Investigating the Growth Characteristics and Infectivity of a Newly Isolated Bacteriophage Against *Mycobacterium smegmatis* mc^2 155

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Abstract: Bacteriophages or phages are specific viruses that are capable of infecting bacteria without harming eukaryotic cells. Mycobacteriophages are a specific type of phage that only infects bacteria in the genus *Mycobacterium*. Mycobacteriophages have been studied for their potential in killing virulent mycobacteria, such as the etiological agents of tuberculosis and leprosy. In this study, we used *Mycobacterium smegmatis* mc^2 155 to isolate a mycobacteriophage from Oklahoma [USA] soil. Mycobacteriophage OKCentral2016 was isolated from soil enrichment obtained at the University of Central Oklahoma. This phage produced transparent plaques and has a morphology consistent with the *Siphoviridae* morphotype. The phage remained stable at temperatures below 55°C and within the pH range of 6-8. The viral replication cycle took approximately 4 hours to complete under standard growing conditions. This phage only infected *M. smegmatis* and decreased biofilm formation in planktonic cultures.

Introduction

The genus *Mycobacterium* is composed of acid-fast, aerobic bacteria that are ubiquitous in soil and aquatic environments (Norby et al., 2007). Two of the more medically significant mycobacterial species are *M. tuberculosis* and *M. leprae*, which are the causative agents of tuberculosis and leprosy, respectively. The most common non-tuberculosis mycobacteria (NTM) agents that are medically relevant are: *M. avium* complex (*M. avium* and *M. intracellulare*), *M. kansasii*, *M. simiae*, and *M. abscessus*. These bacterial pathogens cause pulmonary disease, lymphadenitis, and soft tissue infections (Johnson and Odell, 2014). There has also been an increase in NTM infections in patients with cystic fibrosis (Leung and Olivier, 2013).

Biofilm formation is one of the key survival strategies used by bacteria to protect them from unfavorable conditions (Kiefer and Dahl, 2015). Biofilms are formed when bacteria aggregate together on a solid surface and become encased in a secreted matrix of an extracellular polymeric substance (EPS) (Harper et al., 2014). Bacteria encased in biofilms have increased resistance to disinfectants and antibiotics (Carter et al., 2003; Steed and Falkinham, 2006). This has become a problem in hospital settings due to the buildup of biofilms on medical equipment; biofilms have contributed to the rise of post-surgical nosocomial infections and chronic infections (Phillips and Reyn, 2001). The National Institute of Health estimates that 80% of all bacterial infections are associated with biofilms (Harro et al., 2010), and biofilm formation of *M. abscessus* has been linked to multiple cases of chronic pulmonary infections (Qvist et al., 2015).

Bacteriophages or phages are viruses that only infect bacteria, and regulate bacterial populations in the environment (Atterbury et al., 2005). Phages are considered the most abundant

and diverse group of organisms in the biosphere. It is estimated that there are approximately 10^{32} phages on our planet (Brussow and Kutter, 2005), with 10^8 different phage species (Rohwer, 2003).

Phage therapy is the therapeutic use of bacteriophages to treat bacterial infections. Currently, phages are being evaluated as an alternative treatment for antibiotic-resistant bacteria (Lin et al., 2017). Bacteriophages have also been used to treat and prevent the formation of biofilms (Fu et al., 2010; Harper et al., 2014).

Mycobacteriophages are bacteriophages that can infect mycobacteria. At the time of this study as per The Actinobacteriophage Database (phagesdb.org) 10,562 mycobacteriophages have been isolated and 1,766 have been sequenced. The bacteriophages on this database are taxonomically organized into clusters based on nucleotide similarity (Hatfull et al., 2010). Mycobacteriophages are studied for their therapeutic potential in treating NTM and *M. tuberculosis* infections (Broxmeyer et al., 2002; Danelishvili et al., 2006). Mycobacteriophages have been demonstrated to prevent mycobacterial contamination on solid surfaces (Kiefer and Dahl, 2015). *M. smegmatis* mc^2 155 is the model organism for studying the genus *Mycobacterium*, because of its non-pathogenicity and increased growth rate (Beltan et al., 2000; Gordon and Smith, 1953). In this study, we isolated a soil-dwelling phage using *M. smegmatis* mc^2 155. We characterized the virion and explored the therapeutic potential of the isolated phage against common NTM and biofilm formation in *M. smegmatis* mc^2 155.

Methods

Culturing bacteria

All mycobacterial strains were grown in 7H9 broth supplemented with albumin dextrose catalase (ADC) (10% V/V) and 1 mM $CaCl_2$. Liquid bacterial cultures were incubated in a shaking incubator set at 37°C. On solid media, the bacteria were grown on 7H10 agar supplemented with 0.2% glucose and 1 mM $CaCl_2$. The bacteria grown on 7H10 agar plates were incubated at 37°C. *Mycobacterium smegmatis* mc^2 155 was obtained from ATCC (# 700084™). *M. avium* (strain DJO-44271), *M. abscessus* subspecies bolletii (strain MC1518), *M. intracellulare* (strain 1956), *M. kansasii strain* (824), and *M. simiae* (MO-323) were obtained through BEI Resources, NIAID, and NIH.

Bacteriophage isolation

Soil was collected from the southwest corner of the University of Central Oklahoma (35.653371 N, 97.474118 W) near the Coyner Health Sciences building. The collected soil was enriched using a double enrichment method (Patton and Kotturi, 2018). Three grams of the collected soil was enriched with 10 mLs of 7H9 broth and 1 mL of host bacterium and was incubated at 37°C for 24 hours (h). Following incubation, 10 mLs of phage buffer (10 mM Tris pH 7.5, 10 mM $MgCl_2$, 68 mM NaCl, and 1 mM $CaCl_2$) was added to the mixture; the mixture was vortexed and centrifuged at 1,589 x g for 10 min. Following centrifugation, 9 mLs of supernatant was removed and added to 1 mL of 10X 7H9 broth, which was supplemented with ADC, and 1 mM $CaCl_2$. One mL of host bacterial culture with approximately 1.2 x 10^9CFU/mLs was also added, and the bacteria were incubated at 37°C for 24 h. Following enrichment, phage lysate was centrifuged at 12,000 x g for 10 min. The supernatant was filtered using a 0.2 µm filter and serially diluted. Fifty µL of diluted phage was added to 450 µL of host bacterium. The phage and host bacterium were mixed and incubated at room temperature for 10 min. Phage and bacteria were plated using the agar overlay method (Hockett and Baltrus, 2017) and incubated for 24 h at 37°C. Following incubation, the plates were examined for plaques and plaques were measured in mm.

Plaque purification

Once a single plaque was identified it was purified by removing the plaque and adding it to a pure culture of *M. smegmatis* mc^2 155 and incubated overnight. Following incubation, the culture was filtered and plated as previously described. This process was done three times to ensure purification of a single type of

bacteriophage.

Amplification and precipitation of phage

A single plaque was purified 3 times and the isolated phage was amplified by seeding 30 plates with a high titer phage of 10^8 plaque forming units (PFU)/mL. Approximately 7 mLs of phage buffer was added to the plates and plates were incubated for 8 h at room temperature. The phage lysate was pooled and centrifuged at 5500 x g for 10 min. at 4°C. The supernatant was transferred to a sterile flask and phage was precipitated by adding 1 M NaCl and polyethylene glycol 8,000 (10% V/V), and incubated overnight at 4°C (Colombet et al., 2007). Following incubation, the solution was centrifuged at 5500 x g for 10 min. at 4°C. The supernatant was decanted and the sediment containing the phage was resuspended in phage buffer. This solution was incubated at 4°C for 24 h with gentle agitation from an orbital shaker. The phage was centrifuged at 5500 x g for 10 min. at 4°C. The supernatant, containing the concentrated phage, was removed and was used as the phage stock. The phage stock's titer was determined, and the phage stock supplied the phage for all experiments.

Electron microscopy

High titer phage lysate of 10^9 PFU/mL was added to a carbon-coated electron microscope grid and negatively stained using 1% uranyl acetate. Micrographs of the virion were obtained using a JEM-2000FX scanning transmission electron microscope (TEM) at 80,000 X total magnification. Bacteriophage measurements of the head and tail were obtained using the ImageJ software package. The diameter of the icosahedral head was obtained by measuring the distance from one vertex to the opposing vertex. Phage tails were measured from the beginning of the tail, which was adjacent to the icosahedral head, to the bottom of the tail. Three measurements were taken for the head and tail and they were averaged. Five different virions were used when measuring the size of the tail and icosahedral head.

DNA extraction and restriction digest

The genomic DNA (gDNA) was extracted using the sodium dodecyl sulfate (SDS)/ phenol:chloroform:isoamyl alcohol (PCI) (25:24:1 V/V) extraction method as described by (Green and Sambrook, 2012). The extracted gDNA was treated with five different restriction enzymes (BamHI, ClaI, EcoRI, HaeIII, and HindIII,) per the manufacturer's recommendation (New England Biolabs). An undigested DNA sample was used as a control. The restriction digest results were confirmed by *in silico* analysis using the DNA Master Software package (http://cobamide2.bio.pitt.edu/).

Determining the thermal stability of phage

Thermostability of the phage particle was determined by incubating phage, at an initial concentration of 10^8 PFU/mL, at three different temperatures (50°C, 55°C, and 60°C). The incubated phage was diluted and plated at three specific time points (20, 40, and 60 min.). Plaques were counted, and the number of PFUs/ mL were determined.

Determining pH stability of phage

The stability of the virion was evaluated by incubating the phage in phage buffer with adjusted pHs (5, 6, 7, 8, and 9) for 1 h. Adjustment of the pH was done using a pH meter and the addition of HCl or NaOH to the phage buffer; the phage buffer, with an adjusted pH, was then autoclaved to ensure sterilization. Phage was incubated in adjusted phage buffer, at an initial concentration of 10^8 PFU/mL, at 37°C for 1 h. Following incubation, the phage was diluted, plated, and incubated using the agar overlay method described above.

One-step growth curve

This method was described by Catalao et al using a multiplicity of infection (MOI) of 1.0. Sulfuric acid (0.4%) was used to inactivate unattached phage. The solution was then neutralized using 0.4% NaOH (Catalao et al., 2010). Every 30 min. for a duration of 4 h, 1 mL of suspension was diluted and plated using the agar overlay method as previously described. Plates were incubated at 37°C for 24 h then plaques were counted. This experiment was repeated four times. Data was pooled and the mean ± standard error was graphed.

Effect of phage on biofilm formation

The biofilm forming media used in these experiments was composed of 7H9 broth (as previously described) supplemented with 100 µM $CuSO_4$ (Nguyen et al., 2010). A MOI of 1.0 was used by initially seeding 50 µL of phage (10^8 PFU/mL) onto a 96-well tissue culture plate. One hundred µL of *M. smegmatis* mc^2 155, at a 0.5 McFarland concentration, was added to a 96-well plate, and plates were incubated at 37°C for 72 h. Planktonic cells were removed and biofilms were washed with 200 µL of sterile nanopure water. Fifty µL of phage and 150 µL of biofilm forming media were added to the wells and incubated at 37°C for 72 h. Biofilms were quantified using crystal violet (CV) staining (Kang et al., 2013). Biofilm growth was analyzed using an ELx808 microplate reader (BioTek) at an OD_{570} nm. Phage-treated biofilms were compared to biofilms treated with phage buffer that lacked phage for both assays. In each experiment, 12 biological replicates were used, and experiments were repeated two additional times (n=36).

Determination of host range

The different hosts used to evaluate OKCentral2016's host range were: *M. avium* (strain DJO-44271), *M. abscessus* subspecies *bolletii* (strain MC1518), *M. intracellulare* (strain 1956), *M. kansasii* strain (824), and *M. simiae* (MO-323). The phage was enriched with the host bacterium for 48 h. Following incubation, the phage and host bacterium were filtered and plated using the agar overlay method as previously described. The plates were incubated at 37°C and plates were inspected for plaque formation every day for a duration of 30 days. Plates were inspected every day because of the diverse generation times of these mycobacterial species. *M. smegmatis* mc^2 155 was used as a positive control. These results were confirmed using a spot assay (Mirzaei and Nilsson, 2015).

Data collection and analysis

All experiments were performed using at least three biological replicates, and experiments were repeated at least two additional times to confirm the results. Student t-tests were used to perform the statistical analyses of the data using the analysis tool pack within the Microsoft Excel software package (2016).

Results

Using the methods described above, bacteriophage OKCentral2016 was isolated and purified from soil enrichment. We named the purified phage OKCentral2016. The name is derived from the location and year this phage was isolated. OKCentral2016 produced transparent plaques that had an average diameter of 4.0 mm (Figure 1). The size and transparency of the plaques remained consistent throughout this study. The phage was observed using TEM (Figure 2). OKCentral2016 possessed a *Siphoviridae* morphotype that contained an icosahedral head with a non-contractile tail. The average diameter of the icosahedral head was 56.152 (±5.187) nm, and the length of the non-contractile tail was 130.485 (±9.294) nm.

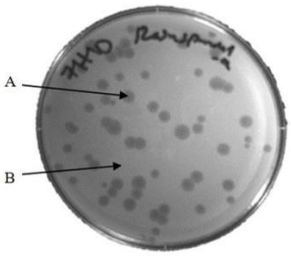

Figure 1. Plaques (A) formed by OKCentral2016 on a bacterial lawn of *M. smegmatis* (B).

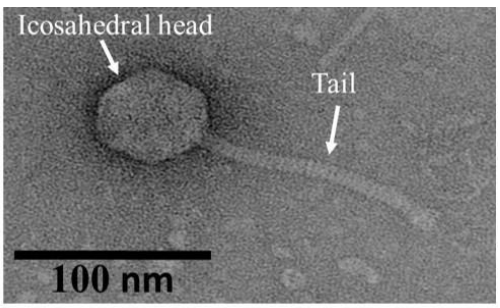

Figure 2. Micrograph of OKCentral2016 stained with 1% uranyl acetate.

The infectious cycle of this phage was characterized by constructing a one-step growth curve (Figure 3) based on the number of PFUs produced at a given time point. We found that the latent period and the rise period took 90 min. Following the rise period, the number of PFUs plateaued, which was indicative that the infectious cycle had concluded. These results are comparable to other mycobacteriophages such as SWU1, which had a latent period that lasted only 30 min (Fan et al., 2015). Whereas phages BO1 and BO2a had latent periods that lasted 150 min. and 260 min., respectively (Kraiss et al., 1973).

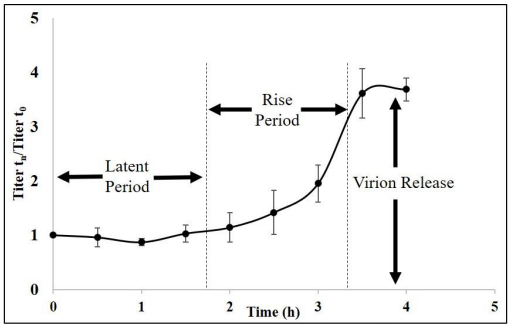

Figure 3. The one-step viral growth curve of OKCentral2016 infecting *M. smegmatis*. Error bars are expressed as ± SE.

A decrease in infectivity was observed after subjecting phage to various thermal (Figure 4A) and pH conditions (Figure 4B). There was a 1 log reduction of PFUs after 60 min. of incubation at 55°C. However, after 40 min. of incubation at 60°C no plaques formed. A significant decrease ($p<0.05$) in phage infectivity was also observed when phage was incubated at an acidic pH of 5.0 or an alkaline pH of 9.0. No reduction in phage infectivity was found within the 6.0 to 8.0 pH range.

The gDNA of OKCentral2016 was approximately 49,000bp on agarose gel and contained restriction sites for two of the five enzymes tested, BamHI, and HaeIII (Figure 5). Digestion by BamHI resulted in the formation of 14 bands at various molecular sizes between 48,000 and 1,000 bp. HaeIII (lane 4)was the other enzyme that cleaved the gDNA with

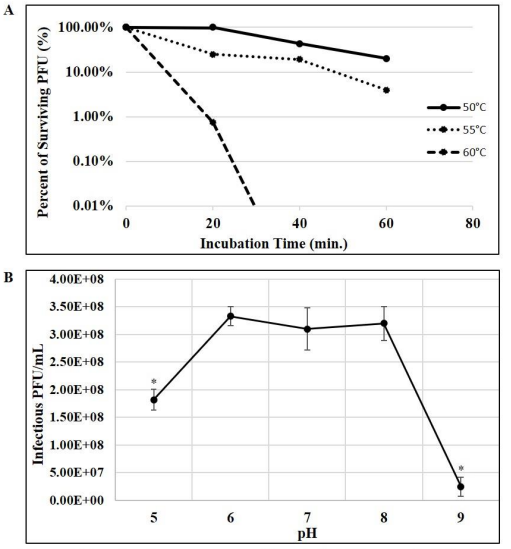

Figure 4. The effect that temperature (A) and pH (B) have on the infectivity of OKCentral2016. Error bars are expressed as ± SD.

***p<0.05**

multiple restriction sites. From the gel image, we can clearly see a DNA smear ranging from 49000 to 500bp in Figure 5. The gDNA did not possess the restriction sites for ClaI, EcoRI, and HindIII, and thus, the DNA was not cleaved. These results were compared to the untreated

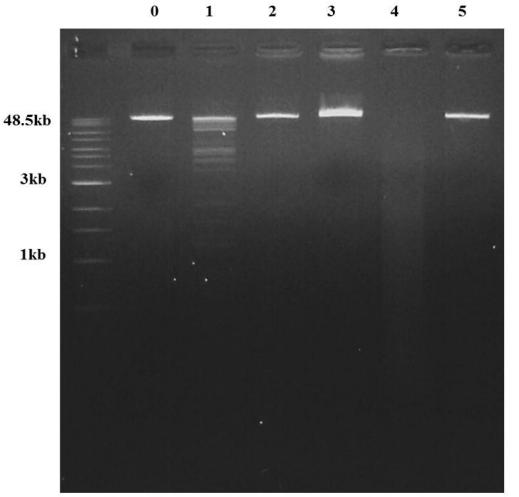

Figure 5. Treatment of OKCentral2016's gDNA with restriction enzymes BamHI (1), ClaI (2), EcoRI (3), HaeIII (4), and HindIII (5). Untreated gDNA served as a control (0).

gDNA. This restriction pattern is common among members of cluster A and subcluster A10. These results were confirmed through genomic sequencing and *in silico* analysis of the genome.

The presence of phage significantly decreased ($p<0.05$) biofilm formation in planktonic cultures of *M. smegmatis* (Figure 6A). However, when phage was added to cells already encased in a biofilm, the biofilm was significantly larger ($p<0.05$) than biofilms lacking phage treatment (Figure 6B). This phage did not infect any other mycobacterial species tested (Table 1).

Discussion

Due to the high pathogenicity and transmission of *M. tuberculosis*, mycobacteriophages have been studied as plausible phage therapy agents. It has been suggested that there is a relationship between the phage cluster and the phages ability to infect other mycobacterial species such as *M. tuberculosis*. The A2 and A3 subclusters have been shown to infect mycobacterial hosts other than *M. smegmatis*. For example, phages Bxb1 and U2 (A1 subcluster), L5 and D29 (A2 subcluster), and Bxz2 (A3 subcluster) infected *M. tuberculosis* (Jacobs-Sera et al., 2012; Rybniker et al., 2006). Treating OKCentral2016's gDNA with five different endonucleases determined that this phage belonged to cluster A (Gissendanner et al., 2014). Genomic sequencing confirmed that this phage belonged to cluster A and subcluster A10 (Patton and Kotturi, 2018). However, it has been shown that single amino acid substitutions on the putative tail fiber protein of mycobacteriophages allowed mutant phages to efficiently infect *M. tuberculosis* (Hatfull, 2014a). Phage infectivity of a specific host is postulated to be determined by the presence of specific bacterial receptors. However, very few phage receptors have been identified (Hatfull, 2014b). Therefore, identifying various phage receptors should be thoroughly investigated.

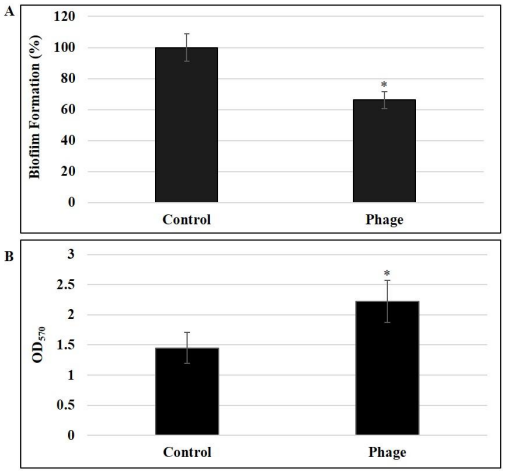

Figure 6. Adding OKCentral2016 to a planktonic culture of *M. smegmatis* significantly decreased biofilm formation (A). However, when OKCentral2016 was added to cells already encased in a biofilm, the biofilm increased significantly (B). These results were graphed ± SE. *$p<0.05$

Bacteriophages have been shown to effectively prevent and degrade various bacterial biofilms, such as those produced by *Campylobacter jejuni*, *Escherichia coli*, and *Proteus mirabilis* (Carson et al., 2010; Chibeu et al., 2012; Siringan et al., 2011). Phages capable of degrading biofilms may possess depolymerizing enzymes such as a glycanase, which breaks down EPS. For this to occur, the phage binds to biofilm polysaccharide, which serves as a secondary receptor. The glycanase degrades the biofilm by hydrolyzing β-glycosidic linkages until the phage has reached the primary receptor on bacterial cell's exterior surface. The phage then adsorbs to the bacterial receptor and begins replicating lytically or lysogenically (Hughes et al., 1998; Rieger-Hug and Stirm, 1981). Some mycobacteriophages have been shown to disrupt *M. smegmatis* biofilms (Kiefer and Dahl, 2015). Our results support the previous observations that treating established mycobacterial biofilms with phage can increase biofilm formation (Hughes et al., 2016). The underlying mechanisms related to enhancement or disruption of the biofilms by phages remain unknown and are not fully investigated. We can speculate that some of the annotated genes with no known function may contribute in some way. Our future work will examine some of these possible mechanisms.

Table 1. The host range of OKCentral2016.

Bacterial Strain	Phage Infectivity (+/-)
M. abscessus subspecies *bolletii* MC1518	-
M. avium DJO-44271	-
M. intracellulare 1956	-
M. kansasii 824	-
M. simiae MO-323	-
M. smegmatis mc^2 155	+

Acknowledgments

The following reagents were obtained through BEI Resources, NIAID, NIH: *Mycobacterium abscessus*, Strain MC1518, NR-44266, *Mycobacterium avium*, Strain DJO-44217, NR-49092, *Mycobacterium intracellulare*, Strain 1956, NR-44267, *Mycobacterium kansasii*, Strain 824, NR-44269, *Mycobacterium simiae*, Strain MO-323, NR-4434. We want 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.

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