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Characterization of a Novel Mycobacteriophage via Sequence Analysis

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Abstract

Bacteriophages provide a relatively new area of scientific research. Approximately 10^{31} viruses on earth are capable of infecting bacteria and thereafter disseminating. Despite their widespread and pervasive nature, bacteriophages have been relatively unstudied. Not only do they provide a new class of organism but they also present potential usefulness to humans, including within the medical field. Bacteriophages represent a new tool in medicine's ever-developing fight against bacterial infections. Notably, bacteriophages may offer a new weapon to battle infections that have developed resistance to multiple antibiotics. The prospect of bacteriophage use against infectious processes will require significant research which begins with a basic understanding of structure and function of the phages themselves.

Introduction

Bacteriophages, colloquially known as phages, represent a relatively unexplored scientific area. First discovered and described in 1915 as "microscopic viruses," phages represent a potential well of new knowledge (Twort). It is estimated that there are 10^{31} bacteriophages inhabiting the biosphere (Wommack and Colwell 2000). Because phages infect a variety of bacteria, the potential for phage application is very broad.

Most directly, phages may be used to infect and destroy harmful bacteria, which offers a clear application in medicine. However, phages can also be used to treat cancer (DePorter and McNaughton 2014), to make bacteria more susceptible to antibiotics (Viertel *et al.* 2014), and also to reverse the effects of agents utilized in biowarfare such as anthrax (Fischetti 2008).

Of the 10^{31} phages on earth, less than 2000 which infect mycobacteria have been characterized (Hawtrey *et al*. 2011). All mycobacteriophages characterized thus far are dsDNA (double-stranded) tailed phages (Hatfull *et al.* 2011). Mycobacteriophages are commonly identified after isolation from infected *Mycobacterium smegmatis*, but due to their versatility the newly isolated phage could also be tested for its ability to infect *M. tuberculosis* or *M. leprae* in order to improve treatment of diseases brought on by these bacteria. This research would require a laboratory with Biosafety Level Two status for containment, and Biosafety Level Three practices and equipment (Herman *et al.* 2006).

Any mycobacteriophages thus isolated and capable of infecting *M. tuberculosis* or *M. leprae* have the potential to treat humans infected by these bacteria without harm to the human host, as evidenced by prior medical use with little to no toxicity (Abedon *et al.* 2011). Global health could be positively impacted by discovery of new treatments for *M.*

tuberculosis as the number of cases diagnosed globally appears to be on the rise (Herman *et al.* 2006).

Perhaps more important is the potential for treatment of Multiple-Drug-Resistant tuberculosis (MDR TB) along with other antibiotic resistant bacterial infections. The presence of drug-resistant organisms among tuberculosis patients in the United States had increased by 7% in the early 1990's. Reasons for this increase center primarily on immunocompromised patients, specifically co-infection with Human Immunodeficiency Virus (HIV) and *M. tuberculosis*. However, incorrect or unmonitored treatment and failure to recognize ongoing infectiousness also played a large role (Jacobs 1994). Current regulations in Western countries present a difficult barrier to overcome prior to clinical phage use (Allen *et al.* 2014). Therefore, further study in clinical trials will be required prior to widespread use of phages in Western medicine. However, as there is a constant increase in antibiotic resistance among bacteria there has been a recent surge in interest in the potential therapy of phages (Viertel *et al.* 2014). Importantly, bacteriophages have already been shown to be useful in treating *Staphylococcus aureus* as well as other bacterial infections in the Soviet Union prior to the invention of antibiotics (Sulakvelidze *et al*. 2001). The Soviet Union continued to utilize phages well into the "antibiotic era," but due to the secrecy associated with the "iron curtain" of the Cold War, as well as a language barrier, much of the Soviets' success was lost upon the West (Abedon *et al.* 2011). Abedon and colleagues note that phage therapy was used in France until the mid-1990's and Poland has persisted in employing phages. However, since 2005 their use has been mostly relegated to cases of antibiotic resistant infections (2011). Such results provide a viable option for the healthcare industry as the race against

MDR TB continues. This research indicates a potential new, and therefore likely *M. tuberculosis* susceptible, avenue for eradication projects worldwide.

Not only do phages potentially provide a new weapon against an old foe but they also attack more specifically than antibiotic therapies, thus minimizing damage to other colonizing bacteria not directly targeted (Allen *et al.* 2014). Phages target only specific bacteria they can infect and as such can be utilized as vectors to deliver exogenous proteins or genes (DePorter and McNaughton 2014, Adhya *et al.* 2014). DePorter and McNaughton describe treatments employing tagged phages to target cancer cells which deliver disruptive proteins ultimately leading to cell death (2014). The ability for phages to deliver DNA to their bacterial host provides an indirect method of phage therapy in antibiotic resistant infections by supplanting the resistance (Adhya *et al*. 2014). Thereafter, antibiotic therapy could continue as before.

Additionally, the specificity of phages could prevent the eradication of "healthy bacteria." In cases where antibiotics are used too frequently, patients become susceptible to infection of *Clostridium difficile*. *C. diff* appears as an infectious agent to supplant recently killed healthy bacteria. As phages are specific to the bacteria they target, widespread killing of healthy bacteria within the human intestinal tract could be avoided, thus preventing the costly treatments associated with *C. diff*.

Phage therapy also includes the opportunity for use of phage components. Injection of endolysins proved to be nearly as effective as whole-phage infection (Viertel *et al.* 2014). The endolysins degrade cell wall peptidoglycan leading to a decreased bacterial cell wall integrity which ultimately causes cell death (Viertel *et al.* 2014). Importantly even Gram-negative bacteria, with their protective outer membranes, can be

infected by *Acinetobacter baumannii* phage endolysin LysAB2 and *Bacillus amyloliquefaciens* phage endolysin (Viertel *et al.* 2014). Unlike antibiotics, bacteria have not been able to build up a resistance to endolysins thus far in laboratory studies (Loeffler *et al.* 2001).

The question of resistance to phages is also related to the potential for personalized treatment (Chan *et al.* 2013, Merril *et al.* 2003). By developing phage cocktails directed to the individual's particular invading bacterial species, fewer phages overall would need to be employed in treatment and the likelihood of insufficient treatment leading to pervasive infection would be significantly lowered (Chan *et al.* 2013). The capability of identifying the bacterial cause of infection is also facilitated by phages: a simple combination of phage and infected tissue or serum will reveal the infecting bacteria and its most successful phage treatment. This process utilizes luciferase which emits light due to the availability of ATP during cell lysis (Merril *et al.* 2003). This method could very well supplant the need for bacterial cultures which often delay proper treatment.

All potential phage applications stem from an understanding of their basic structure and function. Prior to application in medicine, phages must be analyzed to test the presence of toxin genes (Merril *et al.* 2003). By analyzing a newly isolated bacteriophage via DNA sequencing, my research may broaden the knowledge base of bacteriophages and provide a new phage for future use in medicine. I hypothesize that analysis of a phage genome from purified phage DNA via DNA Master as outlined in the Annotation Guide, available from the Phages database (phagesdb.org), will reveal a unique mycobacteriophage with mosaic characteristics.

Materials and Methods

Phage genomic DNA was provided by Dr. Kirk Anders at Gonzaga University and Dr. Marisa Pedulla at Montana Tech of the University of Montana. This DNA was isolated from phages following infection of soil samples with *M. smegmatis*. The DNA was sent for sequencing at North Carolina State University. The completed sequences were returned as FASTQ files.

One phage, Tollulah, was selected for analysis and annotation. Tollulah's FASTQ file was comprised of the results of all sequences obtained by North Carolina State University. As such, the file contained a multiplicity of genome segments which required alignment prior to analysis. The in silico protocol developed by the Kentucky Biomedical Research Infrastructure Network suggests use of Consed for generation of a consensus sequence via segment alignment. Consed opens the file as a project which then undergoes alignment via identification of consensus sequences and identifies the most common bases at each position. Unfortunately, in this case Consed was unable to complete alignment of the sequences. As such, an alternate program, Codon Code Aligner, was utilized due to similar accessibility and functionality with the available FASTQ sequence. Tollulah's FASTQ file was opened in Codon Code Aligner and also only partially aligned.

Multiple approaches were used, including alignment of portions of the genome with subsequent addition of genome segments, in an attempt to align the genome in a piecemeal fashion. In this mode, the original FASTQ file was separated into ten smaller files containing the genome segments. This was minimally successful and a single

consensus sequence still could not be successfully generated. Additionally, a partially aligned sequence could not be generated by the program.

Results and Future Directions

At this time, the Tollulah genome has not been successfully annotated due to lack of a consensus sequence. Therefore, the characteristics and genes contained within Tollulah are still unknown at this time. Analysis of the genome requires further attempts to generate a consensus sequence. As both alignment programs, Consed and Codon Code Aligner, were unable to fully align the genome, this likely represents limitations within the programs or irreconcilable errors within the genome itself.

An option for further research includes submitting another DNA sample for sequencing and thereafter attempting annotation. Due to the errors encountered within the alignment programs, there is a possibility that the program was unable to reconcile differences between some of the genome segments and therefore did not complete the alignment protocol. The programs may not be able to identify exogenous DNA, from the vector or host, and therefore cease the process of alignment. Renewed infection of *M. smegmatis* with the Tollulah phage prior to isolation of DNA for a second round of sequencing could aid in characterization of its genome to ensure portions of the sequencing are not derived from the host utilized in the original isolation. This would also aid in assessing whether the genome itself contains errors that cannot be reconciled by Consed or Codon Code Aligner or multiplicities preventing proper alignment.

For future sequence analysis, isolated phage samples should be amplified and the purification should be carried out in triplicate. Samples could also be run on an agarose gel prior to submission for sequencing in order to evaluate the expected genome size. Samples revealing bands incongruent with the expected size of a phage genome should be eliminated prior to sequencing. Those genomes which cannot be successfully aligned

should undergo more rigorous purification methods prior to resubmission for sequencing in an attempt to remove impurities and significant incongruities in the various segments produced via sequencing.

Although Tolullah could not be aligned or annotated at this time, the Phages Database continues to expand with submissions from partner institutions. Therefore, the ability for research involving toxicity and potential medical application can still be assessed. The Phages Database provides access not only to sequences, but also the annotation of numerous phages which infect a variety of hosts. Further research of infectious capabilities and utilization of phage components may significantly speed the timeline for potential human trials, thus providing an entirely new field of medicine.

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