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Thomas Scott Brady
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Bacteriophages for Treating American Foulbrood and the
Neutralization of *Paenibacillus larvae* Spores

Thomas Scott Brady

A thesis submitted to the faculty of
Brigham Young University
in partial fulfillment of the requirements for the degree of
Master of Science

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ABSTRACT

Bacteriophages for Treating American Foulbrood and the Neutralization of *Paenibacillus larvae* Spores

Thomas Scott Brady

Department of Microbiology and Molecular Biology, BYU
Master of Science

The causative agent of the most devastating honeybee disease, American foulbrood (AFB), is the spore-forming bacterium *Paenibacillus larvae*. To prevent AFB outbreaks beekeepers prophylactically treat their hives with antibiotics even though it decreases the overall health of uninfected hives. A new treatment for AFB is needed due to recent legislation against using antibiotics, antibiotic resistance developing in *P. larvae*, and the resilience of *P. larvae* spores. Bacteriophages, or phages, are an attractive alternative to traditional antibiotics because of their specificity and ability to evolve alongside their target bacterium. In this study, two phage cocktails were developed for the treatment of AFB. The first cocktail was comprised of *Brevibacillus laterosporus* phages. *B. laterosporus* is a commensal microbe in most honeybee guts. When treated with *B. laterosporus* phages, *B. laterosporus* is induced to produce an antimicrobial toxin to which *P. larvae* is highly sensitive. Treating AFB infected hives with *B. laterosporus* phages was able to clear active infections at a rate of 75% as opposed to untreated hives that did not recover. However, *B. laterosporus* phages did not clear latent *P. larvae* spores and recovered hives relapsed after treatment. The second cocktail was comprised of *P. larvae* phages and hives treated with the second cocktail recovered at a rate of 100%, protected 100% of at-risk hives, and treated hives did not relapse with AFB suggesting neutralization of *P. larvae* spores. A *P. larvae* phage used in the second cocktail was examined to identify any spore-phage interactions. Results from modified plaque assays, fluorescence from FITC-labeled phages bound to spores, and electron microscopy images all confirm that phages bind to *P. larvae* spores. Phage therapy for the treatment of AFB is an exciting avenue not only as an alternative to chemical antibiotics, but rather a treatment that can neutralize *P. larvae* spores.

Keywords: *Paenibacillus larvae*, American foulbrood, spores, phage therapy, honeybees, *Brevibacillus laterosporus*, antimicrobial toxin, bacteriophage, phage binding

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SPECIFIC AIMS

Aim 1: Identify the effectiveness of bystander phage therapy as a treatment for AFB.

1. Generate a phage cocktail that hits a wide range of *B. laterosporus* that also induce toxin production in the bacterium.
2. Test the phage cocktail on uninfected hives to observe any adverse effects of removing *B. laterosporus* from hives and treat AFB infected hives to gauge ability to treat vegetative bacteria as well as latent spores.

Aim 2: Identify the effectiveness of traditional phage therapy as a treatment for AFB.

1. Generate a phage cocktail that affects a wide range of *P. larvae* strains.
2. Test the phage cocktail on uninfected hives to observe any adverse effects in comparison to prophylactic antibiotic treatments and treat AFB infected hives to gauge ability to treat vegetative bacteria as well as latent spores.

Aim 3: To determine the relationship between *P. larvae* phages and *P. larvae* spores.

1. Prepare a stock of pure *P. larvae* spores on which experiments involving phage binding can be performed.
2. Develop assays that quantify the binding of *P. larvae* phages to spores in relation to related and unrelated bacteria.

INTRODUCTION

1.1 American foulbrood: a significant threat to honeybees

American foulbrood (AFB) is the most devastating bacterial disease in honeybees (1). AFB was first distinguished from European foulbrood in 1907 and has since been identified on all inhabited continents (2-5). The firmicute that causes AFB, *Paenibacillus larvae*, is a spore-forming facultative anaerobe that only germinates from spore form in the gut of honeybee larvae (6-10). The spores do not affect adult honeybees as they do not germinate in their guts, however, spores can accumulate in the gut of nurse bees; inadvertently passing the spores on to larval honeybees during feeding (11-13). The spores germinate and become vegetative (8), rapidly divide, and produce chitinase, an enzyme that degrades larval exoskeleton, resulting in the death and liquefaction of the larvae (6,14). Nurse bees clean the spore-laden slurry and spread the spores as they feed the rest of the brood. Within a week without treatment, an active AFB infection results in the collapse of an infected hive (3,15). The collapse of hives allows robber bees from other hives to steal spore-contaminated honey, which allows for rapid spread of the disease within an apiary. The only way to ensure no reinfection by latent spores requires beekeepers to burn affected hive boxes and tools (16). Other methods for decontaminating beekeeping supplies such as gamma radiation and portable autoclaves show promise but are not yet practical and do not save the honeybees (1,17,18). *P. larvae* spores can survive for decades in hive boxes and will build up in a hive over time and cause seemingly spontaneous outbreaks (9,10,13).

Current treatments for American foulbrood are harmful to honeybees and are becoming ineffectual. It is common practice for beekeepers to dose their hives with antibiotics to prevent brood diseases. The antibiotics do not harm *P. larvae* spores but can kill susceptible vegetative

bacteria. There are two protein synthesis antibiotics used on honeybee hives; terramycin (a tetracycline) and tylosin (a macrolide), which are typically administered prophylactically to beehives in the spring and fall to prevent outbreaks. Antibiotic use causes the overall health of treated hives to diminish by altering honeybee gut microbiota (19-21). Furthermore, tylosin degrades into a secondary antibiotic, desmycosin, which remains in hives for years longer than tylosin contaminating honey of treated hives (22,23). However, many *P. larvae* strains are now resistant to terramycin due to overuse and recently strains have been isolated that are resistant to tylosin (24-29).

1.2 Phages as an alternative to conventional antibiotics

Bacteriophage cocktails are an exciting alternative to chemical antibiotics for the treatment of American foulbrood. Bacteriophages, or phages, are viruses that infect and can kill bacteria. Phages use tail fibers to attach to specific receptors on the surface target bacteria and inject their DNA into the bacterial cell. Once infected, the bacterial cell begins to transcribe and replicate the phage genome by hijacking the bacterium's energy and protein synthesis equipment to generate more phages. Lytic phage proteins lyse the bacterium after generating progeny, killing the bacterial cell. Phages naturally only target one species of bacteria and are often limited to a small number of strains within that species (30-32). Some phages produce enzymes on their tail fibers or tail fiber sheaths that are active against specific polysaccharides or proteins to allow the phage to inject its DNA into a target cell (33,34). Due to this host selectivity, phages cannot infect anything else – that is to say they cannot infect plants, animals, insects, or even non-target bacteria, such as commensal bacteria present in the gut of honeybees.

Using phages to treat bacterial disease is not a new idea. Félix d'Herelle, credited with the discovery of phages, recognized their potential as a treatment of bacterial infections and

employed them in the world's first documented phage therapy in 1919 against *Salmonella gallinarum* in rural France (35). Since then, the popularity of phage therapies has waxed and waned through time and geography, remaining popular in the former Soviet Republic of Georgia for the past 80 years (36). In the face of increasing antibiotic resistance in bacteria, phage therapies have become an attract alternative. Today, phage cocktail therapies have been shown to be safe and effective for treating many bacterial infections including *E. coli* infections in humans, *E. amylovora* in fruit orchards, and with *P. larvae* infections in honeybees (36-39).

Honeybees benefit from phage specificity because other gut bacteria are not affected, unlike antibiotic treatments that can increase the chances of fungal infections, nosema, due to disruption of the normal balance of bacteria in the bee gut (20,40-42). Bee keepers typically apply two to four treatments of antibiotics to their hives prophylactically to prevent AFB infections, decreasing the overall health of their hives (43). However, in 2016 the FDA banned prophylactic antibiotics, requiring beekeepers to obtain a prescription from a veterinarian in order to dose their bees (44). This highlights the need for an effective alternative to traditional antibiotics such as phage therapy.

1.3 Phages binding to bacterial spores

Research involving phages that target *Bacillus anthracis* and *Bacillus subtilis* has shown interesting results for phages that bind to spores. In 2003, researchers used modified *E. coli* phages to biopan for phages that are selective for *Bacillus* spores (45). They identified phages that selectively bound *Bacillus* spores and from those derived phages with high affinity for *B. subtilis* and *B. anthracis* spores (46). Subsequent research has shown that the phages for *B. anthracis* can be used as bio-markers to ascertain the presence of spores. In 2003, a research team isolated naturally occurring phages using a mixed lysate biopanning method to identify

them. They discovered that the isolated phages are active against vegetative *B. anthracis* and tested their ability to decontaminate surfaces with *B. anthracis* spores. They found that the phages were able to kill the spores after germination, effectively decontaminating certain surfaces (47). In 2011, the same researchers characterized the mechanism by which the spore-binding phage 8a inserts its DNA into *B. anthracis* spores (48). These successes with spore-binding phages leave a pattern that can now be followed while approaching *P. larvae* spore-binding phages.

We hypothesize that spores can be covered with phages and then, upon spore germination, the bound phages can inject their DNA to replicate in the now vegetative cell. If conditions allow for phage neutralization of *P. larvae* bacterial spores, then treatment with phages will be an even more powerful method of preventing AFB. Phages that neutralize spores could lead to a future treatment of AFB-contaminated hive components and equipment as well as a long-acting preventative treatment for AFB. If the phages that target *P. larvae* do not neutralize the spores, then a limit on the effectiveness of the phage cocktail treatment can be set.

1.4 *Brevibacillus laterosporus* bystander phage therapy

Bystander phage therapy using *Brevibacillus laterosporus* as a target is another method by which phages could be used to treat AFB. *B. laterosporus* is a ubiquitous bacterium found in soil, milk, cheese, and insect bodies, the most notable of which being honeybees (49). *B. laterosporus* is a spore-forming firmicute and is genetically similar to *P. larvae*, both belonging to the family Paenibacillaceae. The role of *B. laterosporus* in honeybee hives is not well understood. Some studies report that *B. laterosporus* is a probiotic that increases brood production, prevents disease, and can treat brood diseases while others show that *B. laterosporus* causes minor disease after a primary infection (50-53). *B. laterosporus* is a known pathogen of

many insects of the order Diptera (honeybees belong to the order Hymenoptera) including common houseflies and mosquitoes and has been used as an insect biocontrol with moderate success (54-59). At least twelve toxins have been identified and classified in *B. laterosporus* that have insecticidal and antimicrobial properties (49). When *B. laterosporus* is challenged with *B. laterosporus* phages the bacterium begins to produce high levels of antimicrobials. *P. larvae* is very sensitive to these phage-induced toxin. These findings led us to believe that the creation of a phage cocktail against *B. laterosporus* could be used as a ‘bystander phage therapy’ where the phages do not directly infect and kill the pathogen. Bystander phage therapy could be the bridge between normal phage therapy and antibiotic use but this hypothesis requires further investigation.

1.5 Summary of chapters 2-4

The following chapters are copies of articles that are published or under review. Chapter 2 follows our investigation into the toxicity of phage-induced toxins produced *B. laterosporus* and their effectiveness as a bystander phage therapy. Chapter 3 reviews the phage selection process for generating a phage cocktail against *P. larvae*. Also investigated was the detrimental nature of antibiotics to healthy beehives and an experiment showing the effectiveness of the phage therapy in treating and preventing AFB. Finally, Chapter 4 is an investigation of the relationship between phages used in Chapter 3 and the spore form of *P. larvae*. This study shows two methods by which phage binding can be quantified as well as provides electron microscopy images of spore-bound phages.

CHAPTER 2: “PHAGE-INDUCED TOXIN FROM *BREVIBACILLUS*
LATEROSPORUS CAN CONTROL AMERICAN FOULBROOD”

The following manuscript “Phage-induced toxin from *Brevibacillus laterosporus* can control American foulbrood” was written for and submitted to the *Journal of Invertebrate Pathology*, and is currently under review. The article describes the use of *B. laterosporus* phages to induce antibacterial toxins to treat American foulbrood.

References for this manuscript are found in chapter 6 and the in text references to figures or sections are to those within this chapter.

Full Title:

Phage-induced toxin from *Brevibacillus laterosporus* can control American foulbrood

Short Title:

B. laterosporus phages to control AFB

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Key words: American foulbrood, bacteriophage, phage, phage therapy, *Paenibacillus larvae*,
Brevibacillus laterosporus, treatment, safety, bystander phage therapy.

Abstract

Brevibacillus laterosporus is a bacterium that is often present in healthy and diseased beehives, with the notable observation of being present in hives infected with the causative agent of American foulbrood (AFB), *Paenibacillus larvae*. In current literature, the role of *B. laterosporus* in honeybees remains ambiguous due to its ability to cause and prevent disease. In this work, phages specific for *B. laterosporus* were found to induce bactericidal toxin production in *B. laterosporus*. Results demonstrated that *P. larvae* is susceptible to the phage-induced toxins from the two field isolates of *B. laterosporus* tested. We report the host range of 12 *B. laterosporus* phages, three of which were selected for their combined ability to infect 11 of 12 *B. laterosporus* strains to create a phage cocktail for the treatment of AFB. Experiments were designed to show 1) how long phages persist in bee larvae 2) their safety to bee colonies over time and 3) the efficacy of *B. laterosporus* phages in treating AFB. Phage presence in bee larvae after treatment rose to $60.8 \pm 3.6\%$ and dropped to $0 \pm 0.8\%$ after 72 hours. Healthy hives treated with *B. laterosporus* phages experienced no difference in brood generation when compared to control hives over eight weeks. Twelve AFB infected hives were treated with the phage cocktail and nine of them recovered, demonstrating a recovery rate of 75%. Despite the success rate of clearing the active infection, all hives eventually manifested AFB again and symptoms were controlled with reapplication of the *B. laterosporus* phage cocktail. These results indicate that this treatment approach can kill vegetative bacteria but not spores. We posit that the effectiveness of this treatment is due to the production of the bactericidal toxin of *B. laterosporus* when infected with phages. Bystander phage therapy may provide a new avenue for antibacterial production and treatment of diseases.

2.1 Introduction

Brevibacillus laterosporus is a gram-positive, spore-forming bacterium that can be found in myriad locations including the gut of honeybees (49,60-63). While typically found at low levels in healthy honeybees, the population of *B. laterosporus* often increases as a secondary infection when a hive is infected with *Paenibacillus larvae* or *Melissococcus plutonius*, the causative agents of American foulbrood and European foulbrood respectively (64). American foulbrood (AFB) is the most devastating bacterial infection in honeybees, killing honeybee larvae and spreading easily from hive to hive within an apiary (8,65,66). In the wake of antibiotic resistance in *P. larvae*, novel methods for controlling AFB outbreaks are needed.

Strains of *B. laterosporus* produce potent toxins that can kill a wide range of organisms (49,51,67). *B. laterosporus* has been used as a biocontrol agent for decreasing the populations of unwanted bacteria and this method yielded modest results in attempts to control American foulbrood (68,69). While typically a symbiont of honeybees (52), *B. laterosporus* can produce toxins with insecticidal properties and certain strains of the bacterium are implicated in causing minor disease in honeybee hives after a primary infection (54,56,57,70). The role of *B. laterosporus* as either a beneficial symbiont or as an opportunistic infector is yet to be fully understood.

Prior to this study, phages that specifically infect *B. laterosporus* were isolated from beehives and the genomes of most have been studied and published (30,71-73). In this study, isolated phages were tested against strains of *B. laterosporus* to determine the most effective combination of phages to be included in a final cocktail. During isolation and experimentation, we discovered that when *B. laterosporus* was treated with phages, the bacteria began to produce

toxins that kill *P. larvae*. These findings led us to believe that *B. laterosporus* phages could be used as a biocontrol for AFB by inducing toxin production and killing *P. larvae*.

The studies presented here show: 1) the host range of identified phages, 2) the phages' presence and persistence in the larval gut after treatment, 3) the phages' ability to induce toxin production compared to other forms of induction, 4) the phages' safety to healthy honeybee hives over time, and 5) the phages' ability to control an active AFB infection. We propose a new approach called "bystander phage therapy" as a method for treating pathogenic bacteria.

2.2 Materials and Methods

2.2.1 Gathering *B. laterosporus* field isolates

Samples of honey and hive material were gathered from local apiaries and used to isolate bacteria. Samples were processed as described previously intended for *P. larvae* isolation (71,74) and isolated bacterial colonies were identified as *P. larvae* or *B. laterosporus* by PCR. Specifically, bacteria were initially streaked on PLA agar (75) and incubated at 37°C. Catalase negative (76) and Gram positive colonies were streaked on LB agar (Becton, Dickinson and Company, Sparks, MD), gathered, archived in 20% glycerol, and stored at -80°C. Bacteria were confirmed as *B. laterosporus* by PCR amplification of the *B. laterosporus* rpoB gene, see Table 2-1. Samples were also PCR tested with primers specific for *P. larvae* rpoB and ftsA to confirm the presence of *P. larvae*. Prior to PCR, bacterial samples were streaked out to single colonies. Template DNA for PCR was extracted by adding part of a colony to 50 µL of ddH₂O in a PCR tube and incubating it at 100°C for 10 minutes. The total PCR reaction volume was 25 µL composed of 22 µL standard PCR reagents (New England Biolabs, Ipswich, MA) plus 3 µL of template DNA. After 30 cycles, PCR products were run in an agarose gel to confirm

Table 2-1. Primer list.

Primers used for amplification and sequencing of *rpoB*, *ftsA*, and 16S rRNA genes of *B. laterosporus* and *P. larvae*. Results were used to positively identify bacterial isolates from beehives.

Primer	Sequence	Direction	Purpose	Reference
27F	5'-AGAGTTTGATCMTGGCTCAG-3'	Forward	16S rRNA universal primer	(77)
907R	5'-CCGTCAATTCMTTTRAGTTT-3'	Reverse		
BLrpoB-F	5'-GCAGGTAAACTGGTCCAGAGCG-3'	Forward	B. laterosporus rpoB	
BLrpoB-R	5'-CACCTGTTGATTTATCAATCAGCG-3'	Reverse		
KAT1	5'-ACAAACACTGGACCCGATCTAC-3'	Forward	<i>P. larvae</i> ERIC-1 or ERIC-2	(78)
KAT2	5'-CCGCCTTCTTCATATCTCCC-3'	Reverse		
PLrpoB-F	5'-ATAACGCGAGACATTCCTAA-3'	Forward	Amplifies <i>P. larvae</i> rpoB	(79)
PLrpoB-R	5'-GAACGGCATATCTTCTTCAG-3'	Reverse		
PLftsA-F	5'-AAATCGGTGAGGAAGACATT-3'	Forward	Amplifies <i>P. larvae</i> ftsA	(79)
PLftsA-R	5'-TGCCAATACGGTTTACTTTA-3'	Reverse		
ERIC1R	5'-ATGTAAGCTCCTGGGGATTCAC-3'	Forward	Generates multiple amplicons to fingerprint the bacteria tested	(80)
ERIC2	5'-AAGTAAGTGACTGGGGTGAGCG-3'	Reverse		

amplification. Amplicons from the reactions were sequenced using BigDye (Life Technologies, Carlsbad, CA). MEGA6 was used to match sequence results with bacterial genomes.

2.2.2 Isolating phages specific for *B. laterosporus*

B. laterosporus phages were isolated from bee debris collected near beehives. Bee debris was crushed and added to a flask containing LB broth and a field isolate of *B. laterosporus*. The bee debris and bacteria were incubated overnight at 37°C. The mixture was spun in a centrifuge and the supernatant was passed through a 0.22 µm filter (VWR, Radnor, PA). Approximately 50 µL of the supernatant were incubated at room temperature with 500 µL of *B. laterosporus* bacteria for 30-60 minutes, mixed with LB top agar, plated on LB agar, and incubated at 37°C overnight. Plaques that appeared were isolated and re-plated a minimum of three times to purify individual phage isolates.

2.2.3 Host range and phage presence testing for isolated phages

B. laterosporus bacterial strains were tested for phage susceptibility using a plaque formation assay and a spot test assay. For the plaque formation assay, phage lysate was incubated at room temperature with 500 μ L of an overnight culture of bacteria for 30 minutes, plated in 0.8% LB top agar, and incubated overnight at 37°C. For the spot test assay, 500 μ L of an overnight culture of bacteria was plated in 0.8% top agar. After the top agar gelled, 3 μ L of phage lysate was placed on the top agar. The plates were incubated agar side facing up overnight at 37°C.

Phage detection in bee larvae was performed by taking one hundred larval samples at each time point and homogenizing them in 500 μ L of LB broth in a 1.7 ml microcentrifuge tube for approximately one minute. Three μ L of the larval homogenate was spotted and incubated on plates *B. laterosporus* strains BL2 and BL6 were plated in top agar as described above.

2.2.4 Electron microscopy

Phages were prepared for electron microscopy by incubating copper grids with 50 μ L of high-titer lysate for 90 seconds, wicking away moisture, incubating with 50 μ L of 2% phosphotungstic acid (pH = 7) for 90 seconds, wicking away moisture, and then allowing the grids to air dry prior to imaging. Electron micrographs were taken on a Helios DualBeam microscope at the BYU Microscopy Center, and images were measured using ImageJ (81).

2.2.5 Creation of bacterial lysate to test for toxin *B. laterosporus* and phage cocktail treatments

Field isolates of *B. laterosporus*, BL-2 and BL-6, were reconstituted from freezer stock by plating onto Porcine Brain Heart Infusion (PBHI) (Acumedia, Lansing, MI) plates and incubating at 37°C for 48 hours. The resulting colonies were streaked to pure culture and

incubated at 37° C overnight. Fawkes and Emery/Abouo were brought out from freezer stock by streaking onto PBHI plates with a lawn of *B. laterosporus* in agar incubated at 37° C overnight. Picked plaques were grown in liquid culture with overnight growths of *B. laterosporus* to generate a high titer lysate. The lysates were centrifuged at 12,000 x g for 30 minutes to pellet bacterial debris and then passed through a syringe a 0.45 µm filter (VWR, Radnor, PA). The controls had no phage added and were processed the same to collect a mock lysate.

Overnight cultures of *B. laterosporus* BL-2/BL-6, *P. larvae* ATCC 9545, *Agrobacterium tumefaciens* field isolate, *Sinorhizobium meliloti* field isolate, and *E. coli* B079 were plated using top agar onto plates of their respective media. Spot assays were conducted on bacterial lawns using three µL of lysate and incubating overnight. *A. tumefaciens* and *S. meliloti* samples were incubated at 30° C and all other cultures were incubated at 37° C.

Phages in the cocktail were generated as described above and then precipitated with polyethylene glycol (PEG) (Spectrum, New Brunswick, NJ) and centrifuged at 10,000 x g for 15 minutes at 4° C to obtain a pure phage stock devoid of toxin. The cocktail was applied to the hives using a spray comprised of phage lysate diluted in a 1:1 sugar/water solution. Control hives received 340 mL of sugar water, while the phage treated hives received 320 mL of sugar water with 50 ml of phages containing a total of 10⁸ pfu mixed into the sugar water.

2.2.6 Phage Beehive parameters

In studies beginning with healthy hives, each had a viable laying queen, approximately 40,000 or more adult worker bees, uncapped brood, and no visible signs of American foulbrood. Sick hives treated in sections 3.5 and 3.6 were identified by a local beekeeper and experimental treatment was approved through the Utah Department of Food and Agriculture.

Population growth was determined in each of the hives based on the amount of racks the bees occupied. A rack was considered full when the space between the racks was fully crowded. In section 3.4 the phage treatment started once all 12 of the hives achieved at least four fully occupied racks.

2.2.7 Statistics

The BYU statistical center analyzed the collected data to generate p-values, standard deviation, and standard error to determine statistical significance using the ANOVA algorithm. Statistical analysis included repeated measures, mixed procedure, two-tailed analysis using the Fisher's exact test for 2x2 contingency tables with $\alpha = 0.05$.

2.3 Results

2.3.1 Phage characteristics and host range

The genome sequences for all of the phages used in these studies, except for Lauren and Fawkes, were previously sequenced and analyzed (30,71). Genbank accession numbers for the phage genomes are as follows: Jimmer1 - KC595515, Jimmer2 - KC595514, Emery - KC595516, Abouo - KC595517, Davies - KC595518, Osiris - KT151956, Powder - KT151958, SecTim467 - KT151957, Sundance - KT151959, Jenst - KT151955.

Electron microscopy images of Jimmer1, Jimmer2, Emery, Abouo, Davies, Osiris, and Powder were previously published (30,71). Figure 2.1a and b include electron microscopy images of the two previously unpublished images of phages used in this study, Lauren and Fawkes, respectively. Figure 2.1c is an image of Fawkes attached to the side of the BL2 *B. laterosporus* field isolate.

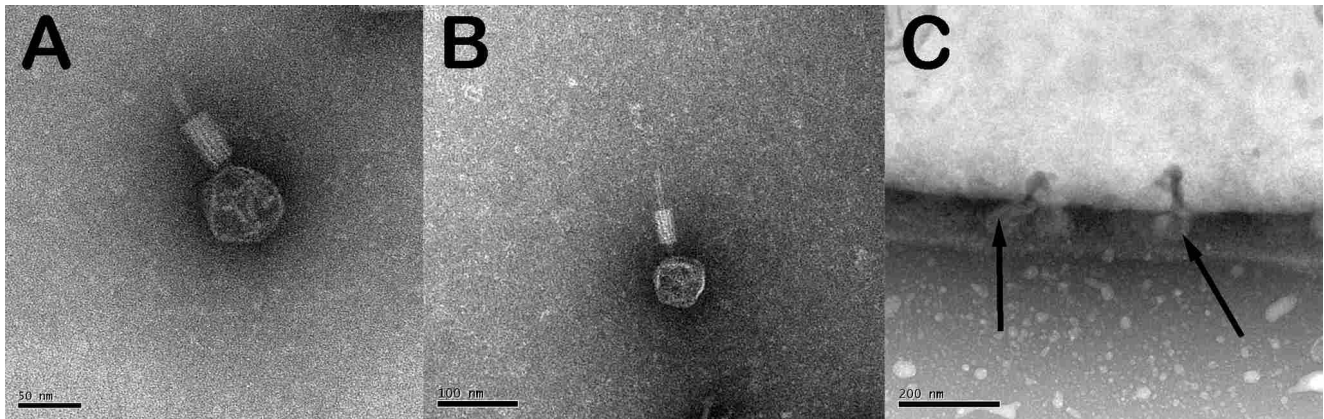


Figure 2.1. *Brevibacillus laterosporus* phages Lauren and Fawkes. (A) Single Lauren phage particle SEM image. (B) Single Fawkes phage particle SEM image. (C) Fawkes phage particles attached to BL2 bacterium SEM image, arrows point to attached phage particles. Images of the other phages mentioned were previously published by (71) and (30).

Upon isolation, *B. laterosporus* phages were challenged for their ability to infect three field isolates of *B. laterosporus* as well as nine type-strains of *B. laterosporus* from the Bacillus Genetic Stock Center (BGSC) by both spot tests and plaque formation assays. Table 2-2 indicates bacterial susceptibility to *B. laterosporus* phage infection using *P. larvae* bacteria as a negative control. Emery/Abouo had the largest host range against archived *B. laterosporus* strains, showing infectivity against eight of the 12 strains. Fawkes showed infectivity against seven strains of which three were not covered by Emery/Abouo. None of the tested *B. laterosporus* phages were capable of forming plaques on lawns of 40A4. Furthermore, no plaques formed on *P. larvae* ATCC 9545, a highly phage susceptible strain, indicating that the isolated phages are specific to *B. laterosporus* and do not have the ability to cross-infect into *P. larvae*.

Table 2-2. Host range of *B. laterosporus* phages.

Twelve *B. laterosporus* strains and one *P. larvae* strain were challenged with 12 *B. laterosporus* phages. The number of plus signs indicate the level of clearing. A minus sign indicates that no plaque formation occurred. BL2-BL14 are our field isolates of *B. laterosporus*, 40A1-40A10 are type strains of *B. laterosporus* from BGSC, and PL ATCC is the type strain of *P. larvae*.

	BL2	BL6	BL14	40A1	40A2	40A3	40A4	40A5	40A6	40A8	40A9	40A10	PL ATCC
Jimmer1	++++	-	++++	+	-	-	-	-	-	-	-	-	-
Jimmer2	++++	-	++++	+	-	-	-	-	-	-	-	-	-
Osiris	++++	-	++	++	+	+	-	+	++	-	++	+	-
Fawkes	++++	-	++	+++	+	-	-	-	+	-	++++	++	-
Lauren	++++	-	++++	+	-	-	-	-	+	-	+	+	-
Powder/Sundance	+++	-	+++	+++	-	+	-	-	-	-	+++	-	-
SecTim467	+++	++	+++	++	+	-	-	-	-	-	+++	-	-
Jenst	-	++++	-	+	-	-	-	-	+	-	+++	-	-
Davies	-	++++	-	++++	++	+	-	+++	+++	+++	-	-	-
Emery/Abouo	-	++++	-	++++	++++	+	-	+++	+++	++	+++	-	-

2.3.2 Phage persistence in the larval honeybee

This study aimed to determine whether phages would reach the larval gut and how long the phages would persist in a larval gut. Five hives were previously established in a single apiary and each hives' brood racks (with the worker bees covering the brood) were sprayed with *B. laterosporus* phage lysate suspended in sugar water. One hundred larval specimens were collected from each hive at spaced time points and were tested for the presence of viable phages, see Figure 2.2.

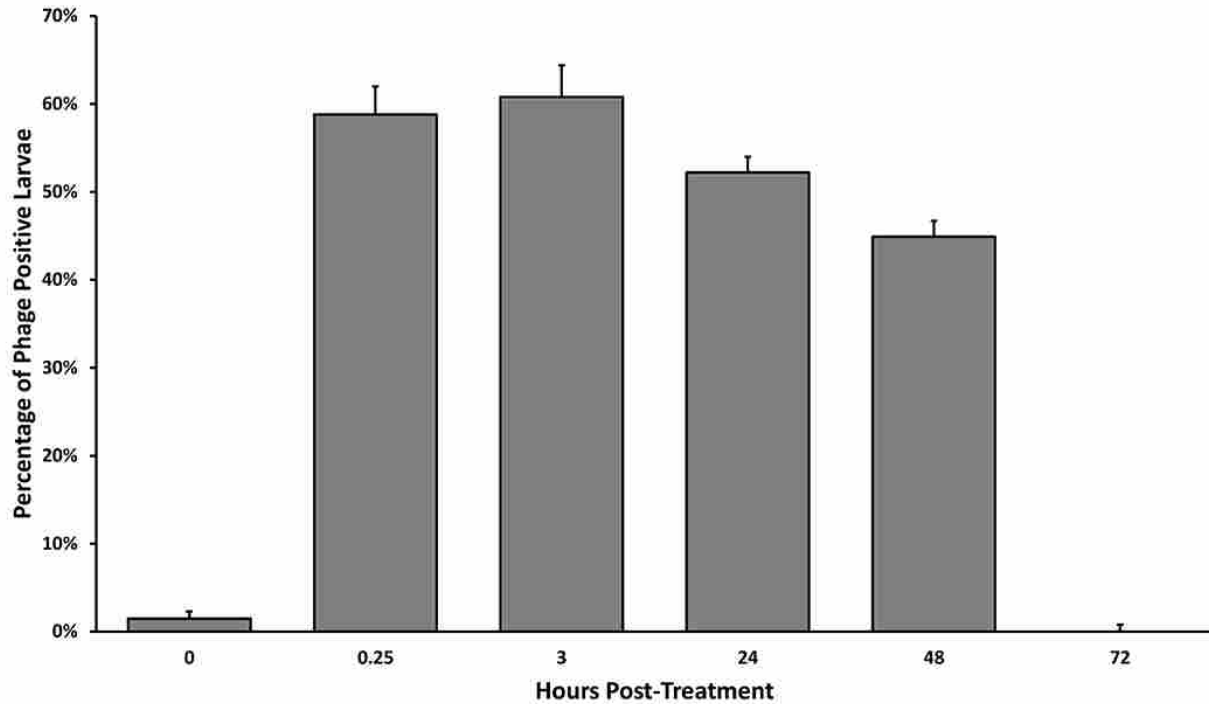


Figure 2.2. Average presence of phages in larvae after treatment. The time 0 sampling was taken just prior to initial treatment to serve as a baseline. Bees and racks were sprayed with phages and larvae were plucked from the racks at each time point and tested for the presence of phages.

The first samples were collected at time 0 immediately prior to treatment with the phage cocktail to establish a baseline for the presence of naturally occurring phages in honeybee larvae. Phage persistence studies showed that phage presence in bee larvae was $1.5 \pm 0.8\%$ before treatment and rose to $58.8 \pm 3.2\%$ 15 minutes after treatment, $60.8 \pm 3.6\%$ after three hours, $52.2 \pm 1.8\%$ after 24 hours, $44.9 \pm 1.8\%$ after 48 hours, and $0 \pm 0.8\%$ after 72 hours. Phages were found in larvae within 15 minutes of the treatment and peaked at three hours where $60.8 \pm 3.6\%$ of larvae contained detectible, viable phages as determined by spot test. Phage presence in bee larvae remained well above the normal untreated control for two days after the treatment was administered. After three days, the phage presence returned to the normal nominal levels.

2.3.3 Phage infection induces *B. laterosporus* to produce antimicrobial toxins

During culture of *B. laterosporus* phages, we observed that bacterial lawns exhibited clearing from phage plaques as well as a diffusion of some type of toxin in the vicinity of a plaque. An experiment was designed to characterize the effects of *B. laterosporus* phage on the production/release of toxins from *B. laterosporus*. Strains BL-2 and BL-6 were infected with the phages Fawkes and Emery/Abouo respectively in duplicate. The resulting lysates were filtered and three μL spotted onto lawns of different bacteria. Toxin was qualified by the creation of a zone of clearing in the bacteria on the plate indicating cell die off distinguished between plaques from phages by observing the shape and size of the clearing (Figure 2.3, Table 2-3). Lysates from Fawkes and Emery/Abouo both contained toxins that were lethal to BL-2, BL-6, *P. larvae* ATCC 9545, and *E. coli* B079. Neither lysate type was effective against *Agrobacterium*

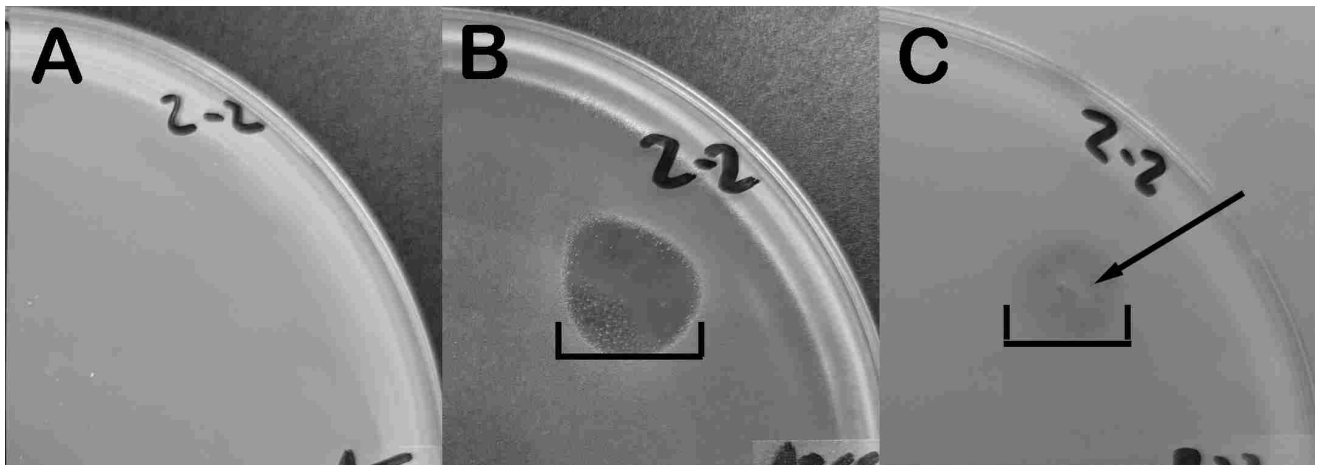


Figure 2.3. *B. laterosporus* toxin spot tests.

Drops of *B. laterosporus* phage lysate were placed and incubated for 24 hours onto (A) a lawn of *A. tumefaciens* that did not respond to the toxin or generate plaque clearings (B) a lawn of *P. larvae* that exhibited toxin death, and (C) a lawn of *B. laterosporus* strain BL2 that showed toxin death as well as phage infection plaque formation. Brackets indicate toxin clearing, arrow indicates phage plaque formation.

tumefaciens or *Sinorhizobium meliloti*. These data indicate the sensitivity of *P. larvae* to the toxin generated by *B. laterosporus*, and that the toxin is not effective against other bacteria.

Control samples recreated various stages of the phage life cycle to verify phage-induced toxin production as opposed to toxin release from other mechanisms. Supernatant from UV-killed bacteria was spotted onto lawns of bacteria to identify if bacterial death alone induces toxin production. The supernatant from mechanically-lysed bacteria was also tested to determine whether phage lysis releases toxins present in the bacterial cytoplasm. Supernatant from untreated vegetative *B. laterosporus* was also tested to identify whether unprovoked bacteria releases toxin. None of the control sample supernatants formed a zone of clearing in bacterial lawns, indicating that these mechanisms did not result in toxin production or release as seen in

Table 2-3. Bacterial susceptibility to *B. laterosporus* toxin. *P. larvae*, *E. coli*, *A. tumefaciens*, *S. Meliloti*, and two strains of *B. laterosporus* were challenged with the supernatant from two phage lysates and the supernatant of live, dead, and mechanically lysed *B. laterosporus*. Toxin-induced death is indicated by plus signs. A minus sign indicates no discernable toxin clearing on the bacterial lawn.

	<i>B. Laterosporus</i> (BL-2)	<i>B. laterosporus</i> (BL-6)	<i>P. larvae</i>	<i>E. coli</i>	<i>A. tumefaciens</i>	<i>S. Meliloti</i>
Emery/Abouo Phage lysate (BL-6)	+++	++*	++++	+	-	-
Fawkes Phage lysate (BL-2)	++*	+++	++++	+	-	-
Supernatant of live <i>B. Laterosporus</i>	-	-	-	-	-	-
Supernatant of UV killed <i>B. Laterosporus</i>	-	-	-	-	-	-
Supernatant of mechanically lysed <i>B. Laterosporus</i>	-	-	-	-	-	-

*Phage plaques were discernable on the bacterial lawns as well as death from toxin

Table 2-3. The lack of toxin-induction via UV killing and mechanical lysis indicates that the bactericidal toxin produced by *B. laterosporus* is not a result of bacterial death or lysis.

Phage-induced toxin production may be the result of expression of toxin genes encoded by the bacteria since no known toxin genes reside in the sequenced phage genomes while several toxins have been identified in *B. laterosporus* (49,67). If the phages carry the toxin gene, then the toxin sequence must be one of the genes of unknown function in these phages. The fact that more than one very-genetically-different *B. laterosporus* phages can induce the bacteria to make an identically acting toxin indicate that the phages do not carry the toxin genes. Table 2-3 and (71) may further suggest that the toxin arises from the bacterial genome instead of the phage genome.

2.3.4 Phage-induced *B. laterosporus* toxin shows inert characteristics against honeybees
This study aimed to determine whether phage treatment for *B. laterosporus* would be problematic for honeybees. Since *B. laterosporus* has been suggested to be a commensal to honeybees, this study was conducted to observe if side effects of phage-induced toxin or phage killing of *B. laterosporus* in the bee gut would decrease the overall health of the hives. Twelve hives, six in a test group and six in a mock-treated group, were installed into new boxes with new frames in spring. New queens and approximately 2.5 pounds of honeybees were installed into each box and weekly inspections were made to follow the bees' progress by observing the amount of bees in the spaces between racks. Hives were allowed to become established for nine weeks before receiving phage or mock treatments. Populations in all treated and untreated hives stayed below four full racks through early-summer. In mid-summer, the bees began to expand to fill the fourth rack, at which point the phage treatment commenced.

All 12 hives were treated three times at weeks nine and eleven, with three days between treatments for each regiment. Our data show that all hives expanded at approximately the same rate during the study, see Figure 2.4. There was no statistical difference between the expansion of the bee populations in mock-treated controls versus the phage-treated group. The data were evaluated statistically using the repeated measures, mixed procedure, two-tailed analysis of the number of bee-filled spaces in the treated and control hives over the 17-week period using an alpha level $\alpha=0.05$ (P-value of 0.1104). These data indicate that toxin in the phage lysate treatment was sufficiently low or not active on honeybees. Further, it shows that the hives were either lacking *B. laterosporus* and thus this bacterium is not essential for honeybee health, and/or that any toxin or killing from phage infection of *B. laterosporus* does not adversely affect

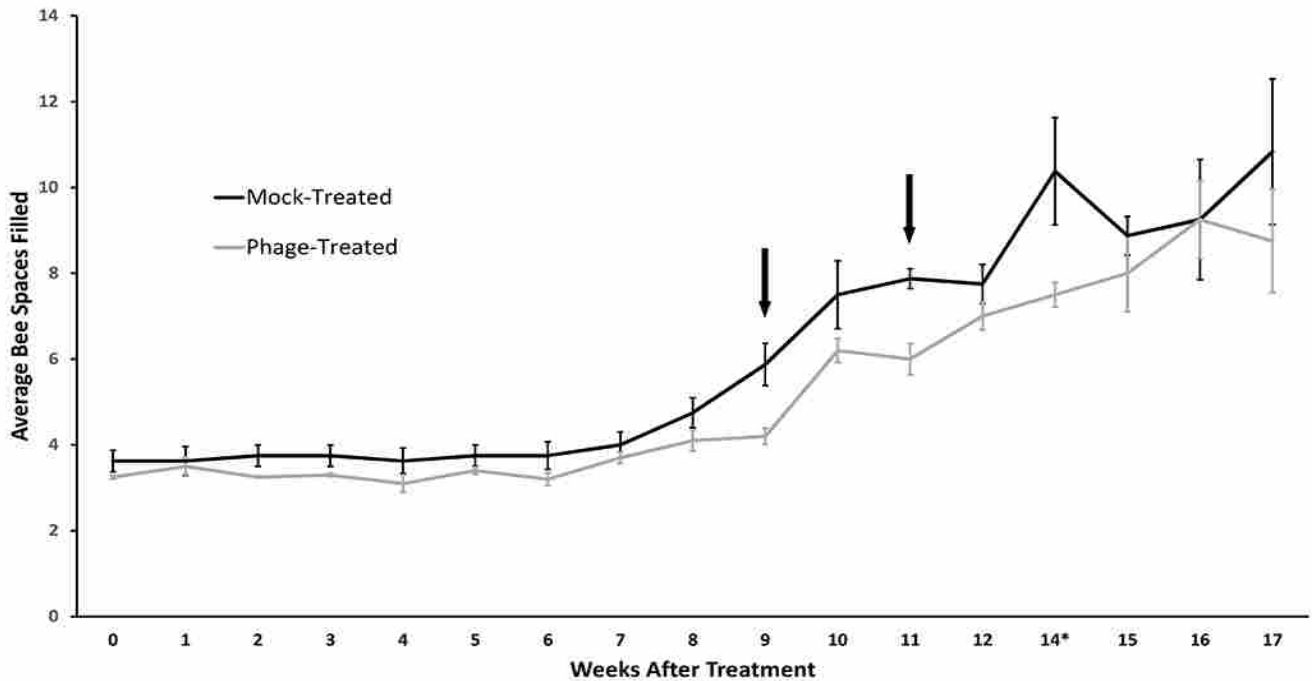


Figure 2.4. Colony expansion after phage treatment in beehives. New packets of bees with a fertilized queen were allowed to establish in new hives. Arrows indicate when phage treatments were administered to the bees and results demonstrate that healthy hives treated with *B. laterosporus* phage cocktail exhibited no difference in colony expansion when compared to healthy control hives. Bee spaces indicate honeybee population within the hive.
*Data were not collected for week 13.

honeybee expansion.

2.3.5 *B. laterosporus* phages can effectively treat an active AFB infection.

The objective of this experiment was to determine the effectiveness *B. laterosporus* phages in curing honeybee hives of American foulbrood caused by *P. larvae*. Forty hives of honeybees (*Apis mellifera*) were previously established in one apiary. Twelve of the 40 colonies presented with American foulbrood, the remaining 28 colonies were relocated to prevent the spread of the disease to the remaining healthy hives. All 12 sick hives were treated three times (each treatment was given three days apart) by spraying each rack on both sides with *B. laterosporus* phages in sugar water. The remaining 28 hives appeared healthy and were treated with antibiotics by the beekeeper. Treatment of the beehives occurred immediately before the onset of winter.

All 40 hives were inspected two weeks after the first treatment. Nine of the 12 infected hives treated with *B. laterosporus* phages recovered and showed no signs of AFB upon inspection at week two, which indicates a 75% cure rate (see Table 2-4). Two of the 28 originally uninfected hives were diagnosed with AFB despite their initial healthy appearance at the beginning of this study. The two hives had received antibiotic treatments along with the other

Table 2-4. Survival rate of hives after treatment in fall and after winter. Infected hives received phage cocktail treatments and uninfected hives were prophylactically treated with antibiotics. Survival rates of the hives were evaluated after two and 16 weeks.

	Total hives	AFB-free post-treatment	Hive Survival over winter
Uninfected hives	28	92.85%*	78.1%†
AFB infected hives	12	75%	62.5%

*Two hives of the 28 uninfected became infected when they were removed from the initial 12 infected

†Results excluding the two hives that became infected with AFB

26 healthy hives; however, both hives collapsed with severe signs of AFB which suggests that the infecting strain of *P. larvae* was antibiotic resistant. Dead-out hives were burned. No further problems were reported with the other 26 hives. Dead larval samples were taken from the hives before the first phage treatment and healthy larvae at two weeks post-phage treatment (healthy larvae were taken post-treatment because no dead larvae were observed). The larval samples were analyzed by PCR for the presence of bacteria. Results of PCR confirmed the presence of *P. larvae* and *B. laterosporus* DNA at pre-treatment, and only *P. larvae* DNA with no amplification of *B. laterosporus* DNA at post-treatment. The nine hives that recovered from AFB were followed through winter. In spring, of the nine recovered hives, five survived, four died. No signs of AFB were found in the four dead hives; three of the hives appeared to have frozen to death, and one hive was destroyed by vandals.

2.3.6 *B. laterosporus* phages do not prevent reinfection by latent *P. larvae* spores.

The five recovered, surviving hives were followed for nine months to investigate the effectiveness of the *B. laterosporus* phage cocktail in the inactivation of latent *P. larvae* spores, see Table 2-5. Two weeks after phage treatment as well as in the following spring, 16 weeks

Table 2-5. Health of five hives after an AFB outbreak, *B. laterosporus* phage treatment, and overwintering.

The surviving hives after *B. laterosporus* phage treatment were monitored for 28 weeks after initial treatment. When hives were seen to relapse, all hives were retreated with the phage cocktail.

	Week 16	Week 18*	Week 20	Week 22*	Week 24	Week 26*	Week 28
Healthy	5	4	5	3	5	0	5
AFB +	0	1	0	2	0	5	0

*Weeks when phage cocktail was administered

after the first treatment, all five hives had no signs of AFB infection. At 18 weeks, one of the five surviving hives experienced an AFB infection and that hive recovered after another treatment of *B. laterosporus* phages. At each time of recurrence, all 5 hives in the apiary were preemptively treated with the phage cocktail. At week 22, the first hive and a second hive experienced AFB symptoms, which were again treatable with *B. laterosporus*, signs of AFB disappearing within a week of the treatment. By week 26, all five hives presented with AFB symptoms and were treated with the phage cocktail, which again cleared all of the hives of AFB symptoms with 2 weeks. All hives were destroyed mid-summer due to the reoccurring infections. These data indicate that *B. laterosporus* phage treatment could kill active *P. larvae* infections, but could not kill *P. larvae* spores nor prevent future infection.

2.4 Discussion

The phage cocktail used in these studies was formulated to specifically infect a wide range of *B. laterosporus* field isolate strains. As seen in (82) and (83), phage cocktails designed in this manner (with phages in the cocktail selected according to the ability to kill as many field strains as possible) are effective at reducing the amount of their target bacteria. Here, we observed that the phages selected for a cocktail using laboratory-generated data of phage efficacy was predictive of the efficacy of phages in field tests, as observed by the reduction in the amount of *B. laterosporus* DNA present in hives. Furthermore, we studied the toxin-inducing capabilities in the laboratory to observe whether or not the toxin could be effective at reducing *P. larvae* bacteria, and then applied our results to safety and efficacy studies in the field. Using *B. laterosporus* phages as a biocontrol comes with some inherent risk. We were concerned to know whether, by inducing toxin synthesis and lysing *B. laterosporus*, the phage cocktail could release toxins with insecticidal properties or other adverse effects in honeybees. No such deleterious

effects were seen in our studies. Firstly, we observed rapid loss of detectable phages in healthy larvae (Figure 2.2), which indicates that a phage treatment has a relatively short exposure time to the bees. Secondly, we observed no short-term or long-term harm to healthy honeybees treated with multiple doses of phages (Figure 2.2, Table 2-4, and Table 2-5). These studies add to the expanding literature that indicates that phage cocktails are a safe alternative to traditional antibiotic use (36,37,84-88).

This study indicates that *B. laterosporus* is not a necessary symbiont for honeybee health, which conclusion is contrary to the postulations of several other researchers (51,69) but supports reports by others (49). The current field of research surrounding *B. laterosporus* is tempestuous as to its merits and disadvantages. However, the research conducted in this article is uniquely equipped to demonstrate the effects of beehives with and without *B. laterosporus* in vivo and the results indicate that there are no significant differences between hives with or without the bacteria. This study also demonstrates advantages to having the bacteria naturally present and using phages to induce toxins to kill pathogenic bacteria.

One aim of our studies was to determine whether a phage cocktail designed for a co-infecting or commensal bacteria (*B. laterosporus*), could reduce the presence of a pathogenic bacteria (*P. larvae*), during a disease state (AFB infection). Figure 2.5 depicts this new “bystander phage therapy” as a phage treatment approach compared to the current dogma of phage therapy. Such situations may be more common than just this *B. laterosporus/P. larvae* system because a co-existing, non-pathogenic bacteria may evolve to secrete toxin in order to out-compete a pathogenic bacteria. The “bystander” bacteria may be poised to produce toxin under stress as we were able to do using phage infection. It is important to note that none of our *B. laterosporus* phages could infect *P. larvae*. Therefore, any activity of a cocktail of *B.*

laterosporus phages against AFB must be either from toxin release to induce bystander killing of AFB or that *B. laterosporus* is responsible for AFB. We do not believe the latter is true. It was already known that *B. laterosporus* can produce antimicrobial toxins (49,67) and results from our laboratory experimentation in section 3.4, and Table 2-3, demonstrate that these compounds are effective against *P. larvae* as well as other unrelated bacteria. As seen in section 3.5 and Table 2-4, the phage cocktail can clear an active AFB infection but is not curative as observed by the recurrent infections presented in Table 2-5, section 3.6. We hypothesize that the toxins released by the phage when infecting *B. laterosporus* are effective against the vegetative bacteria that infect the larval brood, but that the toxins are not strong enough to eradicate *P. larvae* spores.

The *B. laterosporus* phage treatment used in these studies demonstrated a 75% success rate in recovering actively-infected beehives from AFB. After *B. laterosporus* phage treatment, the recovered hives were sufficiently healthy to overwinter, albeit at a slightly lower rate than the national overwinter average. This particular AFB outbreak was sufficiently virulent to cause complete collapse of a beehive, as observed in the two hives that were misidentified in the healthy control group that must have been infected at the start of the study. The virulence of the bacteria is evident by the loss of these two colonies within two weeks of the study start time, despite antibiotic treatment, and during the same time period that the diseased colonies completely recovered after *B. laterosporus* phage treatments. AFB-diseased beehives treated with *B. laterosporus* phages retain bacterial spores from the infection and required ongoing maintenance to prevent recurrent AFB the following spring and summer.

These results indicate that the AFB infection was caused by a *P. larvae* infection as would be expected, and further that *B. laterosporus* phage treatment can clear a *P. larvae* infection despite it not infecting *P. larvae*. The recurrent infections indicated that *P. larvae* spores remained in the hives after the *B. laterosporus* treatment in a similar manner as occurs after antibiotic treatments of AFB-infected hives.

Bystander phage therapy has an advantage over typical phage therapy because the range of targets affected by the toxin can be much greater than traditional phage therapy that has limited host range. For instance, bystander phage therapy does not rely on the phage killing all of its targets. Rather, the phages only need to infect and induce enough bacterial toxin to kill the pathogen (Figure 2.5). By this method, a hive could be infected with several strains of *P. larvae* that could include phage resistant *P. larvae* because of the limited host range of the individual phages, but the bacteria could still be killed by the phage-induced *B. laterosporus* toxin. This bystander effect could occur regardless of whether or not all strains of the non-pathogenic

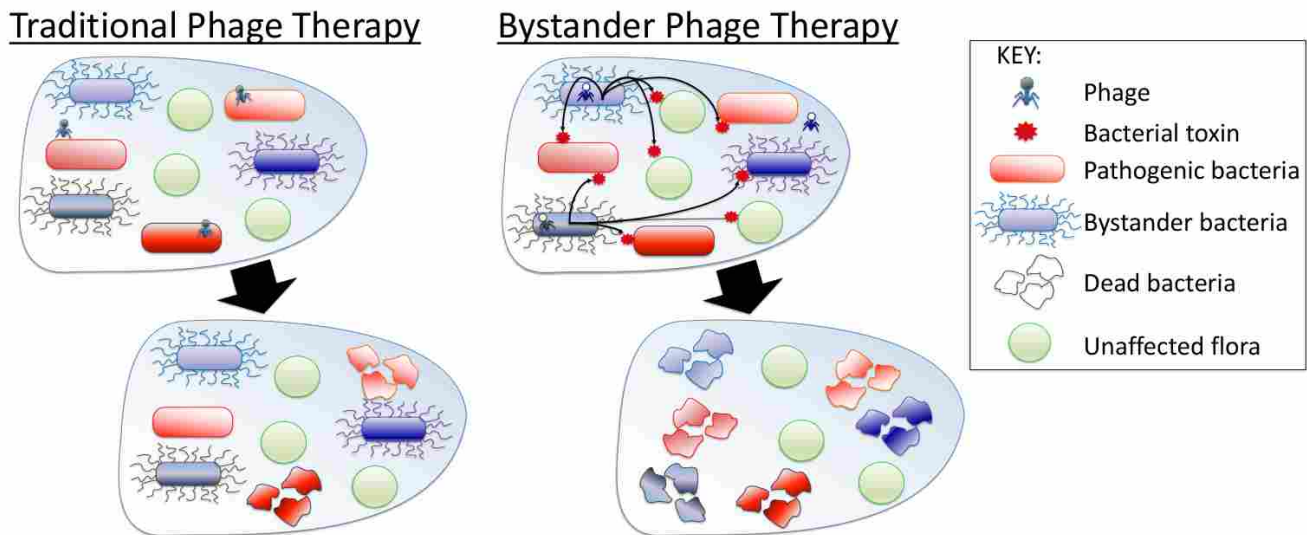


Figure 2.5. Mechanism of pathogen killing using phages for traditional phage therapy versus bystander phage therapy.

Phages against a pathogenic bacterium bind and lyse some bacterial strains, but may leave others unscathed. Phages against a bystander induce the bystander to make a toxin that kills all versions of the pathogenic bacteria and possibly other strains of itself that were not infected by phages.

bacteria (*B. laterosporus*) are killed. An option not to kill all target bacterium is useful and desirable for a phage therapy approach because it means that the cocktail for bystander phage treatment would not need to include phages to kill every possible bacterial strain of its target. This simplifies the cocktail itself, and increases the chances of the treatment being functional since it is not dependent on killing all bystanders, but simply on activating the bystander to kill the pathogen.

Due to the nature of the antimicrobial effects of the toxin produced by *B. laterosporus*, bystander phage therapy could function as treatment against other bacterial infections in beehives such as *M. plutonius*, the causative agent of European Foulbrood. If the phage-induced toxin is lethal to other pathogens such as *M. plutonius*, then it would be an attractive alternative to standard phage therapies because of its ability to treat various diseases. This approach is especially helpful in the case of misdiagnoses, since *B. laterosporus* could be present regardless of the pathogen causing symptoms in the hive. Culture of certain bacterial pathogens, such as the anaerobic bacterium *M. plutonius*, can be difficult to accomplish in the lab and therefore make it difficult to isolate phages for traditional phage therapy treatment. By inducing a bystander bacterium to produce a toxin, phages can remain a treatment option even for difficult-to-culture bacteria.

2.5 Conclusions

Phage therapies are an attractive alternative to traditional antibiotic use in the face of antibiotic resistance in pathogens. This study presents bystander phage therapy as a new alternative approach for phage therapy. The phages used in this study did not target the pathogen causing the disease that it treated, but rather targeted a known co-infecting bacterium and induced the co-infecting bacteria to produce toxins to which the pathogen is sensitive.

The properties of phage-induced toxins produced by *B. laterosporus* can be characterized to establish the extent of their host range. This research demonstrated that phages can induce *B. laterosporus* to produce bactericidal toxins and demonstrated how phages that kill bystander bacteria can also result in killing of off-target, pathogenic bacteria. This approach could be useful as a single treatment for different diseases caused by different pathogens with overlapping symptoms provided that the phage-induced toxin can kill both pathogens, and that the loss of the toxin-producing bystander bacteria is not vital to the organism. In this case, *B. laterosporus* is not a vital commensal and treatment of healthy bees with *B. laterosporus* phages did not result in any detectable health consequences in the bees. Use of *B. laterosporus* phages rescued a significant number of sick hives from succumbing to an antibiotic-resistant form of AFB. The use of bystander phage therapy is an exciting and new avenue of study that merits further investigation in the field of phage research.

2.6 Acknowledgments

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CHAPTER 3: "BACTERIOPHAGES AS AN ALTERNATIVE TO CONVENTIONAL
ANTIBIOTIC USE FOR THE PREVENTION OR TREATMENT OF *PAENIBACILLUS*
LARVAE IN HONEYBEE HIVES"

The following manuscript "Bacteriophages as an Alternative to Conventional Antibiotic use for the Prevention or Treatment of *Paenibacillus larvae* in Honeybee Hives" was written for and submitted to the *Journal of Invertebrate Pathology*, and was published November 2017. The article describes the creation of a phage cocktail to treat and protect at risk hives from American foulbrood.

References for this manuscript are found in chapter 6 and the in text references to figures or sections are to those within this chapter.

Full Title:

Bacteriophages as an Alternative to Conventional Antibiotic use for the Prevention or
Treatment of *Paenibacillus larvae* in Honeybee Hives

Short Title:

Bacteriophages for Treating AFB

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Keywords: American foulbrood, bacteriophage, phage, phage therapy, *Paenibacillus larvae*, antibiotics, tylan, prevention, treatment, safety

Abstract

American foulbrood (AFB) is an infectious disease caused by the bacteria, *Paenibacillus larvae*. *P. larvae* phages were isolated and tested to determine each phages' host range amongst 59 field isolate strains of *P. larvae*. Three phages were selected to create a phage cocktail for the treatment of AFB infections according to the combined phages' ability to lyse all tested strains of bacteria. Studies were performed to demonstrate the safety and efficacy of the phage cocktail treatment as a replacement for traditional antibiotics for the prevention of AFB and the treatment of active infections. Safety verification studies confirmed that the phage cocktail did not adversely affect the rate of bee death even when administered as an overdose. In a comparative study of healthy hives, traditional prophylactic antibiotic treatment experienced a $38\pm 0.7\%$ decrease in overall hive health, which was statistically lower than hive health observed in control hives. Hives treated with phage cocktail decreased $19\pm 0.8\%$, which was not statistically different than control hives, which decreased by $10\pm 1.0\%$. In a study of beehives at-risk for a natural infection, $100\pm 0.5\%$ of phage-treated hives were protected from AFB infection, while $80\pm 0.5\%$ of untreated controls became infected. AFB infected hives began with an average Hitchcock score of 2.25 out of 4 and $100\pm 0.5\%$ of the hives recovered completely within two weeks of treatment with phage cocktail. While the n numbers for the latter two studies are small, the results for both the phage protection rate and the phage cure rate were statistically significant ($\alpha=0.05$). These studies demonstrate the powerful potential of using a phage cocktail against AFB and establish phage therapy as a feasible treatment.

3.1 Introduction

American foulbrood (AFB) is one of the most widespread and destructive bee brood diseases. AFB is caused by the spore-forming bacterial pathogen *Paenibacillus larvae* and is spread by worker honey bees inadvertently collecting *P. larvae* spores from the environment or contaminated hives (65). If worker bees retain spores in their honey stomach, they can infect the bee larvae (brood) while regurgitating the contents of their honey stomach (including the *P. larvae* spores) during larval feeding (16). When infection occurs, the spores germinate and kill the bee larvae. The bacteria liquefy the larvae, producing a viscous, spore-laden fluid. The disease spreads rapidly within a hive and can destroy entire hives if the infection is left untreated (66). Antibiotics, Oxytetracycline and Tylosin Tartrate (Tylan® Soluble™), are commonly used to prevent and treat AFB infections. However, antibiotic treatments have several disadvantages. For instance, many wild strains of *P. larvae* have antibiotic resistance to Oxytetracycline (24,28,40,43,89,90). In a 2006 study, 58% of field samples were resistant to oxytetracycline (28). The only alternative to oxytetracycline for treating AFB is Tylosin Tartrate, which has resulted in Tylosin being the most commonly used conventional antibiotic for the treatment of AFB in the United States today. Antibiotic residue in honey also poses health risks to children and developing babies (41). Antibiotic treatments can increase the chances of fungal infection, nosema, due to disruption of the normal balance of bacteria in the bee gut (20). Furthermore, recent legislation prevents beekeepers from purchasing antibiotics over the counter, requiring a veterinarian visit and prescription to receive any antibiotics. Hives must be burned when antibiotics fail to cure AFB infections to prevent its spread to other hives in an apiary.

P. larvae, like all bacterial species, has natural opponents called bacteriophages (phages). Phages are viruses that only infect and replicate in bacteria and have the potential to overcome

the disadvantages posed by antibiotics. A single type of bacteria will have many phages that are specific for that bacterium (91,92). The extreme specificity of phages is observed as their ability to bind and infect only their target bacterium and leave other cell types, bacterial and eukaryotic, unharmed. The specific killing activity of phages makes them an ideal replacement for antibiotics.

In this study, 39 phages were isolated that infect *P. larvae*. Each phage was tested against 59 bacterial strains of *P. larvae* and the results indicate a variety of infection capacity of each phage. Based on the results of phage infectivity and bacterial lysis in-vitro, three phages were selected for testing in beehives. The selected phages were cultured and concentrated to generate a phage cocktail treatment. The phage cocktail was then used in live beehives to explore the phages' effect on the overall health in beehives compared to traditional antibiotics, and the phages' ability to clear and protect against AFB in a naturally occurring outbreak. In the beehive studies, the phage cocktail appears to be safe for bee consumption, the phages can protect hives from an impending infection and the phages are capable of quickly curing infected hives.

3.2 Materials and Methods

3.2.1 Bacterial and phage infectivity.

Isolated phages were grown from the bacterial strain *P. larvae* ATCC 9545. The bacteria were grown in Porcine Brain Heart Infusion (PBHI) (Acumedia, Lansing, MI) broth overnight in a shaking incubator at 37 °C and 120 rpm. Colony forming units (cfu) determination was made using the equation $C=A/E$ where A is absorbance at 580nm, E is 6.6×10^{-9} , and C is cfu of *P. larvae*/mL. Flasks prepared with 1/4x PBHI broth were inoculated with 10^7 cfu/mL of sterile broth. A well-titered phage lysate is added to the broth such that the final number of pfu of phage in the flask is 1/3 of the number of cfu of *P. larvae*. The mixture was allowed to incubate for 12-

18 hours as described above. After incubation, the solution was filtered through a 0.2 µm sterile syringe filter and the lysate containing phages was stored at 4 °C.

The titer of the lysate was tested as previously described (71). Briefly, a serial dilution of the phage lysate is made to the 10^{-5} dilution. The dilute phages are added to bacteria grown overnight in 10 mL incubation tubes and were allowed incubate at room temperature. PBHI top agar was added to the mixture after 30 minutes and then the entire mixture was plated onto previously prepared PBHI agar plates (Fisher Scientific, Fair Lawn, NJ). Plaques were counted 12-24 hours later to calculate titer with the equation (plaque number)/(phage dilution)(mL infected) = pfu/mL.

3.2.2 Control sugar water mixture, phage cocktail treatment, and antibiotic treatment preparation.

The treatment mixture for trough feeders was comprised of 2:1 volume:volume sugar water. Sugar water was poured into the feeder trough for the control hives. Phage lysate was added to 500 ml sugar water, at a 1x recommended treatment consisting of 20mL, titered at 5×10^8 plaque forming units per ml, and was then poured into the feeding troughs of the hives receiving treatment. For spray on phage treatment a 1:1 sugar water was used instead of 2:1. Control hives received 340 mL of sugar water, while the phage treated hives received 320 mL of sugar water with 50 ml of phages mixed into the sugar water. For antibiotic treatment, 200mg Tylan® Soluble™ (Elanco™, Greenfield, IN) was mixed in 20 grams of powdered sugar for each hive and the mixture was dusted onto the top bars of the brood chamber.

3.2.3 Criteria for healthy hives to be included in studies.

Each of the hives had to meet the following four criteria: a viable laying queen, contain approximately 40,000 or more adult worker bees, have uncapped brood, and have no visible signs of American foulbrood. All hives used in the healthy hive studies met these criteria.

3.2.4 Three-brood rack test, Hitchcock scale test, and non-AFB illness tests qualifications.

These tests were to assess the AFB infection level of a hive. Observation of three full brood racks from a single hive constituted a complete AFB hive inspection. Any hive that had a brood rack that showed signs of illness were inspected for signs of AFB beyond the 3-brood racks using the Hitchcock scale of infection. For each indication of AFB, the hives were rated on a 0-4 scale based on a modification of the method proposed by Hitchcock, 1970 (93). Briefly, hives are examined and each of the frames rated as follows: 0= no signs of disease, 1= <10 cells per frame affected, 2= 11-100 cells per frame affected, 3= > 100 cells per frame affected, and 4= total hive collapse/death. In non-AFB illness tests, a hive was counted as “diseased” if it developed AFB, chalkbrood, European Foulbrood, or struggled to thrive when a queen stopped laying well and the hive could not maintain population even if there were no other visible signs of disease. All hives were inspected using these methods and scores were determined during inspections.

Statistics used. The BYU statistical center was used to generate p-values, standard deviation, and standard error to show the statistical significance of the data collected using the ANOVA algorithm. Statistical analysis included repeated measures, mixed procedure, two-tailed statistical analysis using the Fisher’s exact test for 2x2 contingency tables with $\alpha = 0.05$.

3.3 Results

3.3.1 Phage infectivity of bacterial strains in culture can be used to select phages for a treatment cocktail.

Our objective was to identify phages for our treatment study that could infect and kill a wide range of *P. larvae* strains. A total of 39 *P. larvae*-specific phages were tested for their infectivity against 59 field strains of *P. larvae* and the results of lytic testing are summarized in Figure 3.1. In Figure 3.1, bacterial field isolates are listed in each column in the order in which the bacterial strain was isolated and numbered. Phages are listed in rows down the table in the order of the number of strains that the phage lysed. For instance, Phage 1 comprises the top row in the table because of its ability to lyse all but four bacterial strains and phage 39 could only lyse one strain of the 39 strains of bacteria. Phage 40 was a negative control using a phage that is not capable of infecting *P. larvae*.

The formulation of the phage cocktail arose from the results of the host range test in Figure 3.1. Three phages were selected (phages 1, 5 and 9) for subsequent work in beehives based on the combined ability of these phages to lyse all field isolate strains. Phage 1 lysed all field isolates of *P. larvae* except for PL314, PL323, PL328, and PL334a. Phage 5 lysed several strains including PL314, PL323, and PL334a. Neither phage 1 nor phage 5 could lyse strain PL328. Phage 9 lysed fewer strains but did lyse strain PL328. The combination of phages 1, 5 and 9 into the phage cocktail meant that all the field isolates tested could be lysed by one or more of the phages when administered to the beehives. To prepare a phage cocktail for testing in hives, a high titer lysate was prepared for each of the three phages. Prior to combining phages, the lysates were sterile-filtered to remove any residual bacteria and the lysate was tested for the

3.3.2 Highly concentrated phages in honeybee feed demonstrate anticipated inert characteristics.

A total of 24 hives of honeybees (*Apis mellifera*) were previously established in three apiaries near one another. The 24 hives were separated into four groups and each group received one of four treatments: a control solution of sugar water or dilutions of phages at 0.5x (2.5×10^8 pfu/ml), 1.5x (7.5×10^8 pfu/ml), and 2.5x (12.5×10^8 pfu/ml) of the recommended concentrations administered via feeding troughs. All hives were treated nine times (three times the amount of a standard preventative treatment) so that all treatment groups received what would be considered an overdose of the treatment. Dead bees were collected in traps below hives so that bodies dropped by workers could be counted each week to determine whether an increased

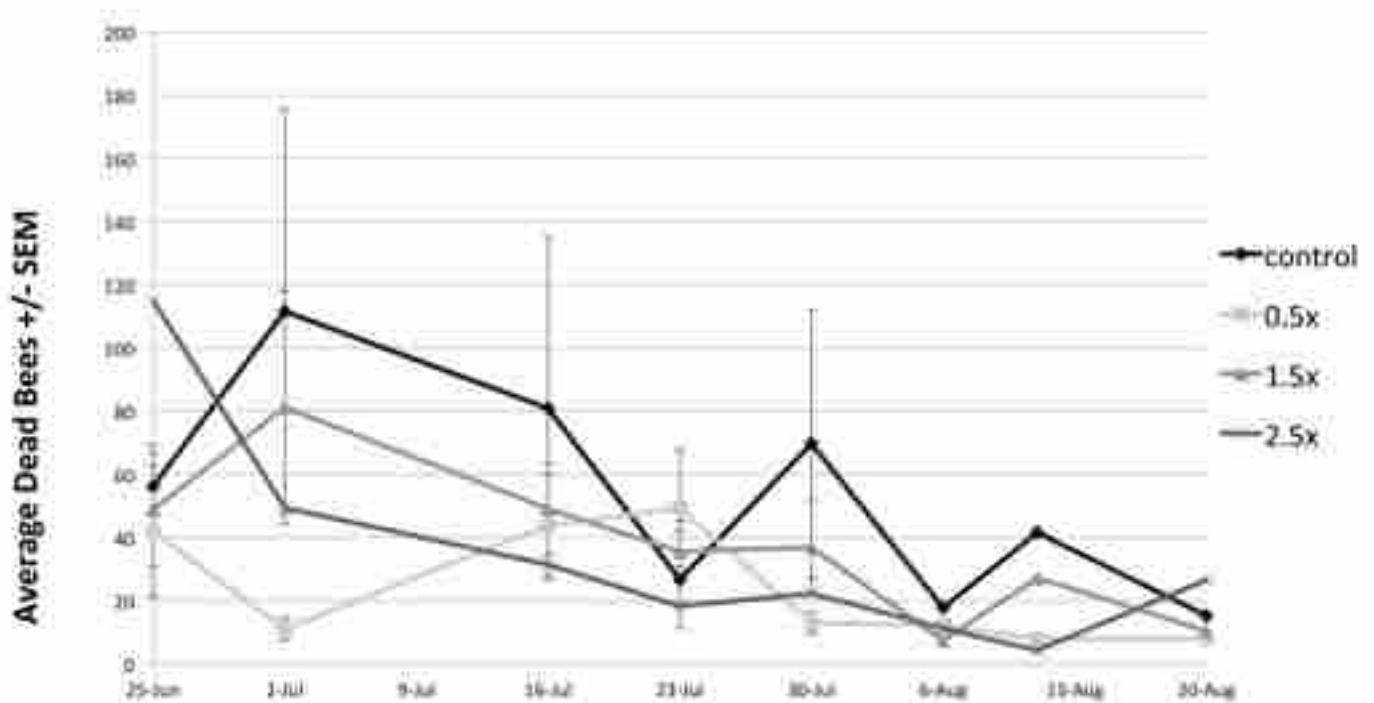


Figure 3.2. Average bee death over time in control and *P. larvae* phage-overdosed beehives. Hives were treated with doses of phage cocktail and average number of dead bees were counted over time. No statistical difference (P-value of 0.639) was observed between any of the test groups versus each other or the control group.

concentration of phages could produce any increase in bee death. Hives were inspected eight times and dead bees were counted. Results of this study are presented in Figure 3.2.

All 24 hives experienced a reduction in bee death over the summer months and remained AFB negative from June 25 to August 20, indicating overall healthy hives. No statistical difference was observed in bee deaths between the different phage concentrations and the control samples (Figure 3.2). The p-value measured for the repeated measures, mixed-procedure, and two tailed statistical analysis was 0.639 with a 95% confidence level. One hive in the 1.5x group was observed to be an outlier and exhibited higher death rates than all other hives. The results of data comparison between groups remained insignificant whether this group was included in the statistical analysis or not. From these findings, we see that dosing and even overdosing bees with phages does not adversely influence the death rate of AFB-free hives, as would be anticipated.

Table 3-1. Hives treated with Tylan® and *P. larvae* phages
Hives at each apiary were assigned to one of three groups to divide the 96 hives equally between treatments.

Group	Apiary 1	Apiary 2	Total # of Hives	Treatment Regimen
Control	16	16	32	Three treatments of 2:1 sugar water in feeding trough.
Tylan®	16	16	32	Three treatments of Tylan in powdered sugar in early spring and in early fall.
PL Phages	16	16	32	Three treatments of PL Phages in 2:1 sugar water in late spring and in early fall in feeding trough*.

*Some hives received spring PL phage treatment as a spray of instead of in the trough but the volume and contents of the treatments were identical for all hives.

3.3.3 Phages maintain hive health while traditional antibiotics are detrimental to honeybees.

A total of 96 beehives were previously established in two neighboring apiaries, 48 hives in each apiary. Each apiary was randomly divided into three equal groups, see Table 3-1. All 96 hives were inspected in spring and fall using a 3-brood rack approach. All hives were healthy at the beginning of the study and were treated in spring with a normal treatment cycle consisting of 3 doses of sugar water, phages or antibiotics according to what the individual hive was assigned. Hives were inspected again at the end of summer. None of the 96 hives in this study became infected with AFB. Three of the 32 control hives became diseased. A hive was counted as “diseased” if at any time it developed any of the following: chalkbrood, European Foulbrood, the

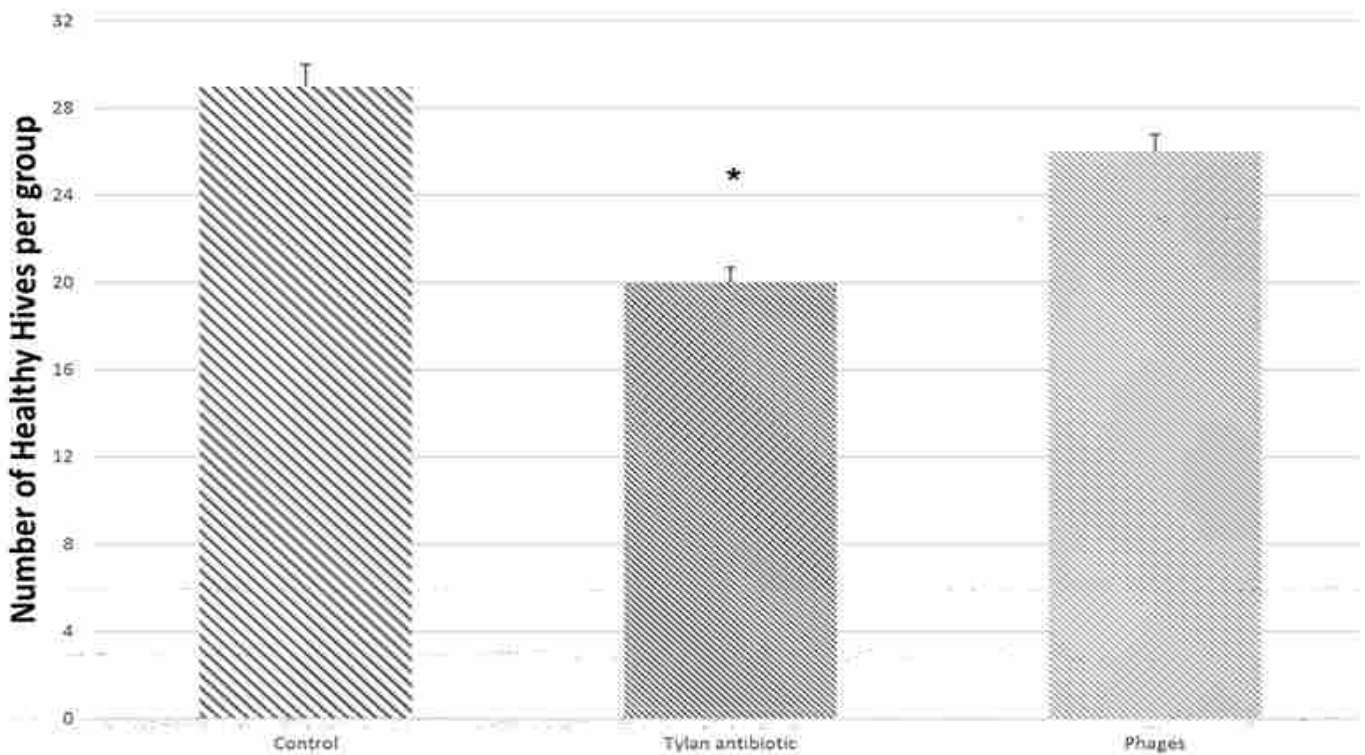


Figure 3.3. Honeybee health in hives that received prophylactic antibiotics, phages, or mock treatments. Treatments were administered in healthy hives during spring. Results depict the number of hives remaining healthy upon inspection at the end of summer with SEM bars indicated for each group. *Statistically significant at $\alpha = 0.05$.

queen stopped laying well, or the hive struggled to survive. Results of this study are presented in Figure 3.3.

Twelve of the Tylosin Tartrate-treated hives became diseased, leaving $62\pm 0.7\%$ of the antibiotic-treated hives remaining healthy. There is a statistically significant difference with a p-value of 0.0146 in the antibiotic-treated group in comparison to the control group using a two-tailed Fisher's exact test for 2x2 contingency tables with 95% confidence, indicating that antibiotic treatment reduces overall hive health. Of the control hives, $90.6\pm 1.0\%$ of the hives remained healthy, and of the *P. larvae* phage-treated hives, $81.3\pm 0.8\%$ of the hives remained healthy. There is no statistical difference between the phage-treated hives and the control hives, having a p-value of 0.4741 using the same evaluation as stated previously, which indicates a lack of evidence of any detriment to the bees due to phage treatment.

3.3.4 Phages Protect At-Risk Hives from AFB

A total of eleven beehives were previously established in a single apiary. One hive became infected with AFB from an unknown (natural) exposure and the other ten hives were still AFB negative at the beginning of this study. The ten originally healthy hives were divided into two groups: five were spray-treated with sugar water as a mock treatment and the other five were spray-treated with the phage cocktail in sugar water. The initially infected hive was also spray-treated with phage cocktail in sugar water (see the next section). All hives were treated three times in the first 10 days and observations and Hitchcock scores were taken at day zero and every two weeks for eight weeks, then again at four months. Any hives that became diseased with AFB during the study were treated with the phage cocktail immediately upon observation of diseased comb. Results are presented in Figure 3.4.

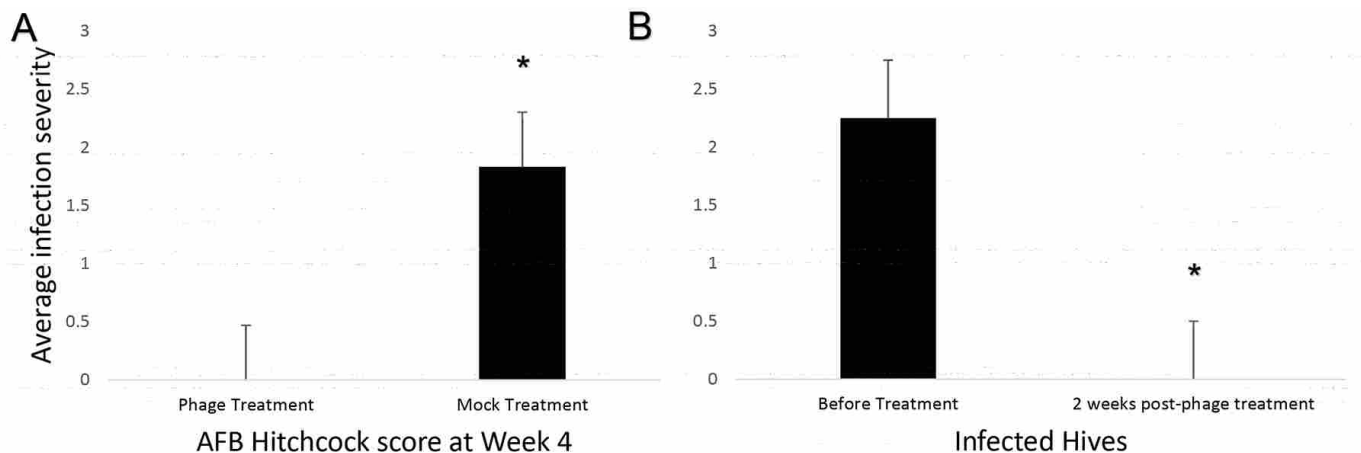


Figure 3.4. Phages effectively prevent and clear AFB.

At risk hives were treated with phages and control sugar water three times within 10 days starting at day 0. At the Week 2 inspection, all of the beehives were healthy (Hitchcock score=0, data not shown). At the Week 4 inspection, mock-treated hives exhibited signs of AFB and the average±SEM Hitchcock score is reported here (A). Any infected hives were immediately treated with phages and their Hitchcock scores before and two weeks after treatments were recorded (B). The Hitchcock infection severity score ranges from 0-4, with 0=no signs of AFB, and 4=hive death from AFB (see Materials and Methods). *Statistically significant at $\alpha = 0.05$.

The ten healthy hives were used to determine the phages' abilities to protect against an infection when housed near a sick hive. At day zero, all ten hives were healthy and had a Hitchcock score of 0. At week two, all ten hives were still healthy and had a Hitchcock score of 0. At week four, four of the five mock-treated hives were sick with AFB. One of the four was already sick beyond recovery (Hitchcock score=4), was abandoned by the queen and had very few adult bees remaining with severe AFB throughout the brood racks. The other mock-treated hives had scores of 1, 1, 3, 0. Results of the mock-treated hives indicate that the infection spread rate was $80 \pm 0.5\%$ for this incident of AFB. In contrast, all five phage-treated hives remained healthy with Hitchcock scores of 0 in all five hives. The average Hitchcock score±SEM for the Week 4 inspection in the phage-treated and mock-treated groups is presented in Figure 3.4A. These data indicate that the phage cocktail was $100\% \pm 0.5\%$ protective for the at-risk hives. The

results between groups were statistically significant with a p-value of 0.0016 using a Least Square Means in a Mixed Procedure analysis.

3.3.5 Phages Clear Hives of *P. larvae* Infection

In the previous study, the originally infected hive that put the other ten at risk, along with the three mock-treated hives that were still alive at the week four observation point, received the phage treatment in an attempt to cure the hives of AFB as immediately as it was observed. Within two weeks of phage treatment, all four of these hives were AFB-free, each with Hitchcock scores of 0. At the beginning of the study, the only one of the eleven hives with AFB had a Hitchcock score of “2.5”, and it recovered fully by week two. Of the five mock-treated hives, the one hive with a score of 4 at week 4 was burned, and the other three AFB-infected hives from the group were phage treated and all three fully recovered by week 6. The recovery of these three hives plus the recovery of the originally sick hive indicate that four of the four *P. larvae*-infected beehives recovered, which is a $100\% \pm 0.5\%$ recovery rate from this study. The average \pm SEM Hitchcock score before and after phage treatment is presented in Figure 3.4B. The difference between the before and after scores is statistically significant, with a p-value of 0.0022 using the same statistics as the previous study. At week eight, all hives remained free of AFB.

In fall, all ten of the surviving hives in the apiary were healthy and lacked signs of AFB. Honey was harvested from all ten hives. The hives were inspected again in October, four months after the infection and just prior to entering winter, and all ten hives remained healthy and lacked any signs of AFB. These data indicate that the phage cocktail successfully treated the infection and prevented recurrence.

3.4 Discussion

The phage cocktail used in this study was the direct result of laboratory host range testing and showed remarkable practical application in the field. Phages are known to infect multiple strains of the same or highly related bacteria. For instance, Jacobs-Sera et. al, identified 220 mycobacteriophages with overlapping ability to infect specific strains of *M. smegmatis* and *M. tuberculosis* (32). Mirzaei and Nilsson performed a host range analysis of phages against 72 strains of *E. coli* in an effort, identical to our approach, to select the best phages for a cocktail treatment (87). Salmonella phages were also isolated and compared for infectivity of 26 different strains of bacteria, with the highest host range phage being able to lyse 25 of 26 of the strains, and the lowest host range phage able to lyse 6 of the 26 strains (95). In our study, the most effective phage lysed 55 of our 59 strains of *P. larvae* and the least capable of our phages only infected one strain.

Only 22 *P. larvae* bacteriophage genome sequences have yet been published (85,94,96,97), compared to over 627 bacteriophage genomes of *M. smegmatis* (98). In this study, genetic comparisons were not attempted, but rather, each phage was tested for its lytic ability across the field isolates in order to identify the functionality of these phages to lyse one or more strains of the bacteria. Using results of the phages vs. bacterial strains study in Figure 3.1, the phage cocktail was based on the physical ability of the phages to lyse *P. larvae* strains rather than their genetic similarity or dissimilarity to one another. Genetic comparisons of phage genomes, such as the expanding work in *M. smegmatis* phages, have revealed complexity in interpretation of phage function to phage genomics. Functional assays, such as lytic tests spanning a variety of strains, are a valuable method to identify appropriate phages for field testing.

The safety of phages to non-target organisms has been well documented (36-39,47,85,99). Phages in the cocktail used in this research showed no discernable negative effects on bee hives as would be expected from the nature of phages. Phage therapy does not alter normal bee deaths nor their gut microbiota besides that they can infect and kill *P. larvae*. This study demonstrates the safety of phages, even when administered in high doses, for prophylactic use against AFB. These results are not surprising given the specific nature of phages, though they do add to the consensus of the literature that phages do not typically infect or harm bacteria for which they are not specific (100-103).

Antibiotics like Terramycin and Tylosin the only currently known antibiotics that have been effective against AFB; however, these antibiotics are deleterious to the overall health of hives when used prophylactically, as demonstrated in our study and also reported by others (20). Furthermore, the tetracycline family of antibiotics, to which Terramycin and Tylosin belong, causes bone and tooth deformities for fetuses and breast feeding infants (41,89,104). Treatment during honey-producing months yields honey with measurable amounts of antibiotics and therefore reduces the safety of honey consumption for those at risk (105). Due to the non-specific toxicity of antibiotics, both harmful and good gut microbes are killed when they are applied to the hives. This toxicity decreases the gut diversity of the honeybee which in turn makes them less healthy (20,43). In our side-by-side trial of phage treatment and Tylosin, the hives that were prophylactically treated with antibiotics had a decrease in hive health while the phage treated hives remained healthy in comparison with the control hives.

Antibiotic resistance in AFB has begun to render the treatment of hives with antibiotics obsolete (40,43). Resistance to phages is more difficult to achieve for the bacteria because the phages also have slight variations from generation to generation, making it possible for phages to

keep in step with any mutant strains of *P. larvae* (32). A concern for the future use and effectiveness of phage cocktails is the possible existence of a strain of *P. larvae* that might not be hit by a phage in the cocktail. However, we believe that the risk of strain specificity is preferential to the health risks and resistance associated with antibiotics. Furthermore, the use of a combination of phages in a treatment cocktail improves the likelihood of the phage treatment being effective against all bacterial strains. Phages are prevalent in the environment and can typically be readily isolated, which makes phages easier to discover than the production of novel traditional antibiotic chemical structures that will also eventually become obsolete due to antibiotic resistance.

We believe that the marked effectiveness of the phage cocktail in the prevention of AFB infection in our studies can be attributed in part to the combined host range of the phages included in the cocktail. Similar studies have been performed in situ with larval bees inoculated with a phage cocktail and then dosed with *P. larvae* spores. The phage-treated larvae showed the same survival rate (84%) as control larvae that did not receive phage nor spores; whereas, mock-treated larvae experienced a 45% survival rate (106). Another study showed that prophylactic phage cocktail dosing was able to increase the chances of larval survival by approximately 59% (85). The results from our study coincide with the results found by Ghorbani-Nezami et al. and Yost et al. that phage dosing can successfully be used prophylactically and after infection against AFB. Our study is the first in vivo phage treatment for AFB and, insofar as the results indicate, it is highly effective as such.

3.5 Conclusions

Phage cocktails have become increasingly popular as a solution to difficult-to-treat bacterial infections over recent years (38,83,86,88,99,106-110). Due to the nature of *P. larvae*

infections, phages are supremely suited for clearing AFB from infected hives. *P. larvae* is introduced to larval bees via nurse bees that feed them. The phage treatment is delivered to the site of infection in the same way. When nurse bees eat phage-laden sugar water from feeding troughs, they take the phages to larvae that may have been exposed to *P. larvae* spores. The phage cocktail treatment works by introducing these naturally occurring phages in artificially-high doses to germinating bacteria. AFB is highly infectious within an apiary, typically spreading to 60-85% of other hives after one hive is infected and, once showing visible symptoms, an infected hive will collapse without treatment (1,4,12,111). This was true in our study, where 80% of the control sugar water treated at-risk hives became infected. The efficacy of phage treatment was pronounced by the fact that 100% of phage-treated at-risk hives were protected from infection, and 100% of phage-treated sick hives became well.

These studies add to collective knowledge about the safety and efficacy of phage therapy. This work also demonstrated that the host range observations made in the lab had powerful correlations to the effectiveness of the field studies. While this publication includes a small prevention and recovery study, the numbers indicate the power behind using phages for prophylactic and curative treatments. Future studies are necessary to expand the overall power of phage treatments for AFB.

3.6 Acknowledgment

The authors thank the students of the Brigham Young University Department of Molecular & members at the Utah State Extension for providing apiaries for the experiments, and Dr. Eggett of the Brigham Young University Department of statistics for statistical assistance. We also thank local and distant beekeepers, and Joey Caputo and Stephen Stanko at the Utah Department of Food and Agriculture.

CHAPTER 4: “PHAGES USED TO TREAT AMERICAN FOULBROOD BIND TO
VEGETATIVE AND SPORE FORMS OF *PAENIBACILLUS LARVAE*”

The following manuscript “Phages used to treat American foulbrood bind to vegetative and spore forms of *Paenibacillus larvae*” was written and submitted to the *Journal of Basic Microbiology*, and is currently under review. The article describes the use of *B. laterosporus* phages to induce antibacterial toxins to treat American foulbrood.

References for this manuscript are found in chapter 6 and the in text references to figures or sections are to those within this chapter.

Full Title:

Phages used to treat American foulbrood bind to vegetative and spore forms of

Paenibacillus larvae

Short Title:

Paenibacillus larvae phages bind vegetative cells and spores

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Key words: American foulbrood, bacteriophage, phage therapy, *Paenibacillus larvae*, bacterial spores, phage-binding.

Abstract

Paenibacillus larvae endospores are the transmissible agent of the honeybee disease, American foulbrood. Previous *in vivo* studies show phage therapy can prevent and control American foulbrood. Here we present evidence that these phages not only bind to vegetative *P. larvae* but also bind to *P. larvae* spores, which likely contributes to the effectiveness of the treatment. *P. larvae*, *Brevibacillus laterosporus*, *Sinorhizobium meliloti*, and spores of *P. larvae* were each challenged with *P. larvae* phages. Plaque counts after a Brady Binding Test were statistically significantly higher from spore samples compared to *S. meliloti* samples ($p = <0.0001$) and negative controls ($p = <0.0001$). The same bacteria were challenged with FITC-labeled phages and fluorescence was measured by flow cytometry to quantify phage binding. Phage binding in spore samples was not statistically different when compared to phage binding in vegetative *P. larvae* ($p = 0.5563$). Phage binding in both vegetative and spore *P. larvae* samples was statistically higher than binding observed in unrelated *S. meliloti* samples and in negative control samples. Electron microscopy images of phage-treated spores provide visual evidence of phages binding to spores similar to that seen on vegetative *P. larvae*. The ramifications of spore-binding phages are discussed.

4.1 Introduction

The spore forming bacterium *Paenibacillus larvae* causes American foulbrood (AFB) in honeybees. An AFB outbreak begins when there are too many *P. larvae* spores in the honey crop of a nurse bee to be cleared naturally and spores get passed to honeybee larvae (10). In the larval intestinal tract, *P. larvae* spores germinate to become vegetative bacteria capable of producing toxins that liquefy the honeybee larvae (112). The resulting degraded larvae becomes laden with *P. larvae* spores which are then tracked to other larvae in the hive by nurse honeybees (1). The disease spreads quickly within a hive, taking just several days from initial infection to decimation of a colony (3,8). After the colony collapses, other colonies may rob the contaminated honey and further spread AFB spores with an 80% transmission rate during an outbreak in an apiary (82).

Due to their narrow host specificity, phage therapies can target pathogenic bacteria while leaving commensal bacteria alone. Phage therapy with an appropriate cocktail has proven to be an effective treatment option for active AFB infections, demonstrating a 100% recovery and prevention rates in treated hives (82). Furthermore, hives treated with *P. larvae* phages had no reinfection of AFB, which may indicate that the phage treatment neutralized latent *P. larvae* spores.

We hypothesize that the ability of *P. larvae* phages to prevent reinfection of AFB is by specifically binding to the spore form of the bacterium. Phages typically bind to and inject their DNA into vegetative bacteria, but some phages are known to attach to spores for which they are specific (47,48). Our data confirm that *P. larvae* phages can bind to *P. larvae* spores. Spore binding was observed in three specific experiments: 1) phages incubated with *P. larvae* spores bound to the spores and subsequently created plaques on lawns of *P. larvae*, 2) bacteria counted

by flow cytometry generated quantitative data of FITC-labeled phages bound to spores and to vegetative bacteria in equivalent percentages, and 3) electron microscopy images of phages bound to the surface of spores.

4.2 Materials and Methods

4.2.1 Spore generation and extraction

Overnight cultures of *P. larvae* ATCC 9545 grown in ½ x liquid porcine brain and heart infusion (PBHI) (Acumedia, Lansing, MI) media were grown in a shaking incubator at 37 °C. The optical density of the culture was taken to estimate the number of cells per milliliter using the equation $C=A/E$ where C is colony forming units, A is absorbance at 580 nm, and E is the e-value 6.6×10^{-9} . A total of approximately 10^4 bacterial cells were spread onto tryptic soy and agar plates with glass beads and allowed to incubate at 37 °C and 5% CO₂ for 8 days. Incubated plates were doused with five mL of cold sterile ddH₂O and allowed to sit for 15 minutes. Colonies on the plates were gently scraped off the plate and into suspension with sterile loops. The solutions from eight plates were combined into a 50 mL tube and centrifuged at 12,000 x g for 20 minutes. Supernatant was poured off and the pellet was resuspended in 40 mL of sterile ddH₂O and centrifuged again as a wash step. The pellet was washed 2 more times. After the last wash step, the pellet was resuspended in 80% EtOH to kill any remaining vegetative bacteria.

Spores were removed from the ethanol immediately prior to the running of any experiment to minimize any chances of any spontaneous germination. Spores suspended in ethanol were centrifuged at 12,000 x g for 5 minutes to pellet the spores and the supernatant containing alcohol and the dead vegetative cells were discarded with the supernatant. The pellet was washed three more times using sterile ½ x PBHI broth and then suspended to a concentration of 10^4 cfu/mL. Spore purity was also confirmed using the Schaeffer-Fulton

staining method: briefly, samples were heat fixed, stained with 5% malachite green for five minutes over heat, and counterstained with 0.2% safranin. (113)

4.2.2 Phage generation

Phages specific for *P. larvae* were previously isolated and confirmed to infect and lyse only *P. larvae* (82) and not *B. laterosporus* (paper in review). Phage lysate was prepared by reconstituting the phage from freezer stock by mixing several ice chunks from the stock with 500 μ L of overnight *P. larvae* and plating the solution in $\frac{1}{2}$ x PBHI top agar and left to incubate at 37 $^{\circ}$ C. After 24 hours, visible plaques were plucked from the plate, suspended in 25 mL of $\frac{1}{2}$ x PBHI broth containing 1×10^6 cfu of *P. larvae* and incubated, shaking at 37 $^{\circ}$ C. After 16 hours the lysate was filtered through a 0.22 μ M vacuum filter (VWR, Radnor, PA).

4.2.3 Phage binding detection using the Brady Binding Test

The Brady Binding Test is designed to identify the ability of a phage to bind to a bacterium or other item and remain viable against its original target. The test relies on incubating the phage with the test bacterium, transferring the sample onto a filter, and then rinsing the trapped bacteria to remove un-bound phages. The trapped, rinsed, bacteria are transferred to incubate with bacteria of the original phage target and a standard plaque assay is done. The Brady Binding Test for this study was setup as follows: overnight cultures of *P. larvae* ATCC 9545, *B. laterosporus* field isolate B-2, *Sinorhizobium meliloti* strain B100, and *P. larvae* ATCC 9545 spores were each diluted to 10^4 cfu/mL. The bacteria were pelleted, supernatant discarded, and the pellets resuspended in 1 mL of phage lysate at a titer of 10^8 pfu/mL, control samples were resuspended in 1 mL of sterile $\frac{1}{2}$ x PBHI broth, and all samples were set to incubate for 30 minutes at room temperature. Each solution was then poured over its own single use 0.22 μ M vacuum filter to catch all bacteria. The filters were then rinsed with 1 L of 1x phosphate buffered

solution to wash away any phages that were not bound to the bacteria. The filters were removed, placed in tubes containing 1 mL of ½ x PBHI broth, and set to vortex on high for 1 hour to dislodge bacteria from the filter. Of the resulting solutions, 100 µL were incubated 5 or less minutes with 500 µL of overnight *P. larvae* ATCC 9545 and then plated in ½ x PBHI top agar. The resulting plaques were counted.

4.2.4 Determination of non-specific FITC staining on bacterial samples

Staining methods similar to those previously described (114,115) were modified to fluorescently label phages and observe by flow cytometry. To prevent false positives where an excess of fluorochromes could bind directly to treated bacteria, the amount of background staining of FITC was determined for seven concentrations of unconjugated fluorescein isothiocyanate (FITC). FITC stain at concentrations of 500, 250, 125, 62.5, 31.25, 15.625, 7.8125, and 0 µg/mL was added to *P. larvae*, *B. laterosporus*, *S. meliloti*, and *P. larvae* spores. Fluorescence of each sample was then measured by a Cytoflex flow cytometer (Beckman Coulter, Indianapolis, IN) and positive samples were observed in comparison to samples containing 0 µg/mL FITC.

4.2.5 Phage binding detection by FITC stain and flow cytometry

Unconjugated FITC was added to a high titer phage lysate suspended in 1x Hepes solution (pH 7.4) at a concentration of 31.25 µg/mL and allowed to incubate for one hour. The high titer lysate (10^{11} pfu) was ultracentrifuged at 25,000 g for one hour to pellet the phages. The supernatant containing the unbound FITC was poured off and the pellet resuspended in Hepes solution to where the FITC concentration would be 15.625 µg/mL to prevent background staining of bacteria. For flow cytometry analysis, bacterial samples were loaded into a 96-well plate containing approximately 5×10^4 cfu in each well. Each well received 200 µL of FITC

labeled phages. Cell fluorescence was measured by a Cytoflex flow cytometer and a minimum of 50,000 cells were counted per sample.

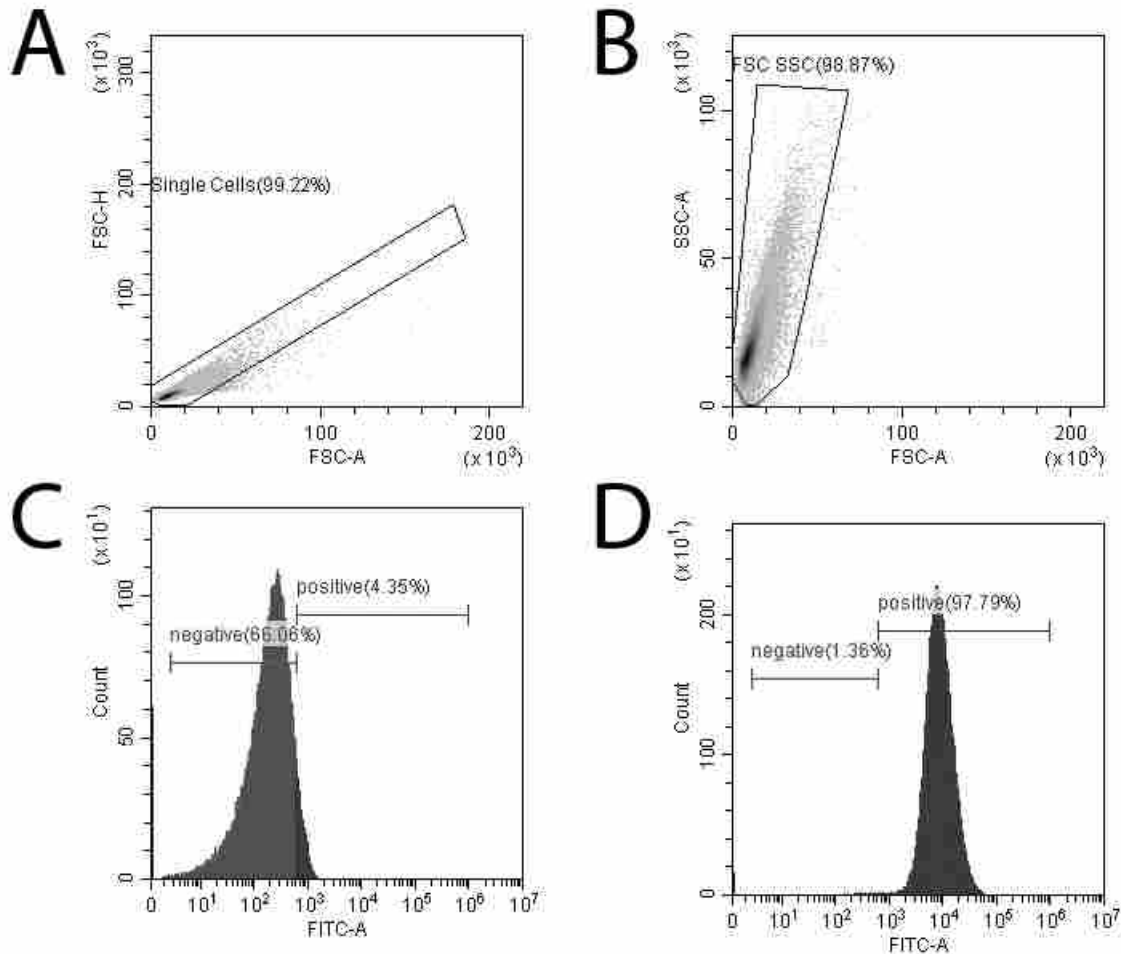


Figure 4.1. Gates used to establish positive samples, eliminate debris, and isolate singlets. The gates used for *Paenibacillus* larvae samples run on a flow cytometer. Each bacterial type had slightly different gates due to individual size, granularity, and autofluorescence. (A) Gate eliminates small and large debris. (B) Gate isolates singlets from the sample to decrease autofluorescence. (C) FITC histogram of a negative sample, the gate is set at the edge of the negative peak. (D) FITC histogram of a positive sample, a shift into the positive bracket indicates a highly positive sample.

4.2.6 Flow cytometry data analysis

Beckman Coulter CytExpert software was used to analyze the flow cytometry data collected on the Beckman Coulter Cytoflex flow cytometer. Three gates were individually set using unstained samples of each bacterial type. The gates were set on FSCxSSC to exclude

debris, FSC-HxFSC-A to isolate singlets, and on the FITC channel to identify positive samples, see Figure 4.1.

4.2.7 Phage binding detection by electron microscopy

Vegetative *P. larvae* and *P. larvae* spores (5×10^5 cfu) were resuspended in 1 mL of 3×10^9 pfu/mL high titer lysate and allowed to incubate for one hour. The phage-treated spores were pelleted at 8,000 rpm for 6 minutes. The supernatant was poured off and the pellet was resuspended in 40 μ L of 1x hepes solution.

Phage/spore samples were incubated with copper grids for 60 seconds and then incubated with 50 μ L of 2% uranyl acetate (pH 7) for 60 seconds. Moisture was wicked away from the grids and then allowed to air dry prior to imaging. Electron micrographs were taken by the BYU Microscopy Center on a Verios STEM machine (81).

4.2.8 Statistics

Data was analyzed using SAS software (SAS Institute Inc., Cary, North Carolina), the ANOVA algorithm, and the mixed procedure method to generate p-values, standard deviation, standard error and to determine statistical significance for Figures 4.2, 3, 4, and 5. For direct count statistics in 3.1, we used Jeffery's 95% confidence interval (116) for binomial proportions. For all experiments $\alpha=0.05$.

4.3 Results

4.3.1 Spores prepared for studies are vegetative-free and viable

Our objective was to establish a bank of viable *P. larvae* ATCC 9545 spores that was devoid of vegetative bacteria from which we could pull to perform our experiments. Spore sample purity was essential to prevent false positive in the experiments performed. All sample

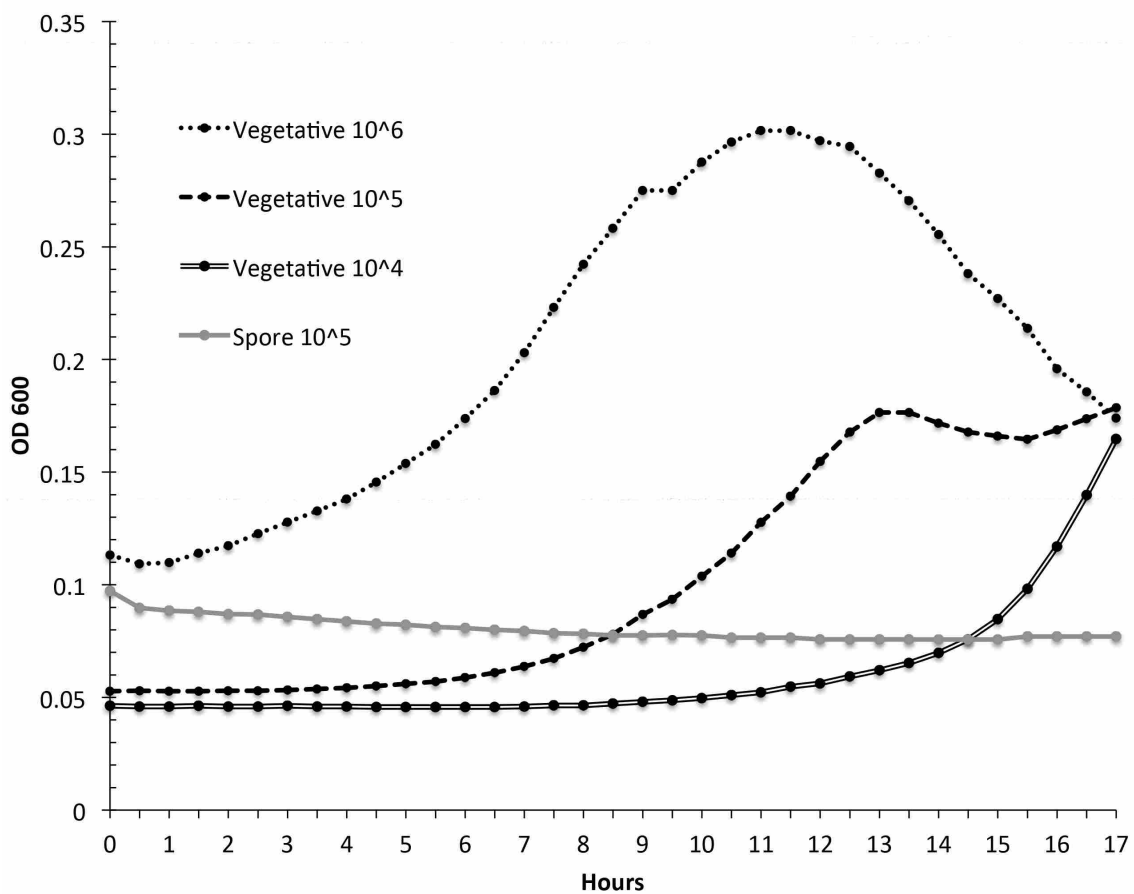


Figure 4.2. *Paenibacillus larvae* growth curves ensure the absence of vegetative bacteria in spore samples.

The optical density was measured for three dilutions of vegetative *P. larvae* and for *P. larvae* spores during a 17-hour incubation in broth that does not allow spore germination. The resulting curves are normal for samples containing vegetative bacteria and no curve was observed from spore samples.

stocks were independently assessed five times to confirm spore purity using multiple methods as follows. After spore isolation, samples were diluted and then stained with Malachite green to verify spore presence and safranin counterstained to identify vegetative bacteria in the sample. At least 100 cells from each of the five samples were observed and counts taken for the number of spore versus spores in mother cell versus vegetative cells. No vegetative cells were identified in the spore samples using this method but an average of $8 \pm 2.62\%$ of spores had not released from their mother cell. Free endospores made up the remaining $92 \pm 2.14\%$ of cells counted.

Spore samples were incubated in ½ x PBHI broth to further confirm the spore purity of the samples. Spores generated from strain ATCC 9545 do not germinate in liquid media; therefore, any increase in optical density of an incubated sample would result from vegetative bacterial growth in the sample. Positive controls of vegetative bacteria were incubated with starting concentrations of 10^6 , 10^5 , and 10^4 cfu/mL. Spores had an approximate concentration of 10^5 cfu/mL. Results of this verification study are presented in Figure 4.2. The optical density of the spore samples did not change significantly over 17 hours in comparison to the vegetative *P. larvae* samples at 10^6 , 10^5 , and 10^4 cfu/mL over the same amount of time. These data indicate that the spore samples did not contain any significant amount of vegetative bacteria.

Purified spore samples were further washed and stored in 80% Ethanol until experimental use as described in the material and methods section. The ethanol wash treatment was also tested on vegetative *P. larvae* to verify that the ethanol treatment would kill any surviving vegetative cells in the spore samples. Killed vegetative cells were pelleted, washed, and resuspended in broth. Ethanol treated vegetative cells were plated in triplicate and incubated for 48 hours. No colonies formed from ethanol killed cell samples. Spore samples were also plated for germination to ensure viability of the spores. After a 48-hour incubation, colonies formed on spore-inoculated plates and the colonies were confirmed to be *P. larvae* by catalase test and gram stain.

4.3.2 Results of the Brady Binding Test indicate that phages bind to spores and related bacteria

We developed the binding test to directly observe phage binding to bacteria and/or spores and to verify phage viability after binding, if binding occurs. Bacterial cells were incubated with a high titer phage lysate and then the bacteria were trapped on a filter for rinsing. After rinsing

non-bound phages through the filters, the bacteria were resuspended and plated with vegetative *P. larvae*. Plates were observed for the formation of plaques.

The phages were challenged with the following bacteria to test for binding: *P. larvae* ATCC 9545, *B. laterosporus* field isolate B-2, *S. meliloti* strain B100, and *P. larvae* ATCC 9545 spores. *B. laterosporus* was selected because of its genetic similarity to *P. larvae* and *S. meliloti* was chosen for its genetic dissimilarity. Vegetative *P. larvae* treated with phages generated the greatest amount of plaques, as would be expected, forming 159 ± 10 on average, see Figure 4.3. However, phages challenged with vegetative *B. laterosporus* generated the next highest amount of plaques at an average of 145 ± 9 . *P. larvae* spores generated 132 ± 9 and *S. meliloti* generated 35 ± 9 plaques on average. Phage-only controls generated 3 ± 10 plaques. The vegetative *P. larvae* and *B. laterosporus* challenges generated numbers of plaques that were not significantly different from each other ($p = 0.1925$). Furthermore, the number of plaques between *P. larvae* spores and vegetative *B. laterosporus* ($p = 0.2494$) were not significantly different. The number of plaques from *P. larvae* spores were statistically different from that of vegetative *P. larvae* ($p = 0.0018$). *S. meliloti* samples were significantly different from all other samples ($p = <0.0001$).

This binding assay uses a short incubation period and a filter rinse of the bacteria prior to a plaque assay. Plaques indicate that binding to the challenge bacteria occurred during the first step. Phages that bind can either infect the bacteria they are challenged with during the first step and then produce plaques on their intended host in the plaque assay, or the phages can exhibit reversible binding wherein the phages bind to the challenge bacteria in the first step and release to infect and produce plaques on their intended host in the plaque assay.

The results in Figure 4.3 suggest that high levels of *P. larvae* phage binding occurs to vegetative and spore forms of *P. larvae*. In addition, phages bind at high levels to vegetative *B. laterosporus* and exhibit a very low amount of binding to *S. meliloti*. *S. meliloti* is not related to

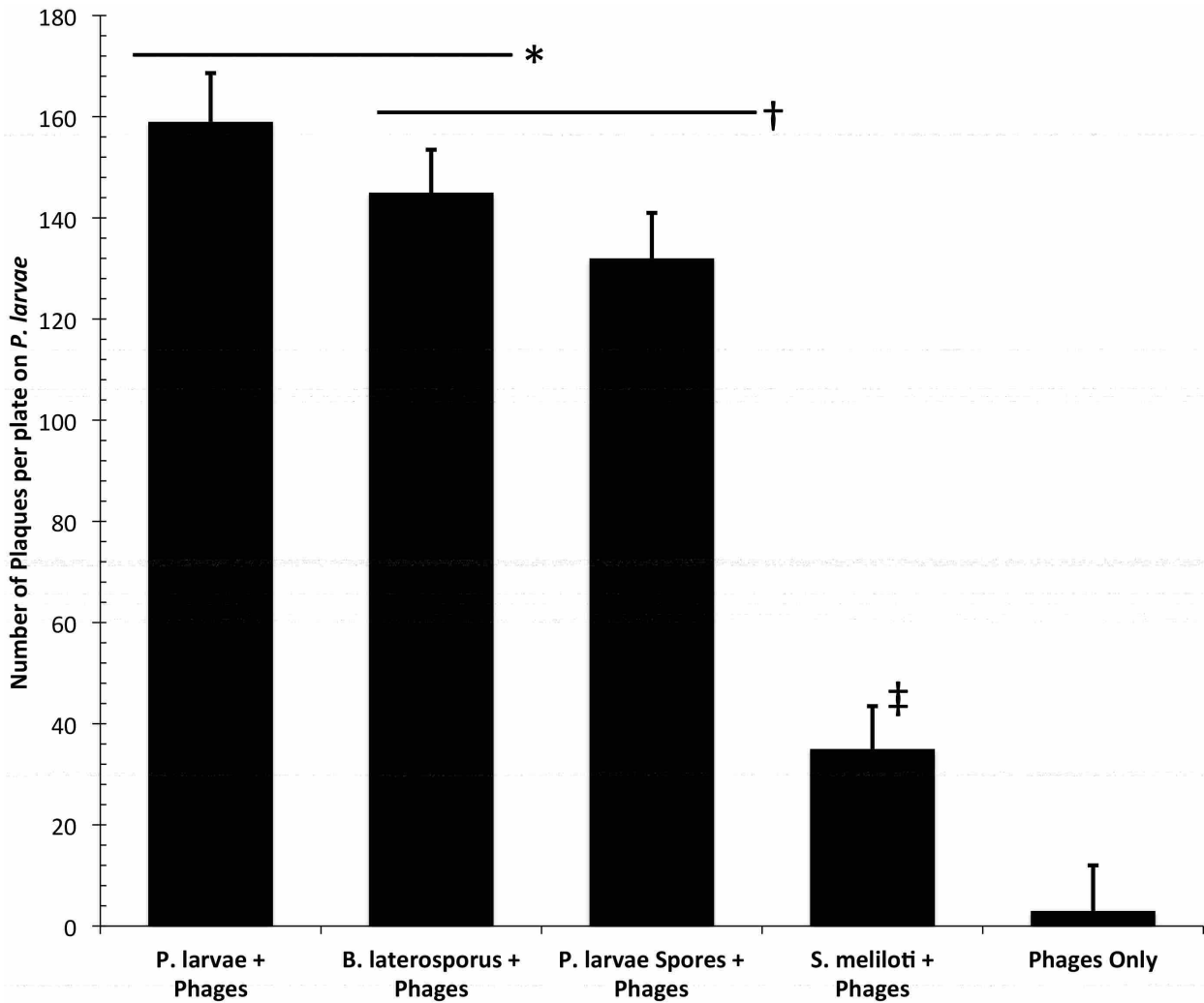


Figure 4.3. Brady Binding Test results indicate that phages bind to *P. larvae* bacteria and spores, and to *B. laterosporus* bacteria.

Phages were challenged for binding with four bacterial types and unbound phages were rinsed away. Resultant samples were plated with vegetative *P. larvae* and incubated overnight for phage infection and plaque development. Plaques were counted and averaged per plate. All samples where phages were challenged with bacteria were statistically different from the phage only control.

* *P. larvae* and *B. laterosporus* were statistically different from the controls ($p = <0.0001$) and not from each other ($p = 0.1925$).

† *B. laterosporus* and *P. larvae* spores were statistically different from the controls ($p = <0.0001$) and not from each other ($p = 0.2494$).

‡ *S. meliloti* was statistically different from all other samples ($p = <0.0001$).

P. larvae, so the low level of phage binding to *S. meliloti* was not unexpected. Binding of phages to *B. laterosporus* was surprising because the *P. larvae* phage used for this study is one that we extensively tested on multiple strains of bacteria and is specific for *P. larvae* and does not infect any of our tested strains of *B. laterosporus* (unpublished). Binding of *B. laterosporus* by the phages seems to be happening at a similar rate as that of it binding to vegetative *P. larvae*. Such binding suggests a reversible binding site of the phages to some cell wall component shared between *P. larvae* and *B. laterosporus*. Furthermore, plaques appeared on plates within 24 hours and yet a spore would take approximately 48 hours or more to germinate, which suggests that the plaques from the phages challenged with *P. larvae* spores are also likely due to reversible binding off of the spore and onto the vegetative cells used in the plaque assay.

4.3.3 FITC can be observed on bacteria at high concentrations and should be diluted if used for flow cytometry detection of phage binding

Puapermpoonsiri et al. published the use of unconjugated FITC to stain phages for fluorescent confocal scanning microscopy (115), and used a concentration of 250 $\mu\text{G}/\text{mL}$. We decided to modify their protocol slightly and use flow cytometry to obtain a quantitative assessment of phage binding to bacteria and spores. First, we needed to determine whether the FITC would also stain bacteria, and if a threshold level could be identified of background staining on bacteria below which we could still stain and detect phages. This study was designed to reduce false positive readings by identifying the concentration of free-FITC that would no longer make bacteria fluoresce. By knowing this limit, we could stain our phages at a higher

concentration, then wash and dilute the phages to a lower concentration and thereby prevent direct FITC binding to the bacterial samples. FITC at concentrations of 500, 250, 125, 62.5, 31.25, 15.625, 7.8125, and 0 $\mu\text{G}/\text{mL}$ were added to *P. larvae*, *B. laterosporus*, *S. meliloti*, and *P. larvae* spores, each at an approximate concentration of 5×10^4 cfu/mL, see Figure 4.4. Each bacterial sample fluoresced when dosed with 500 $\mu\text{G}/\text{mL}$. *B. laterosporus* fluoresced to a

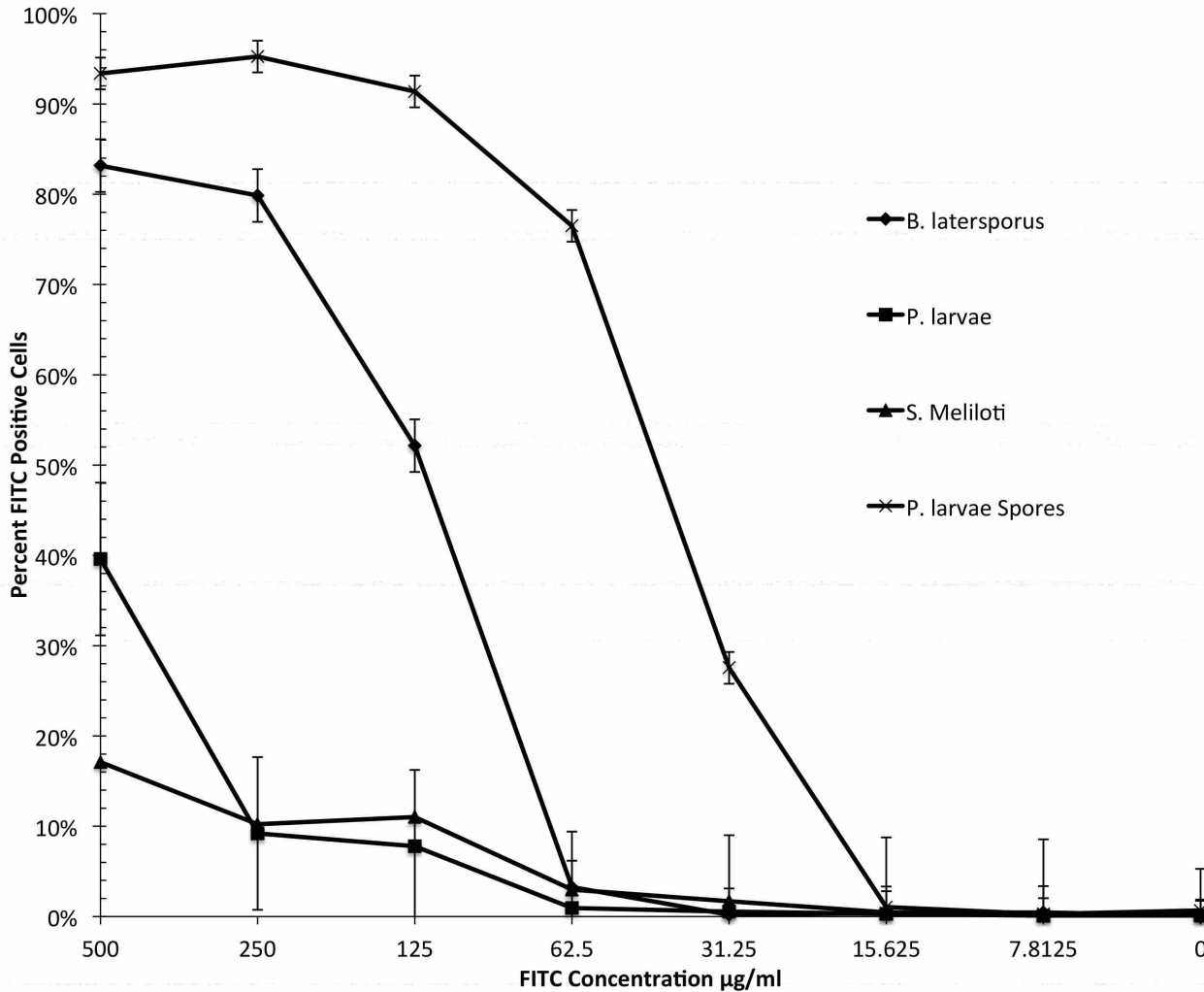


Figure 4.4. FITC stain can be diluted to a concentration below where bacteria will absorb detectable stain directly. Bacterial samples were dosed with seven FITC concentrations to determine fluorescence of bacteria labeled directly. Samples were compared to untreated groups to establish a positive range. All bacteria stopped fluorescing when dosed with 15.625 $\mu\text{g}/\text{mL}$ or less FITC.

significant degree at concentrations of 250 and 125 $\mu\text{G}/\text{mL}$. Spores fluoresced at staining concentrations down to 31.25 $\mu\text{G}/\text{mL}$. From this data, we determined that bacteria should not be exposed to more than 31.25 $\mu\text{G}/\text{mL}$ for the purpose of identifying phage binding.

4.3.4 Results of flow cytometry studies indicate that phages bind to spores and related bacteria

A flow cytometer reads single-cells and reports the fluorescence intensity of each individual bacterium. The flow cytometer can rapidly count and report fluorescence results of

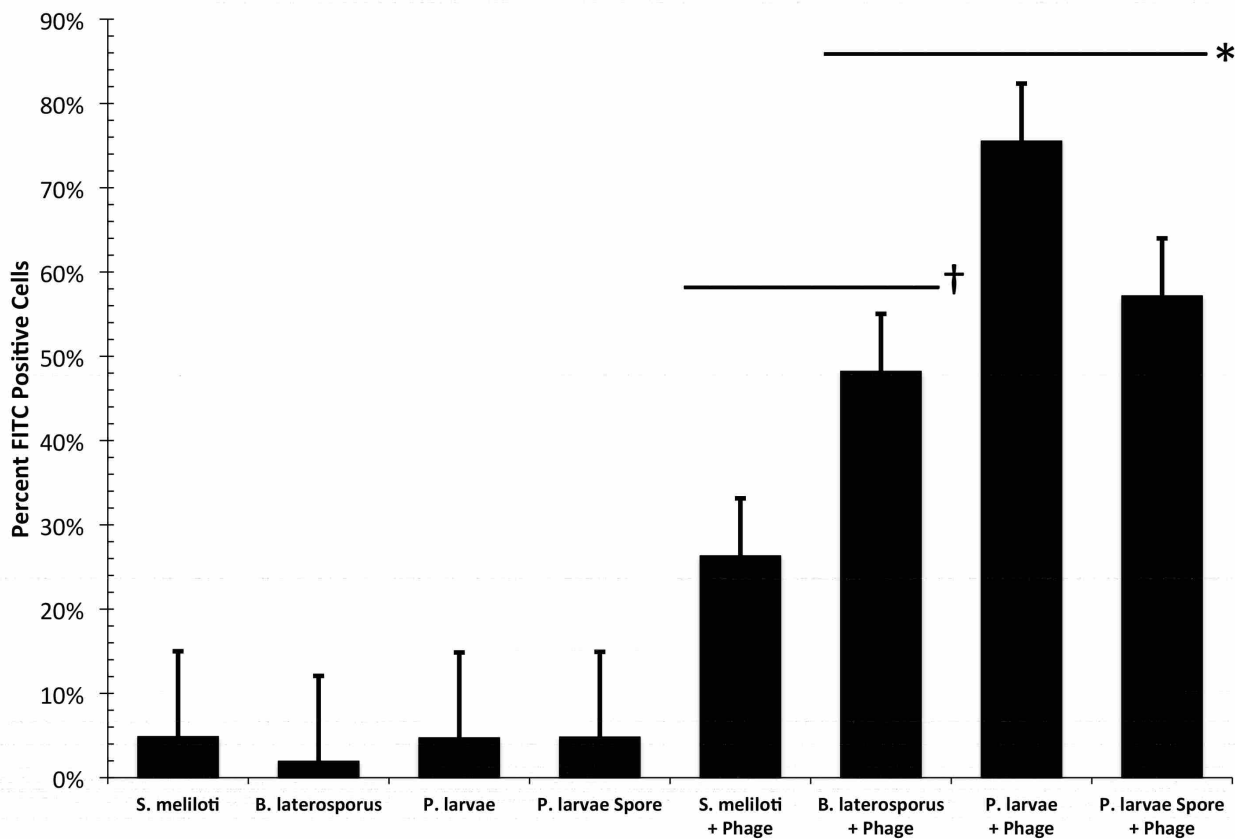


Figure 4.5. Flow cytometry results detect phages bound to bacteria and spores.

Bacteria and spores were incubated with FITC-labeled phages. Fluorescence of the cells was measured via flow cytometry and positive cells were reported as a percentage of the total population of bacteria in the sample. Negative controls (in FITC stain without phages), were low and all samples with phages were statistically significant compared to the controls excluding *S. meliloti* ($p = 0.2494$).

* *P. larvae*, *B. laterosporus*, and *P. larvae* spores were statistically different from their controls ($p = <0.0001$; 0.0084; 0.0017) and not from each other.

† *B. laterosporus* and *S. meliloti* were not significantly different from each other ($p = 0.3297$).

thousands of individual cells in a sample. Phages were first stained with FITC and then incubated with challenge bacteria and analyzed by flow cytometry to identify binding according to fluorescence. Positive and negative regions on the histogram for FITC were determined according to results of the negative control samples treated with FITC without phages for each bacterium with a minimum of 50,000 cells analyzed by flow cytometry per sample. Figure 4.1 shows how flow cytometry data was analyzed and gives an example of a bacterial sample lacking phages (Figure 4.1C) and an example of a bacterial sample with phages attached (Figure 4.1D). FITC-stained phages were challenged with vegetative *P. larvae*, *B. laterosporus*, *S. meliloti*, and spores of *P. larvae*. The average percentage of FITC-positive bacterial cells for each of these samples is reported in Table 4.1.

Table 4-1. Percent of positive fluorescence on FITC histogram.
Percent fluorescence of untreated and phage-treated bacterial samples.

	% FITC-positive Bacteria	
	Negative Control	Phage Treated
Vegetative <i>P. larvae</i>	5±20%	76±14%
<i>P. larvae</i> spores	5±20%	57±14%
Vegetative <i>B. laterosporus</i>	2±20%	48±14%
Vegetative <i>S. meliloti</i>	5±20%	26±14%

As indicated in Figure 4.5, *P. larvae* ($p = <0.0001$), *B. laterosporus* ($p = 0.0084$), and *P. larvae* spores ($p = 0.0017$) treated with labeled phages are significantly different from untreated samples where *S. meliloti* treated with phages did not have a statistical difference from an untreated sample ($p = 0.6513$). These data support the results of the Brady Binding Test because phages were observed to bind to vegetative and spore *P. larvae*, as well as to *B. laterosporus*, and not to *S. meliloti*. The results also confirm that FITC staining and flow cytometry can be

used to observe quantitative differences in phage binding to bacteria.

4.3.5 Electron microscopy yielded images of phages attached to spores

The objective of electron microscopy was to capture visual evidence of the reported results in sections 3.2 and 3.4. Spores treated with phages were imaged using a Verios STEM machine on formvar coated copper grids. Figure 4.6a is an image of vegetative *P. larvae* treated with phages. The arrows on the left of the micrograph show phage tails without capsids bound at a slant to the cell wall of the bacterium. The arrows to the right indicate intact phages; one phage is about to attach to the bacterium, one is attached to the cell surface at a slant, and one phage is attached and erect on the cell. Figures 4.6b and 4.6c show phages bound to *P. larvae* spores. Figure 4.6b shows phages bound at a slant to the spore and Figure 4.6c shows phages erect on the surface of the spore. Electron microscopy studies by others indicate that phages can bind in a slanted orientation and then move upright for injection (117). Both horizontal and vertical binding was apparent in our electron microscopy samples. The alternative phage orientation on the bacterium and spores in our images may indicate differences between reversible and

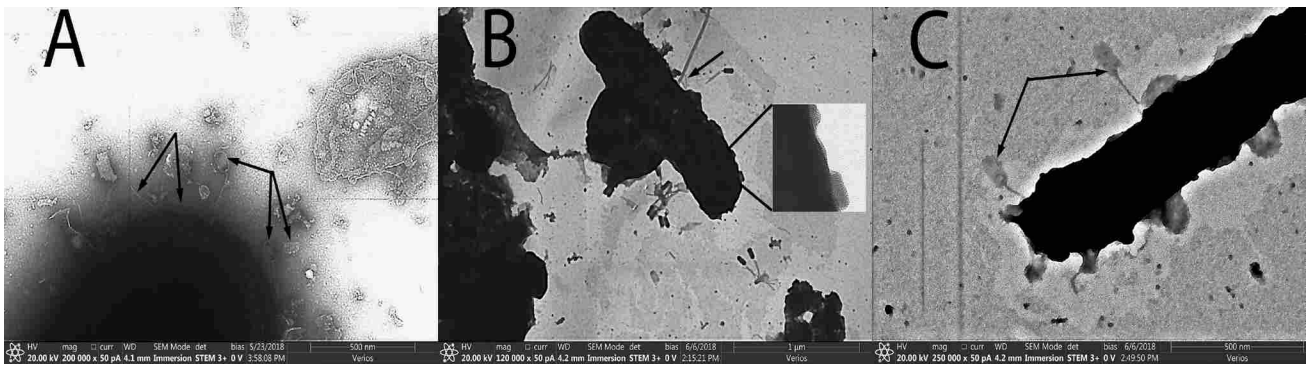


Figure 4.6. STEM images of phages bound to vegetative and spore form *Paenibacillus larvae*. (A) Arrows pointing to phages (right) and phage tails bound (left) in several orientations in relationship to the vegetative bacterial cell. (B) Arrows indicate and box show phages bound to the surface of a spore at a slanted position to the spore. (C) Arrows point to two phages bound to the surface of a spore in an upright orientation to the spore.

irreversible binding, and/or may indicate that DNA injection occurs with both the vegetative and spore forms of *P. larvae*.

4.4 Discussion

Pure spores are vital for any study that will involve a comparison between responses in spores versus responses in vegetative bacteria. Eliminating vegetative bacteria and reducing endospores residing in mother cells that could have vestigial receptors for phage binding was paramount to the experiments we conducted to prevent phages binding to vegetative cell wall proteins and producing false positives. The spores were inert in liquid media meaning that they did not germinate when put into nutrient broth and thus they did not generate vegetative cell membrane proteins. Collected spores were found to be clear of vegetative cells, but did contain a small percentage of spores that had not exited the mother cell and thus may contain small amounts of vegetative cell receptors to which phages could bind. This likelihood is not great due to small number of unreleased endospores were found especially because In the electron microscopy images no cells were identified that were still within mother cells. Further, spore samples run through the flow cytometer were gated to exclude doublets and endospores remaining inside of mother cells should show up as much larger cells much as a doublet would appear.

Detecting phage binding with fluorescence is an exciting new tool to quantify the binding potential of phages to target and non-target bacteria. In these studies, FITC-labeled phages act like large fluorescently labeled antibodies, creating a positive peak on a flow cytometer when bound to bacteria. Our findings indicate a low level of off-target binding of *P. larvae* phages to *S. meliloti* and moderate levels of off-target binding to *B. laterosporus*. Similar levels of phage binding to its target vegetative and spore bacteria were also seen showing that the *P. larvae*

phage used in these studies bind equally to both bacterial states. An excess of fluorochrome in solution with labeled phages leads to direct background staining of bacteria. Unconjugated FITC covalently bonds to primary amines and sulfhydryls via standard NHS isothiocyanate chemistry. Phages incubated with FITC are not individually detected via flow cytometry because they are too small. However, When several phages are bound to the surface of a bacterium the phages' collective fluorescence is read as the bacterial cells' own fluorescence. The results of the background tests allowed us to identify at what concentrations we could label our phages with FITC without worrying about background interference. Spores stained the strongest during our background tests did not stain the strongest during phage binding experiments. Similarly, vegetative *P. larvae* was did not stain strongly during background stain experiments but was the most fluorescent during phage binding tests. These results suggest that phage binding is cause of the fluorescence measured and not due to background FITC staining of bacterial samples.

The Brady Binding Test is a new method that can be used to detect viable phage binding to target and non-target bacteria. *P. larvae* phages bound to the four tested bacterial types generated plaques in lawns of *P. larvae* suggesting phage binding to target and off-target bacteria and spores. The low amount of binding seen with *S. meliloti* in 3.2 could be explained by phages being trapped on top of or in between the bacteria and no actual binding occurred. The differences between *P. larvae* and *S. meliloti* could mean that reversible binding sites on the phage tail did not have a strong interaction with the *S. meliloti* cell wall. In either case, *P. larvae* phages seem to bind to *P. larvae* spores as well as to *B. laterosporus*, the close relative to *P. larvae*. These results are surprising because the phages do not cause plaques on lawns of *B. laterosporus* but create similar amounts of plaques to *P. larvae* challenged with phages. This may be explained by a reversible binding site located on the close relative, *B. laterosporus*,

which allows the phages to attach to the bacterium but lack critical irreversible binding sites for the phage. Baptista et. al presented findings about the siphovirus SPP1, showing that the phage reversibly binds to the cell wall teichoic acid before irreversibly binding to the YueB protein on *Bacillus subtilis* (118,119).

Our data supports what we have seen in field studies where AFB infected hives were treated with *P. larvae* phages. In those previous studies the hives recovered in less than two weeks and did not become reinfected (82). *P. larvae* phages that bind to *P. larvae* spores and *B. laterosporus* in a reversible fashion as seen in 3.2 could account for the long term protection phage therapy lends to hives. By having reversible binding sites on spores and *B. laterosporus* the phages increase their likelihood of coming into contact with vegetative *P. larvae* as the spores germinate or *B. laterosporus* expands as a secondary infection to AFB.

Electron microscopy images show phages bound to the surface of spores in various orientations suggesting interesting possibilities for the *P. larvae* spore/phage relationship. Other researchers using cryotomography on T4 phages revealed different phage orientations during the infection process. Their data suggests that long tail fibers bind to target bacteria first and the strain from the bound tail fibers triggers the release of short tail fibers from the baseplate, leaving the phage to the side of the bacteria. Then, short tail fibers bind to specific receptors on the surface of the bacteria, which erects the phage and triggering the injection of DNA into the cell (117,120,121). Figure 4.6a shows phages bound to the surface of vegetative *P. larvae* bacteria shows phages in two orientations in relation to the bacteria similar to those described in previous research. Both orientations are also seen in Figures 4.6b and 4.6c where phages are bound to the surface of *P. larvae* spores. *P. larvae* phages may have a similar infection initiation as evidenced by images of phages bound to vegetative cells and spores. If the erect phages have bound to their

secondary target and ejected DNA into the cell, then *P. larvae* phages may be able to directly infect *P. larvae* spores similar to other spore infecting phages (48).

Our findings show a relationship between the spore form of *P. larvae* and one of its phages. Although this study did not show that the phage directly killed spores, other phages have been biopanned to do just that (48). By hunting for phages that specifically target and destroy spores, phage cocktail therapies have a greater potential for functionality because of the likely ability to prevent recurrent infections caused by spores.

Acknowledgments

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CONCLUSION

Due to the emergence of antibiotic resistance, phage therapies are becoming an increasingly more attractive treatment method for bacterial infections. This study explored two phage therapy types that can be employed in the treatment of AFB. It was shown that *B. laterosporus* phages can infect and induce toxin production in *B. laterosporus*. When AFB infected hives were treated with *B. laterosporus* phages, 75% of the hives recovered. It was also seen that the recovered hives relapsed with AFB after the completion of treatment indicating that the toxins produced by *B. laterosporus* do not neutralize the spore form of *P. larvae*. The second phage therapy described in this work was comprised of *P. larvae* phages. The *P. larvae* phage therapy showed no detrimental effect on hives, whereas the antibiotic control group experienced reduced hive health. Further, the phage therapy proved to be 100% effective at clearing and preventing AFB. *P. larvae* phage therapy treated hives never relapsed with AFB which suggests that the phages not only killed vegetative bacteria but also neutralized spores remaining in the hive. An investigation was launched into the relationship between the *P. larvae* phages and *P. larvae* spores to understand the full effectiveness of *P. larvae* phage therapy. Several assays were developed to gather evidence of phage binding to spores. It was seen that phages bind to spores and are able to produce plaques when plated onto vegetative *P. larvae*. Furthermore, a modified method to label phages with FITC was developed to quantify phage binding to target bacteria by using flow cytometry. We observed similar FITC fluorescence between vegetative and spore forms of *P. larvae* when treated with labeled *P. larvae* phages while there was not a significant shift for samples of unrelated *S. meliloti* vegetative bacteria treated with labeled phages. STEM images also showed phages binding to *P. larvae* spores.

The findings of this research provide strong evidence that phages bind to and neutralize *P. larvae* spores. These results are exciting as they suggest that phages could potentially be used to decontaminate tools or hive boxes contaminated with *P. larvae* spores. Phages that bind to the spore form *P. larvae* may be evolutionarily favored due to the AFB infection cycle. AFB is caused by nurse bees inadvertently feeding *P. larvae* spores to larval honeybees and *P. larvae* spores, the infectious agents of AFB, only germinate in the gut of larval honeybees after their cells are capped. After liquefying the pupal larvae, the vegetative *P. larvae* sporulate. By binding to spores, naturally lytic *P. larvae* phages are more likely to encounter vegetative *P. larvae* after spore germination. Furthermore, the data shows reversible binding to *B. laterosporus* as well as to spores. *B. laterosporus* is a commensal in many beehives and could act as a reserve for *P. larvae* phages in the gut of honeybees. This work also provides foundational precedence for treating infections with bystander phage therapy. By inducing toxins in nearby bacteria, this method could be taken in many directions for treating a broader range of bacterial, fungal, or parasitic infections.

My contributions to the field of phage research development of lab / research proposed new hypothesis 1) bystander phage therapy, two new protocols, 2) Brady Binding Test, and 3) flow cytometry for phage binding. For applied sciences, in vivo studies where 1) phage safety information & antibiotic use, 2) phage treatment and prevention of AFB, observational differences between treatments that affect spores (i.e. bystander doesn't kill spores, direct phage treatment does).

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