ISOLATION, CHARACTERIZATION AND COMPLETE GENOME SEQUENCE OF LY218: A BACTERIOPHAGE OF *Pseudomonas aeruginosa* ATCC 27853.

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ABSTRACT

It has become widely accepted that bacteriophages are extremely abundant and are enormously influential on the biosphere. Bacteriophages kill between 4-50% of the entire bacteria population daily (Suttle, 2005). The aim of this study was to screen soil samples for *Pseudomonas aeruginosa* lytic phages, select and characterize an isolate morphologically for genome analysis using next generation lllumina sequencing. The lytic isolate was detected using the spot method on lawns of *P. aeruginosa*. Using the described procedure (Jordan, et al. 2010), LY218 was successfully isolated from soil samples. Transmission electron microscopy (TEM) performed by the University of Alabama at Birmingham (UAB), High Resolution Imaging Facility (HRIF) and Illumina Next Generation Sequencing (NGS) also at UAB, were performed to determine the morphology of the phage. The TEM images reveal that this isolate has a short tail when compared to other *Myoviridae* phages. Upon performing the Blast NCBI sequence comparison analysis, it was determined that this isolate is 73083 bp and it is 97% identical to the *Pseudomonas* phage Phi176, a phage isolated and sequenced in Bath, England. Phi176, was submitted to the GenBank database with accession number KM411960. A total of 88 presumptive genes have been identified. Presumptive function of 86 proteins were called based on homology determined by Predict Protein. The 3% difference between these two phage sequences is centered on two proteins of indeterminant function. Sequencing of the LY218 genome revealed a dsDNA with 54.9% G+C content and 356 open reading frames (ORFs) were predicted to be coding sequences. The full nucleotide sequence of the genome of LY218 has been determined, and this has been deposited (the NCBI accession number in MN906996).

INTRODUCTION

After returning to France in 1915, the Franco-Canadian microbiologist Félix d'Herelle noticed some mysterious clear spots within his cultures of coccobacilli which he concluded were zones of dead bacteria (Keen, 2012) and (Fruciano, 2007). It was d'Herelle who first used the term *plaque* to describe the circular area of clearing caused by a single phage infection on bacterial colonies on double-layered agar plates (Fruciano, 2007). He also proposed the name *bacteriophages* which is derived from 'bacteria' and the Greek word 'phagein' which means 'to eat'. In Belgium in 1921, Bruynghe and Maisin reportedly published the first paper on the clinical use of phages after treating cutaneous furuncles and carbuncles in some patients by injection of staphylococcal-specific phages near the base of the cutaneous boils (Wittebole et al, 2014). These patients were reported to have noticable clinical improvement with a reduction in swelling, fever

and pain within 48 hr (Wittebole et al, 2014). Bacteriophages (phages) are a broad class of viruses that infect bacterial cells and can be defined as obligatory intracellular bacterial parasites which lack an independent metabolism. Recent studies of phages have revealed viruses as extremely diversified and ubiquitously present in the biosphere (Curtis, 2005). Accordingly, the sizes of the genome for these phages vary enormously, from a few thousand base pairs up to 234,900 bp (phage vB_AbaM_ME3) which is the largest *Acinetobacter baumanni* bacteriophage sequenced to date (Buttimer et al, 2016). Phages are a relatively untapped reservoir representing the greatest genetic diversity in the biosphere. They contribute to regulation of bacterial population growth and are involved in microbial ecology which drives many global geochemical cycles on earth (Suttle, 2005). Between 20-40% of the bacteria produced every day get killed by phages (Suttle, 2005). Phages just like other viruses consist of nucleic acids surrounded by a protein or lipoprotein coat. The nucleic acid contains ether RNA or DNA but not both; however, the common type in most phages is DNA and it can be double-stranded (ds) or single-stranded (ss). Phages are powerful agents for controlling the composition of bacterial communities. However, not all types of phages can inhibit bacterial growth and some of them may have the side effect of facilitating genetic transfer within the complex microbiomes. Gene transfer between bacteria is a process known as transduction and some of these genes may be toxic and can contribute to bacterial virulence resulting in human diseases (Novik & Savich, 2015). Bacteriophages can be differentially expressed based on environmental factors which can contribute to the survival or clearance of specific bacterial groups from a community (Fister et al, 2016). Phage phiCTX which is a temperate *Pseudomonas aeruginosa* phage has been shown to carry a cytotoxin gene that is expressed in prophage state (Krylov et al, 2013). Some phages can remain in a dormant state allowing the host bacteria to replicate their genomes in a process called the lysogenic cycle. Phages are referred to as prophages, in this state. Moreover, both temperate and virulent phages are involved in the evolution of bacteria through different kinds of transduction (general and specialized) and horizontal gene transfer (HGT), (Krylov et al, 2013). Viruses occupy a unique position in biology, representing an absolute majority of all organisms in the biosphere. With multiple different strains of phages having the ability to infect an individual bacterial species, they are believed to be globally more numerous than bacteria (Essoh et al, 2015). Recently, there has been an increased interest in the use of bacteriophages as antimicrobial agents since the specter of antibiotic resistance has become a larger problem in clinical treatment of patients (La Scola, 2003). Although antibiotics have been successful for treatment of many bacterial infections, the emergence of multi-drug resistant (MDR) bacteria and the failure of drug discovery programs over the last few decades to provide new broad-spectrum antibiotics has highlighted the need to look for alternative treatment methodologies. Many pharmaceutical companies have closed or severely reduced research into novel antimicrobial drugs due to the huge cost of research and development (Thiel, 2004).

MATERIALS AND METHODS

Collecting Samples

Samples were collected from soil surrounding Alexandria Creek, which is located in Alexandria, Calhoun County, Alabama (Latitude: 33.770473° N, Longitude: -85.882815° W, Elevation 630 ft). Ten soil samples were randomly collected in 15 mL sterile conical tubes from five different sites at the location. Field measurements including pH, temperature, depth of sample, as well as other sample descriptions were recorded. Equipment was sterilized before and after collecting samples to avoid any contamination. Samples were transported to the laboratory within an hour of collection on ice.

Bacterial Strains and Culture Conditions

This study was included an ATCC 27853 strain of *P. aeruginos*a. The bacteria were cultured on Luria-Bertani (LB) medium containing 1% tryptone, 0.5 % yeast extract and 0.5 % NaCl (pH 7.2). The bacteria were streaked and incubated at 37°C for 18 hours. All phage exposure/selection was performed using bacteria grown under these conditions.

Phage Isolation and Characterization

Using enrichment culture technique, a 1 mL aliquot of each sample was transferred to a 250 mL baffled Erlenmeyer flask. Using sterile pipettes, 40 mL of autoclaved distilled water, 5 mL of sterile 10X 7H9/glycerol broth, 5 mL AD supplement, 0.5 mL of 100 mM $CaCl_2$ and 5 mL of an 18 hours culture of *P. aeruginosa* were added to the same flask. Flasks then were incubated at 37°C in a shaking incubator at 220 rpm for 24 hours. Next day, 50 mL of each sample was centrifuged at 3,000rpm (2,000 xg) for 10 minutes to pellet. Supernatant was filter-sterilized with 0.22 µm filter and transferred into 50 mL conical tubes and stored at 4°C. Samples were diluted (10^0-10^4). Filtered sterile phage buffer was used as negative control for all samples. Plaque screening was performed using 50 µm of each sample (including controls) added to 5 mL of *P. aeruginosa* into 12 mL culture tubes. Using sterile 5 mL pipettes, a 4.5 mL top agar TA was transferred to the tubes. Immediately mixtures were transferred to LB agar plates and incubated at 37°C for 24 hours.

Electron Microscopy

A high-titre (10^9 p.f.u. ml-^{1}) lysate was sedimented for 60 min at 10,000 x g at 4^{o}C in a SORVALL RC 5B high-speed centrifuge using a SS-34 fixed-angle rotor and was washed twice under the same conditions in phage buffer. Phages were stored at 4^{o}C for 6 hours before staining to allow the pellets to completely dissolve. A 10uL of phage preparation was put onto the grid and kept for two minutes to allowed phages to be adsorbed and examined in a Thermo Fisher TECNAI T12 EM300 electron microscope at the UAB High Resolution Imaging Facility (HRIF).

Sequencing Genomic Characterization

A promega DNA Clean Up Kit was used to isolate and purify phages' genomic DNA. Randomly, 5µL of the DNA was sent for sequence and subsequent bioinformatics analysis. DNA sequencing was performed at the Hudsonalpha Genome Sequencing Center (HGSC) using the MiSeq sequencing system (Illumina, Inc.).

Annotation

The basic features of the LY218 genomic sequence were analyzed using DNAMaster 5.23.2, which was used to align the whole genome of LY218 and compare it with other genomes in the database and GeneMark for positional annotations. Putative ORFs were predicted using ORF Finder with a threshold of 75 amino acids as a minimum length of the protein. Among the 88 protein coding genes, presumptive function of 86 proteins were called based on homology determined by Predict Protein. The 3% difference between Phi176 and LY218 sequences is centered on two proteins of indeterminant function. Sequencing of the LY218 genome revealed a dsDNA with 54.87% G+C content and 356 open reading frames (ORFs) which were predicted to be coding sequences. Gene prediction was also performed using DNAMaster. Similarities with a minimum confidence level of 97%, was used with the BLAST algorithm available in NCBI.

RESULTS AND DISCUSSION

Isolation of Phages

Ten samples of soil were screened for the presence of LY218 phage. Two were positive for LY218 phage. The phage was virulent and formed clear plaques of 3-4 mm diameter on lawns of *P. aeruginosa* with LB soft agar (Fig 1). *P. aeruginosa* strain ATCC 27853 was sensitive to LY218 in both the drop titration test and with soft agar plating assay. Plaques formed within 18 hr.

Electron Microscopy

Under transmission electron microscopy, LY218 showed an isometric head of 84-85 nm between opposite apices, and a neck with a collar of 6x8 nm and a tail of 10x12 nm in extended state (Fig 2).

Genome Characterization and Sequence Analysis.

This isolate is 73083 bp and it is 97% identical to the *Pseudomonas* phage Phi176, a phage isolated and sequenced in Bath, England. A total of 88 presumptive genes have been identified. Presumptive function of 71 proteins were called based on homology determined by PredictProtein. The 3% difference between these two phage sequences is centered on two proteins of indeterminant function. Sequencing of the LY218 genome revealed a dsDNA with 54.87% G+C content and 356 open reading frames (ORFs) were predicted to be coding sequences.

This is a newly discovered double-stranded DNA (dsDNA) phage of the family *Podoviridae* of short tailed phages and the order *Caudovirales*. Phages of this genus typically have genome size of 18,000 bp to 500,000 bp. The order *Caudovirales* constitutes ~ 96% of all known phages (Fokine & Rossmann, 2014). About 94.2% of the known phages that have been used in phage therapy to treat *Pseudomonas* species have been found to belong to the *Caudovirales* order, which includes three families of dsDNA phages (Diana P. Pires, Sillankorva, & Azeredo, 2015). These three phage families differ in the physical characteristics of the phage tail: *Myoviridae*, are known to exhibit a long and contractile tail, *Podoviridae*, has a short and nonconcontractile tail, and *Siphoviridae*, a long and nonconcontractile tail (Diana P. Pires, Sillankorva, & Azeredo, 2015).

The full nucleotide sequence of the genome of LY218 has been determined, and this has been deposited (The NCBI accession number is MN906996). A BLAST analysis revealed that this isolated phage (LY218) genome had a query cover of 100%, and a highest identity of 97% with *Pseudomonas* phage Phi176. Phi176 was submitted to the International Committee on Taxonomy

of Viruses (ICTV) in May 2015. Also, it was submitted to the GenBank database with accession number KM411960, and a total linear genome length of 73,048 bp. However, it has not been annotated yet. Phi176 was first discovered in Bath, England (NCBI, n.d.). To reveal the relationship between LY218 and other members of *Podoviridae*, a phylogenetic tree was constructed using VICTOR, and LY218 was closely related to *Pseudomonas* phages Phi176, Pa2 and PhiCB2047-B (Fig 3).

A total of 88 presumptive genes have been identified. Presumptive function of 71 proteins were called based on homology determined by PredictProtein. The 3% difference between these two phage sequences is centered on two proteins of indeterminant function. In fact, there were no homologous protein structures in the NCBI protein database. Sequencing of the LY218 genome revealed a dsDNA with 54.87% G+C content and 356 open reading frames (ORFs) that were predicted to be coding sequences. The high homology between LY218 and Phi176, could raise an important question; Could it have been transported by individuals? Sea, air and land transport networks increasingly continue to expand in reach. Also, volume of passengers, speed of travel and goods carried significantly increased in the last few years. This could make pathogens and their vectors disseminate more broadly. Furthermore, immigration of birds and stratospheric movements of winds, dust and clouds overseas would be another possible way for viruses to be transmitted through the atmosphere. For instance, during the Summer, African dust storms transport across the Atlantic to the northern Caribbean and North America (Griffin, 2007).

Phages ϕIBB-SL58B and ϕIBB-PF7A, which belong to the *Podoviridae* family, have a short and non-contractile tail. Both phages have been tested on biofilms of *Pseudomonas fluorescens* and *Staphylococcus lentus* and showed efficient lysis of biofilms (Sillankorva, Neubauer, & Azeredo, 2010). This suggests that LY218, could potentially be effective on biofilms of *Pseudomonas aeruginosa* and potentially other bacterial species. Furthermore, Phi176, LUZ7, LIT1, Pa2 and KPP21 belong to the N4-like phages (Shigehisa, et al., 2016). This indicates that LY218 belongs to the N4-like phage group and it is sharing a similar gene order and possessing all 18-core gene that were found among all N4-like members (Buttimer, et al., 2018). This group of phages is highly conserved worldwide because N4-like viruses are assumed to have high adaptation level to *P. aeruginosa* as a new host bacterium (Shigehisa, et al., 2016).
Three of the currently available fifty-nine complete genome sequences of *P. aeruginosa* are closely related to ATCC 27853 strain (Huiluo Cao, Lai, Bougouffa, Xu, & Yan corresponding, 2017). Those strains can cause many human infectious diseases including burn infections, sepsis, cystic fibrosis (Forti, et al., 2018) keratitis, urinary tract infections (UTIs), as well as acute and chronic infections of human airways (Huiluo Cao, Lai, Bougouffa, Xu, & Yancorresponding, 2017). LY218 could be potentially used to design a phage cocktail that reduces *P. aeruginosa* biofilms and acute infections in humans.

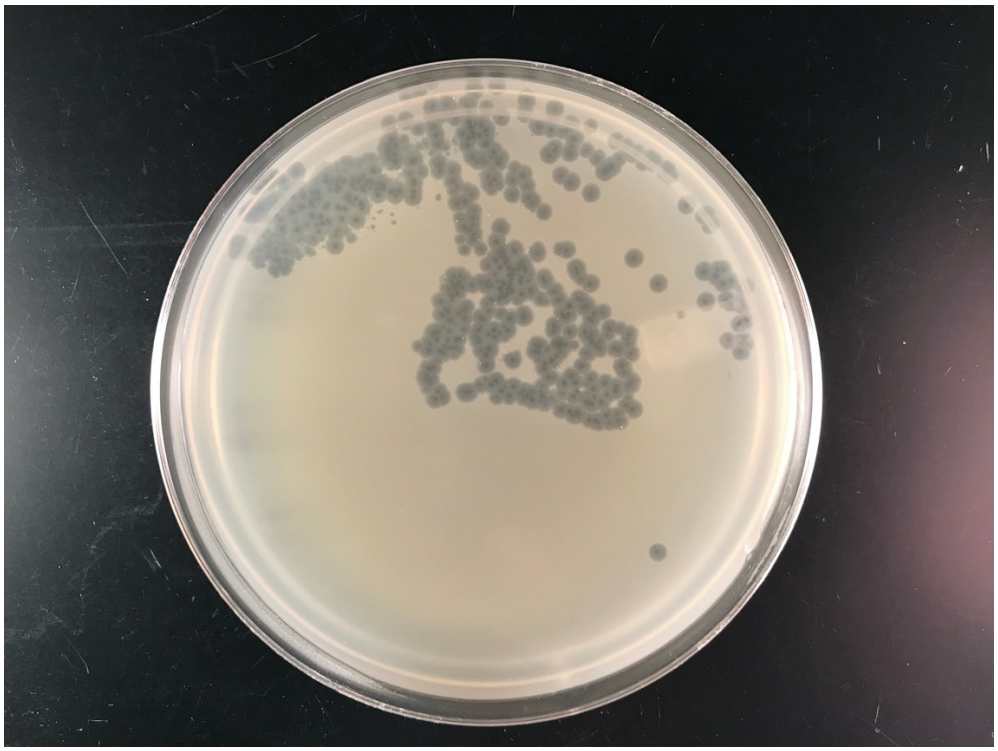

Figure 1: Bacteriophage plaques of LY218 on a Luria-Bertani (LB) agar plate.

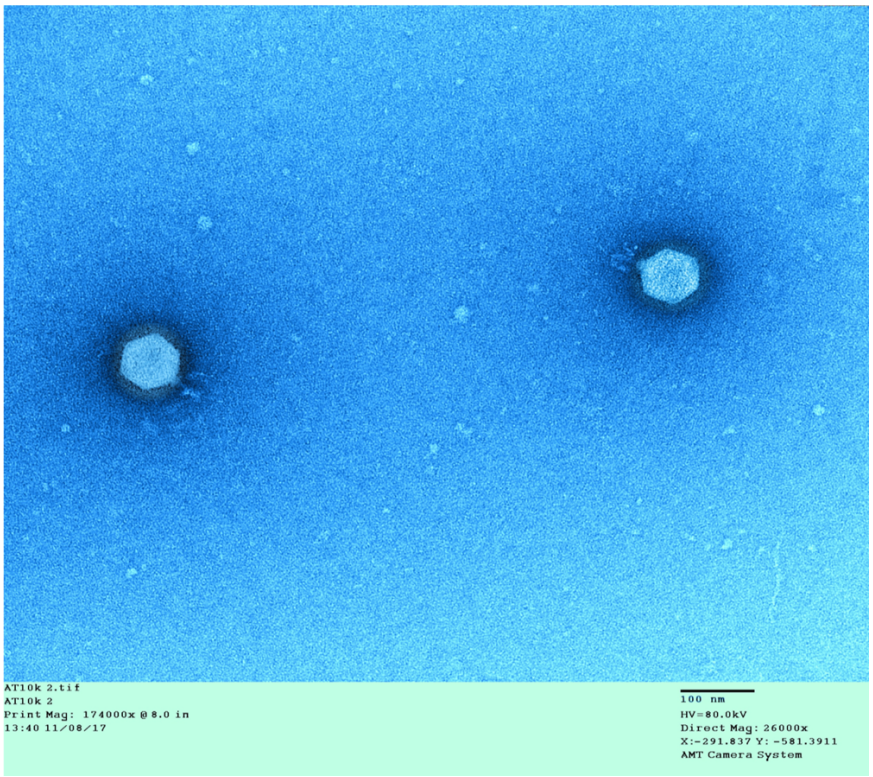

Figure 2: Electron micrograph of LY218, a bacteriophage of Pseudomonas aeruginosa ATCC 27853.

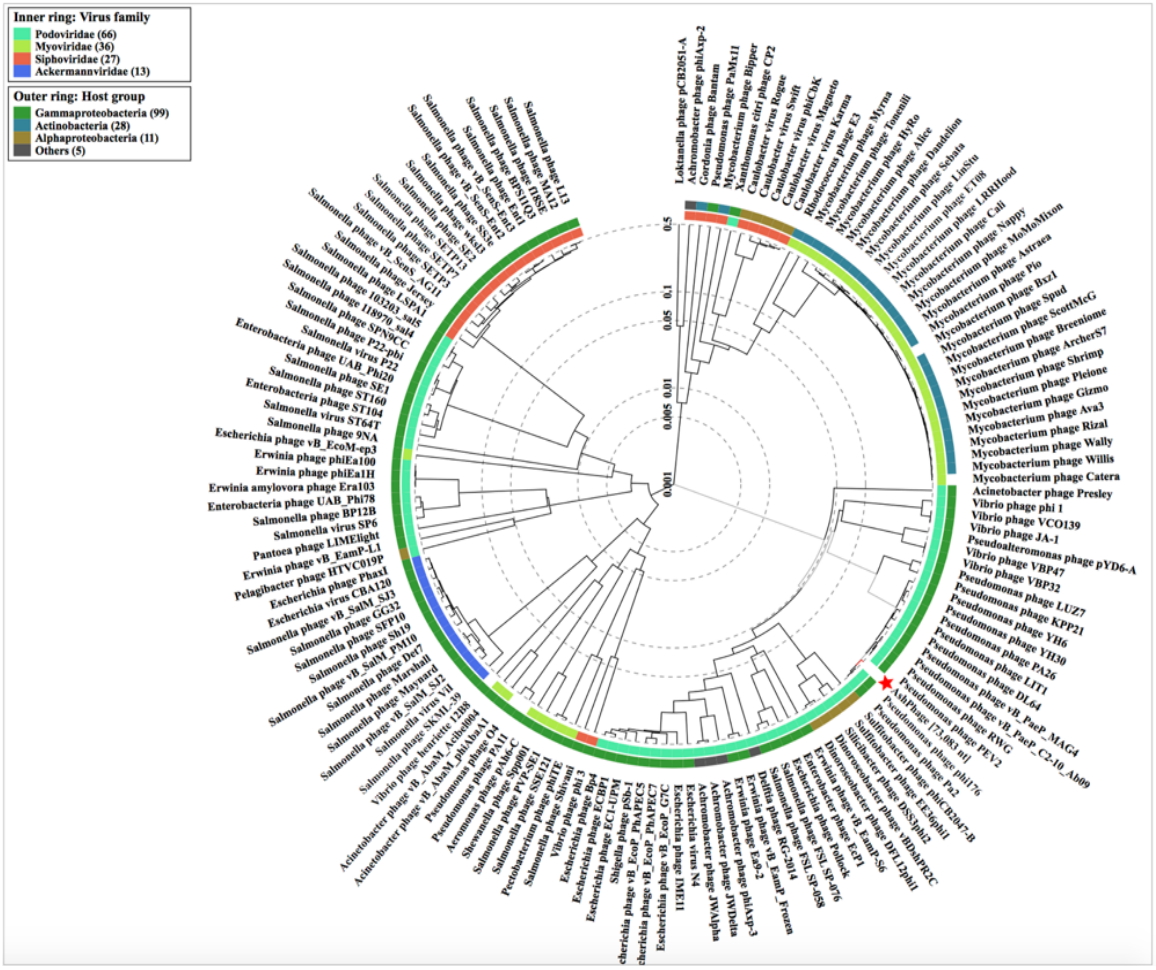

Figure 3: Dendrogram showing the phylogenetic affiliation of LY218 a bacteriophage of *Pseudomonas aeruginosa* ATCC 27853, Jan P. Meier-kolthoff and Markus Goker (2017). Red star on the dendrogram shows LY218.

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