Characterization of Mycobacteriophage Fulbright Isolated from Oklahoma Soil

Charmaine Lopez-Davis
Department of Biology, University of Central Oklahoma, Edmond, OK 73034

Cameron Kedy
Department of Biology, University of Central Oklahoma, Edmond, OK 73034

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: Increasingly unsuccessful antibiotic remedies against emerging drug resistant bacteria make research on alternative treatments paramount. In this project, we characterized the mycobacteriophage Fulbright, evaluated the phage's efficacy against *M. smegmatis* and *M. abscessus*, and explored the possibility of using it for phage therapy applications. We found that Fulbright is stable at pH range 4-9 and temperatures ranging 20-60°C. We observed a 90-minute latent period and a lytic burst plateau 3 h after adsorption. We observed that Fulbright can infect *M. abscessus* at a concentration of $1x10^9$ PFU/mL and higher, but loses efficacy at concentrations lower than $1x10^9$ PFU/mL. In an effort to demonstrate the feasibility of using mycobacteriophage Fulbright in phage therapy applications, we electrospun Fulbright with polycaprolactone (PCL) nanofiber to serve as a model wound dressing and observed PCL_Fulbright successfully infecting *M. smegmatis.*

Introduction

The genus *Mycobacterium* is part of the order Actinomycetales and the phylum Actinobacteria and is composed of bacteria that are aerobic, acid-fast, rod-shaped, and non-spore forming. Some species have evolved into potential human pathogens, presumably due to genomic events such as genome reduction, critical gene acquisition, gene transfer, mutations, and recombination (Singh et al. 2011; Röltgen et al. 2012; Prasanna et al. 2013; Bottai et al. 2014; Franco-Paredes et al. 2019). Mycobacterial species are typically present in water and soil that humans encounter (Stinear et al. 2007; Falkinham 2009; Franco-Paredes et al. 2019), and mycobacterial infections predominantly occur by entering through open skin and mucosal barriers, leading to cutaneous or pulmonary infections, respectively (Adjemian et al. 2012; Wu & Holland 2015; Szymanski et al. 2015; Ryu et al. 2016).

Nontuberculosis Mycobacteria (NTM) are divided into two distinct groups: slow-growing mycobacteria (SGM) and rapid-growing mycobacteria (RGM) (Kim et al. 2013). *M. smegmatis* is a model organism that allows scientists to study the genus *Mycobacterium*. Using *M. smegmatis* is advantageous because it is not pathogenic and is an RGM (Gordon & Smith 1953; Beltan et al. 2000); additionally, it has been observed to be a part of the normal flora

in human sebaceous gland secretions (Brooks et al. 2010). The strain *M. smegmatis* mc^{2}155 is one of the most studied strains of *M. smegmatis* that was derived from the carbenicillin resistant parent strain, mc^{2}6 (Snapper et al. 1990).

Nontuberculosis Mycobacteria (NTM) cutaneous infections occur via direct inoculation through skin barrier damage (Griffith et al. 2007; Wang and Pancholi 2014; Forbes et al. 2018). Treatment of NTM *M. abscessus* infections typically include antibiotics such as azithromycin and imipenem (Marion et al. 2014); however, a recent case of *M. abscessus* infection on a 15-year-old patient with cystic fibrosis showed that the bacteria were not responsive to the antibiotic regimen given. Researchers genetically engineered a cocktail of phages that were collected by students in the Science Education Alliance Phage Hunters Advancing Genomics and Evolutionary Science (SEA-PHAGES) program that were observed to infect *M. abscessus* in vitro. Intravenous administration of the cocktail was given over several months and patient sera did not show evidence of phage neutralization and weak cytokine responses (IFNγ, IL-6, IL-10, TNF α) were observed (Dedrick et al. 2019). Phage Fulbright discussed in this manuscript was also found under the SEA-PHAGES program and has the potential to be used in therapy against *M. abscessus* as described above.

As bacteria continue to develop resistance to antibiotics, there is a growing need for alternative treatments. Bacteriophage therapy presents as a promising alternative; however, the very nature of bacteriophages is still poorly understood. Mycobacteriophages are phages (viruses) that specifically infect mycobacteria. Because mycobacteriophages can act as bactericidal agents, they could be used directly to destroy bacterial hosts, or phage-encoded products such as lysins could be used against pathogens (Samaddar et al. 2016). Phages can also inhibit the metabolism of their hosts (host inactivation) (Miller et al. 2003; Samaddar et al. 2016), and deeper mechanistic understanding could lead to another potential therapeutic strategy.

To determine suitable phages for phage therapy applications, isolation, identification, and full characterization of the phage, while using reliable and reproducible methods, is necessary (Montso et al. 2019). Extreme temperatures can denature the protein capsid shell of phages; for example, experiments on a phage infecting *B. thailandensis* showed that the phage undergoes a lytic cycle at higher temperatures (37°C) and remains temperate at lower temperatures (25°C) (Shan et al. 2014). Extreme pH values cause hydrogen and hydroxyl ions to be highly concentrated in the water, resulting in viral inactivation (Feng et al 2003). Because highly reactive radicals in aqueous environments (such as hydroxyl and superoxyl ions) have a long lifespan, they oxidize materials in the environment and can affect phage capsid shell by removal, deformation, or denaturation of critical ligand sites and overall dissociation of the capsid. Protein shell degradation could cause RNA hydrolysis inside or outside of the phage particle, resulting in the loss of infectivity (Feng et al. 2003)

In this study, we characterized a previously isolated and sequenced mycobacteriophage Fulbright (Kotturi et al. 2021) by testing its stability in various pH and temperature conditions. We observed phage-host interaction using a one-step growth curve assay and evaluated its ability to infect *M. smegmatis* and *M. abscessus.* Lastly, we incorporated phage Fulbright into polycaprolactone (PCL_ Fulbright) to serve as a potential antibacterial wound dressing and tested its efficacy against *M. smegmatis* and *M. abscessus* bacterial lawns.

Materials and Methods

Culture medium and bacterial culture preparation

Mycobacterium smegmatis mc^{2}155 provided by the Hatfull lab at the University of Pittsburgh (Pittsburgh, PA, USA) was grown in the standard 7H9 liquid medium complete (7H9 broth base, 0.2% glycerol, albumin dextrose catalase (ADC) (10%V/V), 1 mM calcium chloride ($CaCl_2$)) and was incubated in a shaking incubator (Fisher Scientific # SHKE4450). On solid media, the

bacteria were grown on the standard 7H10 agar plates (0.5% glycerol, 0.2% dextrose, and 1 mM calcium chloride ($CaCl_2$)). Cultures grown were incubated at 37 °C. 50 µg/mL of cycloheximide and 50 µg/mL of carbenicillin were added to the liquid culture medium and 7H10 agar plates to reduce contamination. 2X Middlebrook top agar (7H9 broth base, 0.8% agar) was diluted at a 1:1 ratio with 7H9 liquid medium neat (7H9 broth base, 0.2% glycerol, 1 mM calcium chloride ($CaCl_2$)) to make 1X Middlebrook top agar, which was used to plate the bacterial lawn. The phage lysate was diluted in phage buffer (pH 7.2, 10 mM Tris, 10 mM magnesium sulfate ($MgSO_4$), 70 mM sodium chloride (NaCl), and 1 mM $CaCl_2$) to quantify the phage. For the agar-overlay method, 10 µL of each dilution was added to 250 µL of *M. smegmatis* mc^{2}155 bacteria and incubated for 10 min. After incubation, 4.5 mL of 1X Middlebrook top agar was added to the bacteria and phage mixture and transferred to a 7H10 agar plate. The plates were incubated at 37°C and assessed for plaques. *Mycobacterium abscessus* ATCC 19977 was grown in similar conditions as *M. smegmatis* but without cycloheximide and carbenicillin. Middlebrook 7H10 Oleic Albumin Dextrose Catalase (OADC) supplementation (cat #MBS652952) was used instead of calcium chloride, dextrose, and glycerol to improve bacterial growth.

Phage Fulbright stability in pH conditions

The stability of the mycobacteriophage was evaluated by incubating the phage (initial concentration of 1×10^{11} PFU/mL) in phage buffer adjusted to a range of pH values (2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12) at room temperature for 1 h following the protocol described in previous publications (Kotturi & Lopez-Davis et al. 2022; Patton 2019). The pH of the phage buffer was adjusted using HCl or NaOH and was 0.22 µm filter-sterilized. Phage was incubated at its respective pH value for 1 h at room temperature. Following the 1 h incubation, the phage solution was serially diluted, plated using the agar-overlay method, and incubated at 37 °C prior to assessment of plaques.

Phage Fulbright stability at various temperature conditions

The stability of phage Fulbright was evaluated at different temperatures (20°C, 30°C, 40°C, 50°C, 60°C, 70°C, 80°C), following the protocol described in previous works (Kotturi & Lopez-Davis et al. 2022; Patton 2019). 1 mL volume of the phage lysate (1×10^{11} PFU/mL) was dispensed onto a 1.5 mL tube and incubated at the temperature conditions for 1 h. Following thermal incubation, lysates were serially diluted in sterile phage buffer and plated using the standard agar-overlay method. After incubation, the number of PFU/mL was determined and plotted. The PFU/mL was measured at 60 minutes of incubation.

One-step growth curve of Fulbright

The one step growth curve was done at a multiplicity of infection (MOI) of 1.0 (one bacterial cell to one bacteriophage) following a protocol from previous work with mycobacteriophages (Patton 2019). The host bacterium and phage were incubated at 37ºC for 50 minutes to allow for complete phage adsorption. Following incubation, 0.4% sulfuric acid (H_2SO_4) was added to inactivate unattached phage particles, and the solution was incubated again for 5 minutes at room temperature. The H_2SO_4 was neutralized by adding 0.4% sodium hydroxide (NaOH). The bacteriophage suspension was diluted in 7H9 broth and incubated at 37 ºC. Every 30 minutes, for a duration of 8 hours, the sample solution was diluted and plated using the double agar layer method. Following incubation, the plaques were counted to determine PFU/mL and plotted.

Determining infectivity of Fulbright

Bacterial lawns were plated on standard 7H10 agar plates by mixing 250 uL of bacteria with 4.5 mL of standard 7H9 Middlebrook 1X top agar. *M. abscessus* ATCC 19977 was plated on standard 7H10 agar supplemented with OADC, while *M. smegmatis* mc^{2}155 was supplemented with 0.5% glycerol, 0.2% dextrose, and 1 mM calcium chloride ($CaCl_2$). 5 uL of serially diluted Fulbright lysate was spotted onto bacterial lawn. Plates were incubated right side up overnight to allow spots to absorb and were flipped upside-

down the next day. *M. smegmatis* plates were incubated for 2 days, while *M. abscessus* plates were incubated up to 6 days.

Comparing efficacy of PCL_Fulbright against host bacteria *M. smegmatis* and *M. abscessus*

Phage Fulbright was incorporated into polycaprolactone (PCL) fiber using methods described in our previous work (Kotturi & Lopez-Davis et al. 2022). After electrospinning, ~2cm^2 of the phage incorporated fiber was cut and plated on agar plates containing host bacterial lawn, *M. smegmatis* or *M. abscessus.* The plates were incubated at 37°C for 48 hours and assessed for clear borders surrounding the fiber.

Data analysis

All experiments were performed in triplicates unless otherwise noted. GraphPad prism was used to calculate one-way ANOVA and Tukey post hoc tests and establish multiple comparisons between samples. A p-value below 0.05 was considered statistically significant.

Results and Discussion

Our results showed that mycobacteriophage Fulbright is stable for up to 60 minutes when exposed to temperatures 20-60°C, and the phage particle is inactivated at 70°C and above (Figure 1). In contrast, other novel mycobacteriophages described in Stella et al. (2013) were isolated at 30°C and were unable to propagate at a higher temperature of 37°C. Phage Cepens was found to be unstable at temperatures 37°C and higher (Cantrell 2019). Our results are comparable to bacteriophages against *Vibrio parahaemolyticus*, which retained infectivity between 25-50°C (Yin et al. 2019).

pH stability assays showed that Fulbright is stable in pH 4-9, with a ten-fold decrease at pH 3. There was a significant difference in phage titer between pH 3 and 4 ($p<0.0001$). No viable plaques were observed at pH 2, 10, 11, and 12 (Figure 2). *Vibrio* phages studied by Yin and others (2019) were found to retain activity at a broad range of pH 2-12. In contrast, mycobacteriophage Cepens showed stability in a narrow pH range of 7-9 (Cantrell 2019).

Because Fulbright can maintain stability in a wide range of temperature and pH conditions, it serves as an excellent candidate for phage therapy. The human body at homeostasis is 37°C, and upon infection and/or sepsis, the body temperature typically climbs to 38°C and higher. Thus, Fulbright can endure these higher temperatures if used for therapy. The human saliva has a typical pH range of 6.2-7.6 (Baliga

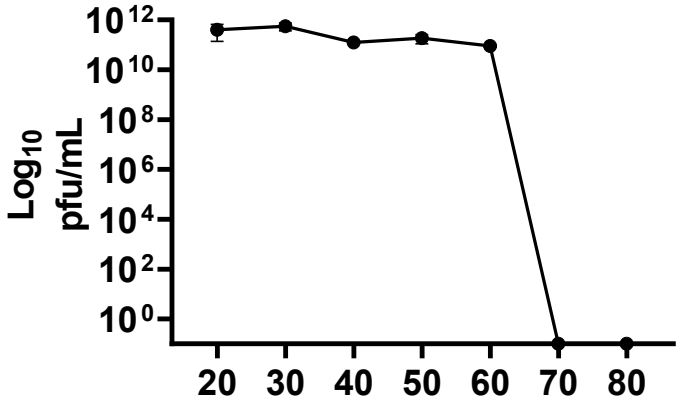

Figure 1. Temperature stability of phage Fulbright. Error bars represent standard error of the mean (SEM).

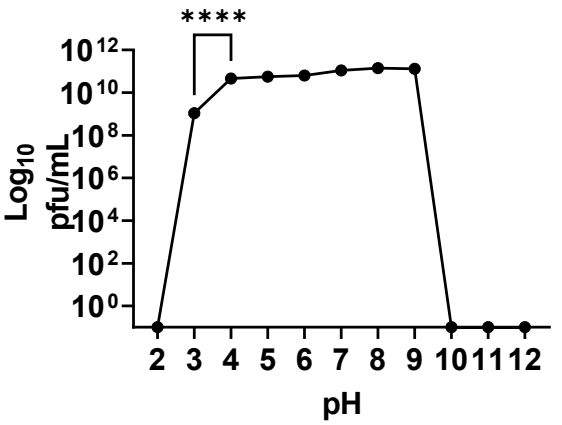

Figure 2. pH stability of phage Fulbright. ****p<0.0001.

et al. 2013). The human blood has a normal pH range of 7.35-7.45 (Hopkins et al. 2021), and values greater than 7.8 (alkalemia) or less than 6.8 (acidemia) often result in death. Lastly, open wounds are characterized to have a neutral to alkaline pH of around 6.5 to 8.5, while chronic wounds exist at a range of 7.2 to 8.932 (Bennison et al. 2017). Fulbright is stable from pH 4-9 and can thus be used for oral, intravenous and cutaneous applications. Ingestion of the phage would not be favorable as Fulbright begins to destabilize at a pH of 3 and lower which are optimal pH conditions for human stomach (Fujimori 2020).

Because a good understanding of a phage's latent period is needed to determine its potential use for phage therapy, we constructed a one-step growth curve for phage Fulbright. We found the latent period of phage Fulbright to be 90 min, followed by a rise period of 90 min (Figure 3). The PFUs plateaued after the rise period, approximately 3 h after adsorption. These results are similar to previous work with mycobacteriophages (Patton 2019). Kalapala et al. (2020) tested the infectivity of mycobacteriophages at various MOIs (10, 1, 0.1, and 0.01), and found that MOI of 10 and 1 both significantly reduced bacterial growth 3 h post treatment. Bavda and Jain (2020) observed a latent period of 60 minutes for mycobacteriophage D29; moreover, when they generated a D29 holin knockout phage, the latent period increased to 90 minutes. Studies by Fan et al. (2016) showed mycobacteriophage SWU1 to have a latent period of 30 minutes and a burst time of 270 minutes. BO1 and BO2a phages had latent periods of 150 min and 260 min, respectively (Kraiss et al. 1973). Samaddar used a different approach to observe mycobacteriophage-mycobacterial host interaction and used flow cytometry to measure bacterial cell viability and observed similar results, with a ~60 min latent period (Samaddar et al. 2016).

To determine whether Fulbright is a good candidate for phage therapy applications for treating human mycobacterial infections, we also tested the infectivity of Fulbright against human pathogen *M. abscessus.* Results showed that at high concentrations, Fulbright can effectively lyse *M. abscessus.* Figure 4b shows Fulbright is effective up to 10^{-2} dilution in lysing the host cell. These results are comparable to preliminary work done with Fulbright (Ali 2019). This limited infectivity against *M. abscessus* has also been observed by the Hatfull lab, where other phages (Muddy, ZoeJ, and BPs) were first isolated using host *M. smegmatis* (The Actinobacteriophage Database). The three phages were genetically modified and engineered to improve infectivity against *M. abscessus* subsp. *massiliense* and were then intravenously administered to a cystic fibrosis patient, making it the first therapeutic use of phages for a human mycobacterial infection. (Dedrick et al. 2019).

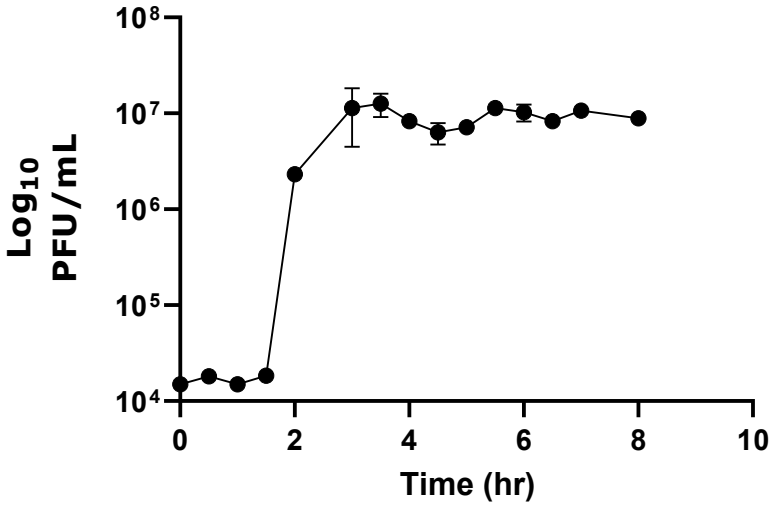

Figure 3. One-step growth curve of phage Fulbright. Error bars represent SEM.

In our previously published work with Fulbright (Kotturi and Lopez-Davis 2022) we successfully incorporated our phage into a PCL wound dressing. Here, we tested phage incorporated fiber against *M. smegmatis* and *M. abscessus.* Our results showed PCL_Fulbright to be effective against *M. smegmatis* (Figure 5a), but not *M. abscessus* (Figure 5b). A possible reason for this is the low-concentration and slow release of phage Fulbright from the nanofiber. We previously observed that ~2cm^2 of PCL_Fulbright released +/−2.1 × 10^5 PFU/mL infectious particles after 1 h incubation in 1 mL of phage buffer. Another reason that PCL_Fulbright was able to infect *M. smegmatis* but not *M. abscessus* could be due to the properties of mycolic acid present. Mycolic acids are a major component of mycobacterial cell walls (Sethiya et al. 2020; Kurosu 2019; Marrakchi et al. 2014; Takayama et al. 2005), and despite providing similar functions in all mycobacterial pathogens (cell protection, virulence, structural

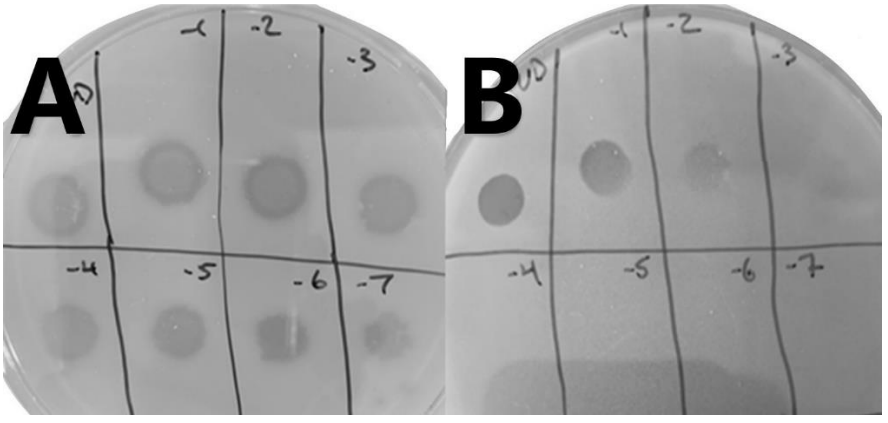

Figure 4. Testing infectivity of phage Fulbright. Fig 4a shows spot test results at ten-fold dilutions against *M. smegmatis;* Fig 4b shows that Fulbright can lyse *M. abscessus* at high titer.

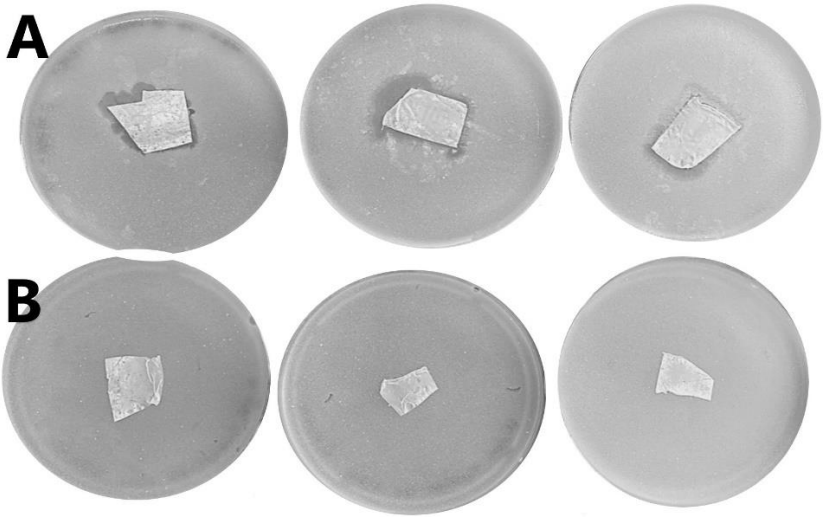

Figure 5. Testing the efficacy of phage Fulbright incorporated PCL against *M. smegmatis* bacterial lawn (Fig 5a) and against *M. abscessus* (Fig 5b). Clear lysis zones surrounding the edges of the fiber on Figure 5a shows that the fiber is effective in lysing *M. smegmatis*; No clear zone is observed in Fig 5b.

integrity), subtle differences in the carbon chain length are found that may contribute to their susceptibility to phage infections (Sethiya et al. 2020). Furthermore, we hope to optimize the efficacy of the phage fiber by improving our electrospinning techniques via increasing porosity and hydrophilicity, or by incorporating other materials such as collagen and hydrogel in order to increase the phage release and effectiveness while promoting optimal conditions for appropriate wound healing.

Bacteriophages have the potential for therapeutic and food safety uses due to their ability to lyse multi-drug resistant pathogens (Montso et al. 2019). They are easy to manufacture and have no documented negative side effects to date. Our research lays a good foundation for continuing research and possible application of Fulbright in the future.

Acknowledgments

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.

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Submitted June 20, 2022 Accepted December 1, 2022