utilize this protein. In addition, phages have acquired the ability to modify their bases by adding chemical groups. For example, *Bacillus* phages utilize DNA methylation encoded by the host to methylate their DNA and bypass restriction nucleases. Common DNA modifications include methylation and the addition of further glucosyl and/or hydroxyl groups. Phage T4 replaces cytosine with glucosyl-hydroxymethylcytosine (glc-HMC), and other phages may have the HM5C/glc-HM5C or HM5U modifications. Additional, much more severe, and extensive modifications exist that likely have further purpose beyond protection against restriction enzymes. The host adapted to overcome these modifications by developing the Type IV restriction enzyme, which only recognizes modified bases—in response the phages utilized the above protein inhibitors, such as the OCR (49–51).

These modifications are also used to combat CRISPR, as well as mutations to the spacer or PAM sequences. In addition, phages can encode for Acr (anti-CRISPR) proteins that are effective at inhibiting CRISPR-Cas9 by blocking crRNA loading, DNA recognition and cleavage. To date, there have been 98 distinct families of Acr proteins found (51, 52).

Another mechanism of defense is abortive infection. This system results in unproductive phage infection that does not produce new viral particles and results in death of the infected cell to protect the rest of the population. There are many systems that have been characterized in bacteria, including RexAB. The protein RexB is a membrane anchored ion channel, that when activated by RexA, results in a drop in membrane potential, causing a depletion in cellular ATP and the arrest of cell replication. The PrrC and Lit systems inhibit protein synthesis, and there are toxin-antitoxin systems also capable of aborting phage infection. Of course, phages have evolved their own mechanisms to combat those of the bacteria, including the RIIAB system in T4 to combat RexAB (49, 51). While the defenses outlined above are numerous, there are many more mechanisms employed by bacteria and phage that have been identified. Given phages have been co-evolving with their host for nearly four billion years, they have had lots of time to adapt to one another (12).

1.6 *Streptomyces*

Streptomyces is the most extensively studied genus of mycelial *Actinobacteria* due to their complex lifecycle and metabolic production (53). These bacteria are gram-positive and grow in soil, marshes, and coastal marine habitats (54). They are filamentous, spore forming, and the largest genus within the Phylum of *Actinobacteria,* which are characterized by their high guanine + cytosine content (55, 56). They are similar to fungi because of their adaptation to similar ecological niches but are evolutionarily distinct. From a spore, *Streptomyces* germinate to form

hyphal filaments that become a vegetative mycelium growing across and downwards into their substrate. This mycelium is segregated by crosswalls that result in multigenomic cells. Given certain environmental triggers, the vegetative mycelium forms aerial hyphae coated in a hydrophobic sheath that grow upwards out of the substrate. These hyphae form septa to parcellate into unigenomic spores. The germination process is highly regulated by a variety of well-characterized genes. These spores are semi-dormant and have low metabolic activity. Spores can withstand nutrient deficient conditions to protect the genetic material, while also serving their purpose of disseminating in the environment. *Streptomyces* spores differ from endospores, like *Bacillus,* that have a thick cell coat and can withstand extremely harsh conditions such as heat, desiccation, and ultraviolet radiation (57–60).

During germination, the process of a spore developing into a hypha, there is production of secondary metabolites that may confer the bacteria a competitive advantage in their environment. These metabolites are commonly used as antifungals, antivirals, immunosuppressants, antibiotics, anti-cancer agents, etc. Currently, 70-80% of the world's natural bioactive products used in agriculture and pharmacology are derived from *Streptomyces* (61, 62). *Streptomyces* chemical compounds are synthesized from biosynthetic gene clusters which are under intense regulatory control and differ greatly across species (57).

Different *Streptomyces* species vary in their process of germination. For example, *S. viridochromogenes* and *S. granaticolor* exhibit fast germination within all the spores of the population. Alternatively, *S. coelicolor* and *S. venezuelae* germinate at a slower rate and only within a fraction of the spores of the population (57). Several factors are essential to initiate hyphal growth, which may include density dependence, the presence of nutrients and/or aqueous conditions. Differences can be seen between germination of young versus mature spores, where

germination is favored within young spores and mature are resistant. In addition, species such as *S. venezuelae* and *S. griseus* have evolved to sporulate in liquid, while other species can only sporulate on solid mediums. Interestingly, species will have the same regulatory gene, but they result in different modes of development, attributed to the gain or loss of regulatory binding sites on their promoters (59).

Streptomyces development has been shown to have a significant impact on phage infection. Phages infect bacteria during the exponential phase; for *Streptomyces*, this occurs during spore germination. Previous studies have shown that older, mature mycelium is resistant to infection compared to what would be seen on newly germinating spores. The mature mycelium normally forms aggregates, which has high rates of phage adsorption without successful infection. However, this is not always the case and certain phages may have preferential infection on older bacteria (63). *Streptomyces* may develop transient resistance to phages following initial infection. *S. coelicolor* contains a unique mechanism deemed the phage growth limitation (pgl) system that results in failed subsequent infections to protect the rest of the bacterial population (64). In addition, *Streptomyces* have been shown to produce chemical metabolites in response to phage infection that can have a limiting effect on phage propagation, such as aminoglycosides that inhibit infection prior to DNA replication (65, 66). Overall, the relationship between a phage and its host is muti-layered, but for the genus *Streptomyces* which has a unique developmental cycle and extensive metabolic activity, the relationship is even more complex and poorly understood.

1.7 Phage Therapy

By the year 2050, there is predicted to be 10 million deaths a year due to infections caused by multi-drug resistant bacteria (67). Pathogenic bacteria are developing resistance to currently available antibiotics faster than they can be adapted to and faster than novel compounds can be

discovered, resulting in untreatable, and often fatal, infections. Therefore, alternative treatments are being sought to treat this global public health dilemma. A very promising avenue is phage therapy, where bacteriophages are used to treat bacterial infections. In compassionate use cases, phages have been used to treat chronic urinary tract infections, severe bacterial infections, and recently, a periprosthetic joint infection, with promising results (68, 69). Phages have potent bactericidal activity and can be very specific for the bacteria they infect, two reasons delineating why they hold incredible promise as a host-specific antibiotic. However, several caveats exist as to why phages are not as prolifically used as they are in European countries where phage therapy has been commonly practiced since its inception. Given treatment must result in bacterial cell death, if phages are being used as the direct treatment, they must be guaranteed to enter the lytic cycle over the lysogenic cycle or else the bacteria will persist. There have been very few completed clinical trials for phage therapy, therefore, the ideal preparation, dosage, and frequency of dosing of the phages for human administration is poorly understood, especially since each infection and phage is unique (70, 71). This remains as a glaring black box when it approaches the clinical applications of phage therapy.

One of the greatest challenges regarding phage therapy is finding a virus to target the pathogen of interest. The limited host range of most phages will be a severe bottleneck in the scalability of phage therapy treatments. Alternatives to overcome this include administration of phage cocktails—doses that contain multiple phages to combat the possibility of the bacteria developing resistance against one of the viruses. Another alternative is engineering phage tails to target a broader range of bacteria. As discussed, a major determinant of specificity between phage and host are the RBPs. Understanding the genetic factors and mechanisms of RBPs in identifying

their host can be manipulated to design a phage that can infect the pathogen of interest or that can infect across a broader range of serotypes.

1.8 Objectives

The focus of this study will be to isolate and characterize bacteriophages infecting the genus *Streptomyces* and to study the mechanism of host range. This genus was selected as *Streptomyces* phages are significantly underrepresented in major phage databases, and these bacteria represent a model system. The antibiotic production and unique development of *Streptomyces* creates complex relationships with phages that are distinct from other hosts. In addition, targeting the intricacies of host range will provide fundamental biology of phage-host specificity and guidance towards engineering phage host range which has implications in phage therapy.

1.9 Aims

The aims of this Master's thesis are as follows:

(1) Establish and optimize phage protocols using the host *Streptomyces*,

(1.1) Isolate novel bacteriophages infecting *Streptomyces,*

(2) Use these phages to screen for and isolate host range mutants, and

(3) Sequence *Streptomyces* bacteriophages and host range mutants using the Illumina MiSeq platform.

Chapter 2

Materials and Methods

2.1 Phage Isolation

Bacteriophages were isolated using a direct isolation method adapted from the SEA-PHAGE Discovery Guide (72). 3-5 grams of environmental soil samples were collected, and Difco Nutrient Broth (DNB) media was added to submerge the sample (3-5 mL). Agitated incubation was performed for ~2 hours at 30°C. Samples were centrifuged at 3000rpm for 5 minutes and the supernatant was collected and filtered through a 0.22μm filter. 500μL of the supernatant was incubated with 100μL of host spores for five minutes and then plated on DNB agar plates using 4mL of Maltose Yeast Malt (MYM) top agar using the double agar overlay method (73). Plates were incubated at 30°C for 1-2 days, a well isolated plaque was picked into phage buffer and then serially diluted 1:10 to a dilution of 10^{-5}. 100µL of dilutions 10^{-1} to 10^{-5} was mixed with 100μL spores each, incubated for five minutes, and plated using a double agar overlay as described above with 3.5mL of top agar. Purification by picking well isolated plaques is repeated for 2-3 rounds to obtain a clonal population of a phage. Phages were stored at 4°C in phage buffer.

2.2 Phage Amplification

Flooding

Once the population of phage is considered "pure", the picked plaque from the last round of purification is used to create "webbed plates" – in which a high density of plaques clears or nearly clears the plate of all host after 1-2 days of incubation at 30°C. The number of phages needed to create a webbed plate depends on plaque size and is determined empirically. Webbed plates are "flooded" with 8 mL of phage buffer, incubated at 30°C for 6≤ hours and the phage buffer is

removed and filtered with a 0.22µm filter. This process is repeated in the double flooding method and lysates are combined. The titer of the phage lysate was calculated by a spot test (see below).

Scraping

Following an initial flooding at 30°C for 6≤ hours and the collection of the lysate, the webbed plates may have also been "scraped". Scraping is performed using a sterile tool fashioned from a Pasteur pipette to remove the top agar layer from the plate into the 15mL conical tube containing the first flood. Agar and lysate underwent agitated incubation at 30°C for 10 minutes, vortexed for 1 minute, and centrifuged at 4000rpm for 10 minutes. The supernatant was collected, 0.22µm filtered, and the titer of the phage lysate was calculated by spot test.

2.3 Phage titering

DNB plates with 100µL spores and 3.5mL top agar are made and allowed to solidify. Phage lysates are diluted 1:10 in phage buffer and spotted as 3-5µL on the bacterial lawn. The plate is incubated for 1-2 days at 30°C. Note: phage dilutions can be made in microfuge tubes or in a 96-well plate. Phage titer is calculated using the following equation:

$$Titer\ (\frac{pfu}{mL}) = \frac{\#\ of\ plaque\ forming\ units}{volume\ of\ phage} \times \frac{1}{dilution\ factor}$$

2.4 Spore Preparation

Agar plugs of bacteria were struck on MYM plates. Single colonies were inoculated into 25mL of MYM and/or DNB liquid media and grown at 30°C for 3-5 days. Cultures were plated on thin MYM agar plates to form a lawn and incubated at 30°C for 8-10 days. (After 3 days, the spore plates are placed in a tray with wet towels to prevent the agar from drying). If starting from an existing spore stock, spores can be plated as 100µL of 4×10^8 CFU/mL using glass beads. Spores

are harvested from the plate by gently scraping the surface with a paintbrush and 6 mL of 0.85% NaCl. The spores were collected into a 50mL conical and an additional 4-6mLs of 0.85% NaCl was added to the plate to collect residual spores. The spores are vortexed on high, filtered through sterile glass wool using a syringe, and centrifuged at 3000rpm for 10 minutes. The supernatant is removed, and the pellets are resuspended in spore buffer (1mL/plate) to create 4×10^9 CFU/mL stocks which are stored frozen at -80°C. Working spore stocks are diluted 1:10 in filtered spore buffer to a final concentration of 4×10^8 CFU/mL.

2.5 Host Range Mutant Isolation

Bacteriophages were spotted as 1:10 serial dilutions on *S. avermitilis*, *S. coelicolor*, *S. venezuelae*, *S. griseus*, and/or *S. tricolor*, which were plated as a double agar overlay. Phages with limited infection on an alternative host (AH) compared to their isolation (native) host (NH) were selected. The selected phage was plated on the NH, and plaques were picked and amplified. High titer wildtype (WT) lysates were collected and spotted as 1:10 dilutions (10^{-2} to 10^{-7}) on both the AH and NH to verify the WT phenotype. Verified WTs were plated as 100µL of 1:10 serial dilutions from 10^{-1} to 10^{-4} on 100µL of the AH. Plaques were picked off the AH and amplified on the NH. High plaque density plates were "flooded" with phage buffer and collected similarly to the phage isolation protocol. This HRM isolation step was repeated by plating on the AH, picking a plaque, and amplifying on the NH. The putative host range mutants (HRMs) were spotted as 3µL from 1:10 dilutions (10^{-1} to 10^{-6}) on the NH and the AH to verify the mutant phenotype.

2.6 DNA Extraction

PEG precipitation

A minimum of 8mL of high titer phage lysate (>1×10^9 pfu/mL) was treated with DNase I (5 mg/mL) to 0.03% and unboiled RNase A (10 mg/mL) to 0.15% and incubated at 37°C for 30 minutes. PEG precipitation buffer is added to a 1:4 ratio and incubated overnight at 4°C. The PEG solution was centrifuged at 15 000 × g for 30 minutes at 4°C, the supernatant is decanted, and the pellets centrifuged for 5 minutes at 15 000 × g. Pellets were resuspended in 0.5-1mL of 15mM EDTA and 0.5% SDS and incubated with 0.2% Proteinase K for 10 minutes at 37°C, then 10 minutes at 55°C.

$ZnCl_2$ precipitation

In lieu of PEG precipitation buffer, 25µL/1mL 2M $ZnCl_2$ can be added to the lysate treated with nucleases. The mixture is incubated at 30°C for 10 minutes, then spun at 10 000 × g for 1 minute. Pellets are resuspended in 15mM EDTA then treated with 2µL Proteinase K and 50µL of 10% SDS per mL and incubated for 10 minutes at 37°C, then 10 minutes at 55°C.

Phenol:chloroform extraction and ethanol precipitation

For both methods, 1:1 water-saturated phenol:chloroform is added to the precipitated phage lysate at a ratio of 1:1, vortexed for 1 minute, then spun at 14 800 rpm for 5 minutes. The aqueous layer is collected, and the previous step repeated. The aqueous layer is mixed with chloroform to a ratio of 1:1, vortexed for 1 minute and then spun at 14 800 rpm for 2 minutes. This step is repeated until there is no white interface between the aqueous and organic layers. $1/10^{th}$ volume of 3M NaOH, pH 5.2 and 2.5X volume of 100% ethanol is added to the supernatant, mixed by inversion, and incubated at -20°C for 1 hour or overnight. Following incubation, the solution is spun at 14 800 rpm for 5 minutes. DNA pellets are washed with 1mL of 70% ethanol, vortexed and spun. The

wash is removed and respun to remove as much residual ethanol as possible. The DNA pellets are left to dry at room temperature for 20-30 minutes, then resuspended in 50-100uL of sterile double-distilled H_2O. The DNA is incubated at 37°C for ~30 minutes, 55°C for 10 minutes, and vortexed throughout. The concentration of DNA was determined by running 1-5µL on a 1% agarose gel containing 5µL/100mL Ethidium Bromide to allow imaging of DNA. Typically, 1-10µg of genomic DNA is purified for downstream uses.

2.7 Illumina Sequencing Library preparation

Libraries were constructed following the NEBNext® Ultra™ II FS DNA Library Prep Kit for Illumina® for use with inputs ≥100ng. Fragmentation at 37°C was optimized to 25 minutes. https://www.neb.com/en/-/media/nebus/files/manuals/manuale6177-e7805

EnoachSoames, Celery, Superstar, Rideau and Wilkos were sequenced on an Illumina MiSeq instrument (150- bp single-end reads). Following Russell, the raw reads were assembled using Newbler v.2.9, resulting in a single linear contig (74, 75). The assembly was checked for completeness, accuracy, and genome termini using Consed v.29.0 (76). Following the SEA-PHAGES Bioinformatics Guide (https://seaphagesbioinformatics.helpdocsonline.com/home), Erla's genome was annotated using DNAMaster v.5.23.5 (http://cobamide2.bio.pitt.edu). Putative genes were identified using GLIMMER v.3.02, GeneMark v.3.25 and v.4.28, and Starterator v.381 (https://seaphages.org/ software) (77, 78). ARAGORN v.1.1 (included in DNAMaster) and v.1.2.38 and tRNAscan-SE v.2.0 were used to search for tRNAs and transfer-messenger RNA (tmRNA) (79, 80). Functional assignments were made using BLASTp v.2.10.1 and HHpred (81, 82). TMHMM v.2.0 and SOSUI v.1.11 were used to gather further information on proteins of no known function (83, 84). Default parameters were used for all software. Phamerator v.381 was

used to determine synteny with other bacteriophages previously sequenced as part of the SEA-PHAGES program, which supported the above functional assignments (85).

2.8 Recipes

Note: for liquid media used in cultures, add 5% w/v PEG8000

DNB media

8g	Difco Nutrient Broth powder
1000mL	Double-distilled H_2O

DNB plates

DNB media
15g/L agar

DNB Supplements

12.5mL of 40% glucose
4mL of 1M $CaCl_2$

MYM media

4g	Maltose
4g	Bacto yeast extract
10g	Bacto malt extract
1000mL	Double-distilled H_2O

MYM plates

MYM media
20g/L agar

MYM top agar

MYM media
4.5g/L agar

MYM Supplements:

2mL/L of trace elements
4mL/L of $CaCl_2$ (only for phage protocols)

Trace elements (1L)

40mg	$ZnCl_2$
200mg	$FeCl_3 \cdot 6H_2O$
10mg	$CuCl_2 \cdot 2\ H_2O$
10mg	$MnCl_2 \cdot 4\ H_2O$
10mg	$Na_2B_4O_7 \cdot 10\ H_2O$
10mg	$(NH_4)_6Mo_7O_{24} \cdot 4\ H_2O$

Phage buffer

10mM	Tris (pH 7.5)
10mM	$MgSO_4$
68mM	NaCl
1mM	$CaCl_2$
10%	Glycerol (optional)

Spore buffer

0.595%	NaCl
15%	Glycerol

PEG precipitation buffer

30%	PEG8000
3.3M	NaCl

Chapter 3

Results

3.1 Establish and optimize phage protocols using the host *Streptomyces*

Streptomyces bacteria are culturable as spores

To establish use of *Streptomyces* bacteria in the lab, I cultured several species which included *S. griseus* as a liquid culture and *S. avermitilis, S. coelicolor, S. venezuelae, S. azureus, S. bicolor*, and *S. tricolor* as spore stocks (fig. 1A). According to the Actinobacteriophage database, these species have been used to isolate for phages and besides *S. griseus*, had few discovered phages. Because *Streptomyces* is a sporulating genus of bacteria, growing most species on solid agar media results in the production of spores along the surface of the lawn (fig. 1B). These spores can be harvested for future use and handled similarly to a liquid culture of cells. For all unculturable species, the greatest production of spores occurred under nutrient starvation conditions, after 8-10 days of growth with no increase in concentration seen thereafter (fig. 1C). In nutrient rich conditions, minimal to no sporulation across the same and extended time course was observed, as the bacteria favored vegetative growth over aerial hypha. Unlike *S. venezuelae* and *S. griseus*, which forms liquid cultures of dispersed cells, liquid cultures of all other *Streptomyces* species resulted in the formation of hyphal networks and aggregates that produced inconsistent lawns when used in double-agar overlays.

New spores are typically made from plating existing spore stocks. Other methods of propagating spores, such as streaking spores, plating culture, or plating a single colony, results in minimal spore concentrations and is more laborious and time consuming.

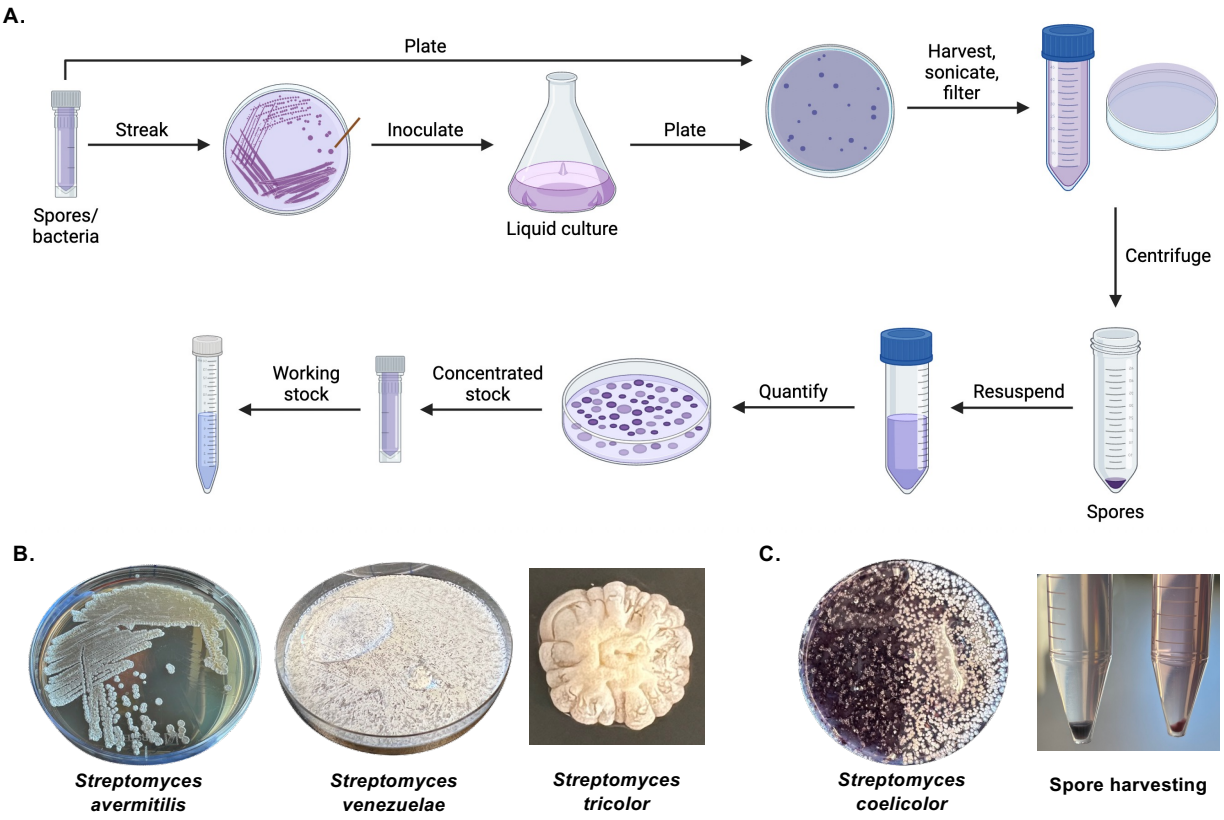


Figure 1. Workflow for harvesting *Streptomyces* spores. (A) Spores/bacteria are struck on an agar plate. A single colony is inoculated into liquid media. The culture (or spores) is plated as a confluent lawn and incubated. The spores are harvested from the plate, filtered, centrifuged, and resuspended to concentrate the stock. Stocks of spores at 4×10^9 CFU/mL are frozen at -80°C. These stocks are diluted to 4×10^8 CFU/mL and used in laboratory protocols. (B) (left) *Streptomyces avermitilis* struck for single colonies on a MYM solid agar plate. *Streptomyces venezuelae* sporulation on a MYM thin agar plate. Colony of *Streptomyces tricolor* on MYM agar. (C) *Streptomyces coelicolor* spore harvesting. Half a spore lawn harvested. Spore pellets collected from thin (left) and thick (right) MYM agar plates.

Confluent lawns are made from working spore stocks

To make a confluent lawn while also limiting bacterial regrowth over plaques, spores are diluted to an optimal concentration (4×10^8 CFU/mL) and plated as a suitable volume (100μL). All species sporulate adequately to form a lawn under these aqueous conditions, temperature, and density. It should be noted that transient resistance to phage infection has been shown in S. venezuelae, where the mature mycelium was resistant and aerial hypha could grow within the plaque interface (63). This resistance was observed when using the host *S. coelicolor* and was partially mitigated by using a lower concentration of spores.

Streptomyces species are sensitive to their growth mediums

Several challenges presented themselves during the growth process of these species. These included sensitivity to the media, meaning certain species would not grow and/or sporulate when in Difco Nutrient Broth (DNB) versus Maltose Yeast Malt (MYM) media. Following four days of growth in MYM++ media (supplemented with trace elements and PEG8000), *S. griseus, S. lividans, S. bicolor*, and *S. mirabilis* all remained translucent in appearance and demonstrated limited to no growth, which appeared in the form of aggregates. The culture for *S. bicolor* had a slight change in colour, representative of the production of secondary metabolites. *S. avermitilis, S. coelicolor, S. venezuelae, S. azureus* and *S. tricolor* produced saturated and translucent to opaque cultures containing aggregates.

S. griseus, S. lividans, S. bicolor, and *S. mirabilis* were then grown in DNB+++ media (supplemented with glucose, calcium, and PEG8000). *S. griseus* formed a saturated culture. *S. bicolor* and *S. mirabilis* demonstrated improved growth in this media, enough to form a lawn for spore plates. *S. lividans* growth did not improve in this media, and so was unculturable in the lab.

The species *S. avermitilis, S. coelicolor, S. venezuelae, S. tricolor,* and *S. azureus* sporulated well on MYM+ agar plates, facilitating the creation of high concentration spore stocks. *S. griseus, S. bicolor,* and *S. mirabilis* did not sporulate on MYM+ agar, which was evident in the concentration of spores after harvest, and by the lack of pigment produced by the bacteria on the spore plate. *S. griseus* grows optimally as a liquid culture, therefore, spores were not pursued. For *S. bicolor* and *S. mirabilis,* once the trace element supplement was removed from the MYM+ agar, both species demonstrated much higher levels of sporulation, enough to produce high concentration spore stocks.

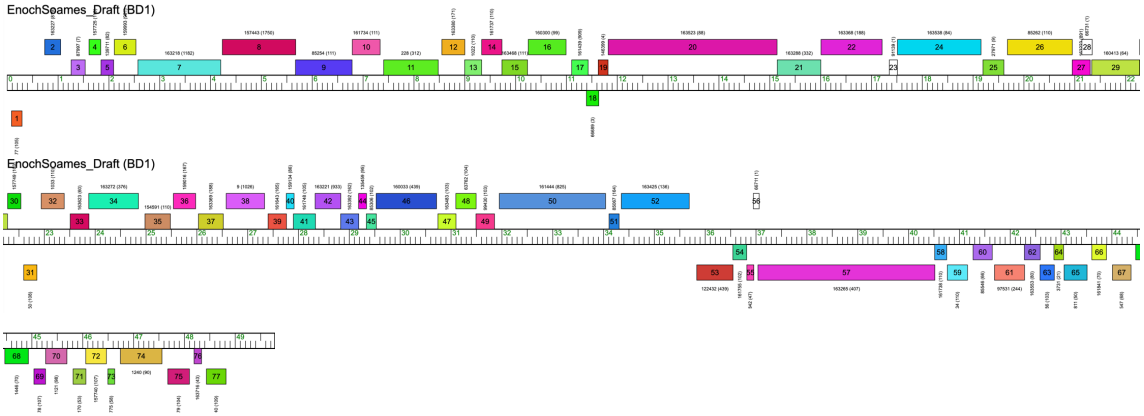
3.2 Isolate novel bacteriophages infecting *Streptomyces*

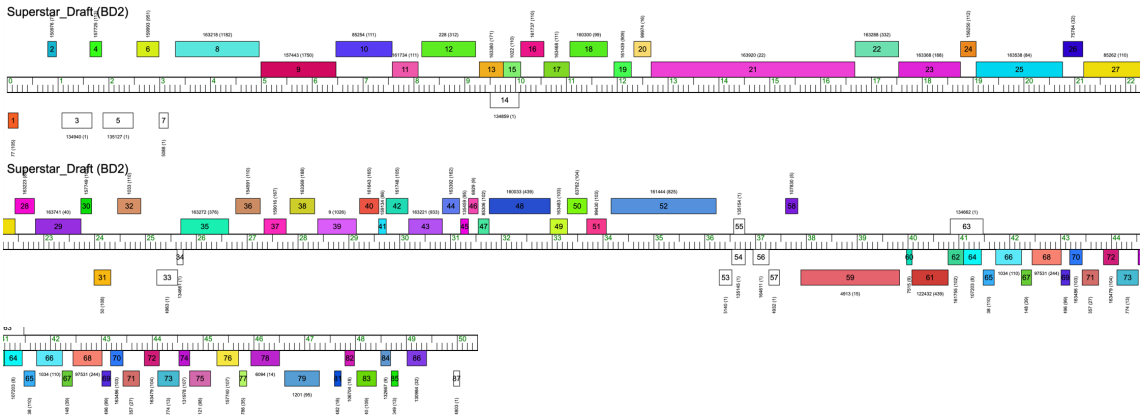
50 novel phages infecting Streptomyces bacteria were isolated

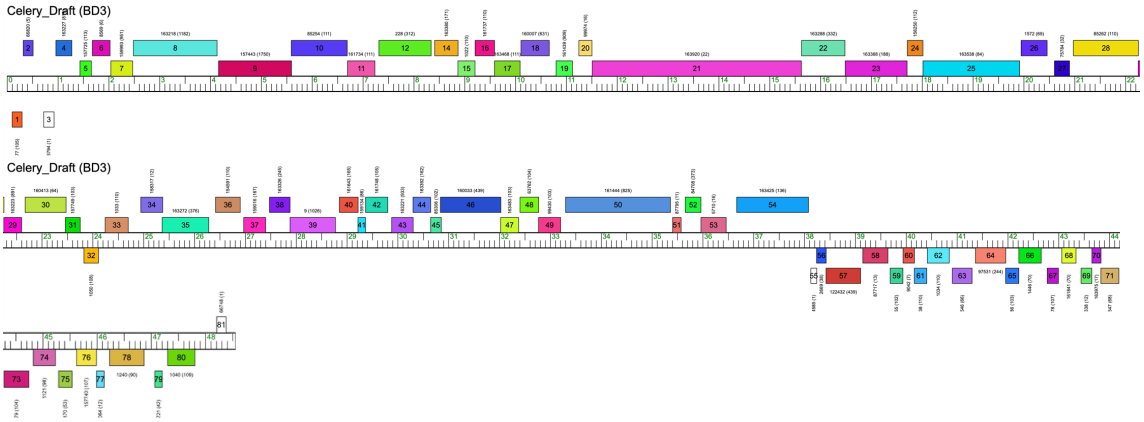
I largely adopted and modified the phage hunting protocols from the SEA PHAGE Discovery Guide to isolate novel bacteriophages infecting the genus *Streptomyces*. In conjunction with the SEA PHAGE Discovery Laboratory course and uOttawa Phage Camp, I facilitated the discovery of 50 novel phages infecting *S. avermitilis* (27)*, S. coelicolor* (21), and *S. venezuelae* (2) that have been characterized by restriction enzyme digestion and transmission electron microscopy (TEM) and included in the Actinobacteriophage database (table 1). 49 phages were of the *Siphoviridae* morphology, and phage Rideau was a *Podoviridae*. Seven phages were sequenced by the University of Pittsburgh or the University of Ottawa, and were revealed to belong to the BD1, BD2, BD3, BE1, BE2 subclusters, and the BF and BN clusters. Phages EnochSoames, Superstar, and Rideau belong to the BD subclusters and demonstrate sequence/functional homology near the start and middle of the genomes, and no similarity toward the 3’ end (fig. 2). Phages Rideau (BF) and Wilkos (BN) however, demonstrate no sequence homology with each other, or with the other BD phages (fig. 2). All sequenced phages except for one (Mugiwara) had a G+C content above

Table 1. uOttawa *Streptomyces* bacteriophage database. 50 novel phages discovered to infect *S. avermitilis* (27), *S. venezuelae* (2) or *S. coelicolor* (21). Found by students in the SEA Phage Discovery Laboratory course, the Rudner lab, and uOttawa Phage Camp participants.

Phage name	Host	Found by	ChaCha	S. avermitilis	Anna Pawelko	Valtteri	S. coelicolor	Adam Rudner
Phox3	S. avermitilis	Adam Rudner	RubyPie	S. avermitilis	Elizabeth Thibeault	Rosita	S. coelicolor	Madeeha Shaikh
Wilkos	S. avermitilis	Lauren Wilkes	Hosilda	S. avermitilis	Hosein Abardeh	EnochSoames	S. coelicolor	Zachary Mitchell
Phadme	S. avermitilis	Katia Koziel Ly	RosieB	S. avermitilis	Hannah Verge	Celery	S. coelicolor	Ayesha Syed
Phox2	S. avermitilis	Adam Rudner	ChiliPhepper	S. avermitilis	Daniel Anderson	Addis	S. coelicolor	Lillian Abebe
Pecan	S. avermitilis	Sara Ladha	Sumeru	S. avermitilis	Lauren Wilkes	Naya	S. coelicolor	Nandini Biyani
JointCustody	S. avermitilis	Larissa Yakoub / Emily Wood	Natlan	S. avermitilis	Lauren Wilkes	Salatiosaurus	S. coelicolor	Rosalie Salati
Rideau	S. avermitilis	Alex Yang	Teyvat	S. avermitilis	Lauren Wilkes	Masr	S. coelicolor	Hoda Osman
Santy	S. avermitilis	Angela Guevara	Anomala	S. avermitilis	Adam Rudner	Moonwalker	S. coelicolor	Tiffany Yang
Mugiwara	S. avermitilis	Gil Toex	Mondstadt	S. avermitilis	Lauren Wilkes	Ottillie	S. coelicolor	Adam Rudner
PinkQuack	S. avermitilis	Larissa Yakoub	Liyue	S. avermitilis	Lauren Wilkes	Pola	S. coelicolor	Selam Yimer
WinterSquash	S. avermitilis	Emma Mahoney	Brownmamba	S. coelicolor	Danyaal Ansari	Kernel	S. coelicolor	Fiona Haugen
Quackie	S. avermitilis	Serena Bezanson	Helian	S. coelicolor	Sabrina Sikka	Resilinacea	S. coelicolor	Tuba Buyuktepe
Laloosh	S. avermitilis	Paul Al Haddad	KayPee	S. coelicolor	Elijah Van Dinther	Jacintha	S. coelicolor	Tamara Synek
Superstar	S. avermitilis	Emily Wood	AlsatianStar	S. coelicolor	Angela Wang	Castel	S. coelicolor	Lauren Wilkes
Invermay	S. avermitilis	Lia Morton	Helian	S. coelicolor	Sabrina Sikka	Inazuma	S. venezuelae	Lauren Wilkes
Tomtom	S. avermitilis	Stephanie Khoury	Phesto	S. coelicolor	Adam Rudner	Yarita	S. venezuelae	Walaa Eid


EnochSoames_Draft (BD1)
EnochSoames_Draft (BD1)


Superstar_Draft (BD2)
Superstar_Draft (BD2)


Celery_Draft (BD3)
Celery_Draft (BD3)

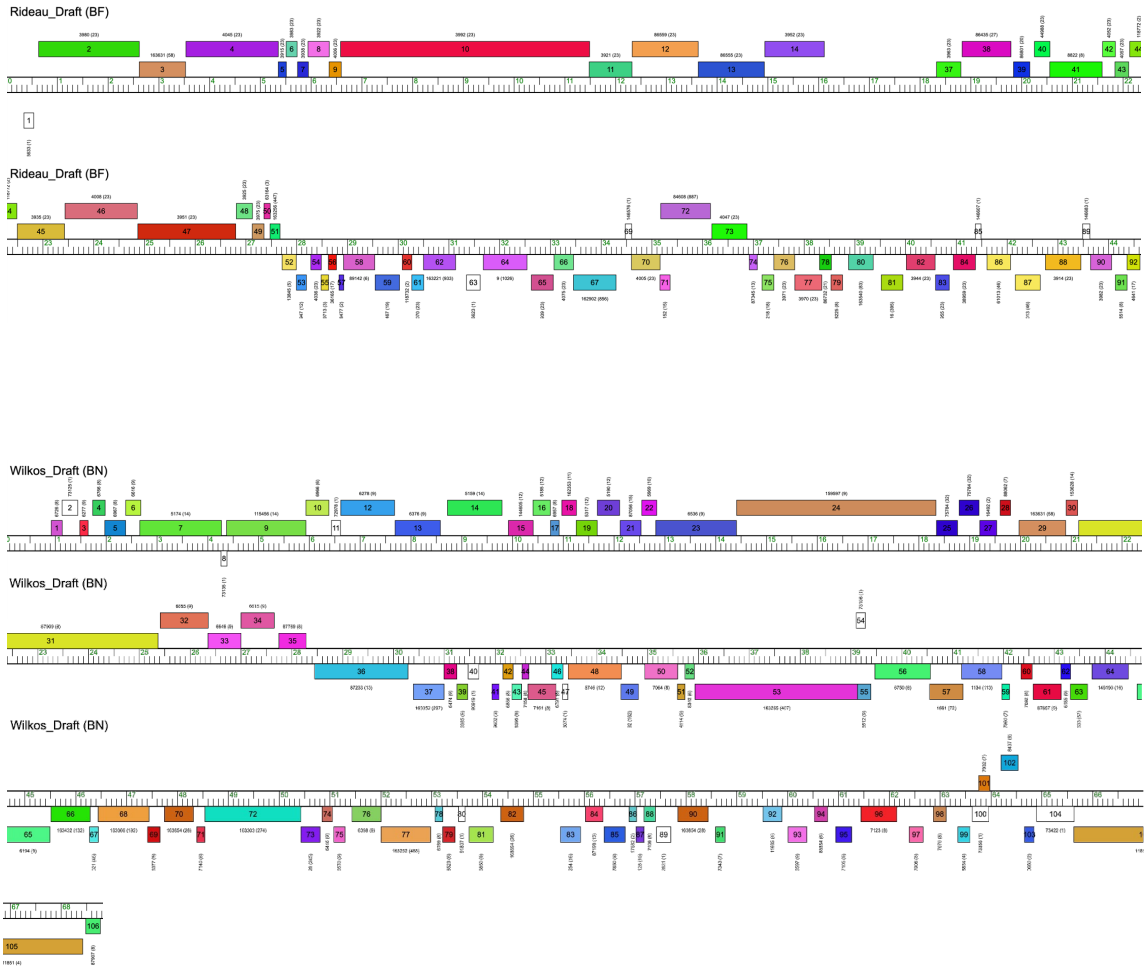


Figure 2. Genome maps of sequenced *Streptomyces* bacteriophages. (Top) EnochSoames (BD1), Superstar (BD2), Celery (BD3), Rideau (BF), Wilkos (BN). Rectangles represent individual genes, colour coded based on pham.

60%, which is consistent with their host. Mugiwara had a G+C content of ~49%; similar values have been observed in other *Streptomyces* phages.

In conjunction with the MSc work of Kieran Furlong, twelve *Streptomyces* phages were sequenced using the Nanopore platform and influenced the selection of Superstar, the only BD2 cluster phage isolate, for Illumina sequencing by the University of Pittsburgh. The non-sequenced phage genomes were characterized based on their restriction digest patterns.

Although *Streptomyces* phages infecting *S. avermitilis, S. coelicolor,* and *S. avermitilis* were abundant in environmental samples, several phages could not be amplified, either because they lost viability on the double-layered agar plate used to visualize phage plaques, or within solution after being picked for purification and amplification. This low viability made these phages impossible to pursue.

Streptomyces bacteriophages are challenging to amplify

Most of these *Streptomyces* phages were difficult to amplify, only reaching a maximal titer of 10^9 pfu/mL. Highest titers were achieved by flooding over-webbed plates with phage buffer and incubating. To solve this issue, I modified the standard protocol for creating high titer phage stocks. Normally, high titer phage stocks, or phage lysates, are harvested from a double agar overlay petri plate that contains a high density of plaques with remnants of a bacterial lawn threaded throughout (named a "webbed" plate) that has been incubated with phage buffer at 30°C for three hours (or overnight at 4°C), followed by harvesting and filtering of the buffer. For these *Streptomyces* phages, it was found that higher titers of phage were reached on over-webbed plates in which the bacterial lawn completely clears and when incubation with phage buffer was at 30°C for 6+ hours. In addition, I discovered that a second incubation with phage buffer ("double flooding") resulted in a second phage lysate of similar titer, effectively doubling the yield of phages. For example,

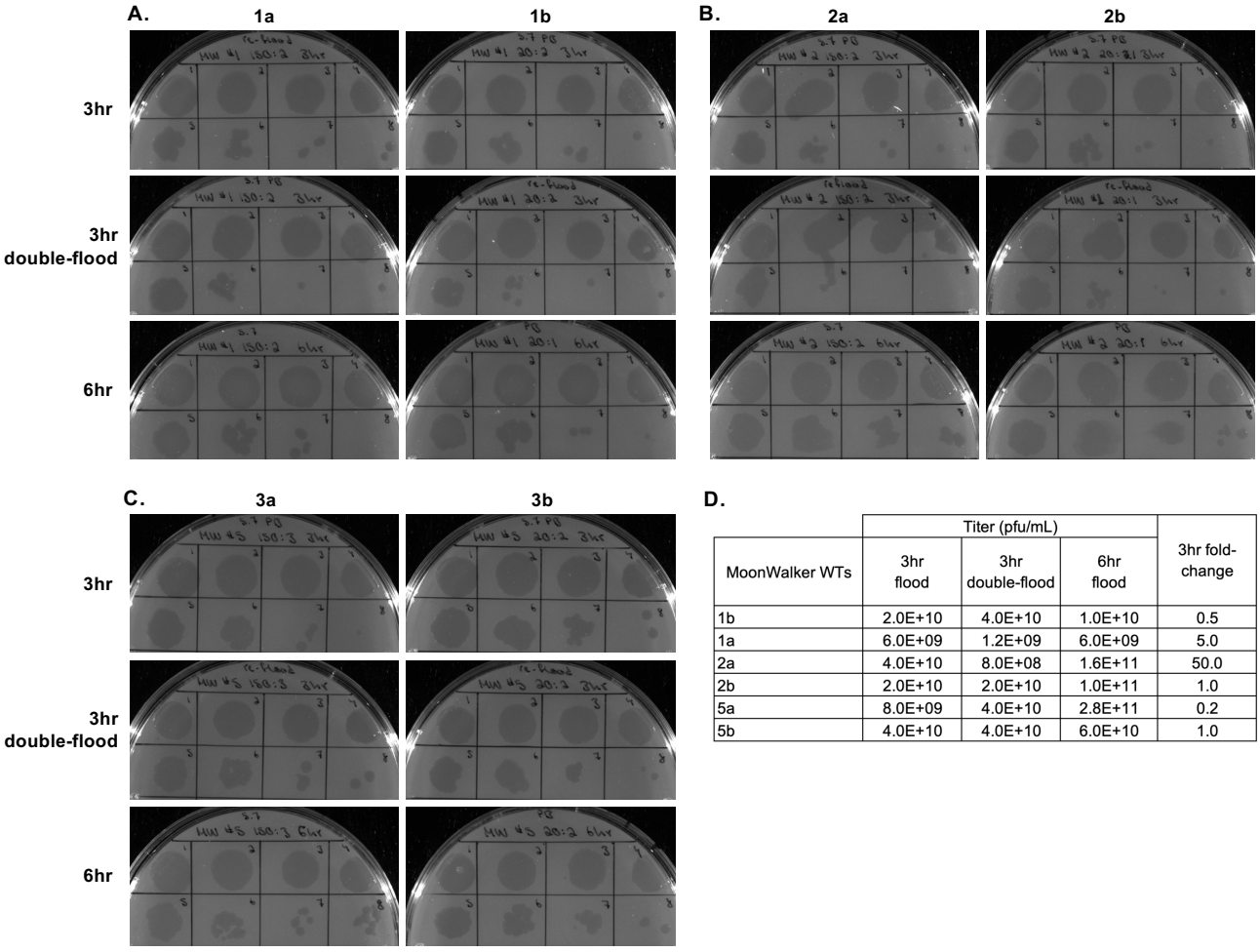
duplicates of Moonwalker WT webbed plates of different dilutions were flooded with phage buffer for 3 hours and incubated at 30°C. This lysate was collected, then the plate was flooded again for the same duration at the same temperature before collection. The duplicate plate was flooded once for 6 hours at 30°C. Of the double-flooded plates, there was an increase in titer in the second flood for 2/6, no change for 2/6, and a decrease for 2/6 (fig. 3A, B, C, D). For the plates that underwent an extended incubation, 5/6 titers were higher than or equal to the 3-hour flood, with only one lysate having a lower titer (fig. 3D).

Additionally, other phages underwent a similar protocol where webbed plates were flooded for 3 hours at 30°C and then underwent a second flooding for either 12+ or 24+ hours at the same temperature. Of the 4 phages tested at 12 hours and the 5 phages tested at 24 hours, a total of 3 phages had a higher titer compared to the 3-hour incubation, one phage had no change, and the other 5 had a decrease in titer ranging from 3 to 100-fold (fig. 4A).

Liquid infection was also tested to achieve high titer phage lysate. Cultures of *S. avermitilis* were infected with phage lysate and allowed to propagate at 30°C for 12 hours. None of the lysates achieved a high titer $\geq 10^9$ pfu/mL (fig. 4B).

Phage precipitation was most successful using PEG

A third challenge was many of these phages yielded low concentrations of genomic DNA when isolated from $ZnCl_2$ precipitated phages followed by a silica-based column purification protocol (Promega Wizard DNA Clean-Up Kit). Higher yields of genome with greater purity were isolated by phenol/chloroform extraction of PEG precipitated phages, followed by ethanol precipitation.



D.

MoonWalker WTs	Titer (pfu/mL) 3hr flood	3hr double-flood	6hr flood	3hr fold-change
1b	2.0E+10	4.0E+10	1.0E+10	0.5
1a	6.0E+09	1.2E+09	6.0E+09	5.0
2a	4.0E+10	8.0E+08	1.6E+11	50.0
2b	2.0E+10	2.0E+10	1.0E+11	1.0
5a	8.0E+09	4.0E+10	2.8E+11	0.2
5b	4.0E+10	4.0E+10	6.0E+10	1.0

Figure 3. Double-flooding and extended flooding incubation of Moonwalker WTs. Webbed plates with different dilutions were either flooded and incubated for 3 hours, then re-flooded and re-incubated another 3 hours, or incubated for 6 hours. Lysates were spotted as 1:10 dilutions from 10^{-1} to 10^{-8}. (A) WT 1a and 1b. (B) WT 2a and 2b. (C) WT 5a and 5b. (D) Titres of each WT lysate in plaque forming units (pfu) per millilitre (mL). Fold change of the titres of the first and second floodings.

A.

	Titer (pfu/mL)			Fold-change
	3hr	12+ hrs	24+ hrs	
Rosita WT 17	1.6E+09	2.0E+09		0.8
Moonwalker WT 9	4.0E+09	1.4E+09		2.9
Moonwalker WT 7a	6.0E+08	4.0E+09		0.2
Moonwalker WT 7b	3.0E+09	6.0E+08		5.0
Ottilie	3.8E+09		1.2E+09	3.2
Moonwalker WT 8a	8.0E+07		8.0E+07	1.0
Moonwalker WT 8b	2.0E+08		4.0E+08	0.5
Moonwalker WT 10	1.2E+10		2.0E+08	60.0
Moonwalker WT 11	2.0E+10		1.8E+08	111.1

B.

	Liquid infection titer (pfu/mL
Rosita WT 11	2.0E+08
Rosita WT 12	1.6E+08
Moonwalker WT 3	6.0E+08
Moonwalker WT 4	8.0E+07
Ottilie	4.0E+06

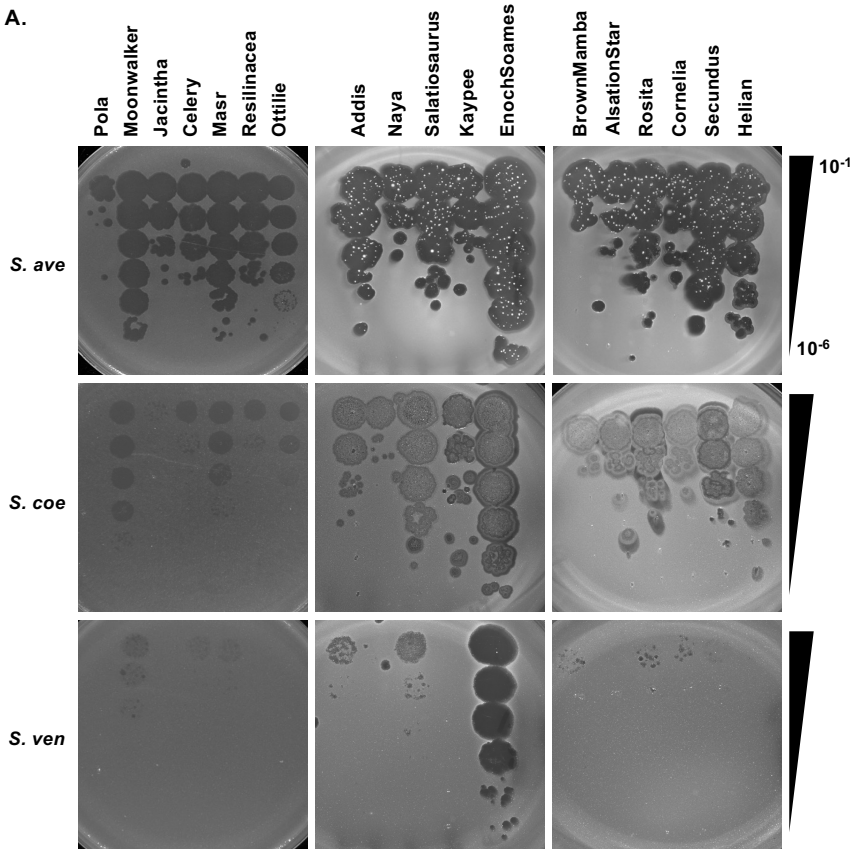
Figure 4. Titres achieved through double-flooding and liquid infections. (A) Webbed plates with different dilutions were either flooded and incubated for 3 hours, then re-flooded and re-incubated another 12+ or 24+ hours. Lysates were spotted as 1:10 dilutions from 10^{-1} to 10^{-8}. (B) Lysates of cultures of *S. avermitilis* infected with phage lysate.

3.3 To use these phages to screen for and isolate host range mutants

Bacteriophage host range is narrow

Our first isolation efforts in the SEA PHAGE Discovery Laboratory course used *S. coelicolor* as a host and we isolated a collection of 18 phages in the Fall of 2022. To characterize the host range of our novel phages (and to identify potential host range mutants) serial dilutions were spotted on lawns of *S. avermitilis*, *S. venezuelae*, and their isolation host *S. coelicolor* (fig. 5A). 17/18 phages demonstrated a higher infective ability on the host *S. avermitilis* compared to *S. coelicolor,* as demonstrated by efficiency of plating (EOP) values greater than 1 (fig. 5B). Seven phages have an EOP of ">1", signifying higher infectivity on *S. avermitilis* than *S. coelicolor*. The plaques of these phages appeared much smaller on *S. coelicolor* and were difficult to quantify, however, it was obvious that infection stopped at an earlier dilution than on *S. avermitilis*, demonstrating the differences in infectivity. Of these seven phages, Moonwalker had an EOP closest to 1, with a ~10-fold difference in infection on both species. In addition to greater infectivity, these 17 phages all produced larger and clearer plaques on *S. avermitilis*.

None of the phages showed a greater infective ability on *S. venezuelae* compared to their isolation host, with only EnochSoames demonstrating nearly full infectivity on *S. venezuelae.* Many phages demonstrate "lysis from without", a phenomenon where at high multiplicities of infection, there is unproductive phage infection that results in death of the bacteria through high levels of phage adsorption depolarizing the membrane (86). Phages Ottilie and Celery demonstrate *lysis from without* on *S. coelicolor*. Their spots on the highest dilutions have completely lysed the bacteria, however, the next closest dilution to this clearing does not have any plaques, supporting that phages were not responsible for cell death (fig. 5A). Other phages such as Moonwalker, Rosita, and Addis contained several individual plaques on the highest dilutions when infecting *S.*



B.

Bacteriophage	EOP
Helian	6.0
Secundus	4.0
Cornelia	10.0
Rosita	5.0
Alsatianstar	3.0
BrownMamba	1.0
EnochSoames	1.4
Kaypee	1.3
Salatiosaurus	0.8
Naya	3.3
Addis	2.0
Ottillie	> 1
Resilinacea	> 1
Masr	> 1
Celery	> 1
Jacintha	> 1
Moonwalker	> 1
Pola	> 1

Figure 5. Host range assay on *Streptomyces* bacteria. (A) Phages Pola, Moonwalker, Jacintha, Celery, Masr, Resilinacea, Ottillie, Addis, Naya, Salatiosaurus, Kaypee, EnoachSoames, BrownMamba, AlsationStar, Rosita, Cornelia, Secundus and Helian were diluted 1:10 and spotted on *S. avermitilis, S. coelicolor, S. venezuelae*. Plates were made using Difco Nutrient Broth (DNB) media, and top agar overlays were made using Maltose Yeast Malt (MYM) media. (B) Efficiency of plating (EOP) values >1 show phages with a higher infective ability on an alternative host. *S. venezuelae* is not represented in the table. On S. venezuelae, EnoachSoames has an EOP of 1 and all other phages have an EOP <0.01.

venezuelae. These plaques may represent phages containing a mutation that broadens their host range (see below).

Genomic similarity is not a determinant of host range

Of this group of 18 phages, EnochSoames and Celery have been sequenced, and are closely related, belonging to the BD1 and BD3 clusters, respectively. Figure 5 demonstrates that they have very different host ranges. Celery infects *S. avermitilis* much better than *S. coelicolor* and does not infect *S. venezuelae*. In comparison, EnochSoames infects all three species equally. Celery has a much narrower host range compared to EnochSoames which has a broad host range on the tested species. This highlights the importance of indicating the isolation host of the phage and understanding there may be a superior infection host that is yet to be tested.

The host range testing of *S. coelicolor* phages suggested *S. avermitilis* may be a more permissive isolation host than *S. coelicolor* and *S. venezuelae* for phage hunting, therefore, in the Summer and Fall of 2023, we isolated 17 additional phages using *S. avermitilis* as a host. The host range of six phages isolated on *S. avermitilis* was tested using the hosts *S. coelicolor, S. venezuelae, S. tricolor*, and *S. griseus* (fig. 6). All phages demonstrate a very high infective ability on their isolation host, with varying infection on other hosts, unlike what was seen on phages isolated from *S. coelicolor*. Of the phages tested, Wintersquash and Rideau infected *S. coelicolor* to the same degree as *S. avermitilis* but demonstrated no other host range beyond these two species. The only other phage demonstrating an expanded host range was Laloosh, which infected *S. tricolor* to the same degree as its isolation host. Comparatively, the phage RubyPie only infected *S. avermitilis* to a high degree and had partial infection on *S. coelicolor* and *S. tricolor* where the infection is strong in the lowest dilutions but quickly diminishes. All phages had similarly turbid plaques with reduced size on *S. coelicolor,* as was seen with the phages that were isolated on this host. Of these six

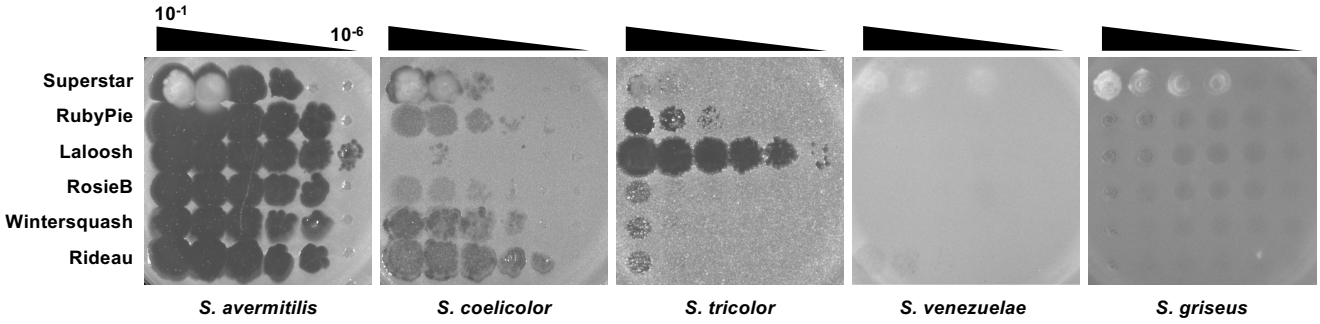


Figure 6. Expanded host range assay on *Streptomyces* bacteria. (A) Phages Superstar, RubiePie, Laloosh, RosieB, Wintersquash and Rideau were diluted 1:10 from 5×10^9 pfu/mL and spotted on *S. avermitilis, S. coelicolor, S. tricolor, S. venezuelae,* and *S. griseus*. Plates were made using Difco Nutrient Broth (DNB) media, and top agar overlays were made using Maltose Yeast Malt (MYM) media.

phages, Superstar and Rideau were sequenced, belonging to the BD2 and BF clusters, respectively (fig. 2).

Host Range mutants arise on alternative hosts

The differences seen in host range among these phages suggested that there may be spontaneous mutations that can expand a phage's naturally occurring host range. Phages Moonwalker and Rosita, while having very strong infection on *S. avermitilis* and *S. coelicolor*, have a very limited infection on *S. venezuelae*. Similarly, RubyPie has strong infection on *S. avermitilis* and limited infection on *S. coelicolor* and *S. tricolor.* This phenotype is weak, with a difference in magnitude by a factor of 4 or 5. The plaque formation on these hosts is representative of the few viral particles within the greater population that likely contain a mutation conferring expanded host range.

Host range mutants are isolated and amplified from alternative hosts

14 Rosita and 11 Moonwalker wildtype (WT) high titer lysates were made through purification and amplification on *S. avermitilis*, as both phages demonstrated the highest infection on this host (fig. 7). All the purified WTs from both phages demonstrated strong infection on their native host and very minimal infection on the alternative host, *S. venezuelae*. A host range assay of Rosita WTs #13-21 shows each WT infecting its native host to a dilution of 10^{-6}, and the alternative host to the 10^{-1} and 10^{-2} dilutions (fig. 8A). WT Rosita and Moonwalker phages cannot infect *S. venezuelae*, however, the limited infection indicates there are mutated phages within their lysates that can. The WTs were used to isolate and purify HRMs by screening for plaques on the alternative host. Plaque formation indicated successful infection. These plaques were picked from the alternative host and amplified on the native host to select for host range mutants (HRMs); this passaging was repeated for two generations. HRMs were successfully isolated from Rosita WT13

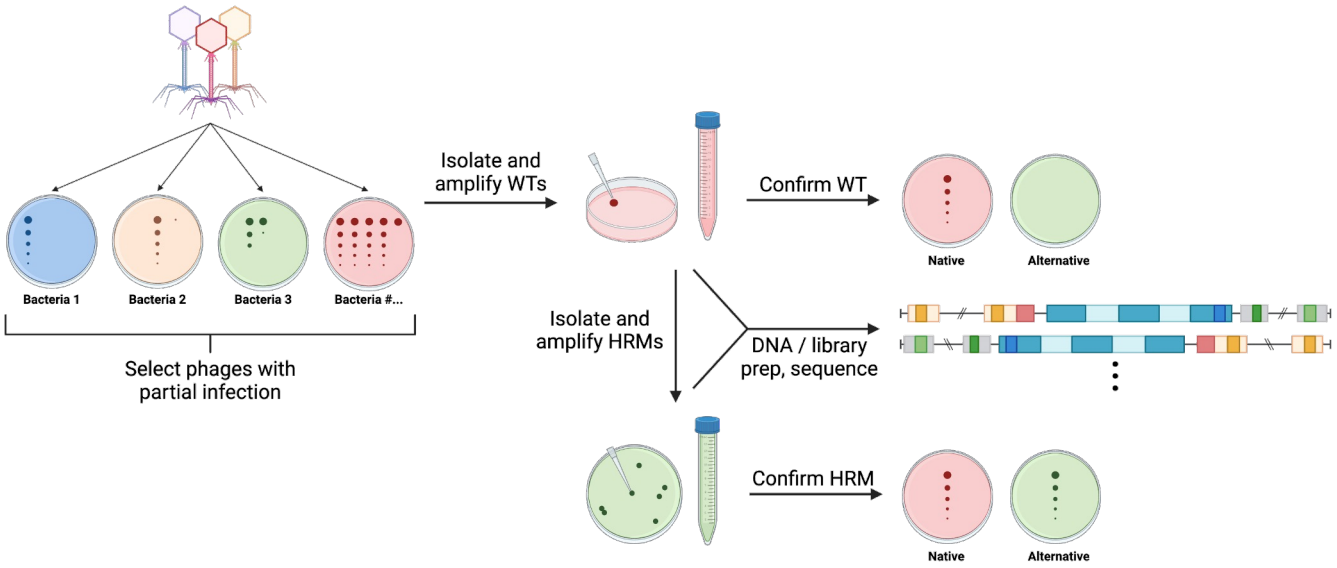
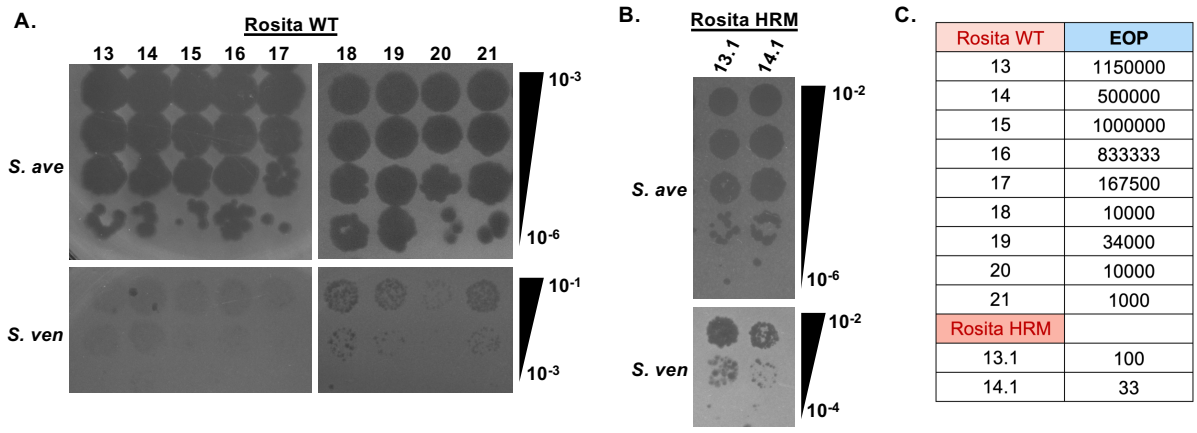


Figure 7. Workflow for isolating phage host range mutants. Phages demonstrating a partial infection on an alternative host are selected. These phages are purified and amplified as multiple WT lysates that display strong infection only on their native host. HRMs are isolated and purified from the WT lysates on the alternative host. HRMs display strong infection on both the native and alternative host.



C.

Rosita WT	EOP
13	1150000
14	500000
15	1000000
16	833333
17	167500
18	10000
19	34000
20	10000
21	1000
Rosita HRM	
13.1	100
14.1	33

Figure 8. Host range assay of Rosita wildtypes and host range mutants. Phages were serially diluted 1:10 and spotted on *S. avermitilis (S. ave)* and *S. venezuelae (S. ven)*. (A) WTs (13-21) from 10^{-3} to 10^{-6} on *S. avermitilis* and 10^{-1} to 10^{-3} on *S. venezuelae*. (B) HRMs 13.1 and 14.1 from 10^{-2} to 10^{-6} on *S. avermitilis* and 10^{-2} to 10^{-4} on *S. venezuelae*. (C) Efficiency of plating (EOP) of WT and HRM phages on each host. HRMs have a minimum ten-fold and maximum 10000-fold difference in efficiency on the native host versus alternative host. Difco Nutrient Broth (DNB) plates and Maltose Yeast Malt (MYM) top agar. Dilutions performed in phage buffer

and WT14. Rosita HRM13.1 and HRM14.1 had the ability to infect the alternative host to a higher degree than the WTs (fig. 8B). The EOP of Rosita WTs ranged from 1000 to 115000 (fig. 8C). There was a great reduction in the EOP of the HRMS, with values of 100 for HRM13.1 and 33 for HRM14.1, supporting that the phage had mutated to expand its host range.

Host range mutants infecting S. venezuelae have low viability

Of the 25 Rosita and Moonwalker WTs tested against *S. venezuelae*, two HRMs from Rosita were amplifiable and maintained their initial phenotype. Multiple attempts at the isolation of HRMs from each WT were performed. Initially, the HRMs were amplified on the alternative host they were picked from. However, the phages resisted amplification, with few plaques appearing on the highest dilutions. In addition, when a sufficiently cleared plate was produced on the alternative host, collection of the lysate resulted in phages with several different phenotypes. One putative HRM collected from the alternative host presented like a WT, another lost the ability to infect the native host and infected the alternative host like a WT. To overcome the insufficient replication on the alternative host as well as the development of strange phenotypes, another method of phage amplification was attempted, where phage and bacteria are grown together in liquid culture, resulting in continuous amplification of the phage. Several ratios of phage to alternative host were attempted, none of which produced HRM lysate. Since the phages consistently resisted amplification on the alternative host, the native host was attempted. This method produced three high titer lysates of potential HRMs from Rosita, two of which (HRM13.1 and 14.1) maintained the phenotype. In addition, attempts were made to purify the HRMs across multiple rounds of plaque picking, however, the phages continued to resist amplification even in the second round. Moonwalker WTs gave rise to HRMs during the isolation, but the phages resisted amplification on both hosts, therefore no HRMs were found for this phage.

3.4 Sequence *Streptomyces* bacteriophages and host range mutants using the Illumina MiSeq platform

Rosita may have DNA modifications

To determine the mutations responsible for conferring expanded host range to the Rosita HRMs, these phages underwent DNA extractions and library preparations (fig. 9A). During library preparation, both phage genomes did not fragment to the desired size (150-200bps) following non-specific enzymatic degradation and most of the genome remained undigested (fig. 9B). Rosita HRM 13.1 particularly resisted digestion, as the band on the DNA gel is above those of the other genomes. Rosita WT 14.1 appeared to be completely digested, as there was no band above 4000bps.

To test whether these genomes were resistant to fragmentation, the length of incubation with the enzyme was extended to multiple time points, surpassing the recommended 40 minutes which should shear genomes to 100bps. Rosita WT14 was used and prepared alongside Laloosh, a phage with a previously successful library preparation. Rosita WT14's genome remained above the 250bp ladder at every time point from 25 minutes to 60 minutes, while Laloosh fragmented to below 250bps at 25 minutes (fig. 9C). In addition, the majority of Rosita WT14 genome remained undigested. At T0, the genome appears to have been partially digested without any incubation, evidenced by a faint band when the gel was run for 30 minutes; this band disappears from all time points when run for 1 hour. When the WT14 DNA was run uncut on a gel, there was a solid band greater than 4000bps, which discredits the digestion seen at T0 and all other time points as being a property of the DNA itself (fig. 9A). At each time point following T0, the band of the genome becomes fainter with a greater smear, indicating greater levels of digestion with longer incubation. This outcome was expected, however, when compared to Laloosh, the genome does not reach the same level of digestion.

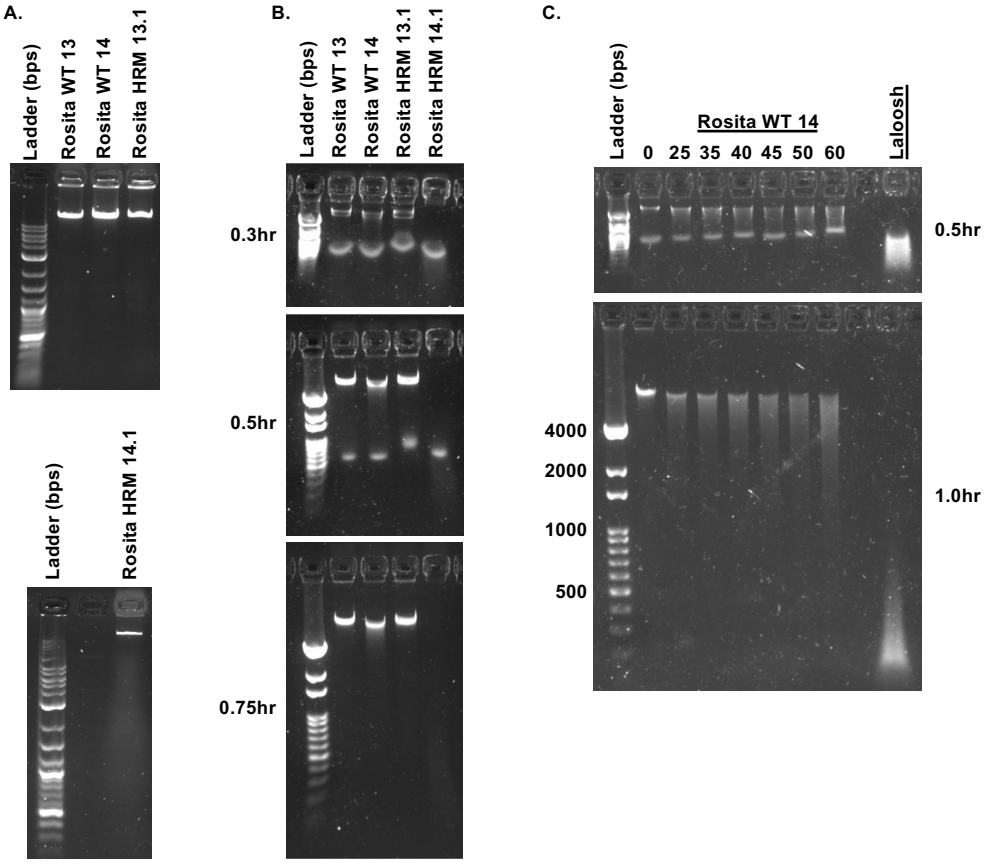


Figure 9. Illumina MiSeq DNA fragmentation. (A) Undigested DNA of Rosita WT13, WT14, HRM13.1 and HRM14.1. (B) Enzymatic DNA digestion of phages Rosita WT13, WT14, HRM13.1 and HRM14.1. (C) Enzymatic DNA digestion of phages Rosita WT14 taken at multiple time points during incubation. Phage Laloosh used as a positive control.

Streptomyces phage libraries can be prepared for Illumina sequencing

To expand the database of sequenced phages, 20/50 of the *Streptomyces* phages isolated in this study were amplified to a high titer lysate and underwent DNA extractions. 5/20 met the minimum DNA concentration for Illumina MiSeq library preparation. 3 additional phages were pursued. According to the Qubit dsDNA Broad Range quantification, the 8 library preparations did not contain DNA. The high sensitivity DNA Bioanalyzer also did not detect any DNA in the samples, which corroborated the results. Slight modifications to the protocol resulted in the preparation of 3 libraries of *Streptomyces* phages (Laloosh, Hosilda, Quackie) with minimal to no loss of DNA.

Chapter 4

Discussion

4.1 *Streptomyces* species have unique culturing methods that utilize spores

The sporulating nature of *Streptomyces* makes it a unique genus of bacteria to work with, with variations between species that call for procedural adjustments. Spore formation is the bacteria's reproductive method, but it is also a defense mechanism. Under nutrient deficient conditions, bacterial survival is at risk, so transforming into a state of low metabolic activity is favorable. In addition, spores can easily disseminate in the environment, facilitating the search for nutrient rich environments. Therefore, when the bacteria were plated on thin (nutrient deficient) versus thick (nutrient dense) agar media, the bacteria were triggered into sporulation (fig. 1C). Spores were harvested from plates throughout the course of incubation to determine the optimal window where spore concentration was highest. This window surpassed the recommended 3-5 days and was determined to be 8-10 days of incubation (87). The time course of sporulation was likely a function of nutrient availability. *Streptomyces* bacteria will sacrifice parts of the vegetative mycelium to provide nutrients for the aerial hyphae (63). Therefore, the bacteria facilitate its own growth in addition to the agar media on the plate. The saturation of the spore layer, initially quantified through the production of a white-grey pigment, varied from species to species and did not appear to be dependent on spatial organization, therefore, while spatial density is a trigger for hyphal formation, it was determined not to be for spore formation (3).

Spore stocks are the preferred method for handling these bacteria over liquid cultures. The working spore concentration meant small volumes could be used and remained viable over several months stored at 4°C, in comparison to liquid cultures that remain viable for 1-2 weeks. While liquid cultures are much easier to prepare and present less risk for contamination, most of these

species cannot grow a saturated, uniform culture. The mycelial nature of this genus resulted in the formation of clumps of bacteria in liquid, which when spread on a plate, did not form a confluent lawn. This was not conducive to phage protocols, where infection across an entire plate is necessary to produce distinct plaques and/or high plaque density plates. *S. venezuelae* and *S. griseus* were two species that sporulate in liquid favored with no formation of hyphal aggregates. This characteristic makes them favorable isolation hosts, which is likely one of the reasons that one third of *Streptomyces* phages recorded in phagesdb.org were isolated on *S. griseus*. The phages infecting *S. griseus* belong to a variety of clusters, including BB-I, BK, and BN, representing a diverse sample. Interestingly, of the six phages isolated on *S. avermitilis*, none could infect *S. griseus* when their host range was tested (fig. 6). This is likely a result of these species being phylogenetically distinct, indicating major differences that phages cannot overcome to infect both hosts (60). We can hypothesize that most phages found to infect *S. griseus* will be unique compared to the 50 phages we have currently isolated. Phage hunting on *S. venezuelae* has produced phages that were resistant to amplification and proved difficult to work with, highlighting how these phages may be interesting but present many obstacles if being pursued. There are currently 45 phages recorded on phagesdb.org which were isolated on various strains of *S. venezuelae*, emphasizing that these phages can be pursued, but that certain strains may be more conducive for phage protocols.

4.2 Isolation of novel bacteriophages is biased towards *Siphoviridae*

The isolation of these bacteriophages was influenced by the selective pressures influencing the dynamics of the environment, including temperature, soil depth, bacterial abundance, and many others.

Phages are not necessarily more or less different in geographically distinct regions. Phages found in Canada can have more genomic similarity with a phage found in Texas than another in the same country. For example, Celery demonstrates higher sequence homology with ElGato and Provolone, phages isolated in North Texas, compared to EnochSoames which was isolated at the same time as Celery in Canada (88).

Of the 50 *Streptomyces* phages found at the University of Ottawa, 49 were of the *Siphoviridae* morphology and one was a *Podoviridae*. Each morphotype has similar modes of infection, varying slightly due to specific functional proteins. Most phages were found in the Phage Discovery Lab which used *S. coelicolor* and *S. avermitilis* as phage isolation hosts in 2022 and 2023, respectively. We speculate that these phages are either not stable when grown on agar plates or within the phage buffer they are stored in. *Streptomyces* species produce a large array of secondary metabolites, some of which have been shown to affect phage infection (65, 89). In addition, growth on agar plates (rather than in the wild) may rapidly inactivate most phages.

Possible explanations could be phage stability, that very few viral particles are infectious following amplification, however this low fitness is not a very favorable characteristic. The bacteria may have been exerting pressure on the phage, either through release of chemical compounds inhibiting phage infection or through defense mechanisms that resulted in defective virions. Lastly, the phage that was picked may have been a mutant for the isolation host that was not very virulent for that species of bacteria.

Three of seven of the phages sequenced belong to the BD cluster, and similarities within restriction digest patterns suggest the majority of unsequenced phages do as well (data not shown). The bias towards BD-*Siphoviridae* phages likely reflects that phages from this cluster are more abundant and successful in this climate. One report found twelve phages from enriched isolations

on soil samples collected from several locations around the world that infect *S. coelicolor* or *S. avermitilis* (65). All twelve phages belong to the BD cluster, suggesting this cluster may be a primary predator of *S. coelicolor* and *S. avermitilis*.

Competition between phages may also be taking place since all the phages extracted from the soil sample are plated on the bacteria at once. A more lytic phage with a faster infection cycle and larger burst size will likely out-compete a temperate phage with a slower infection cycle and smaller burst size. Although most of these phages were found by direct isolation to minimize competition, when students find several phages in their samples with different morphologies, they are likely to purify the phages that have larger and clearer plaques, assuming (often correctly) that these will be easier to work with. Finally, our method of phage isolation selects for phages that are smaller than 0.22μm and for those that can diffuse well within agar, so our isolation technique may exclude large jumbo phages. Increasing the filter size to 0.45μm and encouraging unique plaque selection would obviously help mitigate some of these factors in future phage hunting.

Some of these traits may be hallmarks for *Siphoviridae*. Isolating and characterizing any phage is a valuable contribution to understanding their biology, however, the bias towards *Siphoviridae* phages with similar genomic structure is a limitation in building an accurate profile of phage diversity. It is important to broaden the scope of phage isolation and uproot the bias towards structurally similar phages, which may require phage hunting on a broader selection of hosts. It is possible that like the BD phages infecting *S. coelicolor* and *S. avermitilis*, *Siphoviridae* are the perfect predators for Actinobacteria.

4.3 Intricacies of *Streptomyces* affect bacteriophage amplification

Several methods to achieve high titer phage lysate exist. Infection in liquid culture, webbed plates, Cesium chloride gradient centrifugation, and agar scraping are several methods that have

been used to great success in phages infecting other *Actinobacteria*. For these *Streptomyces* phages, webbed plates accomplished titers of 10^9 pfu/mL but rarely much higher. For comparison, *Mycobacterium* phage lysate collected from webbed plates routinely can reach titers of $\geq 10^{11}$ pfu/mL and as high as 10^{15} pfu/mL. The genus *Streptomyces* is unique because of its mode of replication and production of secondary metabolites but how these two factors influence phage infection and amplification is not well understood. Phages preferentially infect immature spores and/or during hyphal growth, likely due to structural differences such as the expression of surface receptors when the bacteria abandon their vegetative state and the accessibility of host replication machinery (90, 91). Therefore, the age of the spores, as well as the ratio of the population actively sporulating will affect the number of bacteria phages can infect (39, 63). In addition, it has been shown that in the presence of mature mycelium, there are high rates of adsorption to the bacteria without productive infections (60, 63). This decreases the multiplicity of infection (MOI), which reduces the likelihood of infection and the resulting titer. It must be taken into consideration that the spores used during phage protocols may continuously be aging when they are not frozen, which would account for differences in infection of the same phage as the same stock is used. It is also likely that plates incubated for longer may result in a lower titer as the phages are not infecting the older mycelium, and the hyphae gain transient resistance to infection. Overtime, phage viability within the plaque when plated on *S. venezuelae* also decreases, suggesting time is of critical importance when harvesting higher titer lysate (63).

Since the bottom agar layer has a much higher density than the top agar layer, most of the viral population will reside within the top layer (92). We showed that double flooding at 30°C produced the highest titers in phage lysates. This result indicates that even after an extended incubation, there is still phage left on the plate, suggesting there is a limiting factor to phage

diffusion. This is supported by the improvement seen with longer incubation times at higher temperatures. A second flooding on the same plate often results in an equally high titer as the first flooding (fig. 3).

We also tested if agar scraping, a technique that captures the entirety of the top agar layer, would result in the highest titers. The titers of these lysates are comparable to those seen in the flooding/double-flooding methods. We conclude that both methods are sufficient to produce high titers, which are generally lower than those seen in other genera.

It has also been shown that *Streptomyces* produce chemical compounds that may be inhibiting viral activity (65, 89). It is an important consideration when using these hosts that they may be killing the phages through a unique mechanism of chemical warfare that results in unexpected low titers.

4.4 The isolated bacteriophages have independent host ranges

Ten phages isolated on *S. coelicolor* were able to infect *S. avermitilis* to the same degree (fig. 5A). This is likely due to the similarity between *S. avermitilis* and *S. coelicolor*. Each species shares ~69% orthologous genes (93), with most of their genetic diversity isolated in metabolic gene clusters and the terminal ends of the linear chromosome. *Streptomyces* express rodlins and chaplins (proteins on the hydrophobic sheath) on their surface during growth of aerial hyphae. Rodlins function to organize chaplins into a “basketwise” appearance. *S. avermitilis* does not express rodlins, resulting in a disorganized arrangement of chaplins (60, 94). Such differences in expression of surface proteins indicates these phages may contain a receptor for the common chaplin on the surface of each bacterium, in addition to mechanisms to bypass defenses and compatibility with host replication machinery.

Only one of the 18 phages, EnochSoames, a BD1 cluster phage, infected the species *S. venezuelae* to the same degree as its isolation host. The phages may not contain receptors that are able to recognize substrates on the spore surface and they may not be compatible with the host defense mechanisms. EnochSoames may be recognizing a different structure that is common to all three species and contains multiple anti-defense systems to enable successful infection. This is particularly interesting considering *S. coelicolor* and *S. venezuelae* share ~64% protein sequence identity. One major difference between these two species is that *S. venezuelae* can complete its developmental cycle in liquid, alluding to differences in their regulatory mechanisms. Differences in life cycle may explain why other phages were not able to propagate on this species, in addition to differences that exist at the terminal ends of the genome and metabolic clusters. Alternatively, these differences in host range may be determined primarily by differences in the phage genome. The way a phage's host range can vary across very similar species of bacteria is remarkable and a future path of this work would be to determine if genes in the non-conserved regions in EnochSoames are needed for its expanded host range in *S. venezuelae*.

The phages EnoachSoames and Celery both belong to the BD cluster but show different levels of infectivity on three *Streptomyces* species. A comparative analysis of both EnochSoames and Celery's genomes shows that while they do share nucleotide homology through the beginning and middle of the genome, within the 3' variable end of the genome, there are many genes that are shared by both phages but do not have a syntenic organization, as they are separated by non-conserved genes. It is possible that the differences in host range might be assigned to these genes, most of which are of no known function, but may function to bypass bacterial defense systems (44). Both phages encode for four minor tail proteins/RBPs at the beginning of the genome that belong to the same protein families. Therefore, if host range is caused by the minor tail proteins,

small sequence changes may have a large difference in the ability of the phage to interact with difference hosts.

4.5 Diverse phenotypes in similar phages

The phages isolated on *S. avermitilis* did not share the same ability to infect *S. coelicolor* as the phages isolated on *S. coelicolor* did for *S. avermitilis*. Infection seen on *S. venezuelae* was similar. Based on the results of figure 2, it was expected that these phages would have similar infection levels on *S. avermitilis* and *S. coelicolor*. However, 4/6 had significantly reduced infection on *S. coelicolor*. This may signify that there are characteristics involved in phage infection that are shared from *S. coelicolor* to *S. avermitilis*, but not vice versa. Therefore, these four phages may be targeting *S. avermitilis* through means that are not shared between the two species. In addition, the challenges we encountered working with *S. coelicolor* phages highlights the possibility that these phages' native host was *S. avermitilis*, and during phage purification, host range mutants that infect *S. coelicolor* were selected but were not able to achieve the same level of infectivity on this alternative host. Therefore, the higher infectivity seen on *S. avermitilis* by the phages isolated on *S. coelicolor* may be because they are more compatible with that host in the first place. As will be discussed later, the Rosita and Moonwalker HRMs are not easily amplified on the alternative host *S. venezuelae.*

Moving forward, a broader range of hosts should be tested to fully encapsulate the phages' infective ability. Should the phage's terminal genes hold the answer to determining host specificity, testing a broader selection of strains and species could support this hypothesis should similar phages not display the same infective ability. Based on our preliminary data on these phages, it is likely that large differences in host range will reveal themselves for dissimilar phages.

The overall finding, that similar phages can demonstrate different infective abilities on very similar hosts, but also that different phages can demonstrate the same infective ability on very similar hosts is a perfect example of the complexity of phage-host specificity. It is also an excellent example of phage diversity, where two genetically similar phages express themselves differently phenotypically and vice versa. Moving forward the sequences of these phages can suggest where genetic variability exists and how it may contribute to host range, as was seen in EnochSoames and Celery.

4.6 Host range mutants arise on alternative hosts

Several phages such as Moonwalker and Rosita infected *S. avermitilis* and *S. coelicolor* to the same degree but had the appearance of only a few plaques on *S. venezuelae*. These plaques were representative of individual phages that were able to infect an alternative bacterium. The outcome of *lysis from without* and abortive infection were ruled out in this case (86). *Lysis from without* is unproductive infection with no release of phage progeny but results in the death of the bacteria, causing a zone of clearing resembling a plaque. With the case of Moonwalker and Rosita, the level of infection is minimal at the lowest dilution and individual clear plaques are seen, ruling out that this is caused by *lysis from without*, and there is the production of viral particles.

The plaques from Moonwalker and Rosita appearing on the highest dilutions on *S. venezuelae* were hypothesized to be individual phages that had gained the ability to infect an alternative species and broaden their host range. This ability would be mediated through a gain of function or a loss of function mutation that occurred spontaneously during phage replication. Given the rate and breadth of phage replication, they are highly susceptible to mutation (95, 96). Amplification of numerous independent WT lysates allowed the opportunity for independent mutations to arise and then be selected on the alternative host.

4.7 Host range mutants have improved infection on alternative hosts

Two HRMs from Rosita WTs 13 and 14 had EOPs with greater than 10000-fold reductions, representing an expansion of host range. HRMs 13.1 and 14.1 lysates came from individual plaques that arose from WT lysates 13 and 14 on the alternative host and amplified on the native host. The reduction in EOP (approaching 1) is a large indication that the phage has gained the ability to infect a new host.

We hypothesize that this expansion was mediated through mutations in either the receptor binding proteins and/or anti-defense mechanism genes. Mutations to receptor binding proteins could expand host range in a multitude of mechanisms. During infection, phages may have two recognition steps mediating adsorption to the bacterial cell wall, an irreversible and a reversible binding through the same or secondary receptors. The probability a phage infects a bacterial cell is random; since phages are immotile, they do not search their environment for a bacterium but rely on the chance encounter and the adherence to the surface through these receptors. A mutation to the first receptor responsible for reversible binding may improve recognition thereby increasing the probability of an encounter and of infection. Alternatively, a mutation to the secondary receptor responsible for irreversible binding may facilitate adsorption through binding to a new substrate. Another possibility is that the phage bypasses the need for one of its substrates and is therefore less specific and reliant on that receptor. Given that both HRMs maintained their infectivity on the native host, this mutation would have altered the receptor in such a way to recognize both hosts.

HRM mutations may arise in genes needed for anti-defense mechanisms. *Streptomyces* contains restriction enzyme systems that target phages and protect against infection by recognizing their DNA. It also produces chemical compounds that act as DNA intercalating agents (97). In turn, phages have developed mechanisms to defend their genomic DNA once inside the cell.

Therefore, it is possible that HRMs 13.1 and 14.1 may have gained the ability to bypass a defense system in *S. venezuelae* that normally protects against phage infection. For example, a base change within a DNA sequence might bypass recognition from a CRISPR spacer or restriction enzyme. To understand how the phage gained infectivity and expanded its host range, genomic sequencing is necessary to visualize changes between the HRM and WT.

It is unlikely that the host itself is responsible for the increase in host range.

4.8 Rosita may have genome modifications

The Rosita WT13, WT14, and HRM13.1 resisted enzymatic degradation and did not fragment to the desired size of 150-200bps. With longer incubation with the nuclease, some degradation of the genomes is observed, but we believe a DNA modification on the Rosita genome is preventing fragmentation. It could be that the Rosita genome does not have a sufficient number of restriction sites for this enzyme—this is unlikely, since the enzyme is capable of binding to the DNA non-specifically. This could also be due to inactivity of the enzyme, which over time lost its catalytic activity, however this is unlikely because the enzyme was used previously to prepare successful libraries of other *Streptomyces* phages.

These results suggest that Rosita may contain DNA modifications that create steric hindrance for the enzyme, inhibiting cleavage of the DNA. Examination of a restriction digest of wild type Rosita DNA with several enzymes supports this proposal, in particular, BstUI, SmaI and SacI, which contain recognition sites that only contain GC, did not digest the Rosita genome. Rosita has not been sequenced, so we cannot know for certain if these sites are present in the genome. It is very rare for any Actinobacteriophage, which typically have high GC content, to lack BstUI sites. Confounding a simple block to any site containing GC base pairs, several enzymes

that contain GC did cut, including HaeIII, which recognizes a different four base recognition site than BstUI.

DNA modifications on phages are common and are a counter-defense to bacterial restriction modification defenses, which has been described in a variety of cases (98–100). New modifications are frequently discovered and a MSc student in the laboratory will be examining Rosita DNA that has been digested to nucleosides by mass spectrometry to detect potential modifications (101). To overcome the challenge of creating Illumina sequencing libraries of digestion-resistant DNA, alternative library preparations that utilize shearing for fragmentation, such as sonication, will be attempted.

Chapter 5

Conclusion and Future directions

Bacteriophages are the most abundant biological entity on the planet with the greatest reservoir of genetic material on Earth. They have incredible genetic plasticity, constantly undergoing mutations, rearrangements, and natural transformations. Their relationship with bacteria in the genus *Streptomyces* is poorly characterized. *Streptomyces* is the largest genus of Actinobacteria, with over 700 species known to date. They are nature's most competent chemists, producing an extensive array of metabolites that are used in agricultural and clinical industries (102). In addition, this genus is mycelial, with a reproductive cycle resembling that of fungi. According to the Actinobacteriophage database, there are ~1500 phages that have been found to infect this genus, with fewer than 400 being sequenced (103). The nature of this bacteria sets the stage for complex and unique relationships with phages that are not seen in other genera.

This study aimed to expand our understanding of this unique phage-host relationship by isolating 50 novel phages infecting *S. avermitilis, S. coelicolor*, and *S. venezuelae*. These phages were characterized and included in the Actinobacteriophage database. All but one of the novel phages belong to the morphotype *Siphoviridae*, with that phage being a *Podoviridae*. Seven phages were sequenced and grouped into clusters based on nucleotide similarity. Three phages belonged to the BD cluster, two to the BE cluster, and one each to the BN and BF clusters.

The host range of these phages were tested against several *Streptomyces* species, and we found that genetically similar phages demonstrate markedly different host ranges, suggesting that in some phages small genetic differences can have a large influence on their ability to infect different hosts. Hypothesized and known genetically similar phages demonstrated opposite phenotypes during infection, those being narrow and broad host ranges. The opposite result was

also seen, where hypothesized and known genetically dissimilar phages demonstrated very similar phenotypes during infection. To gain further insight into the possible mechanisms of host range, an expanded reservoir of strains and species should be tested, particularly those that are more distantly related. In addition, genomic characterization of these bacteriophages is important to identify the genomic regions and genes implicated in host specificity.

Host range mutants were isolated from the wild-type Rosita phage. These HRMs, termed 13.1 and 14.1, gained the ability to infect an additional host through a hypothesized genetic mutation. Sequencing efforts have not yet been effective, as the phages and their WT genomes resisted the enzymatic digestion used in library preparation, possibly due to DNA modifications in Rosita's genome.

We can conclude that *Streptomyces* phages have unique characteristics compared to phages infecting other *Actinobacteria* that have caused multiple challenges for isolation and characterization. These differences highlight the diversity of phages and the significance of expanding our reservoir of bacteriophages to understand the expanse of phage diversity and their fascinating evolutionary history.

Moving forward, all novel isolated phages should undergo sequencing to expand the number of sequenced phages and provide insight into novel gene functions and phage diversity. In addition, HRMs can be isolated from hosts other than *S. venezuelae,* which may prove more agreeable towards amplification and library preparation. Identifying specific mutations within the HRMs will allow us to begin studying the mechanisms of host range using tools that modify phage or host genomes, explore the phenotypes of deletion or overexpression of candidate genes, identify complexes of phage/host proteins during infection using IP/mass spectrometry, examine the

dynamics of phage infection by RNAseq and live imaging. The specific approach will largely depend on the genes found mutated in the HRMs.

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