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Isolation and Characterization of Paenibacillus larvae Bacteriophages for Use as a Treatment of American Foulbrood Disease in Honeybees

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ISOLATION AND CHARACTERIZATION OF *PAENIBACILLUS LARVAE* BACTERIOPHAGE FOR USE AS A POTENTIAL TREATMENT OF AMERICAN FOULBROOD DISEASE IN HONEYBEES

By

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ABSTRACT

Isolation and Characterization of *Paenibacillus larvae* Bacteriophage for Use as a Potential Treatment of American Foulbrood Disease in Honeybees

By

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American foulbrood disease is a destructive honeybee illness cause by the bacterial pathogen, *Paenibacillus larvae*. Current methods of treatment are either destructive or do not completely eradicate the infection, and as such, undertaking further research to determine the effectiveness of alternate treatment methods is of consequence.

The therapeutic use of bacteriophage that are capable of lysing host bacterial cells, or phage therapy, is one such potential treatment. Phage are viruses that infect bacteria, and are generally very host-specific. As such, targeting a pathogen would require obtaining phage specific for *P. larvae*. Therefore, obtaining *P. larvae* phage requires screening environmental samples likely to have phage capable of infecting *P. larvae*, enriching samples with *P. larvae*, isolating any phage present, and characterizing the phage. After host range characterization, the phage with the broadest host ranges of *P. larvae* strains but inability to target other species of bacteria would be selected for use in a multi-phage cocktail. This cocktail would be used in experimental treatments to determine the effectiveness of phage therapy in increasing survival of honeybee larvae infected with *P. larvae* and eradication of AFB in an infected honeybee hive.

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CHAPTER 1

INTRODUCTION

Honeybees, *Apis mellifera*, are affected by a multitude of diseases including colony collapse disorder, nosema, varroa mites, wax moth infestations, viral infections, and chalkbrood, among others (Shimanuki and Knox, 2000). Such a variety of diseases is causing a decline in honeybee populations (vanEngelsdorp and Meixner, 2010), a devastating reality for agriculture. Honeybees pollinate approximately 35% of food crops worldwide (Klein *et al.*, 2007) and provide the agricultural industry in the United States an economic value of approximately $9 billion (Delaplane and Mayer, 2000). A decline in bee populations leads to a decline in pollination, crop yield, and food supply (Potts *et al.*, 2010). Hence, researching these diseases, including potential treatments and preventative measures, is beneficial to the agricultural industry and conservation strategies in general.

One honeybee infection, American Foulbrood disease (AFB), is caused by the bacterium, *Paenibacillus larvae* (Shimanuki and Knox, 2000). This Gram-positive, rod-shaped bacterium is capable of forming spores which are picked up by adult bees and brought back to the hive (Genersch, 2010). Adult bees are resistant to the bacterial disease; however, they are vectors that transmit the infectious agent to larvae that are susceptible (Hitchcock *et al.*, 1979; Wilson, 1971). Spores are resistant to antibiotics and heat; studies have shown that the spores of *Paenibacillus larvae* in particular can persist for at least several decades (Genersch, 2010), which makes eradication of this disease difficult. As few as 10 spores can cause the disease in a larva (Woodrow, 1942; Woodrow and Holst, 1942). Once spores enter the gut of larvae, they germinate and become vegetative bacterial cells (Bamrick, 1967) that proceed to grow and divide in the larvae. In doing so, however, they compete with the larvae for nutrients, as well as propagating to the point of rupturing the midgut lumen (Yue *et al.*, 2008). The larvae disintegrate

into ropey masses, which deteriorate into scales; this condition is a characteristic trait of larvae killed by this particular disease (Genersch, 2010). The death of one larva can produce millions of spores which are then released in the hive (Sturtevant, 1932). As the larvae of a hive succumb to the disease, and fewer bees are able to reach adulthood, the hive collapses as it is unable to maintain its population. Currently, methods of treatment include the direct use of antibiotics in hives as well as burning infected hives (Genersch, 2010). The former method has caused increased antibiotic resistance in strains of *P. larvae* (Evans, 2003), as well as residual antibiotics in honey that is sold for human consumption (Ortelli *et al.*, 2004; Saridaki-Papakonstadinou *et al.*, 2006). Additionally, there is evidence of decreased hive immunity to protect the bees from future recurrence of AFB following antibiotic treatment (Hawthorne and Dively, 2011). The latter method of treating the disease is incredibly costly to the beekeeping community and results in the decrease of hive materials, equipment, and productive hives, leading to significant economic loss (Genersch, 2010). Therefore, the prevalence and seriousness of the AFB, combined with the lack of effective and safe methods of treatment, has created the need for research into alternate methods of treatment.

One potential method of treatment is bacteriophage, or simply phage, therapy. Phage therapy is the therapeutic use of viruses, or phage. Phage are capable of infecting and lysing bacteria (Carlton, 1999), with specificity for targeting certain bacterial species. Having a specific host range means the bacteriophage can lyse the species of interest without killing even closely related species, ensuring the survival of beneficial or harmless bacteria. A phage cocktail, capable of infecting *P. larvae* but specific to this species, would potentially treat a beehive infected with AFB without harming the native fauna or beneficial microbiota of the hive. Previous work has explored the characteristics of single phage isolated from lysogenic strains of *P. larvae* with the intent of developing a potential treatment strategy for AFB (Gochnauer, 1955; Gochnauer, 1970; Drobnikova and Ludvik, 1982, Popova *et al.*, 1976; Valerianov *et al.*, 1976;

Dingman *et al.*, 1984; Bakheit and Stahly, 1988; Campana *et al.*, 1991); however, none of the previous research led to a treatment regime for AFB. Bacteriophage are natural entities that already exist in the environment, are not genetically modified, are self-propagating, would decrease the need for chemicals, and would be harmless to bees or humans. Therefore, researching the potential for use of phage therapy in treating AFB is of interest.

Because no recent isolates of phage to infect *P. larvae* are available, the first step in developing a potential AFB phage therapy treatment is conducting an environmental search for phage capable of lysing *P. larvae*. An extensive environmental search for *P. larvae* phage has neither been done nor is available; therefore, conducting an environmental search should include investigating both materials related and unrelated to beehives to discover where appropriate phage are found in nature and how prevalent they are. Noting the prevalence and sources for any phage found would be useful for phage ecology, and preserving such phage would be crucial in testing the ability for phage therapy. Therefore, in this research, both environmental and commercial materials will be screened for *P. larvae* phage, any positive samples will be characterized, and then these isolates will be used for experimental treatments.

Characterization of the phage is important to determine if multiple isolates might be several different phage or are actually the same phage isolated from different sources, meaning that a single phage could be ubiquitous. Characterization could also help to determine if newly isolated phage have previously been characterized by other researchers as well as to evaluate the potential for treating AFB. A spot test can be used to access the lytic capabilities of each phage on as many strains of *P. larvae* as possible. This would allow the host range of each individual phage to be determined and allow the patterns of lytic capabilities between phage to be compared. Transmission electron microscopy will be utilized to visualize individual phage for the purpose of comparing the morphologies of each.

After phage characterization, a preliminary experimental treatment needs to be conducted. Honeybee larvae will be infected with the spores of *P. larvae*, as described by Gochnauer and L'Arrivee (1969) and by Brodsgaard *et al*. (1998). Treatments will include: a negative control without either spore infection or phage treatment, a positive control with spore infection only, a positive control with phage treatment only, an experimental treatment with phage cocktails administered after spore infection, and an experimental treatment with phage cocktails administered prior to spore infection. The survival rates of larvae with phage therapy will be compared to survival rates of the controls. Additionally, it will be useful to determine the ability of phage cocktails to prevent infection of AFB by introducing phage prior to infection. Because germination of spores occurs in the gut of the larvae, both spores and phage will be added to their food for ingestion.

Ultimately, the goal of the project is to determine the possible effectiveness and feasibility of utilizing newly isolated bacteriophage from natural sources as a means to treat American Foulbrood Disease in honeybees.

CHAPTER 2

METHODS FOR DEVELOPING TREATMENT STRATEGIES AGAINST THE HONEYBEE PATHOGEN *PAENIBACILLUS LARVAE*, THE CAUSAL AGENT OF AMERICAN FOULBROOD DISEASE

FORWARD

This chapter is a compilation of work conducted by multiple research labs. Ms. Jasmin Khilnani is responsible for the work concerning low abundance compounds under the direction of Dr. Helen Wing and Mr. Israel Alvarado is responsible for the work concerning endospore formation and collection under the direction of Dr. Ernesto Abel-Santos. Dr. Helen Wing is responsible for extensive writing and editing of this manuscript. I do not take credit for the research presented nor the written text in the aforementioned sections other than my own. My contribution to the research of this manuscript was conducted under the direction of Dr. Penny Amy and is solely the research concerning bacteriophage acquisition, Figure 4, Table 2, and Table 3.

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Methods for developing treatment strategies against the honeybee pathogen *Paenibacillus larvae*, the causal agent of American Foulbrood disease

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Paenibacillus larvae, American Foulbrood Disease, AFB, Isolation, Identification, Collection, Assay, Development, Bacteriophage, Spore Production, Antimicrobial peptides

Abstract

Background

Paenibacillus larvae is a Gram-positive, spore-forming bacterium that is the causal agent of American Foulbrood (AFB), a disease which affects honeybee larvae. AFB leads to significant losses in honeybee populations, which drastically affects the agricultural economy each year. Consequently, there is a pressing need to develop new treatment strategies against *P. larvae* and the disease it causes.

Results

The methods described in this report were developed as a first step in the investigation of new treatment strategies against *P. larvae*. Three methodological advances are described and compared to current methods, where they exist. A protocol is described that consistently yields higher numbers of *P. larvae* spores than previously described protocols, regardless of the strain tested. Furthermore, microscopic examination of the spore preparations reveals that the newly devised protocol yields purer spores than previously described protocols. Secondly, two enhanced protocols that test the antimicrobial activity of low abundance compounds found naturally in resistant adult honeybees are presented. The advantages of these protocols are discussed. Thirdly, protocols to isolate, enrich and purify bacteriophage that specifically kill vegetative cells of *P. larvae* are fully described.

Conclusions

We anticipate that this new collection of methods will assist others working to investigate the biology of and develop treatment strategies against *P. larvae*.

Background

According to a recent survey of American bee keepers, one-third of the country's honeybee (*Apis mellifera*) colonies did not make it through the 2012-2013 winter season (Charles, 2013 and

Spleen *et al*., 2013). This decrease in honeybee populations is alarming considering the role that honeybees play in the pollination of many major crops and the potential impact they have on the global economy (Crailsheim and Riessberger-Galle, 2001; Morse, 2000; and Breeze *et al.*, 2011).

American Foulbrood Disease (AFB) is a contributing factor to the decline in honeybee populations (Evans and Schwarz, 2011). AFB is the most widespread and destructive of the bee brood diseases and, despite its name, is found worldwide wherever honeybees are kept (Matheson, 1993 and Calderone, 2001). The Gram-positive, spore-forming bacterium *Paenibacillus larvae* was found to be the causal agent of American Foulbrood Disease (AFB) in 1907 (White, 1907). The spores of this bacterium infect honeybee larvae within 72 h of eclosion (hatching from the egg). This typically occurs when brood food contaminated with infectious spores is fed to larvae by the nurse bees, which, like all adult honeybees, are resistant to AFB (Wilson, 1971).

Paenibacillus larvae was originally classified in the genus *Bacillus* but was reclassified as a separate genus in 1993 (Ash *et al.*, 1993). Formerly, two subspecies, *P. larvae pulvifaciens* and *P. larvae larvae* (Heyndrickx *et al.*, 1996) were described, but more recently the sub-species classification has been removed (Genersch *et al.*, 2006).

The infectious spores of *P. larvae* are known to remain viable for at least 35 years (Haseman, 1961). Currently, eradication of *P. larvae* from infected hives can only be achieved by burning the entire hive and all associated equipment (Matheson, 1993 and Calderone, 2001). However, this highly destructive solution increases the economic losses associated with the disease. In some cases, antibiotics offer an effective prophylactic treatment (Calderone, 2001), but antibiotics are not recommended for the treatment of hives that are actively infected. This is because antibiotics can select for resistant strains of *P. larvae* (Murray *et al.*, 2007) and do not completely clear the pathogen from the hive. Instead, vegetative *P. larvae* cells sporulate and remain quiescent until antibiotic levels drop, at which time a new infection will occur (Calderone, 2001). The hives may

appear healthy until there is a new stressor, such as onset of winter, at which time the disease reoccurs (Karen Bean, personal communication). In addition, antibiotic use has the potential to lead to traces of the antibiotic in the honey of treated hives. For these reasons, the registered use of antibiotics in the treatment of AFB has been withdrawn in many countries (Chan *et al.*, 2011). Clearly, there is a pressing need to improve upon the current strategies used to decrease the incidence of this disease.

The methods described in this report were developed as a first step in the investigation of new treatment strategies against *P. larvae*. Three methodological advances are described; 1) an improved protocol that yields a significantly higher number and purity of *P. larvae* spores, the infectious form of *P. larvae*, 2) enhanced protocols that test the antimicrobial activity of low abundance compounds found naturally in resistant adult honey bees, and 3) the isolation, enrichment and purification of bacteriophage isolates that specifically kill vegetative cells of *P. larvae*. We anticipate that this new set of methods will assist others working on *P. larvae*.

Results & Discussion

An improved protocol for the development and collection of *P. larvae* endospores

Three procedures for *in vitro* sporulation of *P. larvae* are commonly used (Dingman, 1983; Dingman and Stahly, 1983; and Genersch *et al.*, 2005) and described in de Graaf *et al.* (2013). Traditional infection studies do not require large numbers of spores because the infectious dose of *P. larvae* spores is extremely low (<10 spores per larva [Woodrow, 1942 and Woodrow and Holst, 1942]). However, our *in vitro* and *in vivo P. larvae* germination assays required abundant and highly purified spore preparations (Akoachere *et al.*, 2007; Alvarado *et al.*, 2013; Howerton *et al.*, 2011; Powell, 1950; Ramirez and Abel-Santos, 2010; Alvarez *et al.*, 2010). In our experience, the published protocols (described in [de Graaf *et al.*, 2013]) do not yield sufficient numbers of *P. larvae* spores for such studies. Consequently, a new method was

developed that reproducibly yields higher numbers of spores from different strains with greater purity. Here, we present a comparison of our newly devised protocol with the protocol (de Graaf *et al.*, 2013) that has traditionally yielded the highest number of spores (Dingman; personal communication).

For this comparative study of *in vitro* sporulation, three *P. larvae* type strains (B-3554, B-3650, and B-3685; see Table 1) were chosen because reports indicate sporulation rates vary from strain to strain depending on the conditions used. Using the previously published method (Dingman, 1983 and de Graaf *et al.*, 2013), the three *P. larvae* strains were induced to sporulate on MYPGP solid growth medium. Previous reports indicate that sporulation efficiency declines when high numbers of colonies are present on a plate (Dingman and Stahly, 1983 and de Graaf *et al.*, 2013), therefore spores were isolated from MYPGP plates with either low (50-200 colonies per plate) or high (colony 1000-5000 colonies) colony densities after 7 days of incubation at 37°C. The resulting spores were repeatedly washed and pelleted as previously described (Dingman and Stahly, 1983 and de Graaf *et al.*, 2013). In contrast, using the newly devised protocol, *P. larvae* strains were induced to sporulate on Tryptic Soy Agar (TSA) that was incubated for 7 days at 37°C in 5% CO_2. Each TSA plate was inoculated with 200 µl of an exponentially growing culture (approximately 1.2 x 10^8 colony forming units, CFUs). *P. larvae* strains produced lawns on TSA medium. A HistoDenz™ density gradient was used to harvest spores from vegetative cells and cell debris (Akoachere *et al.*, 2007).

The yield of viable spores from each protocol was determined after the spore-containing sample was heated (to kill any remaining vegetative cells), serially diluted and plated on MYPGP. The resulting number of CFUs represents an estimate of the number of viable spores present in each sample (Figure 1A). Regardless of the strain or whether the spores were isolated from either high or low density plates, the newly developed sporulation protocol yielded significantly higher numbers of spores per TSA plate than the established MYPGP plate method (for strain B-3554,

4.66 x 10^9 CFU per TSA plate versus 3.12 x 10^2-1.21 x 10^7 CFU per MYPGP plate; for strain B-3650, 9.73 x 10^6 CFU per TSA plate versus 9.08 x 10^5-3.61 x 10^6 CFU per MYPGP plated; and for strain B-3685, 6.06 x 10^8 CFU per TSA plate versus 6.64 x 10^5-4.27 x 10^6 CFU per MYPGP plate). Limiting the number of colonies per MYPGP plate increased spore yield over those harvested from MYPGP plates with high colony densities for *P. larvae* strains for B-3554 and B-3650, but not for strain B-3685. These data demonstrate that regardless of the strain used, the newly developed protocol routinely yielded higher numbers of viable spores.

The purity of spore stocks was next determined by microscopic analysis of spore smears stained via the Schaeffer-Fulton method. Percent purity was defined as the ratio of spores to total count of vegetative cells and spores in a sample (Figure 1B). Microscopic analysis of spore stocks prepared for strains B-3554 and B-3685 on MYPGP plates consisted of less than 20% spores. In addition the spores, prepared using the MYPGP protocol, were frequently contaminated with cell debris, although the amount of debris was difficult to quantify by microscopy. For example, spore stocks generated from strain B-3650 had 100% purity, even though cell debris could be observed by microscopy (Figure 2A). These results indicate that multiple washes used in the previously reported protocol (Dingman and Stahly, 1983 and de Graaf *et al.*, 2013) are not sufficient to remove vegetative cells and cell debris from the spore stocks. Furthermore, lysis of bacterial cells in spores stocks by heating is not recommended because nutrient release from vegetative cells has been shown to trigger spore germination (Shah *et al.*, 2008). In contrast, spores isolated using a HistoDenz™ density gradient to separate the spores from vegetative cells and cell debris, worked exceptionally well and yielded virtually pure preparations of spores (Figure 2B). We therefore recommend the use of a HistoDenz™ density gradient to purify spores because of the ease and non-disruptive nature of the procedure.

These experiments demonstrate that our newly devised *in vitro* sporulation method is more effective than established protocols (Dingman and Stahly, 1983 and de Graaf *et al.*, 2013).

While we obtained significantly more spores using our method with multiple *P. larvae* strains, the use of a density gradient during spore preparation ensured that we also obtained a pure spore stock. Furthermore, use of a commercially available medium, TSA, facilitated spore production since other protocols describe the use of specialized media that often require extensive preparation and may not support the growth of all *P. larvae* strains. In sum, the newly devised *in vitro* sporulation experiments described above will facilitate research into identifying alternative treatments for American Foulbrood Disease in honey bee larvae. They are already being used to generate spores for spore germination assays (Alvarado *et al.*, 2013), larval exposure assays, and spore decontamination studies (work in progress).

Assays to measure antibacterial activity of low abundance compounds against *P. larvae*

Typically, the susceptibility/resistance of *P. larvae* to antimicrobial agents is measured using either disk diffusion assays or by determining the minimal inhibitory concentration (MIC) on a series of agar plates (agar dilution) or in broth (broth dilution) which contain different concentrations of the antimicrobial agents (as described in de Graaf *et al.*, 2013). The primary drawback of each of these methods is that large amounts of the candidate compound are required for testing. To test the antibacterial activity of low abundance compounds against *P. larvae*, two new methods were developed.

The first protocol is a modified zone of inhibition assay. Two microliters of low abundance compounds were applied to a small hole punched into the center of a 3x R2 agar (described in Methods) inoculated with *P. larvae*. The plates were incubated at 37°C and observed zones of inhibition were measured after 24 and 48 h. By adding the candidate antibacterial compound directly to the agar, diffusion was unimpeded, which does not always happen when molecules are applied to paper disks. To aid diffusion of the compound through the agar plates, the concentration of agar was lowered from 2% (w/v), which is traditionally used in disk diffusion assays on *P. larvae* (de Graaf *et al.*, 2013), to 1.5% (w/v). Rather than using MYPGP agar for the

zone of inhibition assays, 3x R2 agar medium was chosen because; 1) *P. larvae* grows well on this medium, 2) it is commercially available, 3) it has similar composition to MYPGP, which has been used previously for similar assays (de Graaf *et al.*, 2013) and 4) unlike other commonly used broths, such as Brain Heart Infusion (BHI) medium, it does not contain sodium chloride, which has been shown to inhibit the function of honeybee antimicrobial peptides (AMPs) (Caseels *et al.*, 1990; Chi *et al.*, 2003; and Suttle and Fhurman, 2010). The primary advantage of these protocol adjustments over currently published methods is that small aliquots of test compounds can be applied to the inoculated agar, allowing compounds that are in low supply or otherwise prohibitively expensive to be tested.

The second protocol developed is a 96-well plate assay. Briefly, 5 µl aliquots of the compound, at varying concentrations, were incubated with 95 µl of *P. larvae* culture grown to logarithmic growth phase in 3x R2 broth (R2B). Samples were incubated for 24 h and optical density measurements were taken over time. Use of 3x R2B in these assays was found to decrease *P. larvae* cell aggregates that are typically seen when this bacterium is grown in BHI broth, thus eliminating inconsistencies in optical density readings. These protocol adaptations generated an assay that is as quantitative as the MIC assay but uses much less compound than currently published methods (de Graaf *et al.*, 2013). These advances will allow large numbers of MIC tests to be run under identical conditions, making these methods more suitable for low abundance or expensive compounds.

Using these approaches, two active AMPs found in adult honeybees, apidaecin and abaecin, were tested against *P. larvae* strain B-3554 and *Escherichia coli* strain MC4100. Previous reports have indicated that each of these compounds is active against *E. coli* strains in liquid assays. The inclusion of *E. coli* in our assays, therefore, provided a useful positive control (Caseels *et al.*, 1990 and Chi *et al.*, 2003).

First, *E. coli* was tested using both the zone of inhibition and the 96-well plate assays. Strikingly, in the zone of inhibition assay that uses semi-solid media, apidaecin was the only AMP to exhibit antibacterial activity (a zone of clearance 8 mm in diameter was observed for apidaecin versus none for abaecin; Figure 3, A & C). Conversely, in our 96-well plate assays using liquid media, both apidaecin and abaecin show modest, although statistically significant, inhibition of *E. coli* growth over a 24 h incubation period (Figure 3, E & G) These observations suggest that the 96-well plate assay is more sensitive for the detection of antibacterial activity of low abundance compounds than the zone of inhibition assay for our control organism, *E. coli*.

We next tested the antibacterial activity of apidaecin and abaecin against *P. larvae*, to determine whether our newly devised assays would be sensitive enough to screen low abundance compounds for antibacterial activity against *P. larvae*. For *P. larvae*, both apidaecin and abaecin were seen to display antibacterial activity in the 96-well plate assay using liquid media at the 12 and 18 h time points (Figure 3, F & H), but neither AMP showed any observable antibacterial activity in the zone of inhibition assays (Figure 3, B & D). These data, once again, indicate that the 96-well plate assay using liquid media is more sensitive than the zone of inhibition assay for the detection of antibacterial activity of low abundance compounds. While we recognized that the zone of inhibition assays, described above, are simple to set up, require cheaper supplies and do not require expensive technical equipment to collect the experimental data (plate reader/ incubator), it is clear from the data collected that the 96-well plate assays provide greater sensitivity and generate quantitative data that can be used to determine the potential of low abundance compounds to be used as antibacterial treatments to inhibit the growth of *P. larvae* populations. These findings also demonstrate that the antibacterial activity of a compound may be masked when tested on semi-solid media, even though the agar concentration had been decreased to 1.5% from the 2% agar concentration typically recommended for disk diffusion assays. This

observation is worthy of consideration and has not been discussed in other protocol descriptions to date (de Graaf *et al.*, 2013; Caseels *et al.*, 1990; Chi *et al.*, 2003).

Acquisition, enrichment and isolation of bacteriophage that specifically lyse vegetative *P. larvae*

Bacteriophage are highly specific to their host bacteria and are extremely abundant in nature (Suttle and Fuhrman, 1984). Lytic phage enter bacterial cells, replicate and kill their host cell by degrading the bacterial cell wall (lysis). In contrast, lysogenic phage reside within bacterial cells in a quiescent state, but may become activated to enter a lytic cycle under certain conditions. Bacteriophage capable of lysing *P. larvae* strains undoubtedly exist in nature and are likely to be found in environments (soil, water) in and around infected beehives and in compounds derived from honeybee products. To determine whether *P. larvae* specific phage have the potential to be used as novel treatment strategies for AFB, protocols that specifically allow the isolation, amplification, screening and purification of bacteriophage specific for *P. larvae* were developed.

Acquisition: To induce lysogenic bacteriophage from *P. larvae*, procedures adapted from those described by Dingman *et al.* (Dingman *et al.*, 1984) were used. Although it is common to induce lysogenic phage to enter the lytic cycle using environmental stressors such as ultraviolet radiation or chemicals (Mayer *et al.*, 1969), in our experience these stressors were not necessary for inducing lysogenic *P. larvae* phage. Cultures of various *P. larvae* strains were simply grown overnight in liquid medium and pelleted by centrifugation. The growth medium was then filtered to remove any remaining cells and the resulting filtrate containing liberated bacteriophage was used as the starting material for phage enrichment.

To acquire phage from environmental and commercial samples derived from honeybee products, a protocol was adapted from a method designed to isolate bacteriophage from soil (Hurst and Reymolds, 2002). This method described agitating a 50 g soil sample in 50 ml of 10% beef extract solution for 30 minutes. Since our environmental and commercial samples ranged in

mass from 1 g to 5 g, we chose to incubate our samples in 10 ml of phosphate buffered saline (PBS) pH 7.1 overnight. PBS buffer was used to facilitate the liberation of phage bound to particulates in the sample without extensive growth of microorganisms. After 18-24 h, bacterial cells and debris were pelleted by centrifugation and the supernatant was collected. Although the original protocol (Hurst and Reymolds, 2002) suggested using a series of stacked filters to remove bacterial cells from the samples, we filtered supernatants through 0.45 µm sterile syringe filters. If samples of filtrates were discovered to contain contaminating bacteria upon plating, the remaining sample was re-filtered using 0.2 µm sterile syringe filters. Filtrates free from bacterial contamination were used as the starting material for enrichment of bacteriophage capable of lysing *P. larvae*.

Enrichment: To enrich for *P. larvae*-specific bacteriophage, a *P. larvae* host strain free of phage was required. Strain B-2605 (Table 1) was found to be phage-free after testing it for lysogenic phage in our studies, and it was also used as a host strain in previous phage research (Dingman, 1983). To test for the presence of phage from environmental and commercial samples, portions of each filtrate were added to exponentially growing *P. larvae* strain B-2605 and the mixtures were incubated at 37°C overnight. The following day, the supernatants containing putative lytic phage were collected and once again filtered.

Screening & Isolation: To isolate independent isolates of phage, material from single plaques was obtained from mBHI (modified BHI; see Methods) agar plates overlain with a soft agar containing *P. larvae* strain B-2605 mixed with the filtrates described above. After overnight incubation, plaques were clearly visible on plates, which contained filtrates positive for bacteriophage capable of lysing *P. larvae* (Figure 4). This technique is similar to that described by Hurst & Reynolds (2002), but uses a double-pass procedure similar to that described by Bakhiet and Stahly [38]. Each isolated plaque sample and 1 ml of overnight culture of *P. larvae* B-2605 was used to inoculate a 20 ml mBHI broth. After overnight incubation at 37°C, the

supernatant containing the amplified bacteriophage was harvested and filtered. This procedure was then repeated to ensure that independent pure isolates of bacteriophage were obtained (Bakhiet and Stahly, 1988).

Using these protocols, a combined total of 157 *P. larvae* strains, environmental samples, and commercial samples were screened for bacteriophage capable of lysing the pathogen. Of the 157 samples, 32 (20%) were found to contain viral particles capable of lysing *P. larvae*. More than half of these independent phage isolates came from samples taken in and around beehives (53%). Nineteen percent of phage were isolated from *P. larvae* lysogenic strains and the remaining isolates were obtained from either commercial products containing honey or royal jelly or other environmental samples (Table 2).

The original titers of 13 of these 32 pure isolates ranged from 10 plaque forming units (pfu) per ml to 1.55×10^4 pfu per ml. These 13 (described in Table 3) were selected for additional amplification to aid further characterization of the pure lysates (summarized in Figure 3). Using the amplification protocol, titers typically increased by ≥99%. The maximum increase was 1×10^8 fold and the minimum was 1×10^6 fold.

Conclusions

The three sets of methods described above were developed as an initial step to aid the investigation of new treatment strategies against *P. larvae* and the honeybee disease, AFB.

The first set of methods increase the efficiency of *in vitro* sporulation and harvesting of *P. larvae* spores compared to current protocols. These advances will aid those studying the disease process itself, because the spore is the infectious unit, but will also facilitate those attempting to block infection by targeting the endospores specifically, either by inhibiting or triggering their germination. The latter approach is currently being investigated (Alvarado *et al.*, 2013).

The second set of methods focuses on adapting existing protocols that allow the MIC of particular compounds to be tested *on P. larvae*, so that low abundance compounds or compounds that are prohibitively expensive for pilot testing can have their MIC established. Since adult honeybees are resistant to AFB, it is likely that compounds found in adult honeybees but absent from larvae will prove to be effective antibacterial compounds against *P. larvae*. This line of investigation is the subject of ongoing research in our laboratories.

The third set of methods center on the premise that phage therapy can be used to eradicate *P. larvae* from contaminated hives and equipment. Using the newly described protocols, naturally occurring bacteriophage that are specific for *P. larvae* can be isolated, enriched and purified from a variety of environmental and commercial sources as well as *P. larvae* lysogens. Using these approaches, 13 pure phage isolates at titers of over 10^5 pfu ml^{-1} have been collected and are being fully characterized to determine their potential to be used as naturally occurring phage therapeutics against *P. larvae*.

We share these methods because we believe that these methodological advances will facilitate research that is targeted towards the development of novel treatment strategies for *P. larvae* and the devastating AFB disease of honeybees.

Methods

Preparation of *P. larvae* spores

Two methods for producing a large number of spores were compared. *Method 1*: A *P. larvae* culture was serially diluted using MYPGP broth to obtain low (50-200 and high (1,000-5,00) CFU per MYPGP plate. Sporulation on solid growth medium was performed as described previously (Dingman, 1983; Dingman and Stahly, 1983; and de Graaf *et al.*, 2013). Briefly, after 7 days of incubation, spores were removed from MYPGP plates by gently scrapping and washing the agar surface with sterile water three times. The spore suspension was concentrated via

centrifugation, the supernatant was discarded, and the remaining pellet resuspended in ice cold water. Alternate centrifugation and pellet suspension steps were performed to clean spores. Spore stocks produced in this manner were stored in sterile water at 4°C. *Method 2*: *P. larvae* strains were grown on Tryptic Soy Agar (TSA; supplied by BD) for 7 days in a 5% CO_2 incubator at 37°C (Akoachere *et al.*, 2007 and Alvarado *et al.*, 2013). The resulting bacterial lawns containing spores were collected by flooding with ice-cold deionized water. Spores were pelleted by centrifugation and resuspended in fresh deionized water. After three washing steps, the spores were separated from their vegetative and partially sporulated forms by centrifugation through a 20%-to-50% HistoDenz™ gradient. The spore pellet was washed five times with water and stored at 4°C.

*Viability Assessment of Spores***:** The concentration of viable spores in each spore stock was measured by heat resistant counts on MYPGP agar plates (Dingman, 1983). We diluted every spore stock to an OD_{580} of 0.2 as a way to normalize the starting concentration of spores. The diluted spore stocks were heated at 68°C for 15 minutes, diluted in water, and plated onto MYPGP agar. Plates with 30-300 colonies per plate were used to calculate the heat resistant spore counts per MYPGP plate used (Dingman, 1983). Heat-resistant counts were performed in triplicate with two different spore preparations. Statistically significant differences ($P<0.05$) for all pairs of mean heat resistant spore counts per plate were determined using a Student's one-tailed *t* test assuming equal variances.

Purity Assessment of Spores: Samples (10µl) of each spore stock were smeared onto glass slides, air dried, and observed after Schaeffer-Fulton staining (Schaeffer and Fulton, 1933). At least 10 images of each spore stock were acquired randomly, and three random images were analyzed using image processing software (ImageJ). Purity of spore preparations was expressed as: (number of spores/number of spores and vegetative cells in images) X 100. Statistically

significant differences ($P<0.05$) for all pairs of mean spore preparations or percent purity were determined using a Student's one-tailed *t* test assuming equal variances.

Assays to measure antibacterial activity of low abundance compounds against *P. larvae*

Growth of bacterial strains: To prepare *P. larvae* inocula for each of our growth inhibition assays, cultures of strain B-3554 were prepared by scraping frozen glycerol stocks (20% v/v glycerol) with a sterile wooden stick and placing it in 5 ml 3x R2B. Cultures were routinely grown for 16 h at 37°C with orbital shaking (MaxQ 4000 Thermo Scientific) at 325 rpm. These cultures were then sub-cultured (1:20) and subsequently grown using the conditions described above because this was found to minimize cell aggregation in liquid culture. *E. coli* strain MC4100 controls were streaked onto a 3x R2 broth with 1.5% w/v agar (3x R2 agar) and incubated at 37°C overnight. Subsequently, a single colony of each strain was used to inoculate 5 ml 3x R2B. The liquid cultures of *E. coli* were grown at 37°C overnight with orbital shaking at 325 rpm.

Zone of inhibition assay: 500 µl aliquots of the *P. larvae* overnight culture were harvested and centrifuged at 16,100 x g for 2 min. The supernatant was discarded and the cells were resuspended in 150 µl of fresh 3x R2B. Each 3x R2 agar plate was spread with a 150 µl aliquot of the resuspended *P. larvae* cells to ensure the growth of a robust bacterial lawn. After the inoculum had been fully absorbed by the agar, a hole was punched into the agar using a sterile, pre-cut pipet tip, and 2 µl of either honeybee AMP at a concentration of 250 mg ml^{-1} (Apidaecin or Abaecin (purchased from AnaSpec and GenScript, respectively)), antibiotic or distilled water was added to the hole. The plates were sealed with Parafilm® to retain moisture and incubated at 37°C for up to 48 h. The diameters of the zones of inhibition were measured after 24 and 48 h of treatment. *E. coli* samples were prepared similarly; except that 250 µl of an overnight culture was used to form the bacterial lawns.

96-well plate assay: Overnight cultures of *P. larvae* were diluted, approximately 10 fold, in 3x R2B liquid medium to an optical density at 600 nm (OD_{600}) of approx. 0.2. The inner wells of a clear, flat-bottomed, 96-well plate (Greiner Bio-One) were filled with 95 µl of inoculum treated with 5 µl of either the AMP at varying concentrations, antibiotic or distilled water controls. The outermost wells were filled with 100 µl water in order to minimize evaporation and other edge effects. Each plate was incubated for 24 h at 37°C in a Tecan m200 plate reader, which shook the plate in orbital mode at 335.8 rpm for 5 min every 15 min (amplitude of 1.5 mm). Five absorbance scans at 600 nm were taken for each well every 15 minutes and the average was reported. Each condition was tested in five wells and tests were repeated three times. Growth curves were plotted and used to determine whether the AMP and/or the antibiotic controls affected cell growth by comparing the antimicrobial-treated samples to the water-treated controls.

Protocols for the enrichment, isolation and amplification of *P. larvae* specific bacteriophage

Growth of Bacterial Strains: The following stock cultures of *P. larvae* were used: B-2605, B-3554, ATCC-25747, ATCC-25748, ATCC-49843, ATCC-25368, B-3688, and ATCC-25367 (Table 1). In addition, two natural cultures isolated from infected hives were used: 2188 and 2231. *P. larvae* strains were routinely grown in BHI broth modified with the addition of 1 mM $CaCl_2$ and 1 mM $MgCl_2$ (mBHI), to enhance viral attachment in our assays (Hurst and Reynolds, 2002). Soft agar overlays of 3 ml mBHI with 0.95% (w/v) agar and 1% (w/v) yeast extract were used for phage screenings and titer platings. Yeast extract was added to enhance the clarity of plaques that formed on the surface of an overlay (Gochnauer, 1970). Agar mBHI plates with 1.5% (w/v) agar were used for experimental platings. Either mBHI or R2A plates were used to maintain stock cultures. Although the *P. larvae* strains took 3 days to grow on R2A at 37°C, the duration of viability of the bacterium on this medium was longer than on mBHI plates. Broth cultures of *P. larvae* were grown at 37°C and shaken at 100 rpm in an environmental shaker

(Barnstead LabLine MaxQ 4000) for 24-48 h to obtain a maximum density ($OD_{600} \approx 0.7$). Plates (both mBHI and R2A) were incubated at 37°C in a NapCo E Series Model 303 Incubator.

Isolation of Lysogenic Phage: To isolate lysogenic bacteriophage capable of infecting *P. larvae*, procedures similar to those described by Dingman *et al.* (Dingman *et al.*, 1984) were used. Cultures of *P. larvae* were grown in mBHI broth at 37°C and shaken at 100 rpm overnight. These were pelleted by centrifugation at 3,220 x g for 10 minutes (Eppendorf Centrifuge 5810) to remove bacterial cells. Supernatants from the cultures were filtered through 0.45 µm sterile syringe filters with cellulose acetate membranes (VWR) to remove any remaining cells. The filtrate served as the starting material for phage enrichment.

Isolation of Phage from Environmental and Honeybee Products: To isolate bacteriophage from environmental (such as soil, beehive propolis, beehive wax, etc.) or commercial samples (such as lip balm containing beeswax or lotion containing royal jelly), samples ranging in mass from 1 g to 5 g were weighed and placed in 10 ml phosphate buffered saline (PBS) pH 7.1. The mixtures were then shaken in 37°C and 100 rpm overnight. The mixtures were spun at 3,220 x g for 15 minutes and the supernatant was collected. Occasionally, hive materials were found to float on the top of the supernatant. Under these circumstances, a needle and syringe were used to puncture the wax or propolis to extract the supernatant underneath. Supernatants were filtered through 0.45 µm sterile syringe filters to remove any remaining cells. If, upon plating, filtrates were discovered to contain contaminating bacteria, they were re-filtered using 0.2 µm sterile syringe filters. Cell-free filtrates were enriched for phage capable of lysing *P. larvae*.

Enrichment of Isolated Phage Samples: Bacteriophage enrichment was achieved using standard techniques, as described by Hurst and Reynolds (2002). Briefly, aliquots of cell-free lysates were added to 1 ml of a freshly grown, overnight culture of *P. larvae* B-2605. Samples were then shaken in an environmental shaker at 100 rpm in 37°C overnight. The resulting

supernatants containing propagated phage were centrifuged at 3,220 x g for 15 minutes and filtered with a 0.45 µm sterile syringe filter.

Screening of Bacteriophage: To screen for bacteriophage, supernatants of the enriched samples were plated onto mBHI plates using a soft agar overlay technique (Hurst and Reynolds, 2002). Phage lysates, lysate dilutions (mBHI), and mBHI agar plates were all warmed to 37°C prior to plating to prevent premature solidification of the agar. Soft agar tubes were melted in a beaker of boiling water, and molten agar was cooled to 60°C prior to use. A volume of 1 ml from an overnight culture of *P. larvae* B-2605 and 1 ml of an enriched filtrate were combined in the molten soft agar overlay, poured onto the surface of an mBHI plate, and it was evenly distributed over the surface. After the overlays solidified, the plates were inverted and incubated overnight at 37°C (representative plate shown in Figure 4). Individual plaques were necessary to ensure a pure phage type had been isolated.

*Purification of phage isolates***:** Individual isolated plaques were scooped from the soft agar overlay with a sterile wooden stick. The plaque and 1 ml of a freshly grown *P. larvae* culture were used to inoculate 20 ml sterile mBHI broth. The bacterial cells from this overnight enrichment were pelleted by centrifugation at 3,220 x g for 10 minutes, and the supernatant was filtered using 0.45 µm sterile syringe filters. Whenever complete clearing of an overlay was observed, higher dilutions of the filtrate were added to additional soft agar overlays in order to visualize individual plaques. To ensure complete isolation and purification of each phage, a single plaque was picked after a second round of plating and amplified again using the procedure described above. This double pass procedure allowed pure independent isolates of bacteriophage to be amplified in the lysate.

*Further Amplification of Purified Phage Isolates***:** Individual broth cultures of 20 ml of mBHI were inoculated with B-2605, B-3554, and ATCC-25748, incubated overnight at 37°C and shaken at 100 rpm. On each day of the following 10 day process, 1 ml of each overnight culture

was transferred to a fresh 20 ml of broth followed by incubation under the same conditions described above. Each day, the cells in the remaining overnight broth culture were pelleted by centrifugation at 3,220 x g for 15 min, the supernatant removed and the cell pellets resuspended together in 15 ml of fresh mBHI broth. This cell mixture was distributed in 1.0 ml aliquots into phage lysates on day 1 as well as each subsequent day for the 10 day period. The amplifying phage lysate was, by this process, supplied with fresh cells of three bacterial strains daily to further the amplification process. At the end of the 10 day amplification period, phage/bacterial mixtures were centrifuged under the same conditions and the supernatant containing phage was collected and filtered through 0.45 um sterile filters to remove cells and cell debris. Phage titers were determined using the soft agar overlay method, as described previously.

*Quantification of phage titers***:** To calculate the phage titer in a lysate, dilutions of the starting lysate were plated in duplicate using the soft agar overlay technique (Hurst and Reynolds, 2002). The two plates from a dilution with numbers ranging from 30-300 were selected to ensure statistical accuracy when counting. Plaques from the chosen dilution were then counted, counts were averaged, and titers were calculated based on the dilution (Miller, 1998).

Competing Interests

The authors declare that they have no competing interests.

Author's Contributions

Research concept was performed by EAS, ME, PSA and HJW. Experiments and method development were performed primarily by IA, DGY and JCK under the guidance of EAS, ME, PSA and HJW. All authors approved the final manuscript.

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Figure 1 - Effect of sporulation protocol on *P. larvae* strains spore production (A) Average number of heat resistant spores for *P. larvae* strains spore stocks produced by two methods. The error bars represent standard deviations from the mean obtained from at least three independent measures of CFU ml^{-1}. (B) *P. larvae* spore stock smears were analyzed via microscopy after Schaeffer-Fulton staining [39]. The error bars represent standard deviation obtained from the mean based on at least three independent measures of purity. In all cases, the number of spores collected using the new protocol was significantly higher ($P < 0.05$) than those collected using the old protocol. Furthermore, the purity of the resulting spore samples was significantly higher when the new protocol was used ($P < 0.05$) compared to the old protocol, with one notable exception; spores harvested from strain B-3650 using the new protocol were quantified to be equivalently pure as spores harvested using the old protocol.

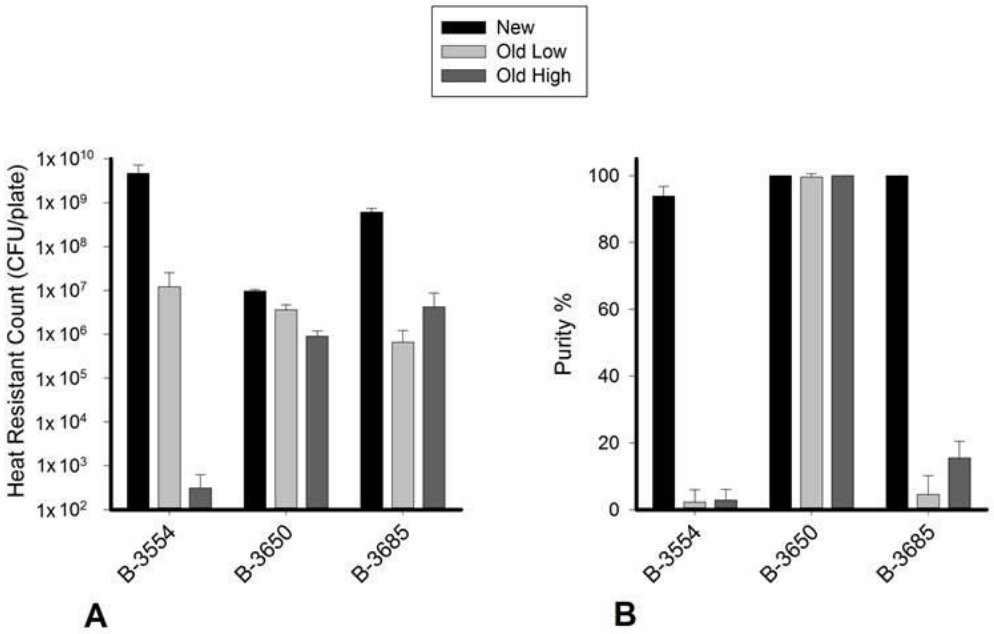

Figure 2 - Purity of spore stocks determined by microscopic analysis (A) Representative image of spore stocks stained using the Schaeffer-Fulton method [39] prepared for strain B-3650 (A) on MYPGP or (B) the new protocol. Cell debris retained safranin-O dye while spores retained malachite green dye. The scale bars indicates 10 micrometers.

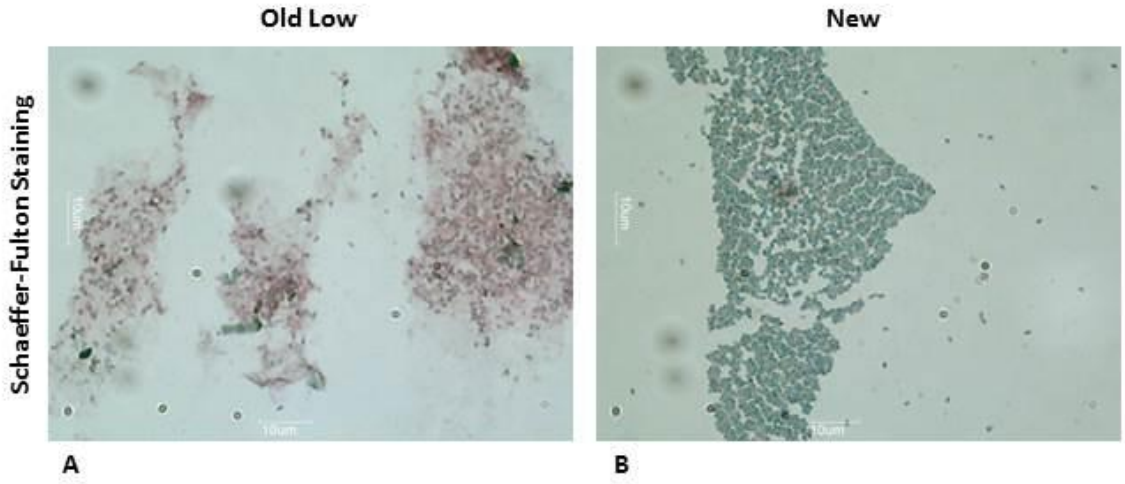

Figure 3 - Comparison of assays used to measure the antibacterial activity of honeybee AMPs. Representative images from zone of inhibition assays (A-D) or data from 96-well plate assays (E-H). Results obtained with the honeybee AMP apidaecin (top four panels). Results obtained with the honeybee AMP abaecin (bottom four panels). Bacterial strains used were *E. coli*, strain MC4100 (A, E, C & G) and *P. larvae*, strain B-3554 (B, F, D & H). Images of zone of inhibition assays (A-D) were taken after 22 h of growth. For the 96-well plate assays (E-H), all samples had an OD_{600} of 0.2 at time 0 (data not shown). Error bars represent standard deviations from the mean for five replicate samples and statistical significance between each AMP-treated sample and the water-treated (0) sample was determined using a Student's t-test assuming equal variance. Stars (*) indicate p-values of $p \leq 0.05$.

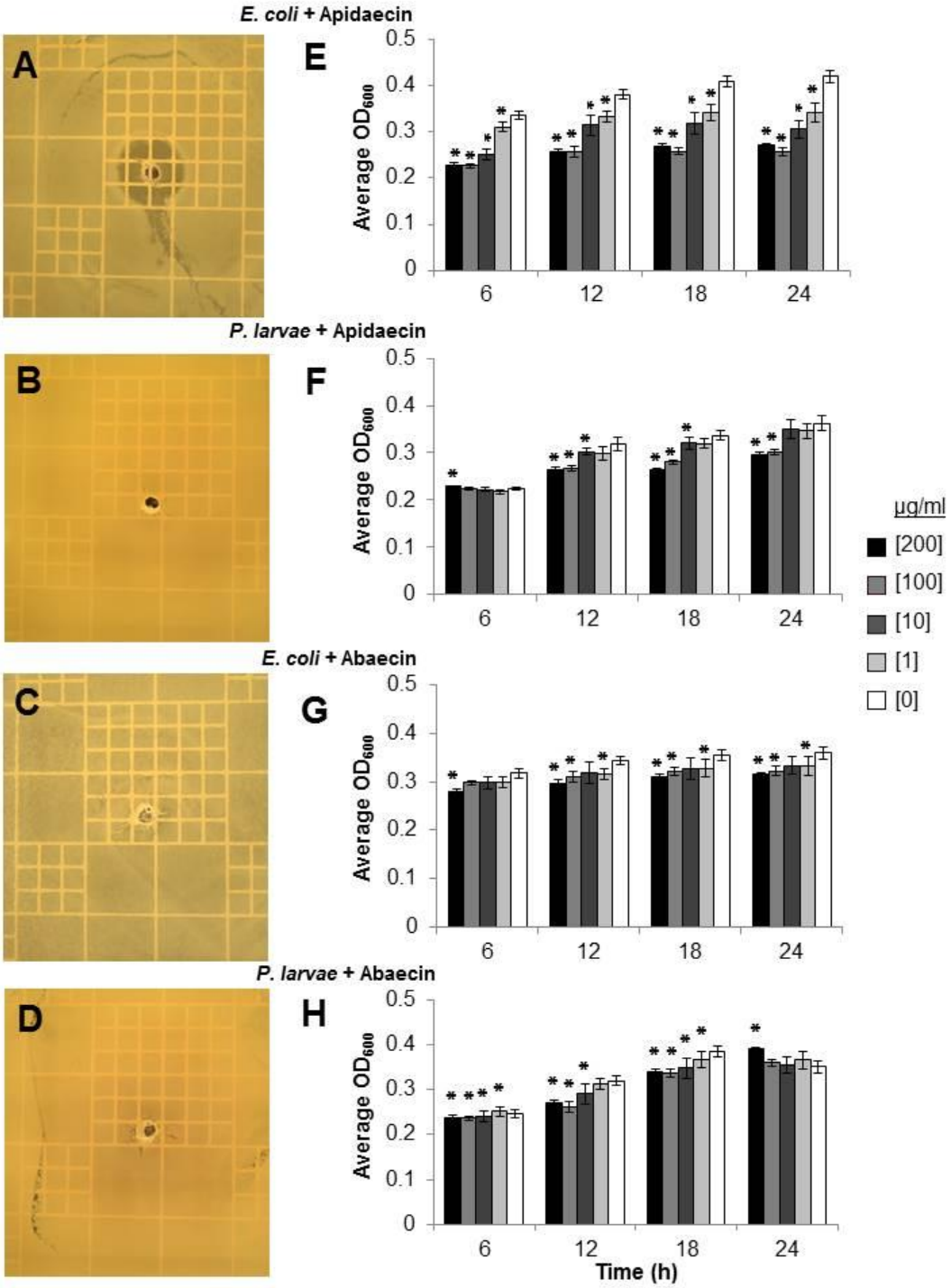

Figure 4 - Representative plate of a soft agar overlay with a bacteriophage capable of lysing *P. larvae.* The image shows a lawn of bacteria containing plaques. This demonstrates that bacteriophage capable of lysing *P. larvae* were present in the filtrate added to the soft agar overlay.

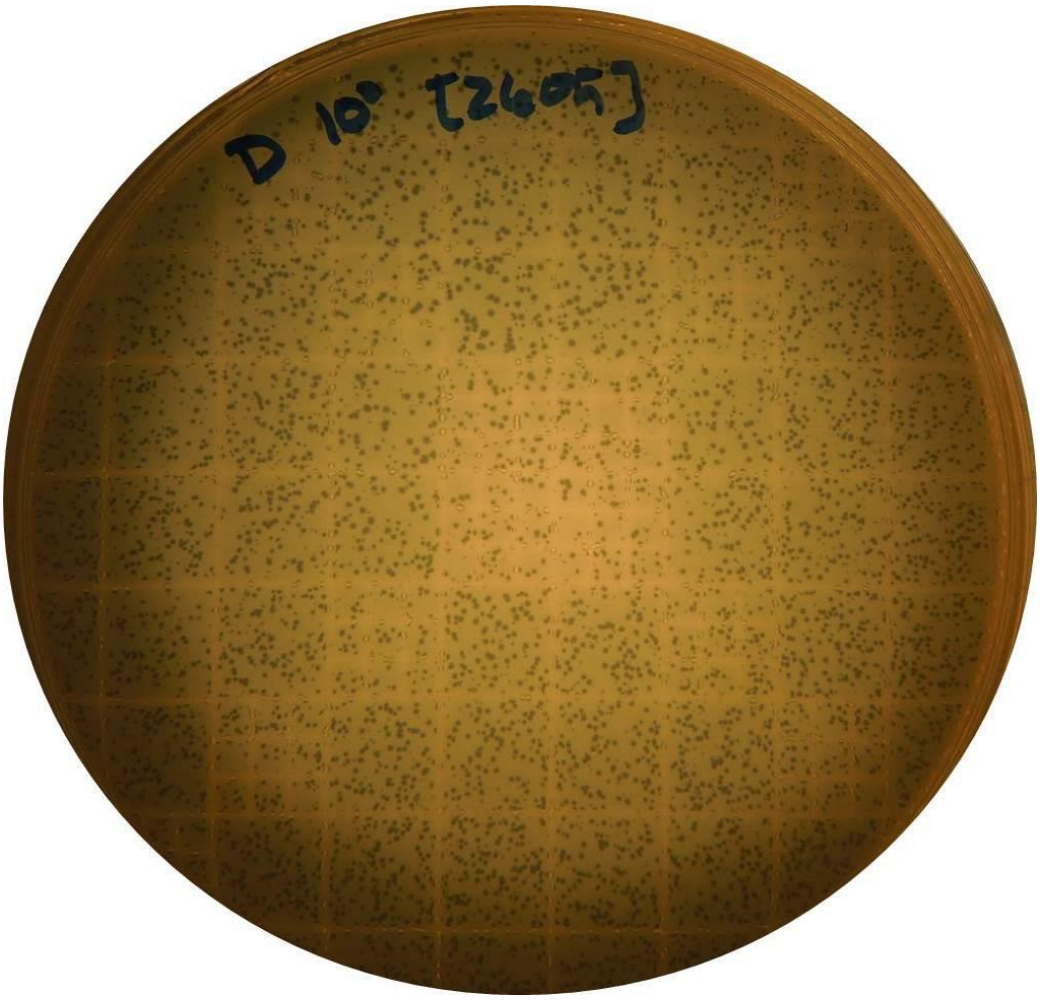

TABLE 1: Strains of *P. larvae* used.

Species	Previous subspecies classification	Source &Strain number[a]	Isolation[a]	Distinguishing features[b]
P. larvae	*larvae*	NRRL B-2605/ ATCC-9545	Isolated from insect, EC Holst	Non-sporeformer
P. larvae	*larvae*	ATCC-25748	Isolated from insect, H Shimanuki	N.D.
P. larvae	*larvae*	ATCC-25747	Isolated from insect, H Shimanuki	N.D.
P. larvae	*larvae*	NRRL B-3650	Isolated from diseased honeybee, RE Gordon	Spore-former
P. larvae	*larvae*	NRRL B-3554	Isolated from diseased honeybee larvae, NRRL	Spore-former
P. larvae	*larvae*	Designated 2188	Isolated from larvae scales, courtesy of Jay D Evans (USDA)	Spore-former
P. larvae	*larvae*	Designated 2231	Isolated from larvae scales, courtesy of Jay D Evans (USDA)	Spore-former
P. larvae	*pulvifaciens*	NRRL B-3685/ATCC-49843	Powdery scale of honeybee larvae, RE Gordon	Spore-former, virulent
P. larvae	*pulvifaciens*	ATCC-25368/ 24027	GW Skyring	N.D.
P. larvae	*pulvifaciens*	ATCC-25367/ 24026	GW Skyring	N.D.
P. larvae	*pulvifaciens*	NRRL B-3688/ATCC 13537	Isolated from diseased honeybee, RE Gordon	N.D.

[a]Info obtained from American Tissue Culture Collection (www.atcc.org), Agriculture Research Services (ARS) Culture Collection Database Server (nrrl.ncaur.usda.gov; formerly known as the Northern Regional Research Laboratories, NRRL) and straininfo.net. [b]Evaluated in house. N.D., none determined

TABLE 2: General characterization of final phage isolates

Category of Sources	Total Number of Isolates in Each Category	Percentage Found in Each Category
Soil around beehives	9	28.13
Beehive materials	8	25
Lysogenic phage from *P. larvae* strains	6	18.75
Other	9	28.12
Totals:	**32**	**100**

TABLE 3: Bacteriophage titers

Purified Phage Lysate (Isolate #)	Titer Before Amplification (ml^{-1})	Titer After 5-day Amplification Procedure (ml^{-1})
1	495	1.66×10^{8}
2	57	6.00×10^{6}
3	20	4.30×10^{7}
4	38	1.04×10^{7}
5	1750	1.84×10^{8}
6	400	3.40×10^{5}
7	150	3.66×10^{7}
8	15,000	3.97×10^{6}
9	10	1.59×10^{7}
10	15,500	1.17×10^{7}
11	500	9.40×10^{6}
12	795	7.45×10^{7}
13	22	1.29×10^{7}

CHAPTER 3

SOURCES OF BACTERIOPHAGE CAPABLE OF INFECTING *PAENIBACILLUS LARVAE*, THE CAUSATIVE AGENT OF AMERICAN FOULBROOD DISEASE IN HONEYBEES

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Sources of Bacteriophage Capable of Infecting *Paenibacillus larvae*, the Causative Agent of American Foulbrood Disease in Honeybees

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Running Head: Sources of bacteriophage that infect *P. larvae*

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Abstract

American Foulbrood Disease (AFB) is caused by an infection of honeybees with the bacterium, *Paenibacillus larvae*. To date, there are no nondestructive, effective treatments for this disease; hives and associated equipment are burned as an extreme measure to prevent contamination. This practice threatens agricultural productivity by both destroying the honeybees that are essential for pollination of crops and, to a lesser degree, by destroying costly equipment. One potential solution to control the pathogen and its spread is the use of bacteriophage that infect *P. larvae*. To find and describe environmental bacteriophage, 157 samples were obtained from many diverse sources including: *P. larvae* strains harboring prophage; various soils; wax, honey, royal jelly and propolis; cosmetics containing products derived from beehives; plants and flowers; compost; water and air samples. Bacteriophage were screened on BHI plates using soft agar overlays containing additional salts and yeast extract (mBHI). High titer lysates were prepared in mBHI broth containing *P. larvae* and a bacteriophage. Of the 157 samples, 30 were positive for at least one bacteriophage capable of growing on *P. larvae* strain 2605 for a total of 32; two sources produced two phage isolates each. The following indicate the percentage of phage-positive samples within each category: lysogens, 54.5%, cosmetics, 22.7%, soil under hives, 18.8%, beehive materials, 15.9%, and other environmental sources, 14.8%. After isolation, bacterial host ranges were analyzed for further characterization.

Introduction

One of many diseases affecting honeybees, *Apis mellifera*, is American Foulbrood disease (AFB) which is caused by the bacterium, *Paenibacillus larvae* (Shimanuki and Knox, 2000). This Gram-positive, rod-shaped bacterium produces spores, which are inadvertently picked up by adult bees and transported back to hives (Genersch, 2010). While adults are resistant to the disease, they are vectors that transmit the infective agent to larvae, which are

susceptible (Hitchcock *et al.*, 1979 and Wilson, 1971). Spores, in general, are resistant to antibiotics and heat, and studies have shown that the spores of *P. larvae* in particular can persist for at least several decades (Genersch, 2010), making eradication of this disease difficult. Furthermore, following infection and death of one larva, millions of spores can be produced and released in the hive (Sturtevant, 1932). Susceptibility of honeybee larvae to *P. larvae* infection is during the first 36 h after hatching (Genersch, 2010). As the larvae of a hive succumb to the disease, and fewer bees are able to reach adulthood, the hive collapses as it is unable to maintain its population. Currently, the only viable method of treatment is the application of powdered antibiotics directly applied and the current extreme measure to control the spread of the disease is burning infected hives (Genersch, 2010). The antibiotic treatment method has caused increased resistance in strains of *P. larvae* (Evans, 2003), as well as produced residual antibiotics in honey that is sold for human consumption (Ortelli *et al.*, 2004 and Saridaki-Papakonstadinou *et al.*, 2006). The method of burning infected hives to treat the disease is costly to the beekeeping community, resulting in loss of hive materials, productive hives, and producing significant economic loss (Genersch, 2010). Therefore, the prevalence and seriousness of AFB, combined with the lack of effective and safe methods of treatment, has created a need for alternative methods of treatment.

One potential method of treatment is phage therapy. Phage therapy is the therapeutic use of viruses, or bacteriophage (also called phage) to kill bacterial cells. Phage are capable of infecting and lysing bacteria (Carlton, 1999), with specificity for targeting certain bacterial species. Previous studies have explored the characteristics of single phage isolated from lysogenic strains of *P. larvae*. Several have suggested using phage as a potential treatment strategy for AFB (Bakheit and Stahly, 1988; Campana *et al.*, 1991; Dingman *et al.*, 1984; Drobnikova and Ludvik, 1982; Gochnauer, 1955; Gochnauer, 1970; and Valerianov *et al.*, 1976); however, no significant treatment strategies using phage have been reported.

Bacteriophage are abundant in many natural environments (Suttle and Furhman, 2010). Further, they are self-propagating only when host bacteria are present as well as being very specific for targeted bacterial species. Phage, if used against *P. larvae*, would decrease the need for chemical treatment of the hives and would not be harmful to bees, humans, or other microbes. Therefore, researching the potential use of phage therapy in treating AFB is of interest in economics and environmental health. Unfortunately, no ready repository of *P. larvae* phage is available for use; therefore, various environments were screened for the presence of *P. larvae*-specific bacteriophage for use as a treatment strategy for AFB.

Materials and Methods

Growth of bacterial strains

The following strains of *Paenibacillus larvae* were used: NRRL B-2605, NRRL B-3554, NRRL B-3650, ATCC-25748, ATCC-25747, ATCC-49843, ATCC-25367, ATCC-25368, ATCC-3688. In addition, two naturally occurring cultures isolated from infected hives were used: 2188 and 2231. Bacteria were grown for phage propagation under the same conditions as described by Alvarado *et al.* (2014, submitted for publication) in a modification of Brain Heart Infusion broth (BHI).

Environmental sampling technique

Environmental samples were obtained using alcohol flame-sterilized metal spoons and placed into sterile Whirlpac bags. Samples were also collected remotely by individuals in other locations using the same sampling methods. After collection, samples were stored at 4 °C until shipment to UNLV.

Sample sources

Lysogenic phage were obtained by screening all 11 strains of *P. larvae*. Procedures adapted from Dingman *et al.* (1984) were used. No special methods were needed to induce

prophage as suggested by Mayer *et al.* (Mayer *et al.*, 1969) from *P. larvae* strains because sufficient numbers of phage became lytic during the growth of their host bacteria. Cells were grown as described by Alvarado *et al.* (2014, submitted for publication). The presence of phage was determined by plaque formation on a bacterial lawn of *P. larvae* 2605 using a soft agar overlay method (Hurst and Reynolds, 2002).

Environmental phage were obtained from screening various soil samples, air samples, cosmetics containing materials derived from beehives, and materials directly from beehives such as royal jelly, wax, propolis, and honey. These samples were obtained from the following geographic locations: Nevada, Washington, New Mexico, Oregon, Pennsylvania, New York, and Iowa. Cosmetic sample sources, obtained from traditional retail settings, included various brands of lip balms with or without honeybee derived additions. A combined total of 157 samples were screened. Methods for preparing environmental samples are fully described in Alvarado *et al.* (2014, submitted for publication). Filtrates free from bacterial contamination were used as the starting material for enrichment of lytic bacteriophage capable of lysing *P. larvae*.

Phage enrichment, screening and isolation

Bacteriophage enrichment was achieved using standard techniques as described by Hurst and Reynolds (Hurst and Reynolds, 2002). To enrich for *P. larvae*-specific bacteriophage, the *P. larvae* host strain 2605 was used. This strain was utilized because it was phage-free after testing for lysogeny using the technique described above, and it was previously used as a host strain in phage research (Woodrow, 1942). Details of phage enrichment, screening and isolation are fully described in Alvarado *et al.* (2014, submitted for publication).

Amplification of phage and determination of phage titers

Phage titers were determined by following the soft agar overlay technique described above. Standard methods using two plates from a dilution with resulting plaque numbers ranging from

30-300 were selected to ensure statistical accuracy. Plaques from the chosen dilution were then counted, counts were averaged, and titers were calculated based on the dilution (Miller, 1998).

Soft agar overlay spot test

After amplification, each lysate was tested to determine its ability to form plaques on each *P. larvae* strain and other bacterial species including: *Paenibacillus polymyxa*, *Paenibacillus alvei*, *Paenibacillus lentimorbus*, *Paenibacillus popillae*, *Escherichia coli*, *Shigella flexneri*, *Bacillus cereus*, *Bacillus subtilis*, *Bacillus anthracis*, *Bacillus circulans*, and *Chromohalobacter* sp. A 1 ml aliquot of sterile broth and 1 ml of an overnight culture of a single bacterial strain were added to a tube of melted GmBHI agar (0.95%) containing 37 g BHI (Difco), 4g dextrose (Sigma), 1 mM of each $CaCl_2$ and $MgCl_2$ in 1L ddH_2O. This mixture was then poured over a GmBHI agar (1.5%) plate to create a bacterial lawn. Plates were divided into quadrants with 10 µl of a single lysate spotted onto the surface of each quadrant creating quadruplicate testing. The ability to lyse a *P. larvae* bacterial strain was measured by clearing. Each phage isolate was tested against each bacterial strain using a scale from no evidence of lysis to complete clearing (Figure 1). All host range results were recorded by the same individual for consistency. The phage with the broadest host range and highest intensity of lysis were of interest as a potential treatment for hives infected with AFB. Therefore, these phage were selected for further characterization. An exception was made for a pair of phage with the same host range pattern but which were isolated from very different sources. Determination of the similarity within these pairs was of interest because they might give some indication of geographic distribution.

EM grid preparation

To prepare a highly concentrated phage lysate, 20 identical soft agar overlay plates were prepared by mixing *P. larvae* strain 2605 with sufficient phage to result in complete lysis of bacterial cells. Plates were prepared with GmBHI (0.4% Difco glucose was added to mBHI)

containing 1.5% agarose and overlays were made of GmBHI with 0.95% agarose. These plates were incubated overnight at 37 °C.

Agarose removal and filtration: Five ml of PBS pH 7.1 was added to the surface of each plate and was allowed to sit for 20 minutes. The top layer of the agarose overlay was then scraped off using a sterile pipette tip, making sure the underlying medium was not disturbed. The scraped agarose plus PBS was collected and transferred to a funnel lined with four layers of cheesecloth to remove the agarose particles. The resulting liquid was then filtered through a sterile 0.2 µm filter (Sartorus) using vacuum filtration to remove bacterial cells.

Concentration of phage: The filtrate was distributed into 50 ml polysulfone centrifuge tubes (VWR) and phage were pelleted by centrifugation for 15 hours at 4 °C and 18,000 x g (Beckman J2-HS). The supernatant was removed and the centrifuge tubes were briefly inverted, being careful to prevent the phage pellet from completely drying. The phage pellet was gently resuspended in 1.0 ml of phage buffer, pH 7.5 with a composition of 10 mM Tris-HCl, 10 mM $MgSO_4$, and 68 mM NaCl (Dr. Malcom Zellars, personal communication), using a cut-off 1 ml sterile, disposable pipette tip, then removed from the centrifuge tube and transferred to a 1.5 ml microcentrifuge tube. The starting volume of approximately 100 ml was concentrated to a final volume of 3 ml. This concentrated phage preparation was used to prepare grids for TEM imaging.

Grid preparation: Using a carbon-coated copper grid (Ted Pella), 10 µl of each concentrated preparation was placed onto the carbon surface and allowed to sit for 10 min prior to wicking away the liquid with Whatman 541 paper wedges. The grid was rinsed (2X) for 2 min with sterile filtered ddH_2O, and the liquid was wicked away. The grid was stained for 2 min with 10 µl 2% uranyl acetate (pH 4.4), and the stain was wicked away before allowing the grid to air dry. Grids were sent to the CAMCOR facilities at the University of Oregon for imaging.

Results

Composition of phage and proportion of positive samples from each category

A total of 157 samples were screened, 32 of which were positive for viral particles. Table 1 displays the proportion of positive samples and Table 2 displays isolate sources.

Table 1. Proportion of samples found to contain *P. larvae* phage from each category.

Category	Screened Samples (#)	Samples Positive for Phage Isolates (#)	Samples Containing Phage (%)
Lysogenic Phage	11	6	54.5
Cosmetics	22	5	22.7
Soil Underneath Beehives	53	10	18.8
Hive Samples	44	7	15.9
Other Environmental Samples	27	4	14.8

Table 2. Sources, descriptions, and current designations of the 32 phage isolates.

Category	Source	Phage Designation
Cosmetics	Hand cream (contains beeswax and honey)	σ
	Body wash (contains royal jelly)	β
	Lipbalm #1	IV
	Lipbalm #2	V
	Lipbalm #3	VIII
Hive Samples	Scale from infected hive	XIII
	Hive sample from Iowa	HU
	Hive sample from Iowa (honey and wax)	YH/W
	Propolis from beehive - Gilcrease Orchards, Nevada	H1P
	Propolis from beehive - Gilcrease Orchards, Nevada	H2P
	Propolis from beehive - Gilcrease Orchards, Nevada	H3P
	Propolis from beehive - Gilcrease Orchards, Nevada	H5P
Soil Underneath Beehives	Soil underneath beehive - Gilcrease Orchards, Nevada	H1S
	Soil underneath beehive - Gilcrease Orchards, Nevada	H2S
	Soil underneath beehive - Gilcrease Orchards, Nevada	H3S
	Soil underneath beehive - Gilcrease Orchards, Nevada	H4S
	Soil underneath beehive - Gilcrease Orchards, Nevada	H5S
	Soil underneath beehive - Pennsylvania	PAIIS1 fl
	Soil underneath beehive - Pennsylvania	PAIS2 fl
	Soil underneath beehive - Pennsylvania	PAIS2 med. cl.
	Soil underneath beehive - UNLV, Nevada	III
	Soil underneath beehive - Washington	WA
Other Environmental Samples	Garden soil - Summerlin, Las Vegas, Nevada	I
	Garden soil - Summerlin, Las Vegas, Nevada	II
	Air sample (gravity plates) - Las Vegas, Nevada	VI
	Air sample (gravity plates) - Las Vegas, Nevada	VII
Lysogenic Phage	Phage from ATCC-49843	A
	Phage from ATCC-25368	B
	Phage from ATCC-25367	C
	Phage from ATCC-25747	D
	Phage from ATCC-49843	E
	Phage from wild strain 2231	F

Plaque morphology

Individual phage filtrates produced plaques in soft agar overlays, which were observed and described based on size and morphology (Table 3). Plaques were classified based on sizes and clarity. Plaque morphologies of the phage were as follows: 4 large, clear; 4 medium, clear; 3 small-medium, clear; 1 small, clear; 1 pinpoint, clear; 1 small, turbid; and 5 pinpoint, turbid. Although there was a distribution of sizes, in general, there were more large, clear plaques than small, clear plaques and more small, turbid plaques than large, turbid plaques.

Table 3. Morphology of plaques of each phage observed in soft agar overlays. Asterisk* formed plaques with a turbid halo around a clear plaque center. Sizes ranged and were described using set plaque diameters in the following classifications: pinpoint (<0.1 mm), small (0.1 mm – 0.5 mm), medium (0.5 mm – 1.0 mm), and large (>1.0 mm).

Phage Designation	Plaque Morphology	
	Size	Clarity
XIII	Large	Clear
H1P	Pinpoint	Turbid
WA	Medium	Clear
HIS	Pinpoint	Clear
F	Large	Clear
V*	Large	Clear
H2S	Small-medium	Clear
H3S	Medium	Clear
E	Pinpoint	Turbid
H5S	Medium	Clear
VII	Pinpoint	Turbid
D	Large	Clear
PA1S2 - fl.	Pinpoint	Turbid
B	Pinpoint	Turbid
VIII	Small	Turbid
PAIS2 - med. cl.	Medium	Clear
Sigma	Small	Clear
IV	Small-medium	Clear
VI	Small-medium	Clear

Host range distribution

The host range of each of the 32 isolated phage on each of 27 different bacterial strains was conducted by spot test experiments (as described in the methods) to determine how specific

each of the isolated phage are to the genus *Paenibacillus* and more specifically the species *P. larvae*. The data is presented in Figure 1.

None of the isolated bacteriophage caused lysis on the following strains: *Paenibacillus polymyxa, P. alvei, P. lentimorbus, P. popilliae, Escherichia coli* C600, *E. coli* W3104, *E. coli* MC4100, *Shigella flexneri* AWY3, *S. flexneri* BS103, *Bacillus subtilis, B. anthracis, B. circulans*, and *Chromohalobacter* sp, indicating host specificity to the genus *Paenibacillus* as well as the species *P. larvae*.

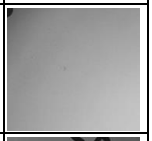
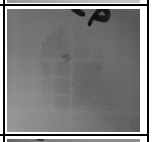
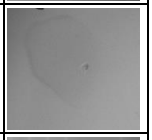
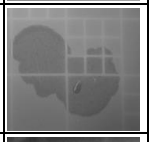
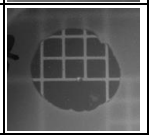
Three phage, H1P, WA, and H1S, lysed all *P. larvae* strains tested, and F lysed all strains with the exception of its host strain, 2231. In addition, these phage with broad host ranges on *P. larvae* were also highly lytic on multiple strains (+++). One exception was XIII, which was highly lytic on only four *P. larvae* strains. The isolated lysogenic phage were generally not capable of lysing the host strain from which they were isolated, with the exception of D and A, and these only produced a +/- result.

There is no apparent correlation between the source and the effectiveness of the phage against *P. larvae* strains.

Figure 1. Host range of 32 isolated *P. larvae* bacteriophage determined by soft agar overlay spot tests. Results are interpreted on a scale from no lysis (blank cell) to complete lysis (black cell). Phage are designated by letters and numbers, corresponding to the source from which they were isolated. The bacterial species are represented across the top and are ranked from left to right in order of susceptibility to lysis by the 32 phage. The isolated phage are listed on the left side of the table and are ranked from top to bottom in order of the percentage of *P. larvae* strains they are capable of lysing.

Isolated Phage	*P. larvae*										
	NRRL B-2605	2188 Isolate	NRRL B-3554	ATCC 25748	ATCC 25747	NRRL B-3650	2231 Isolate	ATCC 25367	ATCC 49843	ATCC 3688	ATCC 25368
H1P											
WA											
H1S											
F											
V											
H2S											
H3S											
E											
H5S											
VII											
D											
PAIS2 fl.											
B											
VIII											
PAIS2											
σ											
A											
III											
HU											
IV											
VI											
I											
β											
H4S											
C											
XIII											
H2P											
H3P											
YH/W											
II											
PAIIS1											
H5P											

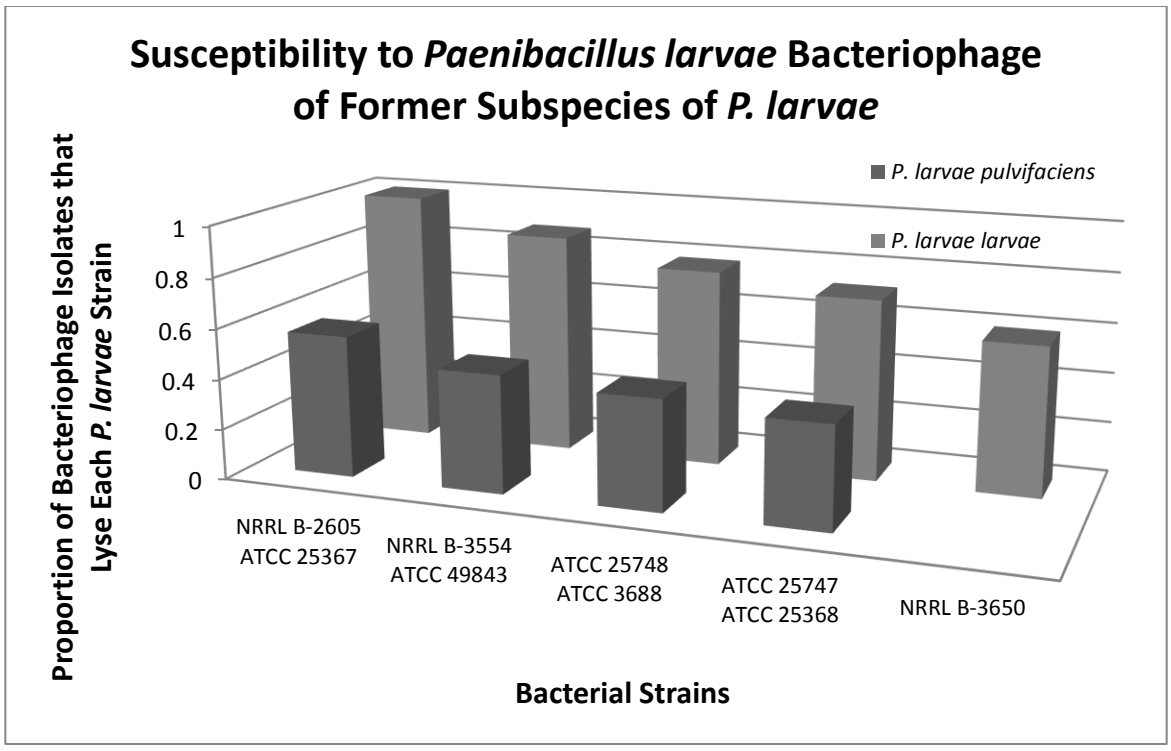
Figure 1 Key. Range of spot test descriptions observed in the host range experiment. The host range results are interpreted on a scale from no clearing to complete clearing. Plated examples show representative images of phage lysis on a bacterial lawn.

Description	Color Code	Result Scale	Plated Examples
No clearing		-	
Faint outline where lysate was placed		+/-	
Visible clearing		+	
Entire area has been lysed but still turbidity within plaque		++	
Entire area is completely lysed; no turbidity		+++	

Susceptibility of phage lysis on former *P. larvae* subspecies *larvae* and *P. larvae* subspecies *pulvifaciens*

There is a distinct difference between the susceptibility to the 32 isolated phage of strains formerly designated as *P. larvae larvae* and *P. larvae pulvifaciens*. Sample variances of *P. larvae pulvifaciens* and *P. larvae larvae* were 0.0237 and 0.0046, respectively. Using Welch's t-test, t=4.169, degrees of freedom ~5.727, and p=0.0087. Assuming $\alpha \leq 0.01$, there is a significant difference between the susceptibility that each group of former *P. larvae* subspecies has to the *P. larvae* bacteriophage. The two strains that were isolated from an infected hive were not classified under the same former subspecies as the repository strains, and were therefore not included in this calculation.

Figure 2. Proportion of susceptibility to isolated phage of *P. larvae* strains, which are grouped by former subspecies. Bacterial strains are listed from back to front.



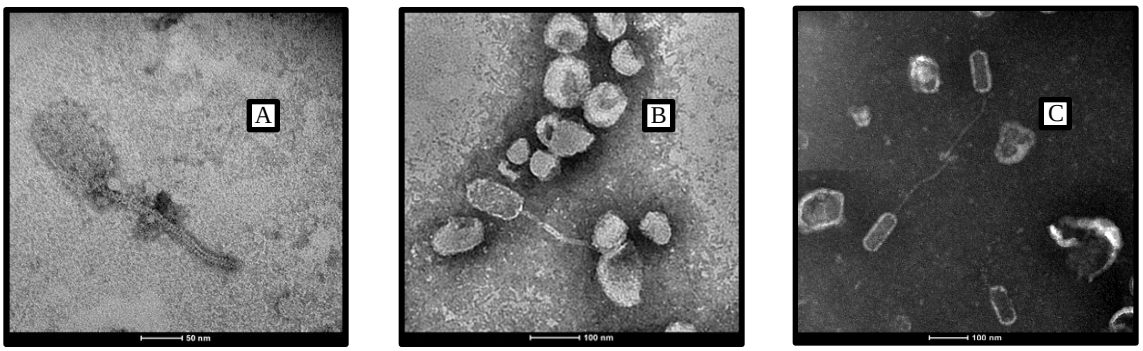
Comparison of phage morphology using TEM.

Results for 16 phage that were confidently imaged are given based on morphological descriptions only, the following are the possible families of these isolated phage: 13 *Siphoviridae*, 1 *Podoviridae*, 1 potential *Inoviridae*, and 1 potential *Tectoviridae* (Table 4). Even among phage potentially classified under the same family, there are size variations of heads and tails. Sample images are presented in Figure 3.

Table 4. Morphologies of chosen phage determined from TEM images. Images were provided by the CAMCOR facilities at the University of Oregon. Measurements are based on the averages of 2-4 images. Question mark indicates uncertainty of classification based on rarity of the family. Family classifications are based on descriptions of morphology only.

Phage Designation	EM Imaging Comparison				
	Head Shape	~Head Length (nm)	~Head Width (nm)	~Tail Length (nm)	Possible Family
H1P	Elongated icosahedron	109	55	227	Siphoviridae
A	Elongated icosahedron	114	71	212	Siphoviridae
WA	Elongated icosahedron	80	35	125	Siphoviridae
H2S	Spherical icosahedron	50	50	200	Siphoviridae
F	Elongated icosahedron	115	65	120	Siphoviridae
H3S	Elongated icosahedron	120	61	138	Siphoviridae
PA1S2 - fl.	Elongated icosahedron	87	41	190	Siphoviridae
D	Elongated icosahedron	94	47	106	Siphoviridae
PAIS2 - med. cl.	Elongated icosahedron	148	74	185	Siphoviridae
V	Spherical icosahedron	56	61	157	Siphoviridae
VIII	Spherical icosahedron	ND	ND	ND	Siphoviridae
H5S	Spherical icosahedron	150	150	225	Siphoviridae
Sigma	Spherical icosahedron	128	109	309	Siphoviridae
HIS	Spherical icosahedron	70	84	40	Podoviridae
E	No evident heads			200-500	Inoviridae?
III	Spherical icosahedron	110	110	No evident tails	Tectoviridae?

Figure 3. TEM images of phage are labeled as follows: A, A; B, H3S; and C, H1P. Scale bars are in the bottom black border, and are 50 nm for A, but 100 nm for H3S and H1P.



Discussion

A total of 32 phage were isolated from 157 sources, suggesting that approximately 20% of the sources screened could yield phage capable of lysing *P. larvae*. While the majority of the isolated phage were obtained from soil under and around beehives, the proportion of isolated phage was highest from lysogenic incorporation in strains of *P. larvae*. However, since a bacterium is likely to be immune to its own lysogenic phage (Lindahl *et al.*, 1970), as supported by the host range table of this work, it would be more beneficial to isolate lytic environmental phage with the broadest host ranges for potential phage therapy. Although the proportion of positive samples from soil was not as high as some other categories, the soil around beehives is easily available and was a reliable source of lytic phage that lysed all or nearly all of the 11 *P. larvae* strains tested. A higher proportion of phage was isolated from cosmetic samples with bee-related ingredients; however, the use of phage isolated from cosmetic sources might entail legal ramifications. Phage appear to be present in hives and soil surrounding hives begging the question of whether they routinely keep *P. larvae* infections in check until either spore numbers overwhelm the hive or environmental stressors such as cold winter temperatures make the bees more susceptible.

Two potentially different lytic phage were isolated from the same soil sample (PAIS2 fl. and PAIS2 med. cl.) and two potentially different lysogenic phage were isolated from a single strain of *P. larvae* ATCC 49843. After using transmission electron microscopy to visualize the phage, it is clear that PA1S2 fl. and PA1S2 med. cl. do not have the same phage morphology and, are therefore, unlikely to be the same phage despite isolation from the same source. As with the first pair, phage A and E, both lysogenic for ATCC 49843, have different phage morphologies determined by EM imaging.

Host range experiments were completed to provide information about the ability of the isolated phage to lyse a broad range of *P. larvae* strains. However, host range can be affected by bacterial receptors (Lindberg, 1973). Therefore, host range is not necessarily an indicator of relatedness, but is useful to determine effective phage for potential AFB treatment. Additionally, plaque morphology distinguishes phage and enables isolation, but is not a characteristic that determines relatedness either. Two isolates, IV and VI, have the same spot test lysis pattern when compared to one another. The plaque morphology of these two isolates is both medium size and clear, indicating the possibility of these two phage being the same, although they were isolated at different times from different sources. DNA sequence comparisons of these and the other phage of interest will need to be made to confirm identity and relatedness. Future work will include DNA characterization of these isolated phage to determine their relatedness as well as whether a single phage was isolated several times from various sources across different geographic locations.

Genersch *et al.* reclassified the former *P. larvae* subspecies as *P. larvae* without subspecies (2006). Host range data (Figure 1) demonstrated a distinct difference between the two former *Paenibacillus larvae* subspecies by susceptibility to *P. larvae* bacteriophage. Former *P. larvae pulvifaciens* (indicated by the numbers 367, 843, 3688, and 368 in the host range table) have a significantly lower susceptibility to the bacteriophage isolates. *P. larvae* former

subspecies *larvae* is significantly more susceptible to lysis by the isolated phage that *P. larvae* former subspecies *pulvifaciens* and, therefore, it would be beneficial to isolate phage on a former *pulvifaciens* strain to ensure effective treatment using a phage cocktail. The fact that there is a significant difference between the proportion of phage susceptibility with each bacterial (former) subspecies, indicates the possibility that there are differences between the two former subspecies not associated with growth or bacterial biochemistry but in biological function in the environment. It may also be that since the phage were all originally isolated using *P. larvae* 2605 (previously classified as *P. larvae larvae*) as the host strain, the phage are more capable of lysing strains from this subspecies. Additional isolates could be obtained using a strain of *P. larvae* former subspecies *pulvifaciens* to ensure that a cocktail would include phage capable of lysing such strains, should an AFB diseased hive be infected with *P. larvae* former subspecies *pulvifaciens*.

In the host range results, the lack of clearing on other genera or species other than *P. larvae*, indicates high host specificity. As a potential treatment for AFB, such severe host specificity is encouraging because the microbial ecology of the hive is not well understood, and it undoubtedly cause problems to harm microbes not intentionally targeted with *P. larvae* phage. A spot test should be undertaken in future work to specifically test phage on the individual microbiota of the natural honeybee microbiota.

By using the most effective phage with the broadest host range on the 11 *P. larvae* strains, a cocktail that is capable of lysing 100% of the strains could be created using as few as the top three isolated phage, H1P, WA, and H1S. A more robust cocktail could be designed by testing the lysing capabilities of these isolated phage on additional strains of *P. larvae*. The use of a cocktail of multiple phage, rather than a single phage, reduces the potential for development of phage resistance (Dr. Vincent Fischetti, personal communication). Therefore, determining selection criteria for the most suitable phage is important. If an arbitrary proportion of strains

lysed is chosen, for example 8 out of the 11, a phage cocktail capable of lysing all 11 strains with multiple phage capable of infecting each of the strains could be designed using 14 phage. Determining the effectiveness of a cocktail consisting of the top ranked 4 isolated phage will be the subject of future work in developing phage therapy as a potential treatment for American Foulbrood Disease.

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Figure Legends

Table 1. Proportion of samples found to contain *P. larvae* phage from each category.

Table 2. Sources, descriptions, and current designations of the 32 phage isolates.

Table 3. Morphology of plaques of each phage observed in soft agar overlays. Asterisk* formed plaques with a turbid halo around a clear plaque center. Sizes ranged and were described using set plaque diameters in the following classifications: pinpoint (<0.1 mm), small (0.1 mm – 0.5 mm), medium (0.5 mm – 1.0 mm), and large (>1.0 mm).

Figure 1. Host range of 32 isolated *P. larvae* bacteriophage determined by soft agar overlay spot tests. Results are interpreted on a scale from no lysis (blank cell) to complete lysis (black cell). Phage are designated by letters and numbers, corresponding to the source from which they were isolated. The bacterial species are represented across the top and are ranked from left to right in order of susceptibility to lysis by the 32 phage. The isolated phage are listed on the left side of the table and are ranked from top to bottom in order of the percentage of *P. larvae* strains they are capable of lysing. **Figure 1 Key.** Range of spot test descriptions observed in the host range experiment. The host range results are interpreted on a scale from no clearing to complete clearing. Plated examples show representative images of phage lysis on a bacterial lawn.

Figure 2. Proportion of susceptibility to isolated phage of *P. larvae* strains, which are grouped by former subspecies. Bacterial strains are listed from back to front.

Table 4. Morphologies of chosen phage determined from TEM images. Images were provided by the CAMCOR facilities at the University of Oregon. Measurements are based on the averages of 2-4 images. Question mark indicates uncertainty of classification based on rarity of the family. Family classifications are based on descriptions of morphology only.

Figure 3. TEM images of phage are labeled as follows: A, A; B, H3S; and C, H1P. Scale bars are in the bottom black border, and are 50 nm for A, but 100 nm for H3S and H1P.

CHAPTER 4

EXPERIMENTAL TREATMENT OF *APIS MELLIFERA* AFFECTED BY AMERICAN FOULBROOD DISEASE USING PHAGE THERAPY

This chapter is formatted for submission to the Journal of Invertebrate Pathology.

Experimental treatment of *Apis mellifera* affected by American foulbrood disease using phage therapy

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Highlights

- Bacteriophage therapy
- American foulbrood disease experimental treatment
- Artificial larvae rearing

Abstract

American Foulbrood disease (AFB) is caused by an infection of the bacterium *Paenibacillus larvae* in European honeybees, *Apis mellifera*. Due to the economic and potential environmental losses of this disease, sustainable treatment regimens should be considered; in particular, phage therapy, or the therapeutic use of bacteriophage that are capable of lysing *P. larvae*. Previously isolated bacteriophage were combined to produce a phage cocktail, then administered to bees under both of the following conditions: lab-reared honeybee larvae infected with cells and spores of *P. larvae*; and a hive infected with AFB located in the state of Washington, USA. The lab-reared larvae were treated with two different phage cocktails, one with a higher number of phage types than the other, as both a prophylactic treatment prior to infection with spores and as a post-infection treatment after infection with spores. Results of the lab-reared larval experiments indicate an increase in survival of infected larvae given phage compared to the survival of infected larvae without treatment. Results of the field experiment are promising as a preliminary study on which to base additional treatments, but are inconclusive because of the unavailability of more than one hive at the time of treatment.

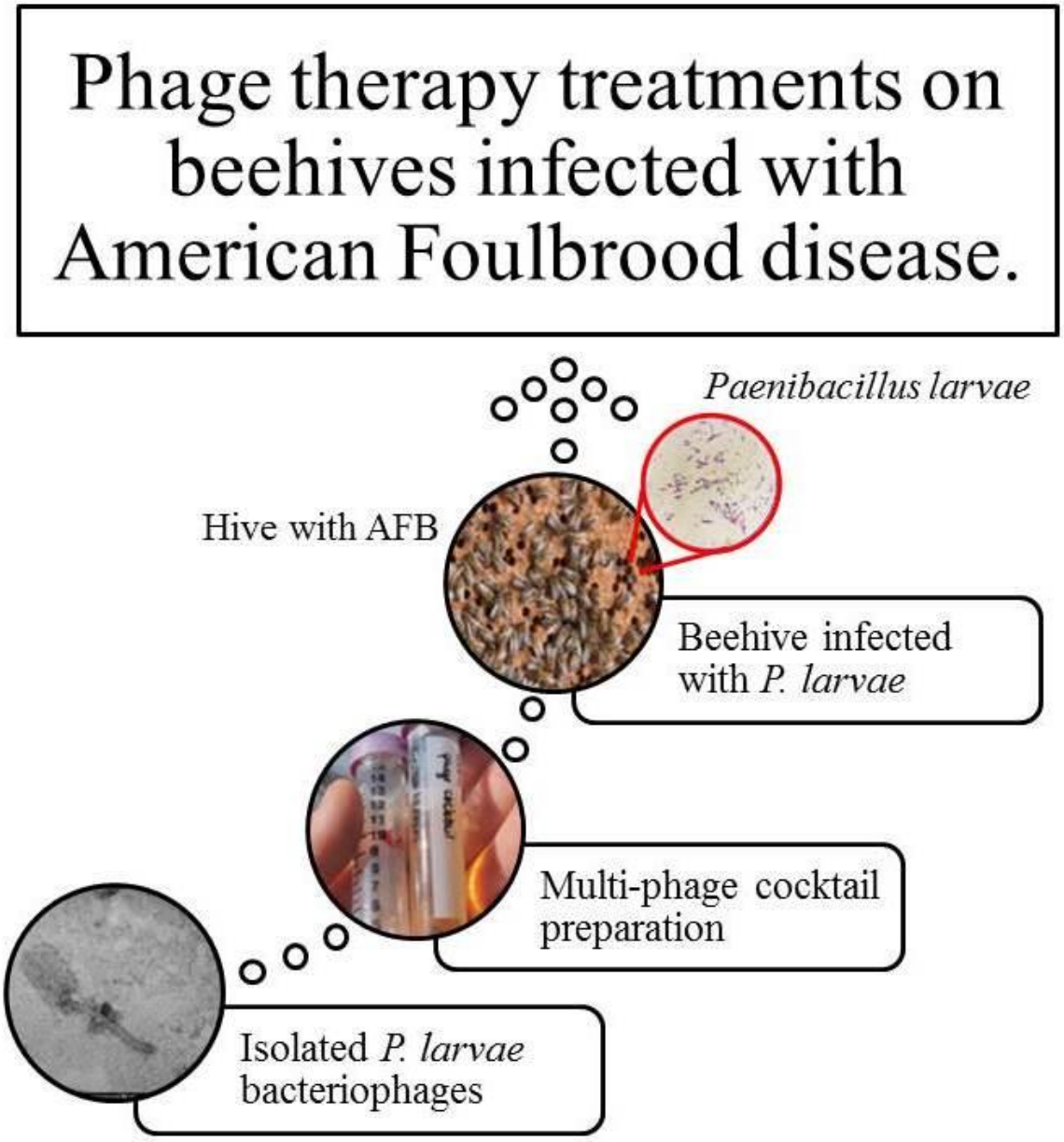
Key words: *Paenibacillus larvae*, American Foulbrood Disease, bacteriophage, *Apis mellifera*

Abbreviations

American foulbrood disease (AFB)

Modified brain heart infusion with glucose (GmBHI)

Graphical Abstract



Introduction (1)

The bacterium, *Paenibacillus larvae*, causes American Foulbrood disease (AFB), one of many diseases affecting honeybees, *Apis mellifera*, (Shimanuki and Knox, 2000). It is recognized worldwide as an important cause of honeybee death and loss of viable hives (Matheson, 1993;

Calderone, 2001). This Gram-positive, rod-shaped bacterium produces spores and it was identified as AFB's causative agent in 1907(White, 1907) . Adults are resistant to the disease, but they are vectors that transmit the infective agent (spores) to susceptible larvae (Hitchcock *et al.*, 1979, Wilson, 1971); the larvae are susceptible during the first 36 h after hatching (Genersch, 2010). In general, spores are resistant to antibiotics, heat and drying, and *P. larvae* spores in particular can remain viable for almost four decades (Haseman, 1961). Following a *P. larvae* infection that results in the death of one larva, millions of spores can be released in the hive (Sturtevant, 1932). As the larvae of a hive succumb to the disease, and fewer bees are able to reach adulthood, the hive collapses as it is unable to maintain its population. These characteristics make eradication of this disease difficult, and currently, the only viable method for curing the hive is the direct application of powdered antibiotics , which is not recommended because antibiotics do not completely eradicate the disease (Calderone, 2001), while an extreme measure to control the spread of the disease is burning infected hives (Genersch, 2010). Unfortunately, antibiotic treatment selects for resistant strains of *P. larvae* (Evans, 2003;Murray *et al.*, 2007) and is also linked to traces of the antibiotic found in the honey of treated hives (Oretelli, 2004; Saridaki-Papakonstadinou, 2006). Because of increased resistance and contaminated commercial products, many countries have ceased antibiotic treatment of AFB (Chan *et al.*, 2011). Additionally, the burning of hives to prevent spread of the disease results in significant economic loss to the beekeeping community (Genersch, 2010). Therefore, the occurrence and significance of AFB, combined with the lack of safe treatment methods, has created a need for alternative strategies to address AFB.

A potential method of treatment is phage therapy, or the therapeutic use of bacteriophage (also called phage) to kill bacterial cells. Phage infect and lyse bacteria (Carlton, 1999), and are generally specific for target bacterial species. Characteristics of single phage isolated from lysogenic strains of *P. larvae* have been explored in previous studies, and several have suggested

using phage as a potential treatment strategy for AFB (Bakheit and Stahly, 1988; Camana *et al.*, 1991; Dingman *et al.*, 1984; Drobnikova and Ludvik, 1982; Gochnauer, 1955; Gochnauer, 1970; Valerianov *et al.*, 1976); however, no significant treatment strategies using phage have been reported.

Bacteriophage are very abundant in many natural environments (Suttle and Furhman, 2010). Further, they are only self-propagating when host bacteria are present and are very specific for their host bacterial species. Phage, if used against *P. larvae*, would decrease the need for chemical treatment of the hives and would not be harmful to organisms other than their bacterial host. Therefore, researching the potential use of phage therapy in treating AFB is of interest for economic and environmental health. Unfortunately, no ready repository of *P. larvae* phage is available for use; therefore, various environments were screened for the presence of *P. larvae*-specific bacteriophage, phage were isolated and characterized, and further experimental treatments of infected larvae and an infected hive are described in this study to evaluate the potential use of phage therapy as a strategy to treat AFB.

Materials and Methods (2)

Bacterial strains and phage isolates (2.1)

The following strains of *P. larvae* were used: NRRL B-2605, NRRL B-3554, NRRL B-3650, ATCC-25748, ATCC-25747, ATCC-49843, ATCC-25367, ATCC-25368, ATCC-3688. In addition, two naturally occurring cultures isolated from infected hives were used: 2188 and 2231. Bacterial cultures were grown with the same media and under the same conditions described in the phage isolation methods from Alvarado *et al.* (2014) (submitted). The phage used in this research had been previously isolated as described in Alvardo *et al.* (2014) (submitted) and were selected from a pool of 32 total isolates based on the broadest host range of *P. larvae* strains (Yost and Amy, 2014) (unpublished data).

Amplification and quantification of phage titers (2.2)

Phage isolates were amplified prior to use in the experimental treatments. The procedures for amplification and quantification of phage titers were the same as those described by Alvarado *et al.* (2014) (submitted).

Larvae experiments (2.3)

Bacterial cell and spore harvesting (2.3.1): Eleven strains of *Paenibacillus larvae* were grown in 20 ml of GmBHI at 37 °C and shaking at 100 rpm. After overnight incubation, the turbid culture was pelleted by centrifugation, the supernatant discarded, and the cells resuspended in 200 µl sterile GmBHI broth. The concentrated cells were plated in serial dilutions using GmBHI agar plates and GmBHI sterile broth dilution blanks, and then colonies were counted to determine the colony forming units (CFU) of the concentrate. A volume of 200 µl of the concentrate was added to 1 ml of prepared larvae food, resulting in a titer of 10^5 cells per total volume. Food was mixed by vortexing, then fed to larvae on a daily basis. New food was prepared with freshly grown bacterial cultures daily. Approximate numbers of CFUs being fed to each larva were calculated according to the final titers in the larvae food and amount of food fed to each larva per day (Table 1). Spores were prepared by first inducing sporulation then harvesting spores as described by the spore methods in Alvarado *et al.* (2014, submitted) with the exception of replacing the HistoDenz (Sigma) density gradient with d-Sorbitol of the same concentrations. Spore concentration was calculated by serial dilution and plating of the final product. Calculations of spore load fed to each larva per day are given in Table 1.

Phage cocktail preparation (2.3.2): Titers per ml of the amplified single phage lysates were determined as previously described and are as follows: H1P, 5×10^4; WA, 3×10^6; F, 5×10^6; V, 4×10^5; H2S, 10^4; H3S, 4×10^5; XIII, 4×10^6; E, 10^4; H5S, 9×10^3; VII, 2×10^6; D, 10^6; PAIS2 fl, 9×10^2; and B, 5×10^6. Two separate cocktails were made, the first (phage cocktail #1 or PC1) containing the following 7 phage: H1P, WA, F, V, H2S, H3S, and XIII; and the second

(phage cocktail #2 or PC2) containing all 13 phage; however, in both cases the final titer of combined phage was approximately the same (phage cocktail #1, 1.8 x 10^6; phage cocktail #2, 1.6 x 10^6). Phage cocktail makeup was determined based on host range capabilities, and represents the broadest range of lysing capability on 11 different strains of *P. larvae* (Chapter 3). A volume of 1 ml of each lysate was combined for the final phage cocktail. The final phage concentration was both calculated from initial titers and confirmed by soft agar overlay platings done in serial dilution after combination. A volume of 200 µL of each cocktail was added to 1 ml of prepared larvae food prior to feeding to larvae. Calculated PFUs fed to each larva per day are listed in Table 1.

Table 1 Volumes of food and titers of phage, bacteria and spores fed to larvae daily.

Days after Grafting	**Day 0**	**Day 1**	**Day 2**	**Day 3**	**Day 4**	**Day 5**	**Day 6**	**Day 7**	**Day 8**
Volume of Larvae Food (µl)	10	10	20	30	40	50	50	60	0
Calculated # of CFUs (any strain)	800	800	1600	2400	3200	4000	4000	4800	0
Calculated # ATCC 49843 Spores	100	100	200	300	400	500	500	600	0
Calculated # NRRL B-3554 Spores	90	90	180	270	360	450	450	540	0
Calculated # Isolated 2188 Spores	90	90	180	270	360	450	450	540	0
Calculated Number of PFUs in PC1	3.00^3	3.00^3	6.00^3	9.00^3	1.20^4	1.50^4	1.50^4	1.80^4	0
Calculated Number of PFUs in PC2	2.67^3	2.67^3	5.33^3	8.00^3	1.07^4	1.33^4	1.33^4	1.60^4	0

Larvae food preparation and treatment (2.3.3): Larvae food consisted of the following: 14.4 ml sterile, distilled water, 4.2 g royal jelly powder (Glory Bee), 0.6 g glucose (Difco), 0.6 g fructose (Difco), and 0.2 g yeast extract (Difco) as described by Peng *et al.* (1992). The sugars and yeast extract were added to the water, this mixture was filtered, and then UV treated for 1 h. The royal jelly powder (4.2 g) was aseptically added to the water mixture but was otherwise

untreated. The mixture was made homogenous by vortexing to ensure complete dispersion of the royal jelly. Food was prepared and stored at -20 °C until needed. Larvae were fed increasing amounts of food each day (Crailsheim *et al.*, 2012), as indicated in Table 1. As a negative control, larvae were fed larvae food without amendments while all other larvae were fed a mixture of food with treatment additives. In each case, 200 µL of concentrated spores, cells, or phage cocktails were added to 1 ml of larvae food as described above. Larvae were given the following treatments: negative control = food with no additives, broth control = food with GmBHI broth added to the same dilution as other additives, water control = food amended with 200 ul sterile water, food containing NRRL B-3554 vegetative cells, food amended with ATCC 49843 vegetative cells, food amended with isolated 2188 vegetative cells, food amended with NRRL B-3554 spores, food amended with ATCC 49843 spores, food amended with 2188 spores, prophylactic phage therapy treatments and post-infection phage therapy treatments (food amended with 200 ul phage cocktail #1 or #2). All larvae in the experimental phage cocktail treatments were infected with spores from *P. larvae* 2188. Two phage cocktails, phage cocktail #1 and phage cocktail #2 were tested in both the prophylactic and post-infection treatment experiments.

Larvae rearing (2.3.4): Larvae were reared by methods similar to those described by Crailsheim *et al.* (2012). Queens were caged using plastic or metal wire mesh approximately one week prior to the intended date of grafting larvae. While the queens were confined, the age and location of larvae in the frame were ensured. Eggs, turned to a horizontal position shortly before hatching, were then closely observed and the hatched larvae were grafted from the frames within a day after hatching. Each treatment included a corresponding negative control consisting of larvae taken from the same frame on the same day. Preliminary experiments were conducted by placing the grafted larvae into 96-well plates, but later were conducted by placing grafted larvae into sterile petri dishes (VWR) because survival rates were higher in larvae reared with more

space. It appeared that higher survival rates were observed because the larvae food was not confined leading to a lower chance of larval drowning. Incubation microcosms were created by placing 1 L of 10% glycerol in the bottom of plastic containers followed by a layer of plastic support on which sat the well plates or petri dishes. The boxes were closed with loosely fitting plastic lids, this allowed the humidity to be maintained at 90% within the microcosms. Metal trays filled with water were placed on the bottom of the incubator to maintain humidity within the incubator's interior at 80%. The temperature was kept at 34 °C. Larvae were fed daily with the amount of food indicated in Table 1. On the eighth day after grafting, larvae were removed from the petri dishes and placed on sterile filter paper in new petri dishes outside the microcosms for pupation.

For the larvae controls, larvae were fed either unamended food, food diluted with GmBHI, or food diluted with water (Figure 1). The negative control data represent 3 replicates with n=20, 21, and 15, the GmBHI data represent 2 replicates with n=20 each, and the water data represents 2 replicates with n=22 and 21.

Each experimental treatment also had a corresponding negative control prepared on the same day from the same frame and fed unamended food. Negative control data for Figures 2-6 represent the average of 10 control replicates with n=12 or 13. The average of all negative controls was determined rather than using individual negative controls because of the small sample sizes, which were limited due to the number of larvae in the hives. During the vegetative cell infection treatments, two replicates for each strain with samples sizes from 32 to 49 (mean size of 45 larvae) were prepared.

Larvae were fed ATCC 49843 and NRRL B-3554 spores daily. Two different treatments with 2188 spores were conducted; one in which larvae were fed spores daily and one in which only one dose of spores was administered on the first day. Spore treatment sample sizes ranged from 48-53 with a mean size of 50 and all spore infection treatments were conducted in duplicate.

Phage preparations were administered to larvae by adding the phage cocktails suspended in GmBHI to the larvae food (as previously described). Phage cocktail experiments were conducted in duplicate and all phage cocktail treatments ranged from 48- 55 with a mean value of 51. Disparate sample sizes were due to larvae available to graft on any given day.

Daily observations (2.3.5): Larvae were viewed under a dissecting microscope (Nikon) daily and observed for signs of life: opening and closing spiracles or food consumption. In the event that no movement could be seen for the first 2 days, larvae were kept until day 3 in the event that larvae were alive but not producing easily visualized movement. On the third day, if no growth or movement was observed, larvae were assumed dead and removed. Samples of dead larvae were kept at -20 C in 20% glycerol stocks for PCR analysis in order to determine presence of bacterial DNA. The number of surviving larvae was recorded daily.

Field experiment (2.4)

Lyophilization of phage cocktails (2.4.1): Between 10 and 15 ml of individual amplified phage lysates were lyophilized separately (LabConco Lyophilizer). Samples were allowed to completely dry overnight; once all liquid was removed, samples were weighed and equal amounts (0.02 g) of each powdered phage preparation was combined. This powdered mixture was easily transported to the field site. Experiments to ensure phage viability after lyophilization were conducted with reconstituted the lyophilized phage. Powdered phage mixtures were resuspended in either water or sugar syrup (8.75 g sucrose/10 ml water) and plated to determine phage viability in diluents proposed for field study.

Field resuspension of phage and spray treatment on hives (2.4.2): Lyophilized phage preparations were taken to the field site near Bellingham, WA, reconstituted with 10 ml of water, and then poured into 400 ml of sugar syrup. After shaking to homogenize the mixture, the entire volume was sprayed directly on alternating frames in the infected beehive. The following day, the sugar syrup mixture had been cleaned by the nurse bees and was no longer visible.

Treatments occurred on 6/26, 6/28, 7/10, 7/23, and 8/6 in the summer of 2013. The first two treatments were administered in the presence of the beekeeper and the remaining three were conducted by the beekeeper. On each date, either odd or even numbered frames were sprayed with the sugar syrup/phage preparation.

Hive observations (2.4.3): Frames were selected on the first treatment day for qualitative visualization of the extent of the infection and were photographed on the first day as well as at each subsequent treatment. Gross comparisons of the frames were made over time, but detailed results were difficult to determine based on visualizations only. Additionally, the beekeeper reported the general state of the treated hive on a regular basis until the end of the treatments.

Post-treatment actions and related observations (2.4.4): One month after the last phage treatment was administered, the beekeeper removed the worst of the diseased frames and replaced them with fresh, uninfected, and unpopulated frames. By October 11, 2013, the beekeeper reported no evidence of AFB in the hive, and as of January 2014, no recurrence has been reported.

Statistical analysis (2.5)

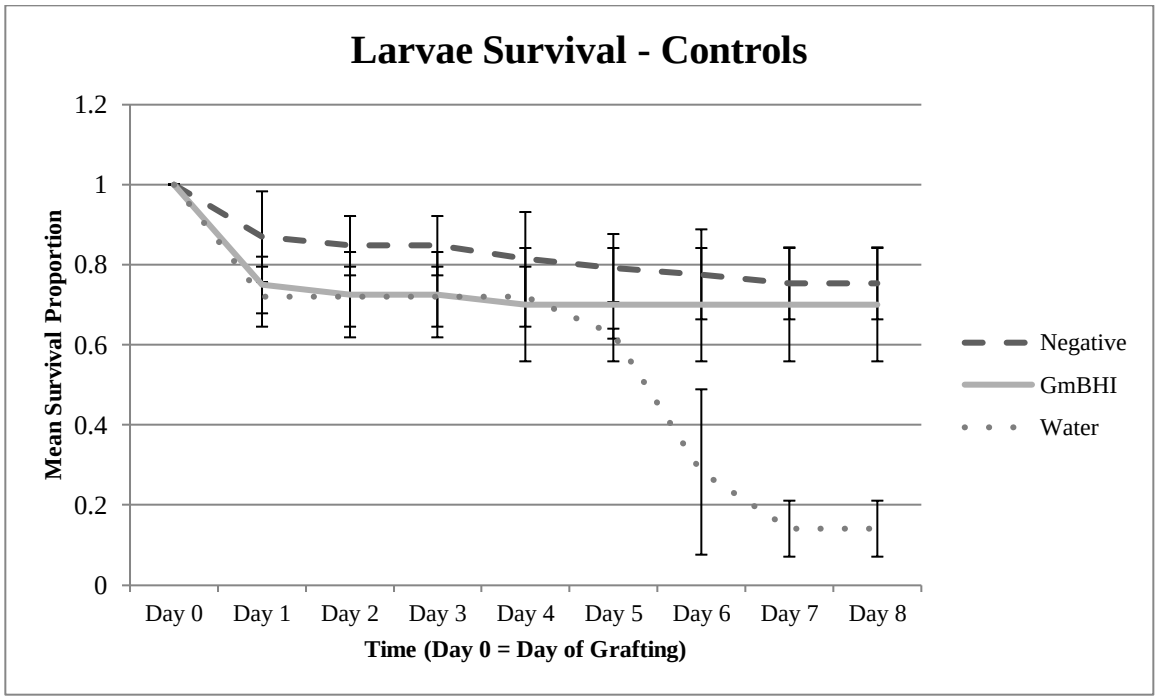
Student T tests were performed on all treatments and controls to determine the statistical significance of their comparisons. A significance value of $\alpha > 0.05$ was used throughout this study.

Results (3)

Lab experiments (3.1)

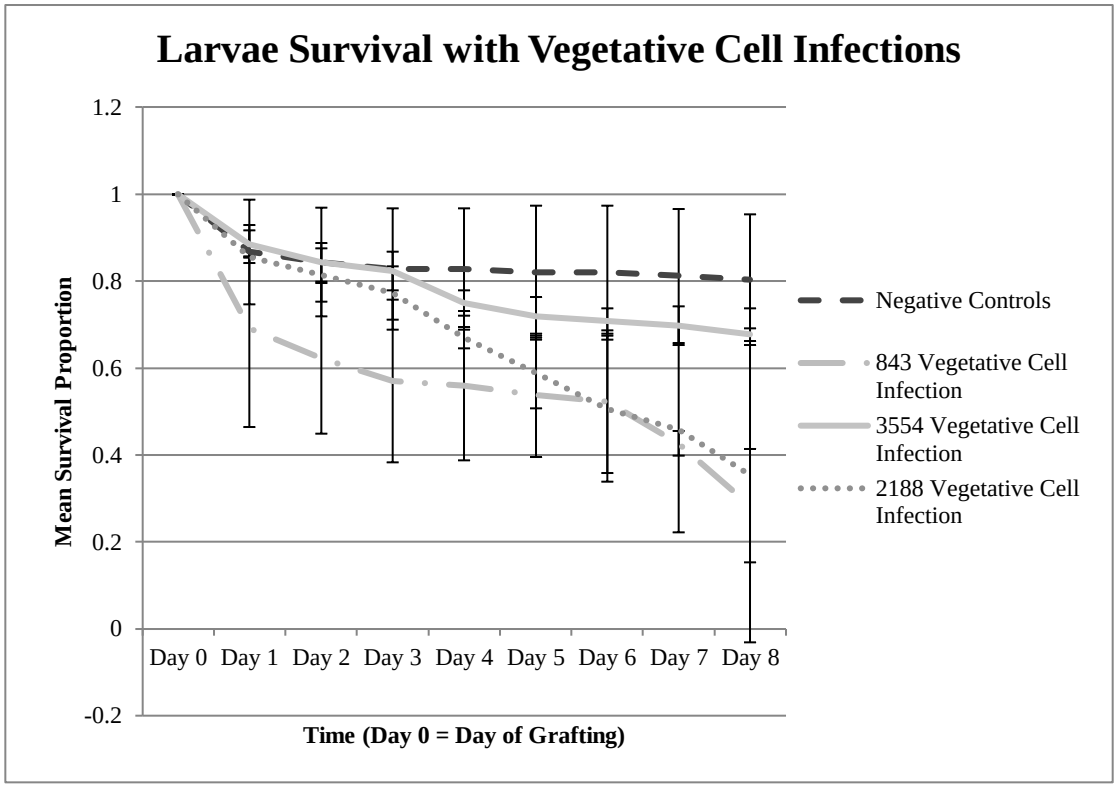
Results obtained from the control experiments are shown in Figure 1. There is a significant difference between both the survival of the negative control and the water control ($p=0.002$) and between the GmBHI broth control and the water control ($p=0.034$), but not between the negative control and the GmBHI broth control ($p=0.347$).

Figure 1 Larvae Survival - Controls. Displays the mean proportion of larvae survival from the following treatments: negative control, food with GmBHI added, and food with water added. Error bars represent the standard deviation.



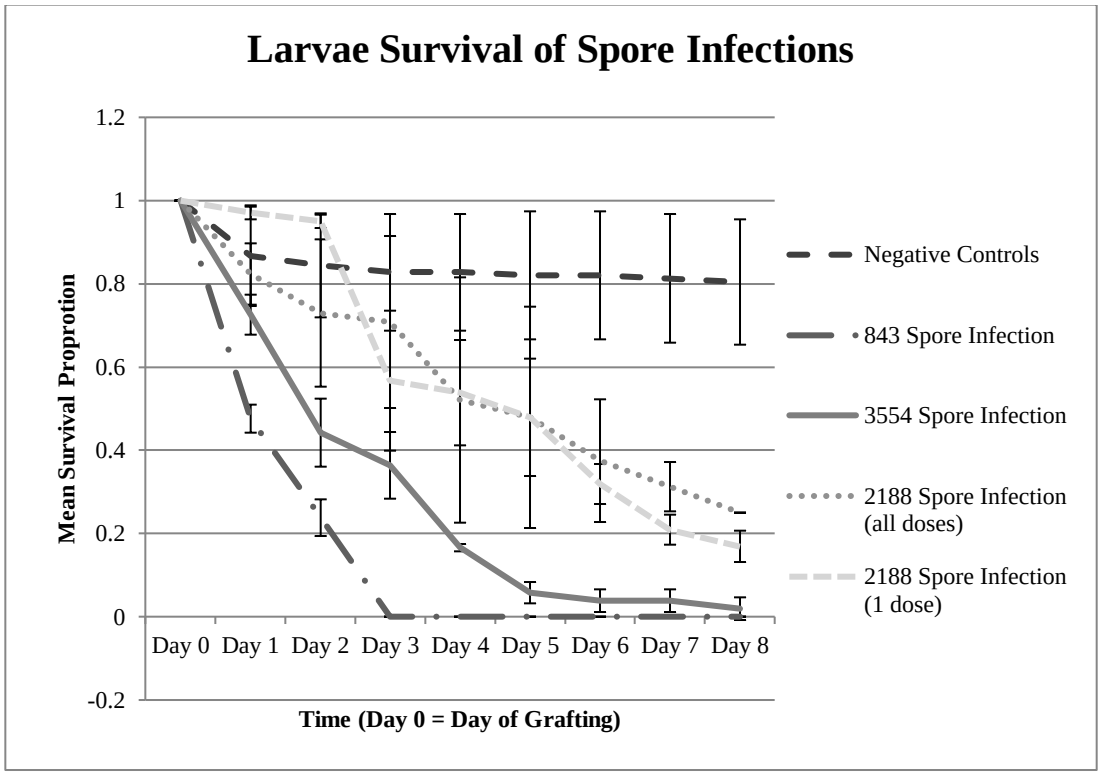
Results from the vegetative cell infection treatments are shown in Figure 2. There is a significant difference in the larvae survival by day 8 between the negative control larvae and those infected with *P. larvae* ATCC 49843 (p=0.000548) as well as between the negative control larvae and those infected with *P. larvae* 2188 (p=0.00560) but not with larvae infected with NRRL B-3554 vegetative cells (p=0.139). The larvae infected with NRRL B-3554 that survived until pupation were incubated until pupation was complete, and the body mass was recorded for each fully pupated bee. Compared to the control bees, the mass of the infected bees was significantly lower (p=0.0035).

Figure 2 Larvae Survival with Vegetative Cell Infections. Larvae infected with vegetative cells from *P. larvae* ATCC 49843, NRRL B-3554, and isolated 2188. Error bars represent the standard deviation.



Spore infection experiments indicate a significant decrease in survival of larvae infected with spores from any of the three bacterial strains compared to the control (Figure 3). There is a significant difference between the survival rates of the larvae infected with any of the spores and the negative control larvae ATTC 49843 (p=1.99E-8; NRRL B-3554 (p=1.79E-8) and the one dose spore infection with 2188 (p=4.97E-7) ; however, there is not a significant difference in the survival rates of larvae fed only one dose of 2188 spores when compared to larvae fed daily doses of 2188 spores (p=0.102).

Figure 3 Larvae Survival of Spore Infections. Data represent the proportion of surviving larvae after infection with *P. larvae* spores from the following strains: ATCC 49843, NRRL B-3554, and isolated 2188. Two infections using spores from 2188 were conducted; one with daily doses of spores and one with a single dose on the first day. Error bars represent the standard deviation.

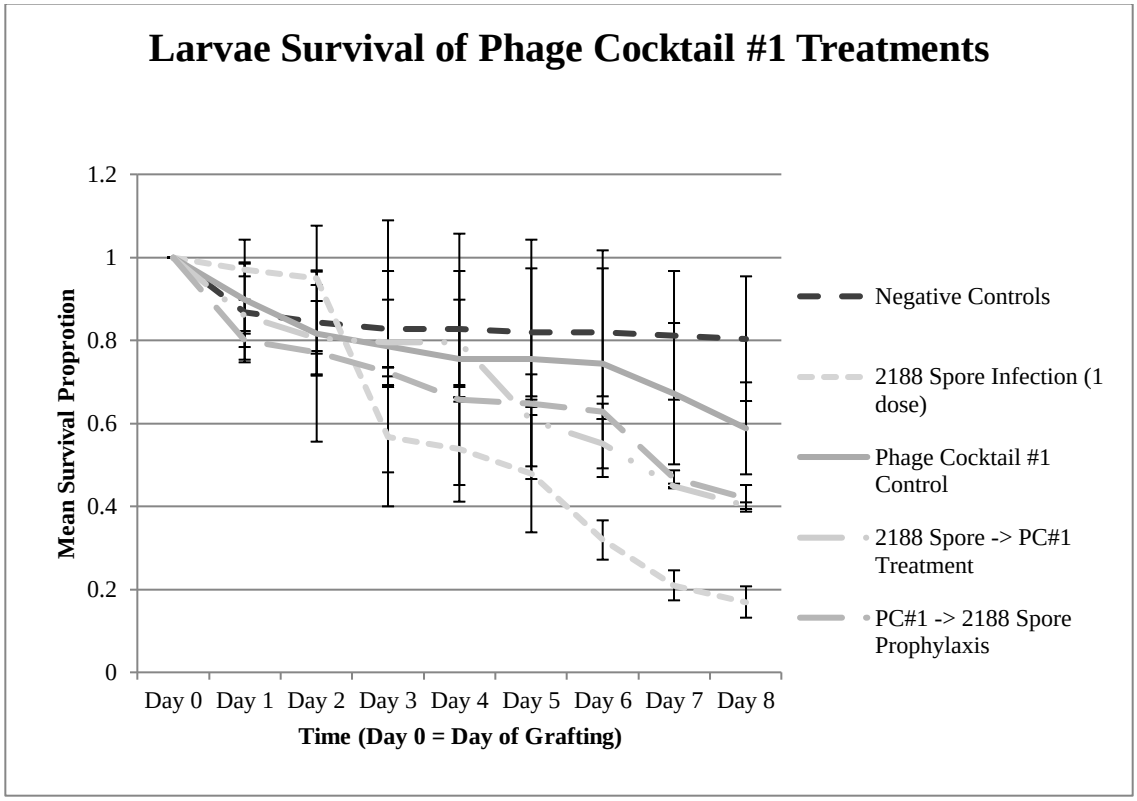


T-test comparisons between the larvae fed spores (Figure 3) or vegetative cells (Figure 2) of the same strains yield the following: the ATCC 49843 vegetative cells compared to spore infection , p=0.010; the NRRL B-3554 vegetative cells compared to spore infection, p=0.002; the 2188 vegetative cells compared to the 1-dose spore infection, p=0.384. There is a significant difference between the survival rates of larvae infected with spores of either ATCC 49843 or NRRL B-3554 compared to larvae infected with vegetative cells of the same strains. There is not, however, a significant difference between the survival rates of larvae by day 8 between those infected with spores or vegetative cells of 2188.

Results from phage cocktail #1 experiments are shown in Figure 4. There is not a statistically significant difference between the negative control and phage cocktail #1 control (

p=0.077). There is a significant difference between the survival of larvae given phage cocktail versus infected with spores of 2188(p=0.045). There is also a significant difference in survival rates between both forms of phage treatment (either administered prior to (p=0.010) or after (p=0.031) infection) and infected larvae without treatment, but not between the survival of the treatments themselves (p=0.293).

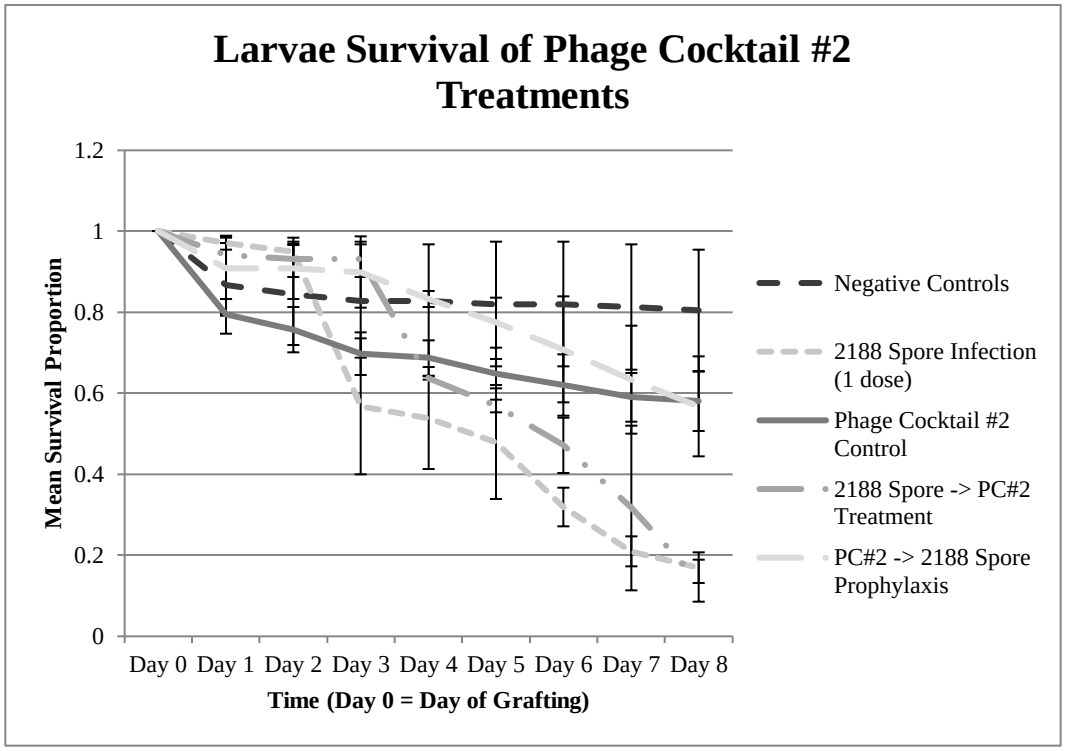
Figure 4 Larvae Survival of Phage Cocktail #1 Treatments. Phage cocktail #1 treatments. Larvae were fed spores, phage cocktail, spores and then phage cocktail, or phage and then spores. Error bars represent the standard deviation.



Results from the phage cocktail #2 experiments are shown in Figure 5. Assuming $\alpha <$ 0.05, there is a significant difference between the phage cocktail #2 larvae and the infection control (p=0.002), but not between the phage cocktail #1 larvae and the negative control (p=0.069). There is not a significant difference between the infection control and the treatment regimen (p=0.271), but there is a significant difference between the infection control and the

prophylaxis regimens (p=0.024). The decrease in survival is most likely due to compromised larvae that were grafted on that day, as the corresponding negative control larvae had a much lower survival than the overall average of all of the negative control experiments. There is a significant difference between the prophylaxis and the treatment regimens using phage cocktail #2 (p=0.044). The survival of larvae treated with the phage cocktail prior to infection increased by 70%, and was comparable with the survival rates of the phage cocktail controls.

Figure 5 Larvae Survival of Phage Cocktail #2 Treatments. Data represents experiments with phage cocktail #2 treatments. Larvae were fed spores, phage cocktail, spores and then phage, or phage and then spores. Error bars represent standard deviation.

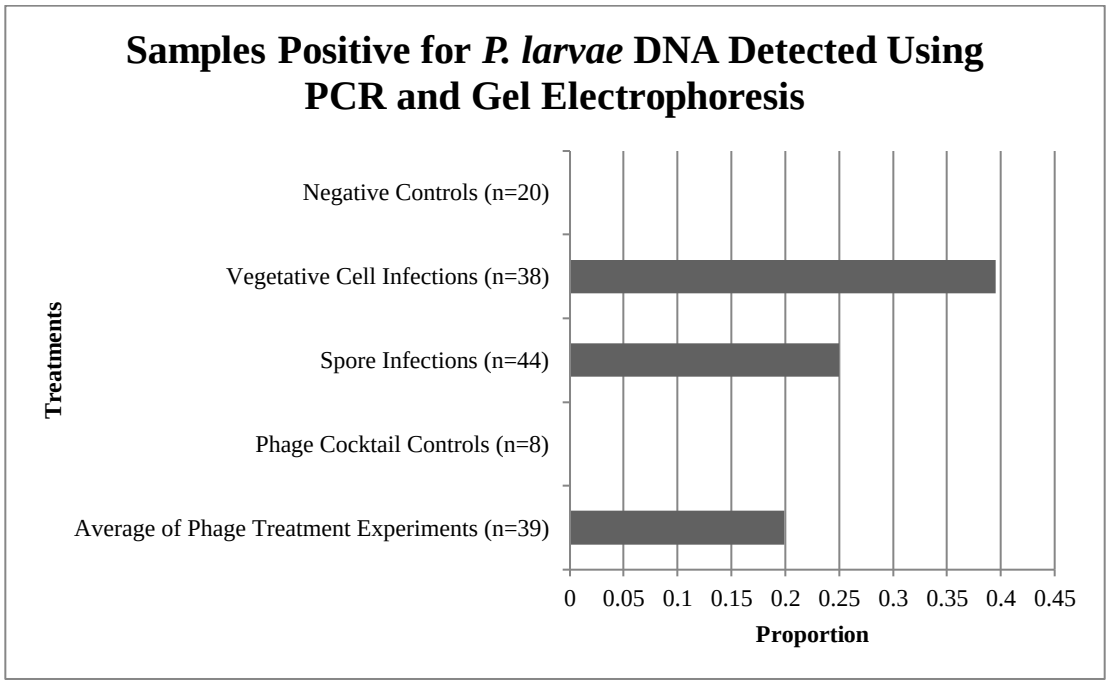


The efficacy of the two different phage cocktails is determined by comparing the data represented in Figure 4 and Figure 5. T-test comparisons yield the following: comparison between the prophylaxis treatment of phage cocktail #1 and phage cocktail #2, p=0.162; and comparison between the treatment regimen of phage cocktail #1 and phage cocktail #2, p=0.041.

Assuming $\alpha < 0.05$, there is a significant difference between the different phage cocktails when used as a treatment, but not when used as a prophylaxis.

Figure 6 displays the proportion of deceased larvae that tested positive for *P. larvae* DNA by PCR and gel electrophoresis (Piccini *et al.*, 2002). Larvae obtained from negative control and phage cocktail control experiments (both of which had no bacteria added) showed no evidence of *P. larvae* DNA. Approximately 40% of the larvae taken from vegetative cell experiments were positive for DNA. Approximately 25% of the larvae taken from spore experiments were positive for DNA. The average proportion of larvae positive for *P. larvae* DNA from phage cocktail treatments, regardless of whether phage was administered prior to or after spore infection, was slightly lower at 20%.

Figure 6 Samples Positive for *P. larvae* DNA Detected Using PCR and Gel Electrophoresis. The proportion of deceased larvae positive for *P. larvae* DNA identified by *P. larvae*-specific primers and gel electrophoresis.



Field experiment (3.2)

Experiments to determine phage viability after lyophilization were conducted to determine whether powered phage lysates were a practical option to use in a field setting. Prior to lyophilization, the average titer of multiple phage lysates was approximately 10^8/ml. After lyophilization, the cocktails were resuspended in either sugar syrup or sterile water and the average of the resuspended cocktails was approximately 10^5/ml. The resuspended phage cocktails were maintained at 4 °C for one month, then titers were determined to be approximately 10^4/ml.

Pictures were taken of the same frames each time a treatment occurred, and observations were determined by the beekeeper. Pictures revealed a slight visual improvement during the treatment process, but not a complete eradication of the disease. The comb was both darker and has more sunken capped cells (both characteristics of AFB) in the image taken on the first day of treatment. The beekeeper reported removing the diseased frames and replacing them with virgin, unpopulated frames after treatments had ended. Four months after the initial treatment, the beekeeper reported no visible sign of infection (Karen Bean, personal communication).

Samples were obtained after the treatment regimen ceased, and the procedures to isolate phage as previously described (Chapter 2) were conducted. It was determined that the phage from the administered phage cocktails were present in the hive after the 5 treatments had ended.

Discussion (4)

Lab experiments (4.1)

Results from the control experiments (Figure 1) indicate that dilution of the larvae food with water decreases the nutrients to the point of starvation, leading to significant larval death after four days of incubation. However, when GmBHI broth is used to dilute food, no significant decrease in larval survival was observed, possibly due to the addition of the nutrients in the

medium. This preliminary experiment is important because phage, spores, and vegetative cells were added to the larvae food in GmBHI broth.

Vegetative cell infection experiments (Figure 2) indicate that the survival of larvae infected with strains 2188 or ATCC 49843 resulted in similar low survival by the 8th day; however, infection with ATCC 49843 rapidly decreased larval survival by the first day after grafting. Although the disease is transmitted by spores (Genersch, 2010) and previous work suggests that vegetative cells do not cause AFB in larvae (Tarr, 1937), our results showing the significant decrease in survival of larvae infected with vegetative cells of strains 2188 or ATCC 49843 compared to the negative control indicate that the vegetative cell of *P. larvae* is also an infective agent. Additionally, although the larvae infected with NRRL B-3554 maintained viability until they entered pupation, the size and weight of the pupae after pupation were significantly smaller than that of the negative control. This indicates the possibility that although bees survived infection with vegetative cells, their development was compromised and begs the question of whether these smaller bees would even be accepted within the hive.

There was a significant decrease in survival of larvae infected with spores from any of the three bacterial strains compared to the control (Figure 3), which is consistent with previous literature (Tarr, 1937; Toumanoff, 1929; Woodrow, 1941; Woodrow, 1942;). The results also demonstrate that there is not a significant difference between the proportion of larval survival when infected with one dose of spores on the first day compared to comparable doses administered each day with the same strain (2188). This indicates a single exposure of *P. larvae* spores causes the same mortality as multiple exposures, which supports the claim that low doses of spores can cause infection of AFB in honeybee larvae (Woodrow, 1942).

Comparisons between data represented in Figures2 and 3 indicate that the efficacy of spores in reducing larval survival is greater than that of the vegetative cells. However, as previously stated, vegetative cells can also decrease larval survival as well as inhibit their

development. This is demonstrated by the similar decreases in survival of larvae infected with spores or vegetative cells of 2188 and by the significantly lower body masses of the fully pupated bees infected with NRRL B-3554.

The phage cocktail #1 control displays a small but insignificant decrease in survival compared to negative controls (Figure 4). In each case using phage cocktail #1, whether administered prior to or following infection with 2188 spores, the larval survival was higher than the untreated infected larvae. Although the survival of larvae under both treatment regimens were significantly lower than the survival of the negative controls, they were also significantly higher than the survival of the larvae infected with spores without treatment. This indicates that the addition of phage cocktail #1 decreased mortality of infected larvae and might potentially be used in prophylaxis or treatment of AFB. The phage cocktail #2 control displays a small but insignificant decrease in survival compared to negative control (Figure 5). The prophylactic treatment using phage cocktail #2 increases survival above the infected larvae with no treatment, indicating it also might be an effective prophylaxis for AFB.

Results of the PCR and gel electrophoresis experiments demonstrate that there was not cross contamination of samples during preparation nor incubation as none of the samples tested that had not received bacteria showed evidence of bacterial DNA (Figure 6). The presence of bacterial DNA in the dead larvae from the vegetative cell experiments suggests that they likely died from a *P. larvae* infection. The low percentage (40%) could be indicative of interference with the PCR process because 100% of the larvae were fed bacterial cells. It is likely that larvae positive for *P. larvae* DNA taken from the spore infections also died from a *P. larvae* infection. The lower proportion of deceased larvae from phage-treated infected specimens that were positive for *P. larvae* DNA is consistent with treated larvae having a lower mortality rate than untreated infected larvae.

Field experiment (4.2)

The opportunity to treat an infected hive presented itself when a beekeeper in the state of Washington, USA, contacted UNLV with questions regarding AFB. She had previous experience dealing with multiple AFB-infected hives, and at the time of contact, had one infected hive. The beekeeper was willing to allow an experimental treatment on her infected hive, and this was undertaken. As such, the results of this study should only be considered as preliminary with clear necessity to carry out further field testing on more hives in the future. Additionally, as the hive was already infected, the study is a post-infection treatment experiment only. It was not deemed ethical to administer phage to a healthy hive prior to purposefully infecting it with *P. larvae*, so a prophylactic treatment regimen was not conducted outside of the lab.

Planning included preparation of phage cocktails by lyophilization for the field experiment, which was a convenient method to store and transport phage. Some loss of phage viability during the lyophilization and reconstitution procedures occurred as seen by reduced phage titers throughout the process. However, enough phage survived to use as a starting material for treatment of the infected hive. Using a phage cocktail with a larger concentration prior to lyophilization would allow for a higher starting titer and perhaps more effective treatment of the hive. The resuspended phage cocktails were maintained at 4 °C for one month, then titers were determined to be approximate 10^4, a loss of only10 fold during that time. This indicates the lasting viability of resuspended, previously lyophilized phage in diluted form and is consistent with long-term storage of phage lysates at either 4 C or at 37 C (data not shown).

Throughout the treatments, the infected hive improved but the disease was not eradicated. This could be because of, among other potential reasons, the severity of the disease in that hive, the need for a higher starting titer of phage cocktail, or the inability of phage to infect spores of *P. larvae*. A combination of a spore germinant administered prior to phage treatment with a higher concentration of phage could be tested for effectiveness . Although the disease was not

eradicated immediately after treatments, the beekeeper reported that at four months after the first treatment (late October 2013) there was no evidence of AFB in the treated hive, and no recurrence of the disease has been reported as of January 2014. These results are based on the observations of the beekeeper, who is familiar with symptoms of AFB, and interpretation of the pictures of hive frames. However, the lack of recurrence of AFB in the hive could be due to the removal of the infected frames by the beekeeper, the addition of the phage cocktail into the hive, or a combination of both. More infected hives should be experimentally treated with phage cocktails to make this determination.

Conclusions (5)

The results of the lab experiments indicate an overall improvement in survival when phage cocktails are administered to infected larvae. Prophylactic treatment with phage cocktail #1 is slightly more effective than the post infection treatment, although not significantly so, while prophylactic treatment with phage cocktail #2 is significantly more effective at increasing larval survival than post-infection treatment. This indicates a prophylactic regimen may be more effective at preventing the disease than a post-infection treatment once a hive was already infected.

The higher survival of larvae that underwent prophylactic treatment with phage cocktail #2 than with phage cocktail #1 indicates that a cocktail with a greater number of different phage is more effective than a cocktail with fewer different phage.

The slightly decreased survival, although not significantly so, of the larvae given food diluted with phage cocktails may be due to the same reason larvae survival is slightly decreased when fed larvae food diluted with GmBHI broth. Experiments should be conducted to add more highly concentrated phage without diluting the food as much and maintaining the same

consistency as the negative control food to ensure that larval death is a result of the food dilution and not of the phage cocktails themselves.

Only one hive was experimentally treated in the field, and confident conclusions cannot be reasonably drawn. However, the fact that the hive has had no recurrence of AFB is promising. More experiments should be conducted to determine whether the apparent disappearance of the disease is the result of phage treatment, the removal of infected frames, or a combination of both. Prophylactic phage treatments on hives in a more natural setting would be useful, but would have to be undertaken careful control to ensure accidental spread of AFB after purposeful infection of an experimental hive would not occur.

The results from these preliminary experiments indicate using phage therapy is a potential option to treat American Foulbrood disease.

The authors declare no known conflicts of interest.

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Figure Legends

Table 1 Volumes of food and titers of phage, bacteria and spores fed to larvae daily.

Figure 1 Larvae Survival - Controls. Displays the mean proportion of larvae survival from the following treatments: negative control, food with GmBHI added, and food with water added. Error bars represent the standard deviation.

Figure 2 Larvae Survival with Vegetative Cell Infections. Larvae infected with vegetative cells from *P. larvae* ATCC 49843, NRRL B-3554, and isolated 2188. Error bars represent the standard deviation.

Figure 3 Larvae Survival of Spore Infections. Data represent the proportion of surviving larvae after infection with *P. larvae* spores from the following strains: ATCC 49843, NRRL B-3554, and isolated 2188. Two infections using spores from 2188 were conducted; one with daily doses of spores and one with a single dose on the first day. Error bars represent the standard deviation.

Figure 4 Larvae Survival of Phage Cocktail #1 Treatments. Phage cocktail #1 treatments. Larvae were fed spores, phage cocktail, spores and then phage cocktail, or phage and then spores. Error bars represent the standard deviation.

Figure 5 Larvae Survival of Phage Cocktail #2 Treatments. Data represents experiments with phage cocktail #2 treatments. Larvae were fed spores, phage cocktail, spores and then phage, or phage and then spores. Error bars represent standard deviation.

Figure 6 Samples Positive for *P. larvae* DNA Detected Using PCR and Gel Electrophoresis. The proportion of deceased larvae positive for *P. larvae* DNA identified by *P. larvae*-specific primers and gel electrophoresis.

CHAPTER 5

GENERAL DISCUSSION

The importance of the European honeybee, *Apis mellifera*, to agriculture, the economy, and environmental health are reasons to consider the overall well-being of the organism, especially when reflecting upon recent declines in honeybee populations (VanEngelsdorp *et al.*, 2013; Charles, 2013). A major contributing factor to poor honeybee health is American Foulbrood disease (AFB) (Genersch, 2010), which is caused by the pathogen, *Paenibacillus larvae*, a gram-positive, spore-forming bacterium. Extreme, destructive methods, such as burning hives and equipment, to prevent the spread of the disease coupled with the problems associated with antibiotic use for treating AFB, including increased bacterial resistance and contaminated honey, are reasons to consider research into alternate methods of treatment.

For example, phage therapy is an alternative to antibiotics in human medicine (Fischetti *et al.*, 2006), and has been proposed as an alternative for treatment of animal diseases, including AFB (Gochnauer, 1955; Gochnauer, 1970; Drobnikova and Ludvik, 1982, Popova *et al.*, 1976; Valerianov *et al.*, 1976; Dingman *et al.*, 1984; Bakheit and Stahly, 1988; Campana *et al.*, 1991). Phage therapy, or the therapeutic use of bacteriophage to target specific bacterial pathogens, would require phage that use the pathogen of AFB, *Paenibacillus larvae*, as its host. The work presented here considered the development of methods to isolate such bacteriophage, the ease of obtaining *P. larvae* phage, phage characterization and selection for further research, and finally, the efficacy of phage cocktails in treating infected larvae and an infected beehive.

Bacteriophage are extremely abundant in nature (Suttle and Fuhrman, 2010), and as such, phage capable of lysing *P. larvae* strains should exist in nature and isolation of such bacteriophage should be feasible. Isolation of phage required acquisition of samples, enrichment with *P. larvae* NRRL B 2605 to allow propagation of phage capable of lysing the pathogen,

screening of each sample for bacteriophage, isolation to obtain pure phage lysates, and amplification of isolated phage (Chapter 2). After screening 157 samples for phage capable of lysing *P. larvae*, 32 phage isolates were found (20%). Completion of the environmental screening revealed that *P. larvae* phage are abundant in easily accessible environmental sources and can be successfully isolated. Such ease of acquiring the phage would be useful to obtain more phage should future researchers decide to pursue phage therapy as an option for AFB treatment.

Chapter 3 reviewed the sources and characterization of the isolated *P. larvae* phage. The majority of the isolated phage were obtained from soil under and around beehives, as indicated by the results outlined in Chapter 3, and lytic environmental phage with broad host ranges on many *P. larvae* strains have potential for phage therapy. The soil around beehives is easily available and was a reliable source of lytic phage that lysed all or nearly all of the 11 *P. larvae* strains tested.

The lack of lysis of other genera and only one incidence of slight clearing on a *Paenibacillus* sp. indicates high host specificity. This is encouraging because it would be undesirable to cause lysis of bacterial species other than the targeted pathogen. This is also important since the natural microbiota is often necessary to maintain both bee gut health and the overall health of the hive (Olosfsson and Vásquez, 2008).

It was not known whether the 32 isolated phage represented one phage type isolated 32 times, 32 different phage, or some combination of isolation of identical phage and new ones from different samples. The phage isolated in this study were characterized by plaque morphology, host range on various bacterial species and by electron microscopy. Since host range can be affected by bacterial receptors (Lindberg, 1973), it is not a very reliable indicator of relatedness, but it is useful to choose effective phage for potential AFB treatment. DNA sequence comparisons of the phage of interest has begun and will need to be further investigated to confirm

identity and relatedness. The methods described in Appendix A outline the procedures required for isolating and purifying the phage DNA. In addition to bacterial species host range data, there is a significant difference between the susceptibility of two former subspecies of *P. larvae*, *larvae* and *pulvifaciens,* to the isolated bacteriophage. The phage isolated using as a host a former *P. larvae larvae* strain had a greater success rate in lysing bacterial strains of that former subspecies compared to the *P. larvae pulvifaciens* strains. Despite the reclassification of these two former subspecies into a single species without subspecies (Genersch *et al.*, 2006), this could indicate differences between the two former subspecies that are potentially distinct enough to reconsider classification. Even if this is not considered, it should be pointed out that in order to create a phage cocktail fully capable of efficiently infecting a broad host range of all *P. larvae* strains that cause AFB, phage isolated by enriching samples with former *P. larvae pulvifaciens* would ensure a more robust cocktail.

The characterization of the isolates in Chapter 3 led to the selection of specific phage for use in both lab and field experimental treatments. Chapter 4 describes the protocols used to determine the efficacy of the selected phage as a phage therapy treatment regimen for infected larvae and an infected hive.

An increase in survival of larvae was observed when phage were administered in conjunction with spore infection compared to spore infection alone (Chapter 4). Dilution of food with water decreased survival of larvae significantly, while dilution of food with media that contained considerable nutrients (GmBHI) decreased survival slightly but not significantly, indicating future experimental design should concentrate any food additives in order to avoid dilution.

Results from experiments feeding vegetative cells to larvae indicate that although mortality was not as high as when larvae are fed spores of the same bacterial strain, there is a significant decrease in survival compared to the negative control for at least two of the strains

tested. Additionally, the strain that did not significantly decrease larvae survival did significantly decrease adult bee body mass after pupation, indicating that although they survived, the larval development was compromised. Comparison of data from vegetative cell infections and spore infections indicate that the efficacy of spores in reducing larval survival is considerably greater. Spore infection experiments indicate a single dose of spores (# of spores) is sufficient to cause a significant decrease in survival of larvae.

Use of phage cocktails (combinations of those phage that can effectively lyse the broadest range of *P. larvae* strains) in larvae infected with *P. larvae* spores increased survival in comparison to the larvae infected with spores only, with the exception of the post-infection treatment which had a corresponding negative control with a low survival. This suggests the ingestion of phage either before or after infection with *P. larvae* spores is beneficial to the survival of the larvae. In particular, the larvae given phage cocktail #2 prior to infection maintained survival so dramatically that it was statistically insignificant from larvae survival in the phage cocktail #2 control. These experiments need to be repeated to affirm their validity.

When the opportunity to treat an infected hive presented itself, an experiment was designed around field practicality to administer a phage cocktail. Through experimentation, it was determined that lyophilization is a feasible way to preserve phage in a convenient form, and that phage retain most of their viability through this process. A high starting phage titer would ensure that the loss of phage viability seen through lyophilization would not be too great as to diminish the effectiveness of the treatment. Although complete eradication of AFB was not witnessed throughout the duration of the field treatment, the hive was reported to be clear of signs of the disease four months after the initial dose of phage was administered, and reoccurrence has not been reported. However, since the beekeeper removed the hive frames with the most severe symptoms, whether the lack of disease is due to the phage, the frame removal, or a combination of both is uncertain at this time. Additional research should be conducted to determine this

question. As such, this study should only be considered a preliminary experiment conducted as a precursor to testing on a larger scale in the future. Furthermore, only one hive was available for this experimental treatment, but should additional infected hives become available for testing, supplementary experiments should be conducted.

The body of this work describes methods to acquire pure isolates of phage capable of lysing *P. larvae*, the causative agent of AFB. Such phage, when combined, are useful to increase the survival of larvae infected with the pathogen under lab conditions, and potentially to help alleviate the disease in the field. Although more experimental field and lab treatments should be conducted, results from the experiments presented here suggest phage therapy is a viable treatment option to of American Foulbrood disease.

APPENDIX A

DNA ISOLATION, CHARACTERIZATION, AND SEQUENCE COMPARISON OF ISOLATED BACTERIOPHAGE CAPABLE OF INFECTING THE CAUSATIVE AGENT OF AMERICAN FOULBROOD DISEASE, *PAENIBACILLUS LARVAE*

FORWARD

The following methods described were those used to prepare phage isolates for DNA analysis. Preparation from raw samples was completed by Diane Yost. After DNA samples were amplified with Phi29 polymerase, they were sent to Andrew Krohn at Northern Arizona University for sequencing procedures. Raw sequence data was analyzed by Philippos Tsourkas. Results are still being fully analyzed and as such, are not presented here.

Materials and Methods

Amplification of Isolated Bacteriophage.

Previous studies were conducted to isolate bacteriophage that are capable of lysing strains of *Paenibacillus larvae* from environmental and commercial sources (Chapter 2 and 3). Based on characterizations conducted that were described in Chapter 3, 18 isolates were chosen for further DNA characterization. Bacteriophage lysates were prepared by inoculation of 50 ml log phase bacterial cultures of *Paenibacillus larvae* 2605 (NRRL) with 1 ml of individual phage isolates and shaking at 100 rpm and 37 °C overnight. Following centrifugation (3220 x g for 10 min), the lysates were filter sterilized using 0.45 um sterile cellulose nitrate filter cartridges (VWR) to remove bacterial cells and debris. This lysate was used to prepare a highly concentrated phage lysate by plating 20 identical soft agar overlay plates. *P. larvae* strain 2605 was mixed with each isolate with sufficient phage to result in complete lysis of bacterial cells. Plates were prepared

with GmBHI (0.4% Difco glucose was added to mBHI) containing 1.5% agarose and overlays were made of GmBHI with 0.95% agarose. These plates were incubated overnight at 37 °C (NapCo E Series Model 303 Incubator). Five ml of PBS pH 7.1 was added to the surface of each plate and was allowed to sit for 20 minutes. The top layer of the agarose overlay was then scraped off using a sterile pipette tip, making sure the underlying medium was not disturbed. The scraped agarose plus PBS was collected and transferred to a funnel lined with four layers of cheesecloth to remove the agarose particles. The resulting liquid was then filtered through a sterile 0.2 µm filter (Sartorus) using vacuum filtration to remove bacterial cells.

Phage DNA Precipitation and Resuspension

The filtrates described above were poured into sterile 50 mL polysulfone centrifuge tubes (Thermo Scientific) and phage were pelleted by centrifugation for 15 hours at 4 °C and 18,000 x g (Beckman J2-HS). The supernatant was carefully decanted from the phage pellet (a thin, transparent film), the tubes were inverted and then allowed to drain for 1-2 minutes but not allowed to completely dry. The phage pellet was gently resuspended in 1.0 ml of phage buffer, pH 7.5 with a composition of 10 mM Tris-HCl, 10 mM $MgSO_4$, and 68 mM NaCl (Dr. Malcom Zellars, personal communication), using a cut-off 1 ml sterile, disposable pipette tip, then removed from the centrifuge tube and transferred to a 1.5 ml microcentrifuge tube. The starting volume of approximately 100 ml was concentrated to a final volume of 3 ml and gently mixed by slowly inverting the tubes 2-3 times.

DNase Treatment

One mL of the concentrated phage lysates were put into 2 mL sterile microcentrifuge tubes (4 tubes per phage isolate). The tubes were incubated at room temperature for 15 min after the addition of 20 µL (40 units total) of DNase to each tube. During incubation, the tubes were carefully inverted 2-3 times every 5 min. The DNase was then heat inactivated by incubation at 75 °C for 20 min before placement on ice for 10 min.

Protein Coat Degradation

To the DNAse treated phage preparations (now in microcentrifuge tubes) was added 20 µL of 20 mg/mL Proteinase K per 1 ml of original lysate and incubated at 55 °C for 2 hours. The tubes were gently inverted several times every 15 min during incubation, then again after incubation prior to starting the DNA extraction procedure.

Extraction and Purification

Each bacteriophage isolate was prepared using two DNA extraction kits. Both Qiagen (DNeasy Blood and Tissue Kit, Cat# 69581) and Norgen (Phage DNA Isolation Kit, Cat# 46700) spin column kits were used according to the manufacturer's instructions. Extracted DNA was stored at -20 °C until further use.

Phi29 Polymerase Amplification

If the concentrations of purified DNA were over 10 µg/µL, the samples were directly sequenced by Illumina sequencing. If they were below 10 µg/µL, the samples were amplified using a GE Healthcare illustra Ready-To-Go GenomiPhi V3 DNA Amplification Kit by following the manufacturer's instructions.

Illumina Sequencing

Prepared purified DNA was sent to Andrew Krohn at Northern Arizona University for sequencing.

Data Analysis

Data obtained from Illumina sequencing is being fully analyzed by Philippos Tsourkas using Geneious, a software program specifically for DNA and protein sequence analysis.

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APPENDIX B

RAPID CHARACTERIZATION OF *PAENIBACILLUS* SPP. BY MATRIX-ASSISTED LASER DESORPTION/IONIZATION TIME-OF-FLIGHT MASS SPECTROMETRY (MALDI-TOF MS)

FORWARD

This appendix is a compilation of work conducted by multiple individuals: Beau M. Grothendick, Lin Zhang, Drs. Penny Amy and Todd Sandrin, and myself. I do not take credit for the research presented other than my own. My contribution to the research of this manuscript was conducted under the direction of Dr. Penny Amy and is solely the isolation and culturing of *Paenibacillus* species sent to Dr. Todd Sandrin for further analysis.

This appendix is formatted for submission to Microbiological Research.

Rapid characterization of *Paenibacillus* by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS)

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Running Head: Profiling of *Paenibacillus* with MALDI-TOF MS

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Abstract

Characterizing *Paenibacillus* species using biochemical tests and molecular methods is time-consuming and cannot effectively control the quick spread of the honey bee disease. Matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry (MS) has been successfully used to characterize many bacteria at the species, and in some cases, strain levels, suggesting that MALDI-TOF MS is a promising tool to rapidly characterize *Paenibacillus*. Thus, the aims of this work were to: 1) develop a method to characterize *Paenibacillus* using MALDI-TOF MS and 2) identify potential biomarkers that afford resolution of *Paenibacillus* at the species and strain levels. Mass spectra from 18 isolates, 12 of which were *P. larvae* strains, were collected and clustered to examine the similarity between isolates. 16S rDNA sequencing was conducted for selected isolates to compare taxonomic resolution of MALDI to PCR-based approaches. Results show that MALDI-TOF MS can generate highly reproducible mass spectra with an average reproducibility of 98.6%. Spectra clustered at the species and strain levels, with the exception of three isolates. In contrast, individual strains were not discernible based on 16S rDNA sequences. Potential biomarkers were identified in three species, including: *P. larvae*, *P. lentimorbus*, *P. polymyxa*, and one previously defined subspecies, *P. larvae pulvifaciens*. Our results suggest that MALDI-TOF MS based characterization is an effective tool to rapidly characterize *Paenibacillus*, and affords higher taxonomic resolution than traditional PCR-based methods.

Keywords: Bacterial characterization, strain, biomarkers, mass spectrometry, *Paenibacillus*, foulbrood disease

Introduction

Paenibacillus are found in a wide array of environments, including soil, water, vegetable matter, and insect larvae (Antúnez et al, 2004; Genersch, 2007; McSpadden, 2004; Rieg et al, 2010), some of which are pathogens. For example, *Paenibacillus larvae*, a gram-positive spore forming bacterium, is the causative agent of American Foulbrood disease (AFB) (Genersch 2008). AFB is a severe honey bee disease that often kills the hive brood (Lindstrom et al, 2008a, 2008b). The spores are the infectious agent of *P. larvae*, which cannot be treated with antibiotics and are resistant to heat (Hamdi et al, 2013). Once the hive is infected, the spores are not easily removed and thus they spread between hives quickly. Infected hives and beekeeping equipment are usually burned to control the spread of AFB (de Graaf et al, 2006), causing massive financial destruction to apicultural industries (Antúnez et al, 2004; Hamdi et al, 2011). Moreover, emergent strains with varied virulence and antibiotic resistance have appeared throughout history in different regions of the world (Genersch et al, 2005; Rieg et al, 2010; Tian et al, 2012). The genetic diversity of *P. larvae* strains makes the control of AFB even more difficult.

To properly diagnose AFB, many methods have been developed to identify *P. larvae* and differentiate it with respect to related *Paenibacillus* species. These methods include catalase test, REP-PCR using BOX primers, and 16S rRNA gene sequencing (Alippi et al, 1998; Genersch et al, 2006; Govan et al, 1999). These methods are effective to identify *P. larvae* at the species level and, in some cases, type the diversity of intra-species. However, they are extremely time-consuming. Honey bee larvae are fed royal jelly contaminated with *P. larvae* spores; the spores germinate within 12 hours of consumption. Obviously, these traditional biochemical and molecular methods cannot be applied in a timely manner to diagnose AFB, since none of them can be easily completed in 12 hours.

A promising technique that can be used to rapidly identify and differentiate *P. larvae* is matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) fingerprint-based

methods. In the past decade, MALDI-TOF mass spectrometry (MS) has been incrementally and successfully utilized to characterize many bacteria at the species, subspecies, and in some cases, strain levels, including *Bacillus* which is closely related to *Paenibacillus* (Diekmann et al, 2010; Mazzeo et al, 2006; Sandrin et al, 2013; Teramoto et al, 2007). Unique fingerprints in the form of mass spectra can be obtained from intact cells or the extracted proteins of pure cultures following previously published protocols (Friewald and Sauer, 2009). The preparation techniques are simple and do not require large amounts of biological materials to perform. The resulting spectra are highly reproducible and can be used to characterize, discriminate, identify, and track bacteria. The entire procedure can acquire only a few minutes, which enables the possibility of analyzing a great number of samples within a short time. Studies also suggest that MALDI-TOF fingerprinting methods have better taxonomic resolution than traditional molecular methods (Böhme et al, 2013; Fujinami et al, 2011). These advantages engendered our interest in applying MALDI-TOF MS to characterize *Paenibacillus* species. Our hypothesis was that with proper protocols, MALDI-TOF MS can rapidly and reliably characterize *Paenibacillus* species and perhaps strains.

As a result, the objectives of this study were to 1) develop and evaluate a method to characterize isolates of *Paenibacillus* using MALDI-TOF MS and 2) identify potential biomarkers that afford resolution of *Paenibacillus* isolates at the species, subspecies, and strain levels. In this work, MALDI-TOF MS was applied to 18 *Paenibacillus* isolates, including 3 species, *P. lentimorbus*, *P. polymyxa* and *P. larvae*, and two previously defined subspecies, *P. larvae larvae* and *P larvae pulvifaciens*, using a protein extraction sample preparation method. A dendrogram was built based on the resulting mass spectra to examine the relationship between species and previously defined subspecies. The resulting spectra were further analyzed for potential species and strain discriminating biomarkers. Results suggest that MALDI-TOF fingerprinting can successfully characterize *Paenibacillu*s at the species level. For the previously

defined subspecies level, results suggest that MALDI-TOF fingerprinting is successful, with the exception of a single misidentified isolate.

Materials and Methods

Paenibacillus isolates and culture conditions

A collection of 18 *Paenibacillus* isolates representing three species and two previously defined subspecies were used in this study (Table 1). Isolates were stored at -80°C in a 20% glycerol/ R2B medium. Isolates were cultured on R2A plates (Difco, BD Diagnostics, Sparks, MD, USA) at 37°C for 48 h. Single colonies were selected to inoculate R2B (Difco, BD Diagnostics, MD, USA). Inoculated broth was incubated at 37°C for 24 to 48 h (due to differing growth rates) on an orbital shaker at 200 rpm.

Sample preparation

A protein extraction sample preparation method was used (Friewald and Sauer, 2009). Cells from R2B cultures were pelleted by centrifugation (17,000 × g for 5 min) and washed with sterile double-distilled water (ddH_2O) (Millipore Corp.; Bedford, MA, USA) to remove pigment. Cells were re-suspended in sterile ddH_2O and the cell density of each suspension was adjusted to 1.0 ± 0.1 $OD_{600.}$ Each cell suspension was pelleted by centrifugation (17,000 × g for 5 min) and the supernatant was carefully removed. Cell pellets were inactivated in 450 µL of absolute ethanol (200 proof) and 150 µL sterile ddH_2O. Each sample was centrifuged (17,000 × g for 5 min) and the resulting supernatant was discarded. Five microliter of 70% formic acid (Sigma-Aldrich, St. Louis, MO, USA) and 5µL of acetonitrile (Alfa Aesar, Ward Hill, MA, USA) were mixed with the pellet by pipetting thoroughly. Each sample was centrifuged (17,000 × g for 5 min), and the supernatant was transferred into a sterile 1.5 mL microcentrifuge tube. Protein extract (0.5 µL) was pipetted onto a polished steel 96-well MALDI target plate (Bruker Daltonics) and allowed to air-dry for ten minutes. Samples were spotted on predetermined, randomly distributed locations

on the target plate and were coded numerically. After the sample had dried, it was overlaid with 0.5 μL of α-cyano-4-hydroxycinnamic acid (ACROS, Fair Lawn, NJ, USA) matrix supplemented with 2.5% trifluoroacetic acid (ACROS, Fair Lawn, NJ, USA). Each isolate was spotted in triplicate.

MALDI-TOF MS data acquisition

MALDI-TOF MS analyses were performed using a Bruker Microflex LRF MALDI-TOF mass spectrometer (Bruker Daltonics; Billerica, MA) equipped with a nitrogen laser (337 nm) under the control of FlexControl software (version 3; Bruker Daltonics). Each spectrum was obtained in a linear positive mode and calibrated externally using Angiotensin II (1046.54 Da), ACTH (1-17) (2094.427Da), ACTH (18-39) (2466.681 Da), Insulin oxidized B (3494.651 Da), Insulin (5734.518 Da), Cytochrome C (1236.974 Da), and Myoglobin (16952.306 Da). Five hundred shots were collected manually in 100 shot steps for each spot within a mass range from 2 to 20 kDa. Laser power was set to the necessary minimum for ionization of selected samples before starting the analyses. After each round of 100 shots, the operator added the spectrum to the sum buffer only if the base peak of the resulting spectrum had an intensity of approximately 1,000 a.u. or greater.

Spectrum cluster analysis

Mass spectra contained in .txt files were extracted from FlexAnalysis (version 3.0; Bruker Daltonics) and then imported into Bionumerics (version 7.1; Applied Maths). Spectra were pre-processed using the following settings: baseline subtraction (rolling disc; disc width 201 points), smoothing (Kaiser Window filter; Window size: 20, Beta: 20), peak detection (CWT Ridges algorithm; minimum wavelet scale: 2, maximum wavelet scale: 16, minimum local ridge length: 2 scale, minimum total ridge length: 12 scale, max number of ridge gaps: 3, edge enhancement), and peak filtering (S:N ratio of 10). Reproducibility of triplicate spectra within a sample was calculated as previously described (Schumaker et al, 2012). Then, the triplicate

spectra were condensed into a single composite (summary) spectrum. For cluster analysis, the composite spectra were further processed using 1% curve smoothing and the Pearson correlation coefficient between composite spectra was calculated. A dendrogram was generated using an unweighted pair group method with an arithmetic mean (UPGMA) algorithm based on the Pearson correlation coefficients. Multidimensional scaling (MDS) analysis was performed as previously described to visualize the similarity between composite spectra (Goldstein et al, 2013). Jackknife analysis was performed using maximum similarities to further quantify the rates of correct classification between species and subspecies.

16S rDNA sequencing

Genomic DNA was isolated by freezing and thawing cell cultures three consecutive times. The cells were centrifuged (17,000 × g for 5 min) and the resulting supernatant was transferred to a new sterile microcentrifuge tube. The primers used for 16S rDNA sequencing were 27f (5' AGA GTT TGA TCC TGG CTC AG 3') and 1492r (5' TAC GGT TAC CTT GTT ACG ACT T 3'). Each 20-µl reaction contained 0.5 µM of each primer, 0.2 mM of each dNTP, 1X phusion HF buffering consisting of 10mM Tris-HCL, 50mM KCL, 2.0 mM MgCL (PH 8.3), 3% DMS0, 0.02 U/µL of taq DNA polymerase, and 10.8 µL UV sterilized ddH_2O. The amplification cycle was 95°C for 5 min, followed by 30 cycles of 98°C for 10 sec, 55°C for 30 sec, and 72°C for 30 sec, and a final extension at 72°C for 10 min. PCR products were purified using a QIAquick PCR Purification Kit (QIAGEN) as recommended by the manufacturer and sent to Arizona State University DNA lab for sequencing. The sequenced products were proofread using FinchTV (Geospiza, Seattle WA). All DNA sequences were assembled and aligned in Bionumerics (version 7.1; Applied Maths). Similarity searches for sequences were performed using BLAST analysis. A pairwise alignment of about 700 base pairs of each isolate was performed (Open gap penalty: 100%, Unit gap penalty: 0%) and a tree was constructed based on alignment scores using

single linkage network creation algorithm with Jukes and Cantor correction (Gap Penalty: 2%). The root was determined using the maximum branch length.

Identification of potential biomarkers

Raw mass spectra were imported into FlexAnalysis software (version 3.0; Bruker Daltonics), and baseline subtraction was applied using the TOPHAT algorithms. Peaks were picked using the following parameters: masses from 2 to 20 kDa, minimum peak resolution at 400 Da, minimum signal to noise at 2, minimum intensity threshold at 100. The picked peaks were manually organized in a list of highest to lowest intensity. Replicate peaks (i.e., potential biomarkers) found unique and exclusively reoccurring in one species, subspecies, or strain were identified and their collective m/z was averaged. The average peak m/z was compared against the individual minimum and maximum m/z values within the exclusive species, subspecies, or strain. If the difference of values was less than or equal to ±2.0 m/z, the potential biomarker was recorded. If the difference was greater than ±2.0 m/z, or a similar peak was found outside of the specific species, subspecies, or strain, the potential biomarker was not recorded.

Results and Discussion

Paenibacillus species, for example, *P. larvae*, are pathogens that cause severe disease (AFB) of honey bees, which results in a huge economic loss in agricultural industries. MALDI-TOF MS is rapid and easy to perform, offering an alternative method to traditional PCR-based methods, which may contribute to rapidly identifying *P. larvae* and limiting the spread of AFB. As a result, the purpose of this study was to evaluate the possibility of MALDI-TOF MS rapidly characterizing the *Paenibacillus* species using a model of eighteen *Paenibacillus* isolates. These isolates were either isolated from larvae scales or purchased from culture collection centers (Table 1). The wild type isolates were identified using specific *Paenibacillus* primers (Piccini et al, 2002) (Table 1).

Method development

MALDI-TOF MS spectra were generated from protein extracts of *Paenibacillus* isolates with three technical replicates for each isolate. While intact cell methods are more favorable than protein extraction for rapid characterization, preliminary experiments using ethanol to inactivate bacterial whole cells failed. Significant colony growth was observed from treated cells streaked onto R2A plates within a period of five days (data not shown). This may be due to that *Paenibacillus* species are spore-forming bacteria and ethanol was not able to kill all the spores. Other inactivation methods, such as increasing inactivation time, using acetonitrile instead of ethanol, and using low pressure plasma, may be used in the future to facilitate the use of more rapid intact cell methods to further increase the efficiency of MALDI-TOF-based characterization of *Paenibacillus*.

Cultivation times for each environmental isolate were not uniform. Visible growth was not apparent in New Mexico strains, NW1 and NW2 (Table 1), for up to five days. *P. larvae pulvifaciens and P. polymxa* cultures consistently showed visible growth after a 24 h period, while *P. larvae larvae and P. lentimorbus* cultures varied dramatically in time required for visible growth (24h – 72h). Some studies suggest that cultivation time affects the resulting mass fingerprints and this may affect reproducibility of replicates (Šedo et al, 2013). However, environmental isolates often have heterogeneous growth rate. To minimize the effect of cultivation time on spectrum reproducibility, the average rate of each isolate's growth, which ranged from 24 h to 120 h, was charted to sync cultivation of cells for MALDI-TOF MS. Specifically, each isolate had visible turbidity after the following estimated times: NM1, NM2, WA (120 h); 16425 (96 h); *P. lentimorbus* 1, *P. lentimborbus* 2, 2605 (72 h); 3554 (48 h); *P.* polymyxa, 232, 367, 368, 747, 748, 843, 2231, 2188, 3688 (24 h).

Representative mass spectra

Previous studies indicate that manual data acquisition can generate more reproducible mass spectra than automated data acquisition (Schumaker et al, 2012). Reproducibility of mass spectra is a very important factor to evaluate the performance of MALDI-TOF MS in bacterial characterization, and high reproducibility is critically important, especially at the strain level (Sandrin et al, 2013). Thus, in this study, manual data acquisition was used to maximize the reproducibility of *Paenibacillus* mass spectra to better evaluate the ability of MALDI-TOF MS to differentiate *P. larvae* strains. The minimum reproducibility of each triplicate across all isolates was 96.4% and the maximum reproducibility was 99.9%. The averaged reproducibility of all technical replicates was 98.6% (± 0.98). The resulting reproducibility was comparable to or better than those reported in other MALDI-TOF fingerprint studies (Schumaker et al, 2012; Goldstein et al, 2013; Wunschel et al, 2005), suggesting that MALDI-TOF MS can generate highly reproducible mass spectra of *Paenibacillus* by using a protein extraction method with manual data acquisition. Our model system contained only 18 isolates. In case of large number of samples, the automated data acquisition is more favorable and applicable with automated parameter optimization.

Representative mass spectra of *P. polymyxa*, *P. lentimorbus,* and two previously defined subspecies, *P. larvae larvae* and *P. larvae pulvifaciens,* are displayed in Figure 1. Spectra produced by different *Paenibacillus* species were readily distinguished from one another based on their mass ranges and base peaks. Specifically, *P. polymyxa* yielded spectra with most peaks in the range of 2,000 – 11,200 Da (Fig. 1A); *P. lentimorbus* yielded spectra with most peaks below 9,800 Da (Fig. 1B); *P. larvae* yielded peaks in a similar mass range, 2000 – 10,300 Da, to that of *P. polymyxa*, but less peaks were shown above 10,000 Da than that of *P. polymyxa* (Fig. 1C and D). With regards to strain level characterization, *P. larvae larvae and P. larvae pulvifaciens* showed similar mass profiles at first glance, but *P. larvae pulvifaciens* has a high intensity peak

(8517.1 Da) in the mass range of 8,000 to 9,000 Da, while no peaks or very low intensity peaks were observed in this range in the mass profiles of *P larvae larvae* (Fig. 1C and D). This suggests that MALDI-TOF MS can support the strain level differentiation of *P. larvae* based on specific peaks or the potential biomarkers. With regards to the base peak, each examined was found to be unique to its taxon. At the species level, the base peaks of *P. polymyxa, and P. larvae* were 4298.8 Da and 4285.3 Da, respectively (Fig. 1). Interestingly, the base peak of *P. lentimorbus* shifted depending on the spectrum analyzed. Its base peak can either be 4289.7 Da or 5106.4 Da (Fig. 1B). This may be due to the protein expression variability during cultivation. At the strain level, the same base peak was observed for *P larvae larvae* and *P. larvae pulvifaciens* (Fig. 1 C and D). These data indicate that, in our model system, MALDI-TOF MS can rapidly distinguish *Paenibacillus* at the species and the strain levels based on the whole mass profiles and/or potential biomarker peaks.

Mass spectra cluster analysis

A dendrogram of mass spectra of *Paenibacillus* was constructed to examine the similarity between isolates. Isolates with similar spectra were grouped together as one cluster (Fig. 2). Each mass spectrum is also represented in a gel-view format (Fig. 2). As shown in Figure 2, most of the species are clearly differentiated by MALDI. Specifically, *P. larvae*, *P. lentimorbus* and the New Mexico isolates (identified as *P. lautus*) formed distinct clusters in the dendrogram (Fig. 2). The *P. polymyxa* isolate was not clustered with any of these groups, suggesting that this species can be distinguished from other *Paenibacillus* species (Fig. 2). Closely related strains, *P. larvae larvae* and *P. larvae pulvifaciens* are also separated as two clusters, suggesting that MALDI-TOF MS is potentially able to characterize *P. larvae* at the strain level (Fig. 2). However, outliers were also observed in the MS data. *P. larvae larvae* isolates 2231, 3554, 2188, 748, and 747 grouped together consistently, while *P. larvae larvae* 2605 and the Washington isolate grouped together as outliers (Fig. 2). The gel views of these two isolates show different

profiles compared with gel representations of other *P. larvae* isolates (Fig. 2). To eliminate the possibility that these two isolates were not *P. larvae* species, 16S rDNA sequencing was conducted and BLAST analysis confirmed that these two isolates belonged to *P .larvae* (Table 2). Similarly, *P. larvae pulvifaciens* isolates 367, 368, and 843 grouped together consistently, while *P. larvae pulvifaciens* 3688 grouped together with *P. larvae larvae* isolates (Fig. 2). The appearance of outlier isolates indicates that other parameters may affect the correct identification and classification of *P. larvae*. It is important to note that the clustering analysis of mass spectra does not establish phylogenetic relationships. The appearance of outliers may be due to the mass shifts of group-specific peaks resulting from amino acid exchanges in the respective proteins (Dieckmann et al, 2005). Another possible explanation is that these two isolates required longer cultivation time than other *P. larvae* isolates and lengthy cultivation times may affect strain classification (Šedo et al, 2013). In addition, studies indicate that processing criteria of mass spectra may affect bacterial identification (Ford and Burnham, 2013). Our preliminary experiments also showed that raw mass spectra processing parameters affected the clustering pattern (data not shown). Future experiments to optimize cultivation conditions and processing parameters are of great interest in our lab to further improve the taxonomic resolving power of MALDI-TOF MS for *P. larvae*.

The Jackknife method was performed to more rigorously evaluate the ability of MALDI-TOF MS to distinguish *P. larvae larvae* and *P. larvae pulvifaciens*. The principle of the Jackknife analysis is to take out one "isolate" and develop a classification function using the remaining isolates. The omitted isolate is then "classified" into one group (in our case, defined as *P. larvae larvae* or *P. larvae pulvifaciens*) using the classification function constructed. This procedure was repeated for all *P. larvae strains*, and a 100% identification score means no mismatch. The jackknife maximum similarity analysis between previously defined subspecies *larvae* and *pulvifaciens* resulted in a 100% match between *P. larvae larvae* isolates and a 75%

match between *P. larvae pulvifaciens* (Fig.2), indicating that *P. larvae pulvifaciens* 3688 consistently clustered with *P. larvae larvae* under our experimental condition.

A multi-dimensional scaled model (MDS) was used to further examine the similarity of each isolate resulting in a visual cue of differences between outliers and grouped isolates (Fig. 3). Similar to the results of cluster analysis, *P. larvae pulvifaciens* 3688 (A) can be seen clustering closer to *P. larvae larvae* isolates and farther away from other *P. larvae pulvifaciens* isolates (Fig. 3). *P. larvae* 2605 (B) and WA (C) have a large difference in space compared to both *P. larvae* subspecies, suggesting these two isolates are outliers (Fig. 3). Both New Mexico isolates have a considerable difference of space between their optimized positions, suggesting that though these two isolates clustered as one group, they show a relatively high variability in their mass profiles. In contrast, both *P. lentimorbus* isolates overlap each other's optimized positions, suggesting a high degree of similarity between the two strains (Fig. 3).

Phylogenetic analysis of 16S rDNA sequences

To compare the taxonomic resolution of MALDI-based and PCR-based approaches for *Paenibacillus* species and strains, the 16S rRNA genes of selected isolates were partially sequenced. BLAST analysis shows that all selected *P. larvae* isolates could be correctly identified at the species level (Table 2). *P. lentimorbus* was only correctly identified at the genus level (Table 2). *P. polymyxa* was identified as *Bacillus* genus (Table 2). This may be due to that *Paenibacillus* was originally included within the genus *Bacillus*. The representative (NM2) of New Mexico isolates was identified as *P. lautus* (100%), which explained the mass spectra clustering result that these two isolates clustered together but separately from other groups (Table 2, Figure 2). With regard to the strain level identification, *P. larvae larvae and P. larvae pulvifaciens* were indistinguishable from each other based on BLAST analysis (Table 2).

Thirteen representative isolates were chosen for pairwise alignment cluster analysis, and constructed into a tree using 16S rDNA sequences (628–897 bp). As shown in Figure 4, there is

no differentiation between *P. larvae larvae* and *P. larvae pulvifaciens* (Fig. 4). All *P. larvae* strains were grouped together with no marginal differences in species identity. *P. polymyxa, P. lentimorbus,* and NM2 have a greater distance from *P. larvae*. The taxonomic resolution was lower than that of MALDI-TOF mass spectrometry in all regards.

Potential Biomarkers

Since outliers were observed in the case of some *P. larvae larvae* and *P. larvae pulvifaciens* strains, we further screened entire spectra for species/strain-identifying biomarkers. Species-specific potential biomarkers were recorded for *P. polymyxa, P. lentimorbus,* and *P. larvae,* which are shown in Figure 5 and tabulated in Table 3. Specifically, *P. polymyxa* has five distinguishing potential biomarkers at 3686.0, 4298.5, 4344.7, 6607.7, and 9809.0 Da (Fig. 5A, Table 3). *P. lentimorbus* has five distinguishing potential biomarkers at 3313.3, 4290.0, 5130.7, 5963.4, and 6444.5 Da, three at relative low intensity (<1,000 a.u.) (Fig. 5B, Table 3). *P. larvae* has five distinguishing potential biomarkers at 3018.6, 4286.0, 5172.2, 7519.3, and 9614.6 Da, one at relative low intensity (<1,000 a.u.) (Fig. 5C, Table 3). The subspecies *P. larvae larvae* exhibited no distinguishing potential biomarkers (Table 3). *P. larvae larvae* has previously been reported to show a range of virulence dependent on the genotype (Genersch E, 2005), which suggests different protein profiles. This may account for the lack of subspecies specific potential biomarkers. The lack of more strain specific potential biomarkers may also be attributed to gradual shifts in mass, affected by distances in preparation time (Keys CJ et al, 2004) over several runs. The subspecies *P. larvae pulvifaciens* has five more distinguishing potential biomarkers beside the potential biomarkers of *P. larvae* at 2675.17, 6134.0, 6347.4, 7311.2, and 7398.5 Da (Figure 5D, Table 3). Four of the potential biomarkers are of relatively low intensity (<1,000 a.u.).

P. larvae pulvifaciens 3688 was an outlier. Its mass spectrum was clustered with the mass spectra of *P. larvae larvae* isolates (Fig. 2). Examination of potential biomarkers showed

that this isolate only had one distinguishing potential biomarker, 6347.4 Da, for identifying it as *P. larvae pulvifaciens*. It lacked the other potential biomarkers that have denoted 75% of all other *P. larvae pulvifaciens* isolates. The lack of potential biomarkers similar to those of *P. larvae pulvifaciens* may explain the misclassification of strain 3688 with *P. l. larvae*. With regard to the two other outliers, *P. larvae larvae* 2605 and WA, each was identified as *P. larvae larvae* using potential biomarkers.

Conclusion

The main objective of this study was to evaluate the ability of MALDI-TOF MS to characterize *Paenibacillus* at the species and strain levels. Results showed that each *Paenibacillus* species yielded highly reproducible spectra and individual characteristic traits using the protein extraction sample preparation method. Based on the spectra, isolates were clustered and differentiated according to their species and subspecies, though outliers were also observed. In contrast, 16S rDNA sequencing only identified the isolates at the genus and species level. Potential biomarkers have been examined for individual species and subspecies to further facilitate the characterization. Our results suggest that MALDI-TOF MS is much more rapid and affords a higher resolution characterization of *Paenibacillus* than PCR-based methods. To our knowledge, this is the first study to use MALDI-TOF MS to characterize *Paenibacillus* at the species, subspecies, and strain levels. Future studies to construct and optimize a more diverse *Paenibacillus* database for discriminating and identifying unknown *Paenibacillus* isolates, as well as typing and tracking *P. larvae* outbreaks are of great interest.

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Table 1 *Paenibacillus* isolates used in this study

Species	Previous subspecies classification	Designated ID	Isolation[b]	Pigmentation
P. larvae	*larvae*	747	ATCC 25747, isolated from insect, OH, USA	-
P. larvae	*larvae*	748	ATCC 25748, isolated from insect, VA, USA	-
P. larvae	*larvae*	2188[a]	Isolated from larvae scales, courtesy of Jay D Evans (USDA)	-
P. larvae	*larvae*	2231[a]	Isolated from larvae scales, courtesy of Jay D Evans (USDA)	-
P. larvae	*larvae*	2605	ATCC 9545, isolated from insect	-
P. larvae	*larvae*	3554	NRRL B-3554, isolated from diseased honeybee larvae	-
P. larvae	*larvae*	16425	Isolated from larvae scales, Belgium	-
P. larvae	*larvae*	WA[a]	Isolated from larvae scales, Washington State	-
P. larvae	*pulvifaciens*	367	ATCC 25367	+
P. larvae	*pulvifaciens*	368	ATCC 25368	+
P. larvae	*pulvifaciens*	843	ATCC 49843, isolated from honeybee larvae	+
P. larvae	*pulvifaciens*	3688	ATCC 13537, isolated from diseased honeybee	+
P. sp.	N/A	NM1	Isolated from larvae scales, New Mexico	-
P. sp.	N/A	NM2	Isolated from larvae scales, New Mexico	-
P. sp.	N/A	232	Wild strain	-
P. lentimorbus	N/A	*P. lentimorbus*1	ATCC strain	-
P. lentimorbus	N/A	*P. lentimorbus*2	ATCC strain	-
P. polymxya	N/A	*P. polymxya*	ATCC strain	-

[a] Positive with *P. larvae* specific primers
[b] Information obtained from ATCC website, ARS Culture Collection (NRRL) Database Server, and straininfo.net
N/A: not applicable/available

Table 2 Nearest relatives of selected *Paenibacillus* isolates by 16S rDNA sequencing

Designated ID	Nearest relative (Accession #) [a]	% Similarity	Representative of group
747	AB073205.1	99	*P. larvae larvae*
748	DQ07623.1	99	*P. larvae larvae*
2188	AB073205.1	99	*P. larvae larvae*
2231	AY530294.1	99	*P. larvae larvae*
3554	AB073205.1	99	*P. larvae larvae*
16425	DQ07623.1	99	*P. larvae larvae*
WA	AY530294.1	99	*P. larvae larvae*
367	AY030080.1	100	*P. larvae pulvifaciens*
843	AY530294.1	99	*P. larvae pulvifaciens*
3688	AB680856.1	99	*P. larvae pulvifaciens*
NM2[b]	FR775438.1	99	*P. lautus*
P. polymyxa	KF512664.1	100	*P. polymyxa*
*P. lentimorbus*1	AB073200.1	99	*P. lentimorbus*

[a] Based on a BLAST search of the NCBI database
[b] Forward and reverse sequences only available for identification; no assembled sequence available

Table 3 Potential biomarker peaks of *Paenibacillus*

Paenibacillus species/subspecies	Mass of potential biomarker (Da)
P. polymyxa	3686.0, 4298.5[a], 4344.7, 6607.7, 9809.0
P. lentimorbus	3313.3[b], 4290.0[a], 5130.7, 5963.4[b], 6444.5[b]
P. larvae	3018.6[b], 4286.0[a], 5172.2, 7519.3, 9614.6
P. larvae larvae	N/A
P. larvae pulvifaciens	2675.2, 6134.0, 6347.4[b], 7311.2, 7398.5

[a] Base peak in the mass spectra of the corresponding *Paenibacillus* species and subspecies
[b] Peak intensity lower than 1,000 a. u.
N/A: not available

Figure 1. Representative MALDI-TOF mass spectra of A) *Paenibacillus polymyxa*, B) *Paenibacillus lentimorbus*, C) *Paenibacilllus larvae larvae* and D) *Paenibacillus larvae pulvifaciens*

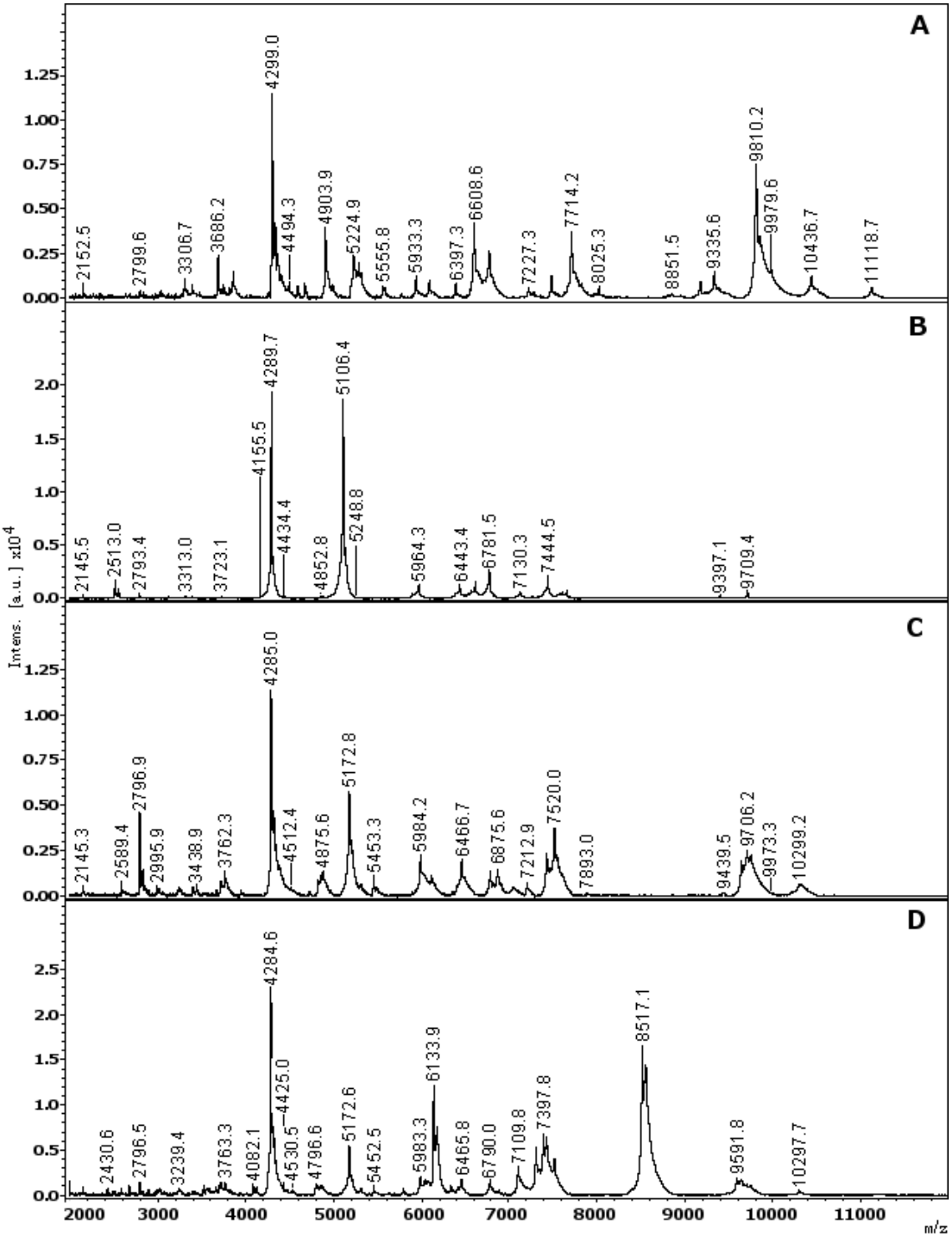


Figure 2. Dendrogram of *Paenibacillus* isolates. Each mass spectrum is represented in a gel-view format. The dendrogram was constructed using the UPGMA method from calculated distances using Pearson correlation similarity matrix in BioNumerics software. The coding colors are *P. polymyxa* (yellow), *P. lentimorbus* (light blue), *P. larvae larvae* (green), *P. larvae pulvifaciens* (red), New Mexico isolates (*P. lautus*) (purple), and *P. sp.* 232 (dark blue).

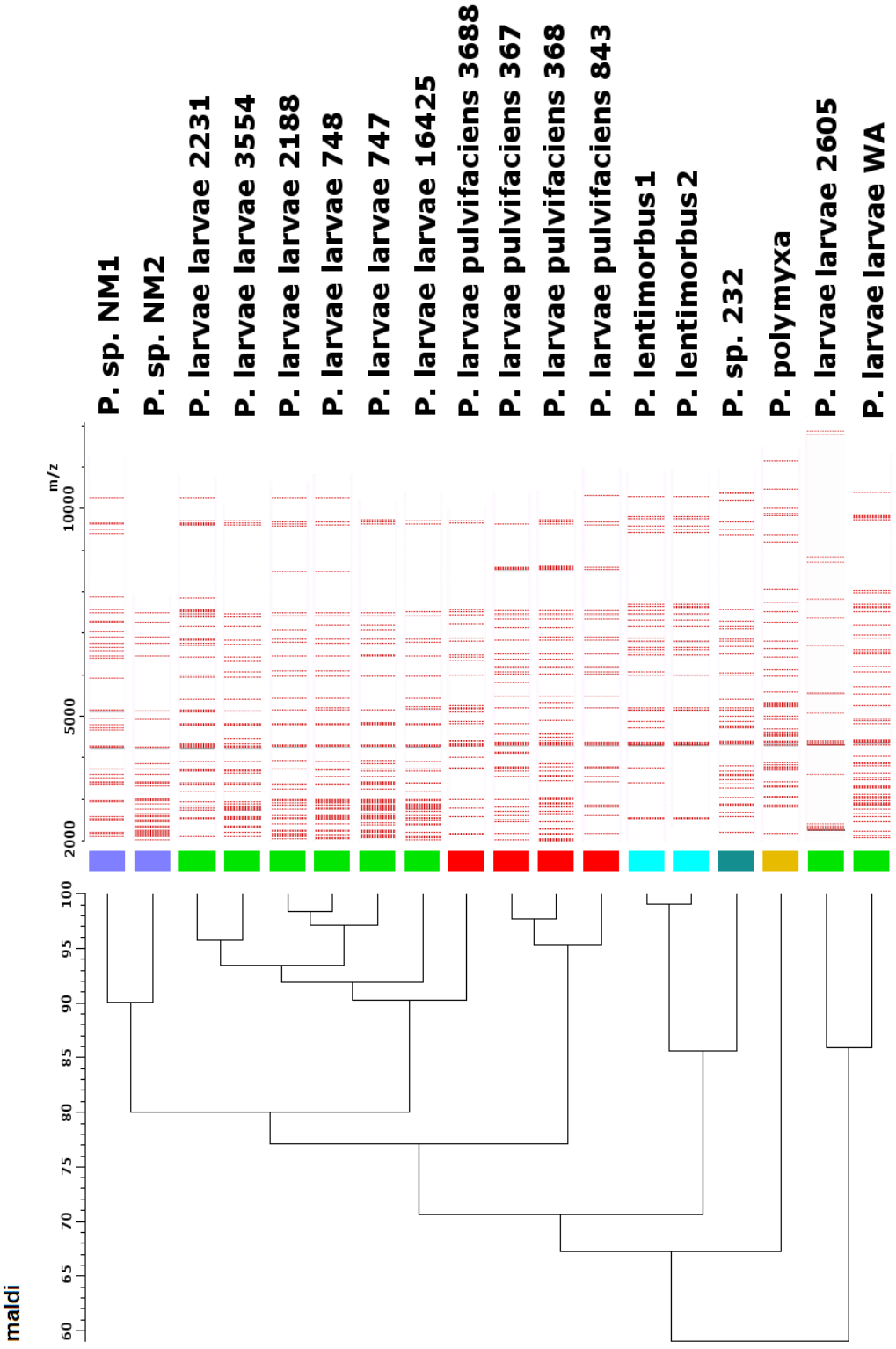


Figure 3. Multi-dimensional scaling (MDS) representation of composite-spectra. The coding colors are *P. polymyxa* (yellow), *P. lentimorbus* (light blue), *P. larvae larvae* (green), *P. larvae pulvifaciens* (red), New Mexico isolates (*P. lautus*) (purple), and *P. sp.* 232 (dark blue).

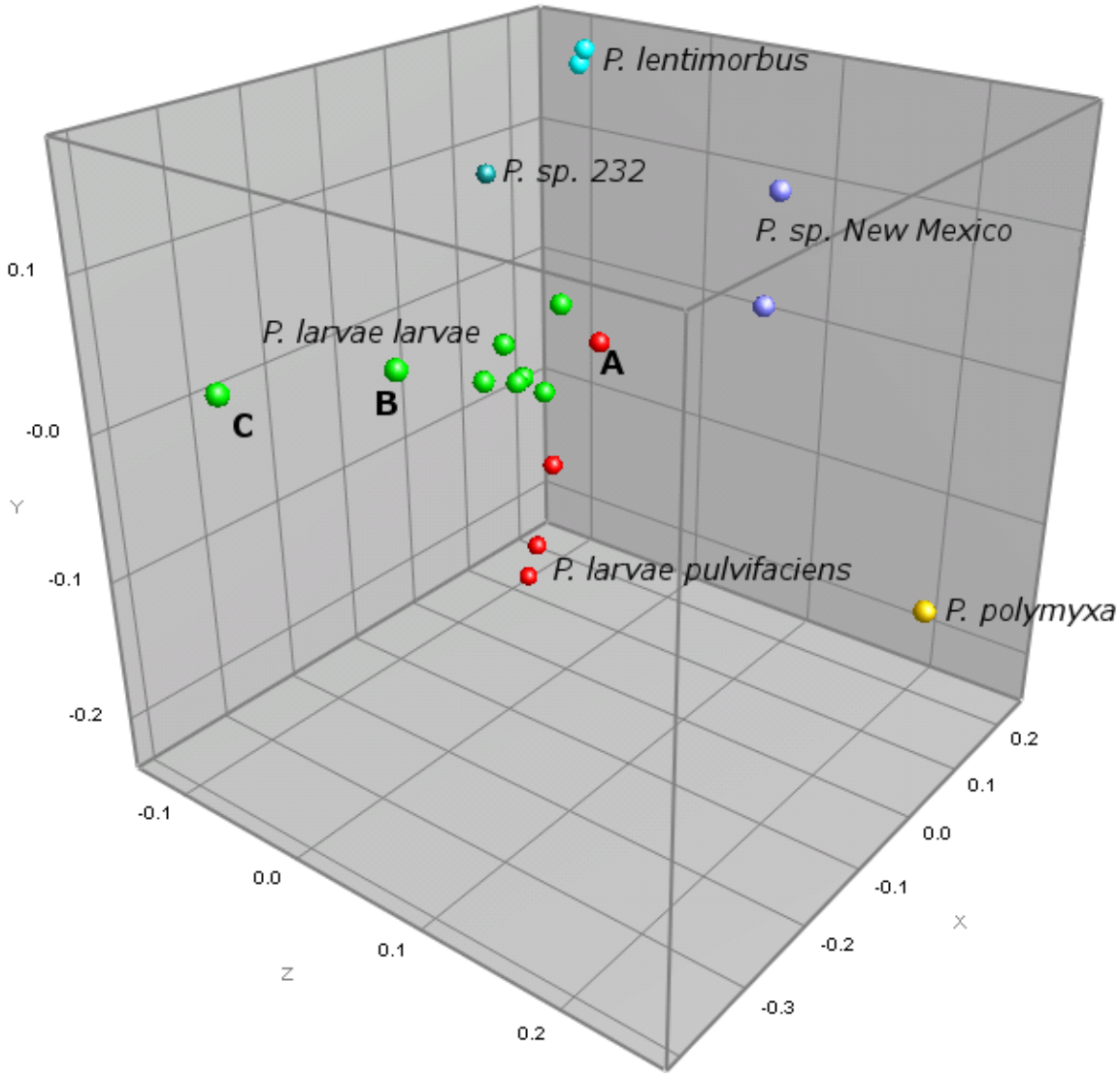


Figure 4. Phylogenetic tree of *Paenibacillus* isolates based on partial 16S rRNA gene sequences. The tree was generated using the single linkage method from calculated distances using Pairwise alignment similarity matrix in BioNumerics software. The coding colors are *P. polymyxa* (yellow), *P. lentimorbus* (light blue), *P. larvae larvae* (green), *P. larvae pulvifaciens* (red), New Mexico isolates (*P. lautus*) (purple), and *P. sp.* 232 (dark blue).

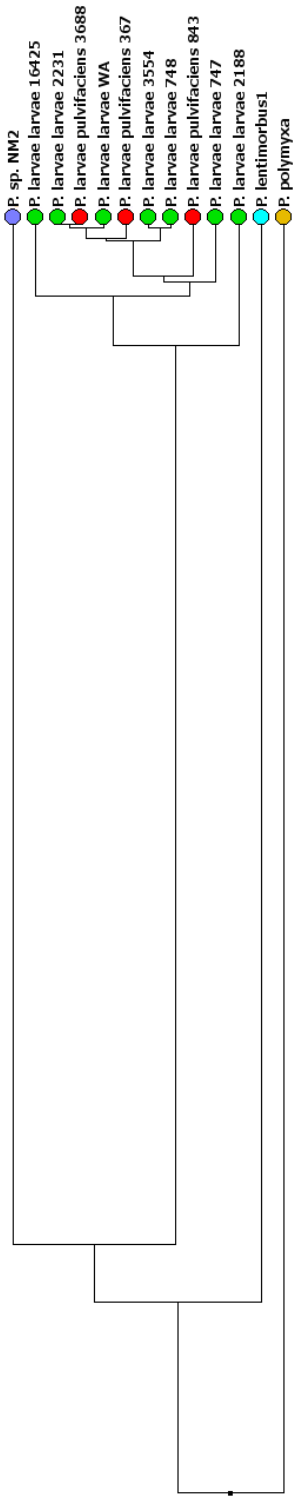
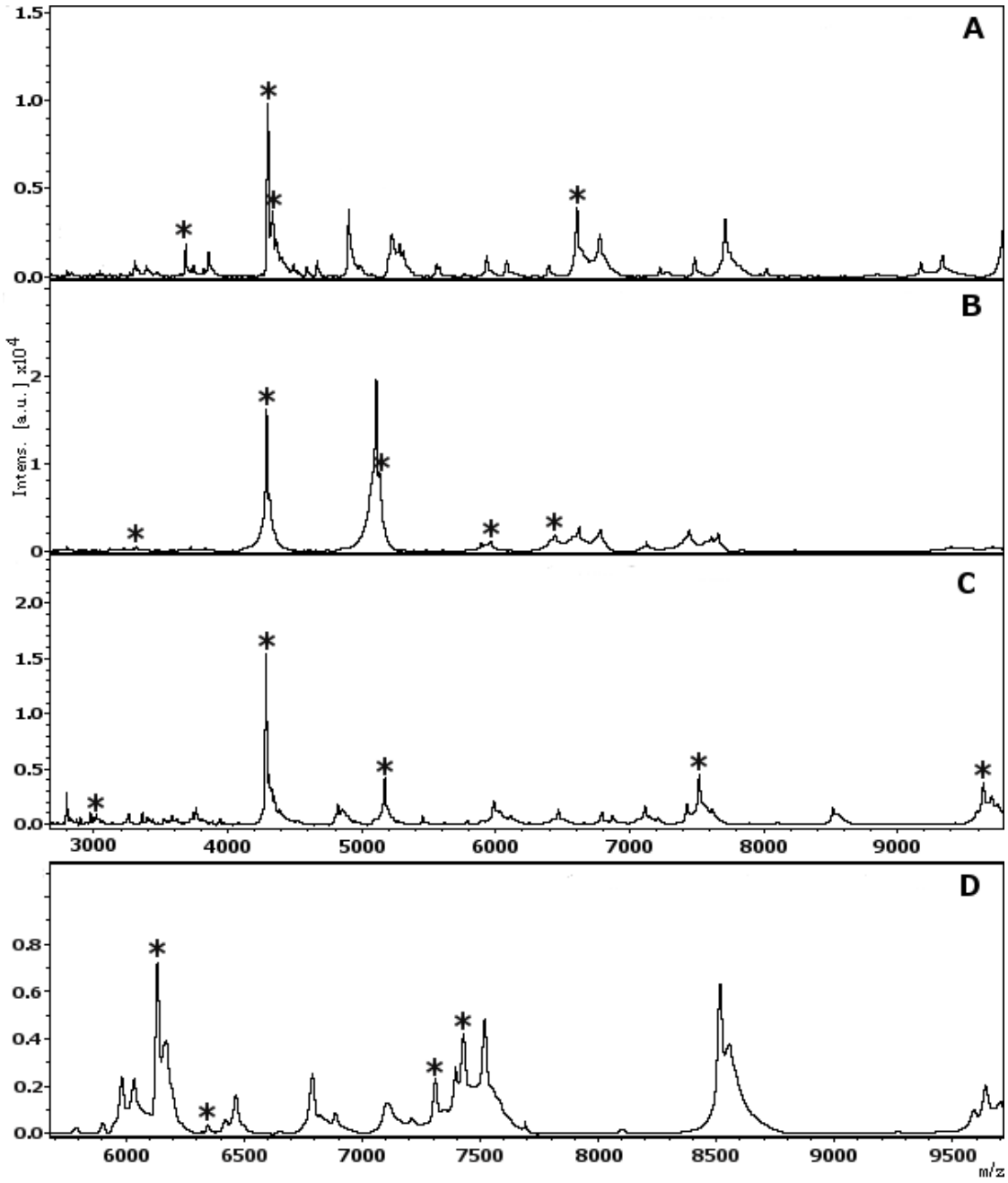


Figure 5. Species/subspecies-specific potential biomarkers (depicted with asterisks) of A) *Paenibacillus polymyxa*, B) *Paenibacillus lentimorbus*, C) *Paenibacillus larvae larvae* and D) *Paenibacillus larvae pulvifaciens*



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Yost DG and Amy PS. 2014. Sources of bacteriophages capable of infecting *Paenibacillus larvae*, the causative agent of American Foulbrood disease in *Apis mellifera*, honeybees. Applied and Environmental Microbiology. Currently in submission.

Manuscripts in Review:

Alvarado I, Khilnani JC, Yost DG, Elekonich M, Abel-Santos E, Amy PS and Wing HJ. 2014. Methods for developing treatment strategies against the honeybee pathogen *Paenibacillus larvae*, the causal agent of American Foulbrood Disease. BCM Microbiology. Currently in review.

Grothendick BM, Zhang L, Yost DG, Amy PS and Sandrin TR. 2014. Rapid characterization of *Paenibacillus* by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS). Currently in review.

Manuscripts in Preparation:

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