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Genome characterization of the "Eaglepride" phage

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Abstract
M. abscessus is a complex pulmonary disease progresses with persistent symptoms leading to decline of pulmonary function, and impaired quality of life; In some cases, the disease can also cause acute respiratory failure and death due to drug/antibiotic resistance. Previous research confirmed that the isolated mycobacteriophage "Eaglepride" will also be able to infect and kill *M. abscessus*, as the *M. abscessus* and *M. smegmatis* bacteria are closely related. Mycobacterium smegmatis is used in place of *M. abscessus*, as it is a non-pathogenic, ubiquitous bacterium of the same genus that can be experimented with in a regular lab. The current research hypothesis is to determine if the genome sequence of the "Eaglepride" has the potential to be applied for human Phage Therapy treatment. The databases DNA master and Phamerator, softwares such as TMHMM and SOSUI were utilized, genome validation with other databases such NCBI BLAST and HHPRED, and coding potential for genome was determined with GeneMark, GeneMarkS and Starterator.
In Eaglepride, genome sequencing located a functional integrase gene (gp 35) in the genome. This gene in a bacteriophage helps the phage become a provirus; once the phage attaches to the host cell surface, it utilizes its tail to inject its genome into the bacterial cell. Before it does this, however, gp 35 is used to facilitate integration of the tail into the bacterial cell membrane through its production of the enzyme integrase. Through gp35's production of integrase, Eaglepride can successfully integrate into host bacterial genomes, although the gene coding is less than ideal for human phage therapy use. Currently, phage-encoded lysins are being investigated for their potential as antimicrobial agents. For future studies, one can clone these lysin genes from Eaglepride phage, purify the lysin protein and investigate their potential as an antimicrobial agent, and test it against mycobacteria that cause human diseases such as Tuberculosis and Leprosy.

Keywords: phage, phage therapy, genome characterization, M. abscessus, M. smegmatis, DNA sequencing

Introduction

Background

My grandfather was diagnosed with pulmonary disease caused by the *M. abscessus* complex, which is a Biosafety Level 2 rapidly emerging pathogen that causes chronic lung disease in immunocompromised patients, leading to pulmonary disease. The *M. abscessus* pulmonary disease progresses with persistent symptoms, resulting in a decline of pulmonary function and impaired quality of life. In some cases, antibiotic resistant variants of the disease can also cause acute respiratory failure and death. My grandfather's tragic death on August 30, 2016, motivated me to learn more about the causes and effects of *M. abscessus*. As I learned more about *M. abscessus*, I understood that there is currently little research being done to find alternative treatments other than antibiotics for diseases caused by *M. abscessus*.
Mycobacterium abscessus is an environmental bacteria type that is found in dust, soil, and water. It can cause further lung infections in people with chronic pulmonary disease, and it has been found to contaminate medical devices or medications. *M. abscessus* bacterial infections are usually transmitted by contaminated substances or devices, and it is not commonly spread from person to person (CDC.gov. *M.abscessus* in the healthcare settings, 2010). The treatment of a serious *M. abscessus* complex disease usually involves antimicrobial therapy with a macrolide (clarithromycin 1,000 mg daily or 500 mg twice daily, or azithromycin 250 mg–500 mg daily) plus intravenous agents for at least 2 weeks to several months followed by oral macrolide-based therapy. The drugs of choice for initial intravenous administration are amikacin (25 mg/kg 3×/wk) plus cefoxitin (up to 12 g/d given in divided doses) or amikacin (25 mg/kg 3×/wk) plus imipenem (500 mg 2–4×/wk).

Antibiotic Resistance

The discovery of antibiotics helped control the infections that once ravaged humanity. However, their liberal use has led to the development of drug-resistant pathogens. Following the introduction of penicillin, S. aureus developed resistance in the early 1940s. Now, the infections caused by these drug-resistant microbes are a global health problem (Boerlin, 2010). Some causes of antibiotic resistance include over-prescription of antibiotics,

overuse of antibiotics in livestock and fish farming, poor infection control in health care settings, and poor hygiene and sanitation.

Societal Impact of Antibiotic Resistance

The most important distinction between bacteria and viruses is that antibiotic drugs usually kill bacteria, but they aren't effective against viruses. In some cases, it may be difficult to determine whether a bacterium or a virus is causing the symptoms. An antibiotic is a drug that kills or slows the growth of bacteria and treats bacterial infections.

The disadvantages of using antibiotics in treating bacterial infections are bacteria can develop antibiotic resistance, it may be difficult to deliver the antibiotic to the site of the infection, some antibiotics can cause severe allergic reactions, and antibiotic resistance can result in progressive symptoms that leads to a patient's death.

Phage Therapy

Before the discovery of the first antibiotic penicillin in 1929, phages have been used to treat bacterial infections. In 1896 phage therapy was used for the first time for treating Cholera which is a severe diarrheal disease caused by bacteria, Vibrio cholera (Horvath et al. 2007). In the early part of 19th century, phages were used to treat a number of bacterial infections such as those caused by Yersinia pestis (plague), Salmonella typhi (typhoid fever), Shigella dysenteriae (dysentery), Neisseria meningitidis (meningitis) (Ackermann and DuBow 1987). After the discovery of antibiotics, the idea of phage therapy as a therapeutic option was abandoned. After the abandonment of phage therapy, antibiotics were heavily used for treating bacterial infections and were also misused for treating viral infections. This antibiotic misuse quickly led to the development of drug-resistant bacteria, which is now a huge problem in modern medicine (Wittebole, 2014). Recent advances in virology and due to drug resistance acquired by most pathogenic bacteria we have seen a renewed interest in phage therapy.

Research Problem

The *M. abscessus* bacterial complex can cause the decline of pulmonary function and impaired quality of life. In some cases, the disease can also cause acute respiratory failure and death due to drug/antibiotic resistance.

The treatment of M.abscessus infection is done through use of powerful antibiotics to kill the bacteria present in the human body. Due to the emergence of antibiotic-resistant strains of M.abscessus and other mycobacteria, alternative treatments are essential in treating mycobacterial infections in pulmonary disease patients.

Over a period of 1991-2007, a total of 28,697 sputum samples were analyzed from 7,940 patients. Of these, 3,988 (50%) were defined as possible cases, and 1,865 (47%) of these were defined as definite cases of nontuberculous mycobacterial lung disease (AJRCCM, Vol. 182, Prevots et al, 2010). Several research publications also confirmed this disease is growing at a rate of 3% per year (NIH, 2015). Bacteriophage/Phage therapy can be way alternative way to treat patients who develop resistance to a M.abscessus pulmonary disease.

Societal Impact of Research

The genus Mycobacterium is composed of acid-fast, rapidly growing aerobic pathogenic bacterium that causes skin and soft tissue infections. Aerobic bacteria require oxygen for their survival (similar to humans). Some of the medically important organisms that belong to this group include M. tuberculosis and M. leprae, which cause tuberculosis (an infectious respiratory illness) and leprosy (an infectious skin disease). The Mycobacterium abscessus bacterium can cause life-threatening chronic pulmonary/lung disease in immunocompromised patients. Due to the emergence of antibiotic-resistant strains of *M. abscessus* and other mycobacteria, alternative treatments are critical in treating Mycobacterial infections.

Safety Requirements

There are no safety risks for completing bioinformatics of a phage because I will be working with Gene databases, phage isolation safety risks are detailed for reference. M.abcessus *M. smegmatis* is commonly used in work on the Mycobacterium genus due to its being a fast grower and non-pathogenic. *M. smegmatis* was a simple model that is easy to work with, and only requires a biosafety level 1 laboratory. This species shares more than 2000 homologous genes with M. tuberculosis and shares the same peculiar cell wall structure of *M. abscessus* and other mycobacterial species. Therefore, I utilized *M.Smegmatis* due its non-pathogenic nature and ability to be experimented on regular work bench during the phage isolation.

Research Question and Hypothesis

The question that arises with this previous background is: can a mycobacteriophage that successfully infects *M. smegmatis* infect and kill *M. abscessus*? My research hypothesis proposes that the isolated mycobacteriophage "Eaglepride" will also be able to infect and kill *M. abscessus*, as the *M. abscessus* and *M. smegmatis* bacteria are closely related. My hypothesis is also supported by previous research, which demonstrated that some mycobacteriophages could infect multiple bacterial hosts (Pope, 2014). *Mycobacterium smegmatis* is used in place of *M. abscessus*, as it is a non-pathogenic, ubiquitous bacterium of the same genus that can be experimented with in a regular lab.

Materials and methods

The process for phage discovery and isolation starts with the collection of the soil samples where there is a high probability of finding the M.smegmatis bacterium. The areas where there is moist soil with not much exposure to sunlight and no foot traffic or sewer pipes are great places to find M.smegmatis.

I collected soil samples near the gutter pipes of our school building. These samples were shipped to University of Central Oklahoma for further analysis.

The following steps have been followed per the Picture 1 in the extraction of the Phage virus.

- **Culturing *M. smegmatis* mc2155 and *M. abscessus*:** Both bacteria were grown in 7H9 broth supplemented with ADC and 1mM CaCl2. On solid media the bacteria were grown on 7H10 agar supplemented with 0.2% glucose and 1mM CaCl2. Bacteria were always incubated at 37°C
- **Soil Enrichment and Phage Isolation:** Soil was collected from the north end of building close to the gutter pipes where foot traffic is minimal and less sunlight (39.014667 N, 77.515417 W) and north end of Lake Overholser in Oklahoma City (35.513554 N., 97.663444 W). Approximately 15g of collected soil was enriched with 15 mLs of 7H9 broth and 1.5mL of *M. smegmatis* in the exponential growth phase and was incubated at 37°C overnight.
- **Phage Isolation:** After incubation, the phage was filtered, diluted, and plated using the soft-agar overlay method.
- **Purification:** Plaques were purified by removing a single plaque and adding it to an exponentially growing culture of *M. smegmatis*. This was repeated three times to ensure the purification of a single virus.
- **Infectivity Against *M.abscessus*:** The spot test was used to evaluate phage infectivity of *M. abscessus*. The isolated phage and other phages isolated in the lab were plated on a lawn of *M. abscessus* to test infection against the pathogen. Phages were also plated on a lawn of *M. smegmatis* as a positive control.
- **Amplification:** The objective of this step is to make more phage lysate. During this step, webbed plates are made, plate lysates are collected, and full plate titer is collected.
- **DNA Extraction:** With enough phage lysate collected, DNA is extracted from the virus.
- **Electron Microscopy:** The concentrated sample is placed under the electron microscope for page characterization/morphology.
- **VII Genome Annotation and Cocktail Trials:** With genome annotation and Genbank submission, higher quantities of Phages can be produced that can be used for Phage trials.

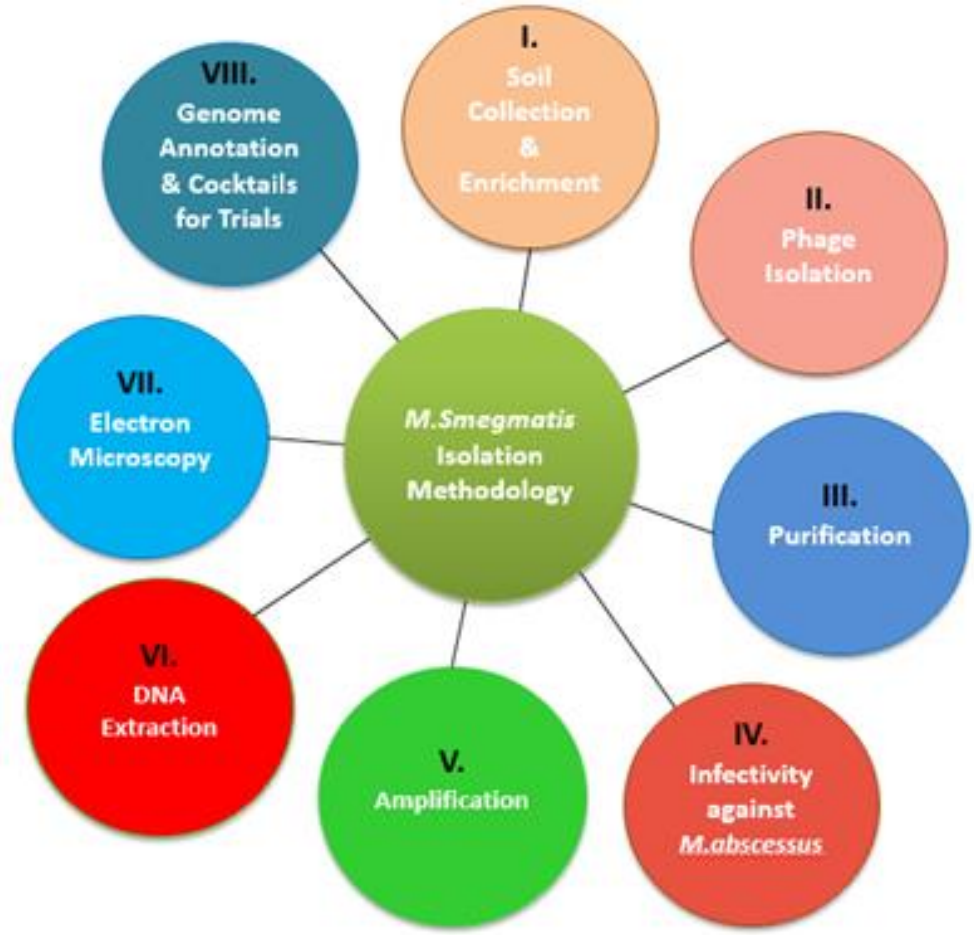


Fig 1: Steps for *M. Smegmatis* isolation methodology

Research Progress

- **Summer 2017**: The newly discovered phage "Eaglepride" was successfully isolated from Oklahoma soil and Virginia soil using the host bacterium *M. smegmatis* mc2 155
- **Summer 2017**: "Eaglepride" phage/virus extracted from Virginia soil showed unique characteristics that were presented and accepted by worldwide phages database http://phagesdb.org/Eaglepride/
- **Summer 2018**: The virus "Eaglepride" was revitalized by following the procedure steps – plague purification, DNA isolation, Phage infectivity against *M. abscessus*. These are replication steps to produce enough phage for producing phage cocktails.
- **Summer 2019/2020**: The virus "Eaglepride" was revitalized by following the procedure steps: plague purification, DNA isolation, phage infectivity against *M. abscessus*. These are replication steps to produce enough phage for producing phage cocktails.
- **Fall 2021/Spring 2022**: Genome Characterization of the "Eaglepride" Phage

Experimental Design

Genome Annotation

Recently there is an increased interest in bacteriophage-based therapy treatments as alternate medical treatment options. As a result of this, analyzing bacteriophage gene expression strategies have been utilized especially due to occurrence of bacterial strains resistant to antibiotics. This further led to the development of effective methods via pairwise sequence alignment such as BLAST. Before conducting the gene annotation of the extracted gene material, gene expression is carried out by identifying the location of genes and all of coding regions in a genome. This process helps in obtaining gene sequence information. For performing the gene annotation and sequence information, the input information that is widely used is the FASTA file which is a test-based format file that represents either nucleotide or amino acid/protein sequences via single-letter codes.

The popular tools that are widely used in the bioinformatics domain include DNA Master and Phamerator. DNA Master is a genome annotation and exploration tool. This tool quickly generates an auto-annotation of the subject genome using GeneMark and Glimmer in making gene calls and Aragorn and tRNAscan-SE in identifying tRNAs. One of the challenges in annotating the bacteriophages genomes is genomes having mosaic architecture with small open reading frames of unknown function. This challenge is addressed with the tool Phamerator database that assorts protein-coding genes into families of related sequences using pairwise comparisons. This database is widely used in generating genome maps of multiple phages that incorporate nucleotide and amino acid sequence relationships, including genes containing conserved domains.

The SOSUI software identifies helices that are relatively easy to predict based on known helical potential of the given amino acid sequence (AAS). The TMHMM software is utilized in membrane topology prediction, powered by Markov model, helps in identifying the fundamental structural knowledge of the TM (transmembrane) proteins, helices and discriminates between soluble and membrane proteins with high degree of accuracy.

The National Center for Biotechnology Information (NCBI) has a program Basic Local Alignment Search Tool (BLAST) that searches for amino-acid sequences of proteins and nucleotides of DNA and/or RNA sequences further allows to compare the subject protein or nucleotide sequence with the database of sequences that resemble the subject sequence above a certain threshold. HHPRED is a suite of open-source search tools that searches similar protein sequences in a database using Markov models. Both NCBI BLAST and HHPRED are the most popular search tools that help to infer further about the subject proteins and nucleotides from similar proteins and nucleotides.

GeneMark database houses many bacterial models allowing us to compare the subject genome based on the start and stop codons and further predict genes via nucleotide probability profiles. The profiles are generated either from an analysis of a subset of Open Reading Frame (ORF) found in the six-frame translation of the genome sequence or from a pre-analyzed set of annotated genes from another, related genome sequence. GeneMark utilizes a second, fourth, or fifth order model to examine all six of the translational frames simultaneously to predict genes. GeneMarkS considers potential ribosome binding sites when predicting gene start positions. Starterator database. Startertator database plays a vital role when a specific start codon for a gene does not agree with scientific evidence. This database analyzes that gene as part of a set of related genes in multiple sequence alignment leading to deciphering the start codon and thus providing the longest possible ORF for each gene within a pham.

Data Analysis

The following steps were followed in understanding gene expression (sequence and characterization, Picture 12) of the "Eaglepride" bacteriophage that can be further utilized for creating phage cocktails in treating pulmonary disease caused by the *M. abscessus* complex

- Utilize DNA Master in analyzing the characteristics of phage "Eaglepride"
- Utilize Phamerator database to identify the groups of proteins that are similar to one another
- Auto annotate the genomes created from the FASTA files and identify the tRNA and tmRNA genes in the phage
- Utilize softwares TMHMM and SOSUI, and identify the transmembrane helices in the genomes

- Validate genome information with the other databases NCBI BLAST and HHPRED
- Utilize GeneMark, GeneMarkS and Starterator and identify coding potential for each of the genes and identify the additional information
- Prepare results, conclusions, and recommendations as part of the research final report that can be presented and published.

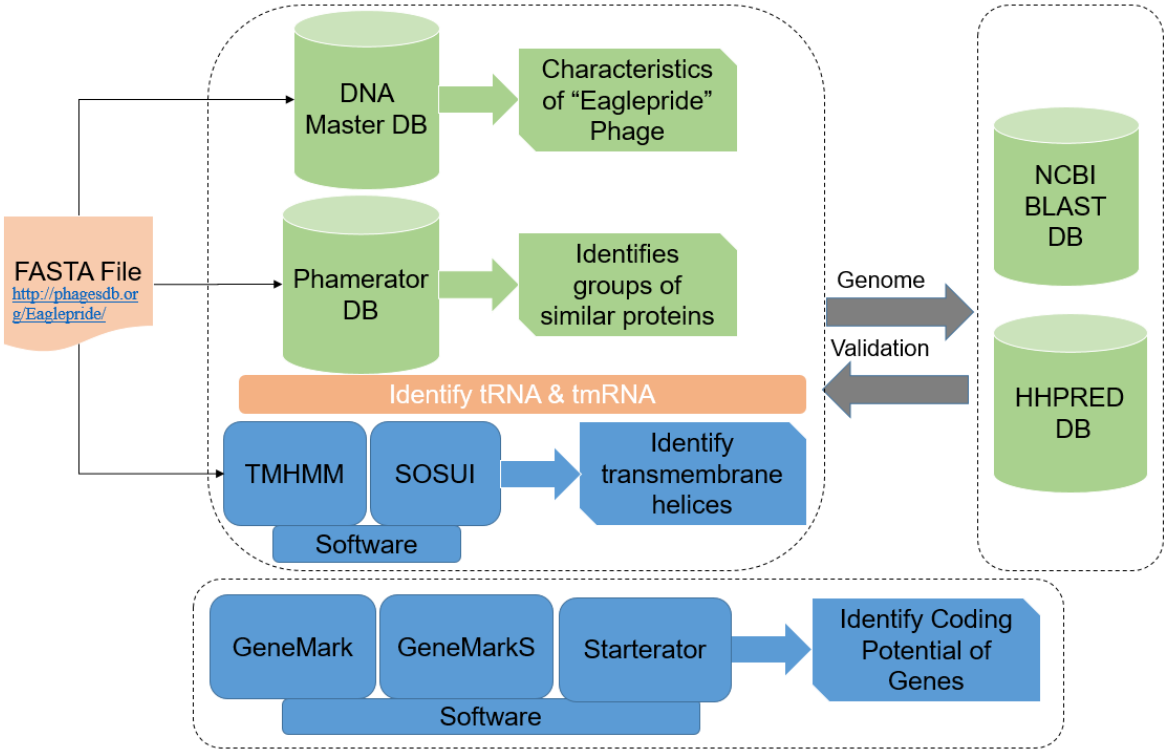


Fig 2: Overview of the databases that were utilized in chacterization of the "Eaglepride" genome

Table 1: Data showing the protein information of Lynsin A, Capsid, Major Tail, DNA polymerase

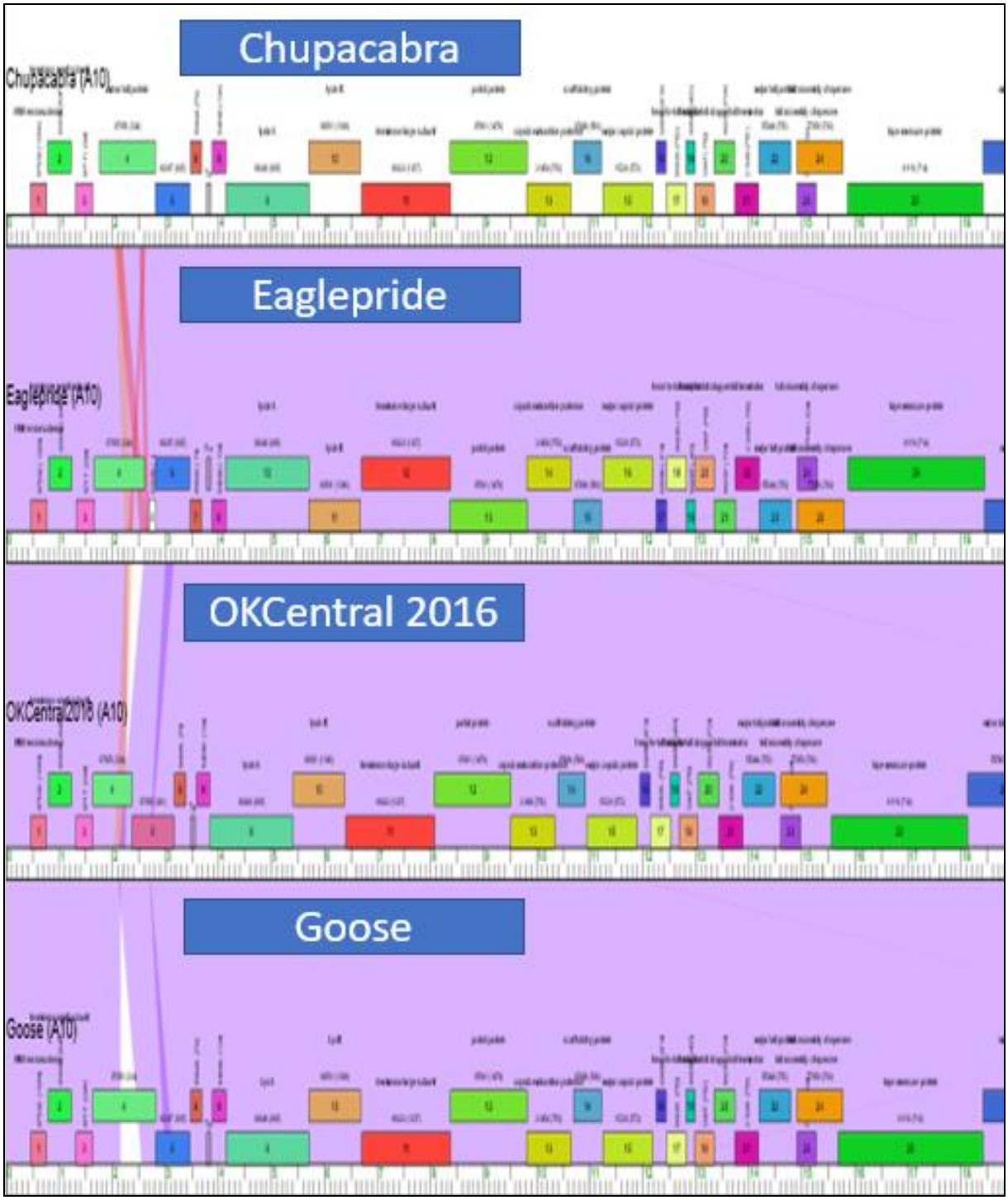
DNA Master – Gene Protein Analysis for Eaglepride															
Lynsin A Gene Protein				Major Capsid Gene Protein				Major Tail Gene Protein				DNA Polymerase Gene Protein			
Name	5' End	3' End	Length		5' End	3' End	Length		5' End	3' End	Length		5' End	3' End	Length
1	473	769	297		473	769	297		473	769	297		473	769	297
2	803	1237	435		803	1237	435		803	1237	435		803	1237	435
…	…	…	…		…	…	…		…	…	…		…	…	…
34	22834	23265	432		22834	23265	432		22834	23265	432		22834	23265	432
35	23262	23453	192		23262	23453	192		23262	23453	192		23262	23453	192
36	23450	25285	1836		23450	25285	1836		23450	25285	1836		23450	25285	1836
Bit Score – 976.5;Identities -494				Bit Score – 592.7;Identities -304				Bit Score – 362.5;Identities -184				Bit Score – 1084.3;Identities -540			
Score – 2523;%Identity – 94.64				Score – 1532;%Identity – 97.44				Score – 929;%Identity – 92.93				Score – 2803;%Identity – 88.52			
E-Value – 0.0E0;Positives - 508				E-Value – 0.0E0;Positives - 306				E-Value – 0.0E0;Positives - 195				E-Value – 0.0E0;Positives - 567			
Length – 522;%Similarity – 97.32				Length – 312;%Similarity – 98.08				Length – 198;%Similarity – 98.48				Length – 610;%Similarity – 92.95			
% Aligned -100%;Gaps - 0				% Aligned -100%;Gaps - 0				% Aligned -100%;Gaps - 0				% Aligned -100%;Gaps - 3			
Query :1- 522				Query :1- 312				Query :1- 198				Query :1- 607			
Target : 1-522				Target : 1-312				Target : 1-198				Target : 1-610			
Note: The table above are examples of annotations that were compiled for all genes															

Null Hypothesis H0: There may be something present in Eaglepride phage that will prevent for human phage therapy (Gene Protein - gp35)

Alternate Hypothesis H1: We can utilize Eaglepride for human phage therapy
Lower E-Value ~0.0 confirms the presence of the Gene Protein (gp35) is significant. We **do not** reject Null Hypothesis

- DNA alignment of the Eaglepride phage is 100% when compared with other **Siphoviridae** family with no significant gaps in the DNA
- Presence of gp35 is highlighted in red in the table 1.
- E-Value – 0.0 E0 = e-179 (0) = 0; Since E-value = 0 approx.; we **do not** reject Null Hypothesis
- The percent similarity with other **Siphoviridae** family is between 92.95% to 98.48% for Lysin A, Major Capsid, Major Tail and DNA polymerase Gene Proteins

- The figures 3 through 8, confirm that in Eaglepride phage, genome sequencing located a functional integrase gene (gp 35) that can integrate into host bacterial genomes, making Eaglepride less than ideal for human phage therapy use (Hyman, 2017)



Source: https://phagesdb.org/Phamerator

Fig 3: Phamerator Report with 3 Closely Matched Phage Genomes OKCentral2016, Goose, and Chupacabra;

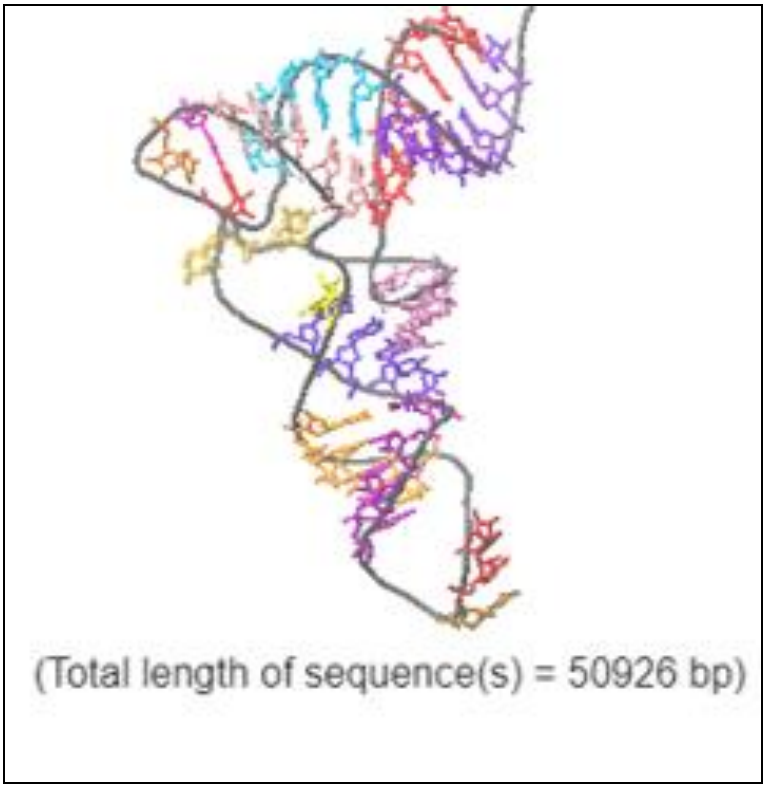
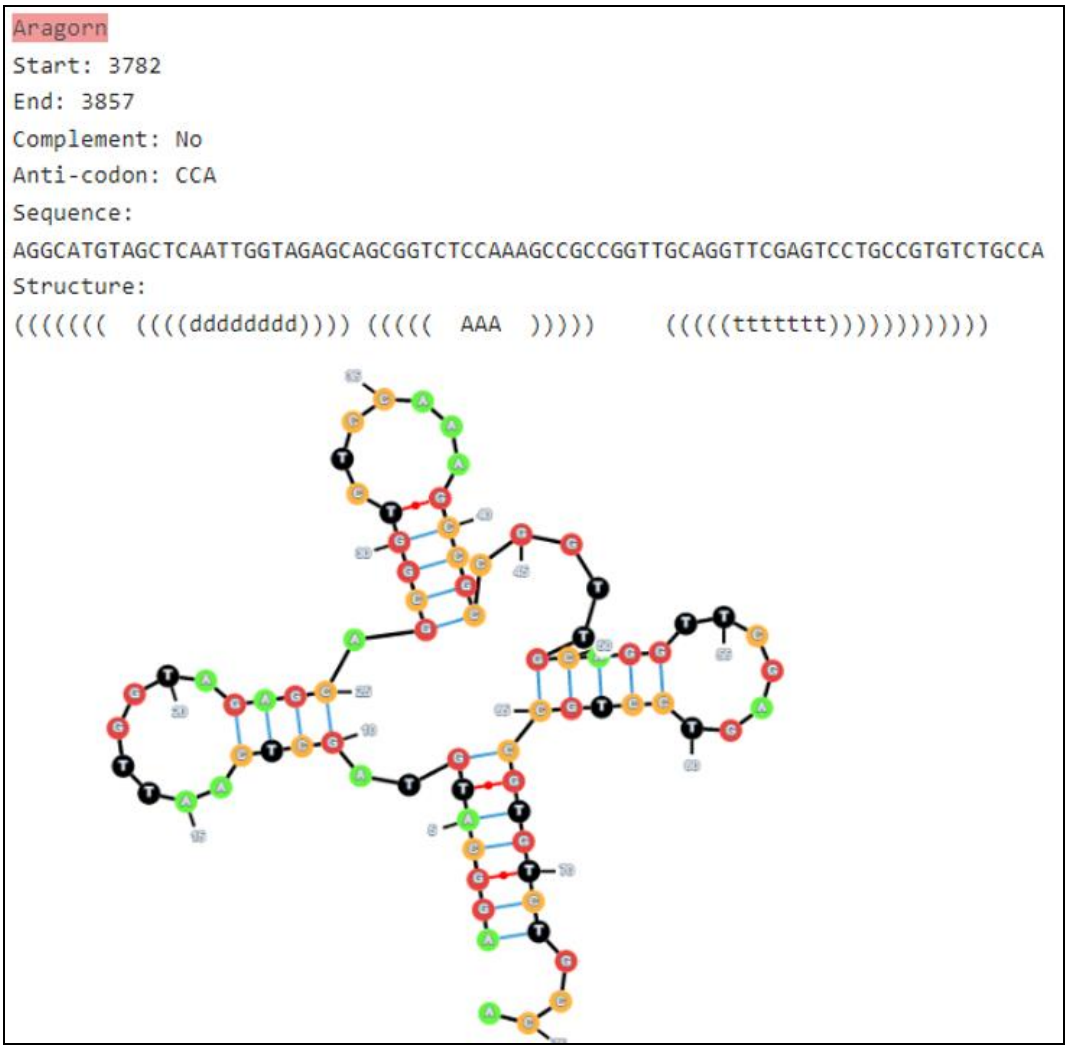


Fig 4: tRNA genes in Eaglepride;Source::tRNA ScanSE 2.0 – http://trna.ucsc.edu/tRNAscan-SE



Source: Aragorn (v1.2.38) http://130.235.46.10/ARAGORN

Fig 5: tRNA & tmRNA genes in Eaglepride

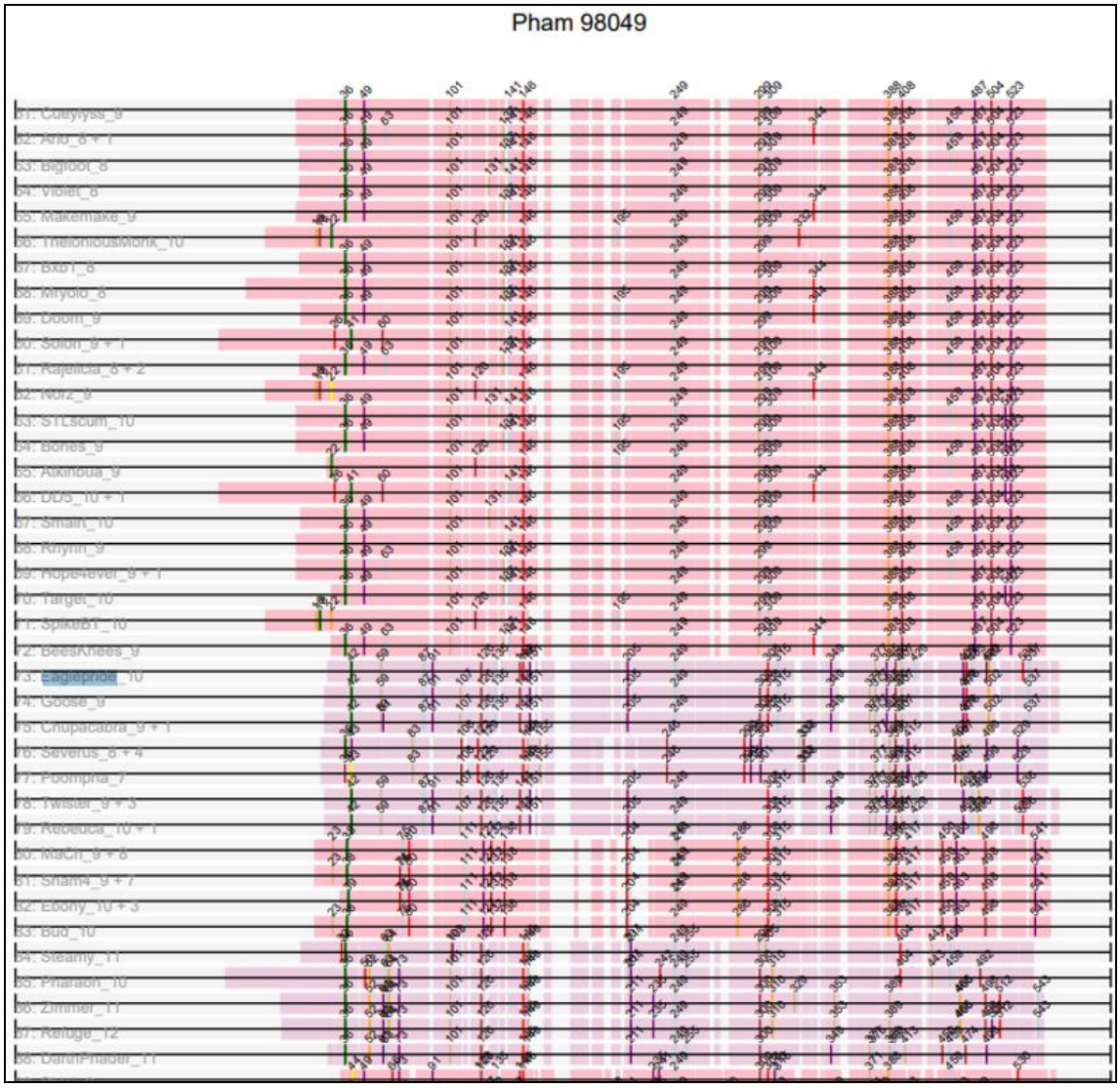


Fig 6: Lysin A gene – Starterator Report Output

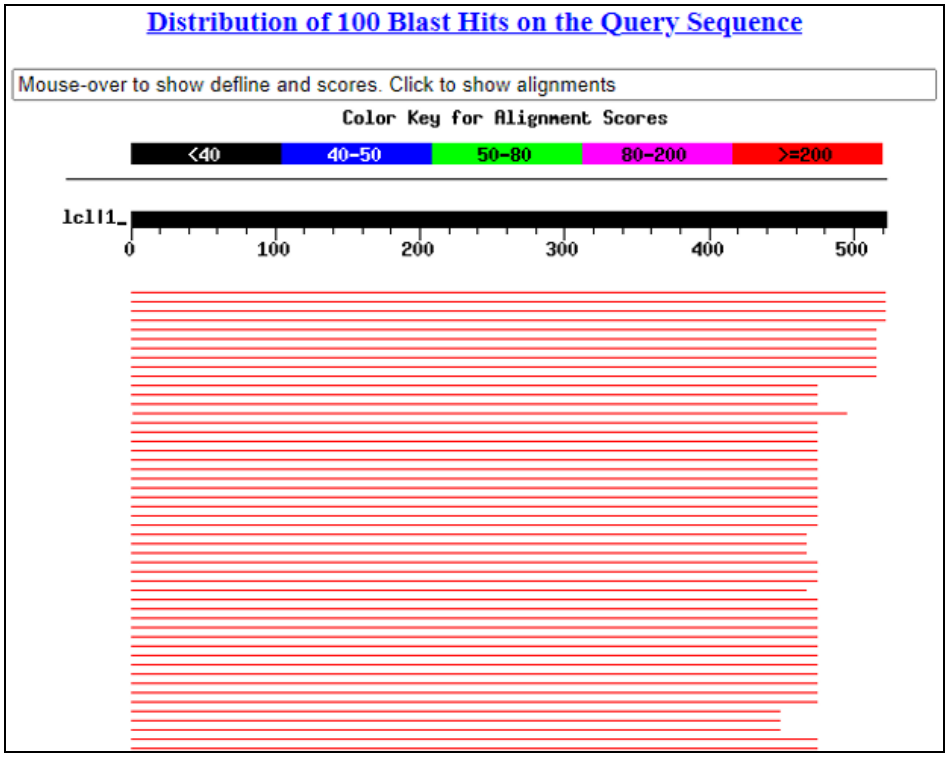


Fig 7: Lysin A gene- PhagesDB BLAST output

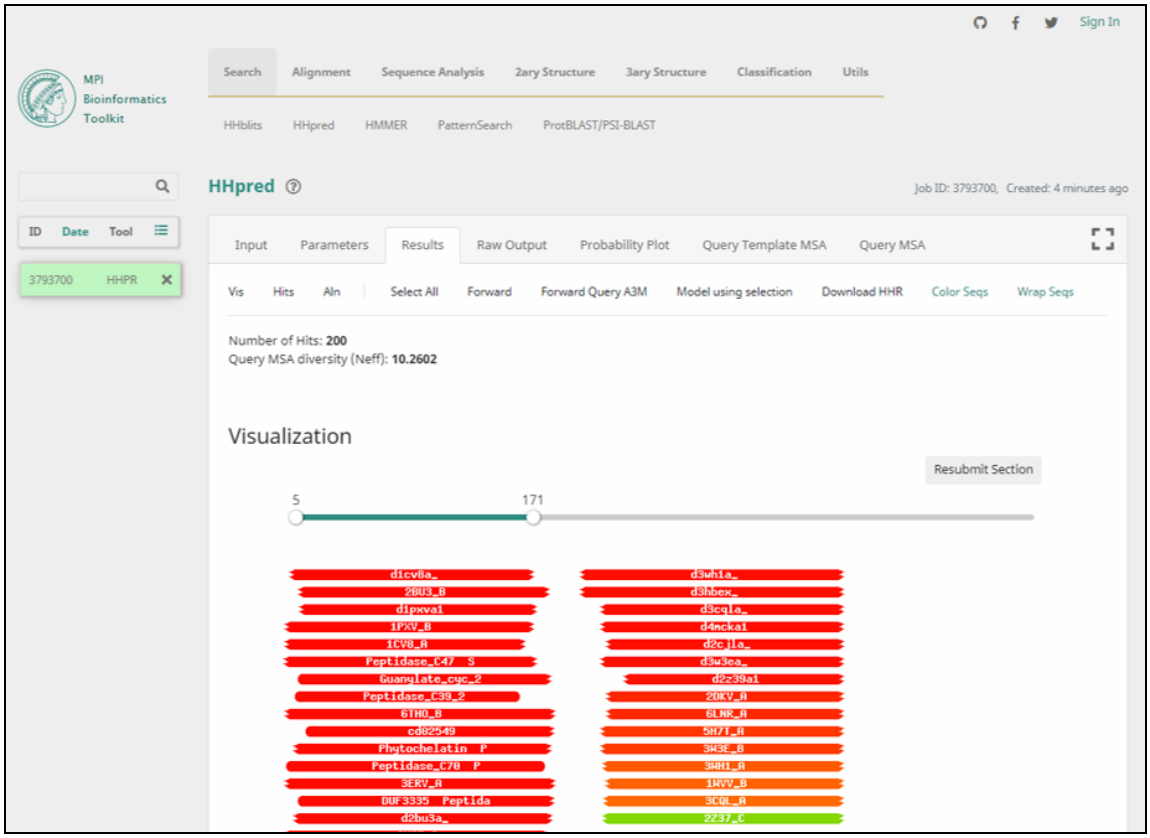


Fig 8: Lysin A gene- HHpred Output

Results and Discussion

- Eaglepride is part of cluster A. The members of this cluster exhibit similar genomic architectures. The left arm of the genome contains the structural and assembly genes, which play a role in building viral particles. The right arm contains genes used in the lytic cycle, which help release the phage particle after its infection cycle. The middle part of the genome contains genes that help this phage enter a lysogenic cycle.
- Eaglepride belongs to the Siphoviridae family, the most abundant family of tailed phages in the public databases.
- Eaglepride is a temperate phage, confirmed through my work while annotating this phage genome. I have located a functional integrase gene (gp 35) in the genome. This gene in a bacteriophage helps the phage become a provirus.
- During my analysis, I determined a programmed ribosomal frameshift (PRF) in the tail assembly chaperone gene. The frameshift occurs at the 15264 bp and was designated as a -1 frameshift. This phenomenon is often seen in viruses where they can use one gene to make two different proteins. In the case of Eaglepride, the ribosomes, while translating the mRNA, at site 15264 base jump one nucleotide to the left and make a different protein.
- Eaglepride genome has one tRNA gene identified as Trp (cca). It is 76 bps in length. tRNA is needed during the translation process to build proteins.
- Once the phage attaches to the host cell surface, it utilizes its tail to inject its genome. The gene gp23, which is a major tail protein, is needed for this process. The length of the tail is determined by gp26 tape measure protein. The phage tail also contains many repeating subunits of gp27 and gp28 minor tail proteins, which play a role in recognizing the correct host. Once the phage genome enters the host cell, its transcription of various genes is initiated.
- Once the phage genome and structural proteins are made, phage particle is assembled with the help of gp15 scaffolding protein, gp16 major capsid protein, and gp13 dodecameric portal protein. As the last step, the phage particle uses gp10 Lysin A and gp11 Lysin B to get out of the host cell.

Conclusions

- In Eaglepride, genome sequencing located a functional integrase gene (gp 35) in the genome. This gene in a bacteriophage helps the phage become a provirus; once the phage attaches to the host cell surface, it utilizes its tail to inject its genome into the bacterial cell. Before it does this, however, gp 35 is used to facilitate integration of the tail into the bacterial cell membrane through its production of the enzyme integrase.
- Through gp35's production of integrase, Eaglepride can successfully integrate into host bacterial genomes, although the gene coding is less than ideal for human phage therapy use (Hyman, 2017).

- Currently, phage-encoded lysins are being investigated for their potential as antimicrobial agents. For future studies, one can clone these lysin genes from Eaglepride phage, purify the lysin protein and investigate their potential as an antimicrobial agent, and test it against mycobacteria that cause human diseases such as Tuberculosis and Leprosy caused by M.abscessus. If Phages can penetrate and access mycobacteria in the human cells, they can be utilized as alternate forms of treatment for patients that do not respond to antibiotics.

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