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ISOLATION, CHARACTERIZATION, AND ANNOTATION OF A NOVEL MYCOBACTERIOPHAGE EAGLEPRIDE

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ISOLATION, CHARACTERIZATION, AND ANNOTATION OF A NOVEL MYCOBACTERIOPHAGE EAGLEPRIDE

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Glossary

Amplification – Acquisition of high titer lysate for downstream applications, including seeding of plates, pooling of phage, and precipitation of phage.

Annotation – The process of identifying the function and location of genes and the coding region within the genome.

Aseptic – Using practices and procedures to prevent contamination from microorganisms.

Bacterial lawn – A uniform and uninterrupted layer of bacterial growth in which individual colonies cannot be observed.

Bactericidal – The killing of bacteria.

Bacteriophages – A virus that parasitizes a bacterium by infecting and reproducing inside it.

Bacteriostatic – The prevention of bacterial growth, often facilitated by restricting the bacteria to stationary growth.

Biofilm – A communal aggregation of bacterial cells encased within exopolysaccharides and attached to a solid surface.

Broad-spectrum – Effective against a large variety of organisms

Capsid – A protein shell that encloses the viral genome.

Capsomere - A self-assembling protein subunits that form a protective outer covering around the capsid.

Colony - A group of bacteria derived from the same mother cell.

Electrophoresis – The movement of particles in a fluid or gel by the influence of an electric field.

Enrichment – A method used to promote the growth of specific species of microorganisms.

Enveloped viruses – Viruses whose nucleoprotein core is surrounded by a lipoprotein envelope consisting of a closed bilayer of lipid-derived cells from the host cell's membrane.

Genome – The complete genetic complement contained in a DNA or RNA molecule in a virus.

High-Titer Lysate (HTL) - High concentration of liquid lysate.

Holin – Diverse group of small proteins produced by the dsDNA bacteriophages to trigger and control the degradation of the host's cell wall at the end of the lytic cycle.

Lysis – Common viral infection outcome consists of cell membrane disruption and cell death and release of cytoplasmic compounds in the extracellular matrix.

Lysogenic phage – A bacteriophage that integrates its nucleic acid into the host bacterium's genome.

Mesa – Represents the growth of host bacterial cells in the presence of bacteriophages.

Microliter (μL) – Unit of volume representing 10^{-6} liters or one-millionth of a liter.

Milliliter (mL) – Unit of volume representing 10^{-3} or one-thousandth of a liter.

Morphotype - The morphology of the bacteriophage capsid.

Mycobacterium– Immobile, slow-growing, rod-shaped, gram-positive bacteria of the Mycobacteriaceae family.

Mycobacteriophage – A bacteriophage that infects hosts within the mycobacterium genus.

Mycobacterium smegmatis– An acid-fast, non-pathogenic species of bacteria in the *Mycobacterium* genus.

Naked viruses – Viruses without an envelope.

Nosocomial infections – Infection(s) acquired receiving health care that was not present during the time of admission.

Open reading frame (ORF) – The portion of DNA between the start codon and termination codon which can be translated into protein.

Phage therapy – The therapeutic use of bacteriophages to treat pathogenic bacterial infections.
Pham – Represents a family of phages genes with related sequences determined by pairwise amino acid sequence comparisons of the predicted gene product.

Phenotype – The observable characteristics of an organism from the interaction of its genotype with the environment.

Plaque – The clear zones formed on a lawn of bacteria due to phage lysis.

Plaque forming units (PFU) – The measure utilized to determine the number of virus particles capable of forming plaques per unit volume.

Plasmid -- A small circular DNA strand in the cytoplasm of bacterium or protozoan that can replicate independently of the chromosomes.

Prophage – The genetic material of bacteriophages, incorporated in the bacteria genome and able to produce phage if specifically activated.

Putative protein function – Genes that share sequence similarities to already characterized genes can thus be inferred to share a similar function.

Purification – Methods utilized to obtain a single type of virus.

Quorum sensing – A process of cell-cell communication that allows bacteria to share information about cell density and adjust gene expression accordingly.

Serial dilution – The stepwise dilution of a substance in solution in which the dilution factor at each factor is constant.

Titer – A procedure used to quantify the density of plaque-forming units.

Transcription – The utilization of the hosts' transcriptional machinery by the phage to direct the expression of its genes.

Virulent phage – Phages that utilize the lytic pathway in which rapid viral replication ends in progeny release and bacterial death.

Web plates – Media plates containing a concentrated number of bacteriophages, resulting in minimal bacterial lawn growth.

Abbreviations

ADC – albumin dextrose catalase

BLAST – basic local alignment search tool

Bp – base pairs

BSL – biological safety level

DI – demineralized

dsDNA -- double-stranded DNA

EPA – environmental protection agency

GRAS – generally regarded as safe

HTL – high titer lysate

LTL – low titer lysate

NC – negative control

NTM – non-tubercles mycobacterium

ORFs – open reading frame

PFUs – plaque-forming units

QS – quorum sensing

RGM – rapid growth mycobacterium

rpm – revolutions per minute

SDS – sodium dodecyl sulfate

SEA-PHAGES – science education alliance-phage hunters advancing genomics

SGM – slow growth mycobacterium

SSC – start-stop codons

ssDNA – single-stranded DNA

TB – tuberculosis

TBE – tris borate EDTA

tRNA – transfer RNA

UD – undiluted

USDA – United States drug administration

ABSTRACT

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TITLE OF THESIS: Isolation, characterization, and annotation of a novel mycobacteriophage Eaglepride

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ABSTRACT: Bacteriophages (phages) are obligate intracellular parasitic viruses that infect bacterial host cells. They are the most abundant biological entity on the planet, with an estimated 10^{31} viral particles in the biosphere. Bacteriophages play various positions in the biosphere by modulating evolutionary and ecological trends in the local and global microbial communities. These roles include controlling the global bacterial host populations through lytic infections, which are critical for biomass turnover by the lysis-mediated release of nutrients from the microbial biomass into the ecosystem. In marine environments, phages play a crucial role in the biogeochemical cycling process of bacterial species. Phage therapy is an important aspect of bacteriophage research due to the phages' narrow host range and inability to infect eukaryotic cells. The use of phages to control bacterial infections is medically relevant due to the rise of antibiotic-resistant bacteria and the adverse side effects of other forms of therapy. Mycobacteriophages can infect *Mycobacterium* genus hosts. *Mycobacterium smegmatis* mc^2155 (*M. smegmatis*) was utilized to isolate mycobacteriophages due to its non-pathogenic and rapid growth properties. The mycobacteriophage Eaglepride was isolated using an enriched soil sample from Ashburn, Virginia (39.014667 N, 77.515417 W). As the soil sample was collected from Eagle Ridge middle school, it was named Eaglepride. The isolation of Eaglepride on host bacteria-seeded 7H10 agar plates produced clear, transparent plaques that were 3 mm in diameter. Electron microscopy imaging of the phage showed a Siphoviridae morphology with a tail length of 175nm, tail width of 20nm, and a head diameter of 55nm. Eaglepride is a temperate phage, capable of using the lytic and lysogenic

life cycle. Eaglepride had stable growth at temperatures below 60^{o}C and pH between 6 and 8. Genome annotation showed Eaglepride to be most closely related to phages in the A clusters and A10 subcluster. Eaglepride has a total genomic length of 50,926 bp and a GC content of 64.9%. Eaglepride has 88 open reading frames (ORFs), and the annotated genome sequence is in the GenBank database with an accession number of MZ322017. Eaglepride is not a good candidate for phage therapy due to its narrow host range and its utilization of the lysogenic life cycle.

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Chapter 1: Introduction

1.1: Bacteriophages – Bacteriophages are viruses that infect bacterial hosts (1). The word "bacteriophage" is derived from Latin, meaning "bacteria eater" due to plaque formation on the bacterial lawn caused by phage lysis of bacterial cells (2). Bacteriophages are composed of a capsid protein structure that encases nucleic acid, consisting of either DNA or RNA (3). The nucleic acid material and protein capsid is the capsomere, composed of protein monomers held together through van der walls interactions, disulfide bonds, peptide bonds, and hydrogen bonds (5, 6). Most phages possess a long, thin tail to attach to the bacteria, penetrate the bacterial cell walls, and inject their viral genome (7). Some viruses also contain an envelope surrounding their capsid which is derived from the host's Golgi membrane, plasma membrane, or endoplasmic reticulum (8, 9, 10). Most enveloped viruses are known to infect animal cells (11). Non-enveloped viruses are referred to as naked viruses (12). Among the differences between enveloped and non-enveloped viruses is the method of entry into the host cell (13). In enveloped viruses, the envelope acts as an anchor for the viral glycoproteins. It facilitates the entrance of the newly budded virus into a host by recognizing and binding to host cell receptors (14). The budding process of enveloped viruses allows them to leave the host cell without killing it (15). Naked viruses, however, replicate in the host cell and escape by killing the host cell through lysis (16). Bacteriophages are often naked viruses that lyse the host cell after the replication cycle is completed (17).

The replication strategies utilized by bacteriophages include the lytic (virulent) and the lysogenic (temperate) pathways. These can be determined by gene expression and environmental factors (Figure 1) (18 19). Both pathways involve cell surface receptor-mediated attachment of the phage, injection of the phage DNA into the bacterial host cytoplasm, and replication of the phage genome by transcription of the phage genes (20,21). However, the phages utilizing the virulent pathway can only replicate through the lytic process, which ends in the lysis of the bacterial host cell upon completion of the phage replication cycle (22, 23). In the lytic pathway, phages utilize holin proteins to hydrolyze the peptidoglycan layer resulting in cell lysis and release of new infectious particles into the local environment (24, 25). Temperate phages, however, can integrate their genome with the host at preferred sites such as tRNA sequences.

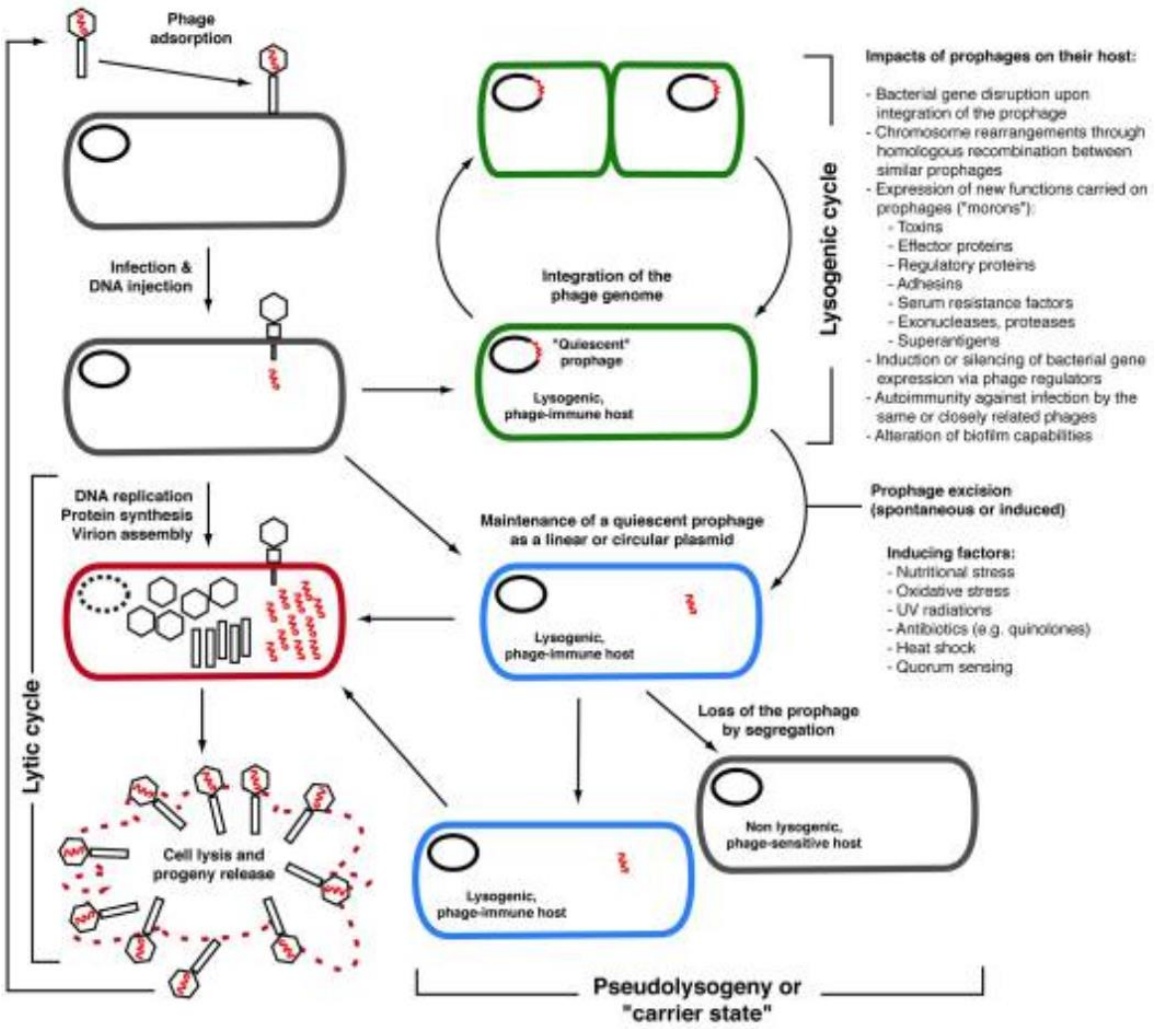


Figure 1: Lytic (virulent) and temperate (lytic and lysogenic) life cycles of bacteriophages.

Source: Fortier LC, Sekulovic O. 2013. Importance of prophages to evolution and virulence of bacterial pathogens. Virulence. 4(5): 354-365.

Integration of the phage genome with the bacterial host is mediated by phage-encoded integrase protein recombination of the *attB* site on the host genome and the *attP* site on the bacteriophage genome (26, 27, 28). Once a bacteriophage becomes integrated into the host genome, it can be maintained within the host and transmitted to the subsequent daughter cells and is called a prophage (29, 30). A prophage can benefit the host if the phage begins expressing superinfection immunity, preventing the host from being infected by another virus (31,32,33, 34). Under unfavorable environmental conditions, the host can deploy the SOS DNA repair mechanism

leading to prophage induction and excision from the bacterial chromosome, after which it enters the lytic pathway (35). Ultimately the life cycle that the phage utilizes can depend upon gene expression, environmental stressors, and competition from other bacteriophages (36).

1.2: Bacteriophage biodiversity and environmental role – Amongst all biological entities in the biosphere, phages are the most abundant, diverse, and ubiquitous (37, 38). There are an estimated 10^{31} total phages and 10^{8} different species in the biosphere (39, 40).

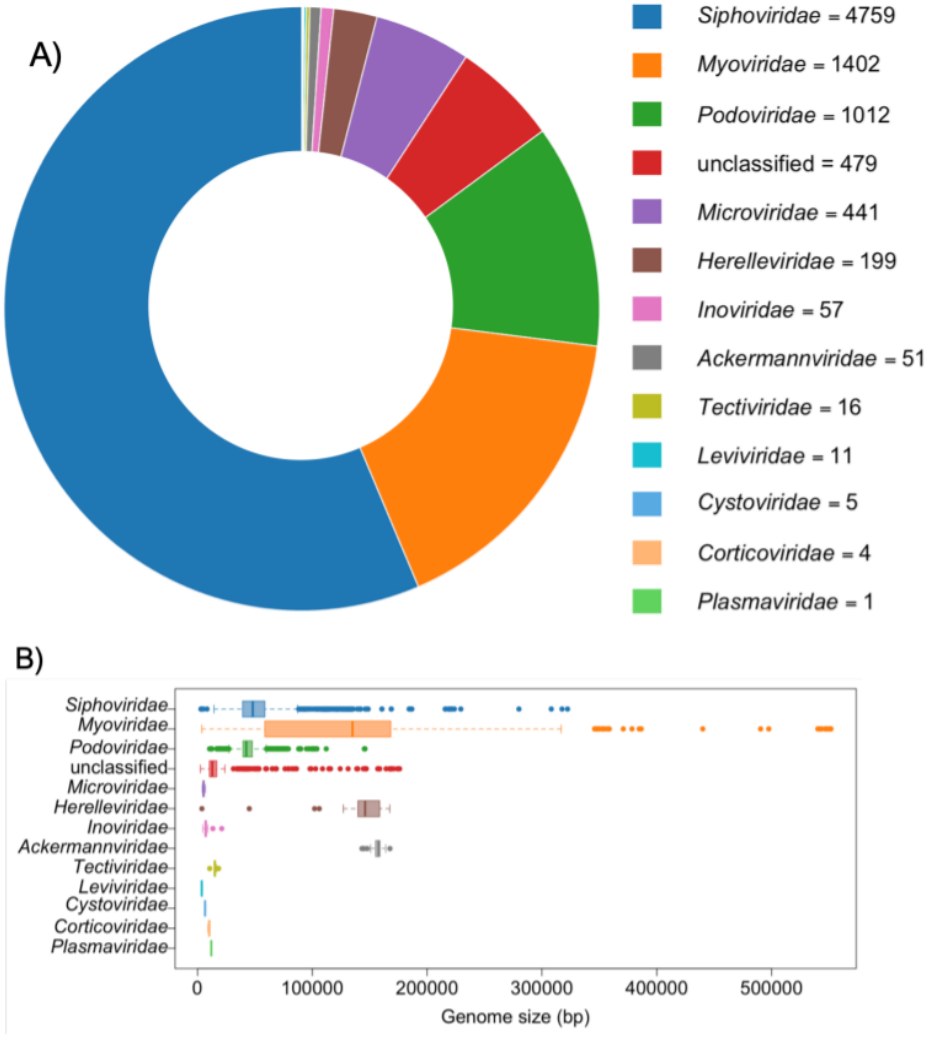


Figure 2: The diversity in bacteriophages (A) and genome size (B).

Source: Dion MB, Oechslin F, Moineau S. 2020. Phage diversity, genomics, and phylogeny. Nature Reviews Microbiology. 18: 125-138.

Direct count studies have shown that one gram of soil can contain 10^{10} phages (41). Bacteriophages play an essential role in regulating bacterial populations in the environment (42). Lytic viruses kill

up to 40% of all marine bacterial cells each day (43). Phage-mediated bacterial lysis plays an essential role in re-routing nutrients away from trophic levels of the marine food web and back into the ecosystem (44). Viruses make up approximately 94% of all nucleic acid-containing particles in the aquatic environment but only 5% of the total biomass (45). Fluorescence and electron microscopy have shown one ml of seawater to contain 10^7 phage particles (46). Phages utilize various methods to find and infect their hosts. For example, in the marine environment, the low availability of iron allows phages to use the iron within their tail to attach to the bacterial siderophore-bound iron receptor, puncture the cell membrane, and inject their nucleic acid into a host (47). Across all genera, bacteriophages display various morphotypes and genetic makeup (Figure 2).

1.3: *Mycobacterium* – This research utilized a *Mycobacterium* host, *Mycobacterium sm*egmatis mc^{2}155. The *Mycobacterium* genus is the only genus within the Mycobacteriaceae family; it contains over 188 species, including several significant human pathogens (48). The *Mycobacterium* genus is composed of acid-fast, obligatory aerobic, Gram-variable bacteria, which are not easily Gram-stained due to the presence of mycolic acid in their cell wall (49). Mycobacteria are ubiquitous and found in a wide array of environments, including garden beds, household plumbing, water samples, and a wide variety of soil types (50). Many of the infections associated with medically relevant mycobacteria cause inflammation of the respiratory system. Some of these medically relevant mycobacterium species include *M. abscessus, M. bovis M. avium, M. intracellulare, M. kansasii, M. chelonae, M. fortuitum, M. marinum, M. simiae, M. ulcerans, M.xenopi, M. smegmatis,* as well as the causative agents for leprosy (*M. leprae)* and tuberculosis (*M. tuberculosis)* (51). *Mycobacterium* species are often categorized as tuberculosis and nontuberculosis mycobacteria (NTM), or rapid-growing mycobacteria (RGM) and slow-growing mycobacteria (SGM) (52). The phages that infect *Mycobacterium* belong to 32 different clusters, and those that specifically infect *M. smegmatis* and *M. smegmatis* mc^{2}155 belong to 32 clusters. Of the 11648 known mycobacteriophages, 11620 infect the *M. smegmatis* species, and of those, 11519 infect the *M. smegmatis* mc^{2}155 strain (Phagesdb.com).

1.4: *M. smegmatis* mc^{2}155 – *M. smegmatis* mc^{2}155 was used to isolate and study mycobacteriophages. The factors that make *M. smegmatis* a model organism are its RGM properties and relatedness to more medically relevant species (53). Although it is a BSL1

organism, *M. smegmatis* can cause human diseases, particularly in the contamination of surgical wounds of immunocompromised patients. Another factor that makes *M. smegmatis* medically relevant is its presence in the normal flora of human sebaceous gland secretions (54). Among the most well-studied strains of *M. smegmatis* is mc^2155, which is derived from the parent strain mc^26 and possesses carbenicillin resistance via plasmid-encoded efflux protein pumps (55). Furthermore, *M. smegmatis* mc^2155 can be grown on media plates containing carbenicillin, which helps prevent the growth of contaminating organisms (56).

1.5: Mycobacteriophages – Mycobacteriophages are phages that infect mycobacterial hosts (57, 58). According to The Actinobacteriophages Database (phagesdb.org), 11,648 mycobacteriophages have been isolated, and 2,056 have been sequenced. Analysis of the GC content for the sequenced mycobacteriophages genomes shows a high degree of variability ranging from 45%-72% GC content. By studying phage and bacterial host interactions, insight into the physiology and genetics of mycobacteria has been found (59). Additionally, these host and phage relationships have played an essential role in clinical applications, including diagnosis, vaccines, drug development, and potential therapies (60, 61, 61). Due to the emergence of multidrug-resistant tuberculosis, mycobacterium tuberculosis phages have been identified as an important investigative tool (63, 64, 65). Additionally, mycobacteriophages have shown the ability to target and lyse biofilm-associated mycobacterium infections (66, 67, 68).

1.6: Pathogenic mycobacteria – The Mycobacterium genus represent numerous pathogenic and medically relevant species. *M. tuberculosis* has caused an estimated one billion deaths over the last two hundred years (69, 70, 71). Recently, many *M. abscesses*, *M. kansasii*, and *M. avium* associated NTM pulmonary infections have become a significant threat to global health (72,73). Although these mycobacterial pathogens can infect many human organs, they are generally linked to infections involving soft tissues, pulmonary, and lymph nodes (74). *M. abscessus* is amongst the most studied species of pathogenic mycobacteria. It is responsible for many skin and soft tissue diseases, central nervous system infections, bacteremia, ocular, and other infections. The *M. abscessus* complex is differentiated into three subspecies, including *M. abscessus* subsp. *abscessus, M. abscessus* subsp. *bolletii*, and *M. abscessus* subsp. *massiliense.* The disease outbreaks caused by the *M. abscessus* complex are often associated with nosocomial transmission and cosmetic procedures (76, 75). The wide distribution of many mycobacterial

species including their presence in water can bring them into contact with the general population (77). The prevalence of these NTM in potable water sources is attributed to biofilm formation (78). Due to the medical significance of mycobacterium species to the global human population, it is relevant to study all biological control methods for the attenuation of these pathogens, including bacteriophages.

1.7: Phage therapy – Phage therapy to treat bacterial infection was first reportedly used in 1896 to treat *Vibrio cholerae* caused diarrheal disease (79, 80). Recently phage therapy has been utilized in the treatment of surgical infections and the causative bacterial species of dysentery (*Shigella dysenteriae*), meningitis (*Neisseria meningitis*), plague (*Yersinia pestis*), and typhoid fever (*Salmonella typhi*) (81, 82). Phage therapy's primary advantages are the self-limiting and self-replicating nature of phages and the lack of adverse effects on the human organ system as compared to antibiotics (83, 84, 85, 86). In addition, phages can treat biofilms and biofilms-associated infections due to their bactericidal activity compared to the bacteriostatic activity of antibiotics (87, 88, 89, 90). Antibiotics often have a broad-spectrum activity that can kill beneficial normal flora, the destruction of which can lead to secondary infections; phages are host specific and do not disrupt normal human flora (91, 92,93). The disadvantages of phage therapy include its inability to treat certain types of infections. Additionally, lysogenic phages are problematic candidates for phage therapy, and prophages that display superinfection immunity can convert phage-sensitive bacteria into insensitive ones and hinder proper treatment (94, 95). Another potential disadvantage of phage therapy is their narrow host range as compared to most antibiotics (96). Bacteria can also develop resistance to bacteriophages through mutations of bacterial surface cell receptors and CRISPR-Cas systems, and antiphage antibodies (97, 98). Among the most significant challenge for phage therapy becoming more widely utilized is the western medical establishment's unfamiliarity with phages as antibacterial agents. Recently, however, some phage products have been classified by the EPA as GRAS (Generally Regarded as Safe), approved by the USDA, or registered by the EPA (99).

1.8: Bioinformatics and phage annotation – Due to the development and availability of various bioinformatic tools, many educational institutions have begun to offer programs geared towards phage research (100, 101, 102). The use of bioinformatics has become a necessary and valuable tool to better study and understand phages at the molecular level. Recent advances in viral

metagenomics have enabled the rapid discovery of an unprecedented catalog of phages across a comprehensive assay of environments, from the human gut to the deep ocean (103). Bacteriophages generally have very compact genomes with numerous overlapping genes; bioinformatics allows for a comprehensive study of the phages at the genetic level, which creates a better understanding of phages and the genes regulating their life cycle (104, 105). The use of bioinformatics databases for phage taxonomy has allowed the identification of evolutionary relationships within phage clusters and their proteins (106, 107). Bioinformatic tools have allowed for identifying specific phage genes, putative gene function, associated stop and start sites, and the confidence values for the results. Other software allows for the identification of possible tRNA and frameshift mutations within the genome. Bioinformatic analysis of phage genomes represents the new frontier in phage research and expands the application and use of phages across the scientific field.

1.8: Research Objectives – The primary objectives of this research were to isolate, characterize, sequence, and annotate a mycobacteriophage from enriched soil and check its viability as a candidate for phage therapy. Upon completing the study, a greater understanding of the mycobacteriophage and its component genes will be deduced.

Chapter 2: Materials

Table 1: The bacterial strains utilized in the research and their source.

Bacterial strain	Source	Species ID
Mycobacterium abscessus mc1518	BEI resources	NR-44266
Mycobacterium smegmatis mc^2155	Hatful Lab	UP000006158

Table 2: Restriction enzymes utilized in the enzymatic digest of the mycobacteriophage.

Restriction Enzyme	Restriction Site	Buffer	Source
BamHI	5′... G▼GATCC...3′ 3′... CCTAG▲G...5′	Buffer 2	New England Biolabs
ClaI	5′... AT▼CGAT...3′ 3′... TAGC▲TA...5′	CutSmart	New England Biolabs
HindIII	5′... A▼AGCTT...3′ 3′... TTCGA▲A...5′	Buffer 2	New England Biolabs
HaeIII	5′... GG▼CC...3′ 3′... CC▲GG...5′	CutSmart	New England Biolabs
EcoRI	5′... G▼AATTC...3′ 3′... CTTAA▲G...5′	CutSmart	New England Biolabs

Table 3: The composition of the buffers and media used in this study.

Buffer/Media	Composition (1 L)	
7H9 Liquid Medium Neat (900mL)	7H9 broth base 40% glycerol stock ddH_20	4.7 g 5 mL To 900 mL

7H10 Agar Plates	dH_2O	490 mL
	anti-bubble	2 drops
	40% glycerol	6.25 mL
	40% dextrose	2.5 mL
	Cyclohexemide	0.5 mL
	Carbenicillin	0.5 mL
	0.1 M $CaCl_2$	5 mL
Phage Buffer	1 M Tris stock (pH 7.5)	10 mL
	1 M $MgSO_4$ stock	10 mL
	NaCl	4 g
	ddH_2O	970 mL
	100 mM $CaCl_2$ stock	10 mL
AD supplement	NaCl	8.50 g
	Albumin	50 g
	Dextrose	20 g
	ddH_2O	To 1 L
100 mM CaCl2	$CaCl_2$	14.7 g
	ddH_2O	1 L
TBE 1X	TBE 10X	100 ml
	ddH_2O	900 ml
Top Agar 2X	7H9 base broth	4.7 g
	Agar	8 g
	ddH_2O	1 L
Phage Buffer	1 M Tris stock (pH 7.5)	10 mL
	1 M $MgSO_4$	10 mL
	NaCl	4 g
	100 mM $CaCl_2$ stock	10 mL
Top Agar 1X	100 mM $CaCl_2$ stock	10 mL
	7H9 liquid Medium	500 mL
	Top Agar 2X	500 mL

Table 4: DNA Extraction Material

Chemical	Effect	Source
1M $MgCl_2$	Lysis buffer components	108
DNAse buffer	Protecting DNA from degradation	109
DNAse (2000 U/ml)	Cleaves host DNA	110
RNAse (100 mg/mL)	Cleaves host RNA	111
0.5M EDTA	Chelating agent	112
Proteinase K (20 mg/mL)	Digest contaminating proteins	113
10% SDS	Denatures secondary proteins	114
95% Ethanol	Remove solvation shell around DNA	115
70% Ethanol	Aggregation of DNA	116
3M Sodium acetate	Neutralizes DNA backbone charges	117
Phenol: Chloroform: Isoamyl alcohol (25:24:1)	Separate DNA (aqueous layer) from contaminants (non-aqueous layer)	118
diH_2O	Medium for dissolving DNA	119

Table 5: The software programs utilized for mycobacteriophage genome annotation

Software	Purpose	Reference
Aragorn	tRNA sequence Identification	120
DNA Master	Genome Annotation	121
Glimmer	Gene coding potential prediction	122
GeneMark	Gene coding potential prediction	123
HHpred	Gene function assignment	124
Phamerator	Gene function prediction	125
Starterator	Gene start codon evaluation	126
tRNAscan-SE	tRNA sequence identification	127

NCBI Blast P	Gene function assignment	145
Unipro UGENE	In-silico enzymatic digest	162

Table 6: The list of *M. smegmatis* mc^2155 infecting mycobacteriophages used for superinfection immunity testing.

Mycobacteriophage	Source
Cads	UCO Virology Class
Dragon	UCO Virology Class
Feline Fury	UCO Virology Class
Fulbright	UCO Virology Class
Micasa	UCO Virology Class
Pleades	UCO Virology Class
Slagle	UCO Virology Class
Wiggle Worm	UCO Virology Class

Methods

2.1: Bacterial and media growth – The experiments used 7H10 complete media plates made using a 7H9 broth supplement (128). Other components added to the growth media were albumin dextrose catalase (ADC)(10% V/V) and one mM $CaCl_2$ (129). In addition, carbenicillin and CHX were added to the mixture to prevent contamination. The plates were incubated at 37^{o}C and stored at 4^{o}C. Liquid media cultures were set in a shaking incubator (environmental shaker) at 37^{o}C (130). The full compositions of the buffers and media utilized in the experiments are listed in Table 2.

2.2: Soil enrichment step – The soil used to isolate the mycobacteriophage was collected from Ashburn, Virginia (39.014667 N, 77.515417 W). The enrichment of the collected soil sample was done using the direct enrichment method (131, 132).

2.3: Isolation of bacteriophage – Following soil enrichment, the phage lysate supernatant was filtered using a 0.22 µm filter to remove contaminants, including cell debris and non-phage

particles. The sample was serially diluted, and 10µl of each phage dilution was added to a tube containing 250 µl of host bacteria and incubated at room temperature for 10 minutes (133). The mixture was combined with 5 ml of 1x top agar and plated on 7H10 media plates. The agar plates were left at room temperature for 20 minutes to solidify and then inverted before being incubated at 37^{o}C for 2-3 days (134). Following incubation, the plates were viewed for the presence of plaques, and if present, their diameter, and appearance on the bacterial lawn were recorded (135).

2.4: Plaque purification – Plaque purification was done to ensure that the subsequent steps and experiments contained only one species of plaque-forming phage (136). A single plaque was selected from the media plate, removed using a 10 µl micropipette tip, and inoculated into 250 µl of host bacteria culture. This mixture was serially diluted, plated, and incubated at 37^{o}C for 2-3 days, following the same steps as before. Three rounds of plaque purifications were done to ensure a single type of phage. The webbed plate was found from the third round of plaque purifications, which contained a concentrated number of mycobacteriophages that resulted in minimal bacterial lawn growth (137). This plate was used in the subsequent amplification step.

2.5: Phage amplification and titer – The low titer lysate (LTL) of Eaglepride was created by covering the web plate with 8 mL of phage buffer and incubating it for 4 hours at room temperature. The liquid lysate from the webbed plates was collected, filtered, serially diluted, plated, and incubated at 37^{o}C for 2-3 days. A high titer lysate (HTL) was created by making five webbed plates from the LTL following the same steps as before (138). To determine the phage concentration in the phage stock, the HTL was serially diluted, plated, and incubated for 2-3 days at 37^{o}C (139). The phage titer was calculated using a countable plate (25-250 pfu) and the following equation: Phage titer = $\frac{\#of\ Plaques\ of\ Plate}{Total\ Dilution\ of\ Plate}$ (140).

2.6: Transmission electron microscopy imaging – Electron microscopy was used to determine the morphotype of the phage. Five µl of the DNA sample was loaded on a 200-400 mesh carbon-formvar-coated copper grid and negatively stained with five µl of 1% uranyl acetate (141). The excess uranyl acetate on the grid's edges was wicked using filter paper and air-dried at room temperature. The grid was placed inside the grid box and taken for electron microscopy. The imagining was done at the Oklahoma Medical Research Foundation (OMRF).

2.7: Thermal stability of the mycobacteriophage – The thermal stability of the mycobacteriophage was determined at six temperature points, including 40ºC, 45ºC, 50ºC, 55ºC, 60ºC, and 65ºC. Triplicates were made of the UD phage sample and incubated for three different time intervals of 20, 40, and 60 minutes for each temperature point. A sample for each temperature and time point was diluted, plated, and incubated at 37ºC for 2-3 days. Once the incubation was complete, the PFUs were counted for each temperature and time point. The Kruskal Wallis test was used to test for significance (142).

2.8: pH stability of the mycobacteriophage – The mycobacteriophage growth stability was tested across five pH buffers, ranging from 5 to 9. Each buffer's pH was adjusted using NaOH, and HCI and verified using an Accumet® AE150 pH measuring machine. For each pH level, 100 µl of mycobacteriophage lysate was mixed with 100 µl of the adjusted pH buffer and incubated for one hour at 37ºC. Next, the sample was serially diluted, plated, and incubated at 37ºC for 2-3 days. Following incubation, the PFUs were counted for each pH level. Kruskal Wallis test was used to test for significance (142).

2.9: Temperate phage testing – The HTL of Eaglepride was used to test if the mycobacteriophage was temperate. A 7H10 plate was used for spot testing, following the same protocol as before. Following incubation, the plate was viewed for the presence of mesa, which represents the overgrowth of bacterial cells in the initial zone of clearing caused by the phage lysis and is an excellent source of lysogens.

2.10: Creating lysogen – The mesa from the temperate phage test plate was streaked for host lysogen isolation using the four-quadrant sequential streak technique. The steak plate was incubated at 37ºC for 2-3 days. The purification process was repeated three times to remove any exogenous phages.

2.11: Patch assay – Patch assays were used for lysogen identification by patching putative lysogens onto host-seeded 7H10 plates. A portion of a colony from the previous streak plate was picked using a sterile inoculating loop and streaked vertically on host-seeded plates. Following incubation at 37ºC for 2-3 days, the lysogens were identified by the presence of a clear area surrounding the patches. The positive lysogen candidates were purified and used for liquid phage release assay.

2.12: Liquid phage release – Liquid phage release assay was done to create a liquid culture of the potential lysogen. A plaque containing a lysogen from the patch assay was inoculated into a culture tube containing 3 ml of the bacterial host. The culture tube was incubated at 37ºC for 3-4 days at 220 rpm. Following incubation, 500 µl of culture was centrifuged at 4,000 rpm for 1 min to pellet the bacterial cells. Then 100 µl of supernatant was diluted in phage buffer and spot tested on plates seeded with *M. smegmatis* mc^2155 following the previously described technique. Following incubation, if plaques formed on higher dilution spots, it indicated the presence of a stable host lysogen and suggested the mycobacteriophage to be temperate. The positive prophage was given the name MSUS1.

2.13: Superinfection immunity assay – The superinfection immunity assay was conducted using eight mycobacteriophages listed in (Table 5). Top agar was mixed with the positive host liquid lysogen to create spot test plates. Each experimental mycobacteriophage was spot tested on a lysogen seeded plate following the previously described techniques. The plates were incubated at 37ºC for 3-4 days and checked for evidence of superinfection immunity denoted by the absence of plaques on the spot plates.

2.14: Host range determination – The host range of the mycobacteriophage was tested against the BSL-2, pathogenic mycobacterium species *Mycobacterium abscessus* mc1518. A 7H10 incomplete plate was utilized for the host range assay due to the sensitivity of *Mycobacterium abscessus* mc1518 to antibiotics. The plate was seeded with a mixture of 250 µl of *Mycobacterium abscessus* mc1518 and 4 ml of 1X top agar and left at room temperature for 20 minutes to solidify. The Eaglepride HTL was used to spot test on the host seeded 7H10 plates (143). The plate was left at room temperature for 20 minutes to allow sample diffusion and then incubated at 37ºC for seven days. Following incubation, the plate was observed for the formation of plaques.

2.15: Bacteriophage genomic DNA extraction – The genomic DNA (gDNA) of the mycobacteriophage was extracted using the phenol:chloroform:isomyl alcohol / Sodium dodecyl sulfate (PCI/SDS) DNA Extraction method (144). The reagents (Table 3) were used in conjunction with the PCI/SDS protocol (Phagehunting Protocols, http://phagesdb.org/phagehunters/) to conduct the gDNA extraction. The extraction resulted in the formation of a DNA pellet which was air-dried and dissolved in 100 µL of ddH_2O. The DNA quality and quantity were found using a UV-spectrophotometer (Nanodrop) (145).

2.16: Genomic sequencing –. The extracted gDNA of Eaglepride was sequenced using the Illumina platform with the approximate shotgun coverage of 1845 bp.

2.17: DNA gel electrophoresis and restriction digest – Gel electrophoresis was done using the extracted Eaglepride gDNA sample. A 1.0% gel was created using 500mg of Agarose and 50.0 mL 1X TBE. Five µl of ethidium bromide were added to the mixture and solidified for 40-45 minutes at room temperature. The gel was transferred into the electrophoresis machine, and 1X TBE was added to the machine until it covered the gel mold. A well was loaded with 7 µl of DNA Ladder and another with a mixture containing 8 µl of dye and 15 µl of gDNA sample. The restriction digest was conducted using five restriction enzymes and their associated buffer (Table 1), following the manufacturer's specifications and guidelines (New England Biolabs). The wells were loaded with the restriction mixtures and the undigested gDNA sample as the control. The electrophoresis machine ran for approximately 1 hour at 100 V. The gel was then moved to the BIO-RAD ChemiDoc MP Imaging System and visualized using the ethidium bromide setting.

2.18: Genome annotation – The annotation of the mycobacteriophage genome was done using DNA Master, following the guidelines of the SEA-PHAGES DNA Master guide. A map of the ORFs within the genome was created. Any ORFs missed by the software blast were added into DNA Master following the annotation guidelines (121). Glimmer and GeneMark programs were used to predict protein-encoding genes within the genome. The NCBI BlastP, PhagesDB, and HHPred tools were utilized to compare the gene product to a global database and determine the putative gene function. The Phamerator program was used to compare the genome, genes, and gene product of Eaglepride with other phages in the database. The Starterator program was then utilized to determine the longest possible ORF for each gene by examining all the genes within its Pham. The Aragorn and tRNA scan-SE programs were used to determine the presence and location of tRNA in the genome.

Chapter 3: Results

3.1: Phage name and plaque morphology – Eaglepride produced clear plaques on the *M. smegmatis* mc^{2}155 bacterial lawn, and the plaques were approximately 3 mm in diameter (Figure 3). The isolated mycobacteriophage was named Eaglepride. The name was derived from the middle school's mascot in Ashburn, Virginia, from where the soil for its isolation was collected.

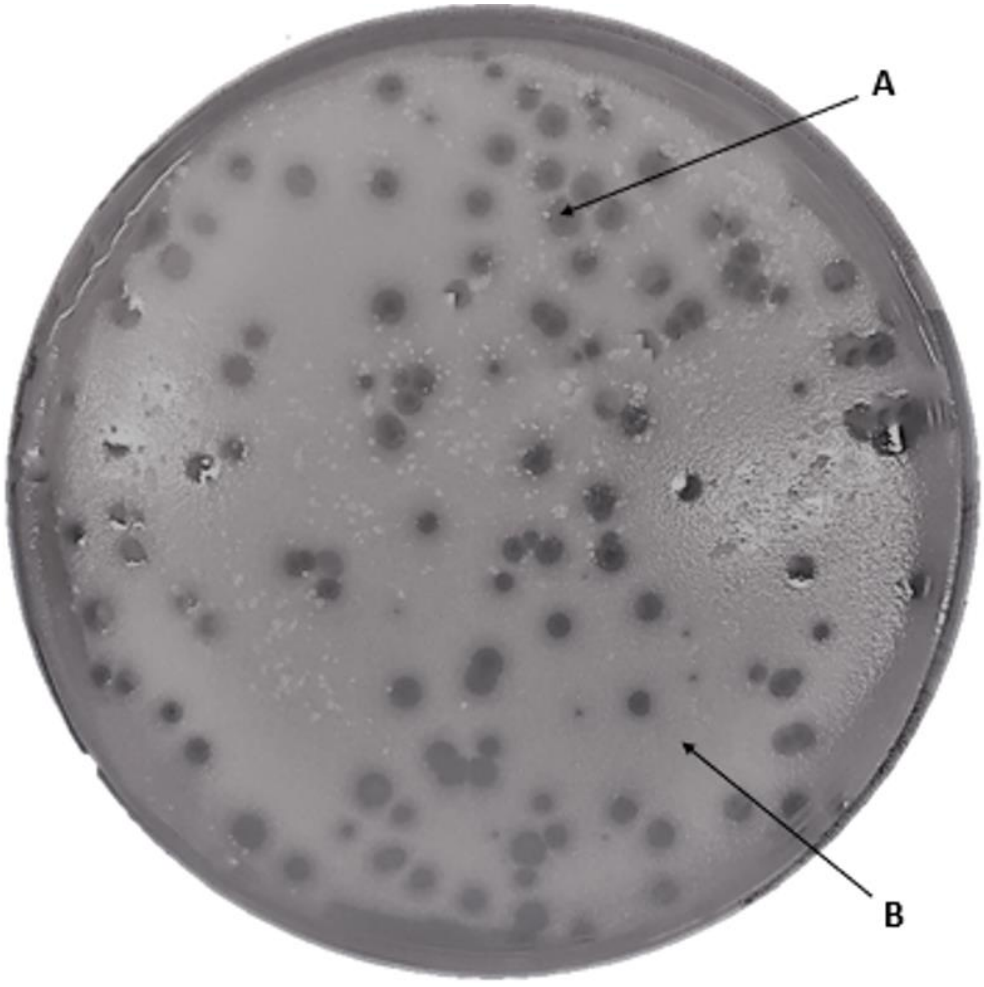


Figure 3: The plaques (A) of Eaglepride on the *M. smegmatis* mc^{2}155 bacterial lawn (B).

3.2: Phage morphology – The utilization of TEM determined Eaglepride to have a *Siphoviridae* morphotype, with an icosahedral head and a thin, flexible, and long tail (Figure 4). Upon measuring three different Eaglepride TEM images, the head diameter was approximately 55 nm, the tail length was about 175 nm, and the width was 20 nm.

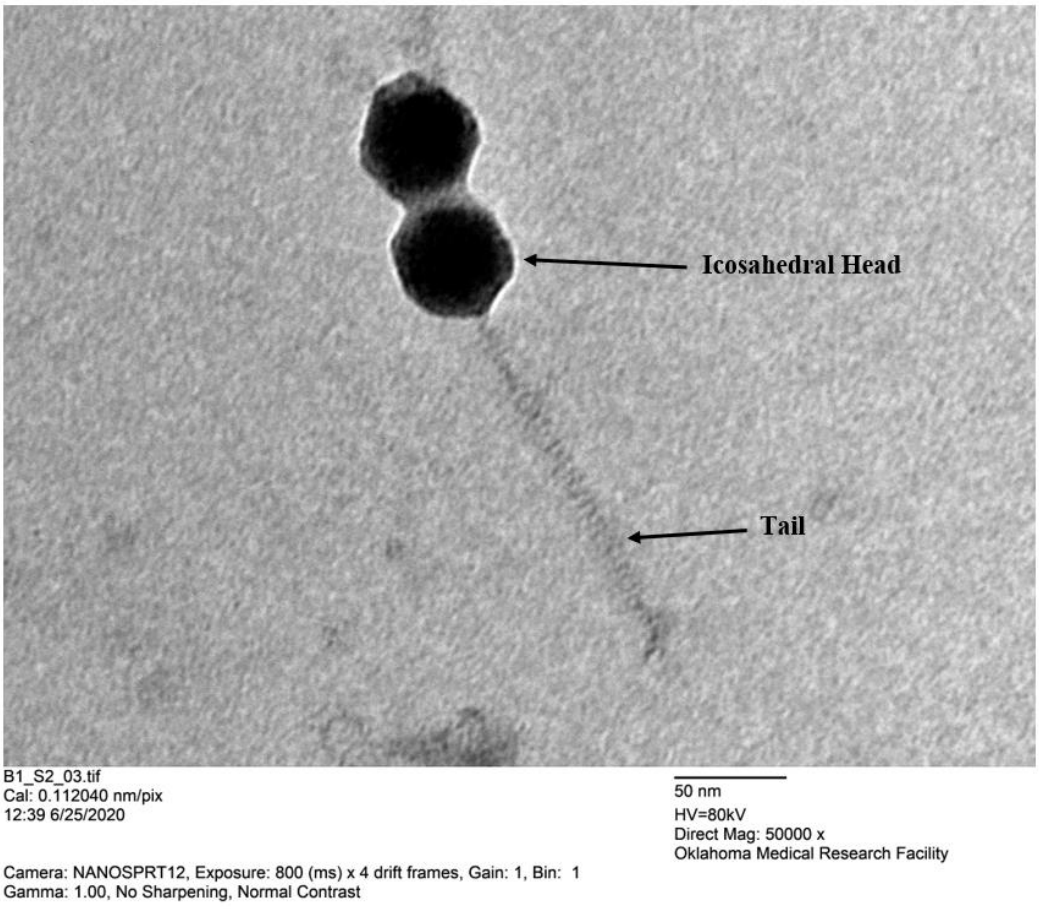


Figure 4: The Transmission electron micrograph of Eaglepride.

3.3: Temperatures and pH stability of the phage particle – The effects of temperature on the stability of Eaglepride showed that the phage particle is stable at temperatures ranging from 40°C to 60°C and is less stable at 65°C. The use of the Kruskal-Wallis statistical significance test also showed a significant difference in the number of surviving PFUs at 65°C, as compared to the other temperatures (Figure 5). Thus, the stability of the particle at the lower temperatures, including 40°C, could be attributed to the stable growth temperature of its host. *M. smegmatis* mc^2155, which is incubated at 37°C (146).

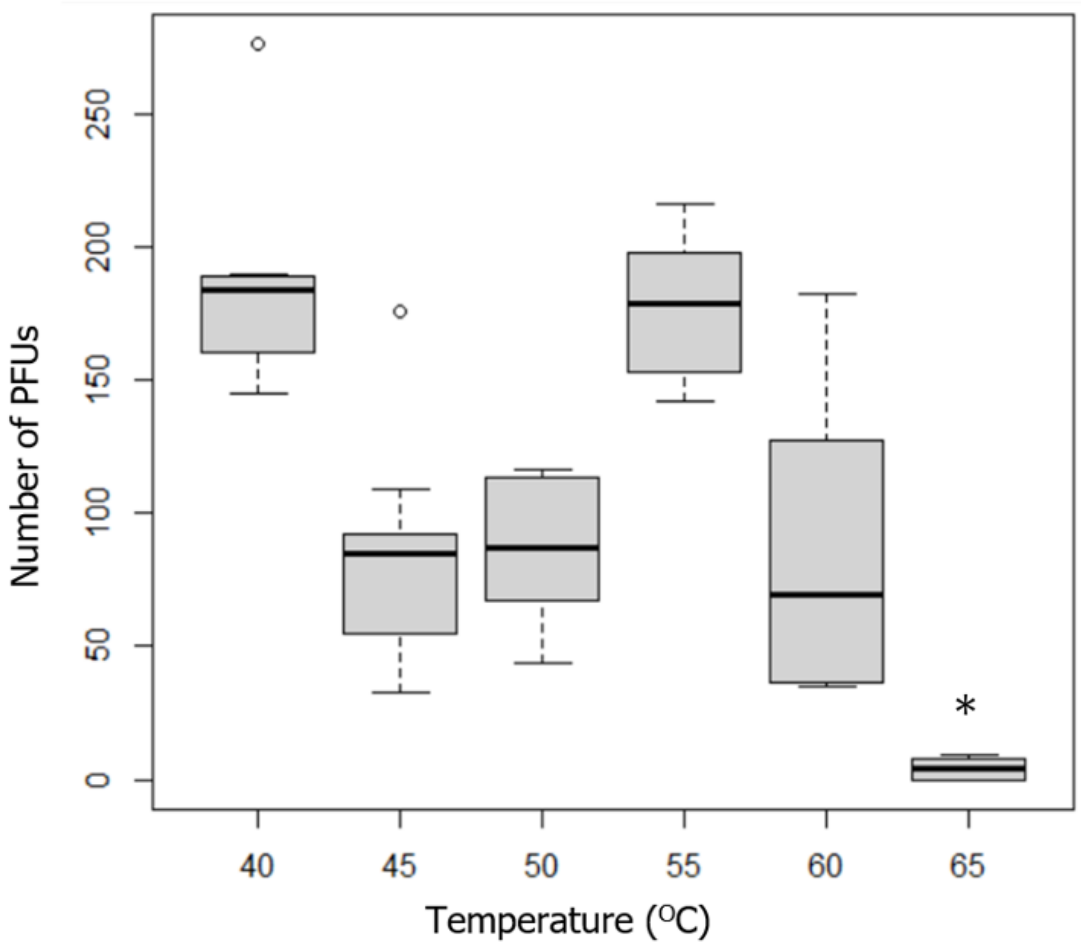


Figure 5: The stability of Eaglepride at varying temperatures. The test results included Kruskal-Wallis chi-squared = 40.218, df = 5, p-value = 1.35e-07.

The stability of Eaglepride at various pHs indicated that the phage particle is stable between pHs 6-8 and less stable at pHs 5 and 9 (Figure 6). The Kruskal-Wallis chi-squared test showed a significant difference in the number of PFUs at pH levels 5 and 9. This could be attributed to the evolution of mycobacteriophages to retain infectivity at pH levels ideal for the growth of the host (147).

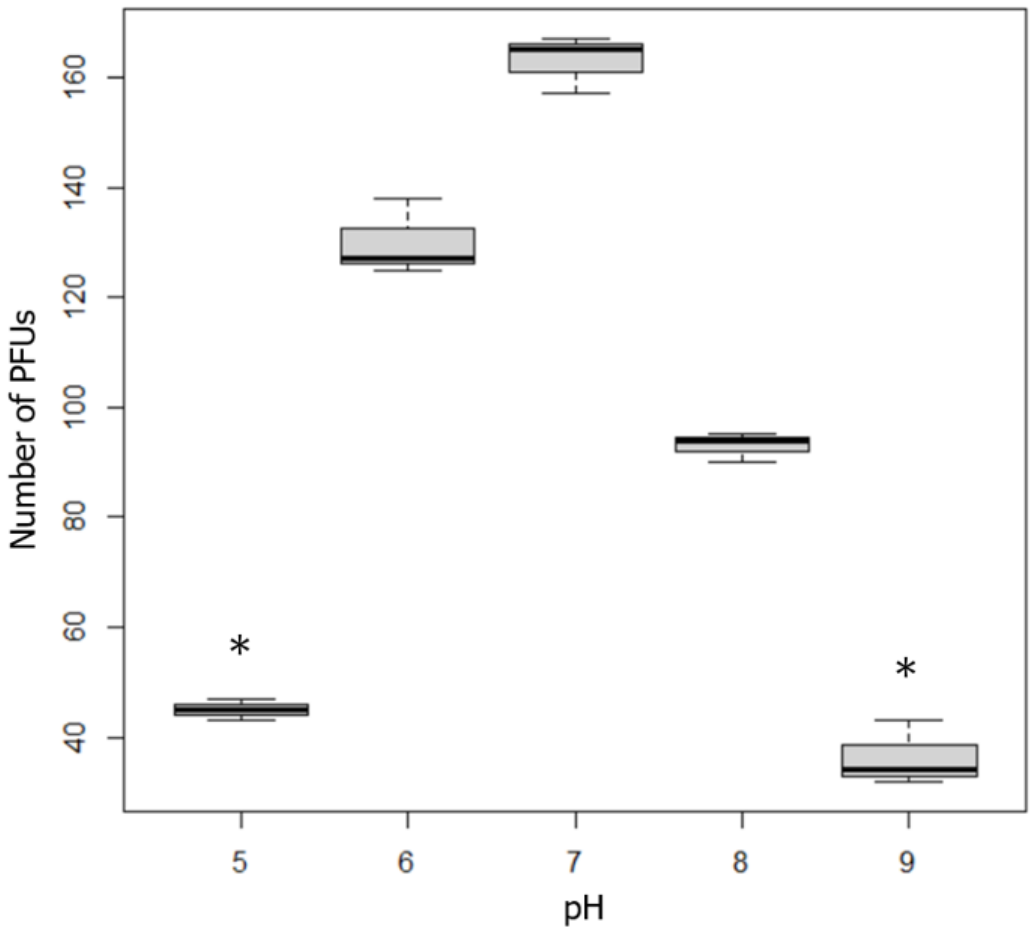


Figure 6: The stability of Eaglepride at varying pHs. The test results included Kruskal-Wallis chi-squared = 13.382, df = 4, p-value = 0.009552.

3.4: Characterizing patterns of phage sensitivity – Results indicate that Eaglepride is a temperate phage due to the presence of a mesa, which is the regrowth of bacteria in the infected area of the host bacterial lawn (Figure 7).

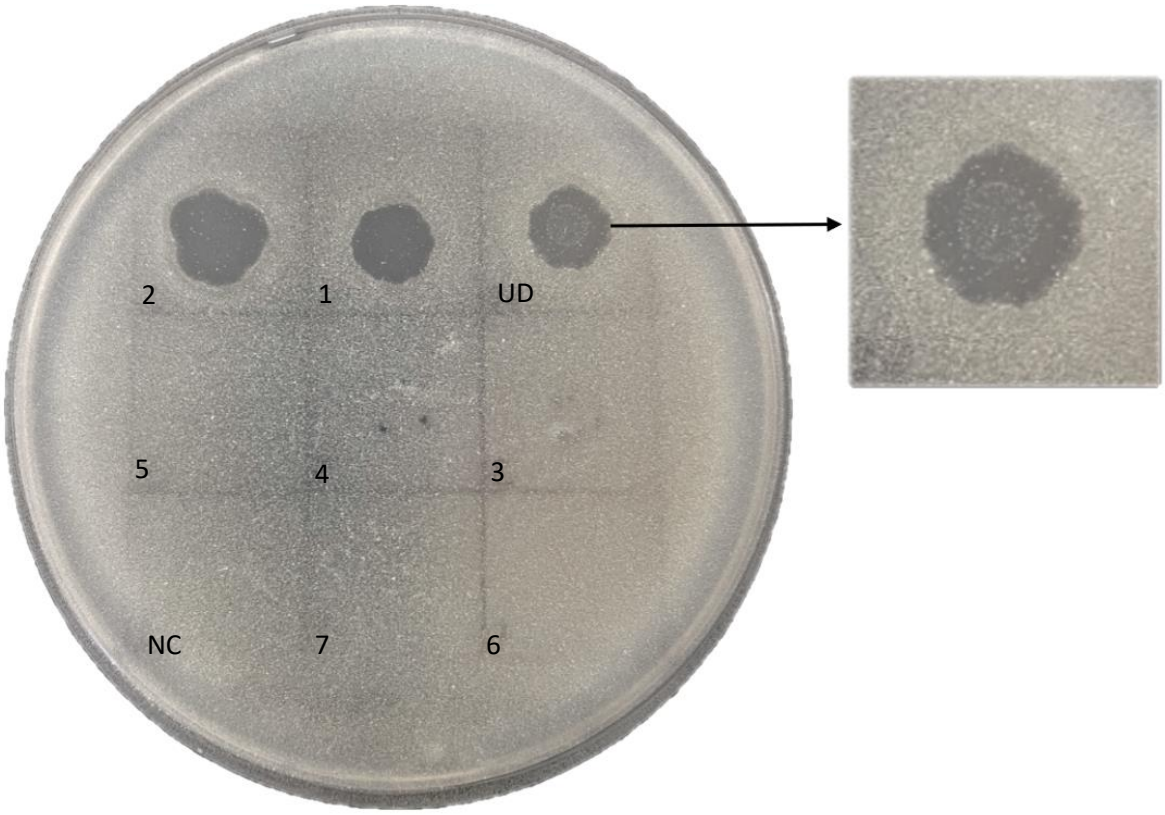


Figure 7: The temperate phage assay of Eaglepride.

3.5: Superinfection immunity – A streak plate was used to purify the host lysogen, and the patch assay was used to confirm the presence of prophage in the host lysogen (Figure 8). The mesa plates of the Eaglepride lysogen did not display superinfection immunity for the host bacteria against other mycobacteriophages. This was supported by the presence of plaques on the lysogen seeded host bacterial lawn (Figure 9).

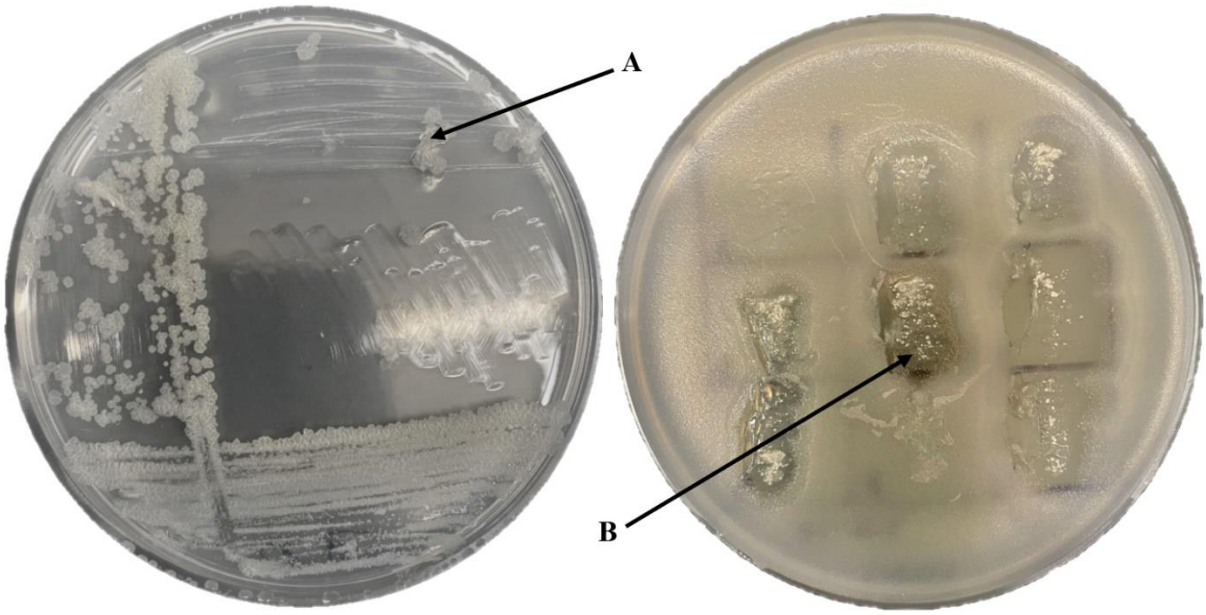


Figure 8: Plate A represent purified lysogen, and plate B represents phage release assay.

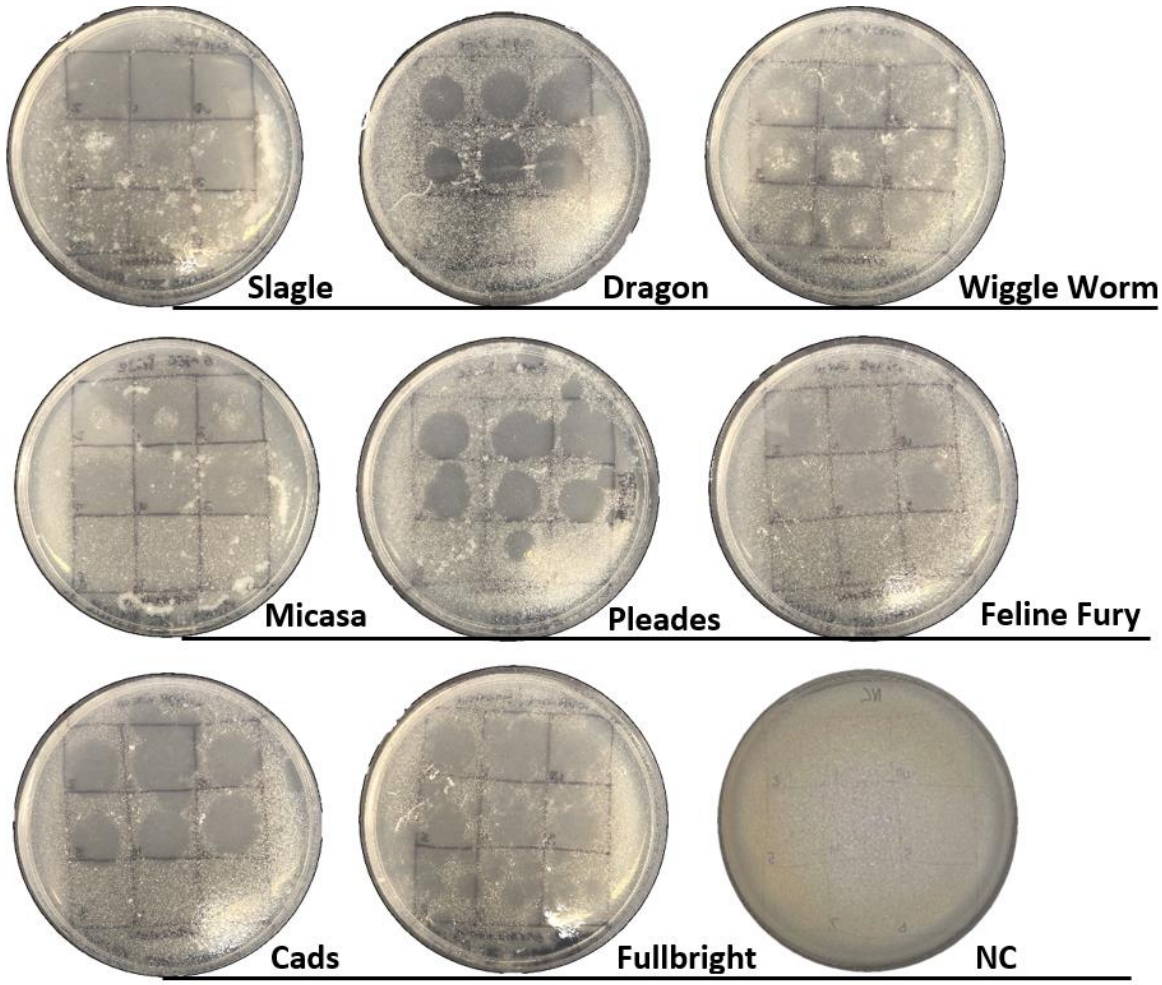


Figure 9: The superinfection immunity assay of MSUS1with the *Mycobacteriophages* Slagle, Dragon, Wiggle Worm, Micasa, Pleades, Feline Fury, Cads, and Fullbright. Phage buffer was the negative control.

3.6: The host range of Eaglepride – Eaglepride did not form any plaques on the lawn of *M. abscesses* mc1518 (Figure 10). These results showed Eaglepride to have a narrow host range (148).

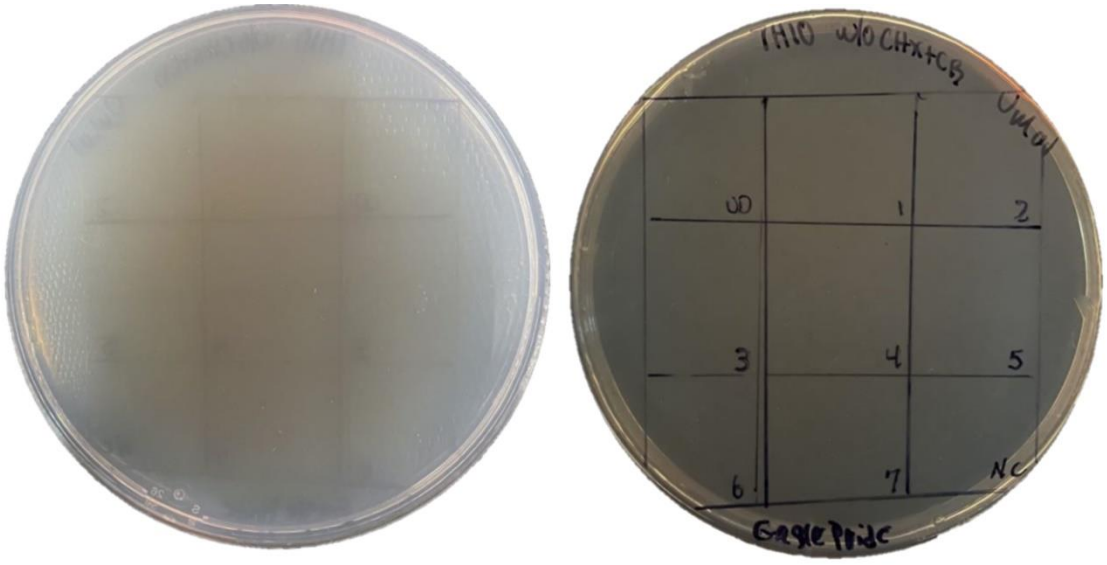
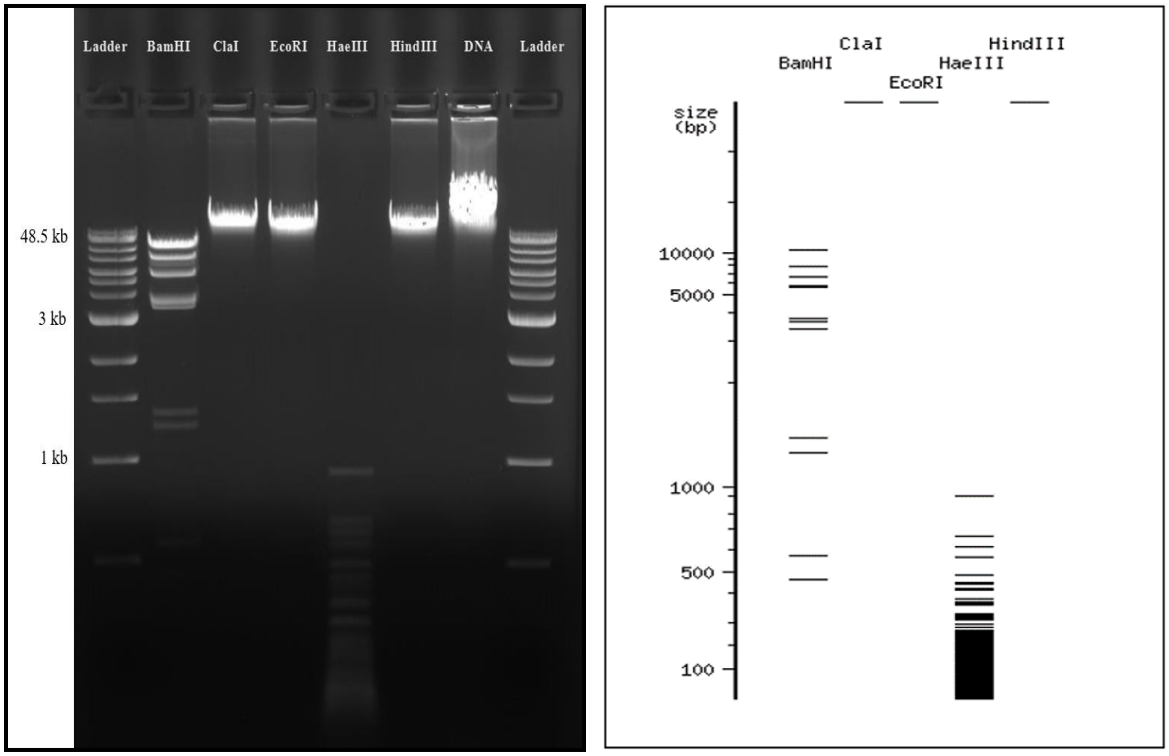


Figure 10: The host range test of Eaglepride on a lawn of *M. abscesses* MC1518.

3.7: DNA gel and restriction digest – The restriction digest produced the following bands (Figure 11). The digestion of Eaglepride gDNA by BamHI created 11 bands at various molecular sizes ranging between 48,000 to 1,500 bps. The digestion by HaeIII produced a DNA smear. The restriction enzymes ClaI, EcoRI, and HindIII were unable to digest the genome, indicating the absence of restriction sites.



A B

Figure 11: The restriction digest of Eaglepride DNA using the restriction enzymes BamHI, ClaI, HaeIII, HindIII, and EcoRI. The undigested gDNA was the control. The gel is represented by (A), and the In-silico analysis is represented by (B).

3.8: Eaglepride annotation and analysis – Eaglepride was found to have a double-stranded DNA (dsDNA) genome with a total length of 50,926 base pairs (bp) and 88 open reading frames (ORFs). Results showed that 34 ORFs are transcribed in the forward direction (5' – 3'), and the remaining 54 are reverse transcribed (3'- 5') (Figure 12). Of the 88 ORFs, 35 have known putative functions (Figure 12). Fifty-nine ORFs have the start codon ATG (67.1%), 23 have GTG (26.1%), and 6 have TTG (6.8%). The total GC content of Eaglepride is 64.9%. Gene 12 was utilized as an example of how the various annotations programs were used and interpreted.

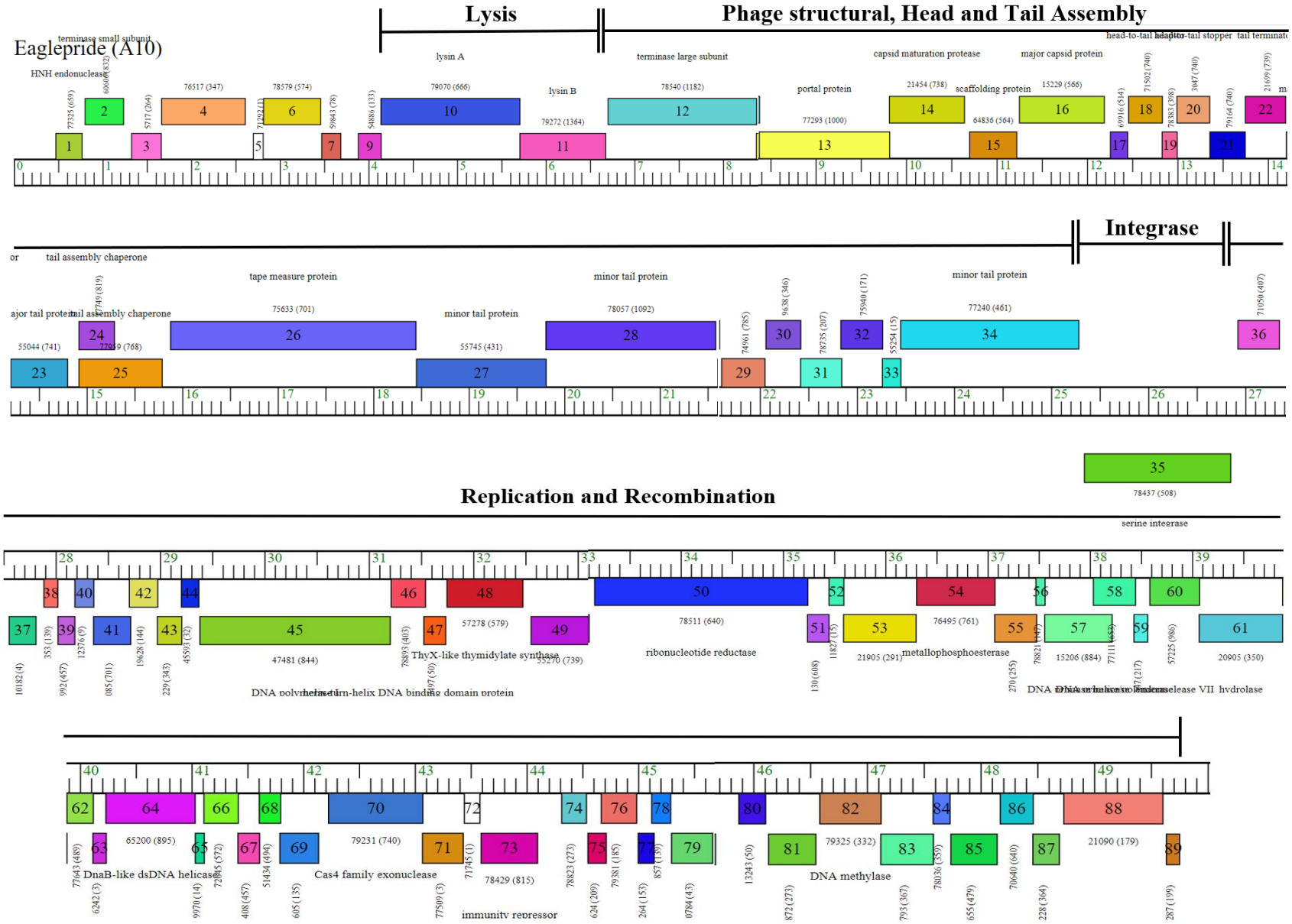


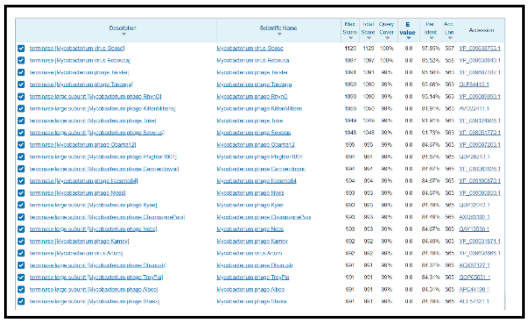
Figure 12: The organization of Eaglepride genome is presented horizontally, with 88 ORFs which are organized from left to right. The ORFs above the measurement line are forward transcribed (from left to right) and those below are reverse transcribed (right to left). Supplemental ORF putative functions are in Table A.

3.9: Preliminary functional assignment – The blasting of the phage genome within PhagesDB provided an initial summary of gene 12, which designated it to be a terminase protein. The use of NCBI blast P further supported gene 12 to function as a terminase protein. The significance values for the function designation by NCBI blast P included 100% quarry coverage, 0.0 E-value, and 97.85% identity (Figure 13). The results of HHPred also showed ORF 12 to be a large subunit terminase protein. The factors for choosing this putative gene function included the high probability (100%) and E-value of the results (1.8e-36).

A

Members (986) of Pham 80197	
20ES_12 (terminase)	40AC_15 (terminase large subunit)
8UZL_5 (terminase, large subunit)	A6_09 (putative terminase)
Aaronocolus_6 (terminase large subunit)	AbbyPaige_14 (terminase)
Abbyshoes_Draft_12	AbbysRanger_11 (terminase large subunit)
Abdiel_11 (terminase large subunit)	Abrogate_120 (terminase)
ACFishhook_10 (terminase)	Achebe_11 (terminase large subunit)
Acme_13 (terminase large subunit)	Acolyte_12 (terminase large subunit)
Adahisdi_12 (terminase)	Adolin_2 (terminase large subunit)
Adumb2043_2 (terminase large subunit)	Adzzy_13 (terminase large subunit)
Aeneas_13 (terminase)	AFIS_11 (terminase large subunit)
Agape74_14 (terminase large subunit)	Agatha_4 (terminase large subunit)
AgentM_9 (terminase large subunit)	Aglet_13 (terminase)

B



C

Nr	Hit	Name	Probability	E-value	Score	SS	Aligned cols	Target Length
1	PF03354.17	; Terminase_1 ; Phage Terminase	100	1.8e-36	314.79	41.6	429	467
2	3CPE_A	DNA packaging protein Gp17; large terminase, Alternative initiation, ATP-binding, DNA-binding, Hydrolase, Nuclease, Nucl	100	4.2e-33	299.18	39	429	592
3	5OEB_A	Large subunit terminase; large terminase, VIRAL PROTEIN; 2.2A (Deep-sea thermophilic phage D6E)	100	1.7e-32	282.54	32.7	388	430
4	4IDH_A	Gene 2 protein; DNA packaging, terminase, ATPase, nuclease, ATP binding, Magnesium binding, VIRAL PROTEIN; 1.69A (Shigel	100	3e-32	285.83	35.1	386	490
5	6M5V_A	Tripartite terminase subunit 3; viral protein; HET: ADP; 4.5A (Human alphaherpesvirus 1 strain 17)	100	5.2e-30	279.89	40.6	439	690
6	4BEJ_B	DNA MATURASE B; HYDROLASE, ATPASE, DNA TRANSLOCATION, SINGLE-PARTICLE RECONSTRUCTION; 16.0A (ENTEROBACTERIA PHAGE T7)	99.97	1.8e-27	248.97	38.2	377	476

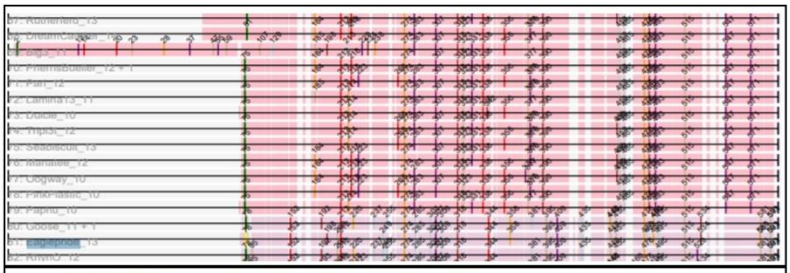
Figure 13: The putative function of Eaglpeide gene 12 determined by PhagesDB (A) NCBI BLAST P (B) and HHPred (C).

4.0: Start site determination – The start site for ORF 12 was initially determined using Phages DB and found to be at the 6703 bp location. Starterator Pham report further supported the start site at the 6703 bp location. Additionally, the start site of Eaglepride ORF 12 at the 6703 bp location was confirmed by the results of GeneMark S (Figure 14).

A

Details for gene Eaglepride_12	
Phage	Eaglepride · Cluster A · 50926 bp
Gene	Eaglepride_12
Pham (click for Pham view →)	80197
Starterator	Pham 80197 report
Genome Position	6703 to 8379 (Forward)
Length	1677 base pairs 558 amino acids
Amino Acid Sequence	Click to View
Notes	terminase large subunit

B



Gene: Eaglepride_13 Start: 6703, Stop: 8379, Start Num: 78
Candidate Starts for Eaglepride_13:
(Start: 78 @6703 has 455 MA's), (Start: 152 @6835 has 2 MA's), (198, 6919), (209, 6955), (212, 6967), (249, 7093), (274, 7153), (285, 7192), (303, 7240), (307, 7261), (309, 7285), (318, 7336), (344, 7459), (358, 7528), (395, 7666), (409, 7711), (435, 7777), (449, 7852), (455, 7876), (478, 7942), (483,

C

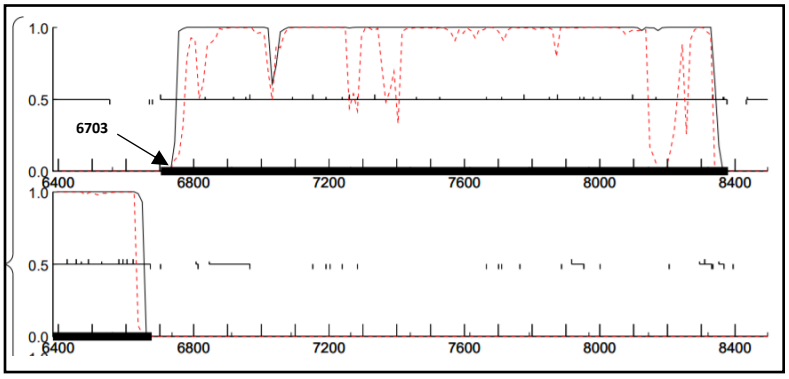


Figure 14: The start site of Eaglepride ORF 12 as determined by PhagesDB (A), Starterator Pham report (B), and GeneMark S report (C).

4.1: tRNA within Eaglepride genome – Aragorn and tRNA scan programs were used to identify a tRNA within the phage genome. The tRNA was 76 bps in length with a %GC content of 59.2 and was an L-tryptophan binding tRNA (Figure 15).

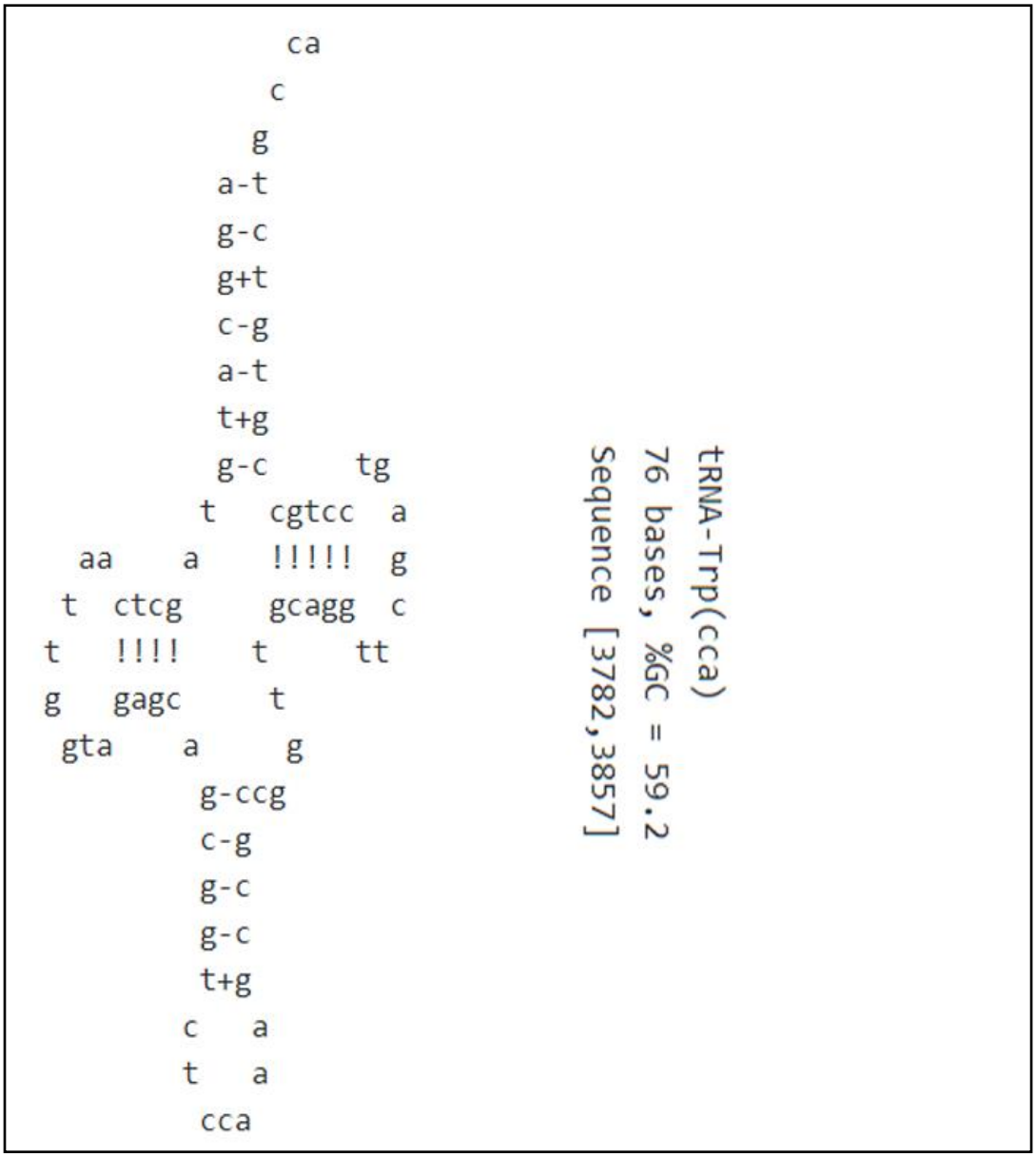


Figure 15: The tRNA within the Eaglepride genome.

Chapter 4: Discussion and Conclusion

4.1: Eaglepride characterization -- Eaglepride belongs to the *Siphoviridae* family, the most abundant family of tailed phages in the public databases (149). Eaglepride produced clear plaques on the host bacterial lawn, and there were no changes in the morphology of the plaques during my research. Due to the ubiquitous distribution of mycobacteriophages across a wide array of environmental habitats, one specific strain of bacteria can be used to isolate phages across different regions (150). While the soil sample used in the isolation of our phage was collected from Virginia, it was isolated in Oklahoma utilizing the host *M smegmatis* mc^2155.

Our analysis indicates that Eaglepride is a temperate phage due to the formation of a mesa. The lysogenic life cycle of Eaglepride was further supported by the presence of an integrase gene (gp 35), which is required for the integration of the phage genome into the bacterial host (151). Additional support for the lysogenic nature of Eaglepride was its placement within cluster A. Amongst the various clusters within the SEA-PHAGES database, cluster A is the largest and contains temperate phages or recent derivatives of temperate parents (152, 153). While Eaglepride often utilized the lytic cycle, the switch to a lysogenic cycle could be attributed to many factors including competition, host genetics, phage genetics, host physiology, and environmental stressors (154). After becoming a prophage, the switch to a lytic cycle can be induced by UV damage or other stressors acting upon the bacterial host (155).

The host range test of Eaglepride showed it to be incapable of infecting *M. abscesses* mc1518. This can be attributed to the host-specific and narrow-spectrum nature of many phages (160). This represents one of the main hurdles in the utilization of many phages for phage therapy.

The stability of the phage particle across varying temperature ranges showed it to be stable between 40^{o}C and 60^{o}C. Eaglepride became less stable and showed no growth at the 60-minute

mark of 65^{o}C (Figure 5). The stability of the phage at various temperatures was indicative of the many ecological zones that the host and phage can inhabit. The phage stability at 40^{o}C could be due to the evolution of the phage to function at the host bacteria's stable incubation temperature, which is 37^{o}C (156). The inability of the phage to form plaques above 60^{o}C could be attributed to the denaturation of phage and host proteins (157, 158).

The effect of pH on Eaglepride showed the phage to be stable at pHs 6, 7, and 8. Eaglepride was less stable at pH 5 and 9 (Figure 6). This could be attributed to the evolution of bacteriophages, like many obligate intracellular parasites, to function at conditions most suitable for their hosts (159, 160).

The superinfection immunity assay showed the prophage of Eaglepride incapable of preventing secondary infection by all eight of the mycobacteriophages tested. The factors allowing for superinfection exclusion of the host are associated with phage-mediated altering of the host cell surface or cell envelope components (161). The inability of Eaglepride to exhibit superinfection immunity could be due to the experimental mycobacteriophages being too genetically dissimilar to Eaglepride, which can play a role in conferring immunity.

The enzymatic digest of Eaglepride gDNA produced restriction sites for BamHI and HaeIII. BamHI generated 11 fragments and HaeIII had multiple fragments. The ClaI, EcoRI, and HindIII enzymes did not digest the gDNA, indicating the absence of restriction sites for these enzymes. In silico analysis using Unipro-UGENE software showed the same results as BamHI and HaeIII digested the gDNA 11 and 562 times (162). ClaI, EcoRI, and HindIII did not cleave the gDNA due to the absence of restriction sites.

4.2: Genomic characteristics of Eaglepride – The annotation of the Eaglepride genome allowed for a comprehensive analysis of its genes and their putative functions. Eaglepride belongs to cluster A, the members of which exhibit a similar genomic architecture. The left arm of the genome contains the structural and assembly genes, the right arm contains genes needed for the lytic cycle and the genome center contains prophage inheritance genes (163). Eaglepride had one programmed ribosomal frameshift (PRF) which was associated with the tail assembly chaperone gene. The frameshift occurred at the 15266 bp and was designated as a -1 frameshift. The PRF is conserved within the genome and found in both *Siphoviridae* and *Myoviridae* families (169). The PRF occurred at the slippery site of the mRNA and conformed to an X_XXY_YYZ 'slippery haptanucelotide' shift site motif. In this motif, XXX represents any three identical nucleotides, YYY represents any three UUU or AAA, and Z represents A, C, or U (163). Viruses generally utilize -1 PRF to code for multiple proteins using the same gene (164).

A tRNA was found within the Eaglepride genome and identified as Trp(cca); it was 76 bps in length, with a GC content of 59.2%. It is hypothesized that bacteriophage tRNAs were acquired through a complex series of recombination events involving bacterial hosts and the dynamics of insertion and retention in phage genomes (165). Amongst the roles attributed to tRNAs is enhanced phage fecundity (166). Phage tRNAs have been shown to compensate for codon bias between the host and phage, especially when the codon is expected within the phage and rare within the host (167). The tRNA genes are also hypothesized to replace genes interrupted by phage insertion into the host genome at tRNA genes. (166). In mycobacteriophage clusters, the tRNA genes are cluster-specific, except for clusters A, E, and K. All members of the clusters either have no tRNA genes or possess at least one copy. The deletion of the tRNA from the phage genome has shown smaller burst size and decreased protein synthesis rates (168). While the purpose and means by which

phages acquire tRNAs are still unclear, it is widely accepted that tRNAs within phage genomes play a significant role in propagation and adaptability.

4.3: Putative genes and their role in Eaglepride – The annotation of the Eaglepride genome predicated 35 ORFs with known putative functions (Table A). The potential roles these genes played in the attachment, replication, assembly, integration, and bacteriophage mediated lysis of the host will be discussed. Once the phage attaches to the host cell surface, it utilizes its tail to inject its genome into the host cytoplasm (169, 170). The tail tube of non-contractile phages like Eaglepride is composed of gp23 major tail proteins. The length of the tail is encoded by the gp26 tape measure protein, which is usually upstream of the major tail protein and separated by the gp24 tail assembly chaperone (171). The injection of the phage genome into the host is facilitated by using an injectosome, the assembly of which involves the tape-measure proteins. The phage tail also contains many repeating subunits of gp27 and gp28 minor tail proteins, which play a role in recognizing the correct host. Before the phage genome is transferred into the host, it is packaged using gp1 HNH endonuclease and gp2 terminase small subunit protein. These genes are highly conserved and located adjacent to each other within the genome (172, 173).

Once the phage genome has been injected into the host, its transcription is facilitated by various phage proteins. The Eaglepride phage possesses dsDNA as its genetic material, which is unwound by the gp57 DNA primase/helicase and cooperatively synthesized by the gp58 DNA primase/polymerase (174). The gp64 DnaB-Like dsDNA helicase catalyzes the separation of the dsDNA into its component ssDNA during replication (175). The gp46 helix-turn-helix DNA binding domain is used to bind the transcription factor to the DNA. The DNA bases to be incorporated are then modified by the gp48 ThyX-like thymidylate synthase and incorporated into the phage DNA strand by the phage-encoded gp45 DNA polymerase I (176). The biosynthesis of

the nucleotides is facilitated by the action of gp50 ribonucleotide reductase, and the hydrolysis of the released pyrophosphate is done by DNA polymerase I associated with gp54 metallophosphoesterase, which shifts the equilibrium towards nucleotide polymerization (177, 178). As the DNA strands replicate, they form Okazaki fragments, holiday junctions, and other branched DNA structures, which are resolved by gp60 endonuclease VII (179). Endonuclease VII has also been shown to successfully restart the transcription if a blockage or break occurs; this is an example of how multifaceted phage genes can often be. Gp81 and gp82 DNA methylase are responsible for the methylation of the viral genome, and it occurs towards the end of phage replication with the replicative intermediate serving as the substrate (175). The methylation plays a vital role in the maturation of the virus and denotes the exons and introns within the genome (180,181). Gp70 Cas4 family exonuclease is thought to stimulate the acquisition of host-derived spacers, exonuclease activity against ssDNA substrate, and evade host immunity (182).

As the genetic material of the phage is copied, it is packaged and transferred into new phage particles. The packaging of the viral genome triggers rearrangement of the coat protein and release of gp15 scaffolding protein, resulting in the expansion of the procapsid lattice. Assembly of infecting particles of tailed phages is facilitated by using an empty precursor capsid (procapsid) composed of multiple copies of coat proteins, gp16 major capsid protein, scaffolding proteins, and one gp13 dodecameric portal protein (183). The portal protein forms a channel facilitating the bidirectional passage of viral DNA, which can move in and out of the virus head and provides an attachment point for the tail apparatus in tailed phages like Eaglepride (184, 185). Once the procapsid is formed, its maturation is mediated by the capsid maturation protease gene, which degrades the parts of the prohead that are required for prohead assembly but must be removed to complete maturation (186).

Once the phage has replicated, it can either undergo the lytic or lysogenic pathway. In the lysogenic pathway, temperate phages like Eaglepride can utilize gp35 serine integrase to integrate their genome with the host genome and block phage gene expression (187). The lysogenic cycle allows the prophage to be replicated and maintained within the host until it switches to the lytic cycle. The lytic activity of phages like Eaglepride is facilitated by the action of several genes, including gp10 Lysin A and gp11 Lysin B. Host *M. smegmatis* mc^2115, which is a gram-positive bacterium containing a mycolic acid-rich outer membrane that is covalently attached to the arabinogalactan-peptidoglycan complex (188). Lysin A and gp61 hydrolase cleave the peptidoglycan layer of the mycobacterial cell wall, and lysin B is used to lyse the mycolylarabinogalactan bonds to release free mycolic acid (188, 189,190). The phage particles are released into the environment and can restart the cycle with a new host.

Conclusion – As part of this thesis I have successfully isolated, characterized, and annotated the Eaglepride genome. The genome analysis showed that 35 of the 88 (39.7%) genes within Eaglepride have a known putative function and 53 (60.23%) have no known function. My work sets the foundation for future research in which genes of unknown function can be cloned and their putative function further investigated. Because of its temperate nature and narrow host range against pathogenic bacteria, Eaglepride is not an ideal candidate for phage therapy.

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Chapter 6: Appendix

Table A: The putative functions of Eaglepride ORFs

Protein	Putative Protein Function
gp1	HNH endonuclease
gp2	Terminase small subunit
gp3	Unknown Function
gp4	Unknown Function
gp5	Unknown Function
gp6	Unknown Function
gp7	Unknown Function
gp9	Unknown Function
gp10	Lysin A
gp11	Lysin B
gp12	Terminase large subunit
gp13	Portal protein
gp14	Capsid maturation protease
gp15	Scaffolding protein
gp16	Major capsid protein
gp17	Unknown Function
gp18	Head-to-tail stopper
gp19	Unknown Function
gp20	Head-to-tail stopper
gp21	Unknown Function
gp22	Tail terminator
gp23	Major tail protein
gp24	Tail assembly chaperone
gp25	Tail assembly chaperone
gp26	Tape measure protein
gp27	Minor tail protein
gp28	Minor tail protein
gp29	Unknown Function
gp30	Unknown Function
gp31	Unknown Function

Protein	Putative Protein Function
gp32	Unknown Function
gp33	Unknown Function
gp34	Minor tail protein
gp35	Serine integrase
gp36	Unknown Function
gp37	Unknown Function
gp38	Unknown Function
gp39	Unknown Function
gp40	Unknown Function
gp41	Unknown Function
gp42	Unknown Function
gp43	Unknown Function
gp44	Unknown Function
gp45	DNA polymerase I
gp46	Helix-turn-helix DNA binding domain protein
gp47	Unknown Function
gp48	ThyX-like thymidylate synthase
gp49	Unknown Function
gp50	Ribonucleotide reductase
gp51	Unknown Function
gp52	Unknown Function
gp53	Unknown Function
gp54	Metallophospho-esterase
gp55	Unknown Function
gp56	Unknown Function
gp57	DNA primase/helicase

Protein	Putative Protein Function
gp58	DNA primase/ polymerase
gp59	Unknown Function
gp60	Endonuclease VII
gp61	Hydrolase
gp62	Unknown Function
gp63	Unknown Function
gp64	DnaB-like dsDNA helicase
gp65	Unknown Function
gp66	Unknown Function
gp67	Unknown Function
gp68	Unknown Function
gp69	Unknown Function
gp70	Cas4 family exonuclease
gp71	Unknown Function
gp72	Unknown Function
gp73	Immunity repressor
gp74	Unknown Function
gp75	Unknown Function
gp76	Unknown Function
gp77	Unknown Function
gp78	Unknown Function
gp79	Unknown Function
gp80	Unknown Function
gp81	DNA methylase
gp82	DNA methylase
gp83	SprT-like protease
gp84	Unknown Function
gp85	Unknown Function
gp86	Unknown Function
gp87	Unknown Function
gp88	Unknown Function