Properties of a Genetically Unique Mycobacteriophage

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PROPERTIES OF A GENETICALLY UNIQUE MYCOBACTERIOPHAGE

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PROPERTIES OF A GENETICALLY UNIQUE MYCOBACTERIOPHAGE

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I dedicate this thesis to my sons, Donovan and Dresden Staples. Life is filled with good times and bad times. I wish you both all the strength and wisdom to face the challenges that come your way. Learn and grow from everything and everyone. Always listen to your heart and don’t be afraid to take the road less traveled. Never forget how much I love you both.
I would like to take the opportunity to express my profound gratitude to all those who contributed to the support and guidance vital in completing this research. Though I am not able to thank all the caring people involved, their assistance has been invaluable.

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the most arduous and merciless journey that exists and I feel like I don’t belong, you remind me, “Nobody exists on purpose. Nobody belongs anywhere…”

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Bacteriophage MooMoo is a temperate phage that was isolated and propagated on *Mycobacterium smegmatis* (*M. smeg*). It typically produces turbid plaques, however spontaneous clear plaque mutants can be readily isolated. Both turbid (MooMoo-T) and clear plaque (MooMoo-C) formers can establish stable lysogens, but the parental turbid plaque forming phage has a higher lysogenic frequency. The phage repressor protein typically plays the central role in regulating the lysis/lysogeny decision. Therefore, we expected that the mutation responsible for the clear plaque phenotype would be located in the repressor gene. Remarkably, whole genome sequencing detected a single base pair mutation in the minor tail protein gene (gp19). The regulatory role of the repressor protein could not be excluded considering it was unclear how the mutation in gp19 was leading to the altered plaque phenotype. To locate the phage repressor, we used bioinformatics to identify several candidate genes with helix-turn-helix and DNA binding motifs (gp42, gp43 and gp44). We also cloned the parental and mutant gp19 genes. Each candidate gene was cloned into a shuttle vector. The clones of gp43, gp44 and both derivatives of gp19 did not prevent MooMoo growth, whereas the clones of gp42 inhibited phage growth. Based on these results, we concluded that gp42 is the phage repressor for MooMoo.

To determine if the presence of gp19 alters lysogenic frequency, lysogeny assays of wild-type (WT) and mutant gp19 clones were evaluated. Compared to the MooMoo-C
lysate, the cloned copy of the mutant gp19 showed a slight increase in lysogeny efficiency. The lysogeny frequencies on strains that carry cloned copies of gp19 (WT or mutant) were similar to those obtained on strains that lacked the plasmids. From these results, we concluded, the presence of either parental or mutant gp19 clones does not affect the lysogeny frequency.

To determine if host cell physiology was affected by lysogeny, carbon, nitrogen, phosphorus and sulfur utilization resources were screened using high-throughput phenotypic microarrays. From these results, we concluded the presence of the WT or mutant prophage had no significant effect on the utilization of the resources tested.
INTRODUCTION

Bacteriophages (phages) are viral parasites that infect bacterial hosts to produce new progeny (1, 2, 3). The phage population represents the microbial “dark matter” that vastly outnumbers any entity on Earth (6, 53). To astronomers and physicists, 85 – 95% of the universe is composed of dark matter — an invisible mass/energy that has yet to be directly measured despite its enormity and influences on the evolution of the universe (74). Analogous to the dark matter of the universe, the phage population is vast and poorly understood (53). Phages are restricted by the availability of their hosts within an environment, yet each phage population is a rich source of genetic variation (2). Both bacteria and their phages drive reciprocal changes within each other in an evolutionary arms race, as bacteria frequently evolve mechanisms to prevent phage infection (i.e. CRISPER/Cas systems) and phages relentlessly counter-evolve to infect their hosts (2, 3, 4, 5, 7, 54, 55). Despite co-evolving with their hosts for millions of years, phages were only discovered a little over a century ago. The first documented accounts of potential phage activity were made in 1896, by Ernest Hankin’s study on the river waters in India. Hankin described how the Ganges and Jumna river waters of India contained a filterable and heat-instable substance capable of killing the bacteria Vibrio cholera (10). However, Hankin’s evidence was difficult to duplicate and several in the scientific community doubted his results (10). One of the scientists who discredited Hankin’s research was Felix d’Herelle (10). Felix d’Herelle was in Mexico, in 1910, assessing diseased locusts when he first came across clear spots on plates covered with a lawn of bacteria (9, 57). When d’Herelle swabbed the spots onto a slide and checked under a light microscope, nothing was seen (56). He hypothesized an unknown factor caused the spontaneous lysis.
of the bacterial cells that created the clear spots (9). After five years of investigating the locusts, he was called to aid in treating the outbreak of dysentery affecting the French soldiers stationed near Paris (9, 56). His work with human dysentery was invaluable, but most infamous was his naming of the filterable bacterial antagonist, the bacteriophage (9, 56, 57). Felix d’Herelle’s research on bacteriophages, in 1917, was not without competition. Frederick Twort was a middle class Englishman researching the metabolic requirements of microbes (9, 57). In 1915, Twort described an unusual glassy appearance of several micrococcus colonies (57). He later ascribed that the glassy transformation occurred by a replicating, filterable and infectious material, but his work was terminated from a lack of funding and his subsequent enrollment into the Royal Army Medical Corps (9, 14, 57, 59). In 1921, Twort’s work was finally recognized eventually resulting in the scientific community acknowledging him as a co-discoverer of bacteriophages (57).

While working with human dysentery, d’Herelle recognized that bacteriophages could be used therapeutically to destroy bacterial pathogens. He was able to generate data that supported his hypothesis by successfully treating multiple cases of enteric diseases around the world with bacteriophages (9, 14, 56, 58). Enteric disorders were prevalent among developed and under developed countries as a result of poor sanitary practices, improper water and waste management measures, along with, inadequate treatment plans and limited access to medical care (15). Though d’Herelle introduced and developed phage therapy, the first documented results of successful treatment of a disease with phages came from Belgium where phages alleviated *Staphylococcal* skin infections (14, 59). Shortly thereafter, d’Herelle’s lab, known later as the L’Oréal
Company, produced large volumes of five phage therapeutics and marketed them as being effective against multiple bacterial infections (14). The Eli Lilly Company, in the United States, began to mass-produce multiple phage therapeutics, which were targeted against a range of ailments, such as vaginitis, abscesses and upper respiratory infections (14). Unfortunately the promise of phage therapy never materialized thanks to poor marketing tactics, limited understanding of phage biology, inefficient phage preparations and reports of side effects (9, 14, 59). In addition, the discovery of antibiotics provided the new miracle cure, as these drugs were relatively inexpensive to produce, specific towards a range of bacteria and lacked the negative side effects sometimes observed with phage injections (11, 12). Companies, like Eli Lilly, that previously produced phage therapies, re-directed their attention to manufacturing antibiotics and phage therapy disappeared in the Western world (11). As antibiotic production increased, the incidences of enteric diseases similar to those d’Herelle treated, were nearly eradicated in the US (15). However, respiratory illnesses proved to more difficult to diagnosis and treat. For example, the disease known as consumption (tuberculosis) or the “white death” was spreading within the population (15, 16).

Although Robert Koch had described *Mycobacterium tuberculosis* (*M. tuberculosis*) as the causative agent of consumption in 1895, early treatments were highly toxic and proved ineffective (15). Antibiotics were initially successful against tuberculosis (TB), however antibiotic resistance occurred almost immediately (38). By mid-1960’s, US federal programs were established to control the spread of TB (15, 16). Unfortunately, TB was not localized to the US and according the World Health Organization (WHO), TB is now a pandemic with nearly one-third of the world’s
population infected (60). Even more alarming, strains of multi-drug resistant (MDR) and extensively drug resistant (XDR) TB are increasing, rejuvenating the therapeutic need for phages once more (5, 13, 18, 38, 60).

*M. tuberculosis* is exquisitely adapted as a human pathogen owing much of its success having complex cell wall and envelope structures that allow the microbe to manipulate host immune responses (8, 61, 62, 63). Evidence of TB infections has been discovered in Egyptian mummies dating back over 4,000 years ago and descriptions of TB-like symptoms were even noted by Hippocrates (38, 61, 83, 84). Studying *M. tuberculosis* in the laboratory has been difficult and time consuming due to its slow growth rate (13).

In the late 1930’s, researchers bypassed the prolonged cultivation time of *M. tuberculosis* in favor of the “fast growing” relative *Mycobacterium smegmatis* (*M. smeg*) as they share numerous genetic, metabolic and physiological features (13, 19, 20, 39). Since then, *M. smeg* has been used as a surrogate for *M. tuberculosis* to enrich for mycobacteriophages. After the isolation of the first mycobacteriophages, the decades of studies that followed, provided crucial information on multiple viral and host aspects, such as phage morphology, infection mechanisms, lysogenic conversion and alterations to metabolic functions (13, 17, 21, 22, 23, 24). In 1993, the whole genome of L5 became the first mycobacteriophage to be sequenced. Subsequent studies on L5 have allowed scientists to uncover a plethora of gene expression processes involved in the phage life cycle and lysogeny (64, 65). The research henceforth describes the discovery and characterization of a clear plaque mutant, along with the in depth analysis on the properties the mycobacteriophage MooMoo.
Phage Properties and Morphology

Unlike systematics for living organisms, viruses are sorted by morphological structures, nucleic composition and method of replication (29). Tailed phages, like those infecting *M. smeg*, belong to the *Caudovirales* group which share a general icosahedral capsid architecture that encases the genomic double-stranded (ds) DNA (29, 30; Figure 1). Capsids vary in size depending on the total length of the genome, however some capsids can have an unusual architecture forming unique prolate heads (13, 25, 26, 66). Prolate capsids are uncommon in mycobacteriophages, yet as Figure 2 illustrates, mycobacteriophage MooMoo has a prolate head measuring roughly 112 nanometers (nm) long (13, 17). Phage tails also vary considerably in length and morphology, especially within mycobacteriophages. The International Committee of Taxonomy of Viruses (ICTV) determined that tailed phages exhibit three morphotypes – *Myoviridae*, *Siphoviridae* or *Podoviridae* (13, 21, 27, 71). Mycobacteriophage tails have only two configurations – *Myoviridae* and *Siphoviridae* – contractile or flexible non-contractile, respectively (13, 17). MooMoo has a Siphoviridae tail measuring approximately 225 nm in length (Figure 2). Phage tails mediate the recognition and binding to host cell receptors, thus they determine host specificity (17, 25, 27, 28). Since these interactions are critical for the first step in the phage infection process, mutations in specific tail proteins can often extend host ranges (5).
Figure 1. General architecture of a tailed bacteriophage.
Figure 2. Electron micrograph of mycobacteriophage MooMoo. Image shows the prolate capsid and siphoviridae morphology.
Mycobacteriophage Genome Clusters

Mycobacteriophages are organized into clusters (Clusters A – Z, AA – AC) based on genetic similarities (6; Figure 3). Each cluster is further divided into subclusters demonstrating genetic heterogeneity even among related phage genomes. Phages with no appreciable homology with any particular cluster are called singletons (6). As Figure 3 shows, MooMoo is a singleton.
Figure 3. SplitsTree phylogeny of sequenced mycobacteriophages (6). Colored circles represent clusters, while singletons are labeled (MooMoo, Patience, Muddy, Wildcat, Sparky, Dori, and Gaia). Electron micrographs illustrate the morphotypes in Corndog (Cluster O), MooMoo (singleton) and Mozy (Cluster F).
Phage Life Cycles – Part I (Lytic v. Lysogenic: The Lambda Paradigm)

The lytic cycle was named for the multi-step phage infection process culminating in cell death upon release of new viral progeny. The infection process begins with recognition and attachment to host cell surface receptors by the phage tail. In conjunction with attachment, tails contain enzymatic activity to degrade the cell wall and provide a tunnel for the phage genome to enter into the host cell cytoplasm (17). Once inside the cell, the phage DNA circularizes and the bacterial biosynthetic machinery is commandeered to transcribe/translate early phage genes. Expression of early phage genes for DNA replication results in multiple copies of the phage genome being generated. As phage DNA is replicating, the capsid and tail structural proteins are then mass-produced. New virions are self-assembled and the phage genome is packaged inside the capsid (32). The host cell fills with mature virions, while phage encoded lysins degrade the cell wall of the host (8, 33). The disruption of the cell wall, along with the formation of channels through the cell membrane by the phage holin proteins, leads to cell lysis and release of newly formed phages (Figure 4). Lytic phages are those that strictly enter the lytic cycle and form clear plaques on a lawn of their host.

Temperate phages can enter the lysogenic life cycle as an alternative infection strategy. Phages that enter the lysogenic pathway do not immediately lyse the host cell. Some temperate phages integrate their entire genome into the bacterial chromosome after entering the host cell cytoplasm (Figure 4). However, before the phage genome integrates, lytic genes are silenced by the expression of the phage-encoded repressor protein. Integration is favored by increased levels of Integrase (int), expressed from the P1 promoter.
Integration events occur between the two genomes at specific homologous attachment sites, one on the phage genome (attP) and the other on the bacterial chromosome (attB). Phage integration proteins and host derived integration host factors (IHF) bind to the attP site and mediate site-specific recombination with the attB site. The outcome is that the phage DNA integrates to become a part of the host chromosome (35). The repressor protein continues to be expressed to maintain the lysogenic state. The integrated phage genome is referred to as a prophage. During host cell division, the prophage is replicated along with the host chromosome allowing the temperate phage to by-pass the infection process with each daughter host cell inheriting a copy of the prophage (Figure 4). Bacterial hosts with integrated phage genomes are called lysogens and these cells exhibit immunity towards super-infection by the same phage. Prophages can spontaneously excise from their host chromosome and enter the lytic cycle, and prophage excision can also be induced by events such as DNA damage.

The archetype for switching between the lysogenic and lytic life cycles is best represented by the coliphage Lambda. The repressor protein is the central regulatory factor for maintaining lysogeny. When the repressor is bound to its DNA recognition sites (operators), lysogeny is promoted and maintained (Figure 5A). When the repressor is not bound, Lambda enters the lytic cycle (Figure 5B).
Figure 4. Illustration of lytic and lysogenic life cycles. Lytic events are depicted by the red arrows. Lysogenic events are shown as two phases – integrated prophage (green arrow) and excised prophage (blue dotted arrow).
Figure 5A-B. Lytic/Lysogenic regulation of Lambda. Both A and B are simplified schematics of the P<sub>R</sub> promoter based off the detailed information provided in “A Genetic Switch” (75). Sites 1 – 3 represent the DNA binding (operator) sites. A) Repressor bound to DNA promotes the lysogenic cycle and maintains lysogeny. B) No repressor bound to DNA allows the expression of the lytic cycle genes.
Phage Life Cycles – Part II (Integration of Mycobacteriophage BPs)

Mycobacteriophage BPs provides an interesting alternate example of establishing and maintaining lysogeny compared to the Lambda paradigm described above. After injection of the phage DNA into the host cell’s cytoplasm, two intergenic promoters are activated. The early lytic promoter, \( P_R \), synthesizes a Cro-like protein, while the more active repressor promoter, \( P_{rep} \), upregulates expression of the repressor and integrase genes (13). The initial increase of Integrase (\( Int \)) proteins stimulates integration between the phage attachment (\( attP \)) site and bacterial chromosome (\( attB \)) site. Unlike Lambda, the \( attP \) is located in the open reading frame (ORF) of the repressor gene, where site-specific recombination creates a truncated repressor expressed from the \( P_{rep} \) promoter (40, 44, 70). After recombination, the integration gene is separated from the \( P_{rep} \) promoter and residual \( Int \) proteins are degraded by host proteases. Lysogeny is maintained by the constitutive \( P_{rep} \) promoter, which expresses the active (truncated) form of the repressor protein (44).

In contrast to Lambda phage, the lytic/lysogenic switch in BPs is determined by the integration event (13). During early gene expression from promoters \( P_R \) and \( P_{rep} \), full-length repressor proteins are synthesized, but are rapidly targeted for proteolysis by the ssrA-like tag located at the C-terminal end of the protein (44, 70). Upon integration, the ssrA-like tag is removed and the active (truncated) repressor is expressed (44). Continuous degradation of the viral form of repressor leads to the expression of genes for lytic growth (44). In this manner, superinfection immunity is not from the viral form of the repressor protein, but from the truncated form (40). The integration-dependent immunity mechanism of BPs illustrates a unique method some mycobacteriophages use.
to lysogenize their hosts and also demonstrates the different mechanism that have evolved to regulate the lytic and lysogenic decision.

**Lysogenic Conversion**

In 1927, researchers demonstrated an unusual phenomenon where previously non-pathogenic bacteria were converted to pathogenic forms after they were grown in the presence of filtered media from a pathogenic strain (3, 67). It would be several decades before phages were proven to be responsible for the pathogenesis of many bacteria, like *Corynebacterium diptheriae* and *Vibro cholera*. Scientists are still uncovering more host behaviors or traits contributed by temperate phages (4, 68). Lysogenic conversion has played an important role in the diversification of bacterial species and is one of the major contributors to bacterial pathogenesis (3, 69). Prophage-induced fitness advantages are under strong selective pressures to be maintained within the bacterial population leading to strain variation and over time possibly speciation (31, 34, 36, 37).
MATERIALS & METHODS

Bacterial Strains and Mycobacteriophages

Bacterial strains and mycobacteriophages used in this study are listed in Table 1.

Growth Conditions

Unless otherwise noted, the growth conditions for all plating and liquid cultures were incubated at 37°C, with liquid cultures shaking at 250 rpm.

Growth Media for Wild-type M. smeg

Wild-type (WT) M. smeg was routinely retrieved from archived frozen stocks and streaked onto appropriate plate media using the standard streak plate technique. The plate was incubated for approximately 4 days. After growth, plates were stored in 4°C and used for several weeks. The inoculation from a plate into liquid media is the first generation of growth (denoted as P1FF). To prepare a P1FF cultures, an independent colony was aseptically transferred into the liquid medium, 7H9 Complete with Tween80 (Appendix I-C), and incubated for 4 days. For bacteriophage plating experiments, a subculture of the P1FF was prepared by diluting the P1FF 1:100 in 7H9 Complete without Tween80 (Appendix I-B). The subculture was the second generation of growth (denoted as P2FF) and was also incubated for 4 days. All cultures were stored at 4°C after growth.

Growth Media for E. coli

Escherichia coli (E. coli.) cells were grown in LB Broth (Appendix I-H). Transformants were grown in LB broth supplemented with Kan (final concentration 50 µg/mL). E. coli cultures were incubated overnight, unless otherwise noted.
Table 1. Host bacteria, lysogens, bacterial strains, vector and mycobacteriophages

<table>
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<th>Host Bacteria</th>
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<td>MC\textsuperscript{2}155</td>
<td>Wild-type <em>M. smeg</em></td>
<td>42</td>
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<tr>
<td>XL1-Blue</td>
<td>endA\textsuperscript{1} gyrA\textsuperscript{96}(nal\textsuperscript{R}) thi\textsuperscript{-1} recA\textsuperscript{1} relA\textsuperscript{1} lac glnV\textsuperscript{44} F\textsuperscript{[}::Tn10 proAB\textsuperscript{+} lacI\textsuperscript{q} Δ(lacZ)M15\textsuperscript{]} hsdR17(r\textsuperscript{K} m\textsuperscript{K}\textsuperscript{+})</td>
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<td>WT phage, Forms turbid plaques</td>
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</tr>
<tr>
<td>MooMoo-C</td>
<td>Mutant, Forms clear plaques; (2017)</td>
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Growth Media Modifications for *M. smeg* Clones

AD supplement and CaCl$_2$ can inhibit the effectiveness of the acetamidase promoter in the vector pLAM-12 (41). There is also evidence to suggest the acetamidase promoter may not be tightly regulated in the presence of succinate (41). For the gp42, gp43 and gp44 clones, P1FF’s were prepared in 7H9 Complete with Tween80 supplemented with Kanamycin (Kan) to a final concentration of 50 µg/mL, then incubated for 4 days. P2FF subcultures were prepared by diluting the P1FF 1:100 into two different medias: 7H9-ADC (Appendix I-J) and Induction Media (Appendix I-K). Cultures were incubated for 4 days. These cultures formed poor lawns with severe clumping of the cells. Subsequently, all clones were grown in 7H9 Complete media (with and without Tween80) supplemented with Kan to a final concentration of 50 µg/mL. All cultures were stored at 4°C after growth.

Plate Media for Wild-type *M. smeg*

WT *M. smeg* was plated on Luria Agar Plate Media supplement with Carbenicillin (CB) and Cycloheximide (CHX; Appendix I-D). These plates are denoted as LA+CB+CHX.

Plate Media for pLAM-12 Clones

Selected *M. smeg* transformants and pLAM-12 clones were plated on Luria Agar Plate Media supplemented with CB, CHX and Kan (Appendix I-E). These plates are denoted as LA+Kan.
Plate Media for Induction of pLAM-12 Clones

Selected pLAM-12 clones were plated on Luria Agar Plate Media supplemented with CB, CHX, Kan and Acetamide (Appendix I-F). These plates are denoted as LA+Acet.

Plate Media for *E. coli*

*E. coli* transformants were plated on LB Agar Plate Media supplemented with Kan (Appendix I-I). These plates are denoted as LB+Kan.

10-fold Serial Dilutions

Ten-fold serial dilutions were performed as required. The undiluted sample is denoted as 10^0. Phage lysates were diluted in Phage Buffer (PB; Appendix II-E), while cells were diluted with the appropriate media. Figure 6 (below) depicts a general 10-fold serial dilutions scheme.

![Figure 6. Illustration of 10-fold serial dilutions.](image)
Preparations with 7H9 Top Agar (Phage Plating)

Phage plating typically involved transferring 4.5 mL of molten 7H9 Top Agar (Appendix I-G) into a glass culture tube containing 0.5 mL of cells and phage. The entire contents of the culture tube were mixed thoroughly by pipetting up and down, and then the contents were transferred onto the appropriate plate media.

Preparations with 7H9 Top Agar (Bacterial Lawn)

Plating of WT *M. smeg* or pLAM-12 clone lawns were performed as described above, however no phages were present in the culture tube. Lawns were prepared on the appropriate plate media.

Phage Growth

Mycobacteriophage MooMoo was grown from a frozen archived stock by scraping some of the frozen material and spotting it onto a lawn of *M. smeg*. The WT *M. smeg* lawn was prepared on a LA+CB+CHX plate as described in “Preparations with 7H9 Top Agar (Bacterial Lawn)”. After 24 hours of incubation, the area of phage growth was transferred into 100 µL of PB using a micropipette tip to stab through the Top Agar layer down to the bottom plate agar. This process is noted as “plugging”. Figure 7 (below) demonstrates how the area of phage growth was plugged. The agar, containing the phage, was incubated in the PB at room temperature for 1 hour to allow diffusion of phage particles into the buffer. A 10-fold serial dilution was performed (to $10^{-6}$). For plating, 10 µL of each dilution $10^{-3} \rightarrow 10^{-6}$ was transferred into 0.5 mL aliquots of P2FF *M. smeg* cells and incubated at room temperature for 30 minutes to allow for phage binding. Molten 7H9 Top Agar was aseptically transferred, mixed, then dispensed onto a LA+CB+CHX plate. After solidifying, the plates were inverted and incubated overnight.
Plaque Assays for Purifications

To purify a clonal population, three rounds of plaque purifications were necessary to ensure consistent plaque morphology. A typical purification involved plugging the most isolated plaque into PB, incubation at room temperature, 10-fold serial dilutions, phage binding to M. smeg cells, plating onto appropriate media and then incubating the plates overnight.

Preparing 1-Plate Lysates (1PL)

After purifying a clonal population, the plate containing the most confluent plaques (denoted as the “webbed plate”) from the final round of purification, was flooded with 6 mL PB and incubated at room temperature for 5 hours. The incubation allowed the phage from the plate to diffuse into the buffer. The PB containing the phage was harvested with a 5 mL syringe and filter-sterilized with a GE Healthcare Whatman™
Puradisc 0.2 μM syringe filter (Fisher Cat. No. 05-713-386) into a 15 mL tube. Plaque assays are used to estimate the concentration, or titer, of phage present in the lysate. Plaques are formed by infectious phage particles, thus noted as plaque forming units (PFU). To titer each 1PL, a 10-fold serial dilution out to $10^{-7}$ was performed. Dilutions $10^{-2} \rightarrow 10^{-7}$ were plated onto LA+CB+CHX plates for each 1PL and then incubated overnight. The titer was calculated as indicated, using a plate with a countable number of plaques. The formula below was used to determine the titer.

$$\text{Titer (PFU/mL)} = \left[ \frac{\text{No. PFU's on countable plate}}{\text{Dilution Volume (μL)}} \right] \times (10^3 \text{ μL/mL}) \times 10^x$$

**Preparation of High Titer Lysates**

The High Titer Lysate (HTL) was used for phage DNA isolation, archiving and electron microscopy. From the 1PL, a 10-plate lysate, also known as a HTL, was prepared. The concentration necessary to achieve 10 webby plates from the 1PL, was empirically determined. From the 1PL, a 10-fold serial dilution was performed out to the determined webbed plate dilution and then 100 μL of that dilution transferred into a 5 mL aliquot of P2FF *M. smeg* cells. As previously described, the phage and cells were incubated at room temperature, mixed with Top Agar and plated onto LA+CB+CHX plates. The plates were incubated overnight, after which confluent plaques appeared on all 10 plates. Each plate was flooded with 6 mL PB and incubated at room temperature.
for 6 hours. The lysate was harvested and filter-sterilized using a 50 mL Corning™ Tube Top 0.22 μM vacuum filter-sterilization unit (Fisher Cat. No. 09-761-34). As described above, plaque assays are used to estimate the titer of the HTL.

**Transmission Electron Microscopy**

Phage morphology was determined by electron microscopy. Prior to imaging and analysis, lysate samples were concentrated by centrifugation. One mL of HTL was transferred into a sterile microcentrifuge tube and centrifuged at 7,200 RCF at 4°C for 1 hour. The supernatant was discarded, after which the invisible phage pellet was re-suspended in 50 µL of PB and incubated at 4°C overnight.

**Phage DNA Isolation**

Phage genomic DNA was isolated from HTL’s in a two-step process. First, remnants of lysed bacterial cells, bacterial nucleic acids, proteins and lipids were removed from the phage lysate. Second, phage DNA was released from the capsid using a chaotropic agent and purified using a DNA binding resin. To begin, a 10 mL aliquot of HTL was transferred into a sterile Oak Ridge tube (Fisher Cat. No. 05-0563-7G) and equilibrated to room temperature. To enzymatically degrade any bacterial DNA and RNA, 40 µL of Nuclease Mix stock (Appendix II-H) was added, mixed by gentle inversion and incubated at 37°C for 30 minutes. After 30 minutes, the treated lysates were removed from 37°C and then incubated at room temperature for 1 hour. To concentrate the phage particles, 4 mL of Phage Precipitation Solution (Appendix II-L) was transferred into the nuclease-treated lysate, mixed by inverting 3 times, then incubated at 4°C for approximately 4 hours. The phage particles were pelleted via centrifugation at 17,217 RCF for 20 minutes at 4°C. The pellet was re-suspended with
0.5 mL sterile water and incubated at room temperature for 10 minutes. To purify the phage DNA from the phage particles, 2 mL of pre-warmed (37°C) Promega Wizard™ DNA Cleanup Resin (Wizard DNA Cleanup System; Fisher Cat. No. PR-A7280) was added and repeatedly pipetted to break up the pellet. The mixture was transferred to a Wizard® Minicolumn attached to a syringe barrel and the material pushed through the minicolumn. The resin contains a blend of microscopic polymer beads and guanidinium thiocyanate. The polymer beads are unable to pass through the fine mesh of the column filter, thus it packs tightly into the column. Contaminants were removed from the bound DNA by washing the column with 80% isopropanol. Wash steps were performed by pushing 2 mL 80% isopropanol through the column. Each wash was followed by a centrifugation step (12,045 RCF for 5 minutes). A final 1-minute centrifugation was done to remove residual isopropanol and the columns were transferred to sterile microcentrifuge tubes. Phage genomic DNA was eluted using 50 µL of pre-warmed (85°C) Elution Buffer (Appendix II-K) and incubated for 1 minute. The eluted DNA was recovered by centrifuging the tube for 1 minute. A second elution using the same volume of Elution Buffer (EB) into a new microcentrifuge tube was performed. The two eluates were then combined into a single tube (approximately 100 µL combined volume). The concentration of the pooled DNA was estimated using the ThermoScientific NanoDrop Spectrophotometer (Fisher Cat. No. ND2000).
Whole Genome Sequencing

Phage genomic DNA samples were sequenced at North Carolina University Genomic Sciences Laboratory (NC-GSL). NC-GSL’s guidelines for sample submission requires phage DNA be submitted at a concentration of 2 µg/120 µL. Two micrograms of purified phage DNA was diluted in EB to the total requested volume of 120 µL and shipped on ice overnight.

Phage Lysogeny Assays

Lysogeny assays were performed as described by the Actiobacteriophage Database (72). This assay is used to determine how readily a phage forms lysogens in a bacterial host. HTL’s were diluted in PB to $10^{10}$ PFU/mL then 100 µL was spread onto LA+CB+CHX plates. A flame sterilized glass L-rod was used to spread the 100 µL of phage over the surface of the plate. These plates are denoted as “seeded” plates. After spreading the phage, selected dilutions of $M.\ smeg$ cells were plated. “Unseeded” plates were the control plates for the lysogeny assay. These plates only have dilutions of $M.\ smeg$ cells on the plate. Ten fold serial dilutions (to $10^{-7}$) of P2FF $M.\ smeg$ cells were plated. The entire volume of each dilution set ($10^{-5} \rightarrow 10^{-7}$) was transferred into a culture tube, mixed with Top Agar, and then plated onto either the seeded or unseeded plates. Once solidified, the plates were incubated for 96 hours. Percent efficiency was calculated by counting the colony-forming units (CFU) on the seeded plates and dividing that number by the CFU on the unseeded plates, then multiplying the quotient by 100%.

Creating Lysogens

To create a lysogen, a 2x2 grid was drawn on the bottom of two LA+CB+CHX (see diagram below).

Lawns of WT *M. smeg* were prepared on each plate as previously described. A 10-fold serial dilution (to $10^{8}$) was prepared from the HTL and 5 µL of each dilution ($10^{1} \rightarrow 10^{8}$) was spotted onto the plates. The plates were incubated and monitored daily until mesas (overgrowth of bacterial cells) formed within the phage spots. Cells from the centers of each mesa were streaked onto a LA+CB+CHX plate using the standard streak plate technique. After incubating for four days, colonies were purified by streak plating. As described in “Growth Media for Wild-type *M. smeg*”, P1FF liquid cultures were inoculated from independent colonies in 7H9 Complete *with* Tween80, incubated for 4 days, subcultured in 7H9 Complete *without* Tween80 and then incubated for 4 days. The presence of the prophage was confirmed before the lysogens were archived (Table 1).

Sequential Restriction Digestion of DNA

Vector (pLAM-12) and plasmid DNA from *E. coli* and *M. smeg* clones was digested sequentially with BamHI and NheI restriction enzymes. The first digestion included: BamHI restriction enzyme (final concentration 5 U), elected amount of DNA,
BamHI enzyme buffer (1/10th volume) and quantum sufficient (Q.S.) with sterile water. Reactions were incubated at 37°C for 2 hours. The enzyme and buffer were removed using the QIAquick® PCR Purification Kit (Qiagen Cat. No. 28104), after which a 5 μL aliquot was reserved for gel observation. The second digestion reaction included: NheI restriction enzyme (final concentration 10 U), digested and purified plasmid DNA, NheI enzyme buffer (1/10th volume) and Q.S. with sterile water. Reactions were incubated at 37°C for 2 hours, followed by removal of the enzyme and buffer with the QIAquick® PCR Purification Kit and another 5 μL aliquot reserved for gel observation. To prepare each sample for gel electrophoresis, a 5 μL aliquot of the digest was added to 3 μL of sterile water, 2 μL of 5X Glycerol Gel-Loading dye (Fisher Cat. No. BP645-5) and gently mixed by pipetting. Gel electrophoresis conditions, as well as the preparation of agarose gels are described in “Gel Electrophoresis” below. All samples were stored at -20°C.

**Simultaneous Restriction Digestion of DNA**

Plasmid DNA harvested from *E. coli* and *M. smeg* clones was simultaneously digested with NdeI and NheI restriction enzymes. The reaction consisted of NdeI and NheI restriction enzymes (final concentrations 5 U and 10 U, respectively), plasmid DNA, CutSmart NdeI enzyme buffer (1/10th volume) and Q.S. with sterile water. Reactions were incubated at 37°C for 2 hours. After incubation, the enzymes and buffer were removed using the QIAquick® PCR Purification Kit and a 5 μL aliquot was reserved for gel observation. All samples were stored at -20°C.

**Gel Electrophoresis**

Fisher Scientific electrophoresis systems were used to run agarose DNA gels (Fisher Cat. No. FB-SB-710, FB-SBR-1316). Agarose gels were prepared at a
concentration of 0.8% agarose in 40 or 100 mL of 1X Tris-Acetate, EDTA buffer supplemented with ethidium bromide (EtBr, final concentration 0.625 μg/mL). The 1X Tris-Acetate, EDTA (TAE) buffer was diluted from a 10X stock (Lonza AccuGene™ 10X TAE Buffer, Fisher Cat. No. BMA50844). DNA standards included a 1 kb ladder (NEB Cat. No. N3232S; 500 μg/mL) and/or a 100 bp ladder (NEB Cat. No. N3231S; 500 μg/mL). Both standards were prepared as 10 μL aliquots (final concentration of 1 μg) in EB and 5X glycerol loading dye. Gels were run in 1X TAE buffer at 100V for approximately 120 minutes.

Table 2. Restriction Enzymes

<table>
<thead>
<tr>
<th>Name</th>
<th>Relevant Characteristics</th>
<th>NEB Cat. No.</th>
</tr>
</thead>
<tbody>
<tr>
<td>BamHI</td>
<td>5’ – G / GATCC – 3’; 20,000 U/mL</td>
<td>R0136S</td>
</tr>
<tr>
<td>NheI</td>
<td>5’ – G / CTAGC – 3’; 10,000 U/mL</td>
<td>R0131S</td>
</tr>
<tr>
<td>NdeI</td>
<td>5’ – CA / TATG – 3’; 20,000 U/mL</td>
<td>R0111S</td>
</tr>
</tbody>
</table>

Polymerase Chain Reaction (PCR)

The Phusion® High-Fidelity DNA Polymerase Kit (NEB Cat. No. M0530S) was used for all PCR reactions. Phusion polymerase has enhanced processivity and 50x higher fidelity than Taq polymerase. A typical 20 μL PCR reaction consisted of Phusion GC Buffer (final concentration 1X), Deoxynucleotide (dNTP’s) Solution Mix (final concentration 200 μM each), selected forward and reverse primers (final concentrations 0.5 μM each), Phusion Taq Polymerase (final concentration 2.5 U/μL), template DNA
and sterile water to volume. The Phusion thermocycling conditions were programmed into the Vappoprotect® Mastercycler® (EppendorfAG Cat. No. 6321) and consisted of an initial denaturation at 98°C for 30 seconds followed by 30 cycles of 98°C for 10 seconds, 56°C for 30 seconds, 72°C for 30 seconds, and a final extension of 72°C for 5 minutes. After completing the cycles, the reactions were held at 4°C indefinitely. Table 3 contains the PCR and cloning oligonucleotides used in this work.

Microfiltration

The Amicon® Ultra-0.5 mL Centrifugal Filters (EMD Millipore Cat. No. UFC510096) were used to concentrate DNA samples and facilitate buffer exchanges. For gel extractions, the filter device was placed in the collection tube and 0.4 mL of sterile water was added to the filter. The eluted DNA processed by gel extraction (see “Gel Extraction” below) was transferred into the 0.4 mL sterile water and gently mixed by pipetting. The filtration device was centrifuged at 12,045 RCF for 10 minutes, after which the filtration unit transferred into a clean collection tube. The filter containing the DNA was washed with 0.5 mL sterile water and centrifugation repeated. Before placing into final collection tube, 15 μL of sterile water was carefully added to each filter membrane in the device. The filtration device was then inverted and inserted into a sterile microcentrifuge tube and centrifuged at 6,708 RCF for 2 minutes to recover the DNA retained by the filter. Approximately 50 μL of the sample was recovered. The procedure was also used to microfilter ligation reactions. The filtration device was directly inverted into the final collection tube after the final wash and approximately 20 μL was recovered. All samples were stored at -20°C.
Table 3. Oligonucleotides

<table>
<thead>
<tr>
<th>Name</th>
<th>Sequence 5’→3’</th>
<th>Specifications</th>
</tr>
</thead>
<tbody>
<tr>
<td>MTP-F</td>
<td>acagc&lt;sup&gt;GATCC&lt;/sup&gt;GGTTCTACAAGCGTGTGAC</td>
<td>Used to amplify gp19</td>
</tr>
<tr>
<td>MTP-R</td>
<td>caaga&lt;sup&gt;GCTAGC&lt;/sup&gt;GTGAGGTCGGCACTTG</td>
<td>Used to amplify gp19</td>
</tr>
<tr>
<td>MTP&lt;sub&gt;1&lt;/sub&gt;</td>
<td>GTCGAGCAAGGGTTGCTG</td>
<td>Forward; Confirm gp19</td>
</tr>
<tr>
<td>MTP&lt;sub&gt;2&lt;/sub&gt;</td>
<td>CGATAGGCACTGATGGTGC</td>
<td>Forward; Confirm gp19</td>
</tr>
<tr>
<td>MTP&lt;sub&gt;3&lt;/sub&gt;</td>
<td>GACGTCGGCGAACAAGGTC</td>
<td>Forward; Confirm gp19</td>
</tr>
<tr>
<td>gp42-F</td>
<td>gata&lt;sup&gt;CATATG&lt;/sup&gt;TTGAGACAAAACG</td>
<td>Used to amplify gp42</td>
</tr>
<tr>
<td>gp42-R</td>
<td>gate&lt;sup&gt;GCTAGC&lt;/sup&gt;CATGTTCTG</td>
<td>Used to amplify gp42</td>
</tr>
<tr>
<td>gp43-F</td>
<td>ttca&lt;sup&gt;GATCC&lt;/sup&gt;CGGCTACTAGGGG</td>
<td>Used to amplify gp43</td>
</tr>
<tr>
<td>gp43-R</td>
<td>cccag&lt;sup&gt;GCTAGC&lt;/sup&gt;CTTTTCTTCGCTG</td>
<td>Used to amplify gp43</td>
</tr>
<tr>
<td>gp44-F</td>
<td>ctt&lt;sup&gt;GATCC&lt;/sup&gt;CAAGCGGTTGCGGTA</td>
<td>Used to amplify gp44</td>
</tr>
<tr>
<td>gp44-R</td>
<td>gtaat&lt;sup&gt;GCTAGC&lt;/sup&gt;CACGCAGCGGTAAG</td>
<td>Used to amplify gp44</td>
</tr>
<tr>
<td>P&lt;sub&gt;LaM&lt;/sub&gt;F&lt;sub&gt;1&lt;/sub&gt;</td>
<td>CCGCAGTTGTTCGCTAC</td>
<td>Confirm vector/inserts</td>
</tr>
<tr>
<td>P&lt;sub&gt;LaM&lt;/sub&gt;F&lt;sub&gt;2&lt;/sub&gt;</td>
<td>CGGTCATCGCAGCGTA</td>
<td>Confirm vector/inserts; GeneWiz sequencing gp19</td>
</tr>
<tr>
<td>P&lt;sub&gt;LaM&lt;/sub&gt;R&lt;sub&gt;1&lt;/sub&gt;</td>
<td>CGGCCTCGAGCAAGAC</td>
<td>Confirm vector/inserts</td>
</tr>
<tr>
<td>P&lt;sub&gt;LaM&lt;/sub&gt;R&lt;sub&gt;2&lt;/sub&gt;</td>
<td>GATTGCCGCCGACATTATCGCG</td>
<td>Confirm vector/inserts; GeneWiz sequencing gp19</td>
</tr>
</tbody>
</table>

For cloning primers, lowercased segments were randomly chosen nucleotides. The restriction site is incorporated into the oligonucleotide and are highlighted as follows: **BamHI, NheI, NdeI.**
Gel Extraction (Phenol-frozen Method)

To purify DNA from agarose gels, two alternative procedures were used (73). After running DNA on an agarose gel (supplemented with EtBr), the gel was placed on a UV light table and a sterile razor was used to cut out the desired bands. The gel fragments were carefully transferred into microcentrifuge tubes and then chopped into smaller pieces inside the microcentrifuge tube before adding 0.9 mL of equilibrated phenol (Affymetrix Cat. No. J75829). After thoroughly blending, the gel-phenol mixture was frozen at -80ºC for 10 minutes then immediately centrifuged at 12,045 RCF for 10 minutes. The aqueous phase containing the DNA was transferred into a sterile microcentrifuge tube. Three successive chloroform wash steps were performed by adding an equal volume to the aqueous phase followed by centrifugation at 12,045 RCF for 30 seconds. After the final chloroform wash, 1/10th volume of 3 M Na(C₂H₃O₂)₂, pH 5.2 and 2 volumes of cold (4ºC) 100% ethanol was added to the aqueous phase. Typically, the solution was allowed to incubate at -20ºC overnight. The next day, a precipitate was formed by centrifuging the sample at 11,337 RCF for 30 minutes at 4ºC. The supernatant was decanted and the invisible pellet air-dried briefly before re-suspending in 0.1 mL EB. Samples were concentrated and purified as described in “Microfiltration”.

Ligations

T4 DNA Ligase (NEB Cat. No. M0202S) was used for each ligation. A typical 20 µL ligation reaction consisted of 2 µL of the 10X T4 DNA Ligase Reaction Buffer (NEB Cat. No. B0202S), 1 µL of T4 DNA Ligase, purified insert and/or vector and Q.S. with sterile water. The amount of each insert was estimated by examining the purified
fragments on agarose gels. Equamolar amounts of vector and insert were mixed in each ligation reaction. A vector only control ligation reaction was also performed. Ligations were incubated at 14°C overnight, and then inactivated by heating at 65°C for 20 minutes. After heat inactivation, the ligation buffer was removed by ultrafiltration (see “Microfiltration”). The recovered volume (approximately 20 µL) was placed in the Labconco Centrivap Concentrator (Centrivap Cat. No. 7810000, Centrivap Rotor Cat. No. 745500) to further concentrate the sample to roughly 10 µL. All samples were stored at -20°C.

**Plasmid DNA Isolation**

Plasmid DNA was isolated from 5 mL cultures of saturated cellular growth from both *M. smeg* and *E. coli* transformants. A 5 mL culture was centrifuged at 1,299 RCF for 10 minutes at 4°C. The supernatant was discarded and the pellet was re-suspended in 2.5 mL 10 mM MgSO₄ (Appendix II-Gi). One mL of the cell suspension was transferred into a sterile microcentrifuge tube and centrifuged at 4,293 RCF for 15 minutes at 4°C. The supernatant was carefully decanted and an additional 1 mL aliquot of the cell suspension was added into the tube containing the pellet. The centrifugation was repeated and the supernatant discarded. Plasmid DNA was isolated using the QIAprep® Spin Miniprep Kit (Qiagen Cat. No. 27104) following the manufacturer’s protocol. Briefly, the cell pellet was re-suspended and heavily vortexed in 0.25 mL of Buffer P1 (which contains RNase A and LyseBlue); 0.25 mL of Buffer P2 was added and mixed by inverting 4-6 times; 0.35 mL of Buffer N3 was added and mixed by inverting until the solution was neutralized and the LyseBlue reagent was colorless. The solution was centrifuged at 11,337 RCF for 10 minutes, after which 0.8 mL of the supernatant was
transferred into a DNA binding column in a microcentrifuge tube. The column was centrifuged at 11,337 RCF for 60 seconds then washed with 0.5 mL of Buffer PB and repeated centrifugation. A second wash was done with 0.75 mL of Buffer PE followed by centrifugation. A final centrifugation for 60 seconds was done prior to placing the column in a sterile microcentrifuge tube. The plasmid DNA was eluted by pipetting 50 µL EB onto the surface of the column followed by centrifugation. The flow through contained purified plasmid DNA. All plasmid DNA samples were stored at -20ºC.

**Plasmid Vector DNA Isolation (pLAM-12)**

Strain RK1291 was streaked onto an LB+Kan plate and incubated overnight. A 5 mL overnight culture was prepared as described in “Growth Media for *E. coli*”. The overnight culture was diluted 1:1000 in 100 mL LB Broth+Kan and incubated for 24 hours. The next day, the culture was divided into 50 mL conical centrifuge tubes. Plasmid DNA was harvested using the QIAfilter Midi Kit (Qiagen Cat. No.12243) according to the manufacturer’s instructions with the exception of the last two steps. After isopropanol was added to precipitate the DNA, 1 mL aliquots were transferred into microcentrifuge tubes and centrifuged at 11,337 RCF for 30 minutes at 4ºC. The supernatants were discarded and the tubes were drained by inverting them onto a clean paper towel for 5 minutes to remove any residual isopropanol. The DNA was re-dissolved by serially transferring 100 µL of EB from tube-to-tube. To improve DNA recovery, a second serial transfer was performed using 50 µL EB. The final microcentrifuge tube contained approximately 150 µL of vector DNA. Vector DNA was stored at -20ºC.
**Plasmid Sequencing**

To confirm the sequence of the DNA constructs generated during this study, purified plasmid DNA was sequenced by GeneWiz. The size of the DNA insert in the gp19 clone required multiple sequencing reactions to obtain the full sequence of the cloned DNA. Forward primers MTP<sub>1</sub>, MTP<sub>2</sub> and MTP<sub>3</sub> were designed to be roughly 700 base pairs apart. Vector specific forward and reverse oligonucleotides P<sub>PLAMF</sub><sub>2</sub> and P<sub>PLAMR</sub><sub>2</sub> were also used to verify the orientation of the sequence within the vector. Primer sequences are listed in Table 3. Per GeneWiz sequencing submission guidelines, plasmid DNA for each construct was calculated to 0.8 µg and sequencing primers were added to a final concentration of 5 µM.

**Preparing *M. smeg* Electrocompetent Cells**

*M. smeg* electrocompetent cells were prepared according to Broussard (52) with the following modifications: a 50 mL P1FF culture in 7H9 complete *with* Tween80 was prepared in a sterile non-baffled flask and incubated for approximately two days. In order to obtain an OD<sub>600</sub> of 0.8, 50 mL overnight cultures were prepared by staggering the inoculation volumes of the P1FF culture and the incubation start/end times. These subcultures were prepared using 7H9 complete *with* Tween80 in a sterile non-baffled flask. The next day, the OD<sub>600</sub> was checked until one of the cultures reached 0.8. The cells were transferred into a 50 mL tube and incubated on ice for 1 hour. After incubation, the tubes were centrifuged at 3,000 RCF for 10 minutes at 4°C. The supernatant was carefully discarded and the cells were washed with ice-cold 10% glycerol (Appendix II-Ai) to remove salts. Three wash steps were performed using 1/2 volume, 1/4 volume and 1/8 volume of ice-cold 10% glycerol, respectively. After the
final wash, the pellet was re-suspended in 3 mL ice cold 10% glycerol and aliquotted in sterile microcentrifuge tubes (100 μL/tube). The competent cells were stored at -80°C until further use.

Preparing *E. coli* Electrocompetent Cells

To prepare *E. coli* electrocompetent cells, a 5 mL overnight culture was prepared in LB broth and incubated. The next day, the overnight culture was diluted 1:100 in LB in a sterile non-baffled flask. The culture was incubated until the OD$_{600}$ = 0.4. After reaching OD$_{600}$ = 0.4, the culture was equally divided into 2 – 50 mL tubes. The tubes were centrifuged for 3,000 RCF for 10 minutes at 4°C. The supernatants of each tube were immediately decanted and the pellets re-suspended with ice-cold sterile water. Two wash steps were performed. For the first wash, an equal volume of ice-cold sterile water was used. For the second wash, 1/2 volumes of ice-cold sterile water used. After the final wash, the pellets were re-suspended with 1.3 mL ice-cold 10% glycerol and transferred into pre-chilled (on ice) microcentrifuge tubes. The microcentrifuge tubes were centrifuged at 5,000 RCF for 10 minutes at 4°C, after which the supernatants were carefully discarded. Each pellet was re-suspended thoroughly with 0.3 mL ice-cold 10% glycerol and aliquotted into sterile microcentrifuge tubes (50 μL/tube), then stored at -80°C until further use.

Electroporation into *M. smeg*

All cells and materials were maintained on ice during this procedure. The electroporation for *M. smeg* was performed using slight modifications to Broussard’s protocol (52). DNA was calculated to a final concentration of 150 ng for each sample. The *M. smeg* electrocompetent cells were removed from -80°C and incubated on ice for
10 minutes. DNA was added to the cells and mixed by gently pipetting. The DNA and cells were incubated on ice for 10 minutes while the electroporation conditions were entered into the BioRad Gene Pulser Xcell™ Electroporation System (BioRad Cat. No. 165-2660) PC module. For *M. smeg*, electroporation conditions were as follows: voltage 2.5kV, capacitance 25 μF, and resistance 1000 Ω. After the 10 minute incubation, the DNA and cells were transferred into a Bulldog 2mm Electroporation Cuvette (Fisher Cat. No. NC04493637), placed into the ShockPod and electroporated. Time constants averaged between 18 – 19 milliseconds. For recovery, the electroporated cells were transferred into a 15 mL tube with 1 mL of 7H9 complete with Tween80 and incubated at 37°C, with shaking at 250 rpm for 2 hours. Afterwards, the cells were spread on selective media plates in increments of 1 μL, 5 μL and 50 μL then incubated for 3-4 days. Transformants were subjected to one round of purification by streaking onto appropriate selective media plates. Liquid cultures (P1FF and P2FF) were prepared as previously described in “Growth Media Modifications for *M. smeg* Clones”. P2FF liquid cultures were used in phage spot plating experiments. P1FF liquid cultures were used for archiving and harvesting plasmid DNA (see “Plasmid DNA Isolation”).

**Electroporation into *E. coli***

All cells and materials were maintained on ice during this procedure. The cells and DNA mixture were transferred into a Bulldog 2 mm cuvette and electroporated using the BioRad Gene Pulser Xcell™ Electroporation System programmed with the following conditions: voltage 2.5kV, capacitance 25 μF, and resistance 200 Ω. Time constants were typically 5.1 milliseconds. For recovery, the electroporated cells were re-suspended in 1 mL Super Optimal Broth with Catabolite (SOC) Repression (Appendix I-L),
transferred to a 15 mL tube and incubated at 37°C, with shaking at 250 rpm for 1.5 hours. Afterwards, the cells were spread onto selective media in increments of 50 µL, 100 µL and 150 µL then incubated for 24 hours. Transformants were subjected to one round of purification by streaking onto appropriate selective media plates. Liquid cultures were prepared as described in “Growth Media for E. coli”. Plasmid DNA was isolated from overnight liquid cultures (see “Plasmid DNA Isolation”) to use in further experiments.

**Phenotypic Microarray Analysis (Biolog)**

Phenotypic microarray analysis was performed according to the manufacturers protocol (Biolog, Inc.). Frozen stocks of WT *M. smeg* and lysogenic strains RK1421 and RK1422 were each streaked onto a LA+CB+CHX plate and incubated for 4 days. Two different phenotypic microarray additive solutions (“PM Additive Solutions”) were prepared for the Phenotypic MicroArray™ (referred to as “PM plates”) Panels. PM 1 and 2A Carbon Utilization Assays (Biolog Cat. No. 12111, 12112) require PM Additive Solution 1 (Appendix II-M). PM 3B Nitrogen Utilization Assay (Biolog Cat. No. 12121) and PM 4A Phosphorus and Sulfur Utilization Assay use PM Additive Solution 3 (Appendix II-N). The plates were removed from incubation and immediately scraped with a sterile swab dipped in 1.2% Tween80 (Appendix II-Di) and mashed along the side of a 50 mL tube. The cells were suspended in 20 mL of Inoculating Fluid IF-0a GN/GP Base (Biolog Cat. No. 72268) and mixed thoroughly by gently pipetting. The OD$_{600}$ of the cell suspension was measured to match that of the 85%T Turbidity Standard (Biology Cat. No. 3441). For PM 1 and 2A, the following were mixed into another 50 mL tube: 1.76 mL cell suspension, 2 mL of the PM Additive Solution 1, 0.24 mL Redox Dye Mix G (Biolog Cat. No. 74227) and 20 mL of IF-0a GN/GP Base. PM 3B was prepared by
mixing: 0.88 mL cell suspension, 1 mL of the PM Additive Solution 3, 0.12 mL Redox Dye Mix G and 10 mL of IF-0a GN/GP Base. PM 4A was prepared the same as PM 3B in a separate 50 mL tube. Plates were inoculated with the mixtures (100 μL/well) and incubated at 37°C. If the cells metabolize the nutrient implanted in the well of the plate, the tetrazolium present in the Redox dye turned purple as a result of a reduction reaction. Every 24 hours for 4 days, the plates were removed from incubation and color changes were visibly noted, then the OD$_{595}$ recorded on a Molecular Devices SpectraMax 340PC Microplate Spectrophotometer (Marshall Scientific Cat. No. MD-S340). After readings, the plates were returned to incubation until the 4th day, where no significant color changes or readings were recognized and the plates were discarded.
RESULTS

The Science Education Alliance – Phage Hunters Advancing Genomics and Evolutionary Science (SEA-PHAGES) program provides research-based curriculum to novice students early in their science career. Through the SEA-PHAGES program, student Cole Blair purified MooMoo from an environmental sample, characterized the morphological features by electron microscopy and analyzed the phage DNA with restriction enzyme digestion. The genomic DNA was eventually sequenced and bioinformatics analysis established that MooMoo was a singleton indicating no appreciable genetic homology with other mycobacteriophages. Because of the novelty of this phage, the goal of this project was to further characterize MooMoo, by exploring its ability for form lysogens, its effect on host physiology and by identifying key regulatory genes.
MooMoo Plaque Phenotype Variation and Purification

Plaques are the clearings formed on lawns of infected bacterial cells. They can vary in size and clarity depending on the phage infection pathway (lytic vs. lysogenic) and plating conditions (e.g. temperature). MooMoo is a temperate phage resulting in turbid, bulls-eye plaques (Figure 8A). However, during the phage growth process, I discovered a clear plaque variant on a plate with confluent turbid plaques (Figure 8B). The clear plaque mutant was purified as described in Materials and Methods under the heading “Plaque Assays for Purifications”. Three rounds of purifications resulted in a homogenous population of the clear plaque mutant phage. The WT turbid plaque forming phage was named MooMoo-T (T = turbid plaque phenotype) and the clear plaque forming mutant phage was name MooMoo-C (C = clear plaque phenotype). MooMoo-T and MooMoo-C 1PL’s and HTL’s were prepared as detailed in Materials and Methods under the sections “Preparing 1-Plate Lysates (1PL)” and “Preparing High Titer Lysates”. The results of 1PL titers for both MooMoo-T and MooMoo-C were $1 \times 10^{11}$ PFU/mL. The HTL titer for MooMoo-T was $1.7 \times 10^{11}$ PFU/mL and MooMoo-C was $1.8 \times 10^{11}$ PFU/mL.
Figure 8A-B. Plaque phenotype identification and purification. A) Individual purified plaques of MooMoo-T plated from the HTL. B) Left side: *Representation of how the clear plaque variant (arrow) appeared on confluent plate prior to purification; Right side: Individual purified plaques of MooMoo-C plated from the HTL. (*Images of the original plate of confluent plaques containing the clear plaque were not taken; this image was obtained by plating 10-fold serial dilutions of the HTL of MooMoo-T.)
**MooMoo-T and MooMoo-C Sequence Comparisons**

In 2012, the Pittsburgh Bacteriophage Institute completed the whole genome sequencing of MooMoo-T (GenBank MH001449.1). Since MooMoo-T typically forms turbid plaques, I sought to identify the mutation responsible for the clear plaque phenotype of MooMoo-C. Genomic DNA for both MooMoo-T and MooMoo-C was isolated and the samples were sequenced as detailed in the Materials and Methods under the sections “Phage DNA Isolation” and “Whole Genome Sequencing”. The MooMoo-T and MooMoo-C genomic DNA sequence alignments revealed a 1 base pair (bp) mutation in MooMoo-C at genome coordinate 21,421. The mutation occurs in gene 19 (gp19) that encodes the minor tail protein gene. (Figure 9A). The thymine to cytosine mutation (T→C) causes a Serine to Proline (Ser→Pro) substitution in the protein sequence (Figure 9B).
Figure 9A-B. MooMoo-T and MooMoo-C sequence comparisons. A) Top: Cropped image from Phage Evidence Collection And Annotation Network (PECAAN) illustrating the minor tail protein gene (name MTP) with outlined mutation in the dark red box. Bottom: Cropped image from Phamerator illustrating the sequencing alignment between MooMoo-T and MooMoo-C and the mutation. A dark red line between the cropped images shows where the mutation occurs on both the top and bottom image. B) Ancillary image illustrating the Ser→Pro substitution between MooMoo-T and MooMoo-C.
Transmission Electron Microscopy

Since the sequencing results indicated the mutation responsible for the clear plaque phenotype occurred in the minor tail protein, electron microscopy was used to determine if morphological differences existed between MooMoo-C and MooMoo-T phage particles. Analysis of the images revealed no gross morphological differences in capsid or tail structures between MooMoo-T and MooMoo-C (Figure 10A-B).

Figure 10A. MooMoo-T TEM images magnified 50,000x; Capsid size = 113 nm x 25 nm, Tail length = 225 nm.
Figure 10B. MooMoo-C TEM images magnified 50,000x; Capsid size = 113 nm x 25 nm, Tail length = 225 nm
MooMoo-T and MooMoo-C Lysogeny Assays

The phage lysogeny assays performed on MooMoo-T and MooMoo-C HTL’s to determine how well each phage will form lysogens in a susceptible host. Both MooMoo-T and MooMoo-C HTL’s were diluted in PB to $10^{10}$ PFU/mL to ensure each seeded plate was spread with the same amount of phage. The amount of HTL and PB were calculated as follows:

<table>
<thead>
<tr>
<th></th>
<th>MooMoo-T – Diluted to $10^{10}$ PFU/mL</th>
<th>MooMoo-C – Diluted to $10^{10}$ PFU/mL</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vol. HTL (mL)</td>
<td>$\frac{1 \times 10^{10} \text{ PFU/mL}}{1.7 \times 10^{11} \text{ PFU/mL}}$</td>
<td>$\frac{1 \times 10^{10} \text{ PFU/mL}}{1.8 \times 10^{11} \text{ PFU/mL}}$</td>
</tr>
<tr>
<td></td>
<td>$= 0.0588$ mL (59 $\mu$L)</td>
<td>$= 0.0555$ mL (55.5 $\mu$L)</td>
</tr>
<tr>
<td>Vol. PB (µL)</td>
<td>$1000 \mu$L - $(1000 \mu$L - 59 $\mu$L)</td>
<td>$941 \mu$L - $(1000 \mu$L - 55.5 $\mu$L)</td>
</tr>
<tr>
<td></td>
<td>$= 941 \mu$L</td>
<td>$= 944.5 \mu$L</td>
</tr>
</tbody>
</table>

Three trials (Trials 1 – 3) were performed and average efficiencies are reported in Table 4. For each trial, colony forming units (CFU) from the same dilution of *M. smeg* cells on seeded and unseeded countable plates were used to calculate the percent efficiency. Figure 11A-B demonstrates one of the plating trials.

<table>
<thead>
<tr>
<th>Trial</th>
<th>MooMoo-T</th>
<th>MooMoo-C</th>
</tr>
</thead>
<tbody>
<tr>
<td>Trial 1</td>
<td>89.10%</td>
<td>4.81%</td>
</tr>
<tr>
<td>Trial 2</td>
<td>79.17%</td>
<td>6.58%</td>
</tr>
<tr>
<td>Trial 3</td>
<td>82.32%</td>
<td>1.24%</td>
</tr>
<tr>
<td>Avg.</td>
<td>83.53%</td>
<td>4.21%</td>
</tr>
</tbody>
</table>
Figure 11A-B. CFU’s on countable plates used to determine lysogeny efficiency. Plates are shown with *M. smeg* cells that were diluted to $10^{-6}$. A) MooMoo-T seeded and unseeded countable plates (CFU’s on seeded plate = 652, CFU’s on unseeded plate = 792). B) MooMoo-C seeded and unseeded countable plates (CFU’s on seeded plate = 8, CFU’s on unseeded plate = 645).
Phage Spot Plating on Lysogenic Strains

To confirm that MooMoo-T or MooMoo-C prophages were present in the presumed lysogens, 10-fold serial dilutions of MooMoo-T and MooMoo-C HTL’s were prepared and 5 µL aliquots of each dilution were spotted onto lawns of each lysogen. The plates were incubated overnight and examined the next day. There were no spots present on either plate (Figure 12A-B), indicating one of two formal possibilities: 1) the phage growth was blocked due to the presence of the prophage, or 2) the bacterial cells were resistant to phage infection. Further testing was necessary to confirm if the cells were lysogenic.
Figure 12A-B. Lysogens prevent superinfection by MooMoo-T or MooMoo-C.  A) Putative lysogen purified from MooMoo-T mesa was negative for MooMoo-T and MooMoo-C growth. B) Putative lysogen purified from MooMoo-C mesa was negative for MooMoo-T and MooMoo-C growth. The dilutions of each lysate are indicated on the plates.
Mitomycin C Treatment Does Not Induce Resident Prophages

Since lysogeny could not be confirmed solely on the absence of phage spotting, I attempted to induce phage release with mitomycin C. Mitomycin C creates lesions in the bacterial DNA, which signals that the cell’s DNA is critically damaged (76). The signal of DNA damage activates the SOS response pathway, a pathway leading to cleavage of the phage repressor and expression of phage lytic genes (76). Duplicated dilutions of the cultures were prepared (1:1 and 1:4 dilutions) in 7H9 Neat (Appendix I-A). The purpose of duplicating the dilutions was to use one as the control (no mitomycin C) and the other as the test (added mitomycin C). Table 6 shows the results of mitomycin C induction. The cultures were labeled as either MT (MooMoo-T) or MC (MooMoo-C) for the resident prophage and with A (1:1) or B (1:4) to indicate the dilution. Cultures that were supplemented with mitomycin C were labeled with “myc”. Mitomycin C stock (Appendix III-D) was added to the final concentration of 2 µg/mL and all cultures were incubated at 37ºC, with shaking at 250 rpm for 24 hours. After growth, the cultures were centrifuged at 3,000 RCF for 15 minutes at 4ºC. The supernatant of each sample was filter-sterilized into a 15 mL tube with a GE Healthcare Whatman™ Puradisc 0.2 µM syringe filter. A 10-fold serial dilution (to 10⁻³) was prepared for each filtrate and 5 µL of each dilution was spotted onto lawns of WT M. smeg host cells. All plates were incubated overnight at 37ºC. The cultures incubated with mitomycin C showed no evidence of lytic activity. The single plaque that appeared in the highest concentration is likely a contaminant or a spontaneously released phage. Control cultures (no mitomycin C) showed significantly more plaques compared to the cultures that received mitomycin C indicating that phage were spontaneously released (Figure 13A-B). The lack of
plaques from the mitomycin C treated cultures indicates MooMoo is not inducible by this treatment. This evidence, coupled with the results of the superinfection immunity assay, indicates that these cells do contain resident prophages. Cultures of the lysogens were archived in Mycobacterium Freezing Media (Appendix II-Q) as strains RK1421 and RK1422, respectively.

Table 5. Mitomycin C induction test

<table>
<thead>
<tr>
<th>Dilution</th>
<th>Name</th>
<th>Mitomycin C Added</th>
<th>Plaques</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1:1</td>
<td>MTAmyc</td>
<td>Yes</td>
</tr>
<tr>
<td>2</td>
<td>1:1</td>
<td>MTA</td>
<td>No</td>
</tr>
<tr>
<td>3</td>
<td>1:4</td>
<td>MTBmyc</td>
<td>Yes</td>
</tr>
<tr>
<td>4</td>
<td>1:4</td>
<td>MTB</td>
<td>No</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Dilution</th>
<th>Name</th>
<th>Mitomycin C Added</th>
<th>Plaques</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1:1</td>
<td>MCAmyc</td>
<td>Yes</td>
</tr>
<tr>
<td>2</td>
<td>1:1</td>
<td>MCA</td>
<td>No</td>
</tr>
<tr>
<td>3</td>
<td>1:4</td>
<td>MCBmyc</td>
<td>Yes</td>
</tr>
<tr>
<td>4</td>
<td>1:4</td>
<td>MCB</td>
<td>No</td>
</tr>
</tbody>
</table>

*1 plaque present
Figure 13A-B. Mitomycin C treatment and confirmation of resident prophage. (myc = mitomycin C added)  
A) Filter-sterilized supernatants from MooMoo-T lysogens.  B) Filter-sterilized supernatants MooMoo-C lysogens. Plaques produced by spontaneous phage release are highlighted with yellow circles or arrows. The green arrow points to the single plaque observed in a culture that received mitomycin C.
**Cloning and Analysis of Selected MooMoo Genes**

To facilitate the cloning and analysis of selected MooMoo phage genes, a shuttle vector that allowed controlled expression of the cloned gene was utilized. The pLAM-12 shuttle vector (Figure 14) is a high copy plasmid with an acetamidase promoter and a Kanamycin (KanR) resistance gene. The pLAM-12 (vector) DNA was purified as detailed in “Plasmid Vector DNA Isolation (pLAM-12)”. The desired MooMoo genes were amplified by PCR, digested and inserted between the promoter and the KanR gene using BamHI or NdeI coupled with NheI restriction enzyme cut sites (Figure 14). To prepare the vector, the plasmid DNA was digested either sequentially with BamHI then NheI or simultaneously digested with NdeI and NheI. Restriction enzyme digestions were verified on an agarose gel (Figure 15). The BamHI-NheI and the NdeI-NheI digested DNA’s were gel purified. A released fragment was visible during gel extractions due to the increased concentration of DNA loaded for each vector (Figure 16A-B). The gel-purified vectors were analyzed on agarose gels to confirm quality and estimate quantity for ligations (Figure 17A-B).
Figure 14. Schematic of the pLAM-12 shuttle vector. The vector size is 6,758 bp. Arrows for BamHI, NdeI and NheI restriction sites show their relative locations.

Figure 15. Restriction enzyme digestions on the pLAM-12 vector DNA. Lane 1 = 1 kb DNA Ladder, Lane 2 = Control (undigested vector DNA), Lane 3 = vector DNA digested with BamHI, Lane 4 = BamHI cut vector DNA after digesting with NheI, Lane 5 = vector DNA simultaneously digested with NdeI and NheI.
Figure 16A-B. Pre-extraction agarose gels of digested pLAM-12 vector. Orange boxes highlight the extracted bands present between the 6 kb and 10 kb ladder marker. Green boxes highlight the released fragment (expected band size approximately 150 bp). A) Vector DNA digested with BamHI and NheI. B) Vector DNA digested with NdeI and NheI.
Figure 17A-B. Analysis of the purified and digested pLAM-12 vector by agarose gel electrophoresis. A) BamHI and NheI digested vector (expected band = 6.7 kb). B) NdeI and NheI digested vector (expected band = 6.7 kb).
PCR Amplification of Candidate Phage Genes

Based on bioinformatic predictions several candidate genes (gp42, gp43 and gp44) were selected as the potential phage repressor. Oligonucleotides for amplifying gp43, gp44 and gp19 were designed with incorporated restriction enzyme sites for BamHI and NheI. Primers for gp42 were designed to include NdeI and NheI (Table 3). The genes were amplified from phage genomic DNA and verified on an agarose gel (Figure 18). PCR products for gp42, 43 and 44 were purified and concentrated using the QIAquick® PCR Purification Kit. The gp19 gene amplified from MooMoo-T and MooMoo-C was gel purified.
Figure 18. Analysis of the PCR amplified MooMoo genes 19, 42, 43 and 44 via agarose gel electrophoresis. Lane 1 = 1kb DNA Ladder, Lane 2 = Negative control (PCR Master Mix without DNA), Lanes 3 & 5 – 7 contain genes amplified from MooMoo-T genomic DNA in the following order: Lane 3 = PCR products amplified with primer set MTP-F/R (expected band size = 3033 bp), Lane 5 = PCR products amplified with primer set gp43-F/R (expected band size = 433 bp), Lane 6 = PCR products amplified with primer set gp44-F/R (expected band size = 998 bp) and Lane 7 = PCR products amplified with primer set gp42-F/R (expected band size = 610 bp). Lane 4 = PCR products amplified from MooMoo-C genomic DNA using primer set MTP-F/R (expected band size = 3033 bp). Lane 8 = 100 bp DNA Ladder.
Cloning of Amplified Phage Genes into pLAM-12 Vector and Electroporation into *E. coli*

The PCR products described above were digested similarly to the pLAM-12 vector. For gp19 (WT and mutant versions), gp43 and gp44, the PCR amplified DNA was digested sequentially with BamHI then NheI. For gp42, the PCR amplified DNA was digested simultaneously with NdeI and NheI (Figure 19A-B). The vector and inserts were ligated overnight, purified and then electroporated into electrocompetent XL-1 Blue cells. Figure 20A-D illustrates the vector with the correctly inserted gene. The electroporated cells were plated on selective media.
Figure 19A-B. Analysis of the digested PCR products by agarose gel electrophoresis. A) Lane 1 = 1 kb DNA Ladder, Lane 2 = BamHI/NheI sequentially digested WT gp19 (expected band size = 3033 bp), Lane 3 = BamHI/NheI sequentially digested mutant gp19 (expected band size = 3033 bp), Lane 4 = BamHI/NheI sequentially digested gp43 (expected band size = 433 bp), Lane 5 = BamHI/NheI sequentially digested gp44 (expected band size = 998 bp), Lane 6 = 100 bp DNA Ladder. B) Lane 1 = 1 kb DNA Ladder, Lane 2 = NdeI/NheI simultaneously digested gp42 (expected band size = 610 bp), Lane 3 = 100 bp DNA Ladder.
Figure 20A-D. Representations of cloned MooMoo genes. A-D) The restriction enzymes are represented by red (BamHI), green (NdeI) or blue (NheI) rectangles on the left or right sides of the labeled gene.
Purification and Confirmation of Selected *E. coli* Transformants

Kanamycin resistant transformants were selected and purified by streaking onto selective media (Figure 21A-J). Pure colonies were then used to inoculate overnight cultures. The overnight cultures were harvested and plasmid DNA was purified. Each plasmid clone was verified using PCR and restriction analysis (Figure 22-25). The WT and mutant gp19 clones consistently showed a 200 bp band in the PCR amplification products (Figure 22). Restriction analysis of the mutant gp19 clones showed a potential incomplete digestion (Figure 23). This was unexpected since the 3 kb product was gel purified from phage genomic DNA. To determine if plasmid DNA purified from WT and mutant gp19 clones contained the expected cloned genes, the plasmid DNA was sequenced using primers MTP<sub>1</sub>, MTP<sub>2</sub> and MTP<sub>3</sub> to obtain complete coverage of the cloned genes (Figure 26). After sequence verification of the cloned genes, the plasmids were electroporated into *M. smeg* competent cells.
Figure 22. Analysis of the PCR products amplified from the gp19 clones by agarose gel electrophoresis. Orange box highlights non-specific amplification product at 200 bp. PCR products were amplified from purified plasmid DNA clones of WT or mutant gp19 using MTP-F/R primer set; Lane 1 = 1 kb DNA Ladder, Lane 2 = PCR products amplified from WT gp19 clone (expected band size = 3033 bp), Lane 3 = PCR products amplified from mutant gp19 clone (expected band size = 3033 bp), Lane 4 = 100 bp DNA Ladder.
Figure 23. Restriction enzyme analysis of the plasmid DNA clones of gp19 by agarose gel electrophoresis. Lane 1 = 1 kb DNA Ladder, Lane 2 = Clone of WT gp19-digested with BamHI (expected band size at 9.7 kb), Lane 3 = Clone of mutant gp19 (expected band size at 9.7 kb), Lane 4 = Clone of WT gp19-digested with BamHI then NheI (expected vector band size at 6.7 kb and released band at 3033 bp), Lane 5 = Clone of mutant gp19-digested with BamHI then NheI (expected vector band size at 6.7 kb and released band at 3033 bp; potential incomplete digestion), Lane 6 = 100 bp DNA Ladder.
Figure 24A-B. Analysis of the PCR products amplified from gp42 and gp43 clones and restriction enzyme analysis of the plasmid DNA by agarose gel electrophoresis. Multiple exposures of the same gels are shown to improve the visibility of faint bands. Orange boxes highlight the vector bands, while green boxes highlight the released bands. A) Lane 1 = 1 kb DNA Ladder, Lane 2 = PCR products amplified from plasmid DNA clone of gp42 using PLAM-F1/R1 primer set (expected band size = 837 bp), Lane 3 = plasmid DNA clone of gp42 simultaneously digested with NdeI-NheI (expected vector band size = 6.7 kb and released band = 837 bp). B) Left side gel: Lane 1 = 1 kb DNA Ladder, Lane 2 = PCR products amplified from plasmid DNA clone of gp43 using gp43-F/R primer set (expected band size = 433 bp); Right side gel: Lane 1 = 1 kb DNA Ladder, Lane 2 = Plasmid DNA clone of gp43 sequentially digested with BamHI then NheI (expected vector band size = 6.7 kb and released band = 433 bp).
Figure 25. Analysis of the PCR products amplified from gp44 clones and restriction enzyme analysis of the plasmid DNA by agarose gel electrophoresis. Left side gel: Lane 1 = 1 kb DNA Ladder, Lane 2 = PCR products amplified from plasmid DNA clone of gp44 using P_{LAM-F}/R_{1} primer set (expected band size = 1131 bp); Right side gel: Lane 1 = 1 kb DNA Ladder, Lane 2 = Plasmid DNA clone of gp44 sequentially digested with BamHI then NheI (expected vector band size = 6.7 kb and released band = 1131 bp).
Figure 26. Representation of gp19 gene sequencing coverage on plasmid. The relative locations for the $P_{\text{LAM}}$-F$_2$/R$_2$ primers are noted by the dark pink lines. The relative locations for MTP-F$_1$, MTP-F$_2$ and MTP-F$_3$ primers are marked by the light blue lines. The T→C mutation in mutant gp19 is noted by the purple box.
**M. smeg Transformant Plating and Purification**

Purified plasmid DNA isolated from each clone in *E. coli* was electroporated into *M. smeg* competent cells and plated onto selective media (Figure 27A-F). *M. smeg* was also electroporated with pLAM-12 plasmid DNA without an insert as a control. Kanamycin resistant transformants were purified by streaking onto selective media and afterwards, pure colonies were used to inoculate 20 mL cultures. Unfortunately, only low concentrations and poor quality of DNA was recovered from the *M. smeg* transformants. Attempts to verify the plasmid DNA clones using PCR typically resulted in multiple bands with significant non-specific amplification products (Figure 28-30). Because of the low yield of plasmid, restriction enzyme digestions were rarely seen on agarose gels (Figure 30). However, the recovery of Kanamycin resistant transformants and the rigorous authentication of the constructs isolated from *E. coli*, strongly supports the presence of each clone in *M. smeg*. 
Figure 28. Analysis of the PCR products amplified from the plasmid clone of *M. smeg* (pLAM-12). Lane 1 = 1 kb DNA Ladder, Lane 2 = PCR products amplified from plasmid DNA clone of pLAM-12 (no insert) using P_{LAM-F2}/R_{2} primer set (expected band 503 bp), Lane 3 = 100 bp DNA Ladder.
Figure 29. Comparative analysis of *M. smeg* and *E. coli* PCR products amplified from the plasmid clones. Lane 1 = 1 kb DNA Ladder, Lane 2 = PCR products amplified from *M. smeg* plasmid DNA clone of gp43 using \( P_{\text{LAM}} \)-F\(_2\)/R\(_2\) primer set (expected band size = 614 bp), Lane 3 = PCR products amplified from *E. coli* plasmid DNA clone of gp43 using \( P_{\text{LAM}} \)-F\(_2\)/R\(_2\) primer set (expected band 614 bp), Lane 4 = PCR products amplified from *M. smeg* plasmid DNA clone of gp44 using \( P_{\text{LAM}} \)-F\(_2\)/R\(_2\) primer set (expected band size = 1223 bp), Lane 5 = PCR products amplified from *E. coli* plasmid DNA clone of gp44 using \( P_{\text{LAM}} \)-F\(_2\)/R\(_2\) primer set (expected band 1223 bp).
Figure 30. Analysis of the PCR products amplified from gp42 clones isolated from *M. smeg* and restriction enzyme analysis of the *M. smeg* plasmid construct. Because of the low concentration of DNA isolated from the *M. smeg* cells, the contrast had to be significantly altered to visualize the DNA (boxed in orange). Lane 1 = 1 kb DNA Ladder, Lane 2 = PCR products amplified from the gp42 clone using P\textsubscript{LAM-F2}/R\textsubscript{2} primer set (expected band size = 929 bp), Lane 3 = Simultaneous digestion of gp42 clone with NdeI and NheI (expected vector band size = 6.7 kb and released band = 837 bp).
Verification of Phage Repressor Activity

After successfully electroporating the gp42, gp43, gp44 and gp19 clones into *M. smeg* and purifying the transformants by the streak plate technique, single colonies were chosen and used to inoculate liquid cultures. After growth, lawns were prepared on appropriate media. As a control, a lawn of cells with pLAM-12 (no insert) was also plated. Serial dilutions of MooMoo-T and MooMoo-C lysates were spotted onto the lawns and plates incubated for 24 hours. Evidence of phage infection was present at all the dilutions except when lawns were prepared with the gp42 clones (Figure 31A-F). This evidence strongly supports gp42 is the phage repressor and indicates that the gp42 clone expresses the phage repressor. It also shows that the other clones (gp19, gp43 and gp44) have no effect on the plating of MooMoo-T or MooMoo-C.
Figure 31A-F. Growth of MooMoo-T and MooMoo-C on cells that contain plasmid clones gp19, gp42, gp43 or gp44. A) Positive control Cells with pLAM-12 (no insert) clone. B) Cells with WT gp19 clone. C) Cells with mutant gp19 clone. D) Cells with gp42 clone. E) Cells with gp43 clone. F) Cells with gp44 clone. (Pictures were taken at different dates.)
**Effect of Lysogeny on Host Cell Metabolism**

High-throughput phenotypic microarray analysis was used to determine if the lysogeny of *M. smeg* with MooMoo-T or MooMoo-C (strains RK1421 and RK1422) alters host cell metabolism. No significant differences in cellular metabolism were observed (Figure 32A-C). Table 6 illustrates the results between each lysogen and WT *M. smeg*. 


Figure 32A-C. Phenotypic microarrays (Biolog plate PM1). Red circles = strongly metabolized, green circles = moderately metabolized, yellow circles = weakly metabolized. A) WT *M. smeg*. B) MooMoo-T lysogen (RK1421). C) MooMoo-C lysogen (RK1422).
Table 6. Phenotypic Microarray data.

<table>
<thead>
<tr>
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<th>PM1 &amp; 2A</th>
<th>PM3B</th>
<th>PM4A</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Carbon Sources</strong></td>
<td>78</td>
<td>87*</td>
<td>51</td>
</tr>
<tr>
<td><strong>Nitrogen Sources</strong></td>
<td>87*</td>
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<td>51</td>
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</tr>
<tr>
<td><strong>Sulfur Sources</strong></td>
<td>30*</td>
<td>32*</td>
<td>31*</td>
</tr>
</tbody>
</table>

*Negative control was strongly metabolized
Does gp19 Affect Lysogeny Frequency?

As noted earlier, lysogeny frequencies for MooMoo-C were significantly lower than MooMoo-T. Since the gp19 clones did not appear to affect phage plating (see Figure 31B-C), we asked if the lysogeny frequency might be affected. A different class of mutations that could result in a clear plaque phenotype are those that interfere with the establishment of lysogeny. Studies with other phages show that a number of genes are required for this process, but the involvement of a minor tail protein has never been reported. *M. smeg* gp19 clones were evaluated for potential changes in the lysogeny frequencies when the gp19 gene is expressed from the plasmid. Table 7 summarizes the results. Though there is some slight variation in the frequencies, the results suggest the gp19 clones from MooMoo-T has a lysogeny frequency that is similar to MooMoo-T lysogeny of *M. smeg* host cells that do not carry a plasmid. It is clear that the presence of cloned copies of WT gp19 from MooMoo-T did not compensate for the low lysogeny frequency obtained with MooMoo-C phage.
Table 7. Lysogeny frequencies on *M. smeg* cells that carry cloned gp19 gene from MooMoo-T or MooMoo-C

<table>
<thead>
<tr>
<th></th>
<th>MooMoo-T lysate</th>
<th>MooMoo-C lysate</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>Seeded</td>
<td>Unseeded</td>
</tr>
<tr>
<td>WT gp19 (cells)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Trial 1</td>
<td>98</td>
<td>122</td>
</tr>
<tr>
<td>Trial 2</td>
<td>59</td>
<td>64</td>
</tr>
<tr>
<td>Trial 3</td>
<td>62</td>
<td>74</td>
</tr>
<tr>
<td>Avg.</td>
<td></td>
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<table>
<thead>
<tr>
<th></th>
<th>MooMoo-T lysate</th>
<th>MooMoo-C lysate</th>
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<tbody>
<tr>
<td></td>
<td>Seeded</td>
<td>Unseeded</td>
</tr>
<tr>
<td>Mutant gp19 (cells)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Trial 1</td>
<td>138</td>
<td>171</td>
</tr>
<tr>
<td>Trial 2</td>
<td>420</td>
<td>762</td>
</tr>
<tr>
<td>Trial 3</td>
<td>485</td>
<td>479</td>
</tr>
<tr>
<td>Avg.</td>
<td></td>
<td>69.08%</td>
</tr>
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</table>
DISCUSSION

Roughly 80 years has passed since the fist mycobacteriophage was isolated. To date, over 10,000 mycobacteriophages have been collected and characterized, and over 1700 of these have been completely sequenced (72). The breadth of mycobacteriophage genomic and morphological diversity has been illuminated by the massive amounts of accumulated data obtained through programs such as SEA-PHAGES (81, 82). Research on the methods mycobacteriophages bind, deliver and manipulate their hosts has been exploited to develop multiple applications, including genome engineering techniques and therapeutics. Examples include: assays that can detect the drug susceptibility of tuberculosis, vectors capable of introducing deleterious genes into Mycobacterium and recombineering systems that allow the introduction of point mutations anywhere in the Mycobacterium genome (44). The therapeutic potential of mycobacteriophages to treat pathogenic Mycobacteria is also being actively explored. However, the number of mycobacteriophages that can cross-infect both non-pathogenic M. smeg, the primary host used in the SEA-PHAGES program, and pathogenic species of Mycobacterium is small (44).

In this study, I discovered a clear plaque mutant of a temperate mycobacteriophage called MooMoo. As previously discussed, temperate phages can enter either the lytic or lysogenic phage life cycles. The lysis-lysogenic decision is regulated by the production of the phage repressor protein that blocks early gene expression by binding to stoperator sites along the genome (44). Because phage repressor mutations are a common class of mutations that produce a clear plaque phenotype, we predicted MooMoo-C had suffered a mutation(s) in its repressor gene.
Surprisingly, whole genome sequencing revealed a single base change in a minor tail protein gene, gp19. To identify the repressor gene and to verify that it was not responsible for the observed clear plaque phenotype, several candidate genes whose products could potentially serve as the phage repressor were predicted using bioinformatic tools. Each candidate gene was successfully cloned into a shuttle vector and transformed into *E. coli* for initial characterization. After the constructs were confirmed, the clones were moved into *M. smeg* where the phenotype could be assessed. The pLAM-12 shuttle vector used in this study is also an expression vector where cloned genes are placed under the control of an inducible promoter. This feature allowed us to determine if overexpression of the cloned gene resulted in an observable phenotype. We have shown that cells that contain a cloned copy of the gp42 gene, and no other phage genes, prevented infection of MooMoo-T and MooMoo-C phage. Based on our evidence, we conclude that gp42 is the repressor for MooMoo (51).

As mentioned above, whole genome sequencing of bacteriophage MooMoo revealed that the clear plaque mutant contained a single amino acid substitution in the minor tail protein gene (gp19). The mutation is located downstream of a D-alanyl-D-alanine-carboxypeptidase domain. Phage carboxypeptidase domains form the largest class of enzymatic domains specifically targeting different components of the bacterial cell wall (47). Published data suggests that the D-ala-D-ala-carboxypeptidase motif assists in phage infection by enzymatically destabilizing the cross-linking covalent bonds within peptidoglycan (5). We suggest that the native protein conformation of WT gp19 may occlude the catalytic D-ala-D-ala-carboxypeptidase domain except during the infection process and that the serine → proline substitution in the mutant gp19 may alter
the conformation and expose the enzymatic domain. The “exposed domain” could potentially increase the presumed degradation activity of the tail leading to the cell wall destabilization and cell lysis. This proposed mechanism explains how a mutation in a tail protein could influence the plaque phenotype.

Mycobacteriophage tails play a critical role in host cell recognition, attachment and penetration and are composed of several different proteins (17). Interestingly, genomic analysis of siphoviridae mycobacteriophage genomes shows that some of these genomes contain five to eight minor tail protein genes (17). Experiments with other phages have shown that binding of phage tail fibers to host cell surface receptors triggers profound structural changes within the phage, bringing secondary tail structures (i.e. tail tip) in contact with the cell surface (46). The enzymatic activity in phage tails may induce host cell lysis without phage production, a phenomenon referred to as “lysis from without” (80). There is precedence for enzymatic activity in some mycobacteriophage tails and this activity appears to alter the host cell surface. Mycobacteriophage Bxb1 is a temperate phage that typically forms turbid plaques with impressively large halos on lawns of confluent WT M. smeg (45). A clear plaque mutant of Bxb1 also forms halos that are indistinguishable from the WT phage. However, only the WT, turbid plaque forming phage could establish lysogeny. Viable phages could be isolated from the halos formed by their respective phages but these phages did not appear to be produced from active lytic infections. The genomic analysis of Bxb1 revealed a unique D-ala-D-ala-carboxypeptidase domain in the minor tail protein gene (45). This led the authors to propose that the viable phage particles diffuse through the agar. The authors also proposed the enzymatic activity of the tail modify the cell surface of the non-growing
Mycobacterium cells and alters the appearance of the lawn (e.g. forming a halo; 45). Other examples of enzymatic activities in phage tails have been described in phages from various clusters and subclusters (A1, C1, D, E, F, H1 I1, J and singletons Corndog and Wildcat) where the genomes encoded tail proteins with β-lactamase-like D-ala-D-ala-carboxypeptidase activity that is presumed to be associated with the tail tip (17).

Both MooMoo and Bxb1 are temperate mycobacteriophages. MooMoo-T forms plaques with a halo (noted as “bulls-eye”) and readily lysogenizes WT M. smeg host cells. Since MooMoo gp19 also contains the D-ala-D-ala motif, we performed the Bxb1 halo experiment using MooMoo-T and MooMoo-C phages. Neither MooMoo-T nor MooMoo-C showed any evidence of halo expansion (data not shown). Our evidence suggests that no enzymatic activity, similar to that observed with Bxb1, is associated with MooMoo phage particles.

Several unique properties of mycobacteriophage MooMoo were characterized in this study and are summarized here. The analysis of the MooMoo-T and MooMoo-C phage particles showed that both are morphologically identical (siphoviridae) with prolate capsids (approx. 113 nm long x 25 nm wide) and long (225 nm), flexible tails. This analysis proved that the mutation in the minor tail protein of MooMoo-C does not affect phage morphology.

How the mutation causes the clear plaque phenotype remains unanswered at this time, but our characterization of MooMoo has identified a number of interesting properties. MooMoo-T and MooMoo-C can each form stable lysogens and are immune to both MooMoo-T and MooMoo-C phage superinfection. This information was used to establish the identity and verify the activity of the phage repressor, gp42. MooMoo-T
and MooMoo-C have significantly different lysogeny frequencies (MooMoo-T, 83.53% vs MooMoo-C, 4.21%). It is unclear how a mutation in the minor tail protein gene could influence lysogeny frequency. However, we did show that the presence of the cloned copy of WT gp19 did not alter the lysogeny frequency of either MooMoo-T or MooMoo-C. If gp19 plays a direct role in the observed difference in lysogeny frequencies, we expected the WT copy to compensate for the apparent lysogeny defect displayed by MooMoo-C. A trivial explanation for the lack of complementation is that the cloned copy of gp19 is not expressed. This possibility is explored further below.

Lysogeny can often alter the physiology of the host cell (68, 69, 78). We detected no changes in host cell physiology when M. smeg is lysogenized with either MooMoo-T or MooMoo-C. The absence of an observed effect could be due to inherent limitations of the chosen assay. A more sensitive assay capable of detecting changes in gene expression (e.g. RNAseq or DNA microarray) may be a better approach.

The research described in this study has established the foundation for future work on bacteriophage MooMoo. The next steps in the characterization of this unique bacteriophage include optimizing the lysis of M. smeg cells so that the expression of the cloned genes can be verified by SDS-PAGE. I made several unsuccessful attempts to efficiently lyse M. smeg cells using a number of different procedures. Quantitative plating of cells after sonication proved the published conditions were ineffective at lysing M. smeg cells (48, 49, 50). However, the E. coli cells that were tested as a control were readily lysed and produced consistent banding patterns on SDS-PAGE gels (data not shown). In the absence of a demonstrable and measureable phenotype, the optimization
of cell lysis conditions will be necessary to conclusively prove that the plasmid clones do indeed express the gp19 protein.

Another important experiment would be to delete gp19 from the MooMoo-T genome to see if its absence affects phage morphology, plaque phenotype and lysogeny frequency. MooMoo-T and MooMoo-C form distinct and different plaque phenotypes (turbid and clear, respectively), but the phage particles are morphologically indistinguishable. Minor tail genes have important roles in mediating phage tail assembly. Published reports have shown that mycobacteriophages (e.g. TM4) that suffer deletions of similar genes have shortened tails (17, 79). I hypothesize that deleting gp19 from mycobacteriophage MooMoo will result in a clear plaque forming phage with a shortened tail.

Lastly, the mosaic nature of MooMoo’s genome suggests that it acquired genes from other phages and/or hosts. To gain insight into MooMoo’s evolutionary history, it would be instructive to determine the host range of mycobacteriophage MooMoo by plating it on a variety of Actinomycetales hosts (i.e. Gordonia spp.). Recent publications have shown that expanded host range mutants can be recovered and that the responsible mutations occur in the phage tail proteins (5, 13).

MooMoo is an enigma. Sequenced singleton mycobacteriophages make up less than 5% of the mycobacteriophage population in the database (72). More singleton bacteriophages need to isolated and further characterized to expand the genomic richness within singleton bacteriophage populations. The research described in this study has provided an important insight into a unique mycobacteriophage and has set the stage for further exploration.


72. The Actinobacteriophage Database at PhagesDB.org. 2010. Online database for access to protocols, data, research about phages infecting hosts in the Phylum Actinobacteria. phagesdb.org/workflow/FurtherDiscovery/


APPENDIX I

Medias

A. 7H9 Neat stock (per 900 mLs): 4.7 g Difco\textsuperscript{TM} Middlebrook 7H9 Broth Base (Fisher Cat. No. DF0713-17-9), 5 mL of 40\% glycerol (Appendix II-A) and Q.S. with sterile water. The media was aliquotted into bottles (100 mL/bottle) then autoclaved. 7H9 Neat stock is stored at room temperature.

B. 7H9 Complete without Tween80: 7H9 Neat stock (final concentration 1X; see above), AD Supplement (final concentration 10\%; Appendix II-B), CB (final concentration 50 μg/mL; Appendix III-A), CHX (final concentration 10 μg/mL; Appendix III-B), 100 mM CaCl\textsubscript{2} (final concentration 1 mM; Appendix II-Ci).

C. 7H9 Complete with Tween80: 7H9 Neat stock (final concentration 1X), AD Supplement (final concentration 10\%), CB (final concentration 50 μg/mL), CHX (final concentration 10 μg/mL), 100 mM CaCl\textsubscript{2} (final concentration 1 mM), 20\% Tween80 (final concentration 0.05\%; Appendix II-D).

D. Luria Agar Plate Media supplemented with CB and CHX (per 1 L): 30.5 g Difco\textsuperscript{TM} Luria Agar Base, Miller (Fisher Cat. No. DF0413-17-2) and Q.S. with sterile water. The plate media was autoclaved and then cooled to 60°C. Prior to pouring, CB and CHX (final concentrations of 50 μg/mL and 10 μg/mL, respectively) were added. Plates were solidified and then cooled at room temperature for two days before storing at 4°C.

E. Luria Agar Plate Media supplemented with CB, CHX and Kan (per 1 L): 30.5 g Difco\textsuperscript{TM} Luria Agar Base, Miller and Q.S. with sterile water. The plate media was autoclaved and then cooled to 60°C. Prior to pouring, CB (final...
concentration of 50 μg/mL), CHX (final concentration of 10 μg/mL) and Kan (final concentration of 50 μg/mL; Appendix III-C) were added. Plates were solidified and then cooled at room temperature for two days before storing at 4°C.

F. Luria Agar Plate Media supplemented with CB, CHX, Kan and Acetamide (per 500 mL): 15.3 g Difco™ Luria Agar Base, Miller, to 493.5 mL with sterile water. The plate media was autoclaved and then cooled to 60°C. Prior to pouring, acetamide (final concentration 0.2%; Appendix II-O), CHX, CB and Kan (final concentrations 10 μg/mL, 50 μg/mL and 50 μg/mL, respectively) were added. Plates were solidified and then cooled at room temperature for two days before storing at 4°C.

G. 7H9 Top Agar (per 1 L): 4.7g Difco™ Middlebrook 7H9 Broth Base, 4 g Difco™ Agar, Granulated (Fisher Cat. No. DF0145-17-0), 2.5 mL of 40% glycerol stock and Q.S. with sterile water. The media was continuously stirred with heating until a rolling boil was achieved. After which, the media was removed from heat, but continued stirring. The Top Agar was aliquotted into media bottles (50 mL/bottle) and then autoclaved. 7H9 Top Agar is stored at room temperature. Prior to plating, 100 mM CaCl₂ is added (final concentration 1 mM).

H. LB Broth (per 1 L): 20 g LB Broth, Lennox (Fisher Cat. No. BP1427-500) and Q.S. with sterile water. The media was aliquotted into media bottles (100 mL/bottle) and autoclaved. LB Broth is stored at room temperature.

I. LB Agar Plate Media supplemented with Kan (per 1 L): 20 g LB Broth, Lennox, 15 g Difco™ Agar, Granulated and Q.S. with sterile water. The plate media was
autoclaved and then cooled to 60°C. Prior to pouring, Kan (final concentration of 50 μg/mL) was added. Plates were solidified and then cooled at room temperature for two days before storing at 4°C.

J. 7H9-ADC (per 200 mL): 7H9 Neat stock (final concentration 1X), CB (final concentration 50 μg/mL), CHX (final concentration 10 μg/mL), 100 mM CaCl₂ (final concentration 1 mM), Kan (final concentration 50 μg/mL).

K. Induction Media (per 200 mL): 7H9 Neat stock (final concentration 1X), CB (final concentration 50 μg/mL), CHX (final concentration 10 μg/mL), 100 mM CaCl₂ (final concentration 1 mM), Kan (final concentration 50 μg/mL), 20% Succinic Acid Solution (final concentration 0.2%; Appendix II-P).

L. Super Optimal Broth with Catabolite (SOC) Repression (per 1 L): 20 g Tryptone (Cat. No.), 5 g Yeast Extract (Cat. No.), 0.5 g Sodium chloride (Fisher Cat. No. BP358-1), 250 mM KCl (to final concentration of 2.5 mM) and 800 mL of sterile water. The pH is adjusted to 7 and then autoclaved. After cooling to 60°C, 20% Glucose (final concentration 20 mM), 1 M MgCl₂ (final concentration 10 mM; Appendix II-R) and 1 M MgSO₄ (final concentration 10 mM; Appendix II-G) were added.
APPENDIX II

Buffers, Reagents and Stock Solutions

A. 40% Glycerol stock (per 200 mL): 80 mL of Acros Organics Glycerol (Fisher Cat. No. AC410985000) and Q.S. with sterile water. The solution was transferred into a media bottle, then autoclaved and stored at room temperature.

i. 10% Glycerol stock (per 1 L): 100 mL of Acros Organics Glycerol and Q.S. with sterile water. The solution was aliquotted into media bottles (100 mL/bottle) and autoclaved. 10% glycerol is stored at room temperature.

B. AD Supplement stock (per 250 mL): 2.13 g Sodium chloride (Fisher Cat. No. BP358-1), 5 g Dextrose (D-Glucose) Anhydrous (Fisher Cat. No. D16-500), 12.5 g Alfa Aesar Albumin, Bovine, Cohn Fraction V, 98% Immunoassay grade, protease and enzyme-free, pH 7.0 (Fisher Cat. No. AAJ6573122) and Q.S. with sterile water. The solution was filter-sterilized into a sterile media bottle using a 0.2 μm SFCA membrane Nalgene™ Rapid-Flow™ sterile disposable bottle top filter (Fisher Cat. No. 09-740-22H). AD Supplement is stored at 4°C.

C. 1 M Calcium chloride (CaCl₂) stock (per 500 mL): 55.5 g Calcium chloride (Fisher Cat. No. C77-500) and Q.S. with sterile water. The solution was aliquotted into media bottles (100 mL/bottle) and then autoclaved. 1 M CaCl₂ is stored at room temperature.

i. 100 mM CaCl₂ stock (per 1 L): 11.1 g CaCl₂ and Q.S. with sterile water. The solution was aliquotted into media bottles (100 mL/bottle) and then autoclaved. 100 mM CaCl₂ is stored at room temperature.
D. 20% Tween80 stock (per 40 mL): 8 mL Tween$^\text{TM}80$ (Fisher Cat. No. BP338-500) and Q.S. with sterile water. The solution was filter-sterilized into a 50 mL Corning$^\text{TM}$ Tube Top 0.22 μm vacuum filter-sterilization unit (Fisher Cat. No. 09-761-34). 20% Tween80 is stored at room temperature.

i. 1.2% Tween80 sock (per 500 mL): 6 mL 20% Tween80 stock (see above) and Q.S. with sterile water. The solution was filter-sterilized into a media bottle using a 0.2 μm SFCA membrane Nalgene$^\text{TM}$ Rapid-Flow$^\text{TM}$ sterile disposable bottle top filter. 1.2% Tween80 is stored at room temperature.

E. Phage Buffer (PB) stock (per 1 L): 4 g Sodium chloride, 1 M Tris-HCl, pH 7.5 stock (final concentration 10 mM; Appendix II-F), 1 M Magnesium sulfate (MgSO$_4$) stock (final concentration 10 mM), 100 mM CaCl$_2$ stock (final concentration 1 mM) and Q.S. with sterile water. The buffer was aliquotted into media bottles (250 mL/bottle) and then autoclaved. PB is stored at room temperature.

F. 1 M Tris-HCl, pH 7.5 stock (per 500 mL): 60.57 g Acros Organics Tris(hydroxymethyl)aminomethane (Fisher Cat. No. AC167620010), half volume with sterile water. The pH was adjusted with 12 M Hydrochloric acid (from the biotech center) prior to bringing the total volume with sterile water. The solution was transferred into a media bottle and then autoclaved. 1 M Tris-HCl is stored at room temperature. (NOTE: Several 1M Tris-HCl stock solutions were made at different pH levels for multiple applications. For this work, four 1 M Tris-HCl stocks were made as described above at pH levels 7.3, 7.5, 8.0 and 8.2.)
G. 1 M MgSO₄ stock (per 200 mL): 24.07 g Magnesium sulfate anhydrous (Fisher Cat. No. M65-500) and Q.S. with sterile water. The solution was filter-sterilized into a media bottle using a 0.2 μm SFCA membrane Nalgene™ Rapid-Flow™ sterile disposable bottle top filter (Fisher Cat. No. 09-740-22H). 1 M MgSO₄ is stored at room temperature.

i. 10 mM MgSO₄ stock (per 50 mL): 0.5 mL of 1 M MgSO₄ stock with 49.5 mL sterile water. The solution was filter-sterilized using the Corning™ Tube Top 0.22 μm vacuum filter-sterilization unit and stored at room temperature.

H. Nuclease Mix (per 10 mL): 88 mg Sodium chloride was dissolved in 4.25 mL sterile water (final concentration 150 mM). Aseptically, DNase I stock (final concentration 250 μg/mL; Appendix II-I), RNase A stock (final concentration 250 μg/mL; Appendix II-J) and glycerol (final concentration 50%) were added to the solution. After thoroughly mixed, the solution was aliquotted (1 mL/tube) into Nalgene® System 100™ Cryogenic Tubes (Fisher Cat. No. 5000-1012). Nuclease Mix is stored at -20°C.

I. DNase I stock (5 mg/mL): The total of MP BioMedicals DNase I, bovine pancreases (Fisher Cat. No. ICN10057520) weighed is divided by 5 to determine the volume of sterile water added. Once in solution, it was filter-sterilized into a sterile 15 mL tube with the GE Healthcare Whatman™ Puradisc 0.2 μM syringe filters, and then aliquotted (1 mL/tube) into Nalgene® System 100™ Cryogenic Tubes. DNase I is stored at -20°C.
J. RNase A stock (10 mg/mL): The total of RNase A, bovine pancreases (Fisher Cat. No. BP2539-1) weighed is divided by 10 to determine the volume of sterile water added. Once in solution, it was filter-sterilized into a sterile 15 mL tube with the GE Healthcare Whatman™ Puradisc 0.2 µM syringe filters, and then aliquotted (1 mL/tube) into Nalgene® System 100™ Cryogenic Tubes. RNase A was stored at -20°C.

K. Elution Buffer (EB) solution (per 50 mL): 0.5 mL of 1 M Tris-HCl stock, pH 8.0 (Appendix II-F) with 45.5 mL sterile water. EB is stored at room temperature.

L. Phage Precipitation Solution (per 100 mL): 19.3 g Sodium chloride, 30 g Acros Organics Poly(ethylene glycol), PEG8000 (Fisher Cat. No. AC418050010) and Q.S. with sterile water. The solution was filter-sterilized into a media bottle using a Corning™ Tube Top 0.22 µm vacuum filter-sterilization unit. Phage Precipitation Solution is stored at room temperature.

M. PM Additive Solution 1 (per 500 mL): 1 M MgCl₂ (final concentration 240 mM), 1 M CaCl₂ (final concentration 120 mM; Appendix II-C), 20% Tween80 (final concentration 1.2%) and Q.S. with sterile water. The solution was filter-sterilized into a media bottle using a 0.2 µm SFCA membrane Nalgene™ Rapid-Flow™ sterile disposable bottle top filter. PM Additive Solution 1 is stored at 4°C.

N. PM Additive Solution 2 (per 500 mL): 1 M MgCl₂ (final concentration 240 mM), 1 M CaCl₂ (final concentration 120 mM), 20% Tween80 (final concentration 1.2%), 1 M Dextrose (D-glucose) stock (final concentration 600 mM; Appendix II-S) and Q.S. with sterile water. The solution was filter-sterilized into a media
bottle using a 0.2 μm SFCA membrane Nalgene™ Rapid-Flow™ sterile disposable bottle top filter. PM Additive Solution 2 is stored at 4°C.

O. 20% Acetamide Solution (per 100 mL): 20 g Acetamide, 99% pure (Fisher Cat. No. AC153631000) and Q.S. with sterile water. The solution was filter-sterilized into a media bottle using the Corning™ Tube Top 0.22 μm vacuum filter-sterilization unit. 20% Acetamide is stored at room temperature.

P. 20% Succinic Acid Solution (per 100 mL): 20 g Butanedioic acid (succinic acid) disodium salt hexahydrate (Sigma Cat. No. S-2378) and Q.S. with sterile water. The solution was filter-sterilized using the ThermoScientific™ Nalgene™ Rapid-Flow™ 0.2 μm PES membrane sterile disposable filter unit (Fisher Cat. No. 09-741-01). 20% Succinic Acid is stored at room temperature.

Q. Mycobacterium Freezing Media (per 100 mL): 43.75 mL 7H9 Neat stock, 5 mL AD Supplement stock, 1.25 mL 20% Tween80 stock and Q.S. with 100% glycerol. The media was filter-sterilized into two 50 mL tubes using the Corning™ Tube Top 0.22 μm vacuum filter-sterilization unit. Mycobacterium Freezing Media is stored at 4°C.

R. 1 M Magnesium chloride (MgCl₂) stock (per 100 mL): 20.33 g Magnesium chloride hexahydrate (Fisher Cat. No. M33-500) and Q.S. with sterile water. The solution was filter-sterilized into a media bottle using a 0.2 μm SFCA membrane Nalgene™ Rapid-Flow™ sterile disposable bottle top filter. 1 M MgCl₂ is stored at room temperature.

S. 1 M Dextrose (D-glucose) stock (per 50 mL): 9.01 g Dextrose (D-glucose) anhydrous (Fisher Cat. No. D16-500) and Q.S. with sterile water. The solution
was filter-sterilized into a 50 mL tube using the Corning™ Tube Top 0.22 μm vacuum filter-sterilization unit. 1 M Dextrose is stored at room temperature.
APPENDIX III

Antibiotics, Antimycotics and Cytotoxic Agents

A. Carbenicillin (CB) stock (50 mg/mL): The total of Carbenicillin, Disodium salt (Fisher Cat. No. BP26485) weighed is divided by 50 to determine the volume of sterile water added. Once in solution, it was filter-sterilized into a sterile 15 mL tube using a GE Healthcare Whatman™ Puradisc 0.2 µM syringe filter. Carbenicillin is stored at 4°C.

B. Cycloheximide (CHX) stock (10 mg/mL): The total of Arcos Organics Cycloheximide (Fisher Cat. No. AC357420050) weighed is divided by 10 to determine the volume of sterile water added. Once in solution, it was filter-sterilized into a sterile 15 mL tube using a GE Healthcare Whatman™ Puradisc 0.2 µM syringe filter. Cycloheximide is stored at 4°C.

C. Kanamycin (Kan) stock (50 mg/mL): The total of MP Biomedicals Kanamycin Monosulfate (Fisher Cat. No. ICN15002901) weighed is divided by 50 to determine the volume of sterile water added. Once in solution, it was filter-sterilized into a sterile 15 mL tube using a GE Healthcare Whatman™ Puradisc 0.2 µM syringe filter and then aliquotted (1 mL/tube) into Nalgene® System 100™ Cryogenic Tubes. Kanamycin is stored at -20°C.

D. Mitomycin C stock (2 mg/mL): 2 mL of a 1:1 solution of ethanol to sterile water solution was prepared and injected through the seal of the Mitomycin C (Fisher Cat. No. BP2531-2) bottle. The solution was transferred into a Nalgene® System 100™ Cryogenic Tube covered with aluminum foil. Mitomycin C is stored at 4°C.