Isolation of Four Mycobacteriophages from Oklahoma Soil and Testing Their Infectivity Against *Mycobacterium abscessus*

Ahmed K. Ali
Department of Biology, College of Science, Mustansiriyah University, Baghdad, Iraq

Hari Kotturi
Department of Biology, University of Central Oklahoma, Edmond, OK 73034

Abstract: Mycobacteriophages are phages that infect genus mycobacteria. Mycobacteriophages have potential application in phage-based diagnostic and phage therapy for identification and treatment of diseases caused by pathogenic mycobacteria. Four mycobacteriophages: Irak (IR), Fulbright (F), Ibrahim (IB), and Ahmed (A) were isolated from soil samples collected from different locations from the University of Central Oklahoma campus using *Mycobacterium smegmatis* mc^2155 as a host. The genomic DNA of the purified phages was subjected to restriction digest using BamH1, Cla1, EcoR1, HaeIII, and HindIII enzymes. The restriction digestion patterns of the four phage genomes were distinct. Fulbright and Ibrahim phage genomes have been sequenced with genome lengths of 42396 (bp) and 42596 (bp) respectively. The ability of the four viruses to infect *Mycobacterium abscessus* was determined using the spot test. Here, we also report the isolation of a mycobacteriophage Fulbright from the Oklahoma soil which can infect *Mycobacterium abscessus*. Transmission electron microscopy showed that all phages have siphoviridae morphology with isometric heads and flexible, non-contractile tails.

Introduction

Mycobacteriophages are viruses that infect the members of genus mycobacteria. *Mycobacterium smegmatis*, a soil-borne, non-pathogenic bacterium was used as a host to isolate the first mycobacteriophage (Gardner and Weiser 1947). Later, mycobacteriophages gained considerable attention due to their application in diagnosis, typing, and control of pathogenic mycobacterial species, such as *Mycobacterium tuberculosis* and *Mycobacterium leprae* (Broxmeyer et al., 2002, McNerney and Traore 2005, Hatfull 2014).

As of August 2018, 9829 mycobacteriophages were isolated using different mycobacterial species as hosts. Approximately, 1623 have been sequenced (The Actinobacteriophage Database 2018). Mycobacteriophages display a large genetic diversity (Hatfull et al., 2006). Depending upon their nucleotide sequence and gene content comparison, mycobacteriophages have been assigned to clusters (A-Z). Some clusters are divided into subclusters based on nucleotide sequence relatedness and phages without any close relatives are referred to as singletons (Hatfull 2012).

The host range of bacteriophages is generally known to be narrow. For example, phages isolated using *Vibrio parahaemolyticus* were able to infect a few strains of this species, but not other *Vibrio* species (Koga et al., 1982). The constraints that restrict the host range include host restriction-modification system, receptor-attachment compatibility, and abortive infection. However, some bacteriophages like P1 (Yarmolinsky and Sternberg 1988), Mu (Harshey 1988), SN and BHR (Jensen et al., 1998) have a broad host range and can infect hosts of other genera. The ability of mycobacteriophages to infect other mycobacterial hosts has been tested (Jacobs-Sera et al., 2012). A wide range

of mycobacteriophages has been proven to traverse the species-barrier and infect other mycobacterial hosts of clinical importance such as *M. tuberculosis*, *M. avium* (Broxmeyer et al., 2002), *M. bovis*, and *M. ulcerans* (Rybniker et al., 2006).

M. abscessus is known to be a causative agent for a wide range of skin and pulmonary infections (Moore and Frerichs 1953, Sermet-Gaudelus et al., 2003, Koh et al., 2010, Lee et al., 2015). The increased resistance of *M. abscessus* to antibiotics (Nessar et al. 2012) has prompted interest in Mycobacteriophage-based phage therapy to control infections (Broxmeyer 2004, McNerney and Traore 2005). To date, prophage Araucaria is the only mycobacteriophage isolated from *M. abscessus* subsp. *bolletii*. This prophage was detected in the cultures of specimens taken from respiratory tract infections of patients with lung disorders. The morphological and genomic characteristics of Araucaria were extensively analyzed following the retrieval of the prophage genome from *M. abscessus* subsp. *bolletii* CIP108541^T (Sassi et al. 2013). We report the isolation, purification and the characterization of four new mycobacteriophages isolated from Oklahoma soil using *M. smegmatis* mc^{2}155 and testing their infectivity against *M. abscessus.*

Methods

Phage isolation and purification

Four soil samples were collected from the University of Central Oklahoma campus. The global positioning system (GPS) coordinates of the collected soil samples that yielded four phages are as follows: IR (35.655^0 N, 97.473^0 W), F (35.658^0 N, 97.474^0 W), IB (35.656^0 N, 97.474^0 W) and A (35.655^0 N, 97.473^0 W). Soil samples were enriched as previously described (Patton and Kotturi 2018). Briefly, three grams of a soil sample were enriched in 10 ml of 7H9 broth (Dubos and Middlebrook 1947) containing 10% Albumin Dextrose Complex (ADC), 1mM $CaCl_2$ and 1 ml of overnight *M. smegmatis* mc^{2}155 culture. The enriched soil samples were incubated at 37^{0}C for 24 hours. After incubation, samples were filter sterilized using a 0.22 µ filter. To screen the filtered sample for the presence of phages, five microliters of the filtrate was plated on *M. smegmatis* mc^{2}155 lawn using an agar overlay method. The plaque forming samples were serially diluted using 10-fold dilution series in phage buffer (10 mM Tris pH 7.5, 10 mM MgSO4, 1 mM $CaCl_2$, and 68.5 mM NaCl). A ten-microliter aliquot of the samples was plated again using the agar overlay method. For phage purification, a single plaque was picked, diluted in phage buffer and plated. Three plaque purifications were performed to obtain a pure clone of each virus. The purified phages were amplified by seeding eight plates with high titer phage lysate and followed by salt precipitation with 1 M NaCl, polyethylene glycol 8,000 (10% V/V). Phage stock solution was prepared by resuspending phage in phage buffer. The phage stock solution was used for all the experiments.

Sample preparation for electron microscopy

100 ul aliquot of high titer phage lysate (5x10^9 pfu/ml) was concentrated at 4^{0}C top speed in a microcentrifuge for 20 min. The supernatant was carefully removed, and then the pellet was resuspended by 50 ul of phage buffer. The phage suspension was placed on a carbon electron microscope grid and stained with 1% uranyl acetate. Images were taken using a ZEISS 10A conventional transmission electron microscope.

Phage DNA extraction, restriction enzyme digestion, and genome sequencing

High titer phage lysate (5x10^9 pfu/ml) was treated with DNaseI and RNaseA for 30 min at 37^{0}C. The genomic DNA (gDNA) was extracted using a sodium dodecyl sulfate (SDS)/ phenol:chloroform: isoamyl alcohol (PCI) (25:24:1 V/V) extraction method as previously described (Green and Sambrook 2012). The extracted gDNA was treated with five different restriction enzymes (BamHI, ClaI, EcoRI, HaeIII, and HindIII,) as per the manufacturer's recommendation (New England Biolabs). The restriction digests were visualized by ethidium bromide-stained agarose gel using Gel Doc. An undigested DNA sample was used as a control. Genomes of two viruses Fulbright and Ibrahim were sequenced using Illumina® Next-

Generation Sequencing Technology on a MiSeq platform.

Phage infectivity against *Mycobacterium abscessus*

The isolated phages were tested against *M. abscessus* using the protocol described before (Jacob Sera et al. 2012). Briefly, 3 ul phage lysates were spotted onto fresh lawns of *M. abscessus* grown on 7H10 supplemented with 10% ADC, 1 mM $CaCl_2$, at 37^0C. The plates were inspected for plaques formation after five days.

Results and Discussion

Four new mycobacteriophages Irak (IR), Fulbright (F), Ibrahim (IB) and Ahmed (A) were isolated from four soil samples in the summer of 2017. As described above, the phages were isolated by enrichment method, in which the soil was incubated with the host, *M. smegmatis* mc^2155, culture. Phage filtrates were serially diluted and plated with the bacterial host. Upon the inspection of plaques, phages (IR, F, and A) formed relatively large plaques (2.5-4 mm); however, the plaque size of phage (IB) was less than 1 mm diameter. IR, F and A formed clear and round plaques. However, (IB) formed plaques with a clear center and fuzzy halo (Figure 1. A, D, G, and J).

The genomic DNA of four isolated phages was digested with five different restriction enzymes (BamHI, ClaI, EcoRI, HaeIII, and HindIII). These restriction enzymes recognize and cut specific nucleotide sequences in the double-stranded DNA. Each phage DNA sample produced a unique restriction pattern (Figure 1. B, E, H, and K, lanes 3, 4, 5, 6, and 7). Untreated phage DNA samples (Figure 1. Lane 2) of each virus was used as a control for comparing restriction digests. The results indicated the presence of multiple restriction sites specific to HaeIII (Figure 1. B, E, H, and K lanes 6) among all phages even though their restriction patterns were distinct. Restriction enzyme digestion is a commonly used technique for determining the genomic fingerprints of the isolated phages and often facilitates grouping isolated phages into specific clusters. Gissendanner et al., (2014) proposed a tool relying on analyzing restriction endonuclease patterns, rather than genome sequencing, to facilitate assigning discovered mycobacteriophages into possible clusters. Restriction analysis also gives an insight into the diversity even though the phages were isolated using a common host (Hatfull 2011). Two of the four phage genomes have been sequenced. The genome length of phage Fulbright is 42396 (bp) with GC content of 66.3% and belongs to cluster N of mycobacteriophages. Phage Ibrahim belongs to cluster T with a GC content of 66.1% and genome length of 42596 (bp).

Morphological characteristics of all four mycobacteriophages were examined using transmission electron microscopy (Figure 1. C, F, I, and L). The four viruses isolated in this study exhibit Siphoviridae morphotype with isometric heads and long, flexible non-contractile tails. The average tail lengths of all four phages are 225 nm. This morphology is one of the most common morphologies in mycobacteriophages with tail lengths ranging from 110 to 300 nm (Hatfull et al., 2010, Pope et al., 2011).

Testing the host range of the isolated mycobacteriophages would contribute to the development of techniques for detecting and controlling pathogenic mycobacteria such as *M. tuberculosis* and *M. avium* in clinical samples (Alcaide et al. 2003, Perkins 2000). In this study, we examined the ability of the four isolated phages (IR, F, IB, and A) to infect and lyse *M. abscessus*. Purified lysates of the phages isolated using *M. smegmatis* mc^2155 were spotted on active cultures of *M. abscessus*. Our results indicate that mycobacteriophage F can effectively infect and lyse *M. abscessus*, but the other three phages (IR, IB, and A) failed to form any plaques (Figure 2. A, C, and D). Previous studies have shown that some mycobacteriophages were able to infect different species of mycobacteria through mutations in tail fiber genes (Jacobs-Sera et al., 2012).

The specificity of the infection process is often restricted by different mechanisms like restriction-modification system of the host, receptor-attachment site interaction (Hyman and

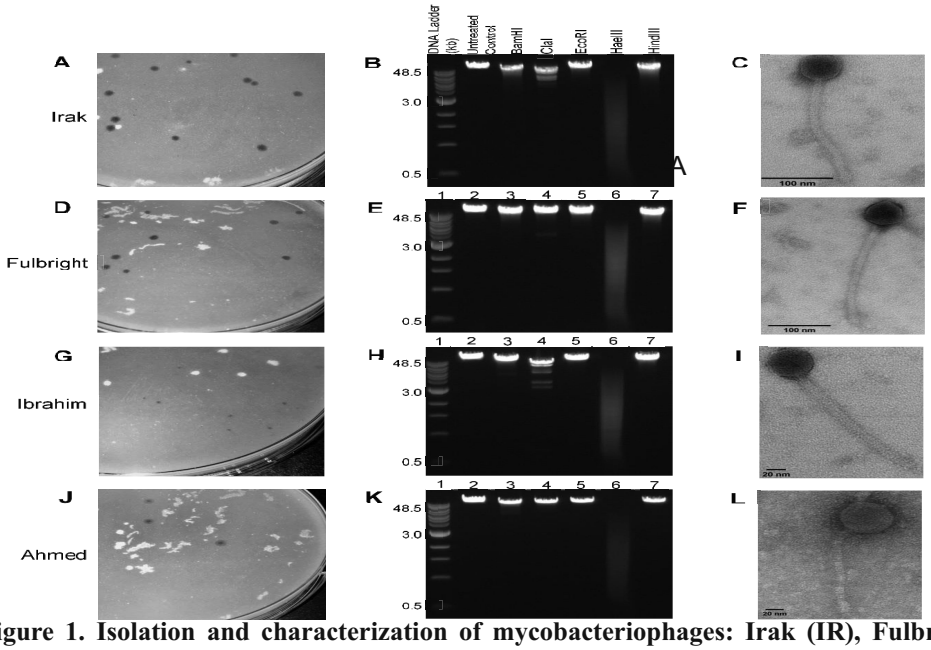

Figure 1. Isolation and characterization of mycobacteriophages: Irak (IR), Fulbright (F), Ibrahim (IB), and Ahmed (A). Panel A, E, D and J represent plaque morphology on *M. smegmatis* mc²155 lawns. Panel B, E, H, and K represents restriction digest patterns of the four phages genomic DNA with five restriction enzymes (BamHI (lane3), ClaI (lane4), EcoRI (lane5), HaeIII (lane6), and HindIII (lane7); Lane 1 is a 1-kb size marker; Lane 2 contains undigested phage genomic DNA. Panel C, F, I and L represent transmission electron microscope images of the four phages with a Siphoviridae morphotype.

Abedon, 2010), and abortive infection (Fineran et al. 2009). Although bacteriophage infection process is specific and its underlying mechanism is poorly understood, testing the host range of newly-isolated phages is a worthy task.

In this work, we report the isolation of characterization of four mycobacteriophages from Oklahoma soil. The genome of two of the four viruses has been sequenced. One of the isolated phages has a broad host range infecting *M. smegmatis* and *M. abscessus*. In our future work, we will further investigate the host range of Fulbright and would explore the possibility of using this phage in phage therapy.

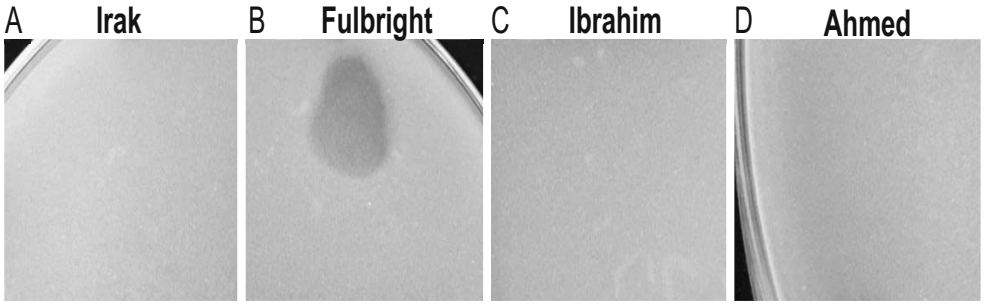

Figure 2. Host range of the four mycobacteriophages against *M. abscessus*. (A, B, C, and D) Irak (IR), Fulbright (F), Ibrahim (IB), and Ahmed (A) phage lysates spotted on *M. abscessus* lawns. (B) Mycobacteriophage Fulbright infects *M. abscessus*.

Acknowledgment

We would like to thank Dr. Graham Hatfull at the University of Pittsburg for sharing *M. smegmatis* mc^{2}155. *M. abscessus*, strain MC1518 was obtained from BEI Resources, NIAID, NIH. We would like to thank Mr. Ben Fowler at the Imaging core facility at Oklahoma Medical Research Foundation for his help with electron microscopy. Funding for this project was acquired through the University of Central Oklahoma, Office of Research and Sponsored Programs – RCSA Grant program and by the National Institute of General Medical Sciences of the National Institutes of Health under award number P20GM103447. The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institutes of Health.

References

Alcaide F, Gali N, Dominguez J, Berlanga P, Blanco S, Orus P and Martin R. 2003. Usefulness of a new mycobacteriophage-based technique for rapid diagnosis of pulmonary tuberculosis. J. Clin. Microbiol. 41: 2867-2871.

Broxmeyer L, Sosnowska EM, Chaco´n O, Wagner D, McGarvey J, Barletta RG, Bermudez LE. 2002. Killing of *Mycobacterium avium* and *Mycobacterium tuberculosis* by a mycobacteriophage delivered by a nonvirulent Mycobacterium: A model for phage therapy of intracellular bacterial pathogens. J. Infect. Dis. 186: 1155-1160.

Broxmeyer L. 2004. Bacteriophages: antibacterials with a future?. Med. Hypotheses. 62: 889-893.

Dubos RJ, and Middlebrook G. 1947. Media for tubercle bacilli. Am. Rev. Tuberc. 56:334-345.

Fineran PC, Blower TR, Foulds IJ, Humphreys DP, Lilley KS, Salmond GP. 2009. The phage abortive infection system, ToxIN, functions as a protein-RNA toxin-antitoxin pair. Proc Natl Acad Sci U S A. 106:894–899.

Gardner GM, and Weiser RS. 1947. A bacteriophage for *Mycobacterium smegmatis*. Proc. Soc. Exp. Biol. Med. 66**:**205-206.

Gissendanner CR, Wiedemeier AMD, Wiedemeier PD, Minton RL, Bhuiyan S, Harmson JS, and Findley AM. 2014. A web-based restriction endonuclease tool for mycobacteriophage cluster prediction. J. Basic Microbiol. 54: 1-6.

Green MR, and Sambrook J. 2012. Molecular Cloning: A Laboratory Manual. Cold Spring Harbor, New York, NY: Cold Spring Harbor Laboratory Press.

Harshey RM. 1988. Phage Mu. In: Callendar R, ed. The bacteriophages. New York, NY: Plenum Press. 193-234.

Hatfull, GF, Hendrix, RW. 2011. Bacteriophages and their genomes. Curr. Opin. Virol. 1: 298-303.

Hatfull GF, Jacobs-Sera D, Lawrence JG, Pope WH, Russell DA, Ko CC, Weber RJ, Patel MC, Germane KL, Edgar RH, Hoyte NN, Bowman CA, Tantoco AT, Paladin EC, Myers MS, Smith AL, Grace MS, Pham TT, O'Brien MB, Vogelsberger AM, Hryckowian AJ, Wynalek JL, Donis-Keller H, Bogel MW, Peebles CL, Cresawn SG, Hendrix RW. 2010. Comparative genomic analysis of sixty mycobacteriophage genomes: Genome clustering, gene acquisition and gene size. J. Mol. Biol. 397(1): 119–143.

Hatfull GF, Pedulla ML, Jacobs-Sera D, Cichon PM, Foley A, Ford ME, Gonda RM, Houtz JM, Hryckowian AJ, Kelchner VA, Namburi S, Pajcini KV, et al. 2006. Exploring the mycobacteriophage metaproteome: Phage genomics as an educational platform. PLoS Genet. 2(6):e92.

Hatfull GF. 2012. The Secret Lives of Mycobacteriophages. In: Lobocka M, and Szybalski WT, eds. Advances in virus research. Vol. 82, Burlington: Academic Press. 179-288.

Hatfull GF. 2014. Mycobacteriophages: Windows into tuberculosis. PLoS Pathog. 10(3): e1003953.

Hyman P, Abedon ST. 2010. Bacteriophage host range and bacterial resistance. Adv. Appl. Microbiol. 70:217–248.

Jacobs-Sera D, Marinelli L, Bowman C, Broussard G, Guerrero C, Boyle M, Petrova Z, Dedrick R, Pope W, Science Education Alliance Phage Hunters Advancing Genomics and Evolutionary Science (SEAPHAGES) program, Modlin RL, Hendrix RW, Hatfull1 GF. 2012. On the nature of mycobacteriophage diversity and host preference. Virol. 434 (2): 187-201.

Jensen EC, Schrader HS, Rieland B, Thompson TL, Lee KW, Nickerson KW, Kokjohn TA. 1998. Prevalence of broad host range lytic bacteriophages of *Sphaerotilus natans*, *Escherichia coli*, and *Pseudomonas aeruginosa*. Appl. Environ. Microbiol. 64 (2): 575-580.

Koga T, Toyoshima S, Kawata T. 1982. Morphological varieties and host ranges of *Vibrio parahaemolyticus* bacteriophages isolated from seawater. Appl. Environ. Microbiol. 44 (2): 466-470.

Lee MR, Sheng WH, Hung CC, Yu CJ, Lee LN, Hsueh PR. 2015. *Mycobacterium abscessus* complex infections in humans. Emerg. Infect. Dis. 21 (9): 1638-1646.

McNerney R, and Traore´ H. 2005. Mycobacteriophage and their application to disease control. J. Appl. Microbiol. 99: 223-233.

Moore M, Frerichs JB. 1953. An unusual acid-fast infection of the knee with subcutaneous, abscess-like lesions of the gluteal region. J. Investig. Dermatol. 20 (2): 133-169.

Nessar R, Cambau E, Reyrat JM, Murray A, Gicquel B. 2012. *Mycobacterium abscessus*: a new antibiotic nightmare. J. Antimicrob. Chemother. 67: 810–818.

Patton CJ, Kotturi H. 2018. Genomic sequence Mycobacteriophage OKCentral 2016. Genome Announc. 6(8): e01208-17.

Perkins, MD. 2000. New diagnostic tools for tuberculosis. Int. J. Tuberc. Lung Dis. 4:S182–S188.

Pope WH, Jacobs-Sera D, Russell DA, Peebles CL, Al-Atrache Z, Alcoser TA, Alexander LM, Alfano MB, Alford ST, et al. 2011. Expanding the diversity of mycobacteriophages: Insights into genome architecture and evolution. PLoS One 6 (1): e16329.

Rybniker J, Kramme S, Small PL. 2006. Host range of 14 mycobacteriophages in *Mycobacterium ulcerans* and seven other mycobacteria including *Mycobacterium tuberculosis* – application for identification and susceptibility testing. J. Med. Microbiol. 55: 37-42.

Sassi M, Bebeacua C, Drancourt M, Cambillaua C. 2013. The First Structure of a Mycobacteriophage, the *Mycobacterium abscessus* subsp. *bolletii* Phage Araucaria. J. Virol. 87 (14): 8099-8109.

Sermet-Gaudelus I, Bourgeois ML, Pierre-Audigier C, Offredo C, Guillemot D, Halley S, Akoua-Koffi C, Vincent V, Sivadon-Tardy V, Ferroni A, Berche P, Scheinmann P, Lenoir G, Gaillard JL. 2003. *Mycobacterium abscessus* and Children with Cystic Fibrosis. Emerg. Infect. Dis. 9 (12): 1587-1591.

The Actinobacteriophage Database. 2018. Host Genus: *Mycobacterium* [online]. Available from: http://phagesdb.org. (Accessed August 7, 2018).

Yarmolinsky RM, and Sternberg N. 1988. Bacteriophage P1. In: Callendar R, ed. The bacteriophages. New York, NY: Plenum Press. 291-438.

Submitted August 15, 2018 Accepted December 3, 2018