2019-07-01

Isolation, Genetic Characterization and Clinical Application of Bacteriophages of Pathogenic Bacterial Species

Trever Leon Thurgood
Brigham Young University

Follow this and additional works at: https://scholarsarchive.byu.edu/etd

Isolation, Genetic Characterization and Clinical Application of

Bacteriophages of Pathogenic Bacterial Species

Trever Leon Thurgood

A thesis submitted to the faculty of
Brigham Young University
in partial fulfillment of the requirements for the degree of

Master of Science

Julianne House Grose, Chair
Steven M. Johnson
Richard A. Robison

Department of Microbiology and Molecular Biology

Brigham Young University

ABSTRACT

Isolation, Genetic Characterization and Clinical Application of
Bacteriophages of Pathogenic Bacterial Species

Trever Leon Thurgood
Department of Microbiology and Molecular Biology, BYU
Master of Science

Bacteriophages (phages) are the smallest biological entity on the planet. They provide vast amounts of valuable knowledge to biologists. Phage genomes are relatively simple compared to the organisms they infect (prokaryotes) and yet continually point to the complexity surrounding molecular- and microbiological mechanisms of life. By studying phages we can learn of the systems of gene expression, protein interaction and DNA organization. Phages are useful not only from an academic perspective, but may also have useful clinical applications. In the face of the rise of antibiotic-resistant bacterial "super pathogens", scientists and researchers turn to phages as alternative treatments to these types of infections. Phages are capable of infecting and killing even the deadliest of bacterial pathogens, such as carbapenem-resistant *Enterobacteriaceae* (CRE) or *Bacillus anthracis*, and may prove increasingly useful in the future for combatting harmful pathogens. This thesis looks at several aspects of phage biology—from the underlying genetics contributing to phage virulence, to the clinical application of phage therapy to treat infections. First, a look at CRE-*Klebsiella pneumoniae* isolates and phages capable of infecting some strains may reveal a potential therapeutic approach in the future. Additionally, genomic analysis reveals interesting features that may explain aspects of phage virulence and evolutionary history. Then, a collection of genetically diverse phages is used in infection assays on pathogenic strains of *Bacillus anthracis* to establish the first-reported phages capable of infecting these strains. Finally, the process of preparing phage samples for therapeutic application is explored in-depth to conclude with discussion of clinical application. During the course of these projects over 25 phages were isolated, as many phage genomes were assembled and annotated, resulting in the preparation of two genome announcements and near-completion of two publishable first-author papers (chapters II and III). In addition, participation in a variety of collaborative efforts may lead to a handful of co-author papers and on various topics, including phage biology and application.

Keywords: bacteriophage, phage, carbapenem-resistant *Enterobacteriaceae*, *Klebsiella pneumoniae, Bacillus anthracis*, phage therapy

ACKNOWLEDGEMENTS

I want to thank my committee chair, Dr. Julianne Grose, for providing many opportunities for growth and learning, as well as providing the resources necessary for these projects. I also want to thank my committee members, Dr. Rich Robison and Dr. Steven Johnson for their continued guidance and encouragement during the past year. Under their supervision of my committee, I have improved as a scientist and scholar and will continue to utilize these skills throughout the remainder of my life. I also want to thank the Grose lab members for their assistance with a variety of tasks and problems throughout my time at BYU, and I appreciate their friendship. I specifically want to thank Kai Li Ong, for training me in wet-lab techniques when I began in the lab, and Ruchira Sharma, for training me in dry-lab techniques. Without their mentorship, I would not have been able to complete this degree. Additionally, I would like to thank Alyssa Applegate for her patience in training me over the course of the year to be able to work safely in the BSL-3 facility, as well as with the variety of antibiotic-resistant strains. I likely would not have survived this experience without her guidance. Finally, I would like to thank the 2018-2019 Phage Hunters class for allowing me the opportunity to mentor and teach my first class. I will remember my experiences in each of these labs and draw from these experiences as I continue to progress.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

CHAPTER I: Introduction to Bacteriophages and Host

Introduction

Pathogenesis of Enterobacteria *and* Firmicute *bacteria*

The first correlation between a specific disease and its causative bacteria was made in 1876 by Robert Koch, who discovered *Bacillus anthracis* to be the cause of anthrax.[1] Since then, numerous pathogenic bacteria have been discovered. The most basic division of bacterial species separates bacteria into three main groups based on cell wall structure: Gram-positive and Gram-negative and Acid-fast (Fig. 1-1). Pathogenic species are found in all three groups. Within Gram-positive bacteria, the phylum *Firmicutes* has a number of pathogenic genera that cause serious human infections including *Clostridium, Staphylococcus, Streptococcus, Listeria* and *Bacillus*. Of the Gram-negative bacteria, the family *Enterobacteriaceae,* also known as *Enterobacteria* or enteric bacteria*,* consists of many common human pathogenic genera, including *Klebsiella, Escherichia, Salmonella, Citrobacter, Serratia, Shigella* and *Yersinia. Mycobacterium tuberculosis* is one of the most well-known Acid-fast pathogenic species. This proposal focuses on 4 species found across Gram-positive and Gram-negative bacteria: *Klebsiella pneumoniae, Serratia marcescens, Citrobacter freundii* and *Bacillus anthracis* (see Table 1-1 for taxonomic summary).

B. anthracis is a non-motile, spore-forming, Gram-positive bacterium capable of causing disease in man and animals. While there is high genetic similarity among

Figure 1-1. Distinction between Gram-positive and Gram-negative cell wall structures.

pathogenic strains, Van Ert, et al. classified *B. anthracis* strains into 12 phylogenetic clades that separates them based on single-nucleotide differences.[2] One representative from each clade has been selected to test susceptibility to phage infection. Common to all pathogenic strains is the production of a tripartite toxin, suitably named anthrax toxin, which is responsible for pathogenicity in hosts.[2] The genes encoding the toxins are found on virulence plasmid *pXO1*.[3] Anthrax toxin is composed of edema factor (EF), lethal factor (LF) and protective antigen (PA), all of which cooperate to invade and lyse host cells.[2,4,5] PA forms a pore on the host cell surface, while EF and LF are transported through the membrane and carry out fatal enzymatic activities

Table 1-1. Taxonomic summary of bacteria in study.

Domain	Bacteria	Bacteria	Bacteria	Bacteria
Phylum	*Firmicutes*	*Proteobacteria*	*Proteobacteria*	*Proteobacteria*
Class	*Bacilli*	*Gammaproteobacteria*	*Gammaproteobacteria*	*Gammaproteobacteria*
Order	*Bacillales*	*Enterobacteriales*	*Enterobacteriales*	*Enterobacteriales*
Family	*Bacillaceae*	*Enterobacteriaceae*	*Enterobacteriaceae*	*Enterobacteriaceae*
Genus	*Bacillus*	*Klebsiella*	*Serratia*	*Citrobacter*
species	*anthracis*	*pneumoniae*	*marcescens*	*freundii*

Figure 1-2. Infection cycle of *B. anthracis.* Sequestration of plasmid *pXO1*- and *pXO2*-containing *B. anthracis* to its sporulated form, subsequent inhalation and infection of immune cells. Sporulation is a reversible process, which is necessary for toxin expression and successive infection.

that lead to apoptosis and subsequent necrosis (Fig. 1-2).[2,4] Anthrax toxin causes disease by interfering with host immune cell signaling pathways such as mitogen-activated protein kinase (MAPK) and chemokine networks, ultimately inhibiting proinflammatory and cellular transcriptional responses that would otherwise activate immunity cascades.[2,4–7] In addition to this virulence plasmid, pathogenic strains of *B. anthracis* also contain a second plasmid, *pXO2,* encoding genes for a poly-γ-D-glutamic acid capsule that aids in host immune evasion.[8,9]

Another trait of *Bacillus* species is the ability to form spores. As a spore, *B. anthracis* is estimated to have a half-life of 100 years.[10] Spore formation protects the cell from environmental dangers, including high or low pH, UV radiation, extreme temperatures, desiccation, nutrient depletion and mutagens.[11–13] Sporulation enhances pathogenicity by allowing *B. anthracis* to become aerosolized and inhaled by the host (Fig. 1-2). Upon inhalation, alveolar dendritic cells and macrophages become the point of entry into the host lymphatic and circulatory systems, leading to rapid sepsis and likely mortality.[14–17] This set of molecular mechanisms empowers *B. anthracis* to be a formidable pathogen and potential bioweapon.[10,18]

Pathogenicity of the *Enterobacteriaceae* species varies greatly from that of *Bacillus anthracis*. Many *Enterobacteriaceae* are opportunistic pathogens that cause secondary infections in immunocompromised individuals and are one of the main causes of nosocomial infections in the United States.[19] Infection with an enteric bacteria begins with invasion of susceptible tissue. Common infection sites include lung tissue, catheter entry sites and urinary tract, surgical wound sites, burn sites and the bloodstream.[20] Once introduced into vulnerable sites, pathogenic enteric bacteria establish infection by adhering to surrounding tissues through adhesive extracellular components such as pili and fimbriae, adhesins and intimins, and extracellular polysaccharides

(EPS).[20–23] Additional virulence factors include type-III secretion systems that inject toxins and host signal transduction disruptors that appropriate host metabolic activity.[23]

Antibiotic-resistance confers protection for bacterial cells

Since the discovery and mass-production of antibiotics in the mid-twentieth century, antibiotic-resistant strains or serotypes of bacterial species have increased globally.[24] While a number of new classes of antibiotics have been introduced over the past half-century, the rate of acquired resistance exceeds the rate of discovery.[25] This precarious reality merits the study of alternative treatments to bacterial infections, which remain among the leading causes of death worldwide.[26]

Carbapenems are a sub-class of ß-lactams capable of potent antimicrobial activity. They are defined as the 4:5 fused ring lactam of penicillins with a double bond between C-2 and C-3, but with the substitution of carbon for sulfur at C-1 (Fig. 1-3).[27] The expanded structure of carbapenems from penicillins directly improves effectiveness against microbes, as ß-lactamases are less

Figure 1-3. Carbapenems. General structure (a) and specific examples of carbapenems (b-d). Additions in R-group side-chains can be as simple as a methyl group or more complex structures that directly affect effectiveness in killing bacteria and susceptibility to ß-lactamases.

effective in hydrolyzing the molecule due to their irregular structure. In modern medicine, carbapenems have served as "last-resort" antibiotics in order to minimize development of antibiotic-resistance. Nonetheless, like every preceding class of antibiotics, continuous exposure

of bacteria to these compounds has yielded resistance, whether through intrinsic or acquired mechanisms.[27,28] These mechanisms of resistance-acquisition are endemic within *Enterobacteriaceae* and are leading to increasing numbers of long-term infections and deaths per year.[24,29,30] These bacteria, Carbapenem-resistant *Enterobacteriaceae* (CRE), have been named as a top health concern by the CDC. Furthermore, horizontal gene transfer has contributed to both the onset and worsening of the antibiotic crisis, with critical resistance genes passing between even the most unrelated bacteria.[27–31] There have not been, however, any reported clinical cases of antibiotic-resistant *B. anthracis* infections. Yet, the ease of genetic engineering may also place this potential weapon on the list of antibiotic-resistant bacteria.

Bacteriophages differ drastically from antibiotics in antimicrobial mechanisms of action

While phages have been known since the beginning of the twentieth century, even before the advent of antibiotics, their use in Westernized medicine has been limited.[32] Nevertheless, in the face of spreading antibiotic-resistance, phage therapy is again garnering interest. In contrast to antibiotic compounds, phages are independent biological entities capable of permanently altering bacterial lifecycle and genetics. Also dissimilar to antibiotics, phages are capable of co-evolution with their host, and undergo genomic alterations favorable to maintain virulence.[33] These phenomena are essential for the successful infection of the host by a phage.

Phage infection begins with virion adsorption onto the host cell via recognition of bacterial surface components. This surface component is typically a trans-membrane protein or signal receptor, though surface enzymes, transport channels, pili, flagella, capsular elements, specific moieties of LPS on Gram-negative bacteria as well as teichoic acids in Gram-positive hosts, can serve as targets.[34] Host targets are recognized by the phage's tail fibers, which serve as attachment points for the viral particle. Post- adsorption to the cell surface, the phage undergo a
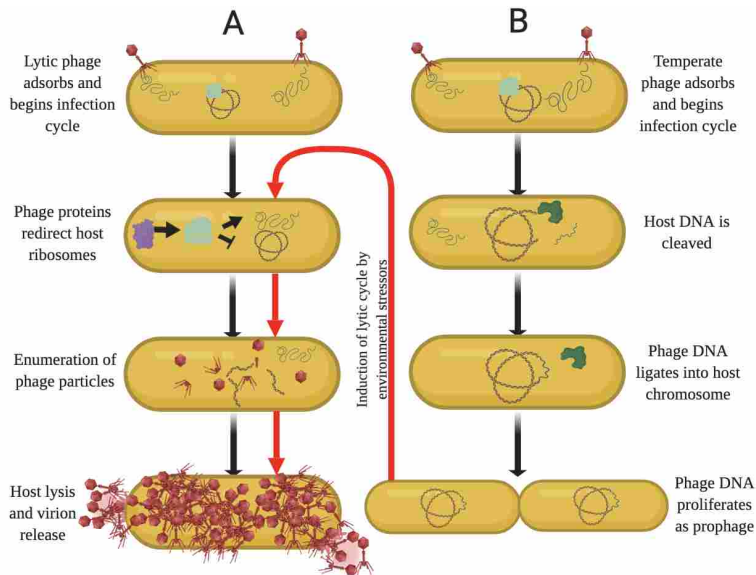
Figure 1-4. Lytic versus lysogenic phage lifecycle. (A) Injection of phage DNA into the host cell, appropriation of host metabolic machinery with use of transcription factors, gene expression and virion production, lysis of the host and subsequent spread into the environment. (B) Injection of phage nucleic acid into the host cell, cleavage of host genome, integration of phage DNA into host chromosome, and subsequent DNA proliferation. Integrated phage DNA now considered a prophage.

conformational change that involves injection of viral nucleic acid into the bacterial cell. Upon entry to the cell, the phage can initiate one of two lifestyles: lytic or lysogenic. In the former, successful infection relies on the timed order of gene expression, commonly referred to as early-, middle- and late-genes. Timed gene expression is essential for successful host infection, as phage lifecycle is dependent on specific proteins being expressed at different timepoints during the infection period. For example, transcriptional regulators designed to take over bacterial metabolic machinery are immediately expressed, which directs bacterial polymerases and ribosomes to replicate and express the viral genome. Then, as the infection cycle progresses, structural and enzymatic proteins are expressed (Fig. 1-4A). In the lysogenic lifecycle, phages initiate infection in a similar manner, but diverge in lifestyle by inserting the viral genome into the host genome (Fig. 1-4B). This can occur via site-specific recombination (SSR) or via homologous recombination (HR).[35] Phage DNA then proliferates with the host and can remain in the host genome permanently.

Bacteria susceptible to infection by a phage are defined as being part of the phage's "host range". As bacteria acquire mutations, phage recognition sites may become unrecognizable to the phage's tail fibers such that the phage is no longer able to adsorb onto the cell surface to initiate infection, and the bacterium is no longer within the host range of the phage. Regardless, as a bacterium evolves out of the host range of one particular phage, it likely will evolve *into* the host range of a different phage (Fig. 1-5). Bacteriophages outnumber bacteria by ten to one.[33] Thus, it almost assuredly follows that there is a phage capable of infecting every bacterial species, strain and serotype.

Figure 1-5. Mutation prevents one phage infection, permitting another. (A) The red phage has tail fibers capable of recognizing the receptor protein (labeled RP1) and adsorbs to the cell surface. (B) After adsorption, the phage infection cycle begins. (C) RP1 is randomly mutated, becoming ΔRP1, and is no longer recognizable by the red phage's tail fibers, thus preventing adsorption and infection initiation; (D) however, the mutation now makes ΔRP1 recognizable by the blue phage, which can adsorb and initiate its infection cycle.

It is for this reason that phage therapy is of great interest. When a bacterial strain becomes resistant to antibiotics, without alternative treatment, a patient is likely stuck with the infection indefinitely; however, utilization of a phage's natural host range may serve as an alternative antimicrobial to otherwise resistant bacteria.

Aside from surface protein mutations that confer resistance to bacteriophage infection, capsule expression and biofilm production may also inhibit phage infections. For example, the poly-γ-D-glutamic acid capsule of *B. anthracis* may play a role in protection against phage recognition of the host cell wall. In 2013, Negus, et al. found that capsule production by *B. anthracis* prevented phage infection of host cells, contradicting previous studies reporting that capsule production played little to no part in inhibiting phage infections.[36] Together, these studies lead us to question which phages have the ability to infect capsule-producing strains of *B. anthracis*.

Bacteriophages are capable of infecting antibiotic-resistant bacteria

Bacteria that become resistant to antibiotics have developed one or more mutations that change the effectiveness of some types of antibiotics. For example, a bacterium may alter some essential metabolic pathway inhibited by the antibiotic, while others may develop efflux pumps that specifically target antibiotic compounds.[37] These adaptations are generally acquired via plasmid transfer.[37] Plasmid acquisition and retention generally come at a cost to the bacteria, but when the plasmid confers resistance to otherwise fatal antibiotics, the benefits outweigh the cost.[38] Despite this beneficiary fitness tradeoff, however, phage challenge can alter the fitness cost of maintaining antibiotic-resistant pathways. In a 2016 study, a *Pseudomonas aeruginosa* bacteriophage, OMKO1, that targets bacterial outer membrane porin M (OprM) which is used in a multi-drug efflux system, forced a mutation of the receptor leading to increased sensitivity to antibiotics.[32] Conversely, phages have also been known to transfer antibiotic-resistance genes between bacteria, though no phages are currently known to be the source of resistance genes.[39] Such occurrences are important considerations in phage therapies, so as not to strengthen resident bacteria in an infected site, when the aim is to eradicate them. Furthermore, because

resistance to phage infection can develop within a bacterial community, having genetically diverse phages can increase the chances of a successful treatment (i.e. due to the fact that phages use different entry mechanisms, and a bacterial population is unlikely to spontaneously mutate multiple receptors at once).

Summary of aims to elucidate bacteriophage infection mechanisms

For the reasons previously detailed, it is the purpose of this project to isolate, sequence and genetically characterize phages capable of infecting a variety of clinical multi-drug resistant bacterial strains. The strains in question include a variety of CREs (*Klebsiella pnuemoniae, Serratia marcescens, Citrobacter freundii*) as well as the Gram-positive, pathogenic *Bacillus anthracis* strains, which heretofore have never been reported to be susceptible to any phage infections outside of the Gamma phage cluster. We seek to uncover some of the mechanisms used by phages to infect antibiotic-resistant bacterial strains, possibly shedding light on novel phage molecular genetics. These studies lead to results that may be clinically useful for treatment of antibiotic-resistant infections via phage therapy. Additionally, the results may have implications beyond the scope of these studies. The specific aims for this project are as follows:

Evaluate phage efficacy against clinical isolates of Klebsiella pneumoniae

Using a library of drug-resistant clinical isolates of *Klebsiella pneumoniae*, susceptibility to bacteriophage infection is analyzed. Then, genomic analysis serves to identify unique properties that may elucidate phage infection mechanisms.

Establish host range of Bacillus anthracis *bacteriophages against pathogenic strains*

Using a collection of newly-isolated *B. anthracis* bacteriophages, define the host range against pathogenic strains of *B. anthracis* for each viral genetic family represented by phages

9

within the collection. A sub-aim includes genomic analysis of phage genetic makeup to further characterize our collection in relation to known phages.

Isolate, prepare and administer phages for treatment of a Serratia marcescens *infection*

Under the guidance of the FDA, phages isolated from sewage using a clinical isolate of *S. marcescens* is be purified, amplified and purged of endotoxin in preparation for use in treatment of a human infection. Genomic analysis and RNAseq of at least one of these phages will reveal genes used during the course of infection.

Experimental plan

Isolate novel phages against clinical and non-clinical strains

Because the bacterial strains being used in this proposal are human pathogens, strains and phages should be obtained from a source abundant with the pathogens. Untreated sewage, also known as primary influent or raw water, contains a high concentration of these pathogens and their relevant phages. Phages, whether lytic or lysogenic, require a host to replicate and are found where bacteria are abundant. By incubating bacteria-of-choice with raw sewage, phages capable of infecting the host bacteria will enumerate. Centrifugation and filtration remove debris and unwanted bacteria, leaving behind phage in the lysate. This lysate can be spotted onto the host bacteria or used in a plaque assay to produce isolated plaques. In either case, plaque purification must be done a minimum of three times for the purpose of "purifying" phage from additional virions, toxins, and unwanted pathogens from the original enrichment culture.

Determine susceptibility of multi-resistant K. pneumoniae *strains to phage infection*

The wet-lab portion of this aim was completed by Olivia Tateoka in summer 2018. Due to sequencing concerns revealing multiple phage genomes in a single sample, phages were separated via plaque purification, phage presence confirmed with PCR and her results replicated.

Determine host range of B. anthracis *phages against pathogenic strains*

Because *B. anthracis* is considered a "select pathogen" by the CDC, there are stringent regulations surrounding its use. For this reason, all pathogenic *B. anthracis* work is be done in the BSL3 facility following all safety protocols established by BYU and the CDC. The extent of the host range testing is to determine the variety of phages that can infect different strains of *B. anthracis*. Van Ert, et al., classified over 400 clinical *B. anthracis* strains into twelve phylogenetic clades.[40] In order to account for this genetic variability, we have selected one strain from each clade as a representative host to test infectability

Figure 1-6. Dot plot comparison of *B. anthracis* phages. Bolded names are already-published phages. Colored squares represent clusters, with sub-clusters separated by dotted lines. Individual phage genomes are separated by black lines (horizontal and vertical). Diagonal lines appear when there is >50% nucleotide similarity between phage genome segments.

by our phages. Additionally, of our 24 *B. anthracis* phages, they can be separated into 6 clusters and 2 sub-clusters, each of which is genetically distinct from the others (Fig. 1-6). Therefore, to account for all variability, a representative phage from each cluster has been selected to infect all twelve strains of *B. anthracis*.

Preliminary results conducted by Hyrum Shumway show that almost all phages are capable of infecting all strains. This finding is particularly interesting because all of the phages used in the study were isolated on the *Sterne* strain of *B. anthracis*. *Sterne* is missing the γ-D-

glutamic acid capsule-encoding plasmid, *pXO2*, whereas the pathogenic strains all produce the capsule. This variance in surface structure does not change susceptibility to phage infection as would be expected and merits further investigation. Additionally, we want to know if the phages are capable of infecting the sporulated form of *B. anthracis*. In contrast, spores produce unique surface components when compared to vegetative cells, which may alter phage recognition sites.

Genetically characterize phage genomes with bioinformatic tools

While phage therapy is the clinical application of phage research, genomic analyses are critical to understanding the underlying molecular genetics driving phage lifecycle. Besides genome sequencing, tools like Phamerator maps, Splitstrees, dot plot comparisons, individual gene sequence alignments, motif identification, and BLAST comparisons elucidate some of these mechanisms. Phamerator maps highlight differences between highly related phage genomes, as well as similarities between highly different phage genomes. Phamerator identifies conserved domains in predicted protein products and categorizes phage genes accordingly. A table of protein products contained within the gene products included in the analysis can be exported and converted to the file-type necessary for SplitsTree usage. SplitsTree phylogenies compare only the presence of protein families in determining relatedness among organisms, rather than the standard way of comparing nucleotide or amino acid sequences. While a dot plot and SplitsTree may predict overall phylogeny, alignments of individual amino acid sequences from select proteins and subsequent phylogenetic tree production can indicate different relationships among organisms. This is particularly pertinent within phage biology, as gene transfer and genome rearrangements are highly common. Thus, MEGA X and Kalign is be used to produce phylogenetic trees for additional analyses.[41] Multiple Em for Motif Elicitation (MEME) is used to predict DNA motifs within phage genomes.[42] DNA motifs tend to indicate protein-binding

sites within a genome, and predicted motifs found in the phage genomes of this study are compared to known motifs through prokaryotic motif databases using TOMTOM.[43]

By performing these analyses we expect to find a number of interesting genomic artifacts that may lead to new understanding of phage genetics. The *K. pneumoniae* and *B. anthracis* phages may contain clues that explain why some phages are capable of infecting certain strains, while others are not. Combined with wet lab techniques, genomic analyses can help identify patterns that coincide with observable patterns (phage infections, in this case) and may provide insights into gene functionality.

RNAseq analysis reveals early, middle and late genes required for infection

A crucial aspect of phage virulence is the expression of essential genes at specific time points during the infection cycle. While variability exists between phages, general classes of proteins are universally expressed at early, middle and late timepoints. Sequencing RNA extracted at early, middle and late timepoints during an infection can provide clues as to the functions of otherwise unknown gene products. Such analysis is particularly interesting with completely novel phages, those which have completely unique genomes. From our own collection, we have selected several such phages, one of which is capable of infecting a multi-resistant strain of *S. marcescens*. RNAseq analysis provides insight into the lytic cycle of the phage and aids in further characterization through protein identification.

Under FDA guidelines, prepare phages for clinical therapy

Our lab was contacted in 2018 by several doctors and a veterinarian seeking assistance in isolating and preparing phages to fight clinical infections. Of these, two requested complete preparation of the phages, from isolation to amplification and elimination of bacterial endotoxin. As Gram-negative bacteria proliferate, they shed LPS into the surrounding culture. LPS is a

13

powerful inducer of the innate immune system, and a phage therapy preparation, if left untreated can lead to septic shock and death.[44,45] With phage infection, additional bacterial lysis occurs, thus increasing the concentration of LPS in the lysate. The human immune system innately responds to and targets LPS, even at low concentrations.[46] Therefore, it is critical that all LPS is removed from a phage lysate before therapeutic use. There are two techniques reported to complete this process with high degrees of efficiency. These are used in the process of preparing phage therapies. The first method is a polyethylene glycol (PEG) 8000 high-speed centrifugation-precipitation protocol, adapted from the Baker lab at University of Washington.[47] The PEG aids in phage conglomeration, which then pellet at high speeds (12,000 $x\ g$) and separates them from the endotoxin-containing supernatant. The supernatant is removed, and the phage resuspended in endotoxin-free buffer. The second method involves organic solvent extraction of the LPS, typically 1-butanol or 1-octanol, and phase separation.[48] Afterwards, the organic solvent is removed through ethanol and sodium dialysis. The PEG precipitation is the faster protocol while the organic solvent phase separation protocol reports lower residual endotoxin levels. In our lab, we use the ToxinSensor[TM] Chromogenic LAL Endotoxin Assay Kit (GenScript; New Jersey, USA) that utilizes limulus amoebocyte lysate and chromogenic substrate to quantify LPS levels, before reporting to the FDA for treatment approval.

Potential problems

Between the wet lab techniques and computational analyses, there are a number of potential problems that may arise during the process of characterization. By comparing phage genomic elements to already-established databases, there is a risk of missing novel genetic components. For example, MEME predicts the presence of motifs, some of which may be novel, but by comparing them to known prokaryotic motifs through TOMTOM, some of the motifs may

not match recognized motifs and appear statistically insignificant. Without extensive lab work to characterize novel motifs, their functions will continue to be unknown.

Additionally, working with pathogenic strains with high mortality rates of infection (*B. anthracis*) or with known multi-drug resistance (*Enterobacteriaceae*) is extremely dangerous. As such, many safety precautions are put into place for the protection of both the experimenters and the general lab population. Increased safety measures can decrease efficiency, particularly in timed assays, such as plaque assays. Therefore, careful planning is required before initiation of experimental procedures. In working with *B. anthracis*, for example, a BSL3 facility is required. All materials must be prepared and ready-for-use before entering the facility. Fortunately, familiarity with the facility can eliminate a plethora of problems.

CHAPTER II: Evaluation of Bacteriophage Against Clinical Isolates of Carbapenem-Resistant

Enterobacteriaceae

Authors: Trever Thurgood, Olivia B. Tateoka, Richard A. Robison and Julianne H. Grose

This chapter is taken from a publication in preparation for submission at the time of writing of

this thesis.

Abstract

Bacteriophage (phage) therapy was an effective treatment against bacterial pathogens that

was discovered nearly a century ago but was quickly abandoned in the western world with the

advent of antibiotics. There has been renewed interest in phage therapy due to increasing

occurrence of antibiotic resistant bacterial pathogens such as carbapenem-resistant

Enterobacteriaceae (CRE), which currently have a fifty percent mortality rate. To explore the

possibility of phage therapy as treatment for these multi-drug resistant infections, fourteen novel

phages were tested against clinical isolates of carbapenem-resistant *Klebsiella pneumoniae*.

Several of the phages were able to infect these clinical isolates suggesting that phage therapy

may be a viable option for treating CREs. Genomic analyses indicates some of the genetic

components that could potentially be responsible for the ability of the phages to infect these

bacterial hosts.

Introduction

Klebsiella pneumoniae, a member of the *Enterobacteriaceae* family, is one of the most

common Gram-negative bacteria responsible for hospital-acquired infections, frequently causing

urinary tract infections, pneumonia, and bacteremia[49,50]. As opportunistic pathogens*, K.*

pneumoniae primarily attack immunocompromised, hospitalized individuals suffering from

severe underlying diseases, such as diabetes. In the United States, *Klebsiella* spp. accounts for 3-

7% of all nosocomial bacterial infections, placing them among the most clinically significant pathogens found in hospitals.[50]

A well-recognized difficulty in treating most *Enterobacteriaceae* infections is their resistance to broad-spectrum antimicrobials.[51] Classically, carbapenems have been the terminal antibiotic in treating these types of infections and carbapenem-resistant strains have been relatively uncommon, until recently.[51] The emergence of carbapenemases that have direct carbapenem-hydrolyzing activity has contributed to an increased prevalence of carbapenem-resistant *Enterobacteriaceae* (CRE), a high mortality rate associated with CRE infections, and the potential for widespread transmission of carbapenem-resistance through mobile genetic elements.[51–54] These issues, combined with the limited therapeutic options available to treat patients with CRE infections, have led to the necessity of alternative treatments, such as phage therapy.

Bacteriophages (phages), are viruses that infect bacteria. Due to the ability to kill their bacterial hosts, phages were first used successfully to treat bacterial infections a decade before penicillin was discovered; however, the ease of production and the broad-spectrum action of antibiotics became more efficient than phage therapy.[55-56] Thus, the advent of antibiotics led to the cessation of phage-based therapies in the Western world, although they continued to be practiced in Eastern Europe and in the former Soviet Union.[57]

Phage therapy has been successful against antibiotic-resistant strains due to the distinct mechanisms by which viruses infect and kill bacterial cells. Phages are often very specific for the species, or even strain, of bacteria that they infect through the recognition of a receptor on the surface that initiates infection. Their DNA is injected into the cell, making them capable of transferring genetic material and altering host DNA through horizontal gene transfer. The

majority of phages studied to date are strictly lytic, using the cell to produce progeny which are released upon lysis [57,58], however some phages can also integrate directly into the host chromosome in what is called the lysogenic cycle of a temperate phage.[58,59] Lysogenic phage are often responsible for the pathogenicity of a particular bacterium, for example pathogenic strains of *Corynebacterium diphtheriae*, *Vibrio cholerae*, and *E. coli* wherein the temperate phage carries the bacterial toxin. Thus, phages for use in phage therapy must be adequately screened for a lytic nature as well as the lack of genes that may contribute to pathogenicity.

An additional benefit of phage therapy includes the relatively limited host range, or natural target cells, of individual phages. By limiting phage therapy to include phages only capable of infecting a single species of bacteria, such as an invasive, antibiotic-resistant strain, the natural host range of the phage will target the harmful bacteria while protecting the normal microflora that is vital to health.[59–61] Phage therapy has already begun to be used in agriculture and food industries, but use in human infections is limited to a case-by-case basis.[57] A number of *in vitro* studies have shown that phage have the potential to lyse targeted bacterial pathogens.[61–63] In this study, we evaluated lytic phages against clinical isolates of carbapenem-resistant *Klebsiella pneumoniae*.

Materials and methods

Bacterial strains and culture conditions

K. pneumoniae ATCC 13883 was used as a control organism and was purchased from American Type Culture Collection (ATCC, Manassas, VA, USA). Clinical isolates of carbapenem-resistant *K. pneumoniae* obtained from the CDC (Centers for Disease Control and Prevention, Atlanta, GA, USA) were designated as *K. pneumoniae* 1002002, *K. pneumoniae* 1300761, *K. pneumoniae* 20080030, and *K. pneumoniae* 1002235. Additional carbapenem-

resistant clinical isolates were obtained from hospital patients at Intermountain Healthcare in Utah County, UT, USA and were designated as *K. pneumoniae* IHC#1, *K. pneumoniae* IHC#2, and *K. pneumoniae* IHC#3. All strains were cultured in Luria-Bertani (LB) broth (Fisher BioReagents, Fair Lawn, NJ, USA) at 37ºC and grown overnight. Following the overnight culturing, strains were aliquoted at 1:10 dilution into LB broth and allowed to recover for 1 hour, ensuring that the bacteria were in exponential phase for subsequent assays.

Antibiotic susceptibility testing (AST)

Testing was done using the microdilution method in 96 well plates to find the minimum inhibitory concentration following the Clinical and Laboratory Standards Institute (CLSI) guidelines.[64] CLSI susceptibility breakpoints (M100-S27) were used to determine susceptibility/resistance status. All of the strains were tested against ampicillin, gentamicin, cefazolin, imipenem, chloramphenicol, and tetracycline. The antibiotics were prepared in two-fold dilutions (e.g. 2,4, 6, 8, and 16 μg/ml). The strains were incubated overnight in cation-adjusted Mueller-Hinton broth (MHB, Sigma-Aldrich, St. Louis, MO, USA) in a shaking incubator at 37ºC. Following overnight culturing, the strains were subcultured to reach an OD600 of 0.01. MHB was mixed with antibiotic and then the subcultured bacteria were added to the well. The plates were incubated for 18 hours at 37ºC and the presence or absence of turbidity indicated the susceptibility of the strain to the antibiotic.

Bacteriophage propagation and titer assay

All fourteen bacteriophages used in this study were isolated by the Phage Hunters program at Brigham Young University (BYU, Provo, UT, USA). All phages were isolated from untreated waste water on K. pneumoniae ATCC 13883. *K. pneumoniae* 13883 was grown overnight at 37ºC in LB in a shaking incubator. Enrichment cultures were created by adding 1

mL of overnight culture into 9 mL of LB, followed by the addition of 100 µL of phage lysate

(provided by Phage Hunters) into the 1:10 bacterial dilution and grown for 24 hours with shaking

at 37°C. The enrichment cultures were centrifuged at 6000 $x\ g$ for 20 minutes, and the

supernatant was filtered through a 0.2-µm filter (Millipore) to remove bacterial debris. To verify

the presence of phage and determine the titer, the supernatant was serially diluted (1:10) to 10^8

dilution, and 50 µL of diluted supernatant was incubated with 400 µL host strain for 30 minutes

at room temperature. After incubation, 4.5 mL of 1% molten LB agar was added to the phage

and host strain and was overlaid on a LB agar plate. The plates were incubated at 37°C for 18-24

hours. Following the incubation period, plaques on the plates were counted to calculate phage

titer.

Bacterial challenge assay

All the strains were incubated overnight in 10 mL of LB broth at 37°C with shaking.

After the overnight incubation, the cultures were diluted 1:10 in LB broth and then allowed to

recover for 1 hour, until OD_{600} reached 0.04-0.05. The strains were aliquoted at 400 µL each and

50 µL of phage were added and incubated for 30 minutes at room temperature. After incubation,

4.5 mL of 1% molten LB agar was added to the mix and overlaid on a LB agar plate. The plates

were incubated for 18-24 hours at 37°C. The presence of plaques indicated the infectivity of

phage. This challenge assay was performed in triplicate.

Phage sequencing and computational analyses

All phages were propagated to reach a high titer, following which the DNA was isolated

using the Norgen phage DNA isolation kit (Norgen, Canada). Quality of isolated DNA was

checked with gel electrophoresis (1% w/v agarose) and quantified with PicoGreen DNA

quantification kit (Thermo Fisher Scientific, Waltham, MA, USA). The DNA was then

sequenced using Illumina sequencing and the genomes assembled with Geneious R8.1.[65] Genome annotations and corrections were made manually using DNA Master[1], Basic Local Alignment Search Tool (BLAST)[2] searches and GeneMark coding potential prediction software.[66]

Genome comparisons were done by aligning phage nucleotide sequences on Gepard to create the dot plot.[67] The GenBank-formatted files of annotated genomes were used in tandem with PhamDB in an online interface to generate a database usable by Phamerator, an open-source program used to create pham maps.[68,69] Kalign was used to generate average nucleotide identity (ANI) tables for the phages.[70] SplitsTree was used to create protein-based phylogenetic grouping of the phages by exporting the pham table of conserved proteins (available on Phamerator) to Janus[A], which converts the table to a nexus (.nex) file-type required for SplitsTree.[71] The maximum likelihood phylogenetic tree of the phages' large terminase subunits was assembled with MEGA X after aligning the amino acid sequences on Geneious R8.1.[41,65]

tRNA genes were predicted by running phage genomes through tRNAscan-SE.[72] DNA motifs in the phage genomes were predicted using Multiple Em for Motif Elicitation (MEME) accessed through the Pasteur Institute's iteration of the Galaxy server, and predicted motifs were run through the TomTom motif database and comparison software to determine potential motif functions.[43,73,74] TomTom settings were set to compare motifs to the Prokaryotic Database of Gene Regulation (PRODORIC) and all motif functions are found on this database.[75] The p-value cutoff for accepting MEME motif predictions as well as the TomTom comparisons in the analysis was 1.00E-3.

---

[1] http://cobamide2.bio.pitt.edu/
[2] https://blast.ncbi.nlm.nih.gov/Blast.cgi

Results

Antibiotic susceptibility testing

The results of a minimal inhibitory concentration (MIC) analysis of several clinical *Klebsiella*

strains are found in Table 1, which indicate that the majority of the isolates are not only

carbapenem-resistant but have multi-drug resistance as well. Six of the seven isolates exhibited

resistance to imipenem with IHC #2 displaying an intermediate resistance, whereas isolate

2008030 showed resistance to imipenem. All of the isolates showed resistance to ampicillin

(AMP) where concentrations as high as 128 μg/mL were insufficient to inhibit bacterial growth.

Similarly, all isolates were resistant to cefazolin (CEF) up to 32 μg/mL. IHC #2 was the only

isolate susceptible to gentamicin (GEN), where the other isolates were resistant up to 64 μg/mL.

Five of the isolates were resistant to chloramphenicol (CAM), with varying amounts of

resistance, depending on antibiotic concentration. Six of the seven isolates were resistant to

tetracycline (TET), with isolate 1300761 showing susceptibility.

Table 2-1: **Antibiotic susceptibility testing results.**

	AMP (≥32)	*CEF (≥ 8)*	*GEN (≥16)*	*IMI (≥4)*	*CAM*	*TET (≥16)*
IHC #1	*R (>128)*	*R (>32)*	*R (>64)*	*R (>16)*	*R*	*R (>64)*
IHC #2	*R (>128)*	*R (>32)*	*S (2)*	*I*	*R (>128)*	*R*
IHC #3	*R (>128)*	*R (>32)*	*R (>64)*	*R (>16)*	*R*	*R*
2008030	*R (>128)*	*R (>32)*	*R (>64)*	*S (0.25)*	*R (>64)*	*R (>64)*
1002002	*R (>128)*	*R (>32)*	*R (>64)*	*R (>64)*	*I*	*R (>64)*
1002235	*R (>128)*	*R (>32)*	*R (>64)*	*R (>64)*	*R*	*R*
1300761	*R (>128)*	*R (>32)*	*R (>64)*	*R (>64)*	*I*	*S*
R, resistant; S, susceptible; I, intermediate; AMP, ampicillin; CEF, cefazolin; GEN, gentamicin; IMI, imipenem; CAM, chloramphenicol; TET, tetracycline						

Characterization of fourteen phages that infect Klebsiella pneumoniae

Fourteen recently isolated and sequenced *Klebsiella* phages were analyzed in this study (Table 2). Whole-genome nucleotide dot plot of the phage genomes is commonly used to assess phage relationships due to its ability to detect relationships among even mosaic, rearranged genomes. A dot plot of these fourteen phages produced by Gepard[67] displays shows clear grouping into five distinct "clusters", typically defined as phages displaying similarity over 50% of their genome.[76–78] Combined with the ANI matrix (supplementary table 1), it becomes obvious that genetic similarity within phage clusters is high, and that diversity between clusters is extensive. These clusters include KaAlpha and Potts1 which are distant relatives (61.48% ANI) , a singleton phage KaOmega, phages Domnhall, IMGroot, Alina, Penguinator, KingDDD, Call and SegesCirculi which are close relatives sharing >85% ANI, a singleton phage Chronis, and phages Sibilus, NahiliMali and Emp27 of which EMP27 is the most divergent.

Table 2-2: **A list of the fourteen phages analyzed in this study**. Includes GenBank accession number and approximate genome size.

Full Phage Name	GenBank Accession Number	Genome Size (kbp)
vB_KpnM_KaAlpha	MN013084	172.3
vB_KpnM_Potts1	MN013081	169.4
vB_KpnM_KaOmega	MN013077	149.5
vB_KpnS_Domnhall	MN013075	54.4
vB_KpnS_IMGroot	MN013076	52.9
vB_KpnS_Alina	MN013083	51.8
vB_KpnS_Penguinator	MN013087	51.7
vB_KpnS_KingDDD	MN013078	51.6
vB_KpnS_Call	MN013079	51.5
vB_KpnS_SegesCirculi	MN013080	50.7
vB_Kpn_Chronis	MN013086	45.7
vB_KpnP_Sibilus	MN013082	40.2
vB_KpnP_NahiliMali	MN013085	39.6
vB_KpnP_Emp27	MN013074	38.6
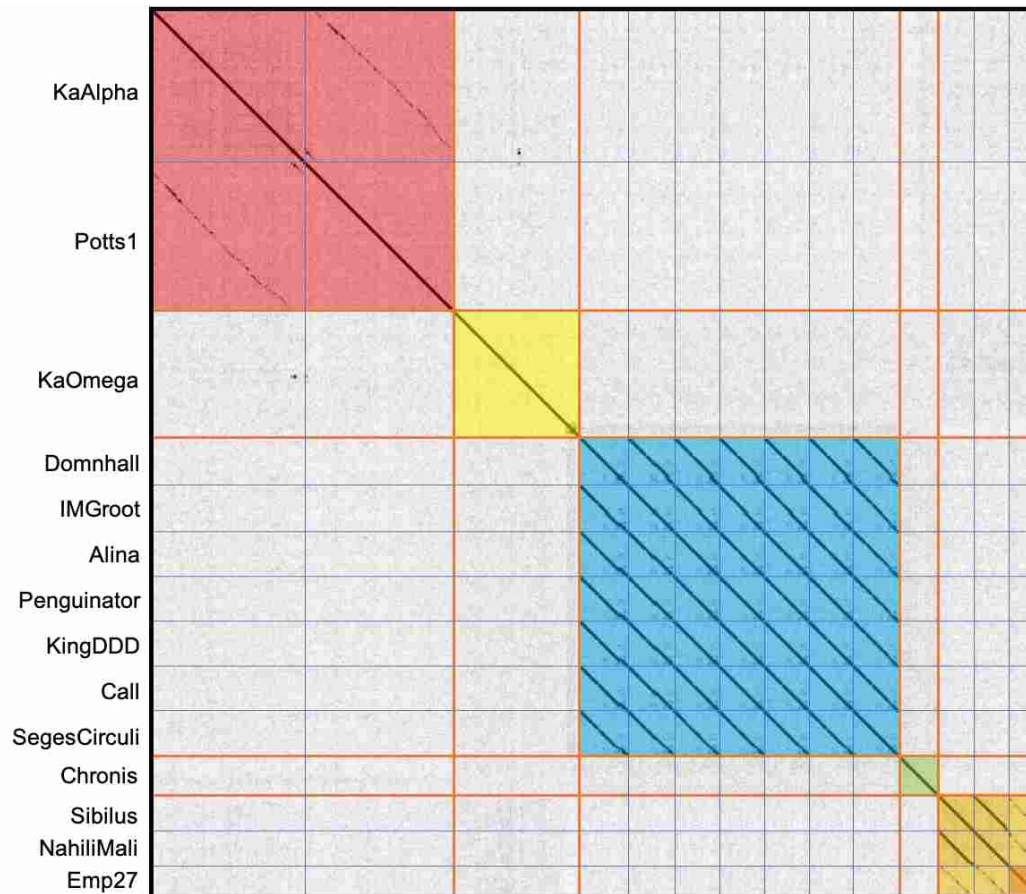
Figure 2-1. Gepard dot plot of K. pneumoniae phages used in this study. A "cluster" is designated when phages share >50% nucleotide identity and is indicated on the dot plot by solid black, diagonal lines. Phage names are written down the side of the plot, separated on the graph by lines. Orange lines (vertical and horizontal) separate clusters, while blue lines separate individual phage genomes. Colored squares indicate "clusters", which are phages with high nucleotide similarity.

A Phamerator[68,69] map was constructed to analyze proteomic similarity between the phages (Fig. 2-2). The coloring in the map between genomes highlights nucleotide similar regions between genomes, and color-coded boxes indicate homologous gene products from the same protein family. Gene products without color do not have homologous gene products in this data set, thus, genomes with the fewest colors (such as KaOmega and Chronis) are the most unique. In addition to distinguishing unique phages, this alignment also makes clear the differences between highly similar phages. In comparing phages Domnhall and IMGroot, for example, insertion, duplication

and rearrangement events are visible on the right half of the genome in IMGroot when compared with Domnhall. In comparing all the phages of this cluster, the beginning of the genomes (left) appears to be fairly conserved, with little variation in the sequences. Towards the ends of the genomes (right), however, there is increased variability, evidenced by numerous indels and at least one inversion event found in IMGroot.
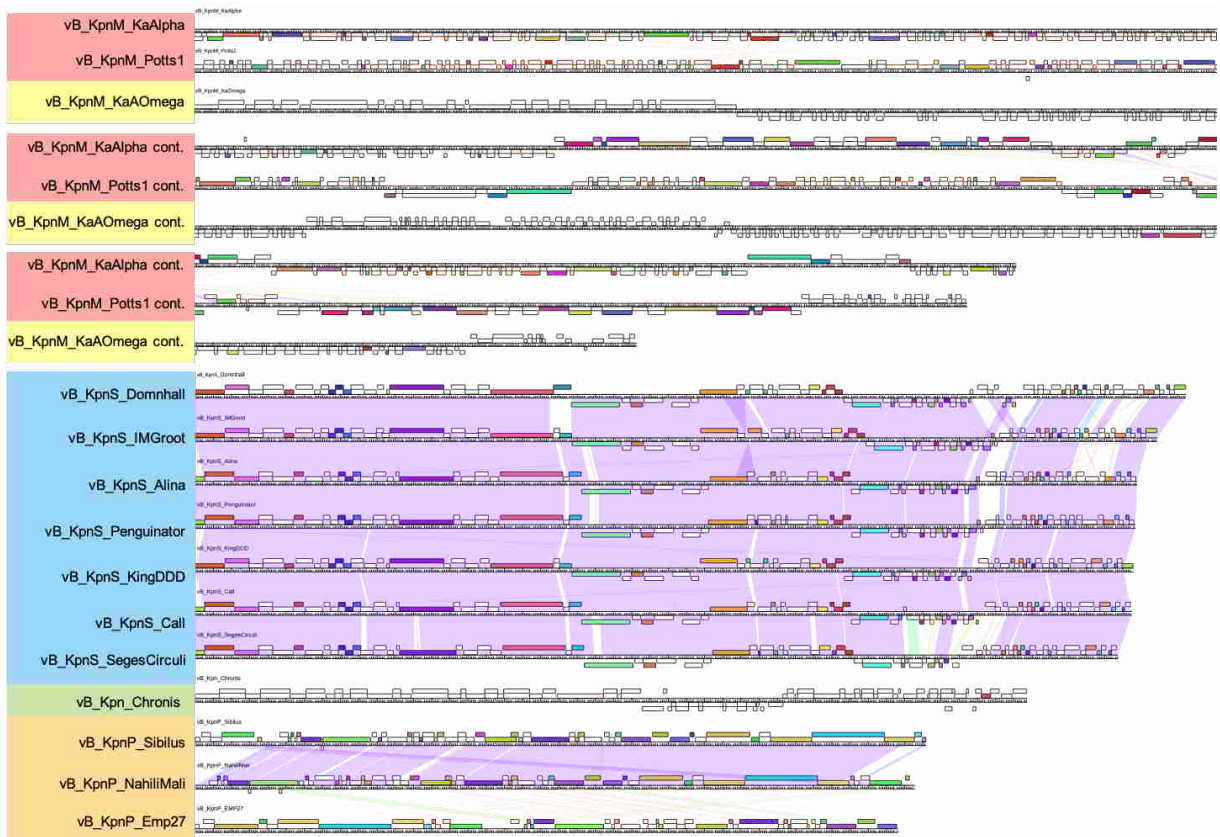
Figure 2-2. Phamerator map of *K. pneumoniae* phages used in this study. Phamerator maps compare nucleotide and protein sequences. The central bar represents the phage genome. The bars above and below the genome represent gene products. Proteins from the same family are colored the same. Nucleotide sequences with >50% identity are connected by purple segments. Coloring of names matches dot plot groupings. Larger image in supplementary data (S1).

In contrast to the nucleotide-based genomic comparisons of Gepard, SplitsTree[79] was used to construct a phylogenetic tree from the protein families identified by Phamerator. The SplitsTree created for these phages shows the same five phage clusters as the dot plot and Phamerator map (Fig. 2-3). The SplitsTree map also includes branching sites between the phages

25

that may point towards potential evolutionarily significant relationships. For this group of

phages, most splits occur at the base of the tree, with limited branching occurring within

individual clusters suggesting they are very distantly related clusters of phages.

To compare the predicted evolutionary relationship between the phages as well as their
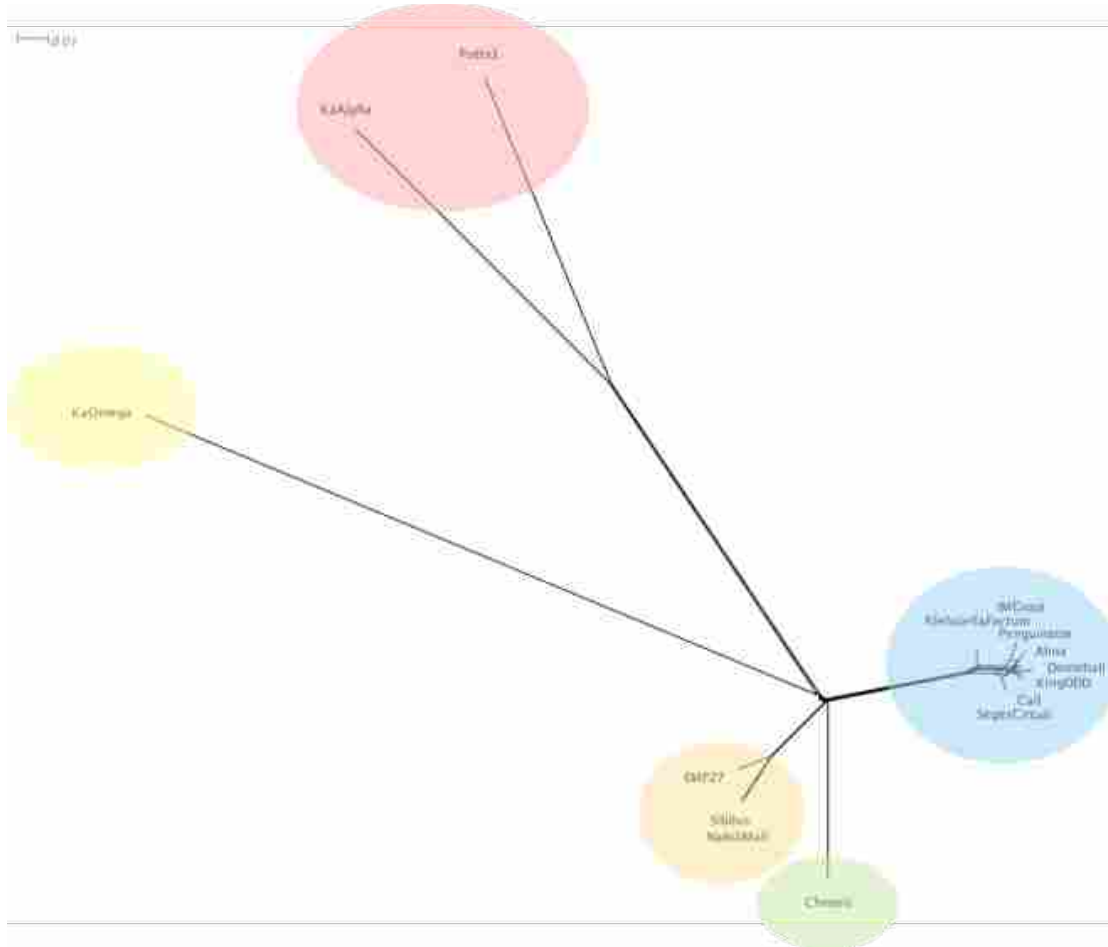
Figure 2-3. SplitsTree map of *K. pneumoniae* phages used in this study. Phages are grouped based solely off of predicted proteins. Grouping is similar to dot plot classification (shown by colors). Larger image in supplementary data (S2).

DNA packaging strategy, the amino acid sequences for the large terminase subunits from each

phage were aligned and run through MEGA X software to calculate the maximum-likelihood

tree (Fig. 2-4).[41] The maximum-likelihood tree, similar to the SplitsTree, predicts most major

branching towards the base of the tree, with few splits occurring more recently at the ends of the

branches. In contrast to the SplitsTree, however, the major grouping differs in which phage

phamilies share a common ancestor. The SplitsTree indicates that Chronis shares a very distant proteomic relationship with the Domnhall cluster, and a very early split with the Sibilus cluster at the base of the tree. The MEGA X large terminase subunit maximum-likelihood tree, however, places Chronis and the KaAlpha cluster under a common ancestor that previously branched from the Sibilus clusters, indicative of common DNA packaging strategies. Individual proteins used for phylogenetic computation that differ from whole-proteome phylogeny point towards apparent mosaicism displayed in the phage phamilies.[80,81]
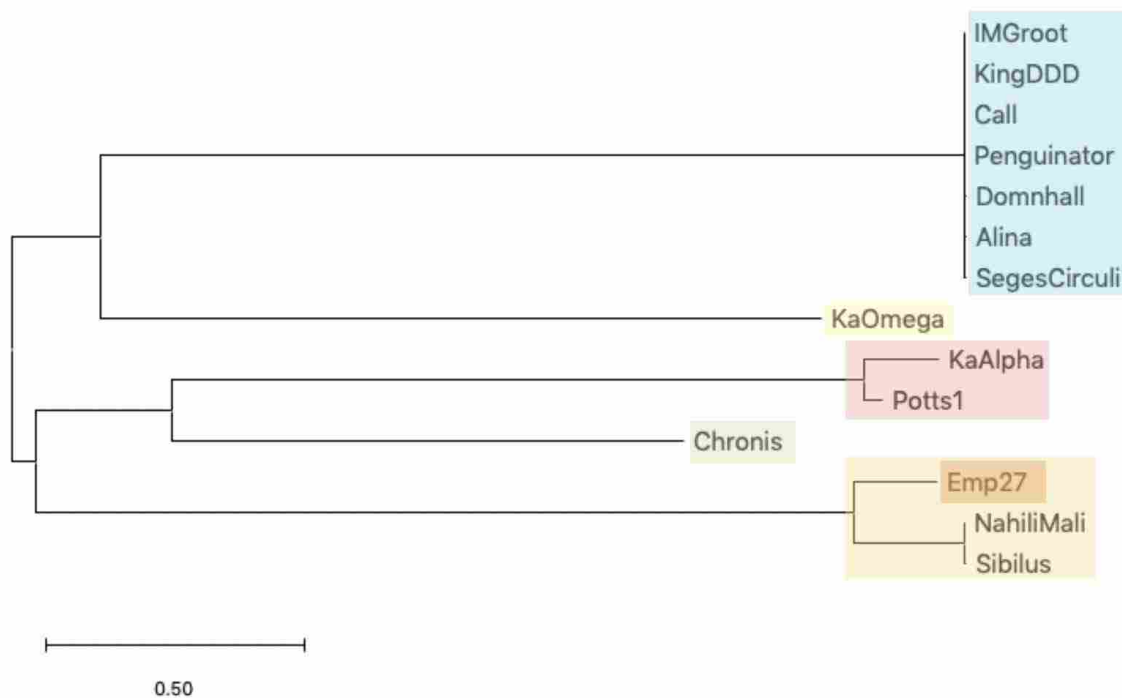
Figure 2-4. Evolutionary analysis by Maximum Likelihood method. The evolutionary history was inferred by using the Maximum Likelihood method and JTT matrix-based model (Jones, et al., 1992). The tree with the highest log likelihood (-8891.46) is shown. Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using a JTT model, and then selecting the topology with superior log likelihood value. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. This analysis involved fourteen amino acid sequences. There were a total of 796 positions in the final dataset. Evolutionary analyses were conducted in MEGA X (Kumar, et al., 2018; Felsenstein, J., 1985).

Analysis of the proteins encoded by these fourteen phages did not reveal any obviously pathogenic genes, such as toxins, biofilm production genes or antibiotic resistance genes. The presence of tRNA genes was detected in only four of the phages: KaAlpha (19), Potts1 (7), KaOmega (20) and Chronis (1). The tRNA genes all occurred in their respective genomes in grouped segments of <4000bp. The predicted tRNA sequences were compared to the *Klebsiella pneumoniae* codon usage table from Codon Usage Tabulated from GenBank (CUTG), accessed online via FTP.[3] One isotype of tRNA, an asparagine-charged tRNA with anticodon 5'-GUU-3', was found in all four genomes, though the coding sequences were different in all four. All seven of the tRNAs in Potts1 and the lone tRNA gene in Chronis encode A/T-rich anticodons (at least 2 out of 3 nucleotides) which varies from *Klebsiella pneumoniae* codon preference for G/C-containing codons (see database table). tRNAs with anticodons 5'-UUU-3' and 5'-CAT-3' are found in three of the genomes (KaAlpha, Potts1 and KaOmega) and match some of the most frequently used codons in *K. pneumoniae* (29.4 and 25.5 out of 1000 codons, respectively).

DNA motifs found within the genomes frequently matched regulatory elements from *Enterobacteriales* species, as well as a number of hits from *Mycobacterium, Pseudomonas* and *Bacillus*, but few had p-values of significance. KaAlpha predicted motifs had best matches to GntR glucose-regulating motifs of *Pseudomonales* and *Burkholderiales* families (p-value 8.20E-04), *E. coli* outer membrane protein synthesis (OmpR) operon motif (p-value 5.55E-04), and DevR-DevS two-component system in *Mycobacterium tuberculosis*. KaOmega contained motifs implicated in *E. coli* metabolism (MhpR and GlnG; p-values 1.85E-04 and 4.39E-04, respectively) as well as a *Pseudomonas putida* integration host factor protein binding site (p-value 5.49E-04). Domnhall showed motifs matching *E. coli* LacI operon family CytR protein-

---

[3] Accessed at https://www.kazusa.or.jp/codon/

binding site (p-value 7.35E-04) and *Enterobacteriales* multi-antibiotic resistance protein (MarA) binding-site (p-value 1.73E-07).

Temperate versus lytic phage analysis

The most common methods for determining if phages are capable of forming bacterial lysogens are to 1) isolate phage resistant bacterial lysogens which result upon phage integration into the host, 2) observe turbid plaques, particularly those with a bacterial lawn in the center containing phage resistant bacterial colonies, 3) identify integrases in the phage genomes, required for integrating into the host genome, and 4) finding close phage relatives in sequenced bacterial genomes. Since wet lab techniques can be biased by laboratory versus environmental conditions and no turbid plaques were observed, we analyzed the temperate nature of these phages by Blasting the MCP from each phage family against the extant protein database to identify close phage relatives that were integrated in bacterial host genomes.[82,83] Only Chronis came up with a close (>90%) match, and had 100% matches in several *Klebsiella pneumoniae* whole genome sequences deposited in NCBI's Genbank.

Bacterial challenge assay

The ability of the fourteen bacteriophages to infect various clinical isolate host strains was evaluated and presented Table 3, with only a unique few able to infect multiple isolates. Out of the fourteen bacteriophages found against *K. pneumoniae*, Sibilus and KaAlpha had the highest versatility and were effective against six of the seven host strains. The next phages that were able to infect the majority of the clinical isolates were Alina, Chronis and NahiliMali. All plaques were clear and combined with the genomic analysis above, indicate that the phages are most likely lytic with the exception of Chronis.

Table 2-3: **List of clinical isolates and the phage that were able to infect them**. Shaded cells indicate infection was observed. Included is a summary of how many isolates a single phage can infect (far right column) and the total number of phages a strain was susceptible to (bottom row).

	2008030	1002002	1300761	1002235	IHC	IHC	IHC	Number of Strains Infected
KaAlpha	■		■	■	■	■	■	6
Potts1					■	■	■	4
KaOmega								0
Domnhall			■					1
IMGroot			■					1
Alina	■	■		■	■	■		5
Penguinator		■						1
KingDDD								0
Call		■	■					2
SegesCirculi				■				1
Chronis				■	■	■	■	4
Sibilus	■	■		■	■	■	■	6
NahiliMali	■			■	■		■	4
Emp27		■						1
Number of Infecting Phages	4	5	4	6	5	6	5	

Discussion

Hospital-acquired infections that are caused by *K. pneumoniae* are a human health problem that are prevalent worldwide.[49,84] Since antibiotic treatments have associated restrictions, shortcomings and potential side effects, phage therapy is now being considered as a potential treatment and prevention for bacterial infections.[49,58] There are several potential beneficial effects of phage therapy, including creating a "cocktail" of phages that have activity against different bacterial pathogens, ability to infect multi-drug resistant pathogens, the potential for minimal side effects, and wide distribution upon systemic administration.[58] Another crucial aspect of phage therapy is the ability of the phage to be applied directly to local microflora without causing harm.[85] One of the criticisms that phage therapy faces is the ability to meet the "gold-standard" of efficacy. The lack of efficacy is likely caused by insufficient funds particularly in terms of clinical trials.[85] At present, there are few phage products that are

currently in use, both in terms of commercial use (e.g., Pyophage, and Instiphage sold in the former Soviet Union) and in the form of biocontrol (e.g., OmniLytics (UT, USA) and Micreos Food Safety (The Netherlands)).

There are other things to consider in using phage therapy as treatment for bacterial infections. The phages being considered for use need to be thoroughly investigated, including determination of the mechanism of host recognition and resistance, determining the phage titer needed to effectively lyse bacteria, or the genetic contents of the phages that could potentially benefit the bacteria, such as antibiotic resistance genes or bacterial virulence factors. It may also be advantageous to examine the bacteria for any genotypic differences within a species since thousands of strains may exist between which the phages are able to distinguish. For example, *K. pneumoniae* has many different capsule types that may affect the efficacy of phage adsorption to the surface of a bacterium and may explain the differences in host infections reported herein. A phage cocktail that is able to infect most of the common clinical pathogenic strains would be optimal.

This study demonstrates that there are several phages that show some efficacy against clinical isolates of carbapenem-resistant *K. pneumoniae,* including some that infect multiple isolates. The bacteriophages Sibilus and Alina showed versatility against several different strains of *K. pneumoniae* (five out of six). An optimal cocktail would contain phages that can infect all of the common clinical isolates. Thus, Sibilus and Alina, combined with Call and IMGroot, could be used to treat *K. pneumoniae* infections in a broad-spectrum cocktail that may infect many clinical isolates. Additionally, a multi-pronged approach by using multiple, unrelated phages to treat an infection, can prevent a bacterium from developing resistance. This study

indicates some of the necessary careful characterizations (genomic and host range) needed for phage therapy to transition from *in vitro* studies and into clinical studies.

Comparisons between phages shows that we can expect some of the virulence differences found between phages to be a result of small genomic differences between phages. For example, the large phage cluster labeled in blue (Domnhall, IMGroot, Alina, Penguinator, KingDDD, Call and SegesCirculi) shows extremely high nucleotide similarity between phages. Six of the seven phages in this cluster are capable of infecting at least one strain of CRE-*Klebsiella,* indicating that the miniscule differences between the genomes must account for the difference in virulence and host specificity. Phages Call, Alina and Penguinator are capable of infecting *K. pneumoniae* strain 1002002 while phages Call, IMGroot and Domnhall are capable of infecting *K. pneumoniae* strain 1300761. Most of the genomic variability between phages of this cluster is found towards the end of the genome, which shows some rearrangements, inversions, repeats, truncations and indels. Further lab work will be required to identify specific host-specificity factors that account for the differences in phage virulence against these strains of *Klebsiella*, as even with high nucleotide and proteomic similarity, we see that there is great variability in virulence.

Phages NahiliMali and Sibilus have a high nucleotide similarity, yet Sibilus was capable of infecting six of the seven strains tested while NahiliMali only infected four. In addition to the four infected by NahiliMali, Sibilus infected *K. pneumoniae* strains 1002002 and IHC#1, while neither was capable of infecting *K. pneumoniae* strain 1300761. There are three complete genes that are present in one, but not both, of the two phages: Sibilus gp3 and gp52 and NahiliMali gp21. All three amino acid sequences have blastp hits to hypothetical proteins from other phages, indicating unknown functions for each. Instead, the variability in host infectivity is most likely

explained by the putative tail fiber proteins (Sibilus gp51 and NahiliMali gp47), which often recognize the host receptor thereby determining host specificity. The tail fiber from Sibilus is smaller (1,641nucleotides, 546 amino acids) than that of NahiliMali (1,746 nucleotides, 587 amino acids). When aligned, the nucleotide sequences have >90% identity for the region that aligns, which covers only the first 962 nucleotides of each sequence. This indicates that the variability in the C-termini of the gene products is likely sufficient to account for the increase in host-recognition by Sibilus, allowing it to infect more strains than NahiliMali. Emp27 is distantly related to Sibilus and NahiliMali and represents its own sub-cluster. Emp27 shows much less host-diversity, capable of infecting only one strain, IHC#1. Emp27 gp8 aligns to Sibilus gp51 (the putative tail fiber) with about 60% amino acid identity, but this similarity is only found in the N-termini 60% of the polypeptides. The C-termini have no similarity, which again may explain the limited host range of Emp27 compared to Sibilus.

While similarities are substantial within individual phage clusters there is significant diversity between the clusters used in this study, with little to no recognizable similarity across clusters' nucleotide and amino acid sequences (i.e. Chronis and KaAlpha; or, Alina and Sibilus). This is indicative of a wide diversity of genes capable of forming viable phages that infect *K. pneumoniae*, a phenomenon that is common in phage biology.[33,86] Genome rearrangement and exchange is a fairly common event in the phage lifecycle, as can be observed here even within a single cluster (Domnhall cluster), and occurs readily with the host chromosome.[87,88] In comparing the two phylogenetic trees (the SplitsTree and large terminase maximum-likelihood tree) it appears that they may be telling two different stories regarding phage evolutionary relationships: the phage Chronis has been placed in two different relative locations on the trees, one based off of the whole proteome and the other based off of a single protein. It is likely that

such genetic exchange events occurring promiscuously in the phages' evolutionary spectrum account for some of the limited genetic crossover seen between clusters and may explain the apparent inconsistencies in the phylogenies. It is possible that phages following the temperate lifecycle left behind certain genetic elements in the host chromosome and were later picked up by new phages during infection. This can occur multiple times within a single phage genome resulting in mosaicism and is at least minimally evident within these phages. Whether the variations in phage infectivity can be explained by horizontal gene transfer or genetic drift (or both) is not directly clear from the results obtained in this study and is beyond the scope the paper. Nevertheless, many of the phages indicate versatility against a variety of CRE-*K. pneumoniae* strains which may prove useful in a clinical setting.

Finally, the presence of many tRNA genes and DNA motifs in the phage genomes has implications for their evolutionary story as well as clinical application. KaAlpha has 19 tRNA genes, whereas one or two is common among phages.[89] Phage genomes tend to be fairly dense with coding sequences, thus the presence of so many tRNA genes is indicative of some evolutionary fitness tradeoff that is—or was—beneficial for the phage. One such tRNA, with anticodon 5'-UUU-3' is among the most highly used of *K. pneumoniae* (fourth most frequent), but among the lowest used of *Mycobacterium tuberculosis.* KaAlpha also has motif matches to known *M. tuberculosis* operons, as well as a number of short nucleotide sequences that match *Mycobacteriaceae* species. While it cannot be concluded with certainty, these results suggest a possible host-change event by this phage, or at the very least some horizontal gene transfer occurrences, which would be surprising for phages that infect such different hosts (Gram-negative versus an acid-fast bacterium). KaOmega similarly has a DNA motif matching a *Pseudomonas* species integration host factor (IHF) protein used for recombination but could also

be used by temperate phages to integrate into the host genome. KaOmega also has an abundance of tRNA genes (20), which is unusual for a phage unless it uses genes with codon biases atypical for the host. Some of the tRNA genes match the most highly used codons in *K. pneumoniae*. Combined, the motif analysis and the presence of commonly used tRNAs is suggestive of a possible host-change for KaOmega, as well. A recent host-change event could explain why KaOmega was incapable of infecting the CRE strains in this study, even though it was isolated on *K. pneumoniae*, as it may not be fully fit to infect variations in the host strain. Domnhall and the other phages in the cluster do not carry tRNA genes, suggestive of long-term host interaction and lack of need to carry their own tRNAs, which are instead abundant in the host cell. Domnhall did, however, carry an interesting DNA motif matching a promoter sequence that binds a transcription factor involved in activating multi-antibiotic resistance genes (MarA) in *Escherichia coli*.[90] The MarA protein has homologues in other *Enterobacteriaceae* family species, as well, including *K. pneumoniae*. Phages have been shown to increase susceptibility to antibiotic compounds even after resistance has developed, and phage therapies are most effective when administered with low levels of antibiotics.[63,91] The MarA-binding DNA motif found in Domnhall and other members of its cluster (all except SegesCirculi) may suggest one such mechanism for restoring susceptibility to antibiotic drugs, that by infecting the cell the MarA transcription factors preferentially bind phage DNA, subsequently reducing transcription of antibiotic-resistance operons in *Enterobacteriaceae*. Thus, Domnhall and its cluster are excellent candidates for phage therapies of *K. pneumoniae* infections due to host specificity (and lack of evidence suggesting a change of host) and possible host abatement, aside from inherent phage virulence.

The evidence herein supports that there are a variety of genetically distinct phages capable of infecting unique antibiotic-resistant clinical isolates. In this study, fourteen phages were evaluated on a genetic level and a functional level, for their ability to infect a variety of CRE-*Klebsiella* strains. The results indicate that even among highly related phages, minute differences in nucleotide or amino acid sequences can affect the host range of the phage. This finding has important implications for clinical application of phage therapy, as individual phages must be tested against specific strains to confirm virulence, since it cannot be predicted from evaluating the phage genome alone, at this time. Furthermore, we identified certain genetic elements that may contribute to phage virulence, mainly the presence of a DNA motif that would competitively bind proteins used by *Enterobacteriaceae* for activation of multi-antibiotic-resistance genes. The presence of such elements may be critical in future applications of phage therapy, as it allows for the combined usage of antibiotics and may increase their effectiveness. While genetic differences help account for some of the differences in phage virulence against the host, more research should be conducted in the future to identify specific phage proteins responsible for host-recognition and specific host proteins that act as phage recognition sites. Such studies will elucidate the phage-host interactions and functions of otherwise unknown genes. In the meantime, genomic computational analyses serve to uncover some of the mysteries found within phage genomes and help explain some of the nuances contributing to differential virulence.

Supplementary data
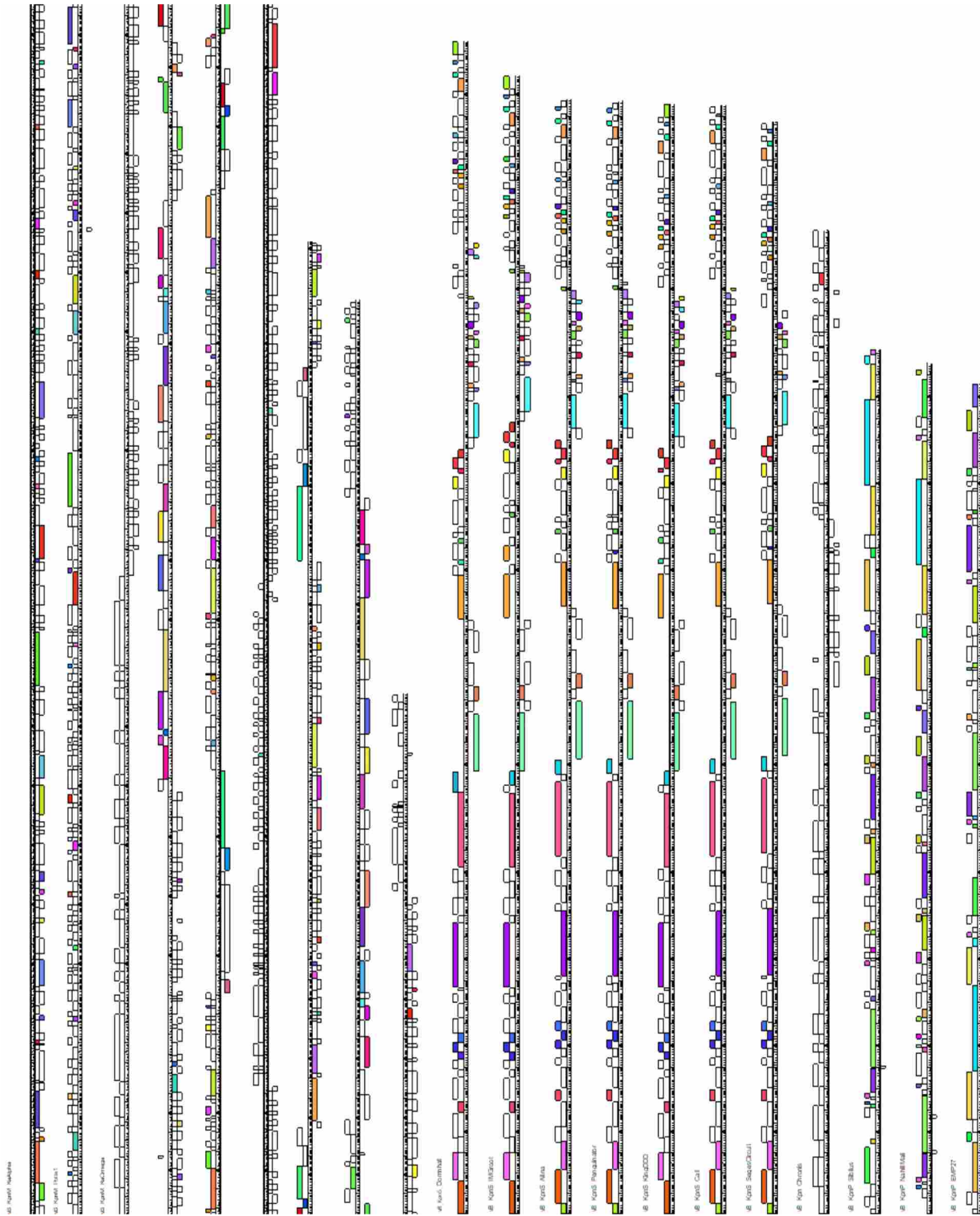
Supplementary Figure 1 (S1): Enlarged Phamerator Map
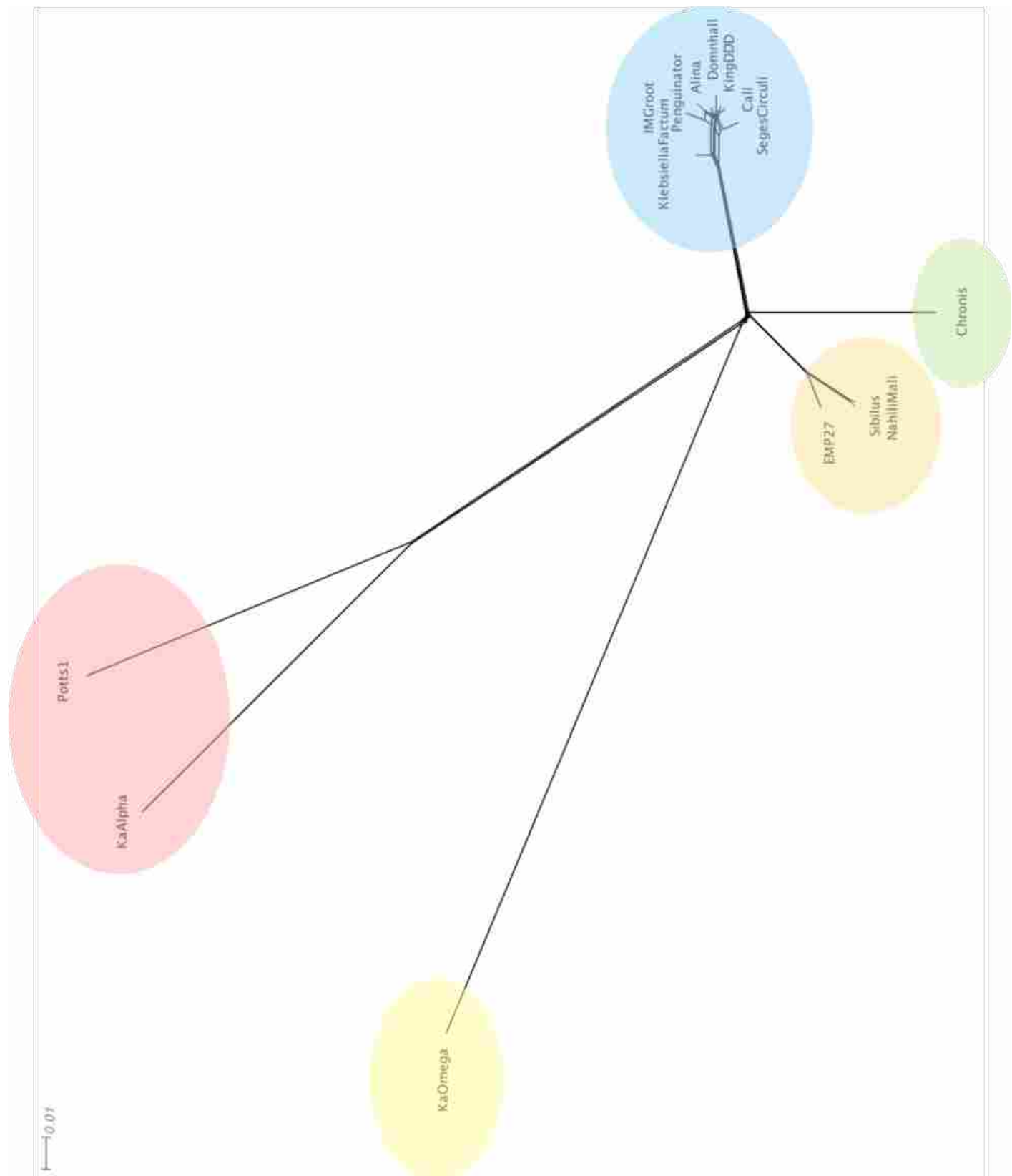
Supplementary Figure 2 (S2): Enlarged SplitsTree Map

Supplementary Table 1: ANI matrix of all fourteen phages.

	1	2	3	4	5	6	7	8	9	10	11	12	13	14
1. KaAlpha	100.00	61.48	42.47	40.98	40.73	40.93	41.07	41.03	41.1	43.24	34.98	34.95	34.55	34.88
2. Potts1	61.48	100.00	42.36	40.8	40.77	40.88	40.94	40.91	40.92	43.1	34.94	34.58	34.25	35.09
3. KaOmega	42.47	42.36	100.00	38.61	38.51	38.68	38.85	38.8	38.79	40.52	33.83	33.51	33.23	33.87
4. Domnhall	40.98	40.8	38.61	100.00	93.33	93.70	94.67	95.65	95.23	87.56	43.9	42.99	42.21	44.09
5. IMGroot	40.73	40.77	38.51	93.33	100.00	92.51	97.94	93.29	95.67	86.65	44.01	42.99	42.21	44.14
6. Alina	40.93	40.88	38.68	93.70	92.51	100.00	94.38	95.18	94.69	88.84	43.96	42.92	42.22	44.05
7. Penguinator	41.07	40.94	38.85	94.67	97.94	94.38	100.00	95.28	97.72	88.72	44.35	43.28	42.51	44.34
8. KingDDD	41.03	40.91	38.8	95.65	93.29	95.18	95.28	100.00	95.93	89.12	44.2	43.14	42.38	44.22
9. Call	41.1	40.92	38.79	95.23	95.67	94.69	97.72	95.93	100.00	88.86	44.27	43.2	42.44	44.28
10. SegesCirculi	43.24	43.1	40.52	87.56	86.65	88.84	88.72	89.12	88.86	100.00	43.03	42.12	41.43	43.37
11. Chronis	34.98	34.94	33.83	43.9	44.01	43.96	44.35	44.2	44.27	43.03	100.00	32.94	32.57	33.12
12. Sibilus	34.95	34.58	33.51	42.99	42.99	42.92	43.28	43.14	43.2	42.12	32.94	100.00	92.8	63.06
13. NahiliMali	34.55	34.25	33.23	42.21	42.21	42.22	42.51	42.38	42.44	41.43	32.57	92.8	100.00	63.12
fourteen . Emp27	34.88	35.09	33.87	44.09	44.14	44.05	44.34	44.22	44.28	43.37	33.12	63.06	63.12	100.00

CHAPTER III: New Phages Infecting Pathogenic *Bacillus anthracis*

Authors: Trever Thurgood, Julianne H. Grose, Richard Robison

This chapter is an excerpt from a publication in preparation for submission at the time of writing

of this thesis.

Abstract

Bacillus anthracis is a well-studied bacterial species that has significant implications for

human and animal health. As a pathogen, *B. anthracis* has increasing mortality rates,

respectively, for each of its three routes of infection: cutaneous, gastrointestinal and respiratory.

Despite high virulence, however, *B. anthracis* has its own natural predators: bacteriophages

(phages). Phages are viruses that adsorb onto the surface of bacteria, inject their nucleic acid, and

lyse the cell or splice into the bacterial chromosome. There are few phages known to infect *B.

anthracis*, namely a select group of phages from the Wß-like cluster. In this study, a set of

newly-isolated *B. anthracis* phages is characterized, and their virulence tested against genetically

diverse strains of pathogenic *B. anthracis*. These are the first phages outside of the Wß-like

cluster reported as capable of infecting multiple pathogenic strains.

Introduction

Bacillus anthracis is an ancient pathogen well-known to humankind. *B. anthracis* was

first identified as the causative agent of anthrax by Robert Koch in 1876.[1] Once the symptoms

and pathogen were formally associated with one another, researchers began to realize that

anthrax-like diseases had plagued humanity for millennia. Reports from ancient Greece, Egypt,

and China, as well as Europe in the middle ages, depict tales of diseases with symptoms identical

to anthrax.[92–95] After its rediscovery, another early microbiologist, Louis Pasteur, created the first

animal anthrax vaccine. This vaccine has been used to protect against the disease, which has

historically caused pestilence amongst livestock, livestock workers and workers in other animal-based professions.[96,97] In 1954, the first human live-attenuated anthrax vaccine was developed, but remains unavailable to the public and requires annual boosters to remain effective.[98,99] In contemporary history, *B. anthracis* has garnered attention due to its potential use as a bioterrorist weapon.[10,18] More recently, bioterrorist attacks in the United States have led to a surge in anthrax research and its mechanisms of pathogenicity. Without rapid medical intervention, *B. anthracis* infection has different mortality rates for the three routes of infection: cutaneous (20%), gastrointestinal (50%), and inhalation (>80%).[99]

Bacillus anthracis is a member of the *Bacillus cereus* group, a small group of pathogenic and non-pathogenic *Bacillus* species. *B. anthracis* is a Gram-positive, spore-forming bacteria capable of causing severe infection in man and animal. Common to all *Bacillus* species are general cell structure, essential metabolic components, and spore-forming capability. The outer structure of the bacterial cell consists of a phospholipid bilayer membrane and a peptidoglycan cell wall encompassing the periplasmic space (Fig. 1A). The peptidoglycan cell wall itself is a complex structure essential for bacterial survival. Some of the functions of the peptidoglycan layer include maintenance of cell turgor pressure, exclusion of extracellular enzymes and toxins, and transport of metabolically important molecules.[1,100] Peptidoglycan consists of repeating units of N-acetylmuramic acid (NAM) and N-acetyl-glucosamine (NAG), interspersed with long repeated chains of teichoic acid that extend through the cell wall as well as out into the extracellular space (Fig 1B). Surrounding the outer edges of the peptidoglycan layer is the surface layer (S-layer) which contains adhesion proteins and genus- and species-specific proteins.[101] Specific to pathogenic *B. anthracis* strains is an additional protective layer, the poly-

A

Poly-D-Glutamic Acid Capsule

S-layer (with teichoic acid, glycoprotein and carbohydrate subunits)

TA

Peptidoglycan (with teichoic acid)

Periplasmic space

Phospholipid bilayer (with membrane proteins)

C

B

Carbohydrate chain

Phosphodiester backbone

TA

N-acetyl-muramic acid
N-acetyl-glucosamine

Figure 3-1. Structure of Gram-positive cell wall and *B. anthracis* capsule. A, cartoon magnification of the Gram-positive cell wall general structure with some cell wall-associated components; B, N-acetyl-muramic acid and N-acetyl-glucosamine structural linkage in peptidoglycan, in addition to the teichoic acid (TA) structure; C, poly-D-glutamic acid capsule structure, showing a single glutamic acid molecule.

D-glutamic acid capsule (PGAC), which plays a role in immune cell evasion when infecting a host (Fig. 3-1C).[102,103]

Genetic similarity among *Bacillus* species is relatively high, with few, but critical genes differentiating *B. anthracis* from other species of this genus. *B. anthracis* is distinguishable from

other *Bacillus* species by its inactive flagellar components, rendering it nonmotile, as well as a mutated, nonfunctional metabolic regulatory enzyme, PlcR, that plays a role in insect and plant pathogenicity.[104,105] ß-hemolysin, an enzyme capable of degrading red blood cells, and which is active in most other *Bacillus* species is also nonfunctioning in *B. anthracis*. Additionally, the *B. anthracis* genome contains four putative, inactive prophage regions not found in most other *Bacillus* species.[106] Furthermore, *B. anthracis* is distinguished from non-pathogenic species by the presence of two virulence plasmids, *pXO1* and *pXO2*. Full pathogenicity of *B. anthracis* is dependent on these two plasmids, which encode a tripartite toxin known as the anthrax toxin and the aforementioned PGAC, respectively. When a vertebrate host becomes infected with *B. anthracis*, the PGAC prevents immune cells from phagocytosing the bacteria, while the tripartite toxin disrupts host cell signaling pathways that lead to cell death and necrosis.[10,17,107] The severity and rate of disease onset of *B. anthracis* makes this bacterium a formidable pathogen.

Bacteriophages are naturally occurring viruses that infect and kill bacteria. Phages have been shown to be viable candidates as an alternative approach to treating bacteria, particularly antibiotic-resistant strains. Phages follow one of two viral lifecycles when infecting bacterial host cells: lytic or lysogenic. The lytic lifecycle begins with phage recognition of and attachment to host cell surface components. A phage can only recognize certain surface receptors, which may limit the number of bacterial hosts it can infect, such as if there are differences between species or strains. Typical receptors include trans-membrane proteins or signal receptors, though surface enzymes, transport channels, pili, flagella, capsular elements, as well as teichoic acids in Gram-positive hosts can serve as targets.[34] After adsorption onto the cell surface, subsequent enzymatic degradation of the bacterial cell wall allows for injection of viral nucleic acid into host cytoplasm. In contrast to lytic phages, temperate phages cleave the host genome and insert

43

themselves as dormant genetic parasites (also known as prophages once integrated into the host genome) and may remain within the host genome indefinitely or until induced into the lytic phase.

There are very few phages reported as capable of infecting a variety of *B. anthracis* strains. The *B. anthracis* phages γ, Wß, Cherry and Fah have all been shown to be capable of infecting encapsulated (*pXO2*-possessing) *B. anthracis*; however, these phages are genetically similar and do not represent the diversity of *B. anthracis* phages.[108–111] Interestingly, it has been reported that the expression of the PGAC inhibits the ability of phages to recognize the bacterium and may inhibit host lysis.[36] To test the ability of genetically diverse phage lineages to infect *B. anthracis*, we utilized a library of genetically diverse *B. anthracis* strains, as characterized by Van Ert, et al., in concert with 18 phages from a newly-isolated collection in our lab. Van Ert, et al., typed over one thousand isolates of *B. anthracis* and categorized the genetic diversity of the strains into three phylogenetic groups, A, B and C, with twelve total branches (Fig. 3-2).[40] The 18 phages represent four clusters and six sub-clusters of *B. anthracis* phages that extend beyond the Wß family (Fig. 3-3).

Materials and methods

Phage isolation

Soil samples were collected from various regions in the Western United States and cultured with *B. anthracis,* Sterne for 48 hours at 30 ºC. One gram of soil was mixed with 10 mL of Luria-Bertani broth (LB) and 1 mL of *B. anthracis* overnight culture grown in the same medium. After the incubation period was complete, the samples were centrifuged at 4,000 $x\ g$ for 50min., the supernatant removed and filtered with 0.45 μm syringe filters. Five μL of the filtered supernatant was added to 500 μL of overnight *B. anthracis* culture and incubated at ambient
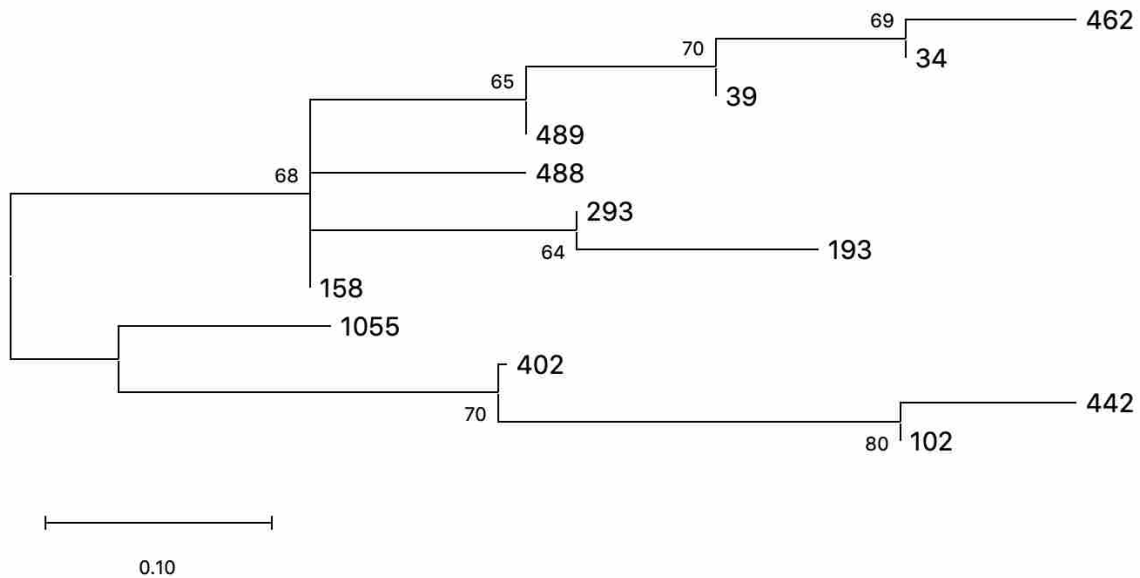
Figure 3-2. Reproduction of phylogenetic tree representing *B. anthracis* genetic diversity. This phylogenetic tree is a reproduction of the phylogenetic tree presented by Van Ert, et al. (2007), using the SNPs from multi-locus variation sequences presented in their study. The evolutionary history was inferred by using the Maximum Likelihood method and Tamura-Nei model. The tree with the highest log likelihood (-71.75) is shown. The percentage of trees in which the associated taxa clustered together is shown next to the branches. Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the Maximum Composite Likelihood (MCL) approach, and then selecting the topology with superior log likelihood value. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. This analysis involved 12 nucleotide sequences. There were a total of 13 positions in the final dataset. Evolutionary analyses were conducted in MEGA X.

temperature for 45 minutes. Then, 4.5mL 1% molten LB top agar was added to the mixture, which was then poured over LB agar plates. The plates were incubated at 30ºC for 48 hours and checked for plaques. If plaques appeared on the plates, they were picked with a pipette tip and resuspended in 200 μL LB broth, of which 50 μL was used to infect another 500 μL of overnight *B. anthracis* culture and the same plating process was repeated. After the second round of plating, plaques were picked, resuspended in 200 μL LB broth, of which 100 μL was used to inoculate a 1:10 dilution of *B. anthracis* culture in LB broth, for a total of 10mL. The enrichment culture was incubated with shaking at 30 ºC for 48 hours. After the incubation period, the same centrifugation and filtration protocol was followed to obtain a purified high titer lysate. Phage

45

titer was assayed using a serial dilution method by moving 100 μL into 900 μL sterile LB broth

until a 1:10^8 dilution was obtained. Fifty μL of the 10^2, 10^4, 10^6 and 10^8 dilutions were used to

infect 500 μL of overnight *B. anthracis* culture and plated in the same manner to determine the

concentration of phage in the lysate.

Phage sequencing

Once high titer lysates were obtained (minimum of 10^8 pfu/mL), phage DNA was

isolated with the Norgen Biotek Phage DNA Isolation Kit (Norgen, Canada). Phage DNA was

then digested with *HindIII* restriction enzyme for 1 hr at 37 ºC and the resulting digestions run on

1% agarose gel electrophoresis to visualize DNA banding patterns and check for diversity. If

phage banding patterns appeared unique on the gel, they were submitted for Illumina sequencing

at the Brigham Young University Sequencing Center (Provo, UT, USA). Phage genomes were

assembled using Geneious R8.1.[65] Genome annotations and corrections were made manually

using DNA Master[4], Basic Local Alignment Search Tool (BLAST)[5] searches and GeneMark

coding potential prediction software.[66]

Genomic analysis

Genome comparisons were done by aligning phage nucleotide sequences on Gepard to

create the dot plot.[67] The GenBank-formatted files of annotated genomes were used in tandem

with PhamDB in an online interface to generate a database usable by Phamerator, an open-source

program used to create pham maps.[68,69] Kalign was used to generate average nucleotide identity

(ANI) tables for the phages.[70] SplitsTree was used to create protein-based phylogenetic grouping

of the phages by exporting the pham table of conserved proteins (available on Phamerator) to

---

[4] http://cobamide2.bio.pitt.edu/
[5] https://blast.ncbi.nlm.nih.gov/Blast.cgi

46

Janus[1], which converts the table to a nexus (.nex) file-type required for SplitsTree.[71] The maximum likelihood phylogenetic tree of the phages' large terminase subunits was assembled with MEGA X after aligning the amino acid sequences on Geneious R8.1.[41,65]

BSL-3 phage infection assay

Each strain selected for the infection assay was streaked from the frozen inventory of *B. anthracis* strains onto LB plates and incubated at least 24 hr at 37 ºC. Liquid cultures were created by inoculating LB broth (5-10 mL) with a single colony from the isoplates and these were incubated with shaking at 37 ºC overnight. Then, in a biosafety cabinet, 500 μL aliquots of the overnight cultures were placed into disposable plastic culture tubes. The aliquots were infected with 50 μL aliquots of phage dilutions ranging from 10^0-10^{-4}, incubated at ambient temperature for 45 min and plated onto LB plates with 1% molten LB top agar. The plates were allowed to solidify at least 10 min before being sealed in plastic bags, removed from the biosafety cabinet and incubated at 37 ºC overnight. Plates were checked for plaques and plaque counts recorded.

Results

Genomic analyses

Eighteen phages were isolated against the host *B. anthracis*, sequenced and annotated as previously described. Dot plot analysis is a common method for comparing phage genomes due to their highly mosaic nature and the ability of the dot plot to detect similarity in rearranged genomes.[80,83] The whole-genome dot plot assembled shows the phages are separated into four clusters, or groups of phages with over 50% genome similarity, two of which have sub-clusters of very closely related phages (Fig. 3-3). The cluster labeled in yellow is related to the *Bacillus* phage known as TsarBomba. This cluster has four sub-clusters, each of which has *Bacillus* phage

relatives, as revealed by comparing nucleotide sequences to phages deposited in GenBank. The

blue cluster is divided by two sub-clusters, but the entire cluster resembles *B. anthracis* phage

Tsamsa. There are two additional clusters consisting of phages with much smaller genomes than

the TsarBomba-like and Tsamsa-like phages and are predicted to be temperate phages. The last

cluster represented on the dot plot resembles genetically the Wß family known to infect *B.*

anthracis.
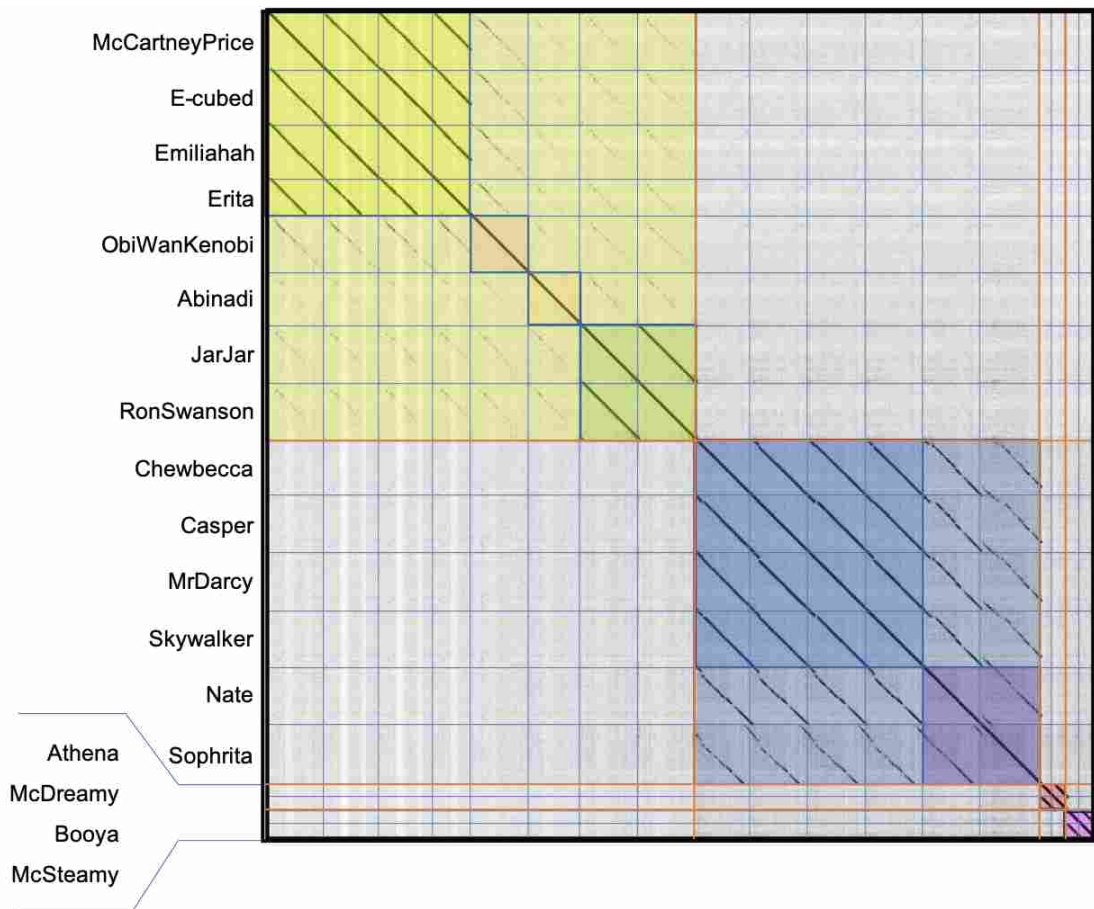
Figure 3-3. Gepard dot plot comparison of *B. anthracis* phage genomes. The phage names are listed down the left side of the figure. On the graph, phages are separated by horizontal and vertical blue lines, while clusters are separated by horizontal and vertical orange lines. Solid black, diagonal lines indicate nucleotide similarity >50%. Clusters are represented with colored squares.

An analysis of the phage proteome confirmed the relationships seen by nucleotide

sequence comparison. Proteins were grouped by relatedness using Phamerator[68,69], and

SplitsTree[79] was used to create a phylogeny based off of protein homologues. The SplitsTree of

the phages used in this analysis shows the protein-based grouping to match the nucleotide-based grouping shown on the dot plot (Fig. 3-4). The cluster and sub-cluster groupings are very distinct, and branching appears to have occurred distantly in the phages' past. Similarly, a genomic nucleotide and protein map generated in Phamerator shows individual nucleotide sequence similarities between phages of the same cluster by highlighting between genomes (Fig. 3-5).[68,69] Protein similarities are displayed with similarly-colored boxes. Differences between otherwise highly related phages are made obvious by regions between genomes that are not highlighted. There is little to no observable similarity between clusters by either SplitsTree of Phamerator analysis.
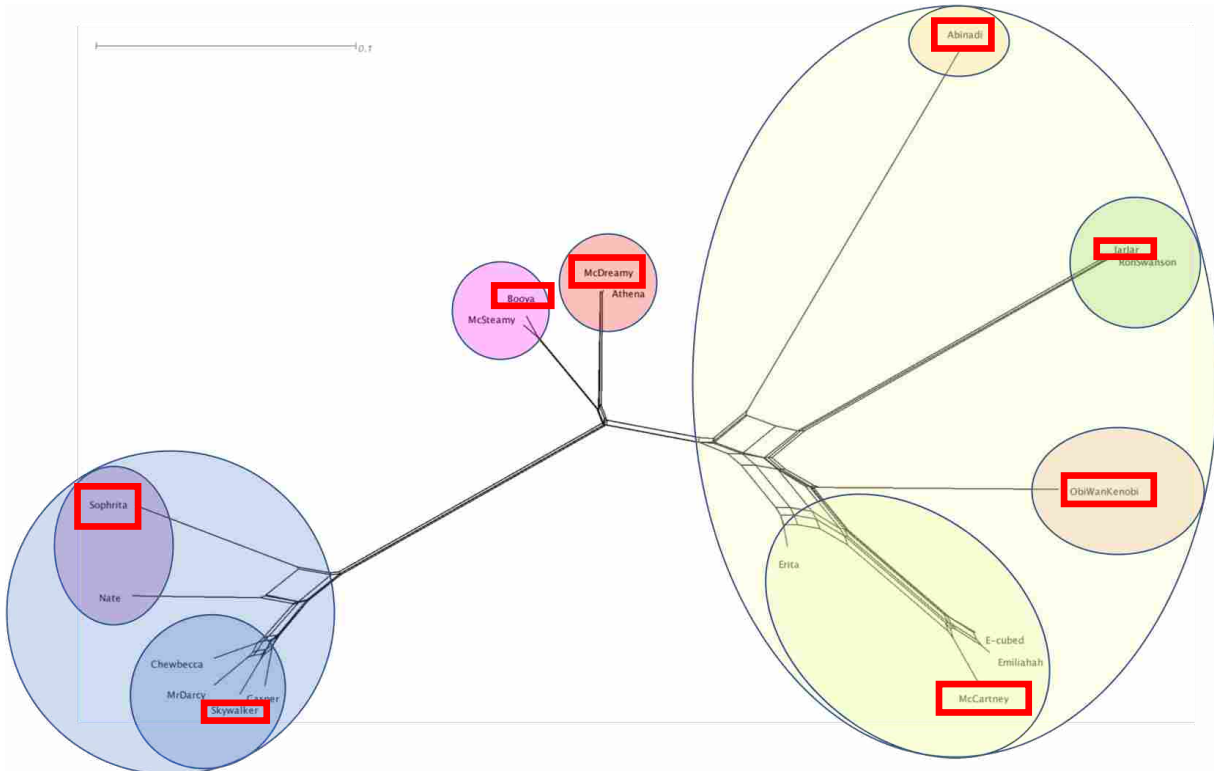
Figure 3-4. SplitsTree comparing proteomic relatedness of *B. anthracis* phages. The phages used for the infection assays are boxed in red, while the circles correspond to the phage clusters and sub-clusters, as shown on the Gepard dot plot.
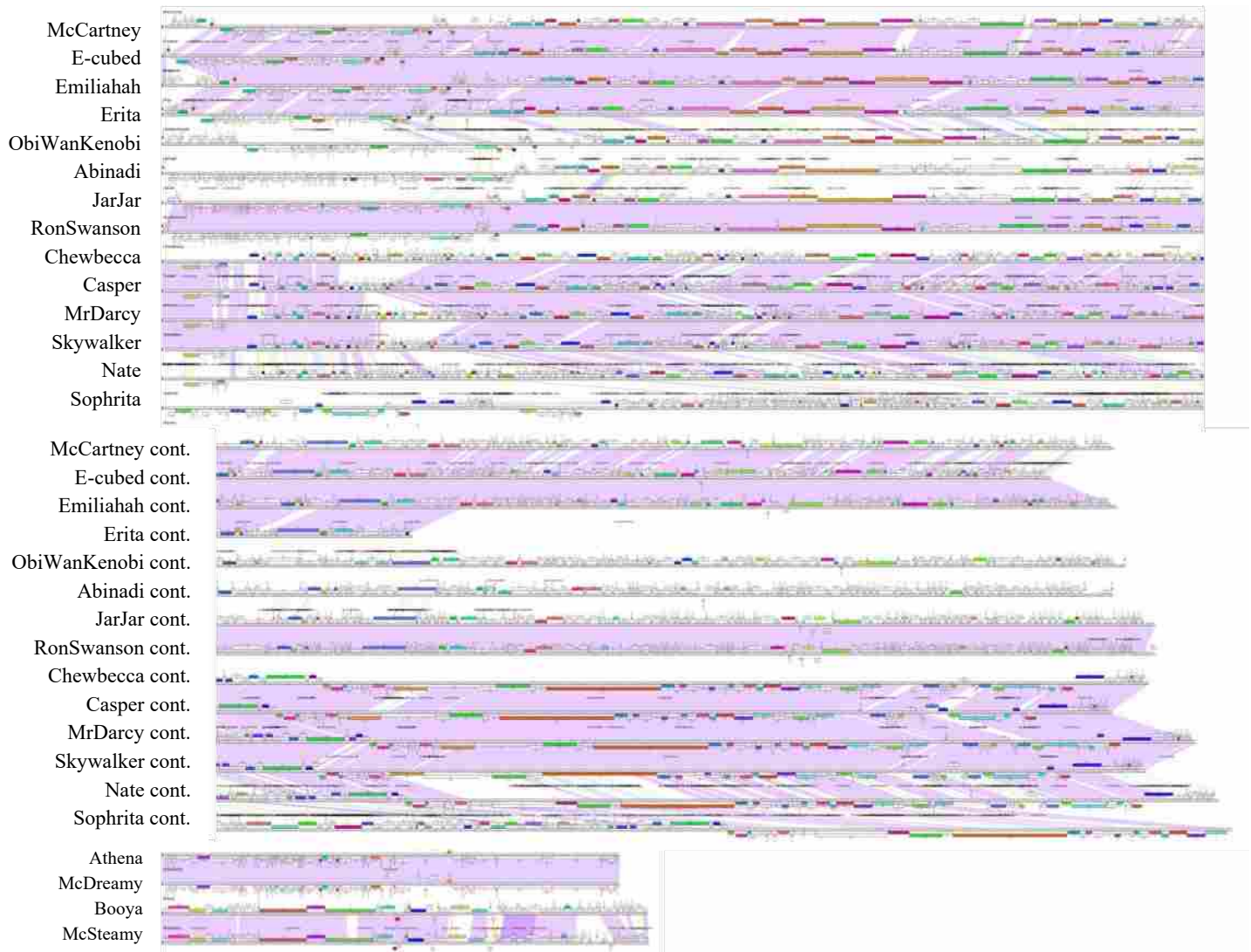
Figure 3-5. Phamerator map of *B. anthracis* phages used in this study. The Phamerator map highlights differences between the genomes of the phages in purple. Similar gene products from conserved protein families are highlighted in similar colors. There is no observable similarity between phages of different clusters.

Finally, the phages' large terminase subunits were aligned using Geneious R8.1 and submitted to MEGA X to create a maximum likelihood phylogenetic tree (Fig. 3-6). Not surprisingly, the phylogenetic tree produced shows the same groupings predicted from the dot plot, SplitsTree and Phamerator map, but does provide some insight into which phages may have experienced more significant genetic drift at the protein level.

Infection assays

The results of the infection assays using the twelve pathogenic strains of *B. anthracis* and eight of the 18 phages we selected for this study are presented in Table 1. One phage was selected from each cluster or sub-cluster from our collection to represent the phages' genetic diversity. While the analysis is still in progress, current results show that every strain is susceptible to a number of phages. Similarly, each phage is capable of infecting a
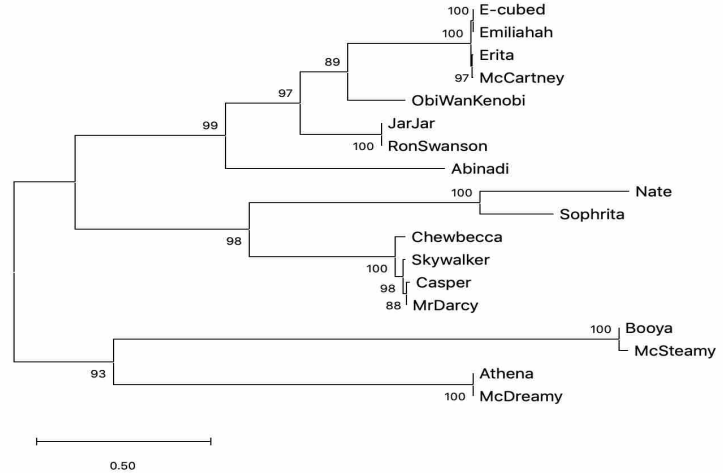
Figure 3-6. Evolutionary analysis by Maximum Likelihood method of phage large terminase subunits. The evolutionary history was inferred by using the Maximum Likelihood method and JTT matrix-based model. The tree with the highest log likelihood (-8535.21) is shown. The percentage of trees in which the associated taxa clustered together is shown next to the branches. Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using a JTT model, and then selecting the topology with superior log likelihood value. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. This analysis involved 18 amino acid sequences. There were a total of 678 positions in the final dataset. Evolutionary analyses were conducted in MEGA X.

Table 3-1. Strain susceptibility to phage infection. Strain numbers are listed across the top and phage names down the left side of the table. Green cells indicate successful infection by the phage observed as plaques. Gray cells indicate no infection observed. Strain 1055 has not yet been assayed. The approximate phage titer is listed under the Sterne column.

(Legend for table below: G = green cell / successful infection; — = gray cell / no infection observed)

	34	39	102	158	193	293	402	442	462	488	489	1055	Total number of pathogenic strains infected:	Sterne
McCartney	G	G	G	G	G	G	—	G	G	G	G	?	10	2e9
ObiWanKenobi	G	—	G	G	G	G	G	G	G	G	G	?	10	4e8
Abinadi	G	G	—	G	G	G	G	G	G	G	G	?	10	3e8
JarJar	—	G	G	G	G	G	—	G	G	—	G	?	8	9e7
Skywalker	—	—	G	G	—	G	—	G	G	G	—	?	6	1e9
Sophrita	—	G	G	G	G	—	—	G	G	—	G	?	7	2e8
McDreamy	—	G	G	G	G	G	—	G	G	—	G	?	8	8e8
Booya	—	G	G	G	G	G	—	G	G	—	G	?	8	3e8
Total number of confirmed infecting phages:	3	6	7	8	7	7	2	8	8	4	7	?		

number of pathogenic strains, while no phage is capable of infecting all strains. The efficiency of plating (EOP) is defined as the ratio between the highest titer obtained on the host strain (Sterne) and the observed infecting titer on non-host strains and is not included in this analysis; however, definitive decreases in infection efficiency (i.e. a ratio <1) have been observed during the course of the infections.

Discussion

This analysis is intended to determine which genetically distinct clusters of phages are capable of infecting a variety of *B. anthracis* isolates with a goal of producing an effective phage therapy cocktail capable of treating an anthrax infection. While the study is still in progress, a number of results indicate that indeed, many of the strains are susceptible to phage infection, despite the observation by Negus, et al., that the PGAC inhibits phage infection. Observed differences in the efficiency of phage infections on the pathogenic strains may be explained by the presence of the capsule but may not account for the decreased infection efficiency of the phages on the pathogenic strains of *B. anthracis*. Additional analysis may be required to determine if the capsule or mutations in surface proteins are responsible for this difference. An additional SplitsTree is included to show how genetically similar phages available on NCBI relate to the phages of this study (Fig. 3-7). Phage phi29, a *B. subtilis* phage that bears no nucleotide similarity to the *B. anthracis* phages of this study, is included as an outgroup. In analyzing this SplitsTree it becomes clear that the proteomic diversity of the phages is substantial. Interestingly, four phages from our collection from the Wß-like group, McDreamy, Athena, Booya and McSteamy, appear to have more proteomic similarity to he intended outgroup (*B. subtilis* phage phi29) than they do to the other *B. anthracis* phages. This could be indicative of possible host changes in the course of the phages' genetic history and may account
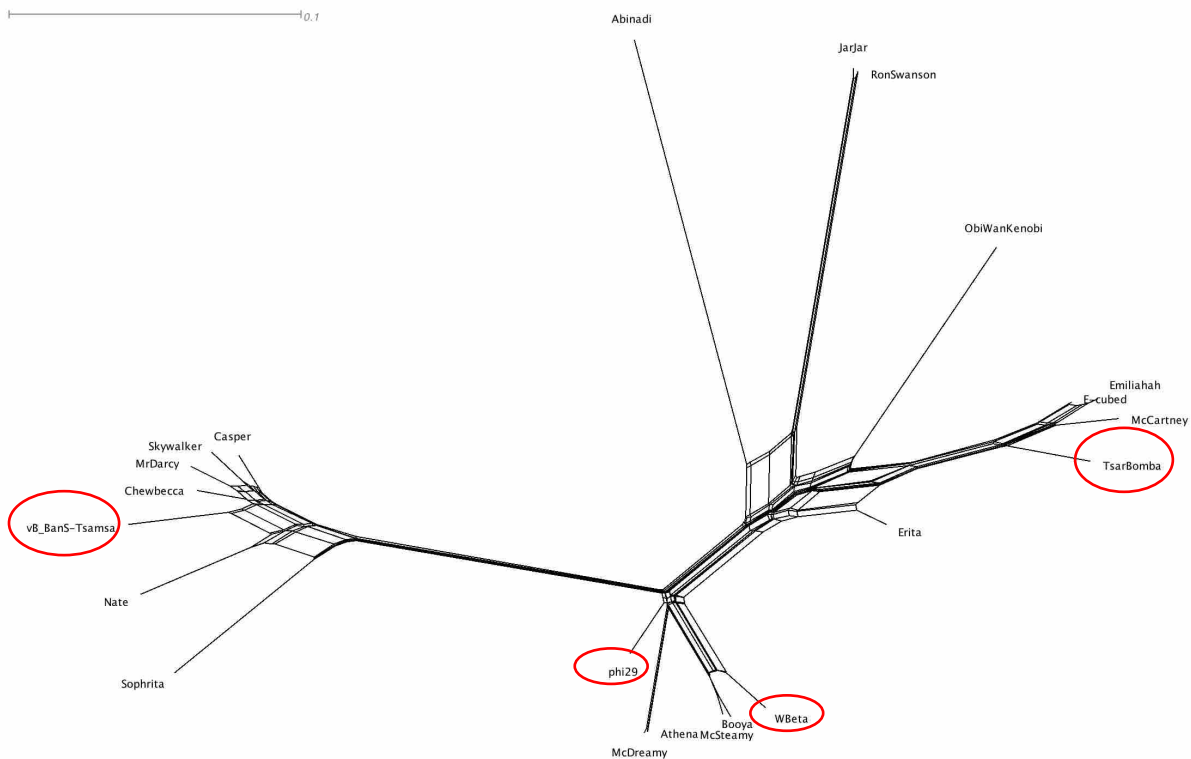
Figure 3-7. SplitsTree of *B. anthracis* phages including previously-characterized phages.
Previously characterized phages are circled in red. Similar to the SplitsTree above (Fig. 4), there are
clear clusters established, based solely off of protein similarity in the phages. In this SplitsTree, the
outgroup, *B. subtilis* phage phi29, is more closely related to two of the cluster (Athena and McDreamy,
and Booya and McSteamy) than these clusters are to other *B. anthracis* phages.

for the broad host range of the phages observed in this study. (Similarly, Wß has a known broad

host range, which may also be explained by genetic divergence from other *B. anthracis* phages).

Strains 34 and 402 seem to be the most resistant to phage infection but are still susceptible to

infection from the TsarBomba-like family of phages. This indicates some genetic components

that increase the host range of this cluster of phages beyond that which is seen from other

clusters. Additional analysis of the genes contained in these phages is needed to elucidate the

permitting factors.

Future directions

Moving forward, there is still much to be learned from these phages. In contrast to

expected results, many of the phages are capable of infecting a variety of strains, and all strains

used in this study are susceptible to at least one phage. While the PGAC does seem to inhibit phage efficiency, it does not prevent phage infection. Perhaps for the strains that seem resistant to a number of phages a higher phage titer is needed to penetrate the capsule. Yet, we see that despite the genetic diversity represented by 11 of 12 strains used in this study, all strains are susceptible to infection from phages outside of the Wß family. As the repository of phages grows annually, perhaps a new cluster of phages will arise that is completely incapable of infecting the variety of *B. anthracis* hosts.

Additional considerations moving forward that must be considered are the ability of these phages to infect sporulated *B. anthracis*. The sporulated form has a completely different and unique cell surface structure than either the avirulent Sterne strain or pathogenic strains exhibit. One phage in particular, SBP8a, is known to infect sporulated *B. anthracis*.[112] This phage bears high nucleotide similarity to phage Abinadi used in this study (>95%) as determined by BLAST nucleotide alignment, and minimal similarity to other phages in the same cluster (TsarBomba-like). Thus, it can be expected that at least Abinadi will also be capable of infecting sporulated *B. anthracis*.

CHAPTER IV: Clinical Application of Phage Therapy for Multi-Antibiotic-Resistant

Enterobacteriaceae Infections

Authors: Trever L. Thurgood, Julianne H. Grose

Abstract

Since the onset of the age of antibiotics, many millions of lives have been saved from the application of antimicrobial compounds to otherwise terminal infections. Yet, with the use of antibiotics comes the inevitability of antibiotic-resistance. Bacteria are living, adapting organisms that are keen to adapt to environmental challenges, and unless alternative treatments are rapidly developed and deployed to fight the dramatic increase in antibiotic-resistant bacterial infections, current methods of treatment will rapidly become obsolete. Phage therapy is one such alternative treatment that has shown to be effective a number of times. In 2018, our lab was contacted for collaboration to prepare a number of phage therapies for multi-drug-resistant antibiotic infections in both humans and animals. In accordance with Food and Drug Administration guidelines, multiple phage therapies were prepared for treatment and are currently in the process of delivery to treat these infections.

Introduction

A number of studies and reviews have analyzed in-depth the oncoming (or, perhaps, the already-onset) crisis of antibiotic-resistant bacterial pathogens.[25,27–30,37,113–115] The CDC has cited this facet of public health as one of its top health concerns.[24] As such, the responsibility of finding alternative treatments to these all-to-common infections falls upon researchers. Microbiologists have deployed a number of resources, one of which comes from nature itself. Bacteriophages have been shown to be effective in treating antibiotic-resistant infections in a number of cases, and show promise going into the future.[57,116–120]

The focus of this thesis has so far been on phage molecular genetics and *in vitro* experimentation of phage therapy on CRE isolates and other pathogenic species. In line with the research methods and techniques developed during the course of this program of study, multiple opportunities presented themselves to apply this research in a clinical setting. Beginning in the summer of 2018, the Grose lab in the Department of Microbiology and Molecular Biology at Brigham Young University (Provo, UT, USA) initiated collaborative efforts with doctors (and a veterinarian) to begin identifying phages capable of treating several multi-drug-resistant bacterial infections. Following stringent protocols, phage research can progress from the lab bench to the hospital room.
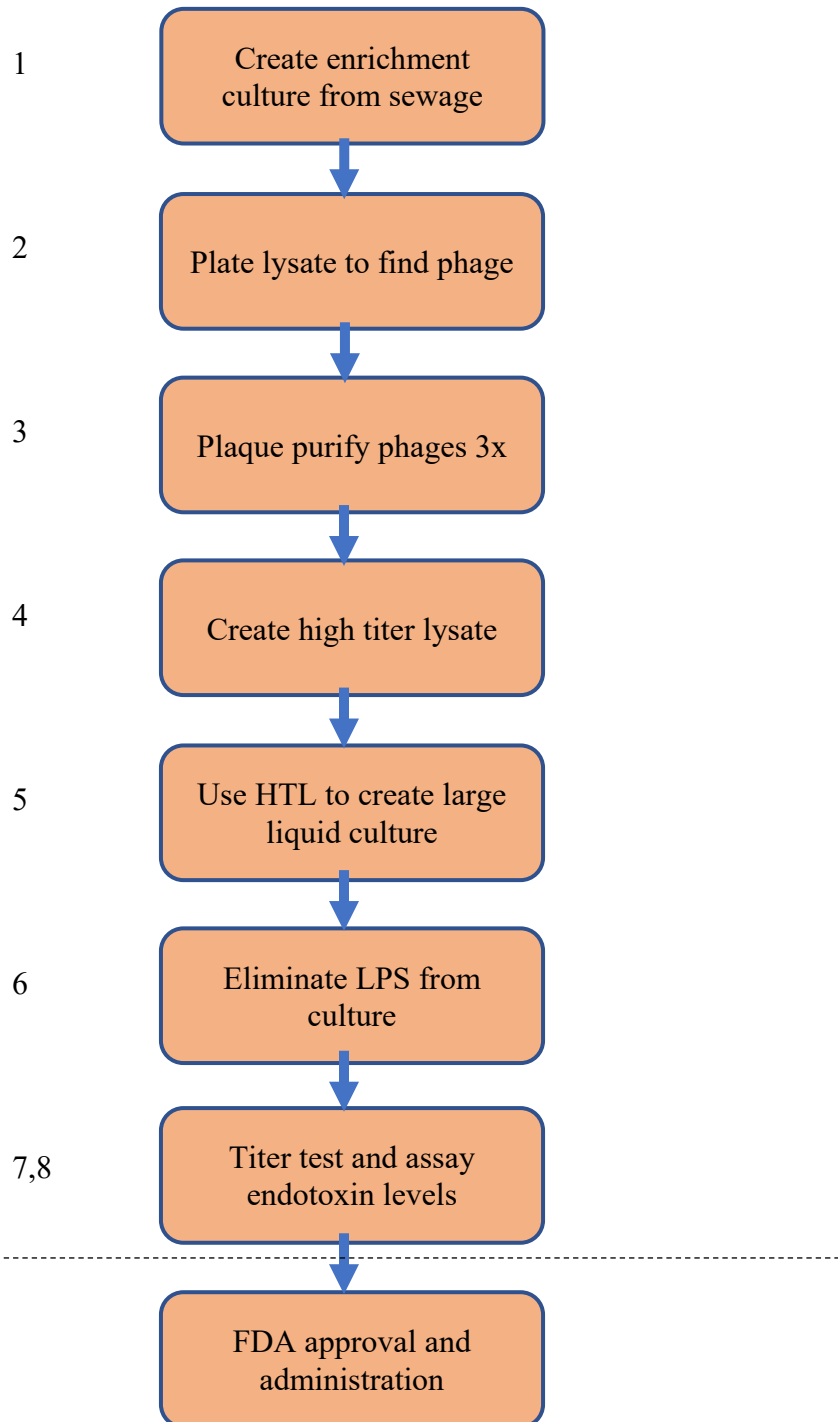
Figure 4-1. General Workflow for Phage Therapy Preparation.

Materials and methods

Enrichment culture: amplify phage from sewage

Bacterial pathogens are abundant in sewage water, and where bacteria are abundant, so, too, are phages. Thus, collection of sewage water is the initial step required for isolation and identification of phages potentially capable of infecting antibiotic-resistant bacteria. To accomplish this, aliquot 0.5mL of sewage samples into culture tubes in a biosafety cabinet. Then, add 4mL LB broth before aliquoting 0.5mL host bacteria (overnight bacterial culture) into sewage + broth mixture. Incubate 24-48 hours at 37°C, with shaking at ~200rpm.

Plaque assay

Using above enrichment culture, centrifuge for a minimum 30 minutes at 4,000 x *g* (4k x *g*) to pellet bacterial cells and debris. Decant supernatant into a clean tube and repeat a 30-minute centrifugation at the same speed. Decant the supernatant again into a clean tube. Use 50uL of the centrifuged lysate to infect 500uL bacterial host for 30 minutes at room temperature. After incubation, add 4.5mL molten LB top agar (LB TA) to the phage infection and pour over an LB agar plate. Incubate overnight between room temperature and 37°C. Check for plaque formation, indicating viable phage presence. Alternatively, a spot test can be done by mixing 0.5mL bacterial host with 4.5mL molten LB TA and pouring over LB agar plate. After setting at least 10 minutes, spot 5uL phage lysate onto plate. Incubate overnight between room temperature and 37°C. Check for plaque formation, indicating viable phage presence.

Plaque purification

Using a sterile micropipette tip, gently touch a single plaque in the center with the end of a pipette tip. Resuspend the phage in 100uL LB by gently swirling and shaking the pipette tip in the LB broth to create a plaque pick suspension. If plaques are too small to pick with a pipette

tip, an alternative is pipetting 10uL sterile LB broth onto the plaque, waiting 10-20 seconds, and decanting the liquid into LB broth for a liquid plaque pick phage resuspension. If using the liquid plaque pick, for best results, the suspension should be centrifuged either at 4k x g for 5 minutes or at full speed on a tabletop centrifuge for 30 seconds before proceeding. Create a dilution series by diluting the 100uL phage suspension into 900uL LB broth, creating a $1:10^1$ dilution. Mix by very gentle vortexing, pipetting or tapping on the desk. Decant 100uL of the 1:10 dilution into 900uL LB broth for a $1:10^2$ dilution. Repeat this decanting and resuspending until desired dilution factor is obtained (typically 10^4 for plaque picks and 10^8 for high titer lysates). Using 2-4 dilutions from the series, infect 500uL aliquots of bacterial host with 50uL phage dilutions (i.e. one aliquot of bacteria will be infected with the 10^2 dilution and one aliquot with the 10^4 dilution, etc.). Incubate at room temperature for 30 minutes. Add 4.5mL molten LB TA to the phage infection and pour over LB agar plates. Incubate overnight between room temperature and 37°C. Check for plaque formation. Alternatively, a spot test may be performed using the same protocol described above. This portion of the procedure (plaque purification) must be completed 3 times before phage can be considered "purified" from sewage samples. This is to ensure removal of superfluous viral/bacterial/toxin particles**.**

Create high titer lysate (HTL)

To create a high titer phage lysate, dilute 0.5mL overnight bacterial culture into 4.5mL LB broth. Then, the bacterial culture was inoculated with a plaque pick suspension by pipetting 100uL of the plaque pick suspension into the bacterial culture. Incubate a minimum of 6 hours at 37°C shaking at ~200rpm. Centrifuge culture for 30 minutes at 4k x g and decant supernatant into a clean tube. Centrifuge supernatant again at 30 minutes, 4k x g. Create dilution series and do a plaque assay to determine the titer.

Create large culture

Large cultures are created because large numbers of virions are needed for a successful phage therapy treatment. Aliquot 5mL overnight bacterial culture into 45mL sterile LB broth. Inoculate bacterial culture with 2-5mL phage HTL. Incubate a minimum of 6 hours at 37°C shaking at ~200rpm. Add 2-3mL chloroform to lyse bacterial cells and liberate additional phage particles; continue shaking ~30 minutes. Centrifuge culture at 4k x g for a minimum of 1 hour. Decant supernatant into clean tubes. Test phage titer via plaque assay or spot test.

Purify phage of LPS

Once a phage culture is successfully separated from bacterial cultures, the lysate is still contaminated with bacterial endotoxin. Lipopolysaccharides (LPS) are endotoxins expressed on the surface of Gram-negative bacteria and are shed into the medium while the bacteria are dividing. Even when bacteria are centrifuged and filtered from a culture, LPS still contaminates the lysate. LPS is a powerful inducer of the innate immune system in vertebrate species, and can lead to septic shock and subsequent death.[44,45] Thus, proper removal from the phage lysate is imperative for the safety and success of the therapy. Fortunately, a number of protocols have been developed for proper removal, and commercial kits are available to test the concentration of LPS in prepared samples.[47,121] For every 30mL clean lysate add 7.5mL PEG-8000, 2.5M NaCl. Incubate on ice for at least 30 minutes. Spin at 12,000 x g to pellet phage. Decant and discard supernatant. Centrifuge again 2-3x (5min) to collect remaining PEG-8000 mixture at the bottom of the tube; decant and discard. Resuspend phage in 10mL phospho-buffered saline (PBS; pH 7.4). Plate 1mL onto LB agar plates to test sterility of the resuspended phage solution.

Titer test

Assay phage titer after resuspension using serial dilution and plaque assay method mentioned above.

Assay for LPS

Assay the concentration of LPS in the resuspended lysate using the ToxinSensor[TM] Chromogenic LAL Endotoxin Assay Kit. As per the protocol on company website, begin by carefully dispensing 100 µl of standard or test sample into endotoxin-free vials.[6] All samples should be mixed thoroughly for 30 seconds with a vortexer. Avoid foaming/bubbles. Each test must include a blank as well as at least four endotoxin standards in duplicate. The blank sample vial contains 100 µl of LAL Reagent Water instead of test sample. Add 100 µl of reconstituted LAL to each vial. Cap the vials and mix well by swirling gently. If the endotoxin concentration in sample is expected in the range of 0.01 - 0.1 EU/ml, incubate the rack with all vials at 37°C±1°C for T1 using a water bath or heating block. If the endotoxin concentration is expected in the range of 0.1 - 1 EU/ml, incubate at 37°C±1°C for T2. Note: The optimal value of T1 and T2 should be referred to the label on the kit. After proper incubation, add 100 µl of reconstituted chromogenic substrate solution to each vial. Cap the vials and swirl gently to mix well. Do not shake or vortex to avoid foaming. Incubate at 37°C±1°C for 6 minutes. Add 500 µl of reconstituted Color-stabilizer #1 (Stop Solution) to each vial and swirl gently to mix well. Do not shake or vortex to avoid foaming. Add 500 µl of reconstituted Color-stabilizer #2 to each vial and mix well. Finally add 500 µl of reconstituted Color-stabilizer #3 to each vial. Gently swirl each vial to mix well. Bubbles must be avoided. Read the absorbance of each reaction vial at 545nm using distilled water as blank to adjust the photometer to zero absorbance.

---

[6] https://www.genscript.com/product/documents?cat_no=L00350&catalogtype=Document-PROTOCOL

Because phage therapy is not approved for clinical use in the United States, even if these steps are completed with accuracy and precision, the Food and Drug Administration must review each request for phage therapy on a case-by-case basis. Therefore, all protocols and preparation data must be stringently recorded and sent to the FDA for approval before use.

Creating phage frozen stock

Dissolve glycerol in distilled H_2O to create a 40% stock solution. Aliquot 600uL 40% glycerol into cryovials. Autoclave the 40% glycerol-containing cryovials on the liquid cycle (121°C, 20 min.). Once cooled, add 1mL phage lysate with high titer to the 40% glycerol, to create a final concentration of 15% glycerol. Cap and mix well by inverting cryovial. Store at -80°C.

Pulling from phage frozen stock

Dilute overnight culture of bacterial host 1:1 in LB broth. Pick a small ice chunk from frozen stock and suspend in the bacterial culture. Incubate overnight at 37°C, with shaking ~200rpm. Centrifuge the lysate at 4k x *g* for 30 minutes in microcentrifuge tube. Decant the supernatant and proceed with dilution series.

Results

Mycobacterium tuberculosis

The hunt for phages to treat an antibiotic-resistant infection began in summer 2018. A young patient with cystic fibrosis had a *M. tuberculosis* infection in their lungs and the doctors were seeking alternative treatment via phage therapy. While a number of mycobacteriophages were in their possession, none infected the strain and could not be used. In order to assist with their efforts, our lab sent a number of sewage samples from our vast library of previously collected sewage to the researchers overseeing the case.

Klebsiella pneumoniae

Similarly, a patient with a persistent urinary tract infection (UTI) caused by *K. pneumoniae* also sought alternative treatment for their infection. A collection of over a dozen phages from the Grose lab was sent to the research team only to discover that none of them infected the strain and were thus inadequate for phage therapy. Similarly, collections from other labs around the world contributed their phages to treat the infection but only a single phage in nearly one hundred partially infected the strain. To circumvent this problem, the multi-resistant strain was sent to the lab and used with our collection of international sewage samples to culture any phages capable of infecting the strain. Out of dozens of samples, only one produced a phage capable of infecting the strain. Once the phage was identified, the plaque purification protocol was followed, and a small sample of the phage lysate was sent to the research team. At this time, the infection has somewhat subsided so as to render phage therapy unnecessary until the infection worsens (again due to lack of government approval for this type of treatment).

Citrobacter freundii

Soon after the contact was made for assistance in treatment of the *K. pneumoniae* infection, another request came for treatment of a *Citrobacter freundii* infection. This time, however, the patient was a well-known sea turtle at an aquarium in Florida, named Shelley. Shelley had had an infection in her carapace and shell for over two years (Fig. 4-2). The infection was

Figure 4-2. Shelley the sea turtle shell infection. This photo was obtained from the Mote Marine Laboratory and Aquarium in Sarasota, FL from the caretakers of Shelley to demonstrate the severity of her condition.

starting to affect her behavior, so the veterinarian reached out. A similar process was followed in identifying phages using sewage samples. This time two sewage samples yielded phages. Once the phages were identified and plaque purified, phage samples were prepped directly for treatment using the above protocols. The phages were prepped, tested for LPS concentrations, and sent to Florida for the treatment of the turtle, who is reported to be doing well.

 Serratia marcescens

Finally, and perhaps most seriously, a request came in for phage therapy treatment of a *Serratia marcescens* infection that has been festering in a patient's leg for an extended period of time. The nature of the infection is that it intensifies, then ebbs with the administration of antibiotics, only to return again. While the antibiotics have limited effectiveness in treating the infection, the patient and doctors seek to eradicate the unwanted pathogen. For this they have turned to phage therapy, to administer in tandem with antibiotics, which seems to be the most effective treatment method.[91] Identifying phages necessary for treatment followed a similar isolation protocol as previously mentioned. Five phages in total were found to be effective against the strain. While the phages were being prepared, however, the bacterial strain seemed to lose virulence and the effectiveness of the phages began to decrease. Eventually, the bacteria was totally nonviable and another isolate had to be requested from the doctors treating the patient. This loss of viability was likely due to the lack of antibiotics in culturing the bacteria. Interestingly, this particular strain is susceptible to a number of antibiotics, with limited resistance to common laboratory antibiotics, such as ampicillin. Fortunately, however, culturing the bacteria with antibiotics the second time around maintained virulence and proved effective enumerating the phages. The phage cultures were purified of LPS and tested for endotoxin a number of times. Most kits are calibrated for LPS concentrations to be quite low (maximum of 1

endotoxin unit per mL). Unfortunately, while effective enough for clinical application, the LPS purification protocol written above does not remove LPS below this level. It was learned in the process of purification and LPS-level testing that samples must be diluted 10,000-fold or more *before* testing for endotoxin levels in order to gain an accurate number from the assay.

Discussion

This study has shown that identifying phages capable of infecting antibiotic-resistant bacterial strains is not only possible, but a viable option for treatment of multi-resistant infections. The process of isolating, identifying, enumerating, and purifying phages from environmental samples is a strenuous and mildly dangerous process (due to repeated exposure to known resistant bacteria), but may become the norm for treatment of bacterial infections. While phages may never have the convenience and simplicity of mass-produced antibiotic compounds, they will always have the reliability of antimicrobial activity. Phages, like bacteria, are adapting organisms, so even when a bacterium develops resistance to a single phage, the phage responds by developing a new infection mechanism. The co-evolutionary relationship of bacteria and their phages has been described as an "evolutionary arms race".[33,122] As researchers, we can utilize this arms race to develop an arsenal of phages capable of infecting even the most resistant bacterial infections, and ultimately save lives.

Figure 4-3 shows a map of the United States where the sewage samples that produced viable phages against antibiotic-resistant bacterial strains was obtained from.

Figure 4-3. Map showing location of sewage samples that produced phages capable of infecting antibiotic-resistant bacterial strains. The majority of the samples were collected from the Western United States in 2018, while one came from the East coast.

CHAPTER V: Conclusion

Review

Despite their size, bacteriophages are incredibly diverse organisms that are constantly teaching us new things about the fundamentals of biology. The genetic reservoir of phages has proven to be an abundant source of knowledge in elucidating the underlying mechanisms that drive biological processes. Additionally, phages have proven to be clinically useful in a number of settings. In this collection of studies, phage genetics has been reviewed extensively for a set of unique phages found to have interesting applications. Phages of *Klebsiella pnuemoniae*, *Bacillus anthracis, Citrobacter freundii* and *Serratia marcescens* have all been reviewed, and each has added a piece to the puzzle of biology that we, as researchers, seek to assemble.

In reviewing the proceedings of this thesis, it becomes clear that while some questions have been answered, even more questions have arisen. The relationship between phage and bacterium is complex and extensive, and it seems that we have only begun to uncover the underlying mechanisms that drive biological processes. Yet, the data included in this thesis have attempted to provide some satisfactory answers to these mysteries. From underlying genetics to clinical application of phage biology, the utility of phages is great in our efforts to learn.

From the phages of *K. pneumoniae,* certain genetic elements, such as promoter regions mimicking the host promoters, may be found in phages that could play a role in decreasing virulence. DNA sequences are essential for taking over host metabolic machinery. The new *B. anthracis* phages can be characterized based on similarity to phages in online databases, and their characterization can also provide clues into the underlying biology of what permits phage infections of this species. Finally, while many aspects of phage mechanisms remain unknown,

they can be harnessed for the eradication of bacterial infections. Thus, while not all is known, they can still be useful for biologists.

Future directions

Moving forward, additional questions need to be answered in an effort to learn more about phage biological processes and molecular mechanisms. The completion of the *B. anthracis* phage infection assays is necessary to understand how the genetic diversity of these phages affects interaction with the host. RNAseq analysis on phages capable of infecting antibiotic-resistant bacterial strains will provide insight into the functions of proteins necessary for host takeover. Bioinformatic analyses on the variety of phages of all hosts will continue to reveal unique and unknown aspects of phage genomes that will provide additional research questions for future microbiologists. In sum, our search for answers will undoubtedly lead to additional questions, as we seek to uncover the mysteries of phage-bacteria interactions.

To elucidate some of the underlying processes of the phages used to infect *K. pneumoniae* clinical isolates, tail fibers of the successful phages can be cloned into unsuccessful phages via homologous recombination to see if progeny can now infect the species of interest. Furthermore, there could be components of the bacterium responsible for preventing phage infection, such as DNA degradation post-injection, or degradation of some essential phage protein. A similar explanation may be found in *B. anthracis*. The strains used in this study nearly cover the complete genetic diversity of global *B. anthracis* strains, therefore some variability in phage infections is expected. Further characterization of the genetic variation could explain phage efficiency. Additionally, phage components may exist that specifically allow for capsule-binding, capsule-degrading or spore-binding and spore-degrading mechanisms that are yet to be characterized. No matter the case, however, there are many doorways opened by these findings.

REFERENCES

1.   Lerner BW and KL. *World of Microbiology and Immunology*. 1st ed. (Narins B, ed.). Detroit: Gale; 2003. https://pharmareview.files.wordpress.com/2011/10/microbiologyimmunology.pdf.

2.   Friebe S, van der Goot FG, Bürgi J. The Ins and Outs of Anthrax Toxin. *Toxins (Basel)*. 2016;8(3). doi:10.3390/toxins8030069

3.   Okinaka RT, Cloud K, Hampton O, et al. Sequence and organization of pXO1, the large Bacillus anthracis plasmid harboring the anthrax toxin genes. *J Bacteriol*. 1999;181(20):6509-6515. http://www.ncbi.nlm.nih.gov/pubmed/10515943. Accessed March 1, 2019.

4.   Brossier F, Mock M. Toxins of Bacillus anthracis. *Toxicon*. 2001;39(11):1747-1755. doi:10.1016/S0041-0101(01)00161-1

5.   Bhatnagar R, Batra S. Anthrax Toxin. *Crit Rev Microbiol*. 2001;27(3):167-200. doi:10.1080/20014091096738

6.   Tournier JN, Rossi Paccani S, Quesnel-Hellmann A, Baldari CT. Anthrax toxins: A weapon to systematically dismantle the host immune defenses. *Mol Aspects Med*. 2009;30(6):456-466. doi:10.1016/j.mam.2009.06.002

7.   Cleret-Buhot A, Mathieu J, Tournier JN, Quesnel-Hellmann A. Both lethal and edema toxins of bacillus anthracis disrupt the human dendritic cell chemokine network. *PLoS One*. 2012;7(8):8-13. doi:10.1371/journal.pone.0043266

8.   Jang J, Cho M, Chun J-H, et al. The poly-γ-D-glutamic acid capsule of Bacillus anthracis enhances lethal toxin activity. *Infect Immun*. 2011;79(9):3846-3854. doi:10.1128/IAI.01145-10

9.   Koehler TM. Bacillus anthracis genetics and virulence gene regulation. *Curr Top Microbiol Immunol*. 2002;271:143-164. http://www.ncbi.nlm.nih.gov/pubmed/12224521. Accessed March 1, 2019.

10.   Goel AK. Anthrax: A disease of biowarfare and public health importance. *World J Clin cases*. 2015;3(1):20-33. doi:10.12998/wjcc.v3.i1.20

11.   Driks A. The Bacillus anthracis spore. *Mol Aspects Med*. 2009;30(6):368-373. doi:10.1016/J.MAM.2009.08.001

12.   Giorno R, Bozue J, Cote C, et al. Morphogenesis of the Bacillus anthracis spore. *J Bacteriol*. 2007;189(3):691-705. doi:10.1128/JB.00921-06

13.   Liu H, Bergman NH, Thomason B, et al. Formation and composition of the Bacillus anthracis endospore. *J Bacteriol*. 2004;186(1):164-178. doi:10.1128/JB.186.1.164-178.2004

14.   Sweeney DA, Hicks CW, Cui X, Li Y, Eichacker PQ. Anthrax infection. *Am J Respir Crit Care Med*. 2011;184(12):1333-1341. doi:10.1164/rccm.201102-0209CI

15.   Albrink' WS. *PATHOGENESIS OF INHALATION ANTHRAX*. https://www.ncbi.nlm.nih.gov/pmc/articles/PMC441103/pdf/bactrev00130-0105.pdf.

Accessed March 1, 2019.

16. Bartlett JG, Inglesby T V, Borio L. *Management of Anthrax*. Vol 35.; 2002. https://www-jstor-org.erl.lib.byu.edu/stable/pdf/4483203.pdf?refreqid=excelsior%3A12ae1087b0a32e83597f03f6a31b3f1d. Accessed March 1, 2019.

17. Guidi-Rontani C, Weber-Levy M, Labruyè E, Le Mock M. *Germination of Bacillus Anthracis Spores within Alveolar Macrophages*. https://s3.amazonaws.com/academia.edu.documents/40633301/Germination_of_Bacillus_anthracis_spores20151204-19861-zwg39k.pdf?AWSAccessKeyId=AKIAIWOWYYGZ2Y53UL3A&Expires=1551435721&Signature=khNePZ4m3dCaEFEALKIQAiJnj4I%3D&response-content-disposition=inline%3B filename%3DGermination_of_Bacillus_anthracis_spores.pdf. Accessed March 1, 2019.

18. Klietmann WF, Ruoff KL. Bioterrorism: Implications for the Clinical Microbiologist. *Clin Microbiol Rev*. 2001;14(2):364-381. doi:10.1128/CMR.14.2.364-381.2001

19. Monegro AF, Regunath H. *Hospital Acquired Infections*. StatPearls Publishing; 2019. http://www.ncbi.nlm.nih.gov/pubmed/28722887. Accessed May 14, 2019.

20. Guentzel MN. *Escherichia, Klebsiella, Enterobacter, Serratia, Citrobacter,and Proteus*. University of Texas Medical Branch at Galveston; 1996. http://www.ncbi.nlm.nih.gov/pubmed/21413290. Accessed May 14, 2019.

21. Li B, Zhao Y, Liu C, Chen Z, Zhou D. Molecular pathogenesis of Klebsiella pneumoniae. *Future Microbiol*. 2014;9(9):1071-1081. doi:10.2217/fmb.14.48

22. HEJAZI A, FALKINER FR. Serratia marcescens. *J Med Microbiol*. 1997;46(11):903-912. doi:10.1099/00222615-46-11-903

23. Luperchio SA, Schauer DB. Molecular pathogenesis of Citrobacter rodentium and transmissible murine colonic hyperplasia. *Microbes Infect*. 2001;3(4):333-340. doi:10.1016/S1286-4579(01)01387-9

24. Antibiotic / Antimicrobial Resistance | CDC. https://www.cdc.gov/drugresistance/. Accessed May 14, 2019.

25. Conly J, Johnston B. Where are all the new antibiotics? The new antibiotic paradox. *Can J Infect Dis Med Microbiol = J Can des Mal Infect la Microbiol medicale*. 2005;16(3):159-160. http://www.ncbi.nlm.nih.gov/pubmed/18159536. Accessed May 14, 2019.

26. The top 10 causes of death. https://www.who.int/news-room/fact-sheets/detail/the-top-10-causes-of-death. Accessed May 14, 2019.

27. Codjoe F, Donkor E. Carbapenem Resistance: A Review. *Med Sci*. 2017;6(1):1. doi:10.3390/medsci6010001

28. Blair JMA, Webber MA, Baylay AJ, Ogbolu DO, Piddock LJ V. Molecular mechanisms of antibiotic resistance. *Nat Rev Microbiol*. 2015;13(1):42-51. doi:10.1038/nrmicro3380

29. Xia J, Gao J, Tang W. Nosocomial infection and its molecular mechanisms of antibiotic resistance. *Biosci Trends*. 2016;10(1):14-21. doi:10.5582/bst.2016.01020

30. Ventola C. The antibiotic resistance crisis: Causes and threats. *P T*. 2015;40(4):277-283. http://www.ncbi.nlm.nih.gov/pubmed/25859123%0Ahttp://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=PMC4378521.

31. Schroeder M, Brooks BD, Brooks AE. The Complex Relationship between Virulence and Antibiotic Resistance. *Genes (Basel)*. 2017;8(1). doi:10.3390/genes8010039

32. Summers WC. The strange history of phage therapy. doi:10.4161/bact.20757

33. Stern A, Sorek R. The phage-host arms race: shaping the evolution of microbes. *Bioessays*. 2011;33(1):43-51. doi:10.1002/bies.201000071

34. Rakhuba DV1, Kolomiets EI, Dey ES NG. Bacteriophage receptors, mechanisms of phage adsorption and penetration into host cell.Bacteriophage receptors, mechanisms of phage adsorption and penetration into host cell. *Polish J Microbiol*. 2010;59(3):145-155. http://www.pjm.microbiology.pl/archive/vol5932010145.pdf. Accessed May 16, 2019.

35. Menouni R, Hutinet G, Petit M-A, Ansaldi M. Bacterial genome remodeling through bacteriophage recombination. *FEMS Microbiol Lett*. 2015;362(1):1-10. doi:10.1093/femsle/fnu022

36. Negus D, Burton J, Sweed A, Gryko R, Taylor PW. Poly-D-Glutamic Acid Capsule Interferes with Lytic Infection of Bacillus anthracis by B. anthracis-Specific Bacteriophages. 2013. doi:10.1128/AEM.02682-12

37. Agnė Giedraitienė1, Astra Vitkauskienė2 , Rima Naginienė3 AP. Antibiotic Resistance Mechanisms of Clinically Important Bacteria. *Medicina (B Aires)*. 2011;47(3):137-146. papers2://publication/uuid/15887EFE-B967-429C-8127-C6FECE1E470F.

38. Thomas CM, Nielsen KM. Mechanisms of, and barriers to, horizontal gene transfer between bacteria. *Nat Rev Microbiol*. 2005;3(9):711-721. doi:10.1038/nrmicro1234

39. Wagner PL, Waldor MK. Bacteriophage control of bacterial virulence. *Infect Immun*. 2002;70(8):3985-3993. doi:10.1128/IAI.70.8.3985-3993.2002

40. Van Ert MN, Easterday WR, Huynh LY, et al. Global Genetic Population Structure of Bacillus anthracis. Petrosino J, ed. *PLoS One*. 2007;2(5):e461. doi:10.1371/journal.pone.0000461

41. Kumar S, Stecher G, Li M, Knyaz C, Tamura K. MEGA X: Molecular Evolutionary Genetics Analysis across Computing Platforms. Battistuzzi FU, ed. *Mol Biol Evol*. 2018;35(6):1547-1549. doi:10.1093/molbev/msy096

42. Bailey TL, Boden M, Buske FA, et al. MEME SUITE: tools for motif discovery and searching. *Nucleic Acids Res*. 2009;37(Web Server):W202-W208. doi:10.1093/nar/gkp335

43. Gupta S, Stamatoyannopoulos JA, Bailey TL, Noble W. Quantifying similarity between motifs. *Genome Biol*. 2007;8(2):R24. doi:10.1186/gb-2007-8-2-r24

44. Alexander C, Rietschel ET. Bacterial lipopolysaccharides and innate immunity. *J Endotoxin Res*. 2001;7(3):167-202. http://www.ncbi.nlm.nih.gov/pubmed/11581570. Accessed July 6, 2019.

45. Rosadini C V, Kagan JC. Early innate immune responses to bacterial LPS. *Curr Opin*

Immunol. 2017;44:14-19. doi:10.1016/j.coi.2016.10.005

46.   Tan Y, Kagan JC. A cross-disciplinary perspective on the innate immune responses to bacterial lipopolysaccharide. *Mol Cell*. 2014;54(2):212-223. doi:10.1016/j.molcel.2014.03.012

47.   Baker Laboratory. Protocol - Phage PEG precipitation/purification. http://depts.washington.edu/bakerpg/protocols/phage_precipitation.html. Published 1999.

48.   Szermer-Olearnik B, Boratyński J. Removal of endotoxins from bacteriophage preparations by extraction with organic solvents. *PLoS One*. 2015;10(3):e0122672. doi:10.1371/journal.pone.0122672

49.   Cao F, Wang X, Wang L, et al. Evaluation of the Efficacy of a Bacteriophage in the Treatment of Pneumonia Induced by Multidrug Resistance *Klebsiella pneumoniae* in Mice. *Biomed Res Int*. 2015;2015:1-9. doi:10.1155/2015/752930

50.   Podschun R, Ullmann U. Klebsiella spp. as nosocomial pathogens: epidemiology, taxonomy, typing methods, and pathogenicity factors. *Clin Microbiol Rev*. 1998;11(4):589-603. http://www.ncbi.nlm.nih.gov/pubmed/9767057. Accessed June 29, 2019.

51.   Gupta N, Limbago BM, Patel JB, Kallen AJ. Carbapenem-Resistant Enterobacteriaceae: Epidemiology and Prevention. *Clin Infect Dis*. 2011;53(1):60-67. doi:10.1093/cid/cir202

52.   Bratu S, Landman D, Haag R, et al. Rapid Spread of Carbapenem-Resistant Klebsiella pneumoniae in New York City. *Arch Intern Med*. 2005;165(12):1430. doi:10.1001/archinte.165.12.1430

53.   Patel G, Huprikar S, Factor SH, Jenkins SG, Calfee DP. Outcomes of Carbapenem-Resistant *Klebsiella pneumoniae* Infection and the Impact of Antimicrobial and Adjunctive Therapies. *Infect Control Hosp Epidemiol*. 2008;29(12):1099-1106. doi:10.1086/592412

54.   Yigit H, Queenan AM, Anderson GJ, et al. Novel Carbapenem-Hydrolyzing -Lactamase, KPC-1, from a Carbapenem-Resistant Strain of Klebsiella pneumoniae. *Antimicrob Agents Chemother*. 2001;45(4):1151-1161. doi:10.1128/AAC.45.4.1151-1161.2001

55.   Hanlon GW. Bacteriophages: an appraisal of their role in the treatment of bacterial infections. *Int J Antimicrob Agents*. 2007;30(2):118-128. doi:10.1016/j.ijantimicag.2007.04.006

56.   Summers WC. Bacteriophage Therapy. *Annu Rev Microbiol*. 2001;55(1):437-451. doi:10.1146/annurev.micro.55.1.437

57.   Sulakvelidze A, Alavidze Z, Morris JG, Jr. Bacteriophage therapy. *Antimicrob Agents Chemother*. 2001;45(3):649-659. doi:10.1128/AAC.45.3.649-659.2001

58.   Wittebole X, De Roock S, Opal SM. A historical overview of bacteriophage therapy as an alternative to antibiotics for the treatment of bacterial pathogens. *Virulence*. 2014;5(1):226-235. doi:10.4161/viru.25991

59.   Międzybrodzki R, Borysowski J, Weber-Dąbrowska B, et al. Clinical Aspects of Phage Therapy. In: *Advances in Virus Research*. Vol 83. ; 2012:73-121. doi:10.1016/B978-0-12-

394438-2.00003-7

60. Jung L, Ding T, Ahn J. Evaluation of lytic bacteriophages for control of multidrug-resistant Salmonella Typhimurium. *Ann Clin Microbiol Antimicrob*. 2017;16(1):66. doi:10.1186/s12941-017-0237-6

61. Pallavali RR, Degati VL, Lomada D, Reddy MC, Durbaka VRP. Isolation and in vitro evaluation of bacteriophages against MDR-bacterial isolates from septic wound infections. Das G, ed. *PLoS One*. 2017;12(7):e0179245. doi:10.1371/journal.pone.0179245

62. Wang Z, Zheng P, Ji W, et al. SLPW: A Virulent Bacteriophage Targeting Methicillin-Resistant Staphylococcus aureus In vitro and In vivo. *Front Microbiol*. 2016;7:934. doi:10.3389/fmicb.2016.00934

63. Chan BK, Sistrom M, Wertz JE, Kortright KE, Narayan D, Turner PE. Phage selection restores antibiotic sensitivity in MDR Pseudomonas aeruginosa. *Sci Rep*. 2016;6(1):26717. doi:10.1038/srep26717

64. M100Ed29 | Performance Standards for Antimicrobial Susceptibility Testing, 29th Edition. https://clsi.org/standards/products/microbiology/documents/m100/. Accessed June 29, 2019.

65. Kearse M, Moir R, Wilson A, et al. Geneious Basic: An integrated and extendable desktop software platform for the organization and analysis of sequence data. *Bioinformatics*. 2012;28(12):1647-1649. doi:10.1093/bioinformatics/bts199

66. Besemer J, Lomsadze A, Borodovsky M. GeneMarkS: a self-training method for prediction of gene starts in microbial genomes. Implications for finding sequence motifs in regulatory regions. *Nucleic Acids Res*. 2001;29:2607-2618.

67. Krumsiek J, Arnold R, Rattei T. Gepard: a rapid and sensitive tool for creating dotplots on genome scale. *Bioinformatics*. 2007;23(8):1026-1028. doi:10.1093/bioinformatics/btm039

68. Lamine JG, DeJong RJ, Nelesen SM. PhamDB: a web-based application for building Phamerator databases. *Bioinformatics*. 2016;32(13):2026-2028. doi:10.1093/bioinformatics/btw106

69. Cresawn SG, Bogel M, Day N, Jacobs-Sera D, Hendrix RW, Hatfull GF. Phamerator: a bioinformatic tool for comparative bacteriophage genomics. *BMC Bioinformatics*. 2011;12(1):395. doi:10.1186/1471-2105-12-395

70. Madeira F, Park YM, Lee J, et al. The EMBL-EBI search and sequence analysis tools APIs in 2019. *Nucleic Acids Res*. 2019;47(W1):W636-W641. doi:10.1093/nar/gkz268

71. Huson DH, Bryant D. Application of Phylogenetic Networks in Evolutionary Studies. *Mol Biol Evol*. 2006;23(2):254-267. doi:10.1093/molbev/msj030

72. Lowe TM, Chan PP. tRNAscan-SE On-line: integrating search and context for analysis of transfer RNA genes. *Nucleic Acids Res*. 2016;44(W1):W54-W57. doi:10.1093/nar/gkw413

73. Bailey TL, Elkan C. Fitting a mixture model by expectation maximization to discover motifs in biopolymers. In: *Proceedings of the Second International Conference on Intelligent Systems for Molecular Biology*. ; 1994:28-36.

74.   Afgan E, Baker D, Batut B, et al. The Galaxy platform for accessible, reproducible and collaborative biomedical analyses: 2018 update. *Nucleic Acids Res*. 2018;46(W1):W537-W544. doi:10.1093/nar/gky379

75.   Munch R, Hiller K, Barg H, et al. PRODORIC: prokaryotic database of gene regulation. *Nucleic Acids Res*. 2003;31(1):266-269. doi:10.1093/nar/gkg037

76.   Grose JH, Casjens SR. Understanding the enormous diversity of bacteriophages: The tailed phages that infect the bacterial family Enterobacteriaceae. *Virology*. 2014;468-470:421-443. doi:10.1016/j.virol.2014.08.024

77.   Pope WH, Jacobs-Sera D, Russell DA, et al. Expanding the Diversity of Mycobacteriophages: Insights into Genome Architecture and Evolution. Aziz R, ed. *PLoS One*. 2011;6(1):e16329. doi:10.1371/journal.pone.0016329

78.   Jacobs-Sera D, Marinelli LJ, Bowman C, et al. On the nature of mycobacteriophage diversity and host preference. *Virology*. 2012;434(2):187-201. doi:10.1016/j.virol.2012.09.026

79.   Huson DH. SplitsTree: analyzing and visualizing evolutionary data. *Bioinformatics*. 1998;14(1):68-73. doi:10.1093/bioinformatics/14.1.68

80.   Hatfull GF. Bacteriophage Genomics. *Curr Opin Microbiol*. 2008;11(5):447. doi:10.1016/J.MIB.2008.09.004

81.   Rohwer F, Edwards R. The Phage Proteomic Tree: a Genome-Based Taxonomy for Phage. *J Bacteriol*. 2002;184(16):4529-4535. doi:10.1128/JB.184.16.4529-4535.2002

82.   Casjens SR, Grose JH. Contributions of P2- and P22-like prophages to understanding the enormous diversity and abundance of tailed bacteriophages. *Virology*. 2016;496:255-276. doi:10.1016/j.virol.2016.05.022

83.   Grose JH, Casjens SR. Understanding the enormous diversity of bacteriophages: the tailed phages that infect the bacterial family Enterobacteriaceae. *Virology*. 2014;0:421. doi:10.1016/J.VIROL.2014.08.024

84.   Okada F, Ando Y, Honda K, et al. Acute *Klebsiella pneumoniae* pneumonia alone and with concurrent infection: comparison of clinical and thin-section CT findings. *Br J Radiol*. 2010;83(994):854-860. doi:10.1259/bjr/28999734

85.   Chan BK, Abedon ST, Loc-Carrillo C. Phage cocktails and the future of phage therapy. *Future Microbiol*. 2013;8(6):769-783. doi:10.2217/fmb.13.47

86.   Koskella B, Brockhurst MA. Bacteria–phage coevolution as a driver of ecological and evolutionary processes in microbial communities. *Fems Microbiol Rev*. 2014;38(5):916. doi:10.1111/1574-6976.12072

87.   Canchaya C, Proux C, Fournous G, Bruttin A, Brüssow H. Prophage genomics. *Microbiol Mol Biol Rev*. 2003;67(2):238-76, table of contents. doi:10.1128/mmbr.67.2.238-276.2003

88.   Brussow H, Canchaya C, Hardt W-D. Phages and the Evolution of Bacterial Pathogens: from Genomic Rearrangements to Lysogenic Conversion. *Microbiol Mol Biol Rev*. 2004;68(3):560-602. doi:10.1128/MMBR.68.3.560-602.2004

89.   Bailly-Bechet M, Vergassola M, Rocha E. Causes for the intriguing presence of tRNAs in

phages. *Genome Res*. 2007;17(10):1486-1495. doi:10.1101/gr.6649807

90. Duval V, Lister IM. MarA, SoxS and Rob of Escherichia coli - Global regulators of multidrug resistance, virulence and stress response. *Int J Biotechnol wellness Ind*. 2013;2(3):101-124. doi:10.6000/1927-3037.2013.02.03.2

91. Torres-Barceló C. The disparate effects of bacteriophages on antibiotic-resistant bacteria. *Emerg Microbes Infect*. 2018;7(1):168. doi:10.1038/s41426-018-0169-z

92. Madle-Samardzija N, Turkulov V, Vukadinov J, Canak G, Doder R, Sević S. [Anthrax-- the past, present and future]. *Med Pregl*. 55(3-4):114-119. http://www.ncbi.nlm.nih.gov/pubmed/12070927. Accessed March 1, 2019.

93. Sternbach G. The history of anthrax. *J Emerg Med*. 2003;24(4):463-467. doi:10.1016/S0736-4679(03)00079-9

94. Simonson TS, Okinaka RT, Wang B, et al. Bacillus anthracis in China and its relationship to worldwide lineages. 2009. doi:10.1186/1471-2180-9-71

95. Tasota FJ, Henker RA, Hoffman LA. Anthrax as a biological weapon: an old disease that poses a new threat. *Crit Care Nurse*. 2002;22(5):21-32, 34; quiz 35-6. http://www.ncbi.nlm.nih.gov/pubmed/12382615. Accessed March 1, 2019.

96. Decker J, Hecht A. *Deadly Diseases and Epidemics: Anthrax*. (Babcock HM, ed.). Chelsea House Publishers; 2008.

97. Swiderski RM. *Anthrax: A History*. Jefferson: McFarland & Company, Inc., Publishers; 2004.

98. Vaccine I of M (US) C to A the S and E of the A, Joellenbeck LM, Zwanziger LL, Durch JS, Strom BL. Anthrax Vaccine Efficacy. 2002. https://www.ncbi.nlm.nih.gov/books/NBK220536/. Accessed March 1, 2019.

99. Ashford DA, Rotz LD. *Use of Anthrax Vaccine in the United States*.; 2000. https://www.cdc.gov/mmwr/preview/mmwrhtml/rr4915a1.htm. Accessed March 1, 2019.

100. Whatmore AM, Reed RH. Determination of turgor pressure in Bacillus subtilis: a possible role for K+ in turgor regulation. *J Gen Microbiol*. 1990;136(12):2521-2526. doi:10.1099/00221287-136-12-2521

101. Fouet A, Mesnage S. Bacillus anthracis cell envelope components. *Curr Top Microbiol Immunol*. 2002;271:87-113. http://www.ncbi.nlm.nih.gov/pubmed/12224525. Accessed June 3, 2019.

102. Koehler TM. Bacillus anthracis genetics and virulence gene regulation. *Curr Top Microbiol Immunol*. 2002;271:143-164. http://www.ncbi.nlm.nih.gov/pubmed/12224521. Accessed April 8, 2019.

103. Mesnage S, Tosi-Couture E, Gounon P, Mock M, Fouet A. The capsule and S-layer: Two independent and yet compatible macromolecular structures in Bacillus anthracis. *J Bacteriol*. 1998;180(1):52-58.

104. Agaisse H, Gominet M, Okstad OA, Kolstø AB, Lereclus D. PlcR is a pleiotropic regulator of extracellular virulence factor gene expression in Bacillus thuringiensis. *Mol Microbiol*. 1999;32(5):1043-1053. http://www.ncbi.nlm.nih.gov/pubmed/10361306.

Accessed June 3, 2019.

105. Davison S, Couture-Tosi E, Candela T, Mock M, Fouet A. Identification of the Bacillus anthracis Phage Receptor. *J Bacteriol*. 2005;187(19):6742-6749. doi:10.1128/JB.187.19.6742-6749.2005

106. Sozhamannan S, Chute MD, Mcafee FD, et al. The Bacillus anthracis chromosome contains four conserved, excision-proficient, putative prophages. 2006. doi:10.1186/1471-2180-6-34

107. Pilo P, Frey J. Pathogenicity, population genetics and dissemination of Bacillus anthracis. *Infect Genet Evol*. 2018;64:115-125. doi:10.1016/j.meegid.2018.06.024

108. Minakhin L, Semenova E, Liu J, et al. Genome Sequence and Gene Expression of Bacillus anthracis Bacteriophage Fah. *J Mol Biol*. 2005;354(1):1-15. doi:10.1016/J.JMB.2005.09.052

109. Fulmer PA. *Susceptibility of Bacillus Anthracis to Gamma and Cherry Bacteriophage*. https://digitalcommons.lsu.edu/gradschool_theses. Accessed July 7, 2019.

110. Schuch R, Nelson D, Fischetti VA. A bacteriolytic agent that detects and kills Bacillus anthracis. *Nature*. 2002;418(6900):884-889. doi:10.1038/nature01026

111. Grose JH, Jensen GL, Burnett SH, Breakwell DP. Genomic comparison of 93 Bacillus phages reveals 12 clusters, 14 singletons and remarkable diversity. *BMC Genomics*. 2014;15(1):855. doi:10.1186/1471-2164-15-855

112. Fu X, Walter MH, Paredes A, Morais MC, Liu J. The mechanism of DNA ejection in the Bacillus anthracis spore-binding phage 8a revealed by cryo-electron tomography. 2011. doi:10.1016/j.virol.2011.08.028

113. De Pascale G, Wright GD. Antibiotic resistance by enzyme inactivation: From mechanisms to solutions. *ChemBioChem*. 2010;11(10):1325-1334. doi:10.1002/cbic.201000067

114. Papp-Wallace KM, Endimiani A, Taracila MA, Bonomo RA. Carbapenems: past, present, and future. *Antimicrob Agents Chemother*. 2011;55(11):4943-4960. doi:10.1128/AAC.00296-11

115. Zaman S Bin, Hussain MA, Nye R, Mehta V, Mamun KT, Hossain N. A Review on Antibiotic Resistance: Alarm Bells are Ringing. *Cureus*. 2017;9(6):e1403. doi:10.7759/cureus.1403

116. Abedon ST, Kuhl SJ, Blasdel BG, Kutter EM. Phage treatment of human infections. *Bacteriophage*. 2011. doi:10.4161/bact.1.2.15845

117. Jassim SAA, Limoges RG. Natural solution to antibiotic resistance: bacteriophages "The Living Drugs". *World J Microbiol Biotechnol*. 2014;30(8):2153-2170. doi:10.1007/s11274-014-1655-7

118. Oechslin F. Resistance development to bacteriophages occurring during bacteriophage therapy. *Viruses*. 2018;10(7). doi:10.3390/v10070351

119. Rohde C, Wittmann J, Kutter E. Bacteriophages: A Therapy Concept against Multi-Drug-Resistant Bacteria. *Surg Infect (Larchmt)*. 2018;19(8):737. doi:10.1089/sur.2018.184

120. Chan BK, Turner PE, Kim S, Mojibian HR, Elefteriades JA, Narayan D. Phage treatment of an aortic graft infected with Pseudomonas aeruginosa. *Evol Med public Heal*. 2018;2018(1):60-66. doi:10.1093/emph/eoy005

121. Szermer-Olearnik B, Boratyński J. Removal of endotoxins from bacteriophage preparations by extraction with organic solvents. *PLoS One*. 2015;10(3):e0122672. doi:10.1371/journal.pone.0122672

122. Shabbir MAB, Hao H, Shabbir MZ, Wu Q, Sattar A, Yuan Z. Bacteria vs. Bacteriophages: Parallel Evolution of Immune Arsenals. *Front Microbiol*. 2016;7:1292. doi:10.3389/fmicb.2016.01292