ABAtE: Active Bacteriophages for AFB Eradication

A thesis submitted in partial fulfilment of the requirements for

the degree of

Doctor of Philosophy

in

Genetics

at Massey University, Auckland, New Zealand

Danielle Kok

2023

Abstract

The European honey bee (*Apis mellifera*) is one of the most important livestock animals in New Zealand. Their value comes from a combination of pollination services and the production of honey for export, notably mānuka honey. American Foulbrood (AFB) is a disease of honey bee larvae and pupae and is caused by the bacterial pathogen, *Paenibacillus larvae.* AFB is the most serious disease that infects honey bees and is present in almost all countries where honey bees are found. AFB has been present in New Zealand since 1877 and spread to all parts of the country within 10 years. Unlike other countries, the use of antibiotics in hives infected with *P. larvae* is prohibited under New Zealand law and infected hives must be destroyed immediately. Bacteriophages (phages) are a well-studied alternative to antibiotics. Phages are simple viruses that kill specific bacteria and are highly abundant in the environment with an estimated 10^{31} globally. Phages have been shown to work effectively as a prophylactic to infection from certain diseases. With the growing antimicrobial resistance crisis, phages are becoming a well-studied and promising alternative to antibiotics. The aim of this research was to investigate the use of phages as a preventative measure against AFB. Previous work undertaken in other laboratories around the world has shown that phages can be isolated from healthy hives and nearby soil and that AFB pathogens are susceptible to destruction by these phages. In this work, we collected soil samples using citizen led science from hives throughout New Zealand. From soil samples provided we isolated 26 novel phages that are destructive to *P. larvae*. Selected phages were combined into a cocktail and tested against vegetative forms of *P. larvae* in in-lab testing. All phages were also sequenced and annotated and compared to other *P. larvae* phages that have been isolated around the world.

This project: ABAtE (Active Bacteriophages for AFB Elimination), provides the groundwork study for an innovative approach to naturally protecting NZ beehives against AFB.

Acknowledgements

Words cannot express my gratitude to my supervisor, Dr. Heather Hendrickson, without her unwavering patience, and support, this thesis would not have been possible. I am grateful for all of our discussions and the feedback and advice provided over the duration of my PhD journey.

Many thanks to the members of the Hendrickson Lab, past and present, for keeping me entertained during lab meetings and long hours working in the lab, particularly Dr. Mike Miller and Dr. Richard Yulo.

I would also like to express my gratitude to my colleagues at the University of Canterbury for their encouragement and moral support during the writing up of my thesis, especially Diana Zhou, Sarah Manners, Andrew McCabe and Holly Welford.

Thanks to my family, especially my parents, for their love and encouragement; their belief in me has kept me motivated during this process. Finally, special thanks to my partner Ash, for believing in me and always supporting my decisions and encouraging me to keep going.

Table of Contents

List of Figures

List of Tables

List of Abbreviations

GDP	Gross domestic product
MPI	Ministry for Primary Industries
AFB	American Foulbrood
AFBPMP	American Foulbrood Pest Management Plan
NAF PMP	National American Foulbrood Pest Management Plan
DNA	Deoxyribonucleic acid
UMF	Unique Manuka Factor
km	Kilometre
mm	Millimetre
nm	Nanometre
NZD	New Zealand Dollars
µm	Micromolar
ERIC	Enterobacterial Repetitive Intergenic Consensus
PFU	Plaque-Forming Units
mL	Millilitres
µL	Microlitres
PCR	Polymerase Chain Reaction
Kbp	Kilo-base pair
µg	Microgram
RPM	Revolutions per minute
CFU	Colony-Forming Unit
min	Minute
g	Gravitational force
bp	Base pairs
ºC	Degrees Celsius

Chapter 1

General Introduction

1.1 Overview

Apiculture is an important industry to New Zealand, due to the substantial monetary contribution of its services and exports. The imported European honey bee (*Apis mellifera*), which was first introduced to New Zealand in 1839, is the driver behind this industry. Apiculture contributes well over 5 billion dollars to New Zealand's GDP via pollination services and the export of honey bee products (Ministry for Primary Industries, 2021; Newstrom-Lloyd, 2013).

Beekeeping in New Zealand has been increasing since 2006 and now has close to 10,000 registered beekeeping enterprises (Ministry for Primary Industries, 2021). With increases in managed beehives we are also seeing increases in overwinter colony losses. Manaaki Whenua, a Crown Research Institute that cares for New Zealand's land environment and biodiversity, has been conducting Colony Loss Surveys for the last seven years and has seen a loss rate increase of 62% since 2015 (Stahlmann-Brown, 2021). Some of these losses are attributed to the parasitic Varroa mite (*Varroa destructor*), invasion of wasp species (*Vespula germanica* and *V. vulgaris*), and the bacterial pathogen, *Paenibacillus larvae*.

P. larvae is a spore-forming, gram-positive bacterium that causes the deadly disease known as American Foulbrood (AFB). AFB has been recorded in New Zealand since 1877 (Lester, 2021). The current goal of the AFB Pest Management Plan, the agency tasked with the management of AFB in New Zealand, is to control and eliminate AFB. Since 1998, when the Pest Management Plan was formed, the reported incidence of AFB in managed hives has been between 0.20% - 0.48%. Recent reports show there are approximately 0.32% of New Zealand hives infected with AFB every year and we are currently no closer to achieving the goal of

elimination (King, 2020). New Zealand has strict biosecurity laws around the management of hives infected with AFB and the use of antibiotics is strictly prohibited. Infected hives must be destroyed by incineration within seven days of discovery (AFBPMP, 2017; *Biosecurity Act 1993 No 95 (as at 01 July 2022), Public Act Contents – New Zealand Legislation*, 2022). Destruction of AFB infected bee hives has a detrimental effect on the New Zealand apiculture industry and an estimated $3.6 million in hive losses will occur in 2022/2023 (Nimmo-Bell & Associates, 2022). Another tool that could be used in the goal of eliminating AFB from New Zealand is to naturally protect hives from this pathogen.

An alternative to antibiotics, that can work in a prophylactic manner, are bacteriophages (phages). Phages are highly specific viruses that are only capable of infecting bacteria. Phages are highly abundant and there has been estimated to be 10^{31} phages on the earth at any given time (Hendrix et al., 1999). Phages specific to *P. larvae* have been studied overseas with positive results (Brady et al., 2017; Ghorbani-Nezami et al., 2015). Another alternative that has recently been studied is the use of an oral vaccine using attenuated bacteria given to queen bees to pass to her offspring (Dickel et al., 2022). Although promising, this method only prevented 30% - 50% of larval deaths in an infected hive, this would ultimately still result in infection and the incineration of the hive under New Zealand laws.

The overall goal of this project is the creation of a novel and robust cocktail of native New Zealand phages that will act as a prophylactic against AFB infection in beehives. To accomplish this, we isolated *P. larvae* isolates from around the country, discovered and characterized native New Zealand phages able to infect these isolates.

The main objectives of this thesis were:

1. Obtain and characterize a collection of representative *P. larvae* isolates from around New Zealand. Previously studied collections in New Zealand have all been destroyed; therefore new representative isolates need to be isolated. These isolates will be sequenced and preliminary annotation for anti-phage defence systems will be undertaken. This aim is contained within Chapter 3.

2. Discovery, characterization, and sequencing of 20-30 phages that kill *P. larvae*. Phage discovery will be conducted using standard methods such as direct plating and environmental enrichment using spot tests on soft agar overlay methods to detect *P. larvae* cell death. Once phages are discovered they will be purified, and high titer lysates will be produced. These lysates will be used for DNA extraction, DNA sequencing, electron microscopy, lysogeny testing and host range analysis. Completely sequenced phages will be carefully annotated. Please see Chapters 3, 5, and 6 for more details.

3. Test host ranges of phages on New Zealand *P. larvae* isolates. Each discovered phage will be tested against all isolated New Zealand *P. larvae* strains to determine phage infectivity. This is described in Chapter 3.

4. Produce a safe phage cocktail of robust *P. larvae* phages. Phages will be combined to make a cocktail that has high infectivity against a broad range of the native strains of *P. larvae*. Contained within Chapter 3

5. *In-vivo* and *in-vitro* testing of phages against *P. larvae*. Testing of phage cocktails against selected *P. larvae* strains *in vivo* using a 96-well plate method. Testing of selected cocktails on bee larvae as a prophylactic. Please see Chapters 3 and 4.

1.2 References

AFBPMP. (2017). *AFBPMP BRIEFING DOCUMENT - MPI Government 01112017*. 1–23.

Biosecurity Act 1993 No 95 (as at 01 July 2022), Public Act Contents – New Zealand Legislation. (2022, July 5). New Zealand Parliamentary Counsel Office. https://www.legislation.govt.nz/act/public/1993/0095/latest/DLM314623.html

Brady, T. S., Merrill, B. D., Hilton, J. A., Payne, A. M., Stephenson, M. B., & Hope, S. (2017). Bacteriophages as an alternative to conventional antibiotic use for the prevention or treatment of *Paenibacillus larvae* in honeybee hives. *Journal of Invertebrate Pathology*, *150*, 94–100.

Dickel, F., Bos, N. M. P., Hughes, H., Martín-Hernández, R., Higes, M., Kleiser, A., & Freitak, D. (2022). The oral vaccination with *Paenibacillus larvae* bacterin can decrease susceptibility to American Foulbrood infection in honey bees-A safety and efficacy study. *Frontiers in Veterinary Science*, *9*, 946237.

Ghorbani-Nezami, S., LeBlanc, L., Yost, D. G., & Amy, P. S. (2015). Phage Therapy is Effective in Protecting Honeybee Larvae from American Foulbrood Disease. *Journal of Insect Science* , *15*(1), 84–85.

Hendrix, R. W., Smith, M. C. M., Neil Burns, R., Ford, M. E., & Hatfull, G. F. (1999). Evolutionary relationships among diverse bacteriophages and prophages: All the world's a phage. In *Proceedings of the National Academy of Sciences* (Vol. 96, Issue 5, pp. 2192–2197). https://doi.org/10.1073/pnas.96.5.2192

King, C. (2020). American foulbrood. *Food Additives & Contaminants, Part B: Surveillance*, *47*(3), 42.

Lester, P. (2021). *Healthy Bee, Sick Bee: The Influence of Parasites, Pathogens, Predators and Pesticides on Honey Bees*. Victoria University of Wellington Press.

Ministry for Primary Industries. (2021). *2021 Apiculture Monitoring Data*. https://www.mpi.govt.nz/dmsdocument/48793-2021-Apiculture-monitoring-report-data

Newstrom-Lloyd, L. E. (2013). Pollination in New Zealand. In J. R. Dymond (Ed.), *Ecosystem services in New Zealand : conditions and trends* (pp. 408–431). Manaaki Whenua Press.

Nimmo-Bell & Associates. (2022). *Cost Benefit Analysis of the National American Foulbrood Pest Management Plan*.

Stahlmann-Brown, P. (2021). *Report on the 2021 New Zealand Colony Loss Survey*. https://www.landcareresearch.co.nz/discover-our-research/environment/sustainable-society-and-policy/nz-colony-loss-survey/2021-colony-loss-survey/

Chapter 2

Literature Review

Save our bees: bacteriophages to protect honey bees against the pathogen causing American foulbrood in New Zealand

Chapter based on the following publication:

Danielle N. Kok & **Heather L. Hendrickson** (2023) Save our bees: bacteriophages to protect honey bees against the pathogen causing American foulbrood in New Zealand, New Zealand Journal of Zoology, DOI: 10.1080/03014223.2022.2157847

Abstract

The European honey bee (*Apis mellifera)* is an important livestock animal in New Zealand. This is due to their combined pollination services and production of honey for export, notably mānuka honey. The honey bee has a long and complicated history as an imported species that has been both praised as a productive partner and accused as an invasive competitive species. Today, this well-entrenched insect pollinator has essentially been driven to extinction outside of managed apiaries by the deadly Varroa mite (*Varroa destructor*). Honey bees, like all animals, are living in a microbial world which includes beneficial microorganisms like the members of the microbiome and pathogens that cause disease. Here we review the biology and prevention of the bacterial pathogen *Paenibacillus larvae* which causes American Foulbrood (AFB). AFB is found across New Zealand and is a devastating disease of honey bees that infects and destroys developing larvae in the brood comb. The use of antibiotics or any other substance to treat or mask AFB is forbidden under New Zealand law and infected hives must be destroyed within seven days of discovery. Bacteriophages, the viruses of bacteria, are abundant in nature and research overseas has demonstrated that bacteriophages can protect hives against AFB infection when applied in advance of exposure. Whilst treating AFB is not allowed, *P. larvae* bacteriophages can be a safe and natural prophylactic for protecting honey bee colonies against AFB infection in New Zealand. Bacteriophages are a potent source of novel solutions to agricultural diseases caused by bacterial pathogens, and we are proponents of their development to protect our domesticated pollinators against AFB here in Aotearoa, New Zealand.

2.1 Introduction

The European honey bee (*Apis mellifera*) is a financially valuable pollinator of many crops worldwide (Klein et al., 2007). Today honey bees are domesticated animals and are widely accepted to be among the smallest livestock raised to produce food and other products. In addition to products such as honey, propolis, and royal jelly, the crops generated globally through pollination have an estimated annual value of $235 - $577 billion US dollars (Pires & Maués, 2020). In 2013, approximately five billion dollars of New Zealand's GDP was estimated to be attributable to honey bees through their pollination of agricultural and horticultural crops. This is likely an underestimate of their true value as it excludes the key role they play in supporting pastoral land and livestock through clover pollination (Newstrom-Lloyd, 2013). Honey bees are a versatile, inexpensive, and productive livestock and the use of these pollinators can sometimes be the easiest way to enhance pollination when other pollinators are sparse. (Elke Genersch, 2010; Morse & Calderone, 2000).

Honey bees are social insects that engage in long range foraging behaviour making their exposure to the microbial and chemical world complex. Honey bee exposures to pests, parasites, antibiotics, and herbicides are not easily controlled as individual foragers can range distances of up to 6 km (Hagler et al., 2011). Despite this, 95-99% of the adult honey bee microbiome is made up of only eight bacterial species dominated by *Lactobacillus, Gilliamella,* and *Snodgrassella* (Kwong & Moran, 2016; Moran et al., 2012). Recent studies have evaluated the effect of honey bee exposure to agricultural stresses on their microbiota. For example, the microbiomes found in colonies in areas with more human influence appeared to have lower health and higher frequencies of Enterobacteriaceae and Rhizobiaceae (Gorrochategui-Ortega et al., 2022). It is also evident that antibiotic resistance genes like *tetL* have been transferred between the gut microbiome and honey bee pathogens like *Paenibacillus larvae* (Tian et al.,

2012). Gene flow of this kind between microorganisms within a host must be front of mind as a biocontrol is being designed and implemented. The bee gut microbiome is clearly a dynamic system responding to internal and external influences, often outside of the direct control of apiarists.

The number of registered beekeepers in New Zealand is increasing and registered apiaries have risen exponentially since 2006. The devastating colony losses noted in many other places are not observed, though some overwinter losses do occur (Philip Stahlmann-Brown et al., 2022). In New Zealand there was an estimated 13.59% total colony loss over the 2021 winter, an increase of 20.2% from 2020 winter losses (P. Stahlmann-Brown & Robertson, 2021). Winter losses within 4% and 15% appear to be normal according to a study undertaken by the German bee monitoring project (Elke Genersch et al., 2010). Pathogens attacking honey bees include bacteria, viruses, parasites and fungi, with the two biggest threats to commercial honey production globally being the parasitic mite, *Varroa destructor,* which reduces hive productivity, and the bacterial pathogen *P. larvae*, the agent that causes the deadly bee disease, American foulbrood (AFB) (Elke Genersch, 2010).

The purpose of this review is to highlight the history and the present status of beekeeping in New Zealand with a special emphasis on the threat of AFB. We will also review what is currently known about *P. larvae* bacteriophages, the viruses that destroy this pathogen. To finish, we will suggest a road map for using prophylactic bacteriophages to aid the AFB eradication goal that is central to AFB management in New Zealand.

2.2 Beekeeping in New Zealand

New Zealand has 41 species of bees, 27 of which are endemic (Donovan, 2007). Most of the native bees in New Zealand are small, solitary bees that have persisted in the presence

of introduced domesticated bees (Donovan, 2007; Newstrom & Robertson, 2005; Newstrom-Lloyd, 2013). Bees in New Zealand fall into four families; the Colletidae are ground nesting solitary bees (28 species), the Halictidae are ground nesting social bees (5 species), the Megachilidae family tend to nest in plant hollows (3 species), and the Apidae family (5 species), which are the most familiar and include various bumble bees and the European honey bee (*Apis mellifera)* (Donovan, 2007).

Before Europeans arrived in New Zealand, modern beekeeping was not practised. According to the kaumātua of the Tūhoe Tuawhenua region, New Zealand's Māori people appreciated the value of pollination services and recognised the role of insects and birds in crop pollination (Tumarae-Teka & Doherty, 2015).

In March of 1839 Mary Bumby, a missionary's sister, arrived at the Mangungu Mission Station in the Hokianga in possession of the first two European honey bee (*A. mellifera*) colonies to arrive in New Zealand (Gillingham, 2008). Live hives were brought across the Tasman from Australia frequently thereafter and New Zealand was reported by one early settler to be "... in an extraordinary degree, seems adapted for bees, and large exports of honey and wax may yet be expected from it" (Barrett, 1995). In April 1842, the first recorded importation of *A. mellifera* was recorded in the South Island (Matheson & Reid, 2018). As early as 1848, William Charles Cotton produced *A Manual for New Zealand Beekeepers* (Cotton, 1987), followed on by his 1849 title *Ko nga pi*, a "treatise on bees in Māori" (Cotton, 1849).

Māori readily adopted the European honey bee once it was introduced to New Zealand. They prized the wild honey produced by the dark-coloured feral European honey bees that had spread across the country, establishing hives in the cavities of massive podocarp trees (Tumarae-Teka & Doherty, 2015). This is documented in particular for the Tuawhenua people of the Te Urewera Ranges in the south-eastern part of New Zealand's North Island

(Tumarae-Teka & Doherty, 2015). Māori also recognised the importance of avoiding honey contaminated by the honeydew produced by passion-vine hoppers (*Scolypopa australis*) feeding on the sap of the tutu tree (*Coriaria arborea),* which contains the deadly neurotoxin, tutin (Tumarae-Teka & Doherty, 2015).

Today, feral honey bee colonies do not persist in New Zealand, lasting for a year at most after a swarm leaves a managed colony (Goodwin & Taylor, 2007). This is commonly attributed to the devastation wrought by the unchecked activities of the vampire-like *Varroa destructor* mite (Donovan, 2007; Lester, 2020). *Varroa destructor* weakens larvae by feeding on their fat bodies and acts as a highly effective vector for many bee viruses such as Acute bee paralysis virus (ABPV) and Deformed wing virus (DWV). It is the impact of these viruses being transmitted by *Varroa destructor* that cause widespread colony mortality. (Traynor et al., 2020).

The introduction of possums (*Trichosurus vulpecula)*, wasp species (*Vespula germanica* and *V. vulgaris*), agricultural pesticides and pest-control toxins are also cited as possible drivers of the decline of both native and introduced feral pollinators (Tumarae-Teka & Doherty, 2015).

Māori are often credited as having been the first commercial honey beekeepers in the nation and apiculture remains an area of growth and sustainable enterprise in the Māori economy (Barrett, 1995; Malmer et al., 2019). Apiculture is an industry that allows Māori to practice Ahikāroa, 'keep the home fires burning', meaning they can develop income streams that utilise the tribal spaces to which they have a right (Tumarae-Teka & Doherty, 2015).

Apiculture has grown dramatically in the 180 years since the introduction of honey bees. It is doubtful that New Zealand's agricultural productivity would have been possible without the influx of non-native pollinators (Newstrom-Lloyd, 2013). There are now 9,891

registered beekeeping enterprises with approximately 806,140 registered beehives in New Zealand (Ministry for Primary Industries, 2021). Apiculture products in New Zealand include honey, beeswax, pollen, pollination services and the export of live bees. In 2021 approximately 12,788 tonnes of honey were exported with a value of $481 million NZD (Ministry for Primary Industries, 2021).

The growth of the industry is due in part to the recognition of the antimicrobial properties of honey produced from high concentrations of Mānuka (*Leptospermum scoparium*) pollen. Whilst all honey exhibits antibacterial properties due to hydrogen peroxide, the source of the additional antibacterial potential of Mānuka honey was identified as methylglyoxal, associated with Unique Mānuka Factor (UMF) (Adams et al., 2008, 2009). Mānuka honey has been demonstrated to have impressive activity against pathogens like *Staphylococcus aureus, in vitro*, when impregnated in bandages to treat an antibiotic-resistant infection recalcitrant to treatment (Cooper et al., 2001), and against *S. aureus, Streptococcus agalactiae* and *Pseudomonas aeruginosa* in a multispecies biofilm (Sojka et al., 2016).

2.3 American foulbrood; a global siege and New Zealand's statutes

In 1877, only thirty-eight years after honey bees were introduced into New Zealand, American Foulbrood (AFB) was first detected in managed hives (Lester, 2020). The source of the introduction event is lost to history. Ten years later, in 1887, AFB had spread throughout the country and was responsible for a 70% decrease in New Zealand's honey production (Goodwin, 2006). Beginning in 1906 the New Zealand government introduced a programme to inspect hives and burn infected colonies (Goodwin, 2005). The importation of live honey bees is strictly prohibited today to prevent additional incursions of diseases, pests, and parasites, such as European foulbrood (EFB). For the past 29 years, the Biosecurity Act 1993

has imposed strict regulations on the import of live honey bees, bee semen, bee products or beekeeping equipment (*Biosecurity Act 1993 No 95 (as at 01 July 2022), Public Act Contents – New Zealand Legislation*, 2022).

AFB is one of the most significant and detrimental honey bee diseases known today (Elke Genersch, 2010). It is wide-spread, highly contagious and if left unchecked, this disease causes destruction of the whole colony (A. M. Alippi et al., 2002; Adriana M. Alippi et al., 2004; Rauch et al., 2009). The name "Foulbrood" was coined in the 18th century due to the characteristic foul smell emanating from the infected colony. It was not until the beginning of the 20th century that foulbrood was recognized to be two separate but similar diseases with distinct etiological agents; EFB, caused by *Melissococcus plutonius,* EFB is found in most countries where honey bees are prevalent but has not been reported in many Oceania countries including New Zealand (Ellis & Munn, 2005; Forsgren, 2010) and AFB caused by *Paenibacillus larvae* (E. Genersch, 2008).

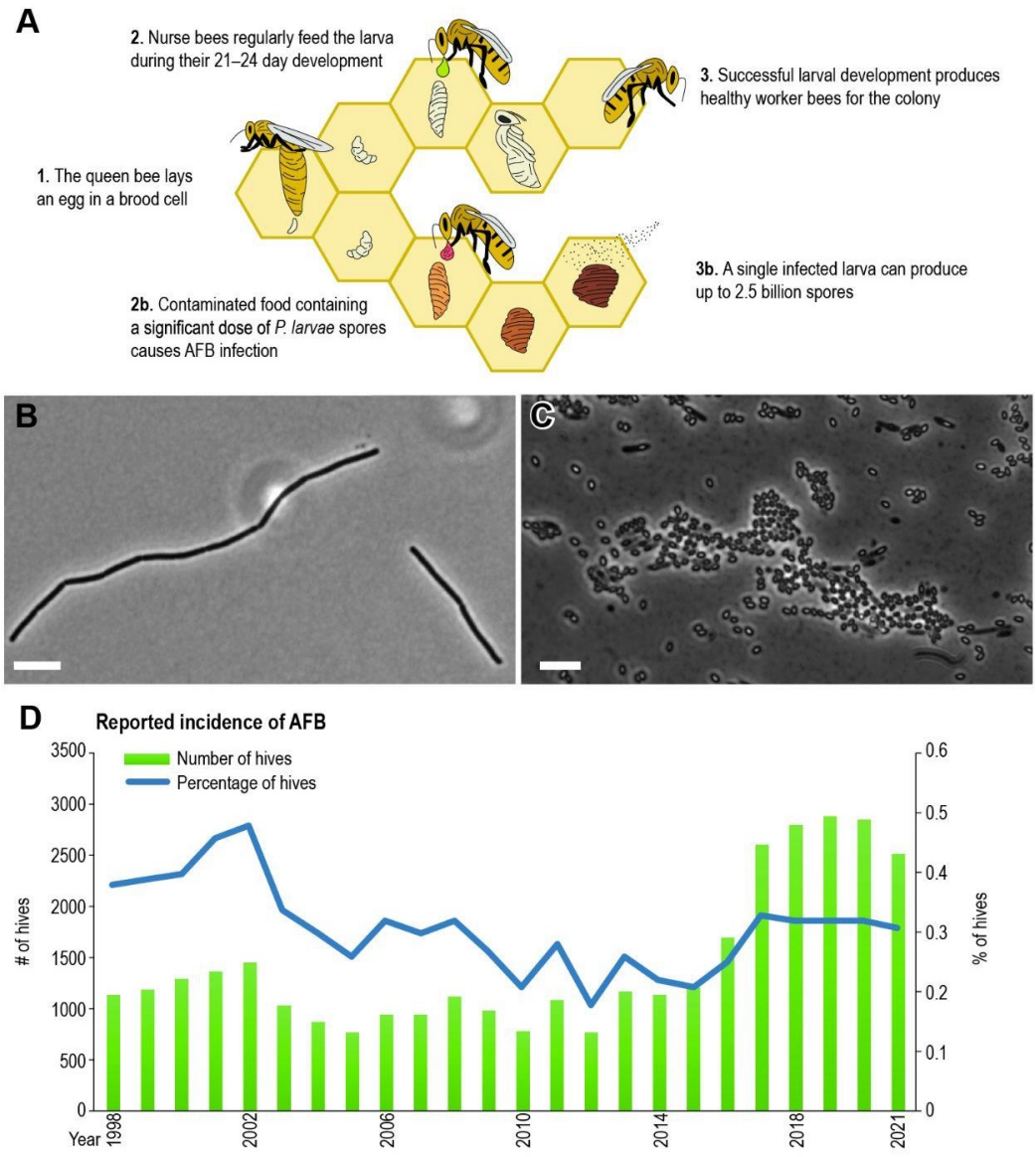


Figure 2.1 American Foulbrood and its effects in New Zealand.

A) The development of a honey bee from egg to bee (top) or through AFB infection (bottom). 1. The queen bee lays a single egg in a chamber in the comb termed a brood cell. 2. Over the course of development, nurse bees feed the developing larvae with a mixture of royal jelly, honey and bee bread or pollen. 2b. *P. larvae* spores can be fed in contaminated food. 3. Successful larval development produces a single honeybee from the brood cell after 21-24 days. 3b. An infected larva becomes a sticky brown sludge in the brood cell that produces as many as 2.5 billion spores. These are cleaned out by nurse bees and can further contaminate the hive. B) The vegetative form of the *P. larvae* bacterium. C) The spore form of the *P. larvae* bacterium (scale bars B & C 5 µm). D) The reported number of AFB found in beehives and the percentage of total hives infected in New Zealand from 1998-2021. (Source, NAF PMP, New Zealand)

As the name suggests, *P. larvae* is a pathogen that infects honey bees at the larval and pupal stages (E. Genersch, 2008) (Figure 2.1A). *P. larvae* is a slim rod-like (2.5–5 µm by 0.5–0.8 µm), gram-positive, spore-forming bacterium with slightly rounded ends (Figure 2.1B) (E. Genersch, 2008). The tough spore is the infectious form of this bacterium, and these spores are reported to be capable of germination after 35 years (Figure 2.1C) (Hansen & Brødsgaard, 1999).

P. larvae spores can reach the interior of the hive through the action of honey bees or by transfer of contaminated hive equipment between colonies (Goodwin, 2005; Hansen & Brødsgaard, 1999). The AFB infection begins when a nurse bee feeds *P. larvae* contaminated food to a larva (Elke Genersch, 2010). Larvae are most susceptible in the first 12-36 hours after hatching and it is estimated that the ingestion of as few as 10 spores is sufficient to produce a lethal infection (Elke Genersch, 2010; E. Genersch et al., 2005). Within twelve hours of ingestion, *P. larvae* spores germinate and the vegetative cells proliferate inside the midgut. Several days post-infection the larval gut is filled with pathogenic bacteria causing damage to the epithelium and resulting in extreme bacteremia and ultimately the death of the larva (Elke Genersch, 2010). Once the diseased larva has died, the *P. larvae* continue to degrade the larval remains until only a semi-fluid, brownish colloid is left, after which *P. larvae* cells sporulate (Figure 2.1A) (Elke Genersch, 2010).

The infection of a single larva produces millions of spores that can be further spread throughout the colony (E. Genersch, 2008). *P. larvae* spores are able to withstand most disinfectants, heat and antibiotics and are extremely difficult to eliminate (Haseman, 1961).

In 1998 the National American Foulbrood Pest Management Plan (NAF PMP) was established in Aotearoa New Zealand. The goal of this plan was ‘to manage AFB so as to reduce the reported incidence of AFB by an average of 5% each year’ (Biosecurity (National American

Foulbrood Pest Management Plan) Order 1998, 1998). In New Zealand, antibiotics cannot be used to treat AFB infection (AFBPMP, 2017; Biosecurity (National American Foulbrood Pest Management Plan) Order 1998, 1998), as antibiotic use can disguise symptoms of AFB infected hives (Locke et al., 2019). Infected hives must be destroyed by the use of petrol fumes and incineration within seven days of detection (AFBPMP, 2017; Biosecurity (National American Foulbrood Pest Management Plan) Order 1998, 1998).

In recent years the NAF PMP have reported the incidence of AFB to be 0.32% of hives in New Zealand (Figure 2.1D) (King, 2020). This was recently affirmed by a 2021 study completed by the Bee Pathogen Programme at MPI, which found the frequency of hives infected with AFB to be between 0.00% and 0.85%, season-dependent (Hall et al., 2021).

2.4 Genetic classification of *P. larvae* and those found in New Zealand

P. larvae can be divided into five distinct genotypes (ERIC I, II, III, IV and V) based on the results of repetitive element sequence based-PCR targeting genomic regions known as enterobacterial repetitive intergenic consensus (ERIC) (E. Genersch, 2006; Versalovic et al., 1994).

The *P. larvae* ERIC I and II genotypes have been isolated in Europe, North America, Asia and Australasia (Morrissey et al., 2014; Papić et al., 2021). *P. larvae* ERIC III & IV have only been seen in laboratory collections in recent years (Adriana M. Alippi et al., 2004; Elke Genersch, 2010; E. Genersch, 2008). The newest genotype, *P. larvae* ERIC V was recently discovered in a honey sample from Spain (Beims et al., 2020).

New Zealand has *P. larvae* ERIC I and ERIC II genotypes. Work undertaken by Morrissey *et al*. using a new typing scheme to distinguish between ERIC groups, isolated 16 strains of *P.*

larvae from New Zealand honey. Of these isolates fifteen belonged to the ERIC I sequence type and one belonged to the ERIC II sequence type (Morrissey et al., 2014).

P. larvae ERIC I & II differ in colony and spore morphology as well as virulence (E. Genersch, 2006, 2008; E. Genersch et al., 2005). Due to the hygienic nature of nurse bees there is a negative correlation between virulence at the larval stage and virulence at the colony level (Table 2.1) (Elke Genersch, 2010; Rauch et al., 2009).

The breadth of *P. larvae* in New Zealand is currently under investigation by the ApiWellbeing project, an initiative of the Ministry for Primary Industries (Ministry for Primary Industries, 2020). This project follows on from the Bee Pathogen Programme and aims to develop molecular tests for a host of honey bee pathogens and pests. The ApiWellbeing project will be cataloguing the most complete record to date of the diversity and distribution of the *P. larvae* in New Zealand. This collection and characterisation of *P. larvae* isolates will be an invaluable tool for the national goal of eradicating this pathogen.

Table 2.1 Morphological and Infectious qualities of the ERIC I and II *P. larvae* found in New Zealand (data from Rauch 2009)

ERIC Type	Colony morphology	Spore morphology	Time to larval death	Infected larval clearance rate	Virulence at larval level	Virulence at colony level
I	non-pigmented	smooth	~12 days	60%	Low	High
II	pigmented	convoluted	~7 days	90-95%	High	Low

2.5 Bacteriophages, from soil to solution

Bacteriophages are simple self-propagating viruses that only infect bacteria. The capsid and tail are made out of protein and these enclose a small, generally DNA based genome (Figure 2.2A). Bacteriophages (or phages) are among the most plentiful biological entities on Earth, with approximately 10^{31} bacteriophages in existence at any one time (Hendrix et al., 1999; Mushegian, 2020). These entities can be found in many different environments and typical discovery protocols are fairly simple to carry out (Figure 2.2B). When a bacteriophage is undergoing the lytic cycle, it can infect a bacterium and produce as many as 200 bacteriophages (Figure 2.2C). At this rate of replication, the 4^{th} cycle produces 1.6 billion new bacteriophages (Carlton, 1999). Some bacteriophages also undergo a lysogenic phase in which lysis of the bacterial cell is delayed in favour of non-lethal co-replication (Figure 2.2C). Bacteriophage replication on agar plates seeded with a lawn of bacteria in top agar produces plaques and the concentration of viable bacteriophages are therefore reported as Plaque Forming Units (PFU) /mL.

The ability of bacteriophages to infect diverse bacteria can not currently be generalised. There are examples of bacteriophages for species such as *Flavobacteria* and *Salmonella* for which host range can be highly limited (Holmfeldt et al., 2007; McLaughlin et al., 2006). By contrast, Bielke *et al*. found a pair of *Salmonella* bacteriophages, WHR 8 and 10, had the ability to infect hosts from other genera or families within the same order (Bielke et al., 2007; Grose & Casjens, 2014). Our understanding of host range is currently limited to a degree by our discovery efforts. For example, *Mycobacterium smegmatis* MC^2155, has been subjected to the largest focused discovery effort to date. Over 11,478 bacteriophages can infect this single strain and 2,011 of these have been sequenced as of this writing (Russell & Hatfull, 2017). Early investigations using this collection were used to demonstrate that

bacteriophages can be either broad or narrow in their host range and that this can and should be determined experimentally (Jacobs-Sera et al., 2012).

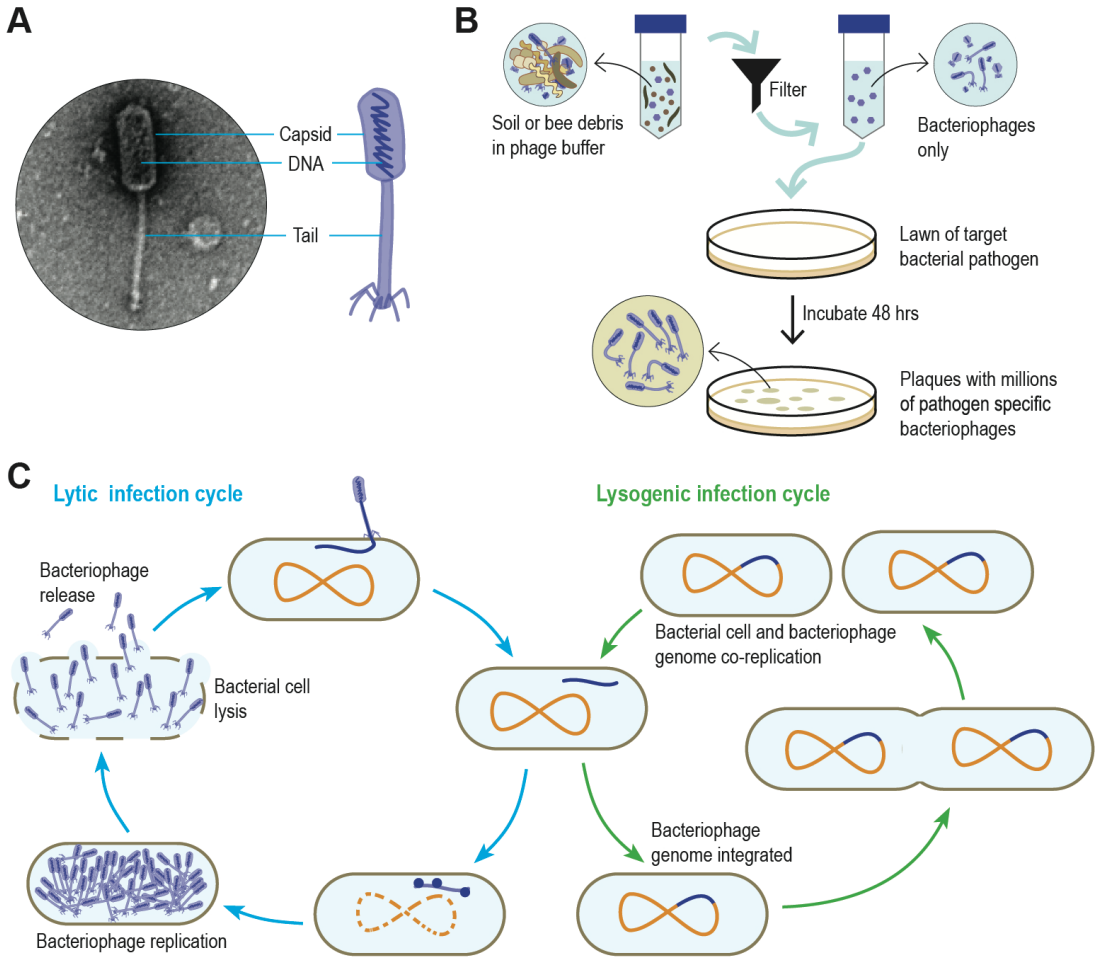


Figure 2.2 *P. larvae* bacteriophages, their discovery and life cycles.

A) A typical *P. larvae* bacteriophage. These are generally Siphoviridae with prolate capsids. B) Bacteriophages can be discovered in soil or bee debris samples by flooding the solid sample in phage buffer followed by very fine filtration (0.45 µm). Filtered particles are plated along with a lawn of *P. larvae.* After 48 hours growth plates are inspected for plaques or areas of cell death in the suspended bacterial lawn. A plaque is an indication that a bacteriophage in the filtered sample has repeatedly infected and lysed the host bacterium. C) Bacteriophages typically have one of two life cycles. Lytic infection (left) is a simple infection cycle in which a single bacteriophage produces a large number of bacteriophage particles and lyses the host cell, releasing the bacteriophages. A bacteriophage undergoing the lysogenic infection cycle (right) will typically integrate into the host chromosome or circularise its genome in the host cytoplasm and follow the replication pattern of the host cell. This can continue until an environmental signal stimulates the bacteriophage to transition to the lytic cycle (centre). Bacteriophages that are in the lysogenic cycle provide resistance to the bacterial cell against infection by that bacteriophage.

The promise of bacteriophages in combating infectious bacteria was recognised early and adopted in an application now known as bacteriophage therapy (Loc-Carrillo & Abedon, 2010; Sulakvelidze et al., 2001). Bacteriophage therapy was utilised in Georgia, Poland, and Russia at a time when the discovery of antibiotics by Alexander Fleming in 1928, was not immediately shared with Soviet Bloc nations. The therapeutic use of these viruses can be effective as either an alternative or an aid to antibiotics, particularly in cases where pathogens have evolved resistance to antibiotics or the use of antibiotics is not possible otherwise (Lin et al., 2017). In the US, many bacteriophage products are approved for use in food processing and the Food and Drug Association has given many such products a designation of 'generally recognized as safe' (GRAS) (Kahn et al., 2019). In Europe there are a growing number of bacteriophage-based products on the market such as Erwiphage manufactured in Pécs, Hungary, for treatment of fire blight (*Erwinia amylovora)* in apple trees, Biolyse manufactured in Dundee, UK, for soft rot (*Pectobacterium spp.)* on potato tubers, and Bafasal®, from Proteon in Łódź, Poland, for *Salmonella* in poultry feed among others (Fernández et al., 2018; Żbikowska et al., 2020).

All bacteria have an ancient history of biological conflict with bacteriophages and this continues when bacteriophages are put to applied use. Bacteria have a suite of anti-bacteriophage adaptations that allow them to resist bacteriophage infection. Some of these provide a general mechanism for identifying novel DNA and destroying it, restriction endonucleases fall into this class. Others are more like an adaptive immune system and an example of this is the CRISPR-Cas systems. These anti-bacteriophage defence mechanisms allow bacteria to take a snap shot of a previously infecting entity which they can then recognise and destroy during a subsequent infection. These are evidence of a prolonged

evolutionary conflict between bacteria and their viruses (Dedrick et al., 2017; Hendrix et al., 1999; Lin et al., 2017). Extant and ongoing development of resistance to bacteriophage infection is, therefore, to be expected. One strategy adopted to counter bacterial resistance is to use a mixture of bacteriophages with different properties that infect the pathogen or pathogens of interest. These 'bacteriophage cocktails' are combinations of infectious bacteriophages that have been shown to reliably reduce bacterial loads whilst safeguarding against the development of resistance (Chan et al., 2013).

2.6 Use of bacteriophages to control *P. larvae*

Previous studies, undertaken in the USA, used bacteriophages to treat and prevent infection by *P. larvae*. In New Zealand AFB infection cannot be treated and infected hives must be destroyed upon discovery (Biosecurity (National American Foulbrood Pest Management Plan) Order 1998, 1998). The following experiments conducted abroad demonstrate that it might be possible to use bacteriophages as a prophylactic treatment to prevent infection of hives.

The first bacteriophages able to infect *P. larvae* were reported in 1953 (Smirnova, 1953) and by 1999 eight *P. larvae* bacteriophages had been discovered (Drobníková & Ludvík, 1981; Gochnauer, 1970). Further work isolated and sequenced 48 bacteriophages for *P. larvae* (Stamereilers et al., 2018). This work established that *P. larvae* bacteriophages could be isolated with relative ease from soil and bee debris samples. There were even examples of bee-related cosmetics having viable bacteriophages in them, implying that these bacteriophages may be stable in propolis or honey (Yost et al., 2016).

P. larvae bacteriophages were demonstrated to be capable of protecting honey bee larvae in a challenge experiment (Yost et al., 2016). A bacteriophage cocktail was prepared

using equal aliquots of seven bacteriophages. This phage cocktail was added to larvae food prior to feeding. The larvae food with phage cocktail was administered to the larvae four hours prior to *P. larvae* spores, survival increased by approximately 59% in comparison to larvae that were only treated with *P. larvae* spores (Yost et al., 2016).

A natural experiment conducted took advantage of an apiary in which a single hive out of eleven had become infected with AFB (Brady et al., 2017). The remaining hives were divided into two groups: a sugar-water control and bacteriophage-cocktail treatment. The control group was treated with 1:1 sugar water administered either in the water trough or as a hive spray. While the phage-cocktail group was treated with sugar water mixed with phage lysate and administered in a similar manner. Hives were treated three times in the first 10 days and inspected every two weeks for eight weeks. By the fourth week, four of the negative control hives showed clear signs of the AFB disease state, while the phage-treated hives were free of disease (Brady et al., 2017).

Together these results show that both during *in vitro* and *in vivo* experiments it is possible to prophylactically protect honey bee larvae from AFB infection when a phage-cocktail is applied prior to exposure to *P. larvae.*

It is worth noting that the *P. larvae* bacteriophages discovered to date have all had genomic characteristics consistent with a lysogenic lifestyle (Figure 2.2C) (Stamereilers et al., 2018). While these were effective in challenge trials as a cocktail, lysogeny is not ideal for therapeutic use. Lysogenic bacteriophages are capable of integrating their genome into that of their host, thereby contributing genes they carry, such as antibiotic resistance or toxin genes. Once integrated these bacteriophages also render the bacteria resistant to any further attack by the same bacteriophage (Fortier & Sekulovic, 2013; Hyman, 2019). A previously isolated *P. larvae* bacteriophage, HB10c2 had been sequenced and this bacteriophage was

demonstrated to be an inappropriate therapeutic agent for *P. larvae,* as a putative protein of the beta-lactamase superfamily was identified which probably confers antibiotic resistance (Beims et al., 2015). This work suggests that genomic characterisation is important in identifying potential toxic or antibiotic resistance genes that may reside in bacteriophage genomes (Beims et al., 2015; Philipson et al., 2018).

The health of honey bees is closely tied to their gut microbiota; therefore characterising *P. larvae* bacteriophages for their ability to infect typical honey bee microbiome constituents is another consideration for cocktail design. Previously, *P. larvae* bacteriophage HB10c2 was found incapable of infecting bacteria from either the larval or adult honey bee microbiomes (Beims et al., 2015). In addition, a major lysis protein (lysin) of bacteriophage Xenia was found to be unable to lyse *Fructobacillus* or *Lactobacillus* species which are known to be found in the microbiome of larval honey bees (LeBlanc et al., 2015). Testing the effect of bacteriophage formulations on bee health will be key to ensuring that microbiomes of these important livestock are not disrupted.

The long-lasting spore form of *P. larvae* is an additional challenge in implementing bacteriophages for apiculture in New Zealand. In a recent study it was found that *P. larvae* bacteriophages are capable of reversibly binding to *P. larvae* spores (Brady et al., 2021). This discovery of bacteriophages able to remain active after binding to spores could have positive consequences for the prevention of infections by spore-forming bacteria such as *P. larvae* (Brady et al., 2021). There are examples in the literature of bacteriophages that can infect spores, such as SBP8a, a *Bacillus anthracis* bacteriophage (Fu et al., 2011). This would be a highly desirable trait for bacteriophages in a *P. larvae* directed cocktail and should be considered whilst screening candidate phages.

2.7 Bacteriophages to control *P. larvae* in New Zealand

Today, the near-total absence of feral honey bee colonies in New Zealand, geographic isolation, and strict rules regarding importing live bees and bee products has set the scene for the possibility of eradicating AFB from New Zealand's shores (Goodwin, 2006). Eradication efforts, even if broadly successful, may still be susceptible to occasional regional flare-ups as long-dormant spores are inadvertently revived. The prophylactic or preventative application of bacteriophage cocktails that are able to infect *P. larvae* is consistent with current New Zealand statutes, approved in principle by the NAF PMP, and supports the goal of eradicating this pathogen in Aotearoa (Biosecurity (National American Foulbrood Pest Management Plan) Order 1998, 1998; Goodwin, 2005; Matheson & Reid, 2018).

In order to bring *P. larvae* bacteriophage prophylactics to New Zealand we have outlined some broadly stated steps that would need to be carried out to bring this application to fruition (Figure 2.3). The groundwork has now been laid for this possibility. MPIs ApiWellbeing team are developing a collection of sequenced pathogen isolates and AFB has been kept in check through monitoring efforts in the face of exponential increases in colony numbers.

The European Honeybee is an important agricultural animal in New Zealand that supports primary production in a host of industries through pollination services in addition to its role in honey production. An eradication strategy is in place that aims to eliminate the bacterial pathogen that causes AFB, the most devastating disease of honeybees on our shores. Bacteriophages are a natural biocontrol agent that can be added to this strategy and deployed to protect honey bees against *P. larvae* by preventing AFB infections. Native bacteriophages provide a microbial tool that can safeguard honeybees and are awaiting discovery in the soil beneath our feet.

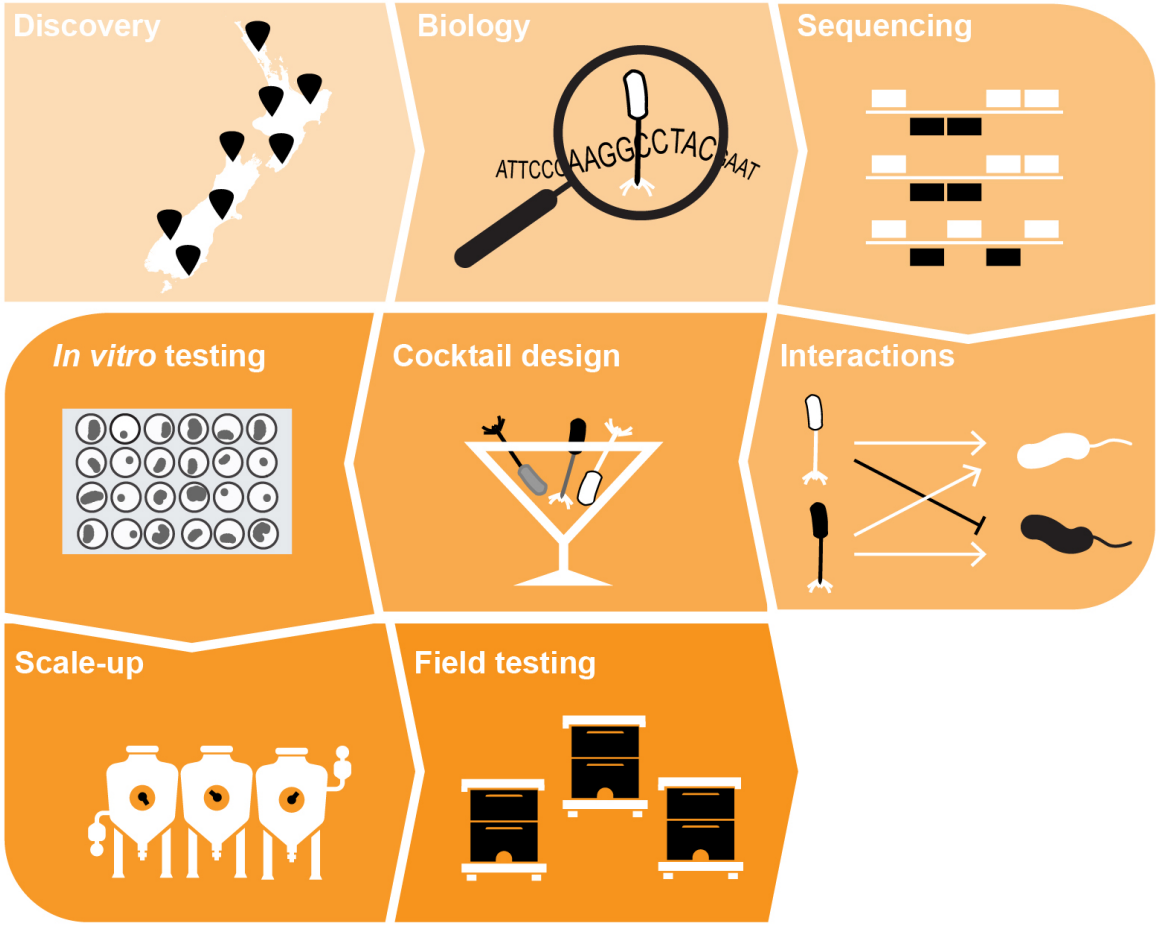


Figure 2.3 Steps to bringing *P. larvae* bacteriophage prophylactics to New Zealand Apiculture.

Discovery: *P. larvae* isolate and bacteriophage discovery. A large number of *P. larvae* bacteriophages must be discovered to construct cocktails of bacteriophages able to infect and destroy the *P. larvae* strains in New Zealand. ***Biology*****:** What is the diversity and distribution of *P. larvae* in New Zealand? Specifically, how many types of this bacteria exist here. ***Genome sequencing:*** Each bacteriophage must be sequenced to analyse the genome for the presence of toxic or antibiotic resistance genes and the propensity for lytic vs lysogenic states. ***Interactions:*** Test the host range of each bacteriophage discovered for its ability to lyse the isolated *P. larvae* pathogens. ***Cocktail design:*** Based on genome sequences and host range tests, a cocktail must be assembled that has a high probability of infecting the pathogens and built-in redundancy to deter resistance. ***In vitro testing:*** *In vitro* testing of the bacteriophage cocktail must be conducted to demonstrate the efficacy and safety of the bacteriophage cocktail. ***Scale up:*** Large volume production protocols must be established for the bacteriophages in the chosen cocktail. ***Field testing:*** Test finalised bacteriophage cocktail in the field and work with the Ministry for Primary Industries (MPI) and the Environmental Protection Authority (EPA) on biocontrol product registration or exemption from the Agricultural Compounds and Veterinary Medicines Act (1997).

2.8 References

Adams, C. J., Boult, C. H., Deadman, B. J., Farr, J. M., Grainger, M. N. C., Manley-Harris, M., & Snow, M. J. (2008). Isolation by HPLC and characterisation of the bioactive fraction of New Zealand manuka (Leptospermum scoparium) honey. *Carbohydr. Res.*, *343*(4), 651–659.

Adams, C. J., Manley-Harris, M., & Molan, P. C. (2009). The origin of methylglyoxal in New Zealand manuka (Leptospermum scoparium) honey. *Carbohydrate Research*, *344*(8), 1050–1053.

AFBPMP. (2017). *AFBPMP BRIEFING DOCUMENT - MPI Government 01112017*. 1–23.

Alippi, A. M., Lopez, A. C., & Aguilar, O. M. (2002). Differentiation of *Paenibacillus larvae subsp. larvae*, the Cause of American Foulbrood of Honeybees, by Using PCR and Restriction Fragment Analysis of Genes Encoding 16S rRNA. *Applied and Environmental Microbiology*, *68*(7), 3655–3660.

Alippi, Adriana M., Reynaldi, F. J., López, A. C., De Giusti, M. R., & Aguilar, O. M. (2004). Molecular epidemiology of *Paenibacillus larvae larvae*and incidence of American foulbrood in Argentinean honeys from Buenos Aires province. *Journal of Apicultural Research*, *43*(3), 135–143.

Barrett, P. (1995). *The immigrant bees, 1788 to 1898: a cyclopaedia on the introduction of European honeybees into Australia and New Zealand: Vol. IV*. P Barrett.

Beims, H., Bunk, B., Erler, S., Mohr, K. I., Spröer, C., Pradella, S., Günther, G., Rohde, M., von der Ohe, W., & Steinert, M. (2020). Discovery of *Paenibacillus larvae* ERIC V: Phenotypic and genomic comparison to genotypes ERIC I-IV reveal different inventories of virulence factors which correlate with epidemiological prevalences of

American Foulbrood. *International Journal of Medical Microbiology: IJMM*, *310*(2), 151394.

Beims, H., Wittmann, J., Bunk, B., Spröer, C., Rohde, C., Günther, G., Rohde, M., von der Ohe, W., & Steinert, M. (2015). Paenibacillus larvae-Directed Bacteriophage HB10c2 and Its Application in American Foulbrood-Affected Honey Bee Larvae. *Applied and Environmental Microbiology*, *81*(16), 5411–5419.

Bielke, L., Higgins, S., Donoghue, A., Donoghue, D., & Hargis, B. M. (2007). Salmonella Host Range of Bacteriophages That Infect Multiple Genera. *Poultry Science*, *86*(12), 2536–2540.

Biosecurity Act 1993 No 95 (as at 01 July 2022), Public Act Contents – New Zealand Legislation. (2022, July 5). New Zealand Parliamentary Counsel Office. https://www.legislation.govt.nz/act/public/1993/0095/latest/DLM314623.html

Biosecurity (National American Foulbrood Pest Management Plan) Order 1998, 1 (1998).

Brady, T. S., Merrill, B. D., Hilton, J. A., Payne, A. M., Stephenson, M. B., & Hope, S. (2017). Bacteriophages as an alternative to conventional antibiotic use for the prevention or treatment of *Paenibacillus larvae* in honeybee hives. *Journal of Invertebrate Pathology*, *150*, 94–100.

Brady, T. S., Roll, C. R., Walker, J. K., Fajardo, C. P., Breakwell, D. P., Eggett, D. L., & Hope, S. (2021). Phages Bind to Vegetative and Spore Forms of *Paenibacillus larvae* and to Vegetative Brevibacillus laterosporus. *Frontiers in Microbiology*, *12*, 588035.

Carlton, R. M. (1999). Phage Therapy: Past History and Future Prospects. *Archivum Immunologiae et Therapiae Experimentalis*, 267–274.

Chan, B. K., Abedon, S. T., & Loc-Carrillo, C. (2013). Phage cocktails and the future of phage therapy. *Future Microbiology*, *8*(6), 769–783.

Cooper, R. A., Molan, P. C., Krishnamoorthy, L., & Harding, K. G. (2001). Manuka honey used to heal a recalcitrant surgical wound. *European Journal of Clinical Microbiology & Infectious Diseases: Official Publication of the European Society of Clinical Microbiology*, *20*(10), 758–759.

Cotton, W. C. (1849). *Ko nga pi: me nga tikanga mo te tiaki i a ratou, mo te mahinga i to ratou honi, i ta ratou ware*. Printed at St. John's College Press.

Cotton, W. C. (1987). *A manual for New Zealand beekeepers*. Southern Reprints.

Dedrick, R. M., Jacobs-Sera, D., Bustamante, C. A. G., Garlena, R. A., Mavrich, T. N., Pope, W. H., Reyes, J. C. C., Russell, D. A., Adair, T., Alvey, R., Bonilla, J. A., Bricker, J. S., Brown, B. R., Byrnes, D., Cresawn, S. G., Davis, W. B., Dickson, L. A., Edgington, N. P., Findley, A. M., … Hatfull, G. F. (2017). Prophage-mediated defence against viral attack and viral counter-defence. *Nature Microbiology*, *2*, 16251.

Donovan, B. J. (2007). Apoidea (Insecta: Hymenoptera). In *Fauna of New Zealand*.

Drobníková, V., & Ludvík, J. (1981). Bacteriophage of *Bacillus Larvae*. *Journal of Apicultural Research*, *21*(1), 53–56.

Elke Genersch. (2010). American Foulbrood in honeybees and its causative agent, *Paenibacillus larvae*. *Journal of Invertebrate Pathology*, *103*(S), S10–S19.

Ellis, J. D., & Munn, P. A. (2005). The worldwide health status of honey bees. *Bee World*, *86*(4), 88–101.

Fernández, L., Gutiérrez, D., Rodríguez, A., & García, P. (2018). Application of Bacteriophages in the Agro-Food Sector: A Long Way Toward Approval. *Frontiers in Cellular and Infection Microbiology*, *8*, E103-5.

Forsgren, E. (2010). European foulbrood in honey bees. *Journal of Invertebrate Pathology*, *103 Suppl 1*, S5-9.

Fortier, L.-C., & Sekulovic, O. (2013). Importance of prophages to evolution and virulence of bacterial pathogens. *Virulence*, *4*(5), 354–365.

Fu, X., Walter, M. H., Paredes, A., Morais, M. C., & Liu, J. (2011). The mechanism of DNA ejection in the Bacillus anthracis spore-binding phage 8a revealed by cryo-electron tomography. *Virology*, *421*(2), 141–148.

Genersch, E. (2006). Reclassification of *Paenibacillus larvae subsp. pulvifaciens* and *Paenibacillus larvae subsp. larvae* as *Paenibacillus larvae* without subspecies differentiation. *International Journal of Systematic and Evolutionary Microbiology*, *56*(3), 501–511.

Genersch, E. (2008). *Paenibacillus larvae* and American Foulbrood – long since known and still surprising. *Journal Für Verbraucherschutz Und Lebensmittelsicherheit*, *3*(4), 429–434.

Genersch, E., Ashiralieva, A., & Fries, I. (2005). Strain- and Genotype-Specific Differences in Virulence of *Paenibacillus larvae subsp. larvae*, a Bacterial Pathogen Causing American Foulbrood Disease in Honeybees. *Applied and Environmental Microbiology*, *71*(11), 7551–7555.

Genersch, Elke, von der Ohe, W., Kaatz, H., Schroeder, A., Otten, C., Büchler, R., Berg, S., Ritter, W., Mühlen, W., Gisder, S., Meixner, M., Liebig, G., & Rosenkranz, P. (2010). The German bee monitoring project: a long term study to understand periodically high winter losses of honey bee colonies. *Apidologie*, *41*(3), 332–352.

Gillingham, A. (2008). Beekeeping. In *Te Ara - the Encyclopedia of New Zealand*. http://www.TeAra.govt.nz/en/beekeeping

Gochnauer, T. A. (1970). Some Properties of a Bacteriophage from *Bacillus larvae*. *Journal of Invertebrate Pathology*, *15*, 149–156.

Goodwin, M. (2005). American foulbrood control: the New Zealand approach. In *Bee World* (Vol. 86, Issue 2, pp. 44–45). https://doi.org/10.1080/0005772x.2005.11099653

Goodwin, M. (2006). *Elimination of American Foulbrood Without the Use of Drugs: A Practical Manual for Beekeepers*. National Beekeepers' Association of New Zealand.

Goodwin, M., & Taylor, M. A. (2007). *Control of Varroa: A Guide for New Zealand Beekeepers*. New Zealand Ministry of Agriculture and Forestry.

Gorrochategui-Ortega, J., Muñoz-Colmenero, M., Kovačić, M., Filipi, J., Puškadija, Z., Kezić, N., Parejo, M., Büchler, R., Estonba, A., & Zarraonaindia, I. (2022). A short exposure to a semi-natural habitat alleviates the honey bee hive microbial imbalance caused by agricultural stress. *Scientific Reports*, *12*(1), 18832.

Grose, J. H., & Casjens, S. R. (2014). Understanding the enormous diversity of bacteriophages_ The tailed phages that infect the bacterial family Enterobacteriaceae. *Virology*, *468–470*(C), 421–443.

Hagler, J. R., Mueller, S., Teuber, L. R., Machtley, S. A., & Van Deynze, A. (2011). Foraging range of honey bees, Apis mellifera, in alfalfa seed production fields. *Journal of Insect Science* , *11*, 144.

Hall, R. J., Pragert, H., Phiri, B. J., Fan, Q.-H., Li, X., Parnell, A., Stanislawek, W. L., McDonald, C. M., Ha, H. J., McDonald, W., & Taylor, M. (2021). Apicultural practice and disease prevalence in Apis mellifera, New Zealand: a longitudinal study. *Journal of Apicultural Research*, 1–15.

Hansen, H., & Brødsgaard, C. J. (1999). American foulbrood: a review of its biology, diagnosis and control. *Bee World*, *80*(1), 5–23.

Haseman. (1961). How long can spores of American foulbrood live. *American Bee Journal*.

Hendrix, R. W., Smith, M. C., Burns, R. N., Ford, M. E., & Hatfull, G. F. (1999). Evolutionary

relationships among diverse bacteriophages and prophages: all the world's a phage. *Proceedings of the National Academy of Sciences of the United States of America*, *96*(5), 2192–2197.

Holmfeldt, K., Middelboe, M., Nybroe, O., & Riemann, L. (2007). Large variabilities in host strain susceptibility and phage host range govern interactions between lytic marine phages and their Flavobacterium hosts. *Applied and Environmental Microbiology*, *73*(21), 6730–6739.

Hyman, P. (2019). Phages for Phage Therapy: Isolation, Characterization, and Host Range Breadth. *Pharmaceuticals* , *12*(1). https://doi.org/10.3390/ph12010035

Jacobs-Sera, D., Marinelli, L. J., Bowman, C., Broussard, G. W., Guerrero Bustamante, C., Boyle, M. M., Petrova, Z. O., Dedrick, R. M., Pope, W. H., Science Education Alliance Phage Hunters Advancing Genomics And Evolutionary Science Sea-Phages Program, Modlin, R. L., Hendrix, R. W., & Hatfull, G. F. (2012). On the nature of mycobacteriophage diversity and host preference. *Virology*, *434*(2), 187–201.

Kahn, L. H., Bergeron, G., Bourassa, M. W., Vegt, B., Gill, J., Gomes, F., Malouin, F., Opengart, K., Ritter, G. D., Singer, R. S., Storrs, C., & Topp, E. (2019). From farm management to bacteriophage therapy: strategies to reduce antibiotic use in animal agriculture. *Annals of the New York Academy of Sciences*, *1441*(1), 31–39.

King, C. (2020). American foulbrood. *Food Additives & Contaminants, Part B: Surveillance*, *47*(3), 42.

Klein, A.-M., Vaissière, B. E., Cane, J. H., Steffan-Dewenter, I., Cunningham, S. A., Kremen, C., & Tscharntke, T. (2007). Importance of pollinators in changing landscapes for world crops. *Proceedings of the Royal Society B: Biological Sciences*, *274*(1608), 303–313.

Kwong, W. K., & Moran, N. A. (2016). Gut microbial communities of social bees. *Nature*

Reviews. Microbiology, *14*(6), 374–384.

LeBlanc, L., Nezami, S., Yost, D., Tsourkas, P., & Amy, P. S. (2015). Isolation and characterization of a novel phage lysin active against *Paenibacillus larvae*, a honeybee pathogen. *Bacteriophage*, *5*(4), e1080787-16.

Lester, P. (2020). *Healthy bee, sick bee; The Influence of Parasites, Pathogens, Predators and Pesticides on Honey Bees*. Victoria University Press.

Lin, D. M., Koskella, B., & Lin, H. C. (2017). Phage therapy: An alternative to antibiotics in the age of multi-drug resistance. *World Journal of Gastrointestinal Pharmacology and Therapeutics*, *8*(3), 162–113.

Loc-Carrillo, C., & Abedon, S. T. (2010). Pros and cons of phage therapy. *Bacteriophage*, *1*(2), 111–114.

Locke, B., Low, M., & Forsgren, E. (2019). An integrated management strategy to prevent outbreaks and eliminate infection pressure of American foulbrood disease in a commercial beekeeping operation. *Preventive Veterinary Medicine*, *167*, 48–52.

Malmer, P., Tengö, M., Fernandez-Llamazares Onrubia, A., Woodward, E. R., Crawhall, N., Hill, R., Trakansuphakon, P., Athayde, S., Carino, C., Crimella, D., & Others. (2019). *Dialogue across indigenous, local and scientific knowledge systems reflecting on the IPBES assessment on pollinators, pollination and food production*. Stockholm Resilience Centre. https://swed.bio/wp-content/uploads/2019/04/7017-0033-SRC-Report-Pollinators-dialouge_WEB.pdf

Matheson, A., & Reid, M. (2018). *Practical Beekeeping in New Zealand*. Exisle Publishing Ltd.

McLaughlin, M. R., Balaa, M. F., Sims, J., & King, R. (2006). Isolation of salmonella bacteriophages from swine effluent lagoons. *Journal of Environmental Quality*, *35*(2), 522–528.

Ministry for Primary Industries. (2020, November). *ApiWellbeing*. Ministry for Primary Industries. https://www.mpi.govt.nz/biosecurity/plans-for-responding-to-serious-disease-outbreaks/bee-biosecurity/apiwellbeing/

Ministry for Primary Industries. (2021). *2021 Apiculture Monitoring Data*. https://www.mpi.govt.nz/dmsdocument/48793-2021-Apiculture-monitoring-report-data

Moran, N. A., Hansen, A. K., Powell, J. E., & Sabree, Z. L. (2012). Distinctive gut microbiota of honey bees assessed using deep sampling from individual worker bees. *PloS One*, *7*(4), e36393.

Morrissey, B. J., Helgason, T., Poppinga, L., Fünfhaus, A., Genersch, E., & Budge, G. E. (2014). Biogeography of *Paenibacillus larvae*, the causative agent of American foulbrood, using a new multilocus sequence typing scheme. *Environmental Microbiology*, *17*(4), 1414–1424.

Morse, R. A., & Calderone, N. W. (2000). The value of honey bee pollination in the United States. *Bee Culture*.

Mushegian, A. R. (2020). Are There 1031 Virus Particles on Earth, or More, or Fewer? *Journal of Bacteriology*, *202*(9). https://doi.org/10.1128/JB.00052-20

Newstrom, L., & Robertson, A. (2005). Progress in understanding pollination systems in New Zealand. *New Zealand Journal of Botany*, *43*(1), 1–59.

Newstrom-Lloyd, L. E. (2013). Pollination in New Zealand. In J. R. Dymond (Ed.), *Ecosystem services in New Zealand : conditions and trends* (pp. 408–431). Manaaki Whenua Press.

Papić, B., Diricks, M., & Kušar, D. (2021). Analysis of the Global Population Structure of *Paenibacillus larvae* and Outbreak Investigation of American Foulbrood Using a

Stable wgMLST Scheme. *Frontiers in Veterinary Science*, *8*, 582677.

Philipson, C. W., Voegtly, L. J., Lueder, M. R., Long, K. A., Rice, G. K., Frey, K. G., Biswas, B., Cer, R. Z., Hamilton, T., & Bishop-Lilly, K. A. (2018). Characterizing Phage Genomes for Therapeutic Applications. *Viruses*, *10*(4). https://doi.org/10.3390/v10040188

Pires, C. S. S., & Maués, M. M. (2020). Insect Pollinators, Major Threats and Mitigation Measures. *Neotropical Entomology*, *49*(4), 469–471.

Rauch, S., Ashiralieva, A., Hedtke, K., & Genersch, E. (2009). Negative Correlation between Individual-Insect-Level Virulence and Colony-Level Virulence of *Paenibacillus larvae*, the Etiological Agent of American Foulbrood of Honeybees. *Applied and Environmental Microbiology*, *75*(10), 3344–3347.

Russell, D. A., & Hatfull, G. F. (2017). PhagesDB: the actinobacteriophage database. *Bioinformatics* , *33*(5), 784–786.

Smirnova, N. I. (1953). Isolation and the use of a bacteriophage of *Bacillus larvae* in the diagnosis of American foulbrood. *Sbornik Nauchnykh Trudov Leningradskogo Instituta Usovershenstvovaniya Veterinarykh Vrachel*, *9*, 85–88.

Sojka, M., Valachova, I., Bucekova, M., & Majtan, J. (2016). Antibiofilm efficacy of honey and bee-derived defensin-1 on multispecies wound biofilm. *Journal of Medical Microbiology*, *65*(4), 337–344.

Stahlmann-Brown, P., & Robertson, T. (2021). *Report on the 2021 New Zealand Colony Loss Survey*. Ministry for Primary Industries. https://www.landcareresearch.co.nz/discover-our-research/environment/sustainable-society-and-policy/nz-colony-loss-survey/2020-colony-loss-survey/

Stahlmann-Brown, Philip, Hall, R. J., Pragert, H., & Robertson, T. (2022). Varroa Appears to

Drive Persistent Increases in New Zealand Colony Losses. *Insects*, *13*(7). https://doi.org/10.3390/insects13070589

Stamereilers, C., Fajardo, C., Walker, J., Mendez, K., Castro-Nallar, E., Grose, J., Hope, S., & Tsourkas, P. (2018). Genomic Analysis of 48 *Paenibacillus larvae* Bacteriophages. *Viruses*, *10*(7), 377–329.

Sulakvelidze, A., Alavidze, Z., & Morris, J. G. (2001). Bacteriophage therapy. *Antimicrobial Agents and Chemotherapy*, *45*(3), 649–659.

Tian, B., Fadhil, N. H., Elijah Powell, J., Kwong, W. K., & Moran, N. A. (2012). Long-Term Exposure to Antibiotics Has Caused Accumulation of Resistance Determinants in the Gut Microbiota of Honeybees. In *mBio* (Vol. 3, Issue 6). https://doi.org/10.1128/mbio.00377-12

Traynor, K. S., Mondet, F., de Miranda, J. R., Techer, M., Kowallik, V., Oddie, M. A. Y., Chantawannakul, P., & McAfee, A. (2020). Varroa destructor: A Complex Parasite, Crippling Honey Bees Worldwide. *Trends in Parasitology*, *36*(7), 592–606.

Tumarae-Teka, K., & Doherty, J. (2015). Tūhoe Tuawhenua (Māori, New Zealand) knowledge of pollination and pollinators associated with food production. In L. P. E. P. M. C. da Cunha and M. Roué (eds.). (Ed.), *Indigenous and Local Knowledge about Pollination and Pollinators associated with Food Production: Outcomes from the Global Dialogue Workshop (Panama 1-5 December 2014)* (pp. 27–37). UNESCO: Paris.

Versalovic, J., Schneider, M., De Bruijn, F. J., & Lupski, J. R. (1994). rep-PRC methods. *Methods in Molecular and Cellular Biology*, 25–40.

Yost, D. G., Tsourkas, P., & Amy, P. S. (2016). Experimental bacteriophage treatment of honeybees \(Apis mellifera\) infected with *Paenibacillus larvae,* the causative agent of American Foulbrood Disease. *Bacteriophage*, *6*(1), 1–15.

Żbikowska, K., Michalczuk, M., & Dolka, B. (2020). The Use of Bacteriophages in the Poultry Industry. *Animals : An Open Access Journal from MDPI*, *10*(5). https://doi.org/10.3390/ani10050872

Chapter 3

Isolation of *Paenibacillus larvae* bacteriophages from New Zealand and their potential to protect honey bees against American Foulbrood (AFB)

Abstract

American Foulbrood (AFB) is a devastating disease of the European honey bee (*Apis mellifera)* and is found throughout the world. AFB is caused by the bacterium *Paenibacillus larvae*. Treatment with antibiotics is strictly forbidden in many regions, including New Zealand. Safe and natural prophylactic solutions to protect honey bees from AFB are needed. Bacteriophages (phages) are a well-studied alternative to antibiotics and have been shown to be effective against *P. larvae* in other countries. We employed a citizen science approach to obtaining samples from around New Zealand to discover novel phages. Herein, we describe the discovery and isolation of eight *P. larvae* bacterial isolates and 26 *P. larvae* phages that are novel and native to New Zealand. We test the host ranges of the phages and formulate cocktails to undertake *in-vitro* testing on a set of representative bacterial strains. These results form the basis of a promising solution for protecting honey bees in New Zealand from AFB.

3.1 Introduction

The European honey bee (*Apis mellifera*) is a valuable livestock animal globally. In New Zealand, this value comes from their role in the pollination of horticultural and agricultural crops, which contributes over five billion dollars to New Zealand's GDP per annum based on 2013 (Newstrom-Lloyd, 2013). The export of apiculture products, including honey, beeswax and live bees, contributes a further $483 million NZD p.a. (Ministry for Primary Industries, 2021). Since 2006, New Zealand has seen a steep increase in the number of beekeepers and apiaries; with these rising numbers, there has also been a rising number of colony losses observed (Ministry for Primary Industries, 2021).

Honey bees are under constant attack by abiotic and biotic factors, including but not limited to herbicides, pesticides, parasites, viruses and bacteria (Li et al., 2018). The two biggest biotic threats to honey bees today are the parasitic Varroa mite (*Varroa destructor*) and American foulbrood (AFB), which is caused by the spore-forming bacterial pathogen *Paenibacillus larvae*. AFB is a serious and destructive disease that attacks honey bees while in their larval and pupal stages (Elke Genersch, 2010; Genersch, 2008). AFB has detrimental consequences at both the larval and colony level (A. M. Alippi et al., 2002; Adriana M. Alippi et al., 2004; Rauch et al., 2009).

AFB has been present in New Zealand for approximately 146 years, after first being discovered in 1877 (Kok & Hendrickson, 2023; Lester, 2021). By 1887 AFB had caused significant damage around the country and led to a 70% reduction in honey production (Goodwin, 2005). The use of antibiotics to treat or mask an AFB infection in New Zealand is strictly prohibited (AFBPMP, 2017; Biosecurity (National American Foulbrood Pest

Management Plan) Order 1998, 1998). Current legislation stipulates beekeepers must destroy hives infected with AFB within seven days of discovery, using petrol fumes and incineration to ensure all traces of AFB are removed (AFBPMP, 2017; Biosecurity (National American Foulbrood Pest Management Plan) Order 1998, 1998). This method is costly to both the beekeeping community and the New Zealand economy.

A potential solution to AFB infection in New Zealand is the prophylactic application of bacteriophages in a phage cocktail. Bacteriophages, or phages informally, are self-propagating viruses that are only able to infect and replicate within bacteria. Phages are ubiquitous and are the most numerous biological entity on Earth, with at least 10^{31} phages in existence globally at any point in time (Hendrix et al., 1999; Mushegian, 2020).

Work undertaken overseas has shown it is possible to protect honey bee larvae from AFB infection by the application of phage cocktails both *in vivo* (Brady et al., 2017) and *in vitro* (Yost et al., 2016). Due to strict biosecurity laws, it is necessary to isolate *P. larvae*-specific phages native to New Zealand. It is doubtful that *P. larvae* phages from elsewhere would be viewed favourably by domestic apiarists or our overseas markets.

In this study, we develop a collection of novel *P. larvae* bacterial isolates and use these to discover *P. larvae* phages native to New Zealand. We report phage host range and design and test phage cocktails as well as *in-vitro* testing of several phage cocktails. This work forms the groundwork to develop an approach to protecting beehives using New Zealand native phages that can be applied to protect hives against infection by a devastating bacterial pathogen that is affecting this industry globally.

3.2 Results

3.2.1. Isolating P. larvae from infected colony material

Previous work suggested that a curated collection of *P. larvae* isolates from New Zealand had been characterised (Graham, 2014). Further investigation revealed that the existing collection had been destroyed (P. Lester private communication). Therefore, a new collection of representative *P. larvae* strains was needed. AsureQuality, a New Zealand government-approved testing facility, provided us with swabs of brood frames or infected larvae material and whole brood frames from beehives suspected of AFB infection. Potential isolates were cultured on semi-selective MYPGP agar plates in order to obtain single colonies. Ultimately, eight *P. larvae* strains were isolated from around New Zealand (Figure 3.1A, Table 3.1). *P. larvae* is a filamentous (2.5–5 µm by 0.5–0.8 µm), spore-forming, gram-positive bacterium (Figure 3.1B, 3.1C) (Genersch, 2008). Isolates were confirmed to be *P. larvae* by positive amplification with 16s rRNA PCR primers (Dobbelaere et al., 2001).

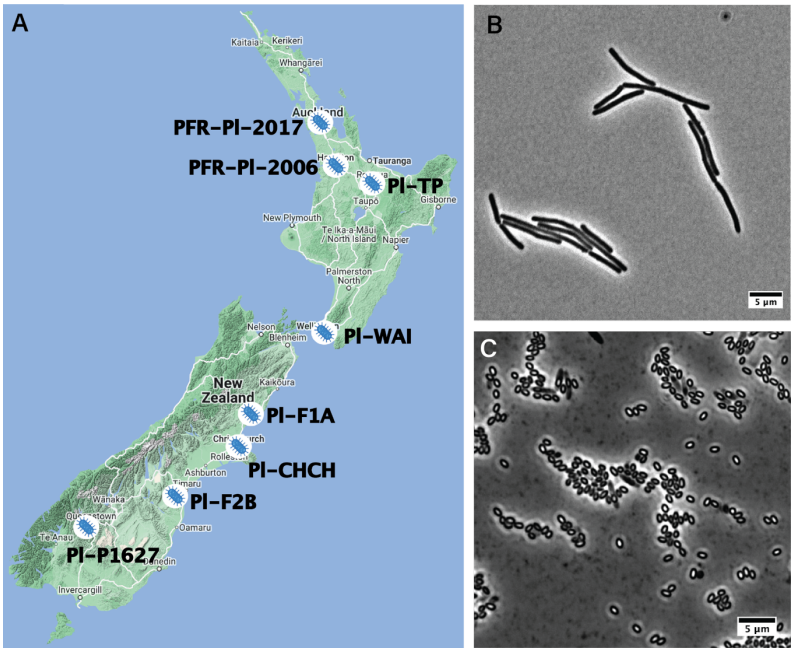


Figure 3.1 *Paenibacillus larvae* bacterial strains

A) Locations of the eight isolated *P. larvae* bacterial strains. B) Vegetative form of *P. larvae* PFR-PI-2006. C) Spore form of *P. larvae* PFR-PI-2006. Scale bar = 5 µm

DNA was extracted from each of the bacterial isolates and submitted for genome sequencing. Genomes were assembled using SPAdes 3.15.3 (Bankevich et al., 2012; Prjibelski et al., 2020) and then annotated using either RAST 1.073 (Aziz et al., 2008; Brettin et al., 2015; Overbeek et al., 2014) or Prokka 1.14.5 (Seemann, 2014). The resulting genome assemblies had between 157 to 219 contigs, which varied in size from 0.12 to 218 Kbp. *P. larvae* strains had GC contents of 44.0% to 44.2% (Table 3.1), this is comparable to several *P. larvae* reference genomes (Douglas W. Dingman, 2017; Djukic et al., 2014; Peréz de la Rosa et al., 2015).

Multilocus sequence typing (MLST) was undertaken using PubMLST (Jolley et al., 2018). MLST for *P. larvae* consist of the following seven housekeeping genes: *ftsA* (cell division protein), *clpC* (catabolite control protein A), *glpT* (glycerol-3-phosphate permease), *glpF* (glycerol uptake facilitator protein), *rpoB* (RNA polymerase beta subunit), *Natrans* (forward sodium dependant transporter), and *sigF* (sporulation sigma factor F) as these offered the most diversity between genomes tested (Morrissey et al., 2014). Seven of the New Zealand isolates belonged to the 18 MLST ST and one belonged to 23 MLST ST (Table 3.1). MLST can also be used to distinguish between ERIC type I and ERIC type II groups, MLST 18 belongs to the ERIC type I group (Morrissey et al., 2014; Papić et al., 2021). MLST 23 has not been ERIC typed in the literature to date.

Table 3.1. *Paenibacillus larvae* bacterial strains isolated from New Zealand

P. larvae strain	Isolation Location	MLST ST	GC%	No. of Contigs	Size Range Contigs (Kbp)	Accession No.
Pl-WAI	Wellington	18	44.2%	219	0.128-218	JARDRH000000000
Pl-TP	Rotorua	18	44.1%	157	0.128-250	JARDRJ000000000
Pl-CHCH	Christchurch	18	44.1%	163	0.128-218	JARDRI000000000
PFR-Pl-2017	Auckland	18	44.1%	176	0.128-218	JARDRG000000000
PFR-Pl-2006	Hamilton	18	44.1%	185	0.128-218	JARDAI000000000
Pl-F1A	North Canterbury	18	44.1%	175	0.5-191	JARDRL000000000
Pl-F2B	South Canterbury	18	44.0%	167	0.5-191	JARDRM000000000
Pl-P1627	Queenstown	23	44.1%	171	0.5-195	JARDRK000000000

We used CRISPRFinder (Grissa et al., 2007) to look for detectable CRISPR Systems and prophages in these eight isolates. Seven of the isolates contained four CRISPR arrays and one isolate contained five. The total number of spacers within the CRISPR arrays for each isolate varied from 15-25 spacers (Table 3.2). Across all eight isolates, 29 unique spacers were observed, Pl-P1627 contained 12 unique spacers that weren't found in any of the other isolates.

We also used DefenseFinder (Abby et al., 2014; Tesson et al., 2022), to search for known anti-phage systems in our bacterial strains. All eight isolates contained the same seven anti-phage systems: both a type I and II restriction-modification system (P. H. Oliveira et al., 2014), a Gao_let system (Gao et al., 2020), two Cas systems (CAS_Class1-Subtype-III-B and CAS_Class1-Subtype-I-B) (Bernheim et al., 2020), a Wadjet_III system (Doron et al., 2018), and a Mokosh_TypeII system (Millman et al., 2022).

Finally, we used Phaster (Arndt et al., 2016; Zhou et al., 2011) to identify prophages contained within the genomes. Phaster designates prophages as either intact, questionable

or incomplete. All isolates contained at least one intact prophage with six containing two intact prophages. The intact prophages were comparable to *P. larvae* Phage Harrison and Phage Vegas (Tsourkas et al., 2015). All genomes also contained 3-4 questionable prophages and 6-9 incomplete prophages (Table 3.3).

Table 3.2 CRISPR Array and Spacer details of the eight *P. larvae* isolates

***P. larvae* strain**	**No. of CRISPR Arrays**	**No. of Spacers**	**No. of Unique Spacers**
Pl-WAI	4	15	0
Pl-TP	4	17	0
Pl-CHCH	4	17	0
PFR-Pl-2017	4	17	0
PFR-Pl-2006	4	16	0
Pl-F1A	4	17	0
Pl-F2B	4	17	0
Pl-P1627	5	25	12

Table 3.3 The number of intact, questionable and incomplete prophages found in the eight *P. larvae* isolates

***P. larvae* strain**	**No. of Prophages**				
	Total	**Intact**	**Name of Intact Phage**	**Questionable**	**Incomplete**
Pl-WAI	14	2	*P. larvae* Phage Vegas *P. larvae* Phage Harrison	3	9
Pl-TP	14	2	*P. larvae* Phage Vegas *P. larvae* Phage Harrison	3	9
Pl-CHCH	14	2	*P. larvae* Phage Vegas *P. larvae* Phage Harrison	3	9
PFR-Pl-2017	13	2	*P. larvae* Phage Vegas *P. larvae* Phage Harrison	4	7
PFR-Pl-2006	15	2	*P. larvae* Phage Vegas *P. larvae* Phage Harrison	4	9
Pl-F1A	12	2	*P. larvae* Phage Vegas *P. larvae* Phage Harrison	3	7
Pl-F2B	13	1	*P. larvae* Phage Vegas	3	9
Pl-P1627	10	1	*P. larvae* Phage Harrison	3	6

3.2.2 Phage Discovery

3.2.2.1 A citizen science approach to national sample collection.

Bee hives are distributed throughout the country in out-of-the-way locations and often on private property. In order to isolate phages from around New Zealand we used a citizen science approach to engage the assistance of New Zealand beekeepers. An infographic (Figure 3.2A) was developed and distributed widely in beekeeping circles via social media, beekeeping magazines, in-person apiculture conferences and posted on our website (http://www.hendricksonlab.co.nz/ABATE/). Beekeepers were encouraged to take samples of soil or hive/bee debris and return them to be processed for the presence of phages in a prepaid and addressed envelope. As part of the citizen science, beekeepers were able to name any phages that were discovered within a sample they had provided to us. A total of 720 sample tubes were distributed, and of these 430 samples were returned and processed, for a return rate of 60%. Samples were taken from a wide distribution of locations in New Zealand (Figure 3.2B). 26 of the samples contained a novel phage able to infect at least one of our bacterial isolates of *P. larvae* (Figure 3.2C, Table 3.4).

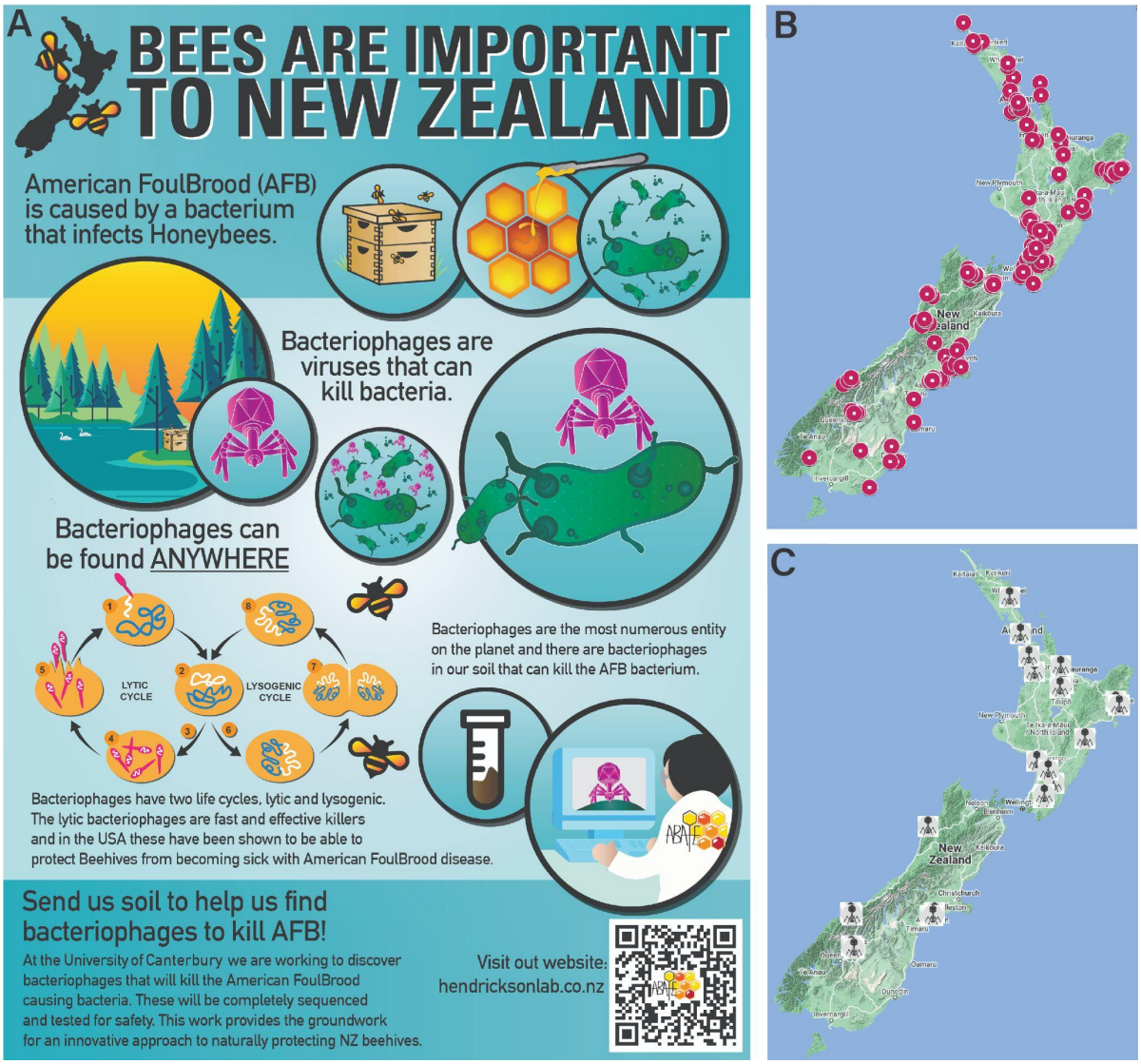


Figure 3.2 Sourcing samples from beehives across the nation.

A) An infographic was distributed to beekeepers to inform them of the research being undertaken and to request their help in sourcing samples. B) Locations of samples that were provided by beekeepers. C) Locations of phages discovered as a result of these efforts.

Table 3.4. Details of 26 *P. larvae* phages discovered

	Isolation Source	Geographic Region	Isolated On	Accession No.
AJG77	Soil	Wanaka	2017	OP503969
GIW2016	Soil	Wanaka	2017	OP503977
BarryFoster_Benicio	Soil	Whangarei	F1A	OP503543
FutureBee	Soil	Hamilton	TP	OP503975
ABAtENZ	Soil	Hamilton	2017	OP503968
Logan	Soil	Tolaga Bay	2017	OP503980
LunBun	Soil	Gisborne	F1A	OP494865
WildCape	Soil	Gisborne	F1A	OP503988
ApiWellbeing	Soil	Masterton	F1A	OP503970
Carlos	Soil	Carterton	F1A	OP503973
Ollie	Soil	Marton	2017	OP503982
Ted	Soil	Napier	2017	OP503985
Rae.2Bee1	Soil	Fairton	TP	OP503983
GaryLarson	Soil	Willowby	F2B	OP503976
Dante	Soil	Elgin	WAI	OP503974
Callan	Soil	West Taratahi	2006	OP503989
Dash	Wax	West Taratahi	2006	OP503990
Lilo	Soil	Pukekawa	F1A	OP503991
TonyLawson77	Soil	Palmerston North	F1A	OP503986
UtuhinaGold_Zacery	Soil	Rotorua	2017	OP503987
Lena	Soil	Rotorua	2017	OP503979
Bob	Soil	Matakana Island	F2B	OP503972
Rosalind	Soil	Westport	F1A	OP503984
Jacinda	Soil	Haast	2017	OP503978
Bloomfield	Soil	Haast	2017	OP503971
NHScienceFair	Soil	Albany	F1A	OP503981

3.2.2.2 Isolating phages from soil/hive material

The 430 samples received were processed using an enrichment technique followed by three rounds of plaque purification (Figure 3.3A, 3.3B). The plaque morphologies of all isolated phages were tiny, pin-prick plaques that appeared clear. When tested by a standard spot titer

plate method, all of our phages had very low effective titers ranging from 6.7 x 10^2 to 2.7 x 10^5. A modified Appelmans technique was used to increase the titer of all phages in order to extract DNA and send for sequencing (Chapter 5). Once these titers were raised by this method to >1 x 10^8 we proceeded with visualisation and complete genome sequencing.

3.2.2.3 TEM of isolated phage

Electron microscopy was undertaken on Phage Lilo (Figure 3.3C) and Phage Ollie (Figure 3.3D). These revealed phages with long, filamentous, non-contractile tails; phages with these types of tails are members of the Siphoviridae family (Ackermann, 2003). All known *P. larvae* phages have this morphotype (Stamereilers et al., 2018). Phage Lilo had a tail of approximately 148 nm in length with a prolate head measuring approximately 105 nm by 41 nm. Phage Ollie had a tail approximately 156 nm in length with a prolate head measuring approximately 106 nm by 43 nm.

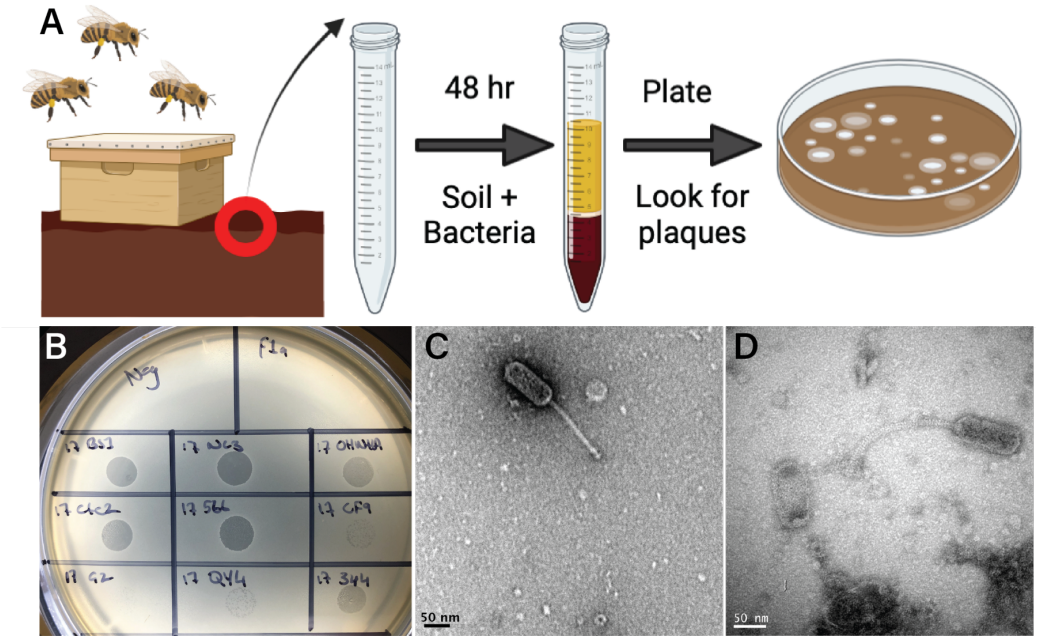


Figure 3.3 Phage discovery

A) Schematic of phage enrichment and isolation process (created with BioRender.com). B) Positive spot tests after enrichment. C) TEM image of Phage Lilo. D) TEM image of Phage Ollie. Scale bars = 50 nm.

3.2.3 Host Range testing

Specificity of each of the 26 New Zealand isolated phages on each of the eight native *P. larvae* isolates identified in this chapter, as well as 22 native *P. larvae* isolates provided by the ApiWellbeing team (Ministry for Primary Industries, n.d.), was carried out using standard spot test assays. Phages were scored as positive or negative for cell lysis. Nine distinct infection patterns were identified (Figure 3.4).

None of the phages were capable of lysing all 30 bacterial isolates, but they are able to lyse between 57% to 87% of them. Bacteria Pl-P1627 and W19_08094, which were both isolated from the Otago region, are not lysed by any of the 26 phages found in New Zealand to date. Pl-P1627 belongs to a different multilocus sequence type than the other seven bacterial strains identified in this chapter, as well as having 12 unique spacer sequences within its CRISPR Arrays.

Phages Dash, Lilo, and Callan are able to infect bacterial strains W19_08078, W19_08082, W19_08091, PFR-Pl-2006, W19_07957, and W19_08023 which are not lysed by any other phage. W19_08099 and W19_08100 are not able to be infected by these three phages, which otherwise infect all non-resistant bacteria. Pl-F1A, Pl-F2B, Pl-WAI, Pl-2017, Pl-TP, Pl-CHCH, W19_07823, and W19_07831 are lysed by all phages in this study. Bacteria W19_08100 is lysed by nine of the phages. Phages TonyLawson77, Bob, and Rosalind have the smallest ranges of infectivity and are only capable of lysing 57% of the strains.

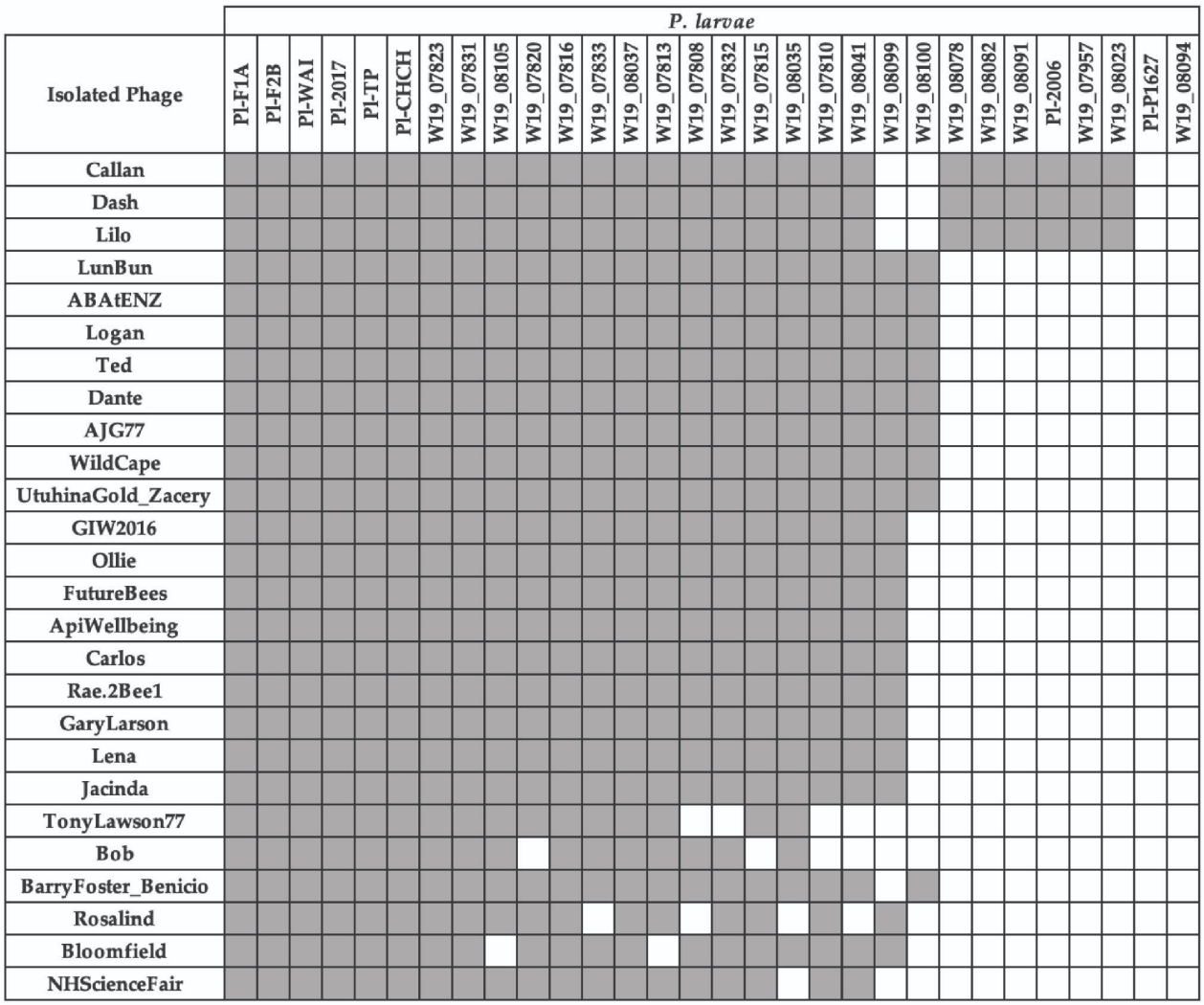


Figure 3.4 Host range of 26 *P. larvae* phages on 30 *P. larvae* bacterial isolates from New Zealand. Grey boxes indicate cell lysis and white boxes indicate no cell lysis has occurred.

3.2.4 Cocktail formulation and in-vitro testing

Four cocktails were formulated based on the host range of the phages to ensure coverage of as many bacterial strains as possible (Table 3.5). The likelihood of a colony being infected with *P. larvae* in New Zealand is 0.0032 (King, 2020), so the likelihood of more than one strain infecting a hive in New Zealand is 0.00001024. Our phage cocktail design therefore focussed on covering the breadth of strains that could infect a colony (Table 3.4).

Cocktails were capable of lysing 93% of the bacterial strains (28/30). Ultimately each cocktail had a 70% - 73% breadth of activity across our 30 bacterial isolates (Table 3.5). The breadth of activity is calculated as the susceptibility of pathogens to at least two phages in the cocktail (Abedon et al., 2021). Higher breadth is an indication of the cocktail's ability to mitigate phage resistance in the pathogen.

Once the four cocktails were decided upon, they were tested against four bacterial strains chosen to represent the types of *P. larvae* present in New Zealand; the bacterial strains selected were Pl-2017, Pl-2006, W19-08100, and Pl-P1627 (Figure 3.5). All four cocktails showed good activity on strain Pl-2017 with cocktails Two and Four showing slightly better lysis potential. Cocktails Two and Four were very effective on strain Pl-2006, while cocktails One and Three only showed good lysis at the highest concentration of phages. On strain W19-08100, cocktails One, Two and Four all showed some lysis while cocktail Three showed very little lysis potential.

As expected, there was no phage activity on our resistant strain, Pl-P1627. Based on these experiments, cocktails Two or Four would be the desirable phage cocktails with which to move forward.

Table 3.5. The phages contained within the four cocktails

Cocktail One	Callan	Logan	ApiWellbeing	Freya
Cocktail Two	Dash	AJG77	UtuhinaGold_ Zacery	NHScienceFair
Cocktail Three	Callan	Ted	FutureBee	BarryFoster_ Benicio
Cocktail Four	Dash	LunBun	Carlos	Bloomfield

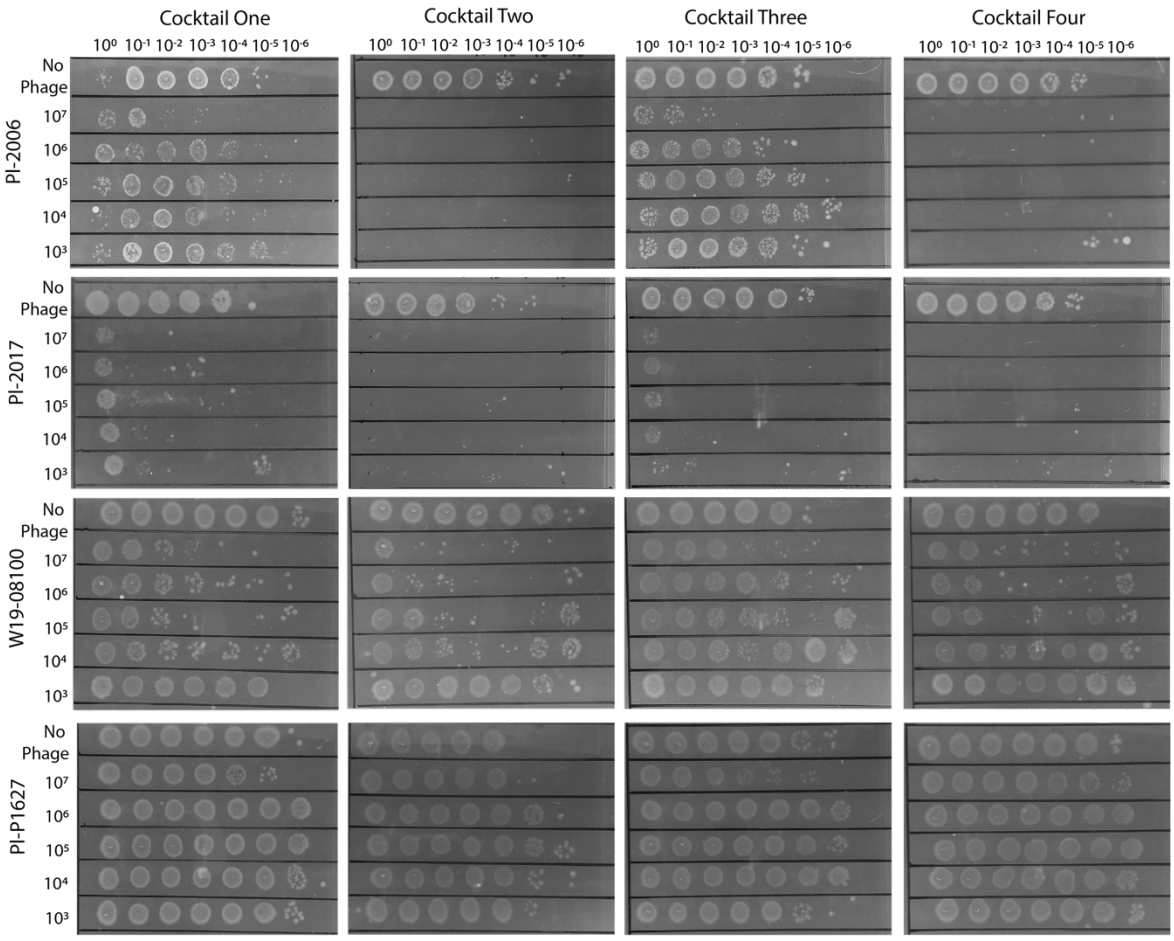


Figure 3.5 *In-vitro* testing of phage cocktails

Four cocktails were tested for their effectiveness against each of four bacterial strains: PI-2017, PI-2006, W19-08100, and PI-P1627. Strain PI-P1627 was known to be resistant to all phages in this study.

3.3 Discussion

In this study, we have set out to lay the groundwork for using phages as a prophylactic against the devastating pathogen *P. larvae* in the New Zealand apiculture industry. Our work began with the isolation and preliminary sequencing of a set of eight novel *P. larvae* strains from across New Zealand. These *P. larvae* bacterial strains were directly isolated from bee larvae or beehives with clinical signs of AFB. This allowed us to start the only collection of *P. larvae* bacterial strains available in New Zealand at the time. These *P. larvae* isolates were assembled to between 157 to 219 contigs and were found to belong to the 18 and 23 MLST.

MLST 18 is consistent with the ERIC type I group and MLST 23 has not been typed in the literature (Morrissey et al., 2014; Papić et al., 2021).

To better understand how these isolates might interact with phages, we screened them for phage defence mechanisms. We found each of our isolates contained CRISPR arrays. Each strain contained four to five CRISPR arrays with a total of 15 to 25 spacers. *P. larvae* strains isolated previously have been found to contain CRISPR arrays as phage defence mechanisms. *P. larvae* ERIC I strains ATCC 9545 and DSM 7030 contained four CRISPR arrays with 17 spacers (Stamereilers et al., 2021).

We also used PHASTER to evaluate the sequenced contigs for prophages and we found each isolate had one or two intact prophages. In another study *P. larvae* ERIC type I strains DSM 25719, MEX14, ATCC 9545, and DSM 7030 each contained eight, three, five, and five intact prophages respectively (Ribeiro et al., 2022).

Seven anti-phage systems were also discovered within our eight bacterial strains. These data suggested to us that these isolates are encountering an active population of phages in nature and are maintaining a suite of defence systems to counter infection when they meet. This is common as previous reports suggest that 50% of bacteria have CRISPR systems (Hille et al., 2018) and other defence mechanisms like restriction-modification systems are widely found within prokaryotes (Loenen & Raleigh, 2014).

The ApiWellbeing project, an initiative of the New Zealand Ministry for Primary Industries, generously gifted us with 22 additional *P. larvae* isolates from their own recent

collection efforts, which brought our collection of hosts to 30 (Ministry for Primary Industries, n.d.). The sequencing and annotation of this collection is underway and will provide a valuable asset in the future.

Since the discovery of the first *P. larvae* phage in 1953 (Smirnova, 1953), 69 *P. larvae* specific phages have been found (Beims et al., 2015; Carson et al., 2015; Jończyk-Matysiak et al., 2021; Bryan D. Merrill et al., 2018; A. Oliveira et al., 2013; Ribeiro et al., 2019; Tsourkas et al., 2015; Walker et al., 2018; Yost et al., 2018). Due to the strict biosecurity laws in New Zealand, it is unlikely that non-native phages would be permitted in the apiculture industry here. We, therefore, sought to discover a suite of native New Zealand *P. larvae* phages to combat AFB.

Previous hunts for *P. larvae* phages have included samples from soil, bee debris, cosmetics and bee wax (B. D. Merrill et al., 2018; Walker et al., 2018; Yost et al., 2018). A large-scale hunt across New Zealand was a daunting task for our small team, we, therefore, approached beekeepers from around New Zealand and received 430 samples of bee debris and soil from both the North and South Islands. These types of citizen science phage hunts have been used previously and there are on-going citizen science projects to isolate new phages for *Pseudomonas aeruginosa* (*Citizen phage library*, n.d.). These samples were processed and led to the discovery of 26 independent phages. Unlike similar efforts overseas (Bryan D. Merrill et al., 2018; Yost et al., 2016) in which phages have been isolated from infected hives, the phages discovered were reported to have been isolated only from hive material or soil associated with healthy hives (B. D. Merrill et al., 2018).

These phages were sequenced to completion, annotated, and their genomes are available publicly (Table 3.4). A more thorough analysis of these genomes and their relationships to the global *P. larvae* phages is outside of the scope of this publication.

To determine if our phage genomes were distinct, we used criteria previously described (Stamereilers et al., 2018). Phages are usually phenotypically identical if they have an ANI greater than 99.975%. We had several groups of phages that had ANIs greater than this cut-off point. However, further analysis showed they all contained at least one amino acid difference, so in these instances they were classed as phenotypically different.

Host range experiments revealed nine distinct infection patterns, these included a subset of six bacterial strains that were only able to be infected by three phages and two recalcitrant bacterial strains that were not infected by any of the phages discovered in New Zealand to date. Overall, there was a 93% host range coverage for our collection of 26 *P. larvae* phages. In similar host-range experiments with *P. larvae* phages, Yost *et al.* found a 100% host range coverage when testing 29 phages on 11 *P. larvae* bacterial strains (Yost et al., 2016). In another experiment, Brady *et al*. tested 39 *P. larvae* specific phages on 59 bacterial strains and also found a 100% host range coverage (Brady et al., 2017). Efforts are ongoing to find phages that can lyse the final resistant strains that are present in New Zealand. We do not currently know if phages found overseas have the ability to lyse the resistant *P. larvae* strains.

Four cocktails were formulated and tested against each of four *P. larvae* isolates. Cocktails One and Three both contained Phage Callan and Cocktails Two and Four both contained Phage Dash, as these were two of the phages that were able to infect six bacterial strains resistant to all other phages.

In-vitro testing using these four cocktails resulted in varying results with two cocktails standing out as the most effective at killing three of the bacterial strains. One *P. larvae* strain, Pl-P1627, was completely resistant to all cocktails. This was to be expected as this strain was not infected by any of our phages. In this limited instance we did not see any evidence of emergent infectivity above and beyond that of the individual phages present in the cocktail.

In our study, cocktails Two and Four were more effective than cocktails One and Three against *P. larvae* strains Pl-2006, Pl-2017 and W19-08100. The reason that cocktails Two and Four were more effective is not currently known. Interestingly, cocktails One and Four had a predicted breadth of activity of 50% (2/4 phages were able to infect at least 2/4 bacterial strains), whilst cocktails Two and Three had a 75% breadth of activity. This suggests that the quality of the phage cocktails tested here cannot be attributed to the breadth of activity alone. The cause of the difference in outcomes of these phage cocktails remains to be investigated.

Several studies have shown that phages within phage cocktails can have an antagonistic relationship. Forti *et al* found combining six phages into a cocktail to lyse *Pseudomonas aeruginosa* showed a lower host range than what had been predicted based on individual phage host ranges (Forti et al., 2018). Another study testing different phage cocktails on *Escherichia coli* O157 showed that not all combinations of phages were as effective as others and phage antagonism was common in certain cocktails (Niu et al., 2021).

These results, taken together with previous studies, show the importance of testing a variety of phage cocktails to find the most effective combination of phages, regardless of their individual host ranges.

This study shows promising results and forms the beginning of the work needed to find a solution to prophylactically protect honey bees in New Zealand from the destructive disease known as AFB. Further work will need to be undertaken to completely understand the characteristics of the phages to ensure the cocktails formulated for this work are the most appropriate available.

3.4 Materials and Methods

3.4.1 Isolation of Paenibacillus larvae

P. larvae was isolated by swabbing suspected brood frames and wiping swabs on MYPGP (D. W. Dingman & Stahly, 1983) with Nalidixic acid (10 μg/mL) and Pipemidic acid (10 μg/mL) plates. Plates were incubated at 37°C for 3-5 days, until colonies had formed. A colony was picked and purified by single colony isolation on another MYPGP plate. A single colony was picked and grown in liquid MYPGP for 48 hours at 37°C and shaken at 100 rpm, then frozen at -80°C.

3.4.2 Bacterial DNA Extraction and 16s rRNA PCR

Bacterial DNA was extracted from overnight cultures in mBHI (Oxoid CM1135B) broth using the commercially available Promega Wizard Genomic DNA Purification kit (www.promega.com/protocols/). The protocol for gram-positive bacteria was followed. A PCR mix was prepared with each tube containing a final volume of 50 μL. The amplification conditions were 95°C (3 mins) followed by 30 cycles of 93°C (1 min), 55°C (30 secs), and 72°C (1 min); and a final cycle of 72°C for 5 minutes. PCR products were visualised on a 1% agarose gels run at 120 volts for 30 minutes.

Primers used were (Dobbelaere et al., 2001):

AFB-F 5'-CTT-GTG-TTT-CTT-TCG-GGA-GAC-GCC-A-3'

AFB-R 5'-TCT-TAG-AGT-GCC-CAC-CTC-TGC-G-3'

3.4.3 Bacterial DNA Sequencing and Assembly

DNA was either sent for sequencing at MicrobesNG, UK, or MiGS, USA, for complete genome Illumina sequencing. Genomes were assembled using SPAdes 3.15.3 (Bankevich et al., 2012; Prjibelski et al., 2020) and then annotated using either RAST 1.073 (Aziz et al., 2008; Brettin et al., 2015; Overbeek et al., 2014) or Prokka 1.14.5 (Seemann, 2014). Average coverage was 30x with 157 - 219 contigs assembled.

3.4.4 Processing Soil/Hive Samples for Phages

Soil samples were processed as previously described (Yost et al., 2016). Only one pass through a 0.45 μm sterile syringe filter was performed. The resulting filtrate was used as a starting material for enrichment. Enrichments were a combination of; 1mL of starting material, 100 μl of each of eight *P. larvae* bacterial isolates, 8 mL mBHI and 0.4% glucose. These were incubated for 48 hours at 37^{o}C shaken at 100 rpm. After 48 hours, enrichments were centrifuged at 3200g for 15 minutes and filter sterilised to 0.45 μm. The resulting supernatants were assayed for phage presence by 3 μl spots on double-layer agar containing one of the *P. larvae* bacterial isolates.

3.4.5 Phage plaque purification

Phages underwent three rounds of purification. Plaques were picked off a double-agar plate using a 200 µl tip, the tip was put in 100 µl of BHI and pipetted up and down to release phage particles. This sample was used to inoculate the next double-agar plate.

3.4.6 Creation of phage stocks

To create phage lysates, ten plates containing plaques of individual phage isolates, together as initiated from a single plaque, were flooded with 8 mL of BHI. Plates were left to sit at room temperature for 2 hours. At the end of 2 hours, plates were swirled and the lysate was removed. Lysates were filtered with a 0.45 µm filter and pooled in a 50 mL falcon tube. Titers were increased using a modified Rapid Appelmans protocol (Chapter 5).

3.4.7 Zinc Chloride DNA Extraction

Phage DNA was extracted using a modified zinc chloride precipitation method (Santos, 1991). Modifications included the addition of 1 µL Proteinase K, incubated at 37°C for 10 min after the TES step. Tubes were left overnight on ice after isopropanol was added. 1 µL of pure glycogen was added to each tube at the beginning of Day 2 before the centrifugation step to aid in pelleting of DNA. DNA pellets were resuspended in 50 µL nuclease-free water.

3.4.8 Host Range Testing

The ability of phages to infect each isolate was assessed by 3µl spots of each phage lysate on double-layer agar containing 500µl of bacterial lawn. Each *P. larvae* bacterial isolate was tested separately. The majority of spot tests showed the presence of individual plaques owing to low phage titers during this testing.

3.4.9 In-vitro Cocktail Assays

Phage titers were normalised to $1x10^8$ PFU/ml and 50µl of each phage was combined into a cocktail. Bacterial cultures were grown in BHI for 48 hours at 37°C to ~$1x10^8$ CFU/ml. The bacteria were serially diluted up to a 10^{-6} dilution and 20µl was aliquoted into 96-well plates containing 90µl 2xBHI and 90µl BHI. The phage cocktail was also serially diluted such that each row contained from 10^7 to 10^3 PFU total phage. 20µl of the phage cocktail was added to each well of the plate. Plates were incubated, shaking at 37°C for 24 hours. Aliquots of 3µl were spotted onto BHI plates and incubated for three to four days at 37°C to observe CFU.

3.5 References

Abby, S. S., Néron, B., Ménager, H., Touchon, M., & Rocha, E. P. C. (2014). MacSyFinder: a program to mine genomes for molecular systems with an application to CRISPR-Cas systems. *PloS One*, *9*(10), e110726.

Abedon, S. T., Danis-Wlodarczyk, K. M., & Wozniak, D. J. (2021). Phage Cocktail Development for Bacteriophage Therapy: Toward Improving Spectrum of Activity Breadth and Depth. *Pharmaceuticals* , *14*(1019). https://doi.org/10.3390/ph14101019

Ackermann, H.-W. (2003). Bacteriophage observations and evolution. *Research in Microbiology*, *154*(4), 245–251.

AFBPMP. (2017). *AFBPMP BRIEFING DOCUMENT - MPI Government 01112017*. 1–23.

Alippi, A. M., Lopez, A. C., & Aguilar, O. M. (2002). Differentiation of *Paenibacillus larvae subsp. larvae*, the Cause of American Foulbrood of Honeybees, by Using PCR and Restriction Fragment Analysis of Genes Encoding 16S rRNA. *Applied and Environmental Microbiology*, *68*(7), 3655–3660.

Alippi, Adriana M., Reynaldi, F. J., López, A. C., De Giusti, M. R., & Aguilar, O. M. (2004). Molecular epidemiology of *Paenibacillus larvae larvae*and incidence of American foulbrood in Argentinean honeys from Buenos Aires province. *Journal of Apicultural Research*, *43*(3), 135–143.

Arndt, D., Grant, J. R., Marcu, A., Sajed, T., Pon, A., Liang, Y., & Wishart, D. S. (2016). PHASTER: a better, faster version of the PHAST phage search tool. *Nucleic Acids Research*, *44*(W1), W16–W21.

Aziz, R. K., Bartels, D., Best, A. A., DeJongh, M., Disz, T., Edwards, R. A., Formsma, K., Gerdes, S., Glass, E. M., Kubal, M., Meyer, F., Olsen, G. J., Olson, R., Osterman, A. L., Overbeek, R. A., McNeil, L. K., Paarmann, D., Paczian, T., Parrello, B., … Zagnitko, O.

(2008). The RAST Server: rapid annotations using subsystems technology. *BMC Genomics*, *9*, 75.

Bankevich, A., Nurk, S., Antipov, D., Gurevich, A. A., Dvorkin, M., Kulikov, A. S., Lesin, V. M., Nikolenko, S. I., Pham, S., Prjibelski, A. D., Pyshkin, A. V., Sirotkin, A. V., Vyahhi, N., Tesler, G., Alekseyev, M. A., & Pevzner, P. A. (2012). SPAdes: a new genome assembly algorithm and its applications to single-cell sequencing. *Journal of Computational Biology: A Journal of Computational Molecular Cell Biology*, *19*(5), 455–477.

Beims, H., Wittmann, J., Bunk, B., Spröer, C., Rohde, C., Günther, G., Rohde, M., von der Ohe, W., & Steinert, M. (2015). *Paenibacillus larvae*-Directed Bacteriophage HB10c2 and Its Application in American Foulbrood-Affected Honey Bee Larvae. *Applied and Environmental Microbiology*, *81*(16), 5411–5419.

Bernheim, A., Bikard, D., Touchon, M., & Rocha, E. P. C. (2020). Atypical organizations and epistatic interactions of CRISPRs and cas clusters in genomes and their mobile genetic elements. *Nucleic Acids Research*, *48*(2), 748–760.

Biosecurity (National American Foulbrood Pest Management Plan) Order 1998, 1 (1998).

Brady, T. S., Merrill, B. D., Hilton, J. A., Payne, A. M., Stephenson, M. B., & Hope, S. (2017). Bacteriophages as an alternative to conventional antibiotic use for the prevention or treatment of *Paenibacillus larvae* in honeybee hives. *Journal of Invertebrate Pathology*, *150*, 94–100.

Brettin, T., Davis, J. J., Disz, T., Edwards, R. A., Gerdes, S., Olsen, G. J., Olson, R., Overbeek, R., Parrello, B., Pusch, G. D., Shukla, M., Thomason, J. A., 3rd, Stevens, R., Vonstein, V., Wattam, A. R., & Xia, F. (2015). RASTtk: a modular and extensible implementation of the RAST algorithm for building custom annotation pipelines and annotating batches of genomes. *Scientific Reports*, *5*, 8365.

Carson, S., Bruff, E., DeFoor, W., Dums, J., Groth, A., Hatfield, T., Iyer, A., Joshi, K., McAdams, S., Miles, D., Miller, D., Oufkir, A., Raynor, B., Riley, S., Roland, S., Rozier, H., Talley, S., & Miller, E. S. (2015). Genome Sequences of Six *Paenibacillus larvae* SiphoviridaePhages: TABLE 1. *Genome Announcements*, *3*(3), 4636–4632.

Citizen phage library. (n.d.). Retrieved February 28, 2023, from https://www.citizenphage.com

Dingman, D. W., & Stahly, D. P. (1983). Medium Promoting Sporulation of *Bacillus larvae* and Metabolism of Medium Components. *Applied and Environmental Microbiology*, *46*(4), 860–869.

Dingman, Douglas W. (2017). Four Complete *Paenibacillus larvae*Genome Sequences. *Genome Announcements*, *5*(22), 270–273.

Djukic, M., Brzuszkiewicz, E., Fünfhaus, A., Voss, J., Gollnow, K., Poppinga, L., Liesegang, H., Garcia-Gonzalez, E., Genersch, E., & Daniel, R. (2014). How to Kill the Honey Bee Larva: Genomic Potential and Virulence Mechanisms of *Paenibacillus larvae*. *PloS One*, *9*(3), e90914-14.

Dobbelaere, W., De Graaf, D., & Peeters, J. (2001). Development of a fast and reliable diagnostic method for American foulbrood disease (*Paenibacillus larvae subsp. larvae*) using a 16S rRNA gene based PCR. *Apidologie*, 363–370.

Doron, S., Melamed, S., Ofir, G., Leavitt, A., Lopatina, A., Keren, M., Amitai, G., & Sorek, R. (2018). Systematic discovery of antiphage defense systems in the microbial pangenome. *Science*, *359*(6379). https://doi.org/10.1126/science.aar4120

Elke Genersch. (2010). American Foulbrood in honeybees and its causative agent, *Paenibacillus larvae*. *Journal of Invertebrate Pathology*, *103*(S), S10–S19.

Forti, F., Roach, D. R., Cafora, M., Pasini, M. E., Horner, D. S., Fiscarelli, E. V., Rossitto, M.,

Cariani, L., Briani, F., Debarbieux, L., & Ghisotti, D. (2018). Design of a Broad-Range Bacteriophage Cocktail That Reduces Pseudomonas aeruginosa Biofilms and Treats Acute Infections in Two Animal Models. *Antimicrobial Agents and Chemotherapy*, *62*(6). https://doi.org/10.1128/AAC.02573-17

Gao, L., Altae-Tran, H., Böhning, F., Makarova, K. S., Segel, M., Schmid-Burgk, J. L., Koob, J., Wolf, Y. I., Koonin, E. V., & Zhang, F. (2020). Diverse enzymatic activities mediate antiviral immunity in prokaryotes. *Science*, *369*(6507), 1077–1084.

Genersch, E. (2008). *Paenibacillus larvae* and American Foulbrood – long since known and still surprising. *Journal Für Verbraucherschutz Und Lebensmittelsicherheit*, *3*(4), 429–434.

Goodwin, M. (2005). American foulbrood control: the New Zealand approach. *Bee World*, *86*(2), 44–45.

Graham, S. A. M. (2014). *American foulbrood and its causative agent, Paenibacillus larvae, in New Zealand's registered hives and apiaries* (P. Lester, Ed.) [MSc]. Victoria University of Wellington.

Grissa, I., Vergnaud, G., & Pourcel, C. (2007). CRISPRFinder: a web tool to identify clustered regularly interspaced short palindromic repeats. *Nucleic Acids Research*, *35*(Web Server issue), W52-7.

Hendrix, R. W., Smith, M. C., Burns, R. N., Ford, M. E., & Hatfull, G. F. (1999). Evolutionary relationships among diverse bacteriophages and prophages: all the world's a phage. *Proceedings of the National Academy of Sciences of the United States of America*, *96*(5), 2192–2197.

Hille, F., Richter, H., Wong, S. P., Bratovič, M., Ressel, S., & Charpentier, E. (2018). The Biology of CRISPR-Cas: Backward and Forward. *Cell*, *172*(6), 1239–1259.

Jolley, K. A., Bray, J. E., & Maiden, M. C. J. (2018). Open-access bacterial population genomics: BIGSdb software, the Pubmlst.org website and their applications. *Wellcome Open Research*, *3*, 124.

Jończyk-Matysiak, E., Owczarek, B., Popiela, E., Świtała-Jeleń, K., Migdał, P., Cieślik, M., Łodej, N., Kula, D., Neuberg, J., Hodyra-Stefaniak, K., Kaszowska, M., Orwat, F., Bagińska, N., Mucha, A., Belter, A., Skupińska, M., Bubak, B., Fortuna, W., Letkiewicz, S., … Górski, A. (2021). Isolation and Characterization of Phages Active against *Paenibacillus larvae* Causing American Foulbrood in Honeybees in Poland. *Viruses*, *13*(7). https://doi.org/10.3390/v13071217

King, C. (2020). American foulbrood. *Food Additives & Contaminants, Part B: Surveillance*, *47*(3), 42.

Kok, D. N., & Hendrickson, H. L. (2023). Save our bees: bacteriophages to protect honey bees against the pathogen causing American foulbrood in New Zealand. *New Zealand Journal of Zoology*, 1–16.

Lester, P. (2021). *Healthy Bee, Sick Bee: The Influence of Parasites, Pathogens, Predators and Pesticides on Honey Bees*. Victoria University of Wellington Press.

Li, G., Zhao, H., Liu, Z., Wang, H., Xu, B., & Guo, X. (2018). The Wisdom of Honeybee Defenses Against Environmental Stresses. *Frontiers in Microbiology*, *9*, 722.

Loenen, W. A. M., & Raleigh, E. A. (2014). The other face of restriction: modification-dependent enzymes. *Nucleic Acids Research*, *42*(1), 56–69.

Merrill, B. D., Fajardo, C. P., Hilton, J. A., Payne, A. M., & Ward, A. T. (2018). Complete genome sequences of eighteen *Paenibacillus larvae* phages. *Genome Announcements*.

Merrill, Bryan D., Fajardo, C. P., Hilton, J. A., Payne, A. M., Ward, A. T., Walker, J. K., Dhalai,

A., Imahara, C., Mangohig, J., Monk, J., Pascacio, C., Rai, P., Salisbury, A., Velez, K., Bloomfield, T. J., Buhler, B., Duncan, S. G., Fuhriman, D. A., George, J., … Tsourkas, P. K. (2018). Complete Genome Sequences of 18 *Paenibacillus larvae* Phages from the Western United States. *Microbiology Resource Announcements*, *7*(13). https://doi.org/10.1128/MRA.00966-18

Millman, A., Melamed, S., Leavitt, A., Doron, S., Bernheim, A., Hör, J., Lopatina, A., Ofir, G., Hochhauser, D., Stokar-Avihail, A., Tal, N., Sharir, S., Voichek, M., Erez, Z., Ferrer, J. L. M., Dar, D., Kacen, A., Amitai, G., & Sorek, R. (2022). *An expanding arsenal of immune systems that protect bacteria from phages*. https://doi.org/10.1101/2022.05.11.491447

Ministry for Primary Industries. (n.d.). *ApiWellbeing*. Retrieved November 29, 2022, from https://www.mpi.govt.nz/biosecurity/how-to-find-report-and-prevent-pests-and-diseases/bee-biosecurity/apiwellbeing/

Ministry for Primary Industries. (2021). *2021 Apiculture Monitoring Data*. https://www.mpi.govt.nz/dmsdocument/48793-2021-Apiculture-monitoring-report-data

Morrissey, B. J., Helgason, T., Poppinga, L., Fünfhaus, A., Genersch, E., & Budge, G. E. (2014). Biogeography of *Paenibacillus larvae*, the causative agent of American foulbrood, using a new multilocus sequence typing scheme. *Environmental Microbiology*, *17*(4), 1414–1424.

Mushegian, A. R. (2020). Are There 1031 Virus Particles on Earth, or More, or Fewer? *Journal of Bacteriology*, *202*(9). https://doi.org/10.1128/JB.00052-20

Newstrom-Lloyd, L. E. (2013). Pollination in New Zealand. In J. R. Dymond (Ed.), *Ecosystem services in New Zealand : conditions and trends* (pp. 408–431). Manaaki Whenua

Press.

Niu, Y. D., Liu, H., Du, H., Meng, R., Sayed Mahmoud, E., Wang, G., McAllister, T. A., & Stanford, K. (2021). Efficacy of Individual Bacteriophages Does Not Predict Efficacy of Bacteriophage Cocktails for Control of Escherichia coli O157. *Frontiers in Microbiology*, *12*, 616712.

Oliveira, A., Melo, L. D. R., Kropinski, A. M., & Azeredo, J. (2013). Complete Genome Sequence of the Broad-Host-Range *Paenibacillus larvae* Phage phiIBB_Pl23. *Genome Announcements*, *1*(5). https://doi.org/10.1128/genomeA.00438-13

Oliveira, P. H., Touchon, M., & Rocha, E. P. C. (2014). The interplay of restriction-modification systems with mobile genetic elements and their prokaryotic hosts. *Nucleic Acids Research*, *42*(16), 10618–10631.

Overbeek, R., Olson, R., Pusch, G. D., Olsen, G. J., Davis, J. J., Disz, T., Edwards, R. A., Gerdes, S., Parrello, B., Shukla, M., Vonstein, V., Wattam, A. R., Xia, F., & Stevens, R. (2014). The SEED and the Rapid Annotation of microbial genomes using Subsystems Technology (RAST). *Nucleic Acids Research*, *42*(Database issue), D206-14.

Papić, B., Diricks, M., & Kušar, D. (2021). Analysis of the Global Population Structure of *Paenibacillus larvae* and Outbreak Investigation of American Foulbrood Using a Stable wgMLST Scheme. *Frontiers in Veterinary Science*, *8*, 582677.

Peréz de la Rosa, D., Pérez de la Rosa, J. J., Cossio-Bayugar, R., Miranda-Miranda, E., Lozano, L., Bravo-Díaz, M. A., Rocha-Martínez, M. K., & Sachman-Ruiz, B. (2015). Complete Genome Sequence of *Paenibacillus larvae* MEX14, Isolated from Honey Bee Larvae from the Xochimilco Quarter in Mexico City. *Genome Announcements*, *3*(4), 95–91.

Prjibelski, A., Antipov, D., Meleshko, D., Lapidus, A., & Korobeynikov, A. (2020). Using SPAdes De Novo Assembler. *Current Protocols in Bioinformatics / Editoral Board, Andreas D.*

Baxevanis ... [et Al.], *70*(1), e102.

Rauch, S., Ashiralieva, A., Hedtke, K., & Genersch, E. (2009). Negative Correlation between Individual-Insect-Level Virulence and Colony-Level Virulence of *Paenibacillus larvae,* the Etiological Agent of American Foulbrood of Honeybees. *Applied and Environmental Microbiology*, *75*(10), 3344–3347.

Ribeiro, H. G., Melo, L. D. R., Oliveira, H., Boon, M., Lavigne, R., Noben, J.-P., Azeredo, J., & Oliveira, A. (2019). Characterization of a new podovirus infecting *Paenibacillus larvae*. *Scientific Reports*, *9*(1), 20355.

Ribeiro, H. G., Nilsson, A., Melo, L. D. R., & Oliveira, A. (2022). Analysis of intact prophages in genomes of *Paenibacillus larvae*: An important pathogen for bees. *Frontiers in Microbiology*, *13*, 903861.

Santos, M. A. (1991). An improved method for the small scale preparation of bacteriophage DNA based on phage precipitation by zinc chloride. *Nucleic Acids Research*, *19*(19), 5442.

Seemann, T. (2014). Prokka: rapid prokaryotic genome annotation. *Bioinformatics* , *30*(14), 2068–2069.

Smirnova, N. I. (1953). Isolation and the use of a bacteriophage of *Bacillus larvae* in the diagnosis of American foulbrood. *Sbornik Nauchnykh Trudov Leningradskogo Instituta Usovershenstvovaniya Veterinarykh Vrachel*, *9*, 85–88.

Stamereilers, C., Fajardo, C., Walker, J., Mendez, K., Castro-Nallar, E., Grose, J., Hope, S., & Tsourkas, P. (2018). Genomic Analysis of 48 *Paenibacillus larvae* Bacteriophages. *Viruses*, *10*(7), 377–329.

Stamereilers, C., Wong, S., & Tsourkas, P. K. (2021). Characterization of CRISPR Spacer and Protospacer Sequences in *Paenibacillus larvae* and Its Bacteriophages. *Viruses*, *13*(3).

https://doi.org/10.3390/v13030459

Tesson, F., Hervé, A., Mordret, E., Touchon, M., d'Humières, C., Cury, J., & Bernheim, A. (2022). Systematic and quantitative view of the antiviral arsenal of prokaryotes. *Nature Communications*, *13*(1), 2561.

Tsourkas, P. K., Yost, D. G., Krohn, A., LeBlanc, L., Zhang, A., Stamereilers, C., & Amy, P. S. (2015). Complete Genome Sequences of Nine Phages Capable of Infecting *Paenibacillus larvae*, the Causative Agent of American Foulbrood Disease in Honeybees. *Genome Announcements*, *3*(5). https://doi.org/10.1128/genomeA.01120-15

Walker, J. K., Merrill, B. D., Berg, J. A., Dhalai, A., Dingman, D. W., Fajardo, C. P., Graves, K., Hill, H. L., Hilton, J. A., Imahara, C., Knabe, B. K., Mangohig, J., Monk, J., Mun, H., Payne, A. M., Salisbury, A., Stamereilers, C., Velez, K., Ward, A. T., … Tsourkas, P. K. (2018). Complete Genome Sequences of *Paenibacillus larvae* Phages BN12, Dragolir, Kiel007, Leyra, Likha, Pagassa, PBL1c, and Tadhana. *Genome Announcements*, *6*(24). https://doi.org/10.1128/genomeA.01602-17

Yost, D. G., Chang, C., LeBlanc, L., Cassin, E., Peterman, C., Rai, P., Salisbury, A., Barroga, N., Cisneros, R., Fersini, J., Juste, J., Ines, J., Leyva, G., Macalinao, D., Muscelli, S., Reyes, G. S., Rhoden, H., Tan, R., Torres, E., … Tsourkas, P. K. (2018). Complete Genome Sequences of *Paenibacillus larvae* Phages Halcyone, Heath, Scottie, and Unity from Las Vegas, Nevada. *Microbiology Resource Announcements*, *7*(12). https://doi.org/10.1128/MRA.00977-18

Yost, D. G., Tsourkas, P., & Amy, P. S. (2016). Experimental bacteriophage treatment of honeybees \(Apis mellifera\) infected with *Paenibacillus larvae*, the causative agent of American Foulbrood Disease. *Bacteriophage*, *6*(1), 1–15.

Zhou, Y., Liang, Y., Lynch, K. H., Dennis, J. J., & Wishart, D. S. (2011). PHAST: A Fast Phage Search Tool. *Nucleic Acids Research*, *39*(suppl), W347–W352.

Chapter 4

In-vivo Apis Mellifera larval experiments

Abstract

Bacteriophage (phage) therapy is the therapeutic use of viruses specific to bacteria to combat bacterial diseases. Phages are slowly becoming a well-researched and accepted alternative to antibiotics. An important step in the process of ensuring phage cocktails efficacy and safety is to undertake *in-vivo* experiments on the organism of interest. To ensure there were no negative effects of the cocktails on the health of honey bee larvae (*Apis Mellifera),* we wanted to test a range of phage cocktails capable of lysing *Paenibacillus larvae,* the bacteria responsible for American Foulbrood. Herein, we describe our experimental design to test two previously described phage cocktails on lab-reared honey bee larvae. Due to unforeseen circumstances, testing occurred later than expected. The results of these larval experiments were inconclusive as large numbers of larval deaths occurred before treatments could be applied.

4.1 Introduction

Bacteriophage therapy is the use of viruses, known as phages, to treat bacterial diseases. Phages are becoming widely researched as alternatives to antibiotics (Lin et al., 2017). *In vivo* testing of phages plays an important role in the pipeline of phage discovery to ensure the safety and efficacy of phages used for therapy. Previous work has shown a positive relationship between *in-vitro* and *in-vivo* testing of bacteria phages on *Pseudomonas aeruginosa* (Henry et al., 2013). In this experimental work, we aimed to test several phage cocktails specific to *Paenibacillus larvae* on lab-reared honey bee larvae, to assess if there is a positive correlation with *in-vitro* experiments previously undertaken.

The phage cocktail selected for this experiment was cocktail Two, which was previously described in Chapter 3 as having the ability to eliminate signs of bacteria of PFR-Pl-2006, PFR-Pl-2017, and W19-08100 in a sub-sampled 96-well plate assay. Cocktail Two was not however effective against recalcitrant *P. larvae* strain Pl-P1627. Cocktail Two contained phages Dash, AJG77, UtuhinaGold_Zacery, and NHScienceFair. Phages AJG77 and UtuhinaGold_Zacery are able to amplify on *P. larvae* W19-08100, whilst phages Dash and NHScienceFair are not capable of amplification. No phages contained within cocktail Two are able to amplify on *P. larvae* Pl-P1627.

In-vivo testing of *P. larvae* on lab-reared honey bee larvae has been undertaken in a laboratory in the US and positive results were obtained (Ghorbani-Nezami et al., 2015). In another experiment, *in-vivo* testing was undertaken in the field and phage cocktails were able to both prophylactically protect beehives from infection and clear established infections (Brady et al., 2017). Herein, we present the methodology used to undertake *in-vivo* testing on honey bee larvae infected with *P. larvae* bacterial spores and the issues we faced due to circumstances outside of our control.

In-vivo experiments were conducted in collaboration with and under the supervision of Dr. Ashley Mortensen at Plant and Food, Ruakura. Dr Mortensen has extensive experience in rearing honey bees in the laboratory and her PhD was on the effects of *in-vitro* larval rearing on adult honey bees.

4.2 Materials and Methods

4.2.1 Bacterial strains and phage isolates

Two strains of *P. larvae* were used in our *in-vivo* testing: Pl-P1627 a natural strain isolated from the Central Otago region and W19_08100 a natural strain isolated from the Otago Lakes region. Cocktail Two contains phages: Dash, AJG77, UtuhinaGold_Zacery, and NHScienceFair. (Refer to Chapter 3 for methods to grow these).

4.2.2 Bacterial spores

To produce bacterial spores to feed to honey bee larvae, a 10-fold dilution series of *P. larvae* bacterial culture was spread onto several MYPGP agar plates. Plates were incubated at 37^{o}C for 6-7 days and plates exhibiting individual colonies were selected. After incubation, spores were removed from the plates by washing with 5 mL cold sterile water. Water was added to the plate, the surface of the plate was gently scraped with a sterile inoculation loop to loosen spores. Water and spores were then removed from the plates using a syringe and transferred into Eppendorf tubes. The spore suspension was concentrated via centrifugation (12,000 x g, 15 min, 4^{o}C). After centrifugation, the supernatant was discarded, and the spore pellet was resuspended in 1 mL of cold water. This step was repeated three times. The final spore pellets from all tubes were resuspended in a total volume of 2 mL cold water. Spores were stored at 4^{o}C (de Graaf et al., 2015).

4.2.3 Lysed bacteria

Lysed *P. larvae* bacteria were produced by growing overnight cultures of the bacterial strain of interest. 1 mL of the overnight culture was transferred to an Eppendorf tube and heated to 80^{o}C for 30 minutes. Heat-treated bacteria were tested to ensure no presence of residual spore formers by plating on MYPGP agar plates and incubating at 37^{o}C for 6-7 days.

4.2.4 Phage Cocktails

Phage cocktails were created as described in Chapter 3. Briefly, phage titers were normalised to approximately 1x10^{8} PFU/ml and 1 mL of each phage was combined into a cocktail. When making larval food, phage lysate was used instead of water. This diluted the concentration of the phage cocktail to approximately 5x10^{7} PFU/ml. Each honey bee larvae was fed 10 μl of food which resulted in the final concentration of phage cocktail administered to each larvae being approximately 5x10^{5} PFU.

4.2.5 Larval Food Preparation

Larval food was prepared as per (Schmehl et al., 2016). Larvae were fed three different diets depending on the number of days post-grafting, changes in food were based on the needs of larvae as they develop. The compositions of the three diets are outlined in Table 4.1. Glucose and fructose were mixed into sterile water until completely dissolved, then yeast extract was added and completely dissolved; finally, the royal jelly was added and mixed until homogenous. Larvae were fed increasing amounts of the appropriate diet type based on the number of days post-grafting. The compositions of the three diets and diet type and amount fed each day are outlined in Table 4.1 and Table 4.2. Larval food was formulated so that phage cocktail, bacterial spores, and lysed bacterial cells were used in place of the water component

of diet A. Phage cocktail was added on Day 0 post grafting and spores or lysed bacterial cells were added on Day 1 post grafting.

Table 4.1. Amount (g) of diet components to feed approximately 400 larvae. Diet changes from A to B to C as larval requirements change due to the development of the larvae

Diet component	A	B	C
Royal Jelly	4.43	4.30	25.00
Glucose	0.53	0.64	4.50
Fructose	0.53	0.64	4.50
Yeast Extract	0.09	0.13	1.00
Water	4.43	4.30	15.00
Total	**10**	**10**	**50**

Table 4.2. Diet type and amount for each feeding

Days after Grafting	Diet	Amount of diet (µl)
0	A	10
1	A	10
2	B	20
3	C	30
4	C	40
5	C	50
6	C	50
7	C	60

4.2.6 Larval Rearing

Experiments were undertaken in late Summer/early Autumn. Larvae were reared in a similar manner to those previously described (Schmehl et al., 2016). Queens from four

different hives were confined on a bee hive frame within a queen excluder cage and released after 24 hours. Frames were left in the hive for a further 48 hours before being brought into the lab (Figure 4.1A). Honey bee larvae were carefully harvested into pre-warmed 48-well plate plates containing queen cell cups, pre-filled with the appropriate food (Figure 4.1B).

The original experimental design was to have three plates per hive, per treatment (Figure 4.2A). Unfortunately, the queens did not lay as many harvestable eggs as expected so a new experimental design was made and four hives were used and a differing number of plates was harvested from each hive (Figure 4.2B).

Each 48-well plate was configured as per Figure 4.3. Negative control larvae were fed unamended food throughout the experiment as per Table 4.2. The negative control had nine replicates with n = 8, for a total of 72 larvae per treatment.

Phage control larvae were fed $5x10^5$ PFU phage cocktail on Day 0 post grafting, they were fed unamended food throughout the rest of the experiment as per Table 4.2. The phage control had nine replicates with n = 8, for a total of 72 larvae per treatment.

Spore control larvae were fed 1000 spores on Day 1 post grafting; they were fed unamended food throughout the rest of the experiment as per Table 4.2. The spore control had nine replicates with n = 10, for a total of 90 larvae per treatment.

Lysed cell control larvae were fed 1000 cells on Day 1 post grafting; they were fed unamended food throughout the rest of the experiment as per Table 4.2. The lysed cell control had nine replicates with n = 10, for a total of 90 larvae per treatment.

Treatment larvae were fed $5x10^5$ PFU phage cocktail on Day 0 post grafting and 1000 spores on Day 1 post grafting, they were fed unamended food throughout the rest of the experiment as per Table 4.2. The treatment larvae had nine replicates with n = 10, for a total of 90 larvae per treatment.

On grafting day (Day 0), negative control, spore control, and lysed cell control wells contained 10 µl of diet A. Phage control and treatment wells contained 10 µl of diet A made with the phage cocktail instead of water to an approximate PFU of $5x10^5$. Plates were incubated in desiccators at 35°C and 90% humidity for 24 hours.

On Day 1 post grafting, negative control and phage control larvae were fed 10 µl of diet A. Spore control and treatment wells were fed 10 µl of diet A with approximately 100 spores per 1 µl. Lysed cell wells were fed 10 µl of diet A with approximately 100 lysed cells per 1 µl. After Day 2 post grafting all wells were fed the appropriate diet type and amount of diet as per Table 4.2.

Plates were checked daily for signs of larval death by microscopy before feeding. Signs of larval death included uneaten food, black spots, deflation, and lack of movement (Schmehl et al., 2016). Dead larvae were removed from plates. After Day 4, samples of dead larvae were placed in Eppendorf tubes with sterile water and stored at -20°C for future analysis of *P. larvae* bacteria. Larval death was recorded daily.

4.2.7 Statistical analysis

Kaplan–Meier analysis and log-rank tests for pair-wise comparison between each group was undertaken using the survival package in R. A *P* value of less than 0.05 was considered significant.

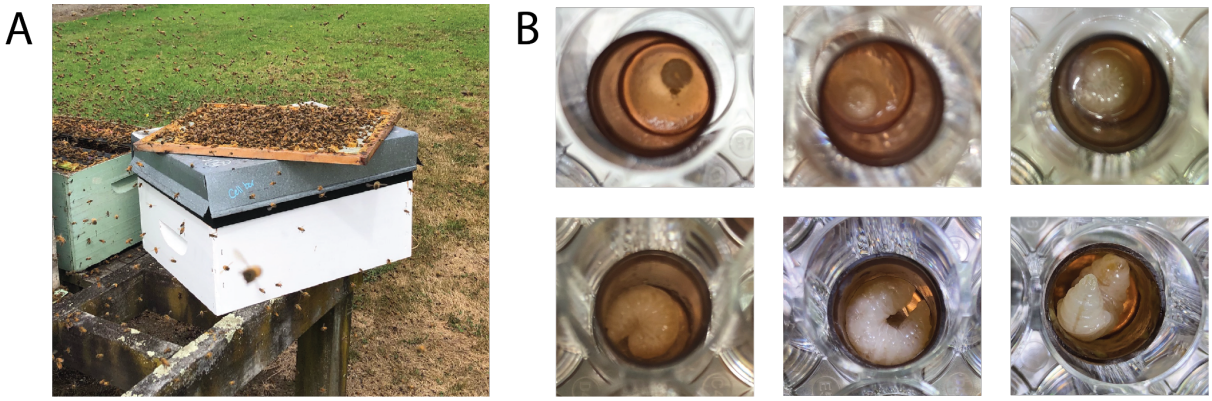


Figure 4.1 Bee Hives and larvae from *in-vivo* experiments

A) Beehive with frame placed on top. B) Honey bee larvae in queen cups filled with food. From left to right Day 2 post grafting until Day 7 post grafting.

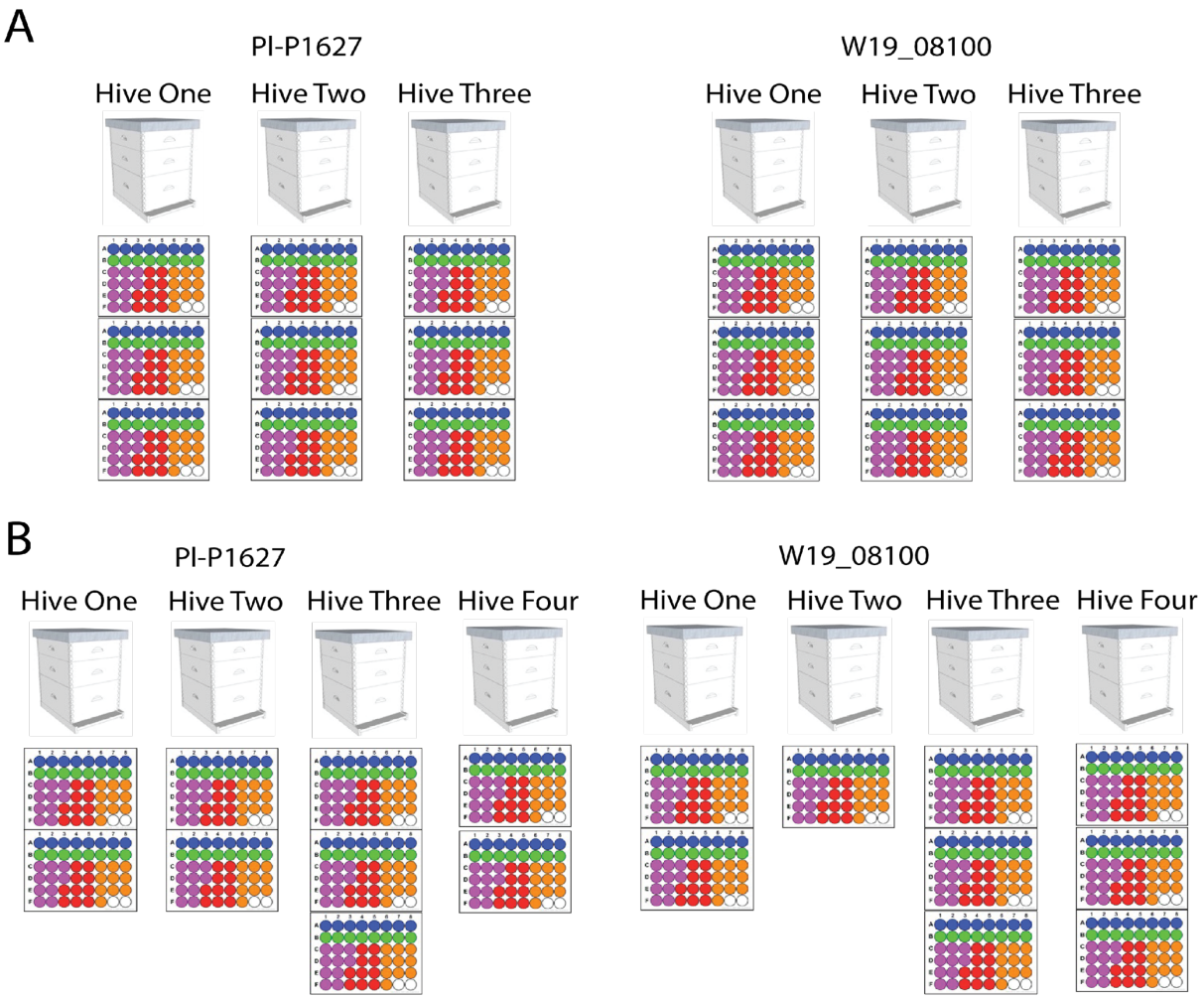


Figure 4.2 Experimental Design of *in-vivo* experiments

A) Proposed experimental design, using three bee hives to propagate three 48-well plates per treatment. B) Actual experimental conditions due to poor queen laying: four bee hives to propagate varying numbers of plates per treatment.

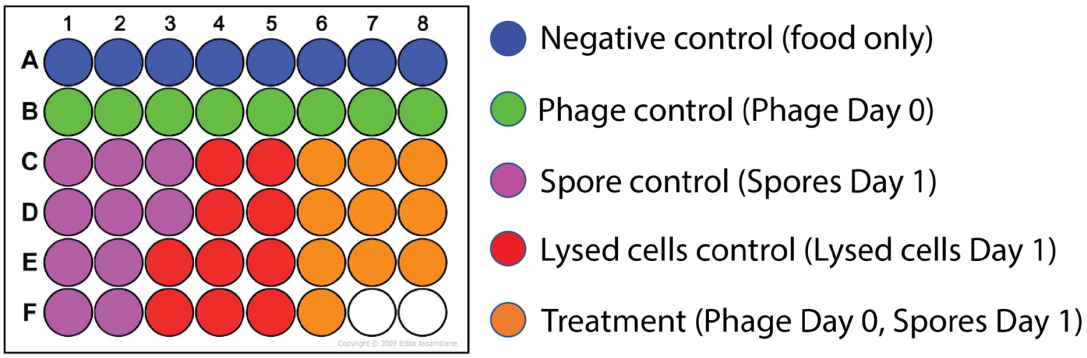


Figure 4.3 Plate design for each plate replicate

Negative control n = 8, phage control = n = 8, spore control n = 10, lysed cell control n = 10, treatment n = 10.

4.3 Results/Discussion

Due to unforeseen circumstances caused by lockdowns during the COVID-19 pandemic, experiments were not conducted during summer as originally planned, and were started in late Summer/early Autumn. It became evident during grafting, that queens had not been laying eggs consistently or of high quality. It was difficult to graft the required number of eggs per hive, per treatment and an additional backup beehive was used. We continued in the hope the larvae that were grafted would be of sufficient health for the experiment to succeed.

Schmehl *et al*. found consistent survival of over 95% when rearing larvae in the laboratory from grafting to bee emergence (Schmehl et al., 2016). When implementing this protocol we observed 10% - 20% death across all treatments only one-day post grafting; this was already two to four fold more larval death than Schmehl *et al*. observed during their whole protocol (Figure 4.4). We decided to continue the experiment and put this initial high number of deaths down to weak larvae post-grafting. We continued to see a steep drop in surviving

larvae, with the median survival across all treatments being 3 days (Figure 4.4). After Day 3, larval death across all treatments continued but at a slower rate. In similar experiments undertaken by a laboratory in the US, survival of negative controls and phage controls was 84.4% - 88.8% and survival of spore controls was 45.5% at Day 8 (Ghorbani-Nezami et al., 2015).

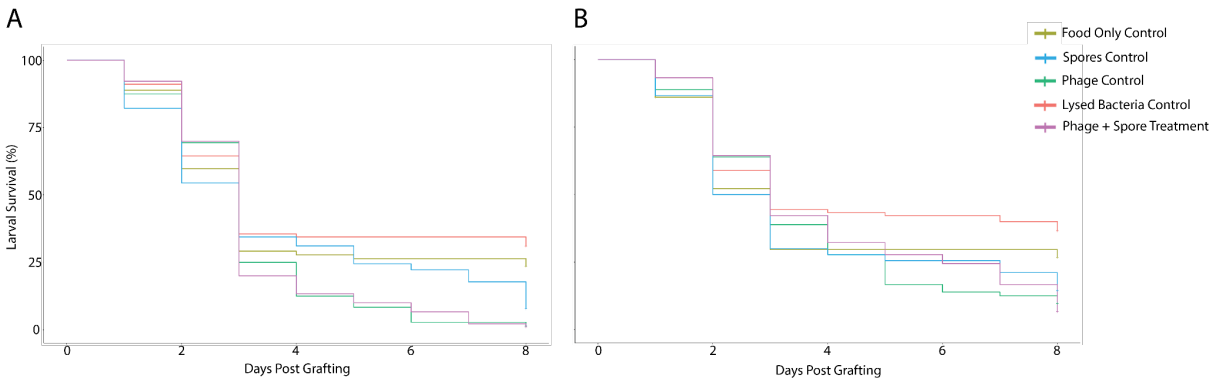


Figure 4.4 Survival rate (%) of honey bee larvae at each day post grafting under five different treatment types.

A) Phage sensitive *P. larvae* W19_08100. B) Phage resistant *P. larvae* Pl-P1627. Phage cocktails were fed to larvae on Day 0 (grafting day) and spores/lysed bacteria were fed to larvae on Day 1 (1 day post grafting).

In the group being tested on *P. larvae* strain W19_08100, survival rate on Day 8 of the negative, phage, spore and lysed controls was 24%, 1%, 8%, and 33% respectively. The larvae treated with phage on Day 0 and spores on Day 1 had a survival rate on Day 8 of 2%.

There was no statistically significant difference in survival rate between the negative and spore-only controls (Log-rank, $P = 0.065$) or the negative and lysed cells controls (Log-rank, $P = 0.29$). In other words, whether the bacterial pathogen was added to the well or not, the larvae were dying in this experiment.

There was, however, a statistically significant difference between the survival rate of the negative and phage control (Log-rank, P = 0.03). This was our first indication that the application of phages in this experiment had a detrimental effect on survival. Similarly, the negative control and phage/spore treatment showed a statistical significance in survival (Log-rank, P = 0.017) with the application of phages and spores decreasing survival.

The observation that the phage-treated groups had a lower survival rate in comparison to the negative control was puzzling. Two hypotheses come to mind. The first is that residual chloroform in the lysates may have killed the larvae. The phage lysates were treated with chloroform as part of the process to remove any remaining bacteria, as described in step 3 (phage clean-up) of the Phage on Tap Protocol (Bonilla et al., 2016). The remaining steps of this protocol were not followed because we are growing a Gram-positive organisms and we therefore did not believe endotoxins would be present. The chloroform was removed by centrifugation of the phage lysates but residual chloroform could have still been present in the cocktails and had a negative impact on the larvae.

The second hypothesis is that a toxin in the lysates may have killed the larvae. Subsequent to these experiments, phage Dash (in cocktail Two) was found to have a toxin that contributes to the virulence of *P. larvae* (this toxin will be described in more detail in Chapter 6). There are two possible ways for this toxin to contribute to the negative effects observed when cocktail Two is applied to larvae, 1) the toxin was present in the Dash lysate or 2) the toxin gene was transcribed and translated in the larval gut. Dash is unable to amplify on either of the two bacterial strains tested, so it is unlikely that Dash produced the toxin during the experiments and the phage-only treatment also had a negative effect, demonstrating the *P. larvae* strains were not required. Transcription and translation of the toxin gene could

however have occurred through infection of a bacterial strain already present in the gut microbiome.

We expected that if the phage cocktail treatment had a deleterious effect on the larvae in the *P. larvae* W19_08100 challenge then similar results would be observed in the *P. larvae* Pl-P1627 challenge trial as well. Whilst we observed a qualitative difference in the survival of the larvae in the presence of phage cocktail, this was not statistically significant, possibly owing to the poor health of the larvae. For example, in the group being tested on the recalcitrant *P. larvae* strain Pl-P1627, the survival rate on Day 8 of the negative, phage, spore and lysed cell controls was 28%, 10%, 14%, and 37% respectively. The larvae treated with phage on Day 0 and spores on Day 1 had a survival rate on Day 8 of only 7%. When comparing the survival rate of the negative control against all other treatments, no statistically significant difference was observed.

The viability at day 8 compared between the negative control compared to the spore control was not distinguishable (Log-rank, $P = 0.19$). Similarly, the negative control vs the phage cocktail alone were too similar to be distinguished (Log-rank, $P = 0.28$). The negative control compared to either the lysed cells control (Log-rank, $P = 0.18$) or the phage cocktail and spore treatment were also not statistically different (Log-rank, $P = 0.2$).

As stated, we had expected that if the phage cocktail had a negative impact on the survival of the larvae in one bacterial strain challenge, it would have a negative impact on the larvae in both strain challenges. One outside possibility would be that in the case of *P. larvae* W19_08100, there are two phages in cocktail Two that are able to replicate on this strain. It

is worth noting that the toxin mentioned above (which will be discussed in more detail in chapter 6) is not present in either of these two phages.

Taken together, it is evident that these experiments were inconclusive. Previous experiments undertaken in the US (Ghorbani-Nezami et al., 2015) suggest that phage cocktails can prophylactically protect honeybees. We have several hypotheses about why we did not observe this here. It is likely that our late start (due to Covid-19 lockdowns in New Zealand) reduced the survival of our larvae. We certainly did not replicate the finding that 95% of lab-reared larvae could be kept alive (Schmehl et al., 2016). Our low larval survival rate was due to conditions other than the treatments each larvae was given.

These experiments need to be repeated to ensure that phage cocktails do not negatively impact larvae. It will be beneficial to undertake steps to remove toxins, which are also present in the *P. larvae* ERIC I bacterial strains, before further challenge experiments. Methods have previously been described to remove protein toxins from phage lysates using both ultrafiltration and anion exchange chromatography (Hietala et al., 2019). It will also be prudent to test cocktails without the toxin-containing phage. These experiments were not tried again as the queens were not ready to start laying again until late spring/early summer and this was beyond the time during which experiments were being conducted for this thesis.

4.4 References

Bonilla, N., Rojas, M. I., Netto Flores Cruz, G., Hung, S.-H., Rohwer, F., & Barr, J. J. (2016). Phage on tap-a quick and efficient protocol for the preparation of bacteriophage laboratory stocks. *PeerJ*, *4*, e2261.

Brady, T. S., Merrill, B. D., Hilton, J. A., Payne, A. M., Stephenson, M. B., & Hope, S. (2017). Bacteriophages as an alternative to conventional antibiotic use for the prevention or treatment of *Paenibacillus larvae* in honeybee hives. *Journal of Invertebrate Pathology*, *150*, 94–100.

de Graaf, D. C., Alippi, A. M., Antúnez, K., Aronstein, K. A., Budge, G., De Koker, D., De Smet, L., Dingman, D. W., Evans, J. D., Foster, L. J., Fünfhaus, A., Garcia-Gonzalez, E., Gregore, A., Human, Hannelie, Murray, K. D., Nguyen, B. K., Poppinga, L., Spivak, M., van Engelsdorp, D., … Genersch, E. (2015). Standard methods for American foulbrood research. *Journal of Apicultural Research*, *52*(1), 1–28.

Ghorbani-Nezami, S., LeBlanc, L., Yost, D. G., & Amy, P. S. (2015). Phage Therapy is Effective in Protecting Honeybee Larvae from American Foulbrood Disease. *Journal of Insect Science* , *15*(1), 84–85.

Henry, M., Lavigne, R., & Debarbieux, L. (2013). Predicting in vivo efficacy of therapeutic bacteriophages used to treat pulmonary infections. *Antimicrobial Agents and Chemotherapy*, *57*(12), 5961–5968.

Hietala, V., Horsma-Heikkinen, J., Carron, A., Skurnik, M., & Kiljunen, S. (2019). The Removal of Endo- and Enterotoxins From Bacteriophage Preparations. *Frontiers in Microbiology*, *10*, 1674.

Lin, D. M., Koskella, B., & Lin, H. C. (2017). Phage therapy: An alternative to antibiotics in the age of multi-drug resistance. *World Journal of Gastrointestinal Pharmacology and Therapeutics*, *8*(3), 162–113.

Schmehl, D. R., Tomé, H. V. V., Mortensen, A. N., Martins, G. F., & Ellis, J. D. (2016). Protocol for the in vitro rearing of honey bee (Apis mellifera L.) workers. *Journal of Apicultural Research*, *55*(2), 113–129.

Chapter 5

In-Vitro Evolution to Increase the Titers of Difficult Bacteriophages: Rapid Appelmans Protocol

Chapter submitted to PHAGE as:

Danielle N. Kok, Joanne Turnbull, Nobuto Takeuchi, Philippos K. Tsourkas, and Heather L. Hendrickson. *In Vitro* Evolution to Increase the Titers of Difficult Bacteriophages: Rapid Appelmans Protocol

Abstract

Bacteriophages (phages) are becoming increasingly important in the race to find alternatives to antibiotics. Unfortunately, phages that might otherwise be useful are sometimes discarded due to low titers making them unsuitable for downstream applications. Here, we present two distinct approaches to experimentally evolve novel New Zealand *Paenibacillus larvae* phages. The first approach uses the traditional agar-overlay method, whereas the other was a Rapid Appelmans Protocol (RAP) modelled after the established Appelmans Method. Both approaches resulted in an increase in plaque-forming units (PFU/mL). The RAP approach was significantly faster and simpler, and allowed us to evolve a set of phages in as little as four days, increasing titers 100-1000 fold relative to their ancestors. The resultant titers were sufficient to extract and sequence DNA from these phages. An analysis of these phages' genomes is provided. We also propose a model that describes the parameters that allow the RAP approach to select improvement of phage titer. The RAP approach is an effective method for experimentally evolving previously intractable phages in a high-throughput and expeditious manner.

5.1 Introduction

Bacteriophages (phages) are the most abundant entities on the planet, with an estimated number of $1x10^{31}$ virus particles, named the 'Hendrix product' (Hendrix et al., 1999; Mushegian, 2020). The discovery, purification, and sequencing of Actinobacteriophages in undergraduate classrooms through the adoption of the Howard Hughes Medical Institute-sponsored SEA-PHAGES (Science Education Alliance Phage Hunters Advancing Genomics and Evolutionary Science) programme has brought personal empowerment through discovery to undergraduate classrooms around the globe (Hanauer et al., 2017; Russell & Hatfull, 2017). This experience has also given some the impression that phages are universally discovered and isolated with ease. The facile and frequent discovery of new Mycobacteriophages belies the lived experience of researchers who have embarked on phage hunts, only to discover that phages, even some Mycobacteriophages, are not amenable to study (LeMieux & Hatfull, 2020). One of the crucial steps in novel phage isolation is to achieve titers of over ~$5x10^9$ PFU mL^{-1}, a commonly used threshold concentration of particles for electron microscopy and DNA extraction. Historically, physiological studies of phages have often been conducted at concentrations of at least $1x10^8$ PFU mL^{-1} (Delbrück, 1940; Ellis & Delbrück, 1939), although these concentrations come with their own challenges (Abedon, 2016). Achieving these high concentrations of phage particles can prove difficult for reasons that are not clear, and can subsequently derail the discovery, description, and application of newly discovered phages.

DNA sequencing is a key step in the evaluation of phages for practical use. DNA sequencing revealed aspects of the life cycle and gene content of novel phages that were not obvious by other means, including their genetic diversity and gene content, genes associated with lysogeny, host toxins, or antibiotic resistance. Genome sequences can and should

influence the selection of phages for application (Philipson et al., 2018; Skurnik et al., 2007). Although genetic sequencing cannot replace *in vitro* or *in vivo* testing, it is an invaluable tool for eliminating inappropriate candidates. Complete genome sequencing of novel phages also contributes to the discovery and development of useful phage encoded enzymes and our understanding of their biology. Finally, sequencing and publishing phages furthers our understanding of the evolution of the most diverse and under sampled entities on the planet (Rohwer, 2003).

In an effort to discover phages for practical use in the apiculture industry in New Zealand, we conducted a phage hunt using the honey bee pathogen, *Paenibacillus larvae*. *P. larvae* is a spore-forming bacterium that leads to the honey bee disease known as American Foulbrood (AFB). AFB is deleterious at both the larval and whole-hive level and can cause irreversible damage to the beehive (Elke Genersch, 2010). There have already been efforts from several laboratories around the world to try and find phages to prophylactically treat AFB, with promising results (Brady et al., 2017; Yost et al., 2016). We encountered a set of plaque-forming *P. larvae* phages that could, with effort, be brought to a titer of $3x10^7$ mL^{-1} but these proved intractable to efforts to further increase their concentrations.

A search of the literature led us to the Appelmans protocol, a 96 well-based procedure that has been used to expand the host range of phages by allowing strains to become simultaneously infected by multiple phages and allowing natural selection to screen recombinant phages for the most successful new chimaeras (Burrowes et al., 2019). Herein, we report two separate approaches we used to experimentally evolve our *P. larvae* phages, which allowed us to significantly increase their effective titer. The first approach was the

experimental evolution and propagation of a phage in four parallel lineages for 25 days using a relatively low MOI of 0.05 in solid media and an agar overlay method. The second approach was a modified Appelmans protocol (Burrowes et al., 2019) that was initially performed for 30 days. We modified this approach further by allowing pure phages to adapt to single hosts rather than propagating mixed populations of phages, thereby allowing natural selection to screen mutant phages for those most able to infect the strain in liquid conditions. Subsequently, we employed this to great effect after only four days (Rapid Appelmans Protocol or RAP).

We describe both these approaches and the quality and speed of the titer improvements achieved. We report on the genomic traits of six separate phages that were evolved and subsequently sequenced using the modified Appelmans protocol. In addition, we modelled the population biology parameters operating in this brief experiment in an effort to understand the conditions under which mutation and selection act in this high-speed adaptive evolution protocol.

5.2 Materials and Methods

5.2.1 Bacterial and Phage Strains

All bacterial strains and phage isolates are listed in Table 5.1. Three strains of *P. larvae* were used, isolated from beehives in New Zealand with symptoms consistent with American Foulbrood disease. *P. larvae*-PaFR-2017 and *P. larvae*-PaFR-2006 were both isolated from infected honeybee larvae provided by Plant and Food Research. *P. larvae*-F1A was isolated from a symptomatic brood comb provided by AsureQuality in December 2018. The phages chosen for the 30-day protocol were all isolated from samples of soil from around healthy

beehives. Phages Callan and Dash were from an apiary in the lower North Island, Phage Lilo was from an apiary in the Greater Auckland region, Phage Logan was from an apiary on the East Coast of North Island, Phage AJG77 was an apiary in the lower South Island, and Phage ABAtENZ was from an apiary in the central North Island. The phages chosen for the four-day RAP protocol were also isolated from around healthy beehives. Phage Wildcape and LunBun from an apiary in Gisborne, Phage Carlos and ApiWellbeing were isolated from an apiary in the Wellington region, Phage FutureBee isolated from an apiary in Hamilton, and Phage Rae2Bee1 isolated from an apiary in Ashburton. Bacterial strains were grown in Brain Heart Infusion Broth (BHI) (Oxoid CM1135). Phages were grown by infecting the bacterial strain they were isolated on (see Table 5.1), using the agar overlay method. Phage titers were established by plating serial dilutions of the phage lysates using the agar overlay method.

Table 5.1. *P. larvae* strains and phage isolates used in this study

Bacterial/Phage Strain	Source	Isolation Source	Geographical Region	Notes	Accession No.
P. larvae-PaFR-2017	Plant & Food Research	Infected larvae	Auckland		JARDRG000000000
P. larvae-PaFR-2006	Plant & Food Research	Infected larvae	Hamilton		JARDAI000000000
P. larvae-F1A	AsureQuality	Symptomatic Brood Frame	North Canterbury		JARDRL000000000
Phage Callan	This work	Soil	Wellington	Isolated on Pl-PaFR-2006	OP503989
Phage Dash	This work	Wax	Wellington	Isolated on Pl-PaFR-2006	OP503990
Phage Lilo	This work	Soil	Waikato	Isolated on Pl-F1A	OP503991
Phage Logan	This work	Soil	Gisborne	Isolated on Pl-PaFR-2017	OP503980
Phage AJG77	This work	Soil	Otago	Isolated on Pl-PaFR-2017	OP503969
Phage ABAtENZ	This work	Soil	Waikato	Isolated on Pl-PaFR-2017	OP503968
Phage Wildcape	This work	Soil	Gisborne	Isolated on Pl-F1A	OP503988
Phage Carlos	This work	Soil	Wellington	Isolated on Pl-F1A	OP503973
Phage ApiWellbeing	This work	Soil	Wellington	Isolated on Pl-F1A	OP503970
Phage LunBun	This work	Soil	Gisborne	Isolated on Pl-F1A	OP494865
Phage FutureBee	This work	Soil	Waikato	Isolated on Pl-TP	OP503975
Phage Rae2Bee1	This work	Soil	Ashburton	Isolated on Pl-TP	OP503983

5.2.2 Experimental evolution to increase the infectivity of P. larvae phage Lilo

Multiplicity of infection (MOI) was established by plating serial dilutions of both colony-forming units (CFU) and plaque-forming units (PFU). Specifically, a single-colony isolate of the *P. larvae* strain was inoculated in 5 mL of BHI, incubated at 37°C and shaken at 100 rpm for 2 days. This *P. larvae* liquid culture was serially diluted to a total dilution factor of 10^{-8} and 5 µL of each dilution was applied to 1.5% agar BHI plates. Plates were incubated without shaking at 37°C for two days. Colonies were counted to determine the number of CFU per mL of culture. PFU determination was similar, the *P. larvae* phage Lilo lysate was serially diluted to a factor of 10^{-8} and 5 µL of each dilution was applied as a spot test to a *P. larvae* bacterial lawn plated in 0.5% BHI top agar. The plate was incubated without shaking at 37°C for two days. The number of plaques observed for each dilution was counted to estimate the titer of the lysate.

Four biological replicates of phage Lilo were serially passaged at a low MOI. At each serial passage, 500 µL of liquid bacterial culture was inoculated with lysate for an estimated MOI of 0.05 ($2x10^6$ PFU were plated with ~$4x10^7$ CFU). These were incubated without shaking at room temperature for 30 minutes to facilitate adhesion before they were plated in 3 mL of 0.5% top agar BHI with 1 mM $CaCl_2$, 1 mM $MgCl_2$ and 1mM thiamine hydrochloride, onto 1.5% agar BHI plates. Plates were incubated without shaking at 37°C for two days. The confluent or cleared plates were flooded with 8 mL BHI broth and incubated without shaking at room temperature for 2-4 hours. The resulting lysate was collected and passed through a 0.45 µm syringe filter, in preparation for use in the next passage. Lysates from each passage were titered by spot plate assay. Plaque counts were used to determine lysate titers from each passage and used to adjust the volumes plated in subsequent passages to maintain the desired

MOI of 0.05. Plaque diameters were measured using ImageJ software (Abràmoff & Magalhães, 2004).

5.2.3 Modified Appelmans Protocol in 96-well plates

We implemented the modified Appelmans protocol as in Burrowes *et al.* with changes to the media and by restricting the horizontal wells in the 96-well plate to a single strain of host and a single strain of phage (Burrowes et al., 2019). Using a 96-well plate, we seeded 100 µL of serially diluted phage lysate in 100 µL double-strength BHI Broth containing 4 µL culture of a single strain (Figure 5.2A). Plates were incubated at 37°C on a shaking platform at 100 rpm. Wells showing complete lysis, plus the first turbid well, were pooled. If no lysis was observed, wells containing undiluted phage were harvested. The pooled lysates were sterilised by vortex mixing with 1:100 $CHCl_3$, left for 10 minutes and subsequently centrifuged at 15,000g for 15 minutes. Lysates were removed, leaving the $CHCl_3$, and transferred to fresh tubes for storage.. These pooled lysates were used to initiate the next round of directed evolution in a similar manner. Pooled lysates were tested for titer after every second round using the agar overlay spot titer method.

5.2.4 Genomic DNA Extraction

Evolved phages were triple purified by selecting a single plaque from a plate and using this to infect in a standard agar overlay. Phages from the final round of purification were used to inoculate a 60 mL culture of the bacterial strain they were isolated on at a multiplicity of infection (MOI) of 0.1. Cultures were incubated at 37°C with shaking at 100 rpm overnight. Phage lysates were purified as described previously (Bonilla et al., 2016). Briefly, an aliquot of phage lysate was transferred into a falcon tube and centrifuged at 4,000 × g for 20 min; the

supernatant was collected and transferred into a new falcon tube. The lysate was filter-sterilised through a 0.22 µm filter and chloroform was added to bring the total volume of the lysate to 1.10 (0.1 of volume in chloroform). The lysate was then vortexed and incubated at room temperature for 10 min. The lysate was then centrifuged at 4,000 × g for 5 min and transferred into Nalgene Oak Ridge High-Speed PPCO Centrifuge Tubes, leaving the chloroform behind. The sterilised phage lysate was then concentrated by centrifugation at 18,000 rpm for 45 minutes. Subsequently, 50 mL of this pelleted lysate was re-suspended into a 5 mL volume of BHI. The phage DNA was extracted using a modified zinc chloride precipitation method (Santos, 1991). Modifications included the addition of 1 µL Proteinase K which was incubated at 37°C for 10 min after the TES step. The tubes were left on ice overnight after the isopropanol was added. On day 2 of the protocol 1 µL of pure glycogen was added to each tube before centrifugation to aid in pelleting and visualisation of the DNA. DNA pellets were resuspended in 50 µL nuclease-free water.

5.2.5 Library Preparation and Sequencing

A total amount of 1 µg DNA per sample was used as input material for the DNA sample preparations. Sequencing libraries were generated using NEBNext® Ultra™ DNA Library Prep Kit for Illumina (NEB, USA) following the manufacturer's recommendations and index codes were added to attribute sequences to each sample. Briefly, the DNA sample was fragmented by sonication to a size of 300 bp. DNA fragments were end-polished, A-tailed, and ligated with the full-length adaptor for Illumina sequencing with further PCR amplification. At last, PCR products were purified (AMPure XP system) and libraries were analysed for size distribution by Agilent2100 Bioanalyzer and quantified using real-time PCR. The clustering of the index-

coded samples was performed on a cBot Cluster Generation System according to the manufacturer's instructions. After cluster generation, the library preparations were sequenced on an Illumina NovaSeq 6000 platform and paired-end reads were generated.

5.2.6 Genome Assembly

Phage genome assembly was carried out using Geneious 9.05 (Auckland, New Zealand) (https://www.geneious.com), using the built-in Geneious assembler, with either Medium Sensitivity/Fast or Medium-Low Sensitivity/Fast, and circularise contigs with matching ends selected.

The genome ends and DNA packaging strategy were identified by sequence similarity to already published *P. larvae* phages (Stamereilers et al., 2018). Phages were searched for the two known 3′ overhang sequences "CGACGGACC" or "CGACTGCCC" near the terminase genes (Stamereilers et al., 2018). Phages AJG77, ABAtENZ and Logan were found to have the "CGACGGACC" sequence, while Dash, Callan and Lilo contained the "CGACTGCCC" sequence. Genomes were rearranged so these 3′ overhang sequences were at the end of the genome. This resulted in the small terminase gene starting at either 50 or 51 base pairs downstream of base 1.

Genes were identified by running the rearranged files through Phage Commander (Lazeroff et al., 2021) with all gene identification programs selected. The GenBank-formatted output files were entered in DNA Master (cobamide2.bio.pitt.edu) to manually check for false positives, missing genes and identify start codons as described in detail in (Salisbury & Tsourkas, 2019). Putative protein functions were assigned as described in previous work (Stamereilers et al., 2018).

5.2.7 Host Range Assays

The ability of phages to infect an isolate was assessed by 3 µl spots of a phage lysate onto a double-layer agar containing 500 µl of bacterial lawn. Each *P. larvae* bacterial isolate was tested separately.

5.2.8 Simulation to Model Mutation Frequency in Phages

We constructed a mathematical model to estimate the number of mutations that could develop in this RAP experiment. The numbers of available cells, phages and time have been input from the experimental protocol. The model to simulate the number of phage mutants at the end of 4 days of experimental evolution assumes the following kinetic equations:

The first equation represents the infection of a bacterium by a phage:

$$\mathrm{B} + \mathrm{P}_0 \xrightarrow{\beta_0} \mathrm{C}_0, \qquad (1)$$

where B is a bacterium, P_0 is a phage with genotype 0, β_0 is the infectivity of P_0, and C_0 is a bacterium infected by P_0.

The second equation represents the lysis of an infected bacterium:

$$\mathrm{C}_0 \xrightarrow{l} (1-U)b_0\mathrm{P}_0 + Ub_0\mathrm{P}_1, \qquad (2)$$

where l is the rate of lysis, U is the mutation rate per replication per genome, b_0 is the burst size of P_0, and P_1 is a phage with genotype 1. The value of b_0 is assumed to be constant.

There are two more equations, which describe the infection and lysis by P_1:

$$\begin{aligned} \mathrm{B} + \mathrm{P}_1 &\xrightarrow{\beta_1} \mathrm{C}_1 \\ \mathrm{C}_1 &\xrightarrow{l} b_1 \mathrm{P}_1, \end{aligned} \tag{3}$$

where β_1 is the infectivity of P_1, and b_1 is the burst size of P_1. We assume that $b_1 = b_0(1+s_b)$ and $\beta_1 = \beta_0(1+s_\beta)$, where s_b and s_β are the selection coefficients. For example, if $s_b = 1$, the burst size of P_1 is twice that of P_0.

For simplicity, we do not assume the spontaneous decay of phages or that there are more than two genotypes of phage. The above kinetic equations are simulated with the Gillespie algorithm. The model assumes 10 tubes, each containing 40,000 B at the beginning as in the experimental evolution described above.

At the start of each simulation, the initial numbers of P_0 in different tubes are set to numbers drawn from Poisson distributions with the following means: 1,000 (zeroth tube), 100 (first tube), 10 (second tube), etc. Thus, about three tubes will have any phage particles at the beginning of Day 0. The initial number of P_1 is set to zero in all tubes. The above initial condition is equivalent to assuming that the concentration of phage particles obtained from the solid medium before the experimental evolution is ten phage particles per µL. All tubes are incubated until all bacteria (B) are lysed in the tubes that have at least one phage particle P_0 or P_1.

The tubes that have at least one phage particle are pooled, plus one tube that has no phage particles. All tubes are assumed to contain 200 µL of media. 100 µL of the pooled media is added to the zeroth tube, 10 µL to the first tube, 1 µL to the second tube, etc. The number of phage particles in each tube is again determined by drawing a number from a Poisson distribution with the corresponding mean number of phage particles.

The tubes are incubated again until all bacteria are lysed in the tubes that have at least one phage particle. In a typical simulation, about five tubes contain phages from Day 1. The above repeats up to three transfers, i.e., four days.

One hundred simulations were run for each condition tested. Any arbitrary large value could be used, we also spot-checked running the model with one thousand simulations with similar results. Parameters were varied within experimentally reasonable values as described in the results.

5.3 Results

Using strains of *P. larvae* as hosts, 26 novel *P. larvae* phages were isolated using standard phage discovery methods. Briefly, direct isolation and enrichment methods were employed and phages able to form plaques were discovered and isolated from hive materials, bee debris and soil from around or within healthy, non-infected hives. The complete process of the discovery of these *P. larvae* phages is described in Chapter 3.

The plaques of the six phages used in the 30-day modified Appelmans Protocol were pinprick size plaques in 0.5% top agar. We employed a suite of standard methods for increasing the titers of these phage lysates but none were successful (webbed plates, liquid infections, large volume infections and enhanced centrifugation). The titers of each lysate generally remained between 5×10^5 mL^{-1} or up to 3×10^7 mL^{-1}, 1,000-10,000 times too low for DNA extraction, electron microscopy, or downstream *in-vitro* testing.

Unable to proceed further in our characterisation, we chose to experimentally evolve these phages in the hopes that by selecting for increased efficiency in host infection we would

be able to increase the titers. We first employed a method that used an agar overlay, reasoning that we had observed plaques but had not had success in propagating these phages in liquid during attempts to perform burst size assays.

5.3.1 Agar Overlay Method of Evolution Improves Titer

We developed an experimental protocol in which phages were serially propagated in top agar overlays with an abundance of host bacteria. We chose phage Lilo and performed infections using a relatively low MOI of 0.05 (1 phage for 20 bacterial cells) reasoning that phages capable of rapid infection and higher burst sizes would be favoured. Four low MOI Lilo lysates were continually harvested and propagated on the ancestral bacterial strains for 25 days (Figure 5.1A). Phage titers were calculated after each overnight passage for all Lilo replicate lines (Lilo E, F, G and H). From the initial titer of 3×10^7 PFU mL^{-1} for the starting lysate, after 15 passages an increase in titer of 4.1 to 14.5 fold was observed in three out of four lines (Figure 5.1B, n = 1). Those three lines maintained an average 5 to 7-fold increase from the starting lysate's titer. Lilo E maintained an average increase of 3 fold throughout the experiment.

5.3.2 Plaque Improvements from Agar Overlay Method

Plaque size changes can indicate a mutation has taken place in a phage of interest (Gallet et al., 2011). The ancestral Lilo phages created plaque diameters that measured at 0.70 mm (+/- 0.05) (mean, n = 10). In order to assess whether the experimental evolution was having an effect on the phages, diameters of the evolved Lilo plaques were measured after each overnight passage. After 15 passages a 56% to 121% increase in the mean diameter of plaques was observed in all four evolved Lilo lines, with lines E-H measuring an average of 1.09

(+/- 0.06) mm, 1.52 (+/- 0.04) mm, 1.55 (+/- 0.11) mm and 1.36 (+/- 0.07) mm respectively (Figure 5.1C). The increase in plaque diameter was maintained throughout the remainder of the 25-day experiment; however, some of these apparent gains were not retained in the course of the selection. Lines F and G saw the greatest increase in plaque size (Figure 5.1D), measuring 1.31 (+/- 0.05) mm and 1.40 (+/- 0.07) mm respectively at the end of the 25 days, while lines E and H measured 0.91 (+/- 0.04) mm and 1.06 (+/- 0.06) mm respectively (Figure 5.1C). Ultimately, the maximum increase in plaque size at the end of the experiment was Lilo G (1.40 (+/- 0.07) which doubled from the initial Lilo plaque size of 0.70 (+/- 0.05) mm.

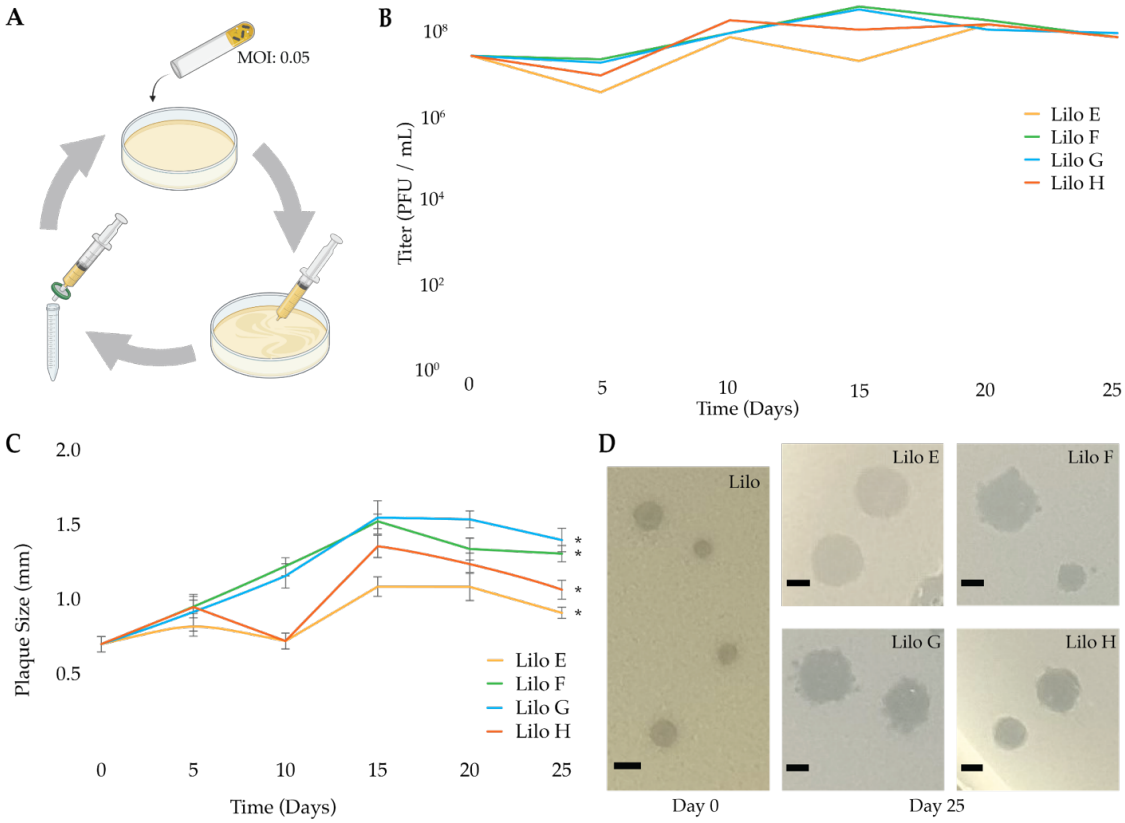


Figure 5.1 Experimental evolution of phage Lilo on solid media

A) Schematic of experimental method (created with BioRender.com). B) Increase in phage titer (PFU mL^{-1}) over the 25-day experimental evolution for four phage Lilo lineages E, F, G and H. C) Increase in plaque diameter from the initial phage lysate until day 25 of the evolution experiment, (*P <0.05; paired t-test). Error bars = standard error. D) Representative plaques for the ancestral Lilo and Lilo lineages E, F, G and H after 25 days. Scale bar = 1 mm.

5.3.3 Modified Appelmans Protocol to Improve Phage Titers

Whilst the top agar overlay evolution experiment showed signs that mutations had likely taken place, the increases in titer achieved (4-14 fold) were not sufficient to justify the effort of propagating phages in top agar overlays for this period of time. We, therefore, turned our attention to the literature and found the Appelmans protocol described by Burrowes and colleagues (Burrowes et al., 2019). The Appelmans protocol is a small volume, high throughput directed evolution experiment administered to foster recombination between similar phages in liquid lysates over a period of 30 days. Historically, this method has been used to increase host range by passaging serial dilutions of mixed phages on several hosts in a 96-well plate format (Figure 5.2A).

We adopted the basic protocol in order to use this passaging procedure to apply selection pressure on populations of a *single* phage on a *single* host to improve infection properties in liquid media over a similar time period (Figure 5.2A). This modified Appelmans protocol was performed on six novel *P. larvae* phages. Phage efficacy was checked by measuring the PFU after every second transfer (Figure 5.2B). Initial phage titers of our 6 phages ranged from 2×10^5 mL^{-1} - 3×10^7 mL^{-1}. After 30 days of the modified Appelmans Protocol the titers of these phages had increased to between 1×10^7 mL^{-1} - 2×10^8 mL^{-1}, a 100-fold increase on average (Figure 5.2B).

5.3.4 Plaque Improvements from Modified Appelmans Protocol

A significant increase in plaque size was observed in four out of the six phages. Plaque sizes were measured on Day 0, Day 15 and Day 30 (Figure 5.2C). AJG77 and Callan did not exhibit larger plaque sizes and measured approximately 0.65 (+/- 0.02) mm and 1.40 (+/- 0.05) mm respectively for the duration of the experiment. ABAtENZ, Logan, Lilo and Dash increased

in their respective plaque sizes from 0.31 (+/- 0.02) mm, 0.33 (+/- 0.02) mm, 0.66 (+/- 0.06) mm and 0.57 (+/- 0.04) mm to 0.50 (+/- 0.01) mm, 0.90 (+/- 0.03) mm, 1.27 (+/- 0.04) mm and 1.52 (+/- 0.09) mm respectively (P <0.05; paired t-test). ABAtENZ and Lilo (Figure 5.2D) achieved 63% and 92% increases in plaque size respectively over 30 days while Dash (Figure 5.2D) and Logan saw the greatest increase in plaque size with an increase of approximately 165% and 170% respectively.

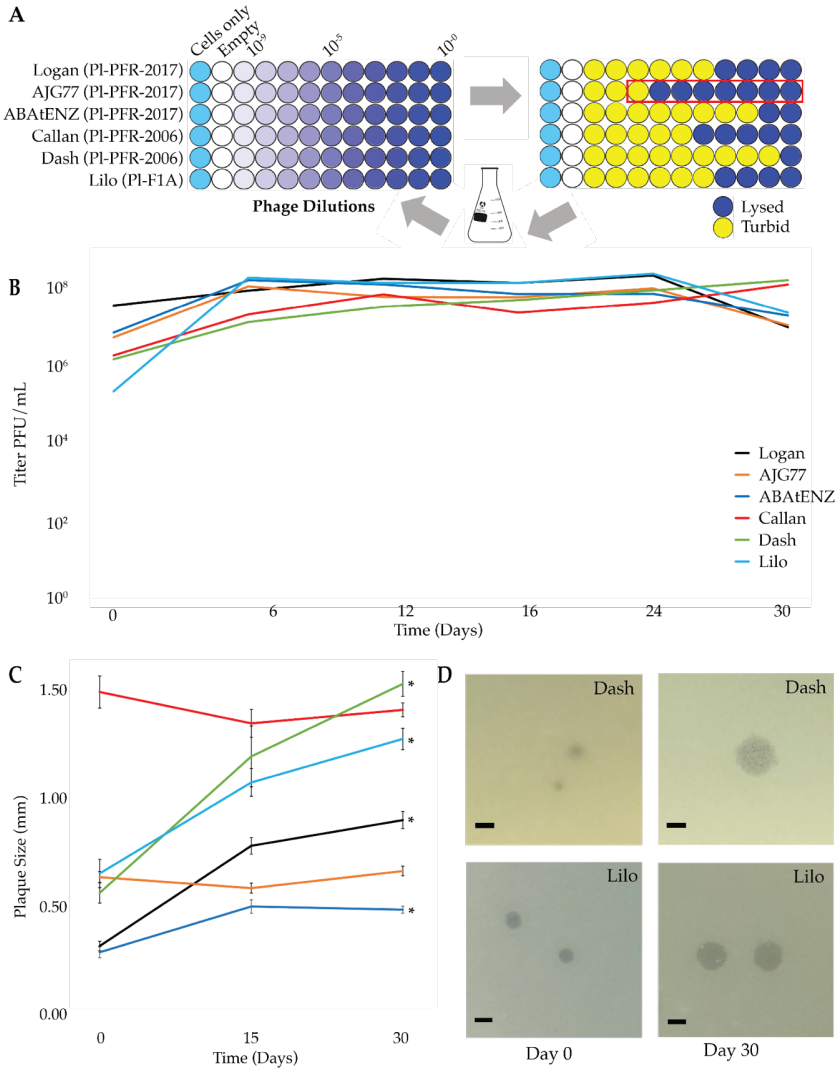


Figure 5.2 Experimental evolution using a modified Appelmans Technique

A) Schematic of 96-well format modified Appelmans protocol. B) Increase in phage titer (PFU/mL) over the 30-day experimental evolution for six phages Logan, AJG77, ABAtENZ, Callan, Dash and Lilo. C) Increase in plaque size from the initial phage lysate until day 30 of the evolution experiment, (*P < 0.05; paired t-test). Error bars = standard error. D) Change in plaque size over 30 days for phage Dash and Lilo. Scale bar = 1 mm.

5.3.5 Similar Results Seen After Four Days

The original Appelmans protocol used a 30-day time period. The Modified Appelmans Protocol experiment described above appeared to yield significant increases in phage titers after the first few rounds of plating (Figure 5.2B). This was particularly evident in Lilo. We, therefore, selected a new set of six novel *P. larvae* phages and subjected them to a 4-day Rapid Appelmans Protocol (RAP) treatment (Figure 5.3). The initial titers of the six phages selected for RAP ranged from 1×10^4 mL^{-1} - 1×10^5 mL^{-1}, with Rae2Bee1 having the lowest titer and Wildcape the highest titer. After four days of RAP evolution, the titers increased to 2×10^7 mL^{-1} - 4×10^8 mL^{-1}, a 1000-fold increase on average (Figure 5.3A). Rae2Bee1 showed the smallest increase in titer, with a 220-fold increase; ApiWellbeing, Carlos, and Wildcape all experienced a 1000 fold increase in titer, and LunBun and FutureBee saw the largest increases in titer with increases of 2,700 fold respectively. An increase in plaque size was also seen in all six phages ranging from an increase of 53% to 514%, FutureBee and Carlos respectively (Figure 5.3B). Interestingly, the average increase in plaque size did not correlate with increases in titer as FutureBee experienced the largest increase in titer but the smallest increase in plaque size. Ultimately, we applied either the RAP or the longer protocol to all 26 of our *P. larvae* phages. The largest improvement observed was NHScienceFair, which had a starting titer of 2.2×10^2 mL^{-1} and achieved a final titer of 4.1×10^7 mL^{-1} after four days of RAP, a 185,000 fold increase in titer. We observed a negative correlation between the starting titer and the titer improvement, which suggests that there is a limit to the titer enhancement that can be achieved in the RAP protocol (Figure 5.3C).

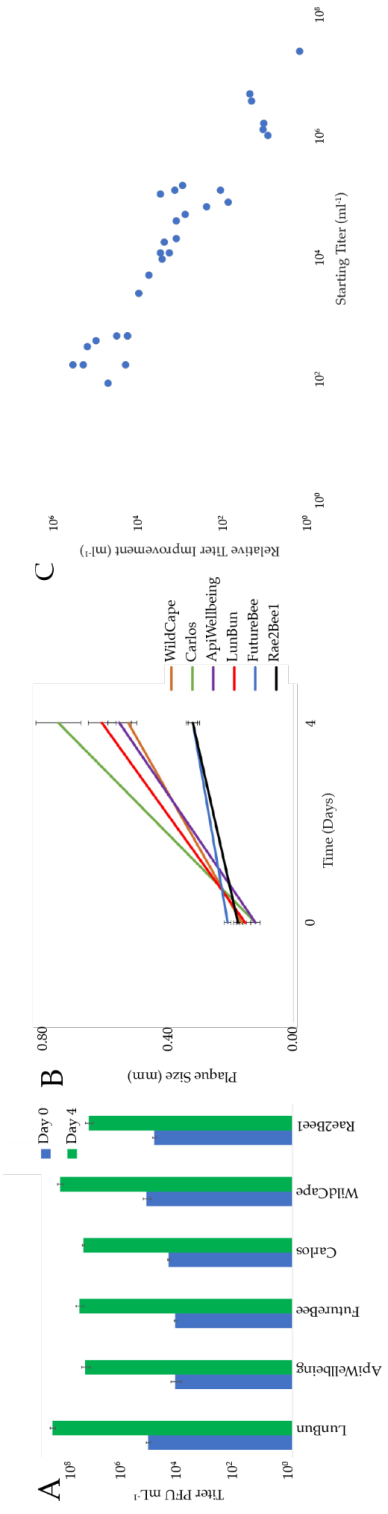


Figure 5.3 RAP Experimental evolution increases lysate titer in as little as four days

A) Increase in phage titer (PFU/mL) in four days for six phages. Error bars = standard error (N=3). B) Colony measurements for the RAP evolved phages in A. C) The RAP protocol appears to limit the potential for phages to improve based on their starting titer.

5.3.6 Appelmans Leads to High Phage Titers and High-Quality DNA

The resulting phage concentrations after both modified Appelmans methods were sufficient to extract high-quality DNA for Illumina sequencing. Sequencing results are shown for phages that underwent the longer 30-day protocol (Table 5.2 and Figure 5.4). The genomes of these phages ranged between 40kbp and 44kbp in length and have 70-82 genes each. Pairwise comparisons of these genomes were carried out using Phamerator, a bioinformatics tool that uses the "Align Two Sequences" program contained within BLAST (Cresawn et al., 2011). The maps show how related or divergent the phages are, showing that Lilo, Callan, and Dash are highly related, whereas ABAtENZ, AJG77, and Logan are highly related to each other but diverge from the other three phages apart from two regions of the structural genes (Figure 5.4). The details of these genomes and their functional genes will be discussed in Chapter 6.

Table 5.2. The genomic characteristics of the first six phage genomes that we sequenced using the Modified Appelmans Protocol to increase titers

	Genome Length (bp)	DNA Packaging Strategy	GC Content (%)	No. of Genes	Genes per 1000bp	Percent Coding (%)
Logan	44,419	3' cos	43.0	82	1.85	94.01
AJG77	44,417	3' cos	43.0	82	1.85	93.46
ABAtENZ	44,419	3' cos	43.0	82	1.85	94.01
Lilo	40,941	3' cos	40.3	70	1.71	91.78
Callan	44,768	3' cos	39.6	77	1.72	91.56
Dash	44,599	3' cos	39.4	79	1.77	93.40

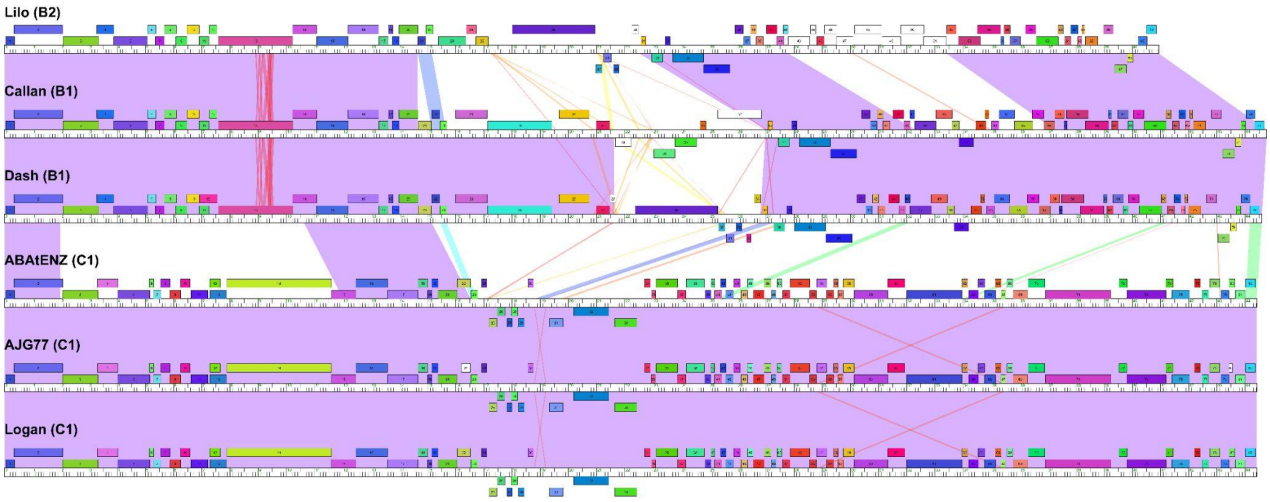


Figure 5.4 Genome maps of phages evolved for 30 days
Maps generated by Phamerator. Shading indicates high sequence similarity between sequences as determined by BLASTN, with purple being the highest. (E-value = 0). Genes are represented by boxes, boxes with the same colour indicate genes which belong to the same families ("phams").

5.3.7 Changes to Host Range of New Zealand P. larvae phages after Appelmans protocol

The host range of all 26 *P. larvae* phages was assessed against 30 New Zealand *P. larvae* bacterial strains before they underwent the Appelmans protocol. The host range was then checked again after the phages had been evolved (Figure 5.5). An expansion in the host range was seen in 32 instances (red asterisks in Figure 5.5) in 11 phages, and there were no instances of a reduction in the host range. Eight instances of phages that expanded their host range to include *P. larvae* strain W19_08100, whereas five evolved phages gained the ability to infect *P. larvae* strain W19_08099. Both of these *P. larvae* isolates previously had fewer potential infecting phages than the majority of bacterial strains in this project. ABAtENZ gained the ability to infect an additional five bacterial strains, which were previously only able to be infected by Callan, Dash, and Lilo before the Appelmans protocol. As we were unable to sequence these phages before evolution, we could not compare their genomes before and after evolution to ascertain what may have caused the expansion of the host range.

We investigated the possibility that mutations might have occurred that allowed escape from CRISPR recognition. Previously sequenced *P. larvae* strains are known to have CRISPR-Cas systems, and *P. larvae* phages may have evolved point mutations to escape their hosts' CRISPR systems (Stamereilers et al., 2021). We subjected preliminary sequences (in contigs) of our eight *P. larvae* strains to CRISPRFinder (Grissa et al., 2007) in order to identify 38 unique CRISPR spacers.

We searched the phage genomes for these *P. larvae* CRISPR spacers allowing up to 80% nucleotide divergence (approximately 7bp changes allowed). Callan, Dash, and Lilo all contain spacers that range from 81% to 100% nucleotide identity to at least one *P. larvae* CRISPR spacer found in our sequenced isolates. However, these three phages did not experience any expansion in their host range after the Appelmans protocol. Based on this analysis, we cannot determine what the mechanism of host range expansion was but it does not appear to be caused by the evasion of CRISPR spacers.

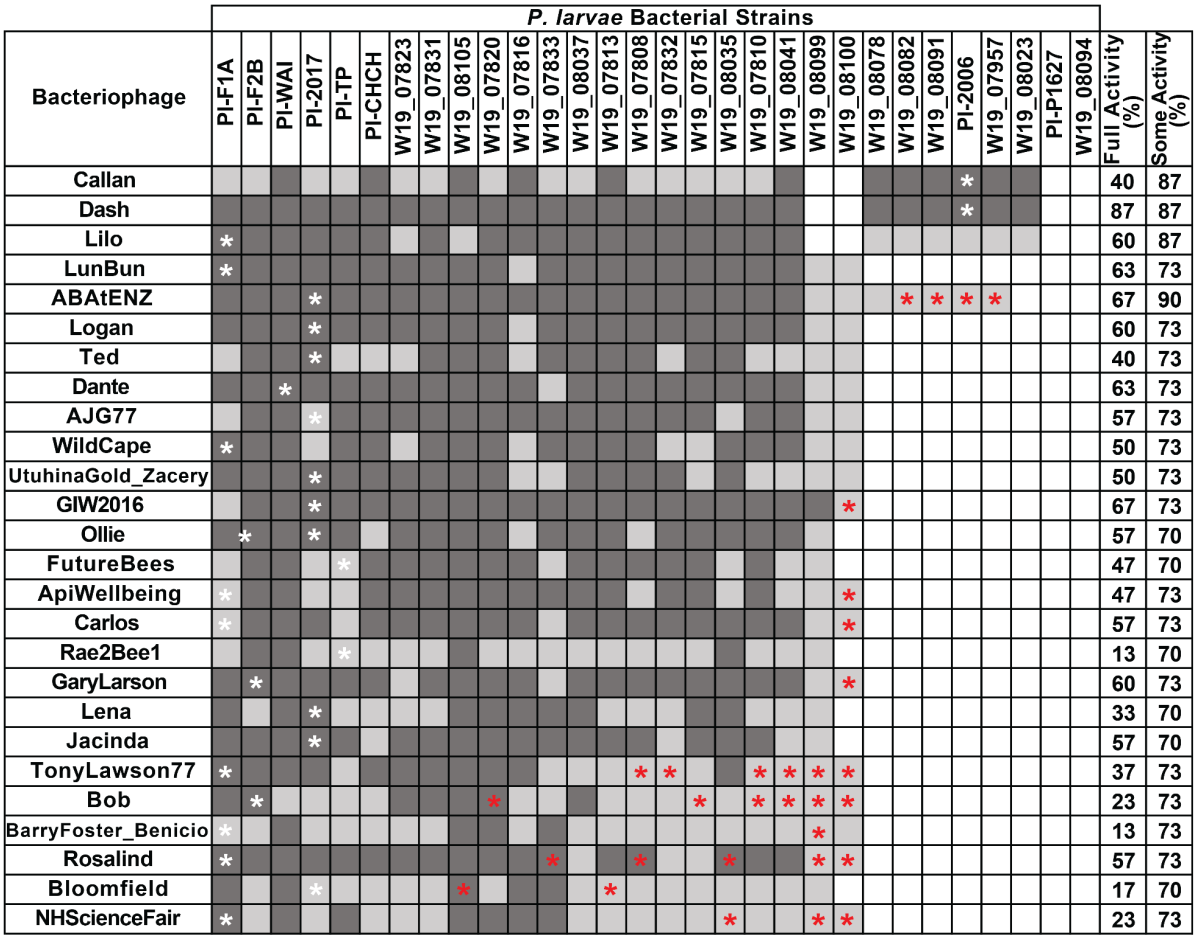


Figure 5.5 Host range of 26 *P. larvae* phages on 30 *P. larvae* bacterial isolates from New Zealand
Dark grey boxes indicate complete cell lysis, light grey boxes indicate some cell lysis and white boxes indicate no cell lysis has occurred. Red asterisks show where host range expansion has occurred. White asterisks show the original *P. larvae* strain the phage was isolated on.

5.3.8 Modelling of Rapid Appelmans Protocol

To model the possibility of a beneficial mutation becoming dominant after as little as four days of evolution in the RAP we assembled a simulation. The initial parameters for the simulation were as follows:

- Rate of lysis: $l = 1/30$ [min^{-1}], meaning that lysis takes, on average, 30 minutes
- Basal infectivity: $\beta_0 = 10^{-6}$ [virion^{-1} min^{-1}]
- Basal burst size: $b = 10$

Under these parameters it would take approximately 10 hours for all 40,000 bacteria to be lysed. The parameters U (mutation opportunity), s_b (burst size coefficient), and s_β (infectivity coefficient) were varied as follows:

Mutation opportunity (U) was set between 0.0×10^0 to 1.0×10^{-5}. This is equivalent to multiplying the mutation rate, 5×10^{-7} per base pair (or Drakes rule (Drake, 1991)) by the number of possible sites that could be mutated (out of ~40,000 bp) that would result in an increase in the burst size of that phage. We set this second part of the U parameter to between 0 and 20 nucleotide sites (eg. $20 \times 5 \times 10^{-7} = 1.0 \times 10^{-5}$). The selection coefficient of burst size (s_b) was set between 1 and 60 (meaning that a mutation increases the burst size by 1 to 60 fold). The selection coefficient of infectivity (s_β) was set between 1 to 10-fold increase in infectivity. This means that depending on the parameters set for any given simulation, the largest potential increase in total fitness due to a single mutation would result if an increase in burst size of 60 fold and an increase in infectivity of 10 fold were chosen, e.g. 60 x 10 = 600 fold increase in phage fitness.

One hundred simulations were run for each chosen set of parameters and a frequency histogram of the fraction of the mutant phage after 4 days was obtained. Histograms showed a bimodal distribution, showing peaks at 0 and 1 (Figure 5.6). This bimodality comes from the fact that the limiting step for the mutant phage to emerge in a population is the occurrence of a fitness-improving mutation. If the mutant occurs, it can spread within a small amount of time. Figure 5.7 shows the percentage of experiments that resulted in populations in which 70% or more of the phages in the simulation were mutants with a fitness advantage after four days of RAP. Setting the mutation opportunity (U) to 0 resulted in zero mutant phages regardless of parameters affecting increased burst size or infectivity.

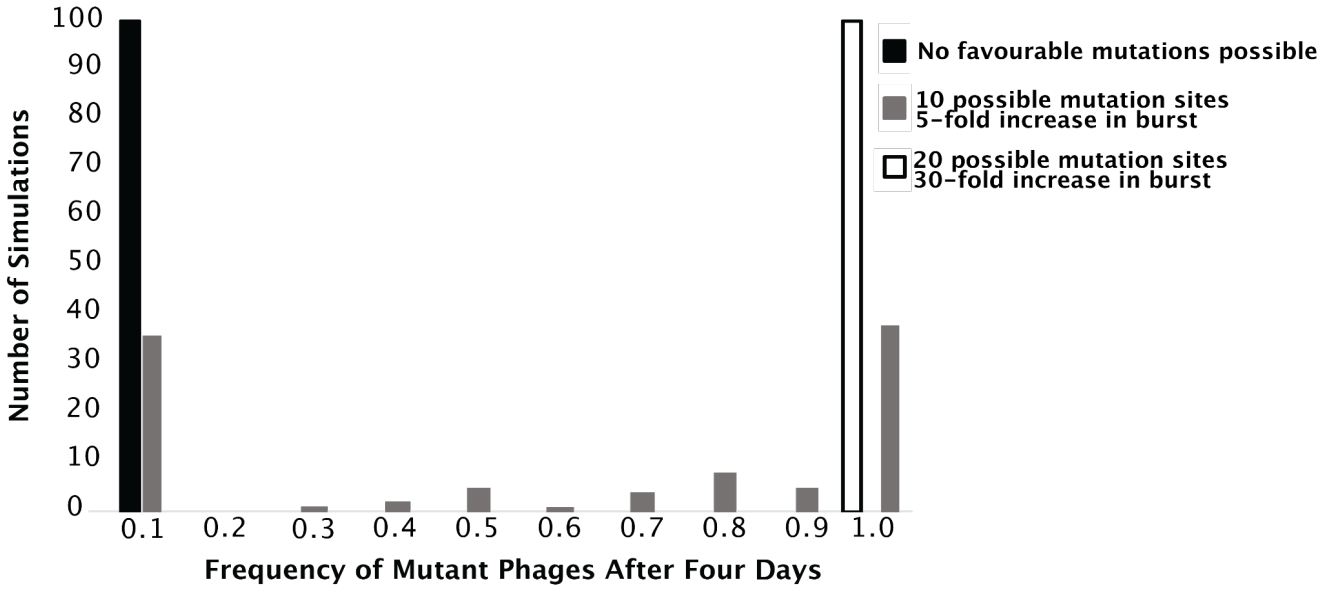


Figure 5.6 Histogram of three representative simulations when infectivity is set to one
Black shows when parameters are set so the simulation results in zero mutant phages (U = 0, s_b = 1, s_θ = 1). Grey shows the transition point or at least 50% of the experiments results in 70% or more of the phages having the fitness-increasing mutation (U = 5.0 x 10^{-6}, s_b = 5, s_θ = 1). White represents the upper limit of the simulation or when all experiments have at least 70% fitness-increasing mutant phage after four days (U = 1.0 x 10^{-5}, s_b = 30, s_θ = 1).

One way to consider these results is to address the transition point, or the point at which at least 50% of the experiments results in 70% or more of the phages having fitness-increasing mutations. If the increase in infectivity was set to 1 (s_θ=1); the transition point appears when the mutation opportunity was set to 5.0 x 10^{-6}, (10 possible nucleotide sites) and the burst size increase for a mutation was set to 5 fold (Figure 5.7). The upper limit (all experiments having 70% mutations after four days) was reached when the mutation opportunity (U) was set to 1.0 x 10^{-5} (20 nucleotide sites) and burst size increased by 30 fold.

If the increase in infectivity in the event of a mutation was set to 5 fold; the transition point appears when the mutation opportunity (U) was set to 5.0 x 10^{-7} (1 nucleotide site) and

burst size for a mutation was set to 10 fold (Figure 5.7). In this case, a single mutation would increase fitness by 50 fold. The upper limit was reached when the mutation opportunity (U) was set to 7.5×10^{-6} (15 nucleotide sites) and the burst size increase for a mutation was set to 5 fold, these settings designated an increase in fitness by 25 fold in the event of a single mutation.

The last set of simulations had the increase in infectivity in the event of a mutation set to 10 fold; the transition point in this instance appears when the mutation opportunity (U) was set to 5.0×10^{-7} (1 nucleotide site) and the burst size increase for the same mutation was set to 5 fold increase above the basal burst size of 10 (Figure 5.7). This effectively increased fitness by 50 fold if the mutation of that single available site occurred. The upper limit was reached when the mutation opportunity (U) was set to 5.0×10^{-6} (10 nucleotide sites) and the burst size increase for a mutation in one of these 10 sites was set to 5 fold increase in burst size; these mutations would increase overall fitness by 50 fold.

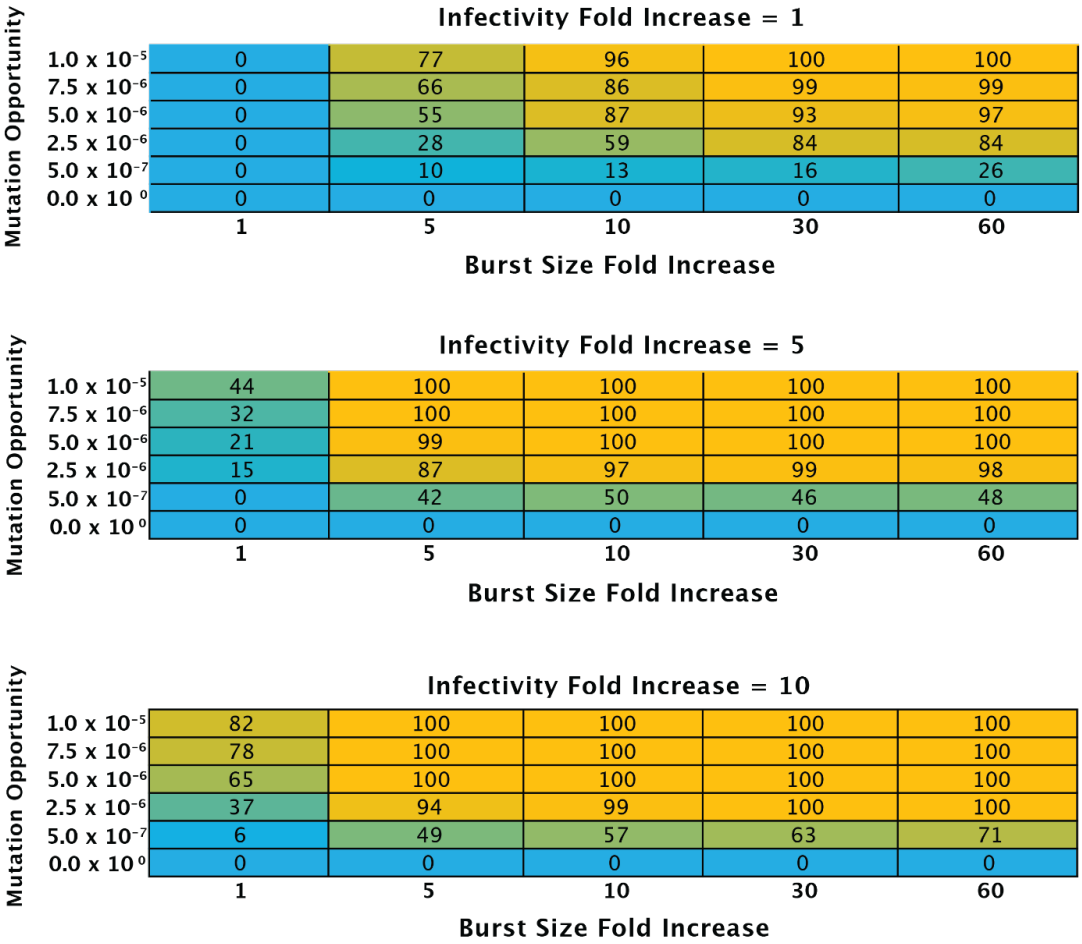


Figure 5.7 Heat maps of simulations of RAP
One hundred simulations were run for each change in parameters. Mutation opportunity, increase in burst size, and increase in infectivity coefficient were varied as follows: Blue indicates the lowest number in the series and orange indicates the highest number.

5.4 Discussion

Like many other phage biologists before us, we discovered *P. larvae* phages isolated from nature and found them to be intractable, meaning they had persistently low titers that could not be increased by standard laboratory procedures. Other *Paenibacillus* phage biologists have had similar issues. Yost and colleagues, working in the USA, described concentrating 100 mL volumes to 3 mL in order to obtain lysates for DNA extraction and EM (Yost et al., 2016). Similarly, a *P. polymyxa* phage isolated in Slovakia required the

concentration of a 200 mL volume of lysate (Halgasova et al., 2010). More recently a similar study from Poland described five *P. larvae* phages, four of which were at a titer of $8x10^6$ or lower and required additional centrifugation steps before DNA sequencing (Jończyk-Matysiak et al., 2021). Similar protocols for tractable phages often require volumes of 1-10 mL and are easily brought to titers above $5x10^9$ PFU/mL.

We endeavoured to evolve one of our New Zealand *P. larvae* phages, Lilo, using traditional agar overlay methods; a 4.1 to 14.5 fold increase in titer was observed across the different replicates in the first 15 days; this increase was maintained for the rest of the duration of the experiment. We also saw increases in plaque size between Day 0 and Day 15 and then saw decreases in plaque size between Day 15 and Day 25. Increases in plaque size are complicated and can be due to a number of different factors. For example, shorter phage latent periods and faster virion diffusion can both lead to larger plaques sizes as well as a larger phage burst size, especially if initial burst size is small (Abedon, 2021). The increases in titer observed were not however sufficient to obtain enough DNA to progress to complete sequenced genomes.

Spatial structure could be a contributing factor to our difficulty in evolution of phages on agar overlay. There are many consequences of spatial structure including resource concentration, barriers and gradients, super-infection and altered gene expression (Bull et al., 2018). It is possible that gene expression changes in the host may determine which method works best for a particular phage (Chapman-McQuiston & Wu, 2008a, 2008b). In addition, the limits to diffusion that are in play when a phage is infecting in an agar overlay are significant.

We subsequently attempted the evolution of six New Zealand *P. larvae* phages using a modified Appelmans method, naturally evolving each phage independently on the bacterial strain on which they were isolated (Burrowes et al., 2019). This method resulted in a 100 fold increase in titer on average across the different phages. We also saw an increase in plaque size of between 63% and 170% in four of our phages. Interestingly, we saw the greatest increase in titer at approximately 4-5 days after commencing evolution. We therefore modified the protocol again and evolved a further six *P. larvae* phages using this new RAP method. From this protocol, we saw an average 1000 fold increase in titer and increases in plaque size ranging from 53% to 514%. The RAP method highlighted the possibility of evolving phages in even shorter timeframes and still obtaining desirable results.

The RAP described appears to facilitate mutations and the increase of these mutants in the population, as indicated by changes in titer and plaque dimensions. We sequenced and analysed the genomes of these experimentally evolved phages, but couldn't analyse their ancestors to determine what mutations led to the observed improvements. We did look at possible changes in CRISPR spacers that might have driven large increases in phage fitness by avoiding bacterial defence (Levin et al., 2013) but we could not find evidence that mutational escape from CRISPR drove these mutants.

Our experience of the RAP protocol and the increased titers of the phages did make us question whether it was possible that mutations could arise so quickly. We have therefore constructed a computer simulation of the population parameters of the RAP in order to determine whether advantageous mutations (and not some other phenomenon) could drive new phages to become dominant in the population in as little as four days.

We varied the target size for a random mutational event by changing the mutation opportunity (U) from 0.0×10^{0} to 1.0×10^{-5}. We allowed single mutations to increase the infectivity from 1 to 10 fold and allowed the burst size to increase from 1 to 60 fold. Ultimately, we observed that with a mutation that would increase the fitness of the phages by only 5 fold, as many as 50% of experiments would have at least 70% mutant phages. This phenomenon is likely similar to that of mutational jackpot events, where beneficial mutations may occur early on in growth experiments resulting in these mutants dominating the population (Fusco et al., 2016; Luria & Delbrück, 1943). These results lead us to believe that this method may be highly relevant for increasing the utility of a wide range of difficult phages.

Serial passage experiments have previously been used to evolve phages. An elegant experiment to evolve four phages infecting *Pseudomonas aeruginosa* PAO1 in liquid media has been reported. At the end of the experiment, two of the phage isolates increased their infection capacity from 80-85% to 100% (Betts et al., 2013). In another experiment, phage FCV-1 was coevolved with *Flavobacterium columnare* in lake water; in the first four days the phage titer increased from 10^{4} PFU/mL to 10^{7} PFU/mL (approximately three log increase) (Laanto et al., 2020). The method that we have put forward here is a generalisable and high-throughput method that can be attempted rapidly with phages that are recalcitrant to other methods for amplification.

5.5 Conclusions

Communication with phage-hunting colleagues suggests that many phages have been discovered only to be abandoned due to issues with low titer. Herein, we have presented a

novel method to experimentally evolve difficult phages to increase their titer and in some cases their host range in as little as four days. We have based this rapid method on the previously published Appelmans protocol (Burrowes et al., 2019). We have used this RAP to evolve 26 New Zealand *P. larvae* phages to rapidly improve stocks that are recalcitrant to high titer lysate creation by normal means. These high-titer lysates enable the extraction of high-quality DNA for sequencing as well as other downstream applications such as large-scale phage production. RAP presents a fast and effective way to experimentally evolve previously intractable phages allowing researchers to study entities that might otherwise be lost to science.

5.6 References

Abedon, S. T. (2016). Phage therapy dosing: The problem(s) with multiplicity of infection (MOI). *Bacteriophage*, *6*(3), e1220348.

Abedon, S. T. (2021). Detection of Bacteriophages: Phage Plaques. In D. R. Harper, S. T. Abedon, B. H. Burrowes, & M. L. McConville (Eds.), *Bacteriophages: Biology, Technology, Therapy* (pp. 507–538). Springer International Publishing.

Abràmoff, & Magalhães. (2004). Image processing with ImageJ. *Biophotonics International*. https://dspace.library.uu.nl/handle/1874/204900

Betts, A., Vasse, M., Kaltz, O., & Hochberg, M. E. (2013). Back to the future: evolving bacteriophages to increase their effectiveness against the pathogen Pseudomonas aeruginosa PAO1. *Evolutionary Applications*, *6*(7), 1054–1063.

Bonilla, N., Rojas, M. I., Netto Flores Cruz, G., Hung, S.-H., Rohwer, F., & Barr, J. J. (2016). Phage on tap-a quick and efficient protocol for the preparation of bacteriophage laboratory stocks. *PeerJ*, *4*, e2261.

Brady, T. S., Merrill, B. D., Hilton, J. A., Payne, A. M., Stephenson, M. B., & Hope, S. (2017). Bacteriophages as an alternative to conventional antibiotic use for the prevention or treatment of *Paenibacillus larvae* in honeybee hives. *Journal of Invertebrate Pathology*, *150*, 94–100.

Bull, J. J., Christensen, K. A., Scott, C., Jack, B. R., Crandall, C. J., & Krone, S. M. (2018). Phage-Bacterial Dynamics with Spatial Structure: Self Organization around Phage Sinks Can Promote Increased Cell Densities. *Antibiotics (Basel, Switzerland)*, *7*(1). https://doi.org/10.3390/antibiotics7010008

Burrowes, B. H., Molineux, I. J., & Fralick, J. A. (2019). Directed in Vitro Evolution of Therapeutic Bacteriophages: The Appelmans Protocol. *Viruses*, *11*(3).

https://doi.org/10.3390/v11030241

Chapman-McQuiston, E., & Wu, X. L. (2008a). Stochastic receptor expression allows sensitive bacteria to evade phage attack. Part I: experiments. *Biophysical Journal*, *94*(11), 4525–4536.

Chapman-McQuiston, E., & Wu, X. L. (2008b). Stochastic receptor expression allows sensitive bacteria to evade phage attack. Part II: theoretical analyses. *Biophysical Journal*, *94*(11), 4537–4548.

Cresawn, S. G., Bogel, M., Day, N., Jacobs-Sera, D., Hendrix, R. W., & Hatfull, G. F. (2011). Phamerator: a bioinformatic tool for comparative bacteriophage genomics. *BMC Bioinformatics*, *12*, 395.

Delbrück, M. (1940). THE GROWTH OF BACTERIOPHAGE AND LYSIS OF THE HOST. *The Journal of General Physiology*, *23*(5), 643–660.

Drake, J. W. (1991). A constant rate of spontaneous mutation in DNA-based microbes. *Proceedings of the National Academy of Sciences of the United States of America*, *88*(16), 7160–7164.

Elke Genersch. (2010). American Foulbrood in honeybees and its causative agent, *Paenibacillus larvae*. *Journal of Invertebrate Pathology*, *103*(S), S10–S19.

Ellis, E. L., & Delbrück, M. (1939). THE GROWTH OF BACTERIOPHAGE. *The Journal of General Physiology*, *22*(3), 365–384.

Fusco, D., Gralka, M., Kayser, J., Anderson, A., & Hallatschek, O. (2016). Excess of mutational jackpot events in expanding populations revealed by spatial Luria–Delbrück experiments. *Nature Communications*, *7*(1), 1–9.

Gallet, R., Kannoly, S., & Wang, I.-N. (2011). Effects of bacteriophage traits on plaque formation. *BMC Microbiology*, *11*, 181.

Grissa, I., Vergnaud, G., & Pourcel, C. (2007). CRISPRFinder: a web tool to identify clustered regularly interspaced short palindromic repeats. *Nucleic Acids Research*, *35*(Web Server issue), W52-7.

Halgasova, N., Ugorcakova, J., Gerova, M., Timko, J., & Bukovska, G. (2010). Isolation and characterization of bacteriophage PhiBP from Paenibacillus polymyxa CCM 7400. *FEMS Microbiology Letters*, *305*(2), 128–135.

Hanauer, D. I., Graham, M. J., SEA-PHAGES, Betancur, L., Bobrownicki, A., Cresawn, S. G., Garlena, R. A., Jacobs-Sera, D., Kaufmann, N., Pope, W. H., Russell, D. A., Jacobs, W. R., Jr, Sivanathan, V., Asai, D. J., & Hatfull, G. F. (2017). An inclusive Research Education Community (iREC): Impact of the SEA-PHAGES program on research outcomes and student learning. *Proceedings of the National Academy of Sciences of the United States of America*, *114*(51), 13531–13536.

Hendrix, R. W., Smith, M. C., Burns, R. N., Ford, M. E., & Hatfull, G. F. (1999). Evolutionary relationships among diverse bacteriophages and prophages: all the world's a phage. *Proceedings of the National Academy of Sciences of the United States of America*, *96*(5), 2192–2197.

Jończyk-Matysiak, E., Owczarek, B., Popiela, E., Świtała-Jeleń, K., Migdał, P., Cieślik, M., Łodej, N., Kula, D., Neuberg, J., Hodyra-Stefaniak, K., Kaszowska, M., Orwat, F., Bagińska, N., Mucha, A., Belter, A., Skupińska, M., Bubak, B., Fortuna, W., Letkiewicz, S., … Górski, A. (2021). Isolation and Characterization of Phages Active against *Paenibacillus larvae* Causing American Foulbrood in Honeybees in Poland. *Viruses*, *13*(7). https://doi.org/10.3390/v13071217

Laanto, E., Mäkelä, K., Hoikkala, V., Ravantti, J. J., & Sundberg, L.-R. (2020). Adapting a Phage to Combat Phage Resistance. *Antibiotics (Basel, Switzerland)*, *9*(6).

https://doi.org/10.3390/antibiotics9060291

Lazeroff, M., Ryder, G., Harris, S. L., & Tsourkas, P. K. (2021). Phage Commander, an Application for Rapid Gene Identification in Bacteriophage Genomes Using Multiple Programs. *PHAGE (New Rochelle, N.Y.)*, *2*(4), 204–213.

LeMieux, J., & Hatfull, G. (2020). Set Phages to Kill: An Interview with Graham Hatfull, PhD. *PHAGE (New Rochelle, N.Y.)*, *1*(1), 4–9.

Levin, B. R., Moineau, S., Bushman, M., & Barrangou, R. (2013). The Population and Evolutionary Dynamics of Phage and Bacteria with CRISPR–Mediated Immunity. *PLoS Genetics*, *9*(3), e1003312.

Luria, S. E., & Delbrück, M. (1943). Mutations of Bacteria from Virus Sensitivity to Virus Resistance. *Genetics*, *28*(6), 491–511.

Mushegian, A. R. (2020). Are There 1031 Virus Particles on Earth, or More, or Fewer? *Journal of Bacteriology*, *202*(9). https://doi.org/10.1128/JB.00052-20

Philipson, C. W., Voegtly, L. J., Lueder, M. R., Long, K. A., Rice, G. K., Frey, K. G., Biswas, B., Cer, R. Z., Hamilton, T., & Bishop-Lilly, K. A. (2018). Characterizing Phage Genomes for Therapeutic Applications. *Viruses*, *10*(4). https://doi.org/10.3390/v10040188

Rohwer, F. (2003). Global phage diversity. *Cell*, *113*(2), 141.

Russell, D. A., & Hatfull, G. F. (2017). PhagesDB: the actinobacteriophage database. *Bioinformatics* , *33*(5), 784–786.

Salisbury, A., & Tsourkas, P. K. (2019). A Method for Improving the Accuracy and Efficiency of Bacteriophage Genome Annotation. *International Journal of Molecular Sciences*, *20*(14). https://doi.org/10.3390/ijms20143391

Santos, M. A. (1991). An improved method for the small scale preparation of bacteriophage DNA based on phage precipitation by zinc chloride. *Nucleic Acids Research*, *19*(19),

5442.

Skurnik, M., Pajunen, M., & Kiljunen, S. (2007). Biotechnological challenges of phage therapy. *Biotechnology Letters*, *29*(7), 995–1003.

Stamereilers, C., Fajardo, C., Walker, J., Mendez, K., Castro-Nallar, E., Grose, J., Hope, S., & Tsourkas, P. (2018). Genomic Analysis of 48 *Paenibacillus larvae* Bacteriophages. *Viruses*, *10*(7), 377–329.

Stamereilers, C., Wong, S., & Tsourkas, P. K. (2021). Characterization of CRISPR Spacer and Protospacer Sequences in *Paenibacillus larvae* and Its Bacteriophages. *Viruses*, *13*(3). https://doi.org/10.3390/v13030459

Yost, D. G., Tsourkas, P., & Amy, P. S. (2016). Experimental bacteriophage treatment of honeybees (Apis mellifera) infected with *Paenibacillus larvae*, the causative agent of American Foulbrood Disease. *Bacteriophage*, *6*(1), e1122698.

Chapter 6

Genomic analysis of 95 *Paenibacillus larvae* bacteriophages including 26 from Aotearoa, New Zealand

Abstract

The bacterium *Paenibacillus larvae* is responsible for the devastating honey bee (*Apis mellifera*) disease American Foulbrood. Research in bacteriophages (phages) that infect *P. larvae* is growing rapidly, due to both the increasing antibiotic resistance of the host and the restrictions on antibiotic use in beehives in some countries. In this study, we present the sequenced and annotated genomes of 26 novel *P. larvae* phages recently isolated in New Zealand, bringing the total number of sequenced and annotated *P. larvae* phages to 95. The 26 novel phages belong to the Vegas or Harrison clusters. We performed a comprehensive genomic analysis of all 95 sequenced *P. larvae* phage genomes, grouping them into four clusters and three singletons using several different clustering methods. Six phages contain the Plx1 *P. larvae* toxin. We performed in-depth analysis of the Plx1 toxin and the N-acetylmuramoyl-L-alanine amidase. This study expands our knowledge of *P. larvae* phages from around the world and represents the state of the art in the field of *P. larvae* phages.

6.1 Introduction

Paenibacillus larvae is the causative agent of American Foulbrood (AFB), a destructive and globally distributed disease of the European honey bee (*Apis mellifera*) (Boncristiani et al., 2021; Elke Genersch, 2010). *P. larvae* is a spore-forming Gram positive bacterium that is easily spread between hives either via beekeepers or by bees themselves. The spore form of the bacteria can persist in the environment for 35+ years (Elke Genersch, 2010; Haseman, 1961) and is resistant to heat and cold. Together, the ease with which AFB spreads and the hardiness of the spores make this disease particularly devastating to Apiculture industries around the world.

Currently, countries take one of two approaches to deal with AFB: treatment with antibiotics, such as Oxytetracycline or Tylosin Tartrate (Tylan® Soluble™) (Brady et al., 2017), or complete destruction of the infected hive via incineration. Both approaches are less than ideal; *P. larvae* can become resistant to commonly used antibiotics, and burning of hives results in massive losses for beekeepers (Alippi et al., 2007; Miyagi et al., 2000; Murray & Aronstein, 2006).

A current alternative, that has been researched by various laboratories over the last 10 years, is the use of bacteriophages (phages) specific to *P. larvae* to either treat the disease or prophylactically protect hives against disease proliferation (Brady et al., 2017; Ghorbani-Nezami et al., 2015; LeBlanc et al., 2015; Yost et al., 2016). Phages that infect *P. larvae* were first isolated in the 1950s, but due to the use of antibiotics, there was little interest in them at the time (Tsourkas, 2020). With the rise of antibiotic resistant strains of *P. larvae*, interest in phages that infect them has grown rapidly in the past decade, with the first *P. larvae* phage

genome sequenced in 2013 (Oliveira et al., 2013). Sequencing and annotation of phage genomes is critical for any potential use of phage to treat AFB. It is especially important to identify genes that make the phages lysogenic (such as integrases, Cro/CI, etc.), CRISPR protospacers that would neutralise the phages (Stamereilers et al., 2021), or worse yet, proteins that would make them unsuitable for therapy, such as bacterial toxins and antibiotic-resistance genes (Philipson et al., 2018). Due to the efforts of several laboratories, including the University of Minho (Oliveira et al., 2013), the Technical University of Braunschweig (Beims et al., 2020), Brigham Young University (B. D. Merrill et al., 2018; Walker et al., 2018), North Carolina State University (Abraham et al., 2016; Carson et al., 2015), University of Nevada Las Vegas (Stamereilers et al., 2018, 2016; Tsourkas et al., 2015; Yost et al., 2018), the Polish Academy of Sciences (Jończyk-Matysiak et al., 2021) and the University of Canterbury (Chapter 3), 95 unique *P. larvae* phages have been isolated and their sequenced and annotated genomes deposited in NCBI Genbank as of this writing. Some of these institutes have not only contributed to the discovery of new *P. larvae* phages, but have also tested the efficacy of phages when treating or protecting beehives against AFB (Brady et al., 2017; Ghorbani-Nezami et al., 2015; LeBlanc et al., 2015; Yost et al., 2016). A comprehensive review of *P. larvae* phage biology is given in (Tsourkas, 2020), while a review of *P. larvae* in therapy applications is given in (Jończyk-Matysiak et al., 2020).

Our New Zealand *P. larvae* phages represent the first sampling effort that we are aware of in the Southern Hemisphere. Beekeeping of *Apis mellifera* was brought to New Zealand in 1839 and *P. larvae* infection was first noted in 1877 (Gillingham, n.d.; Kok & Hendrickson, 2023; Lester, 2021). There have likely been many incursions of the pathogen since these dates, as the importation of live bees and bee products only became strictly regulated 30 years ago,

with the introduction of the Biosecurity Act 1993 (*Biosecurity Act 1993 No 95 (as at 01 July 2022), Public Act Contents – New Zealand Legislation*, 2022). These incursions of the pathogen likely brought the *P. larvae* phages as well; this is evidenced by the shared ancestry between New Zealand native phages and phages found around the world.

This study aims to further expand our knowledge of the global genomic diversity of the *P. larvae* phages discovered to date, to group them into clusters, and to examine two genes of particular interest: the Plx1 toxin that confers virulence to *P. larvae* and an N-acetylmuramoyl-L-alanine amidase endolysin, which is present in all sequenced phage genomes. Presence of the toxin automatically makes a phage unsuitable for therapy, while the amidase is critical for host lysis. Previous work undertaken by Stamereilers et al. examined the initial 48 *P. larvae* phages sequenced (Stamereilers et al., 2018); the work herein expands on this previous work and includes an additional 47 *P. larvae* phages discovered and sequenced since then.

6.2 Materials and Methods

Phage genomes included in this publication discovered in New Zealand were sequenced and annotated as described in (Chapter 5) using the programs Phage Commander (Lazeroff et al., 2021), DNA Master (Pope & Jacobs-Sera, 2018) and the manual curation protocol in (Salisbury & Tsourkas, 2019). Phage genomes published by other laboratories were obtained from NCBI GenBank (Supplementary Table S6.1).

Clustering of *P. larvae* phages was performed in three different ways as described in (Hatfull et al., 2010): Average nucleotide identity (ANI), dot plot similarity, and the

bioinformatics tool Phamerator (Cresawn et al., 2011). ANI between whole genomes was calculated by running multiple alignment using MAFFT (Katoh et al., 2002; Katoh & Standley, 2013) with default settings on Geneious 9.0.5 (https://www.geneious.com) (Kearse et al., 2012). Default settings were: auto algorithm, scoring matrix of 200PAM / k=2, and preserving original sequence order. Phages were clustered together if their ANI was equal to or greater than 60%, and phages were included in a sub-cluster if their ANI was equal to or greater than 90%. Singletons were identified as having less than 60% similarity to any other phage.

A phylogenetic network of phage genomes was created using total Average Nucleotide Identity (tANI) as the distance metric. In brief, tANI is a whole genome distance approach that calculates evolutionary distances by using a modified ANI method which incorporates both an alignment fraction and percent identity cut-off (Gosselin et al., 2022). Distances were imported to SplitsTree4 (4.18.2) (Huson & Bryant, 2006). The network was constructed using a NeighbourNet transformation, ordinary least squares variance, and a lambda fraction of 1 (Bryant & Moulton, 2004).

Dot plots were created using Gepard 2.1 (https://cube.univie.ac.at/gepard) (Krumsiek et al., 2007). Genome maps were made using Phamerator, a tool used for comparative genomics of phages (Cresawn et al., 2011). Phamerator uses the "Align Two Sequences" program contained within BLAST and sets the BLAST E value threshold at $1e^{-4}$. Trees were created using iTOL (https://itol.embl.de) (Letunic & Bork, 2021).

Gene level ANI analyses were performed by taking the nucleotide sequence of the gene of interest and running multiple alignment using MAFFT (Katoh et al., 2002; Katoh &

Standley, 2013) with default settings on Geneious 9.0.5 (https://www.geneious.com) (Kearse et al., 2012). Default settings were as above.

Repetitive elements were found in genomes containing a toxin using the Find Repeats tool in Geneious 9.0.5, minimum repeat length was set to 20 bp and maximum mismatches was set to 25%. Hairpins were visualised using VectorBuilder (https://en.vectorbuilder.com).

6.3 Results

6.3.1 Phage Geographical Locations, Sources and Life Cycle

A total of 95 *P. larvae* phages have been isolated, sequenced, and annotated to date (Supplementary Table S6.1). We have contributed 26 to this collection (Table 6.1). Of the 95, 65% are from the United States (Figure 6.1A), predominantly three institutes (Brigham Young University, North Carolina State University, and the University of Nevada), and 27% are from New Zealand. The remaining 8% were discovered in Poland, Portugal, and Germany (Figure 6.1A). Phages have been isolated from several different sources (Figure 6.1B), with 35% isolated from soil samples collected around beehives and 24% isolated from inside the beehive, including frames and combs. Bee debris (dead bee material) accounted for 23% of the samples, and interestingly 3% came from cosmetics containing bee products. The remaining 15% were prophages isolated from various *P. larvae* strains, meaning that they have been induced or released from a strain.

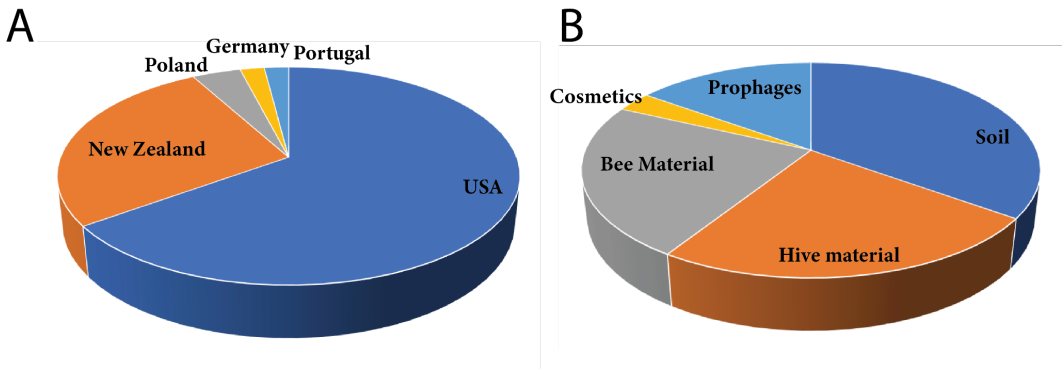


Figure 6.1 Distribution of geographic locations and sources of 95 *P. larvae* phages
A) The geographic locations of the 95 *P. larvae* phages. B) Sources the *P. larvae* phages were isolated from.

6.3.2 New Zealand P. larvae Phages

The 26 new *P. larvae* phages isolated from New Zealand were between 40 kbp - 44 kbp in length with 70 - 83 genes (Table 6.1). The packaging strategy used by all 26 New Zealand phages is the 3' cohesive end strategy, as previously described (Stamereilers et al., 2018). All 26 phages appeared to be lytic *in vitro,* but contained an integrase indicating that they have a temperate lifestyle and have the potential to be lysogenic.

Table 6.1. *P. larvae* phages isolated in New Zealand

	Genome Length (bp)	No. of Genes	GC Content (%)	Cluster	Accession No.	Ref.
ABAtENZ	44,419	82	42.97	Vegas	OP503968	Chapter 3
AJG77	44,417	82	42.98	Vegas	OP503969	Chapter 3
ApiWellbeing	44,429	82	43.01	Vegas	OP503970	Chapter 3
BarryFoster_ Benicio	44,421	82	42.98	Vegas	OP503543	Chapter 3
Bloomfield	44,419	82	42.98	Vegas	OP503971	Chapter 3
Bob	43,553	80	43.03	Vegas	OP503972	Chapter 3
Callan	44,768	77	39.69	Harrison	OP503989	Chapter 3
Carlos	44,430	83	42.98	Vegas	OP503973	Chapter 3

	Genome Length (bp)	No. of Genes	GC Content (%)	Cluster	Accession No.	Ref.
Dante	44,420	82	42.98	Vegas	OP503974	Chapter 3
Dash	44,599	79	39.39	Harrison	OP503990	Chapter 3
FutureBees	44,417	83	42.98	Vegas	OP503975	Chapter 3
GaryLarson	44,420	82	42.98	Vegas	OP503976	Chapter 3
GIW2016	43,555	80	43.01	Vegas	OP503977	Chapter 3
Jacinda	44,419	82	42.97	Vegas	OP503978	Chapter 3
Lena	44,420	82	42.97	Vegas	OP503979	Chapter 3
Lilo	40,941	70	40.33	Harrison	OP503991	Chapter 3
Logan	44,419	82	42.99	Vegas	OP503980	Chapter 3
LunBun	44,421	82	42.97	Vegas	OP494865	Chapter 3
NHScienceFair	44,419	82	42.98	Vegas	OP503981	Chapter 3
Ollie	44,420	83	42.98	Vegas	OP503982	Chapter 3
Rae.2Bee1	44,420	82	42.97	Vegas	OP503983	Chapter 3
Rosalind	43,556	80	43.00	Vegas	OP503984	Chapter 3
Ted	44,419	82	42.99	Vegas	OP503985	Chapter 3
TonyLawson77	44,420	82	42.96	Vegas	OP503986	Chapter 3
UtuhinaGold_ Zacery	44,420	82	42.97	Vegas	OP503987	Chapter 3

6.3.3 Clustering of New Zealand P. larvae Phages

6.3.3.1 Clustering using ANI

Clustering of the New Zealand *P. larvae* phages was based on ANI (Figure 6.2). The New Zealand *P. larvae* phages formed two distinct clusters; Callan, Dash, and Lilo were part of one cluster, with Callan and Dash forming a sub-cluster and Lilo forming its own sub-cluster within this cluster based on % identity thresholds. The remaining 23 phages formed a single cluster; phages in this second cluster had a very high degree of similarity ranging between 97.3% - 99.9% (Figure 6.2). An ANI similarity of > 99.9% indicates that there are only approximately 40 base pairs that differ between the two phages based on a 40 kb genome.

We used the criteria described by (Stamereilers et al., 2018) to determine whether to publish two phages with a high ANI (>99.975%). Phages with an ANI greater than 99.975% were usually phenotypically identical (no AA changes); there were several exceptions to this assumption and all phages described here after had at least one amino acid difference. ABAtENZ, Rae2Bee1, Lena, LunBun, TonyLawson77, and Jacinda had ANI's between 99.965 - 99.987% but all differed by one to several amino acids. BarryFoster_Benicio, GaryLarson, UtuhinaGold_Zacery, and NHScienceFair had ANI's between 99.982% - 99.991% but also differed by at least one amino acid. Bloomfield, Ollie, FutureBee, and Dante also had ANI's between 99.982% - 99.991% and differed by at least one amino acid residue. Lastly, Logan and Ted had an ANI of 99.977% but had three amino acid differences.

From these results, we determined that there are two distinct clusters of phages in New Zealand, with phages belonging to the larger cluster being very closely related, sometimes with very few amino acid differences. In the smaller cluster, Callan and Dash were very closely related, while Lilo had an ANI of 71.37% and 78.37% respectively with each of these two phages.

	ABAtENZ	Rae2Bee1	Lena	LunBun	TonyLawson	Jacinda	BarryFoster_Benicio	GaryLarson	UtuhinaGold_Zacery
ABAtENZ		99.99	99.99	99.97	99.97	99.97	99.94	99.95	99.95
Rae2Bee1	99.99		99.98	99.98	99.97	99.96	99.95	99.95	99.96
Lena	99.99	99.98		99.97	99.97	99.96	99.94	99.94	99.95
LunBun	99.97	99.98	99.97		99.99	99.97	99.96	99.96	99.97
TonyLawson77	99.97	99.97	99.97	99.99		99.98	99.95	99.95	99.96
Jacinda	99.97	99.96	99.96	99.97	99.98		99.94	99.95	99.95
BarryFoster_Benicio	99.94	99.95	99.94	99.96	99.95	99.94		99.99	99.98
GaryLarson	99.95	99.95	99.94	99.96	99.95	99.95	99.99		99.99
UtuhinaGold_Zacery	99.95	99.96	99.95	99.97	99.96	99.95	99.98	99.99	
NHScienceFair	99.95	99.95	99.94	99.95	99.95	99.95	99.99	99.99	99.98
Bloomfield	99.96	99.96	99.97	99.95	99.94	99.94	99.91	99.92	99.93
Ollie	99.96	99.96	99.96	99.95	99.94	99.94	99.91	99.92	99.93
FutureBee	99.96	99.96	99.96	99.94	99.94	99.94	99.91	99.91	99.93
Dante	99.96	99.96	99.96	99.95	99.94	99.94	99.91	99.92	99.93
AJG77	99.92	99.92	99.92	99.91	99.90	99.89	99.88	99.88	99.89
Logan	99.83	99.82	99.82	99.83	99.84	99.84	99.86	99.87	99.86
Ted	99.81	99.81	99.81	99.82	99.82	99.82	99.85	99.85	99.84
ApiWellbeing	99.65	99.66	99.65	99.67	99.67	99.68	99.70	99.70	99.69
Carlos	99.83	99.83	99.83	99.82	99.81	99.83	99.79	99.79	99.80
Wildcape	99.79	99.79	99.79	99.81	99.80	99.81	99.84	99.84	99.83
GIW2016	97.68	97.68	97.67	97.68	97.68	97.68	97.72	97.72	97.71
Rosalind	97.69	97.69	97.69	97.70	97.71	97.70	97.73	97.74	97.73
Bob	97.41	97.42	97.41	97.43	97.43	97.42	97.46	97.46	97.45
Callan	40.94	40.94	40.93	40.95	40.95	40.95	40.95	40.95	40.95
Dash	41.92	41.93	41.92	41.93	41.93	41.94	41.93	41.93	41.93
Lilo	41.05	41.05	41.05	41.06	41.07	41.07	41.06	41.06	41.06

	NHScience Fair	Bloomfield	Ollie	FutureBee	Dante	AJG77	Logan	Ted	ApiWellbeing
ABAtENZ	99.95	99.96	99.96	99.96	99.96	99.92	99.83	99.81	99.65
Rae2Bee1	99.95	99.96	99.96	99.96	99.96	99.92	99.82	99.81	99.66
Lena	99.94	99.97	99.96	99.96	99.96	99.92	99.82	99.81	99.65
LunBun	99.95	99.95	99.95	99.94	99.95	99.91	99.83	99.82	99.67
TonyLawson77	99.95	99.94	99.94	99.94	99.94	99.90	99.84	99.82	99.67
Jacinda	99.95	99.94	99.94	99.94	99.94	99.89	99.84	99.82	99.68
BarryFoster_Benicio	99.99	99.91	99.91	99.91	99.91	99.88	99.86	99.85	99.70
GaryLarson	99.99	99.92	99.92	99.91	99.92	99.88	99.87	99.85	99.70
UtuhinaGold_Zacery	99.98	99.93	99.93	99.93	99.93	99.89	99.86	99.84	99.69
NHScienceFair		99.92	99.92	99.91	99.92	99.88	99.87	99.85	99.70
Bloomfield	99.92		99.99	99.99	99.99	99.94	99.80	99.79	99.63
Ollie	99.92	99.99		99.99	99.99	99.94	99.80	99.79	99.63
FutureBee	99.91	99.99	99.99		99.98	99.94	99.80	99.78	99.62
Dante	99.92	99.99	99.99	99.98		99.94	99.80	99.78	99.63
AJG77	99.88	99.94	99.94	99.94	99.94		99.76	99.74	99.59
Logan	99.87	99.80	99.80	99.80	99.80	99.76		99.98	99.82
Ted	99.85	99.79	99.79	99.78	99.78	99.74	99.98		99.83
ApiWellbeing	99.70	99.63	99.63	99.62	99.63	99.59	99.82	99.83	
Carlos	99.79	99.81	99.82	99.80	99.81	99.77	99.66	99.65	99.81
Wildcape	99.83	99.76	99.76	99.76	99.76	99.72	99.71	99.70	99.85
GIW2016	97.72	97.65	97.65	97.64	97.64	97.61	97.84	97.85	97.70
Rosalind	97.73	97.67	97.67	97.66	97.67	97.63	97.81	97.83	97.67
Bob	97.46	97.39	97.39	97.38	97.39	97.35	97.58	97.59	97.44
Callan	40.95	40.93	40.93	40.93	40.93	40.91	40.97	40.97	41.11
Dash	41.93	41.91	41.91	41.91	41.91	41.90	41.95	41.95	42.09
Lilo	41.06	41.04	41.04	41.04	41.04	41.04	41.08	41.09	41.23

	Carlos	Wildcape	GIW2016	Rosalind	Bob	Callan	Dash	Lilo
ABAtENZ	99.83	99.79	97.68	97.69	97.41	40.94	41.92	41.05
Rae2Bee1	99.83	99.79	97.68	97.69	97.42	40.94	41.93	41.05
Lena	99.83	99.79	97.67	97.69	97.41	40.93	41.92	41.05
LunBun	99.82	99.81	97.68	97.70	97.43	40.95	41.93	41.06
TonyLawson77	99.81	99.80	97.68	97.71	97.43	40.95	41.93	41.07
Jacinda	99.83	99.81	97.68	97.70	97.42	40.95	41.94	41.07
BarryFoster_Benicio	99.79	99.84	97.72	97.73	97.46	40.95	41.93	41.06
GaryLarson	99.79	99.84	97.72	97.74	97.46	40.95	41.93	41.06
UtuhinaGold_Zacery	99.80	99.83	97.71	97.73	97.45	40.95	41.93	41.06
NHScienceFair	99.79	99.83	97.72	97.73	97.46	40.95	41.93	41.06
Bloomfield	99.81	99.76	97.65	97.67	97.39	40.93	41.91	41.04
Ollie	99.82	99.76	97.65	97.67	97.39	40.93	41.91	41.04
FutureBee	99.80	99.76	97.64	97.66	97.38	40.93	41.91	41.04
Dante	99.81	99.76	97.64	97.67	97.39	40.93	41.91	41.04
AJG77	99.77	99.72	97.61	97.63	97.35	40.91	41.90	41.04
Logan	99.66	99.71	97.84	97.81	97.58	40.97	41.95	41.08
Ted	99.65	99.70	97.85	97.83	97.59	40.97	41.95	41.09
ApiWellbeing	99.81	99.85	97.70	97.67	97.44	41.11	42.09	41.23
Carlos		99.94	97.52	97.54	97.26	41.07	42.05	41.19
Wildcape	99.94		97.56	97.59	97.30	41.08	42.07	41.20
GIW2016	97.52	97.56		99.96	99.53	41.64	42.65	41.79
Rosalind	97.54	97.59	99.96		99.50	41.64	42.65	41.79
Bob	97.26	97.30	99.53	99.50		41.64	42.64	41.80
Callan	41.07	41.08	41.64	41.64	41.64		91.60	71.37
Dash	42.05	42.07	42.65	42.65	42.64	91.60		78.37
Lilo	41.19	41.20	41.79	41.79	41.80	71.37	78.37	

Figure 6.2 Clustering of New Zealand *P. larvae* phages based on ANI presented as a percentage (%)

6.3.3.2 Clustering using Dot Plot analyses

Another way to cluster phages is to use dot plots. Dot plots are a way of comparing two sequences using dot matrix analysis and observing their degree of similarity. As per Hatfull *et al.* (Hatfull et al., 2010), two phages are placed in a cluster if they have sequence similarity of at least 50%. Dot plot analyses using Gepard (Krumsiek et al., 2007) showed two distinct clusters in our New Zealand phages (Figure 6.3), in agreement with the clustering determined by ANI (Figure 6.2). The 23 phages within the large cluster (red box in Figure 6.3) showed a very high degree of similarity. The smaller cluster (green box in Figure 6.3) contained Callan, Dash, and Lilo, Callan, and Dash were highly similar and showed a reasonable degree of similarity with Lilo. The two clusters identified in the dot plot showed very little similarity between them, it should be noted however that two regions at the beginning of the genome are highly conserved between all the New Zealand phages; this region usually includes 14 assembly and structural genes.

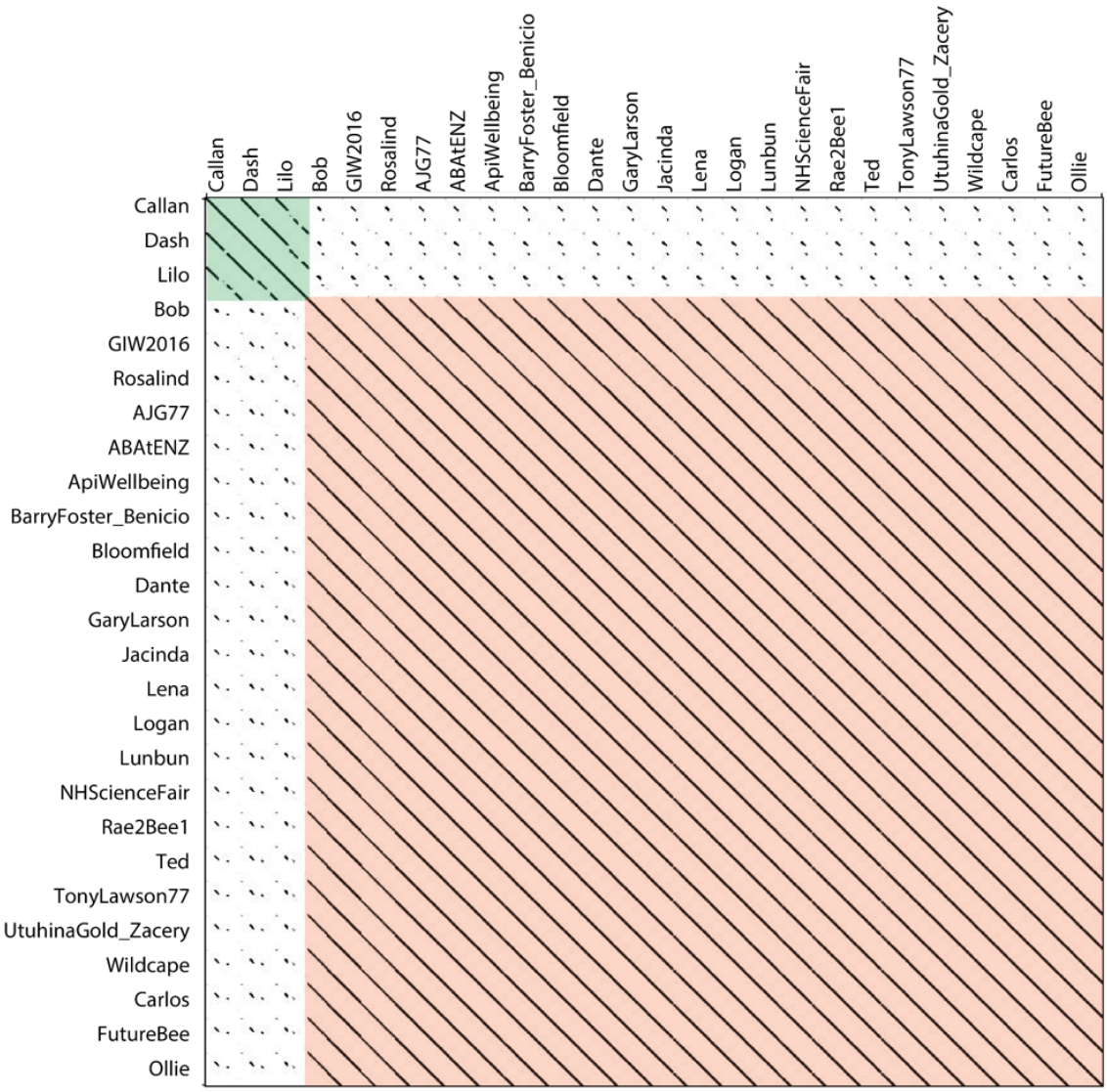


Figure 6.3 Dot plot of 26 New Zealand *P. larvae* phages displayed using Gepard (Krumsiek et al., 2007)

Phages have been grouped into two clusters. The cluster highlighted in red contains 23 phages with a high degree of similarity. The cluster highlighted in green contains three phages with Callan and Dash showing a high degree of similarity and Lilo showing slightly less similarity.

6.3.3.3 Clustering using Phamerator

The final approach we used to assign clusters to the New Zealand *P. larvae* phages was pairwise comparison using Phamerator (Cresawn et al., 2011). This clustering method resulted in the same two clusters as those determined in previous methods. Callan, Dash, and Lilo formed one cluster, and the other 23 phages formed another highly interrelated cluster (Figure 6.4).

Callan and Dash are highly conserved across the majority of the genome, with only one region of difference in the middle of the genome, containing five and three annotated genes, respectively. The region that differs between Callan and Dash is conserved between Dash and Lilo and contains a *P. larvae* toxin. Lilo also had two other regions that differ from Callan and Dash. Two regions of the genome are highly conserved between the two clusters, including the small and large terminase and a region containing tail proteins, a holin and a N-acetylmuramoyl-L-alanine amidase. Within the larger cluster Bob, GIW2016 and Rosalind were different from the other phages due to the deletion of two genes in the middle of the genome.

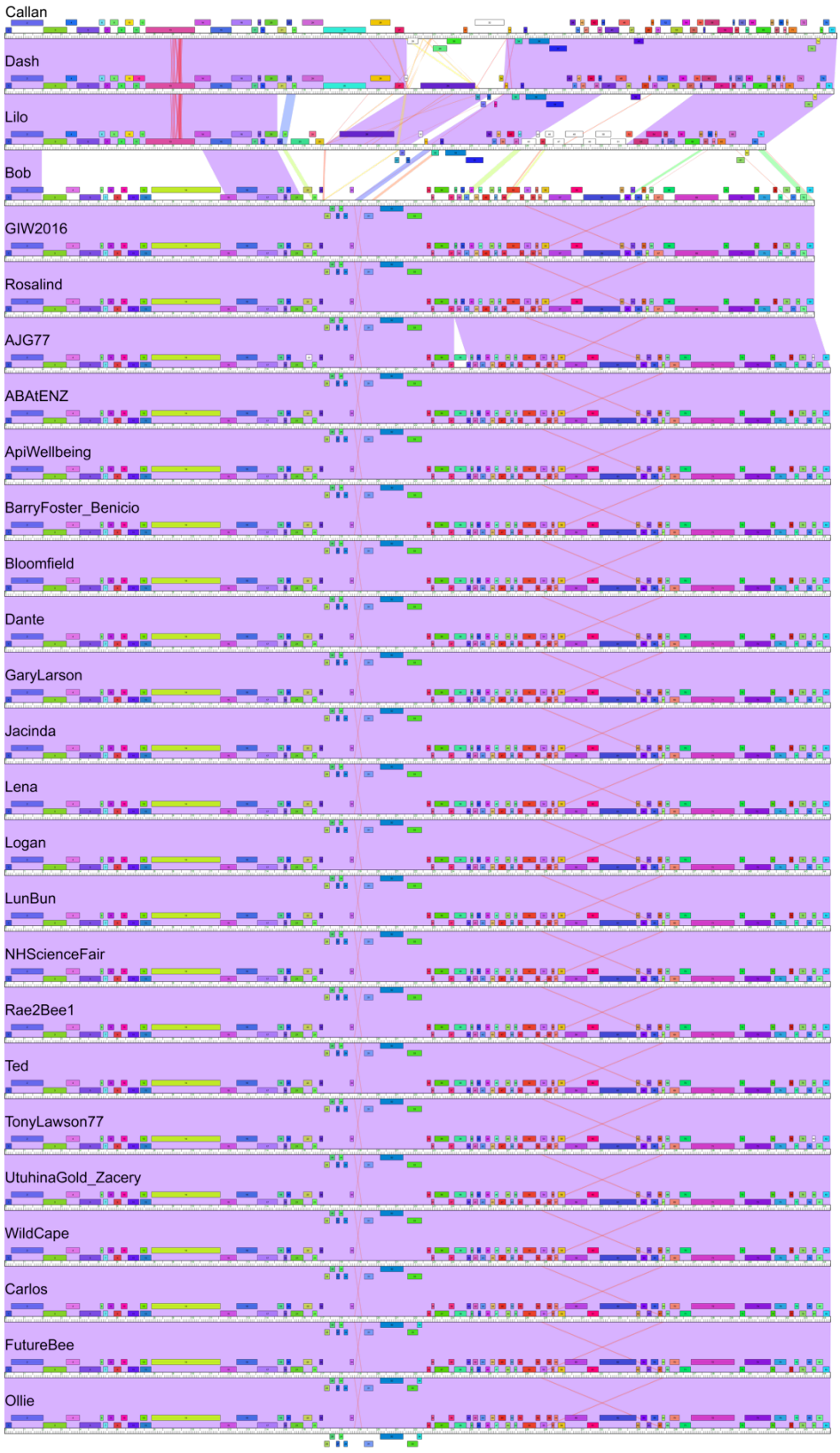


Figure 6.4 Genome maps of 26 New Zealand *P. larvae* phages displayed using Phamerator
Coloured boxes represent genes and genes of the same colour indicate they belong to the same pham. Shading between the genomes indicates how similar an aligned region is at the nucleotide level according to the E-value, with purple depicting an E-value of zero, white indicating no recognisable similarity, and red indicating similarity at the cut-off threshold of 1E-4. Callan, Dash, and Lilo form one cluster and the remaining phages are in a single second cluster.

6.3.4 Clustering of all 95 P. larvae Phages

Once we had established clustering of the 26 New Zealand *P. larvae* phages, we wanted to cluster them with the other 69 *P. larvae* phages available on NCBI. We used the same three clustering methods mentioned above: ANI, dot plot similarity, and the bioinformatic tool Phamerator (Cresawn et al., 2011).

6.3.4.1 Global *P. larvae* phage clustering using ANI

Clustering of all phages was performed in the same way as for the New Zealand phages. All clusters have previously been named based on (Stamereilers et al., 2018). Four main clusters were created using this method, with three singletons (Supplementary Table S6.2). The systematics picture is thus largely unchanged since 2018, given that all phages discovered since then fall into the previously discovered clusters, with the exception of two new singletons. ANI greater than 99.975% was assessed between the 95 phages and other than the New Zealand phages mentioned earlier and two subclusters previously mentioned in Stamereilers *et al*. (Stamereilers et al., 2018), there were two additional sets of phages that had an ANI greater than 99.975%: Newport and Fitz had an ANI of 100%, indicating that they were identical phages; Riker and Norbert had an ANI of 99.990%, but had an amino acid difference.

P. larvae phage Lily remains a singleton but our analysis established that phiERICV (a prophage of recently discovered *P. larvae* ERIC V (Beims et al., 2020)) and vB_PlaP_API480 are new phage singletons. Lily has less than 50% similarity to any other *P. larvae* phage, while vB_PlaP_API480 has less than 30% similarity. The most divergent phage was prophage

phiERICV, which showed less than 25% similarity to any other *P. larvae* phage. We did not discover any singletons in the New Zealand *P. larvae* phages.

6.3.4.2 *P. larvae* phage clustering using Total - ANI (tANI)

Total ANI (tANI) was also used to create a SplitsTree of all 95 phages (Figure 6.5). The Fern cluster (blue in Figure 6.5) was the largest cluster and contained 49 phages within 16 subclusters, including nine subclusters that contained only one phage. The Fern cluster did not have any New Zealand *P. larvae* phages.

The next largest cluster was the Vegas cluster (red in Figure 6.5), which now contains 30 phages within four sub-clusters, including Dragolir, which formed its own sub-cluster. The large cluster of 23 New Zealand phages falls in the Vegas cluster, bringing this former cluster of seven phages to 30, more than quadrupling the size of the Vegas cluster.

The Halcyone cluster (purple in Figure 6.5) contained eight phages within four sub-clusters, with Tripp and Unity forming their own sub-clusters. The Halcyone cluster did not contain any New Zealand *P. larvae* phage or any other new phages since 2018, and remains unchanged since then.

The Harrison cluster (green in Figure 6.5), previously contained two phages. This analysis joins three novel New Zealand phages (Callan, Dash, Lilo) to this cluster bringing it to five phages in three sub-clusters, and a new sub-cluster containing only Lilo.

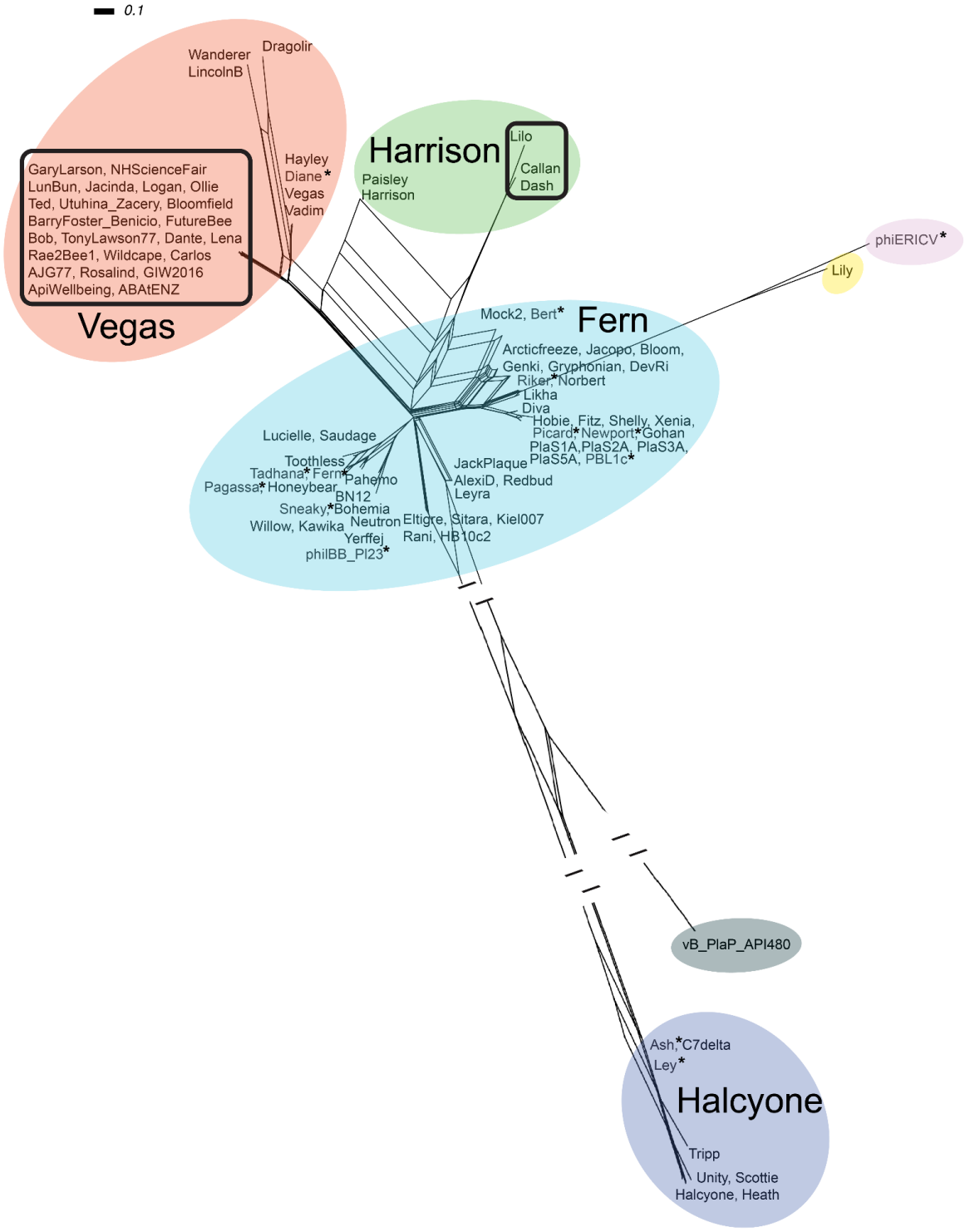


Figure 6.5 Phylogenetic network of 95 *P. larvae* phage genomes using NeighborNet transformed tANI distances, visualised and constructed via SplitsTree

Coloured circles indicate phages grouped into a cluster. Blue = Fern cluster, Red = Vegas cluster, Purple = Halcyone cluster, Green = Harrison cluster, Yellow for singleton Lily, Pink for singleton phiERICV and Grey for singleton vB_PlaP_API480. Asterisks indicate prophages. New Zealand *P. larvae* phages are circled. Dashes (//) indicate shortened branches.

6.3.4.2 Clustering 95 *P. larvae* phages using Dot Plot analyses

Dotplot analyses of all 95 *P. larvae* phages were performed in the same way as described above for the New Zealand phages. The clusters created using this technique were the same as those created using ANI (Figure 6.6). The Halcyone cluster and the singleton vB_PlaP_API48 show very little similarity to any of the other phages. The other phages show some degree of similarity with the other clusters.

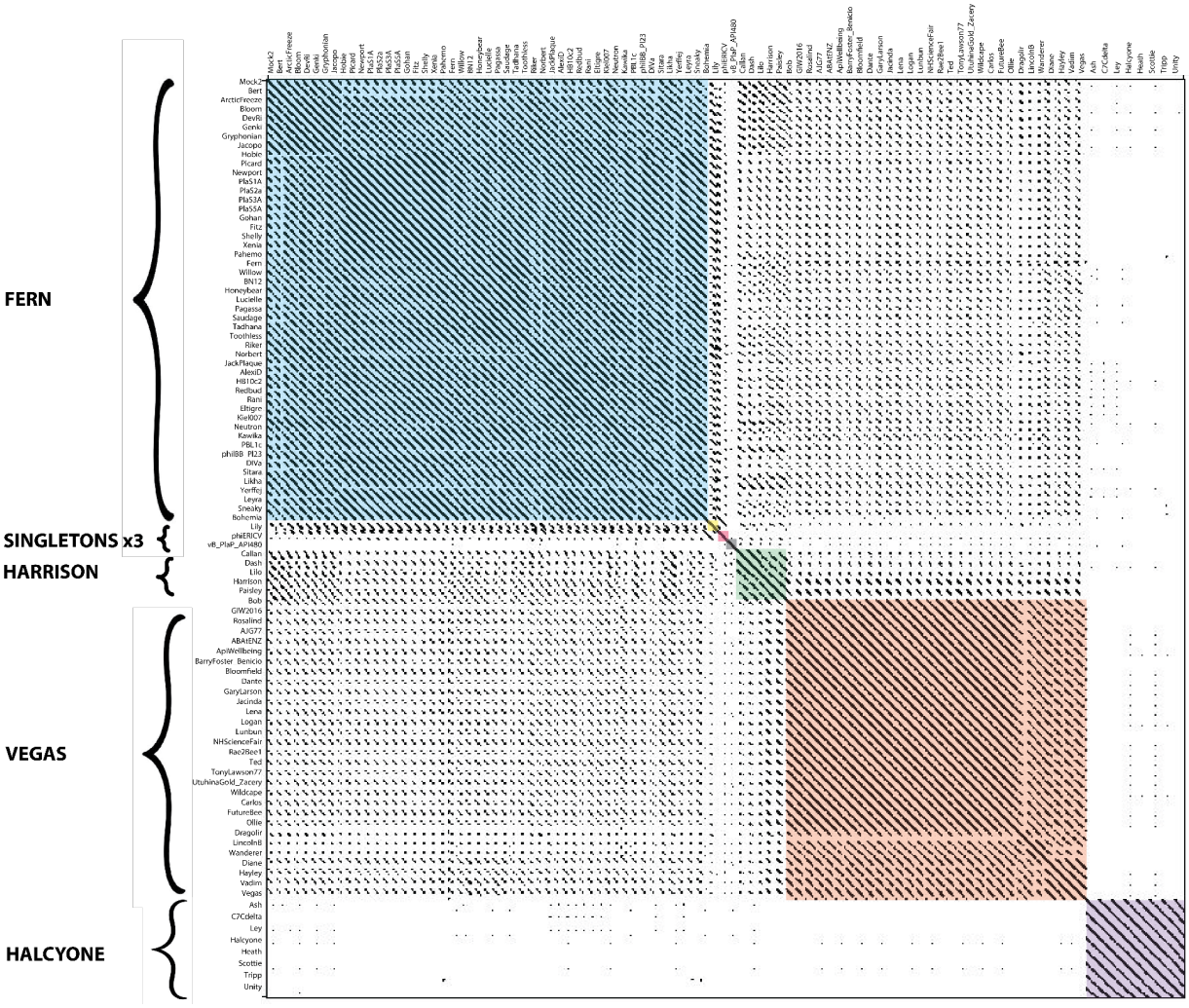


Figure 6.6 Dot plot of 95 *P. larvae* phages displayed using Gepard (Krumsiek et al., 2007)
Phages have been grouped into four clusters and three singletons. Colours indicate phages grouped into a cluster. Blue = Fern cluster, Red = Vegas cluster, Purple = Halcyone cluster, Green = Harrison cluster, Yellow for singleton Lily, Pink for singleton phiERICV and Grey for singleton vB_PlaP_API480.

6.3.4.3 Clustering 95 *P. larvae* phages using Phamerator

The last method for assessing the clustering of the 95 *P. larvae* phages was pairwise comparison using the Phamerator tool. The maps (Supplementary Figure S6.1) show where the genomes are similar and where they diverge. The first 22 genes of the 49 phages within the Fern cluster are highly conserved. The Mock2 and Bert subclusters have conservation across the whole genome. The sub-cluster that contains ArcticFreeze and five other phages is also highly conserved and differs from the Mock2 sub-cluster by a large region towards the end of the genomes. The sub-cluster containing Hobie and ten other phages is largely conserved; Hobie and Picard were highly conserved, whereas the other genomes in that sub-cluster differ by the insertion of a large gene at the end of the genome.

The ten Pahemo sub-cluster genomes show a high degree of conservation throughout the first half of the genomes and variability throughout the second half. The Norbert and Riker sub-cluster are highly conserved. The sub-cluster containing JackPlaque and six other phages is made up of genomes that are conserved, with small regions of divergence in the second half of the genomes. The Sneaky and Bohemia sub-clusters also consist of phages with a high degree of conservation along their lengths.

The rest of the Fern cluster contains individual phages, each in their own sub-cluster. The first half of these genomes are highly conserved, whereas the second half are highly divergent. Lily, phiERICV, and vB_PlaP_API480 are all singletons, which is clearly shown in the Phamerator maps with little genome conservation (Supplementary Figure S6.1). Lily has small

areas of genomic similarity with the phages in the Fern sub-cluster and phiERICV. Phage vB_PlaP_API480 shows almost no genome conservation with other phages.

The Harrison cluster has conservation at the very beginning of the genome, at the end of the genome, and a section through the middle of the genome. Dash, Lilo, and Callan, all New Zealand phages, have sequence similarity in the first half of their genomes with the phages in the Fern cluster, whereas Harrison and Paisley, two phages discovered in the US, have sequence similarity in the first half of their genomes with the Vegas cluster. Harrison and Paisley have similar genomes.

The 23 New Zealand phages contained within the Vegas cluster have been described previously in this chapter. The remaining phages within the Vegas cluster are similar to the New Zealand phages by a region in the second quarter of the genome. Diane, Hayley, Vadim and Vegas form a sub-cluster and are highly similar. The final cluster, Halcyone, contains eight phages; this cluster of phages is highly divergent from the other *P. larvae* phages, as seen in the dotplots, but these phages show a high degree of conservation within this cluster.

6.3.5 Analysis of P. larvae Phage Toxin Plx1

The *P. larvae* toxin Plx1 was detected in two of the Harrison cluster *P. larvae* phages from New Zealand. This toxin had previously been noted in four *P. larvae* phages (Yerffej, philBB_Pl23, Harrison, and Paisley) (Ebeling et al., 2021). Harrison and Paisley are in the Harrison cluster, we therefore decided to make a study of the toxin in these phages. This phage-borne toxin, known as Plx1, is also found in the genome of *P. larvae* ERIC I (CP019687) in a Harrison cluster prophage (Ebeling et al., 2021). Plx1 has been shown to contribute to the

virulence of *P. larvae* ERIC I, but is not the sole source of its pathogenicity (Fünfhaus et al., 2013).

Plx1 is an AB-toxin, which contains an A-subunit with an ADP-ribosylating domain. This domain is thought to induce apoptosis in eukaryotic cells. The protein also has a B-subunit containing four ricin B-like lectin domains. These help mediate the entry of the toxin into the eukaryotic host cells (Fünfhaus et al., 2013).

This highly conserved toxin protein contains 975 amino acids and the amino acids differ between toxins by a maximum of three between any two peptide sequences. In our analysis, phage philBB_Pl23 is the most divergent with only three amino acid differences, caused by three nucleotide changes, compared to Dash, Harrison, Paisley, and *P. larvae* ATCC 9545 ERIC I.

To dig deeper into these mutations we performed an ANI of Plx1 from the six phage genomes (Figure 6.7). We found pairwise identity between these homologs of 99.69% - 100% across the length of the toxins 2928 bp (Figure 6.7). We note that there is 100% nucleotide identity in this toxin between Harrison, Paisley, and Dash, two of which were phages found in Nevada and one of which (Dash) was found in our work in New Zealand. In addition, the toxin gene is 100% identical to the toxin in the *P. larvae* ATCC 9545 strain, from Argentina.

	Dash	Lilo	Harrison	Paisley	philBB_Pl23	Yerffej	P. larvae
Dash		99.97	100	100	99.90	99.93	100
Lilo	99.97		99.97	99.97	99.93	99.97	99.97
Harrison	100	99.97		100	99.90	99.93	100
Paisley	100	99.97	100		99.90	99.93	100
philBB_Pl23	99.90	99.93	99.90	99.90		99.97	99.90
Yerffej	99.93	99.97	99.93	99.93	99.97		99.93
P. larvae ERIC I	100	99.97	100	100	99.90	99.93	

Figure 6.7 Average nucleotide identity of the six phage toxins and the toxin from *P. larvae* ERIC I (ATCC 9545) expressed as a percentage (%)

The remaining phages containing this Plx1 toxin are Yerffej and philBB_Pl23 in the Fern cluster; both these phages show a mere 50% ANI across the length of the genomes with the other four toxin bearing phages from the Harrison cluster (Figure 6.8 and Supplementary Table S6.2).

Of the three New Zealand phages in the Harrison cluster; Dash, and Lilo have this highly conserved Plx1 toxin but Callan does not (Figure 6.8), rather, Callan has a set of five genes in this location, some of which, are present in the Fern cluster and two of which are not otherwise found in the *P. larvae* phages in our Phamerator database. Neutron is a Fern cluster phage, isolated from North Carolina, that bears a high degree of similarity in its gene order or synteny to phage Yerffej with the exception of the toxin region which appears excised. In addition, the Phamerator maps show evidence of repeat regions in the region flanking the toxin gene (Figure 6.8 orange crosses). Together, the phylogenetically heterogeneous appearance of a large toxin, flanked by repeat sequences, and synteny disruption, strongly suggests that the toxin is highly mobile and we sought to further investigate this phenomenon.

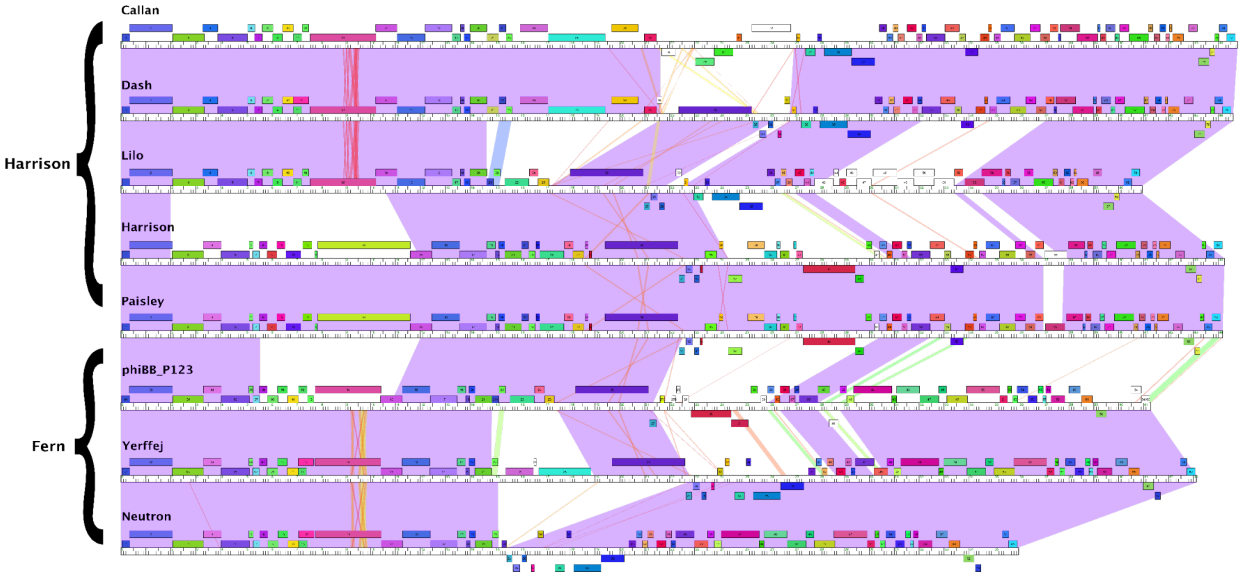


Figure 6.8 Genome maps of eight *P. larvae* phages displayed using Phamerator
The Plx1 toxin is shown as dark purple genes located close to the middle of Dash, Lilo, Harrison, Paisley, philBB_P123, and Yerffej and absent in Neutron and Callan. Coloured boxes represent genes and genes of the same colour indicate they belong to the same pham in the Phamerator database containing 95 *P. larvae* phage genomes. Colouring between genomes indicates how similar pairwise aligned regions are at the nucleotide level, purple indicates an E-value of zero and white shows no recognisable similarity. Other colours represent other levels of similarity (Cresawn et al., 2011).

In order to further characterise this putative mobile region we first identified the repeat regions in the vicinity of the toxin in the six phage genomes and the *P. larvae* ATCC9545 genome. We identified evidence of repeat regions around the toxins and named them according to the genome in which we found both left and right versions (Figure 6.9A, Table 6.2). Both repeat sequences contain inverted repeats that have the potential to form hairpins (Figure 6.9B).

In the Harrison cluster phages Lilo, Harrison, and Paisley all contain direct repeat elements flanking their toxins that come in two slightly distinct types, the Left and Right, Plx1FHL and Plx1FHR respectively (Table 6.2). They all also contain a single copy of Plx1FDR

discovered as a pair in Dash (Table 6.2). This pattern of repeat regions is the same as that contained within *P. larvae* ATCC 9545 ERIC I (Figure 6.9A).

Dash contains the toxin flanked by a longer repeat element termed Plx1FDL and Plx1FDR. In addition, Dash also has a copy of the Plx1FHL and Plx1FHR repeats from Harrison (Figure 6.9A and Table 6.2). When both the Harrison and Dash repeats are present, they are always found in the same order, Dash-Harrison.

Phage Callan is highly syntenic with Dash with the exception of this toxin region and neighbouring genes (Figure 6.8). Callan contains a 100% match to the repeat sequence Plx1FDL which is in close proximity to an incomplete Plx1FHL. A short distance from these repeats, an incomplete and degenerate Plx1FDR is found in a gene that is also present in 10 other Fern cluster phage genomes and the singleton Lily (Figure 6.9A).

Interestingly, in the Fern cluster phages that carry the toxin, Yerffej and PhilBB_P123, do not contain a complete set of either the Harrison or Dash repeat sequences. Phage Yerffej carries a copy of both the right hand repeat sequences Plx1FDR and Plx1FHR, but no left hand repeat sequences. PhilBB_P123 has a Plx1FHL and an incomplete Plx1FDR (Figure 6.9A). Phage Neutron, which is highly syntenic with Yerffej, and has a notable deletion in the vicinity of the toxin, carries two of the right hand repeat regions in this location, Plx1FDR and Plx1FHR (Figure 6.9A).

Table 6.2. Repeat regions found flanking Plx1 toxin. Differences between the Left and Right versions of the repeats are shown in bold

Repeat Name	Bp	Repeat Sequence	Direction	Start in Phage*	Start in *P. larvae* ERIC I
Plx1FHL	**39**	ACAGAAAGAGCCTTTGAG**T**AAGCACCTCAAG**A**CTCTTTT	F	18,634	602,646
Plx1FHR	**39**	ACAGAAAGAGCCTTTGAG**G**AAGCACCTCAAG**G**CTCTTTT	F	22,583	606,593
Plx1FDL	**55**	TCTTGACAAAA**G**CTCTTT**G**CAACCATCTCACCCAAAAACCC**T**TG**T**TTTTGCAACC	F	21,490	-
Plx1FDR	**55**	TCTTGACAAAA**A**CTCTTT**T**CAACCATCTCACCCAAAAACCC**G**TG**A**TTTTGCAACC	F	25,425	606,481

*Phage position is specific to the named phage D = Dash and H = Harrison.

The *P. larvae* ATCC 9545 ERIC I Plx1 toxin also has some evidence of these repeats. Plx1FHL and Plx1FHR flank the toxin on the left and right sides respectively and Plx1FDR is present at the right side of the bacterial toxin gene. (Figure 6.9A).

To learn whether these repeats might have previously been recognised, we submitted the complete region between the sets of repeats to the ISFinder database (Siguier et al., 2006). There was a single significant hit, but this was within the toxin gene and did not include our repeat sequences. Further research will be required to understand the nature of these repeat elements and what role they may play in mobilising the toxin.

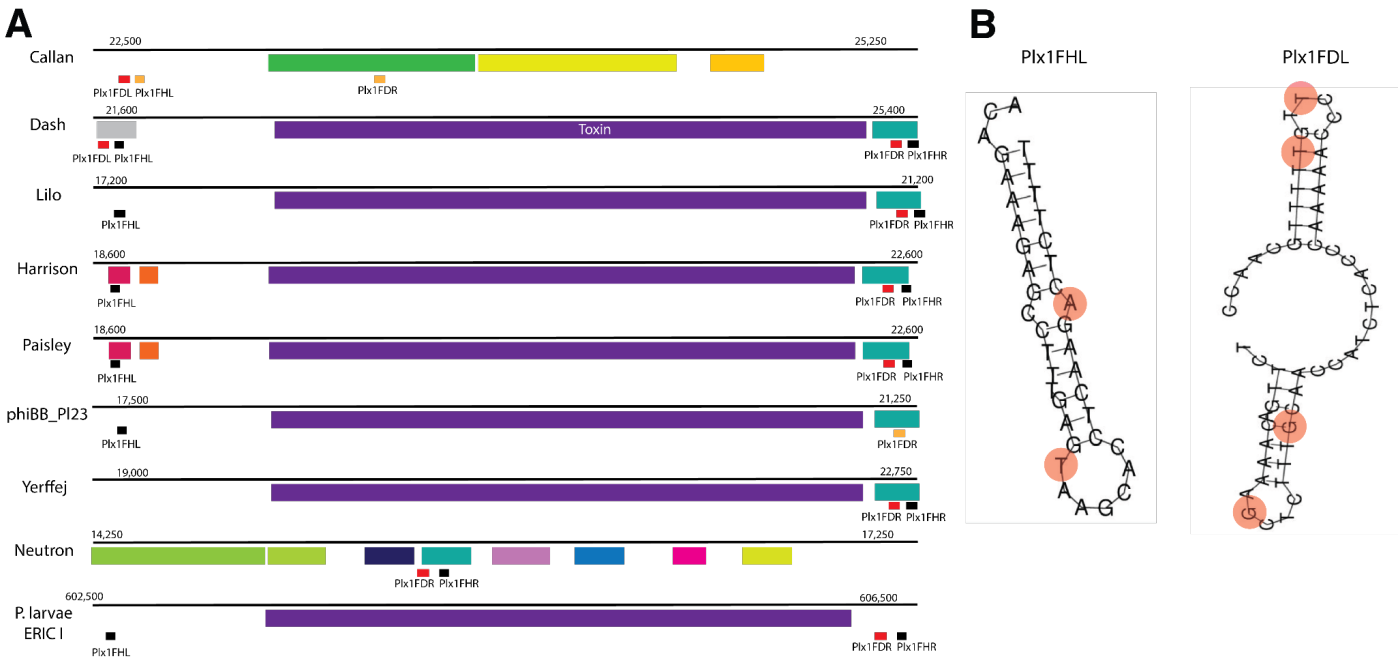


Figure 6.9 Repeat sequences in eight *P. larvae* phage genomes and *P. larvae* ATCC 9545 ERIC I
A) Genome sequences of Callan, Dash, Lilo, Harrison, Paisley, philBB_Pl23, Yerffej, Neutron, and *P. larvae* ATCC 9545 ERIC I showing the repeat sequences that flank the toxin (Purple). Similar genes are the same colour. Red = Dash repeat sequences, Black = Harrison repeat sequences, Orange = <100% match repeat sequence**.** A cost matrix of 70% was used to detect degenerate or truncated repeats. B) Hairpins formed by repeat sequences for Plx1FHL and Plx1FDL made in VectorBuilder (https://en.vectorbuilder.com). Red circles indicate bases that differ between the left and right repeat sequence.

6.3.6 Analysis of P. larvae Phage N-acetylmuramoyl-L-alanine Amidase

In the previous work, 12 amidases were described, this work doubles the number of amidases found in the *P. larvae* phage genomes (Stamereilers et al., 2018). Each of the new 47 new *P. larvae* phage genome contains an N-acetylmuramoyl-L-alanine amidase. The N-acetylmuramoyl-L-alanine amidase is an endolysin responsible for cleaving the host cell wall and has been the subject of several studies, including one that shows it has promise as a therapeutic agent (LeBlanc et al., 2015; Oliveira et al., 2015; Santos et al., 2019). According to an ANI comparison, there are 26 different types of N-acetylmuramoyl-L-alanine amidase in

the 95 *P. larvae* phages sequenced to date. Endolysin types were classified as dissimilar if at least one nucleotide difference was present that resulted in an amino acid change. A single phage endolysin representative was chosen from each of these types and an ANI of these 26 types (Figure 6.10). The ANI also revealed two distinct groups with very little sequence similarity, <14%.

Within the two distinct groups, these endolysins were fairly similar, with the larger of the two groups containing genes with an ANI of 89-99% to one another and the smaller group, with only four phage endolysins having an ANI of 94-98%.

The phages in the smaller group all belonged to the Halcyone cluster, which is the most genetically distinct of the clusters of *P. larvae* phages discovered to date, with 20% longer genomes and Direct Terminal Repeats (DTR) DNA packaging strategy as opposed to 3' cos (Stamereilers et al., 2018). The two N-acetylmuramoyl-L-alanine amidase type groups are shown in an rooted gene tree, in which the two branches are very far apart (Figure 6.11). The amidases of all phages discovered since 2018 are all in the larger cluster, and highly similar to those of other phages in the cluster, even for singletons Lily, phiERICV and vB_PlaP_API480. This indicates the amidase is conserved even in very divergent phages, except for the longer DTR phages. The two versions of the amidase have different 3D structure, with the DTR phage amidase being 60 aa longer, as shown in [Tsourkas 2020]. It remains to be discovered if there is a functional difference between the two versions of the amidase.

	ABAtENZ	AlexiD	BN12	Bohemia	Arctic Freeze	Bert	Lily	Fern	Honey Bear	Tadhana	Likha	Diva	Kawika	phiERICV	ApiWell being	Callan	Lilo	Harrison	LincolnB	Wander	Dragolir	vB_PlaP	Ash	Ley	C7Cdelta	Halcyone
ABAtENZ		94.87	94.44	95.29	92.22	91.78	92.22	93.97	94.42	94.87	93.11	92.19	94.47	94.44	95.31	94.20	94.20	91.11	92.00	91.56	93.56	93.97	13.47	13.48	13.67	13.67
AlexiD	94.87		97.99	97.54	96.65	95.76	95.31	96.21	95.31	95.76	95.31	94.87	95.78	94.87	93.97	92.86	92.86	92.41	93.75	94.20	92.63	93.08	13.47	13.48	13.67	13.67
BN12	94.44	97.99		96.21	97.11	96.22	95.78	97.11	96.22	96.67	95.78	95.31	95.35	95.33	93.56	92.44	92.44	93.33	92.89	93.33	92.22	92.63	13.76	13.77	13.95	13.95
Bohemia	95.29	97.54	96.21		94.87	94.87	94.42	96.19	95.74	96.19	94.42	93.08	94.44	93.97	93.95	92.83	92.83	91.96	92.41	93.30	92.19	93.08	13.51	13.53	13.71	13.71
Arcticfreeze	92.22	96.65	97.11	94.87		98.89	98.44	97.56	95.78	96.22	96.22	95.31	93.14	93.11	92.22	91.11	91.11	93.78	93.78	94.22	93.56	91.74	13.76	13.77	13.95	13.95
Bert	91.78	95.76	96.22	94.87	98.89		99.33	97.56	96.22	96.67	96.22	95.76	93.58	92.22	92.67	91.56	91.56	94.22	93.33	94.22	94.00	92.19	14.43	14.44	14.62	14.62
Lily	92.22	95.31	95.78	94.42	98.44	99.33		97.11	95.78	96.22	95.78	95.31	93.14	91.78	93.11	92.00	92.00	94.67	93.78	94.67	94.44	92.19	14.43	14.44	14.62	14.62
Fern	93.97	96.21	97.11	96.19	97.56	97.56	97.11		97.54	97.99	97.56	95.31	94.03	93.56	93.08	91.96	91.96	93.33	92.89	93.78	92.22	92.19	14.48	14.49	14.67	14.67
Honeybear	94.42	95.31	96.22	95.74	95.78	96.22	95.78	97.54		99.33	95.33	95.76	94.47	93.56	93.53	92.41	92.41	93.78	92.00	92.44	93.56	92.63	14.48	14.49	14.67	14.67
Tadhana	94.87	95.76	96.67	96.19	96.22	96.67	96.22	97.99	99.33		95.78	96.21	94.91	94.00	93.97	92.86	92.86	94.22	92.44	92.89	94.00	93.08	14.14	14.16	14.33	14.33
Likha	93.11	95.31	95.78	94.42	96.22	96.22	95.78	97.56	95.33	95.78		94.87	93.14	94.44	92.22	91.11	91.11	92.00	93.33	94.22	92.22	92.19	14.09	14.11	14.29	14.29
Diva	92.19	94.87	95.31	93.08	95.31	95.76	95.31	95.31	95.76	96.21	94.87		94.89	92.19	93.08	91.96	91.96	93.75	91.96	93.30	93.97	92.19	13.47	13.48	13.67	13.33
Kawika	94.47	95.78	95.35	94.44	93.14	93.58	93.14	94.03	94.47	94.91	93.14	94.89		92.70	95.35	94.25	94.25	92.92	90.71	92.04	91.81	94.44	14.38	14.40	14.57	14.57
phiERICV	94.44	94.87	95.33	93.97	93.11	92.22	91.78	93.56	93.56	94.00	94.44	92.19	92.70		92.67	92.89	92.44	91.11	91.11	91.56	92.67	91.74	13.42	13.44	13.62	13.62
ApiWellbeing	95.31	93.97	93.56	93.95	92.22	92.67	93.11	93.08	93.53	93.97	92.22	93.08	95.35	92.67		98.66	98.66	95.56	91.11	91.56	92.22	93.53	14.81	14.83	15.00	15.00
Callan	94.20	92.86	92.44	92.83	91.11	91.56	92.00	91.96	92.41	92.86	91.11	91.96	94.25	92.89	98.66		98.22	95.13	90.71	91.15	92.00	92.41	14.88	14.90	15.07	15.07
Lilo	94.20	92.86	92.44	92.83	91.11	91.56	92.00	91.96	92.41	92.86	91.11	91.96	94.25	92.44	98.66	98.22		96.68	90.93	91.37	92.00	92.41	14.77	14.78	15.28	14.95
Harrison	91.11	92.41	93.33	91.96	93.78	94.22	94.67	93.33	93.78	94.22	92.00	93.75	92.92	91.11	95.56	95.13	96.68		91.81	92.26	92.89	90.18	14.38	14.40	14.90	14.57
LincolnB	92.00	93.75	92.89	92.41	93.78	93.33	93.78	92.89	92.00	92.44	93.33	91.96	90.71	91.11	91.11	90.71	90.93	91.81		97.12	92.44	89.73	13.71	13.73	13.91	13.91
Wanderer	91.56	94.20	93.33	93.30	94.22	94.22	94.67	93.78	92.44	92.89	94.22	93.30	92.04	91.56	91.56	91.15	91.37	92.26	97.12		92.00	90.63	14.05	14.06	14.24	14.24
Dragoli	93.56	92.63	92.22	92.19	93.56	94.00	94.44	92.22	93.56	94.00	92.22	93.97	91.81	92.67	92.22	92.00	92.00	92.89	92.44	92.00		91.29	13.76	13.77	13.95	13.95
vB_PlaP_API480	93.97	93.08	92.63	93.08	91.74	92.19	92.19	92.19	92.63	93.08	92.19	92.19	94.44	91.74	93.53	92.41	92.41	90.18	89.73	90.63	91.29		13.80	13.82	14.00	14.00
Ash	13.47	13.47	13.76	13.51	13.76	14.43	14.43	14.48	14.48	14.14	14.09	13.47	14.38	13.42	14.81	14.88	14.77	14.38	13.71	14.05	13.76	13.80		98.81	95.00	94.31
Ley	13.48	13.48	13.77	13.53	13.77	14.44	14.44	14.49	14.49	14.16	14.11	13.48	14.40	13.44	14.83	14.90	14.78	14.40	13.73	14.06	13.77	13.82	98.81		95.38	94.69
C7Cdelta	13.67	13.67	13.95	13.71	13.95	14.62	14.62	14.67	14.67	14.33	14.29	13.67	14.57	13.62	15.00	15.07	15.28	14.90	13.91	14.24	13.95	14.00	95.00	95.38		95.00
Halcyone	13.67	13.67	13.95	13.71	13.95	14.62	14.62	14.67	14.67	14.33	14.29	14.33	14.57	13.62	15.00	15.07	14.95	14.57	13.91	14.24	13.95	14.00	94.31	94.69	95.00	

Figure 6.10 ANI of the 26 different representative types of N-acetylmuramoyl-L-alanine amidase expressed as a percentage (%)

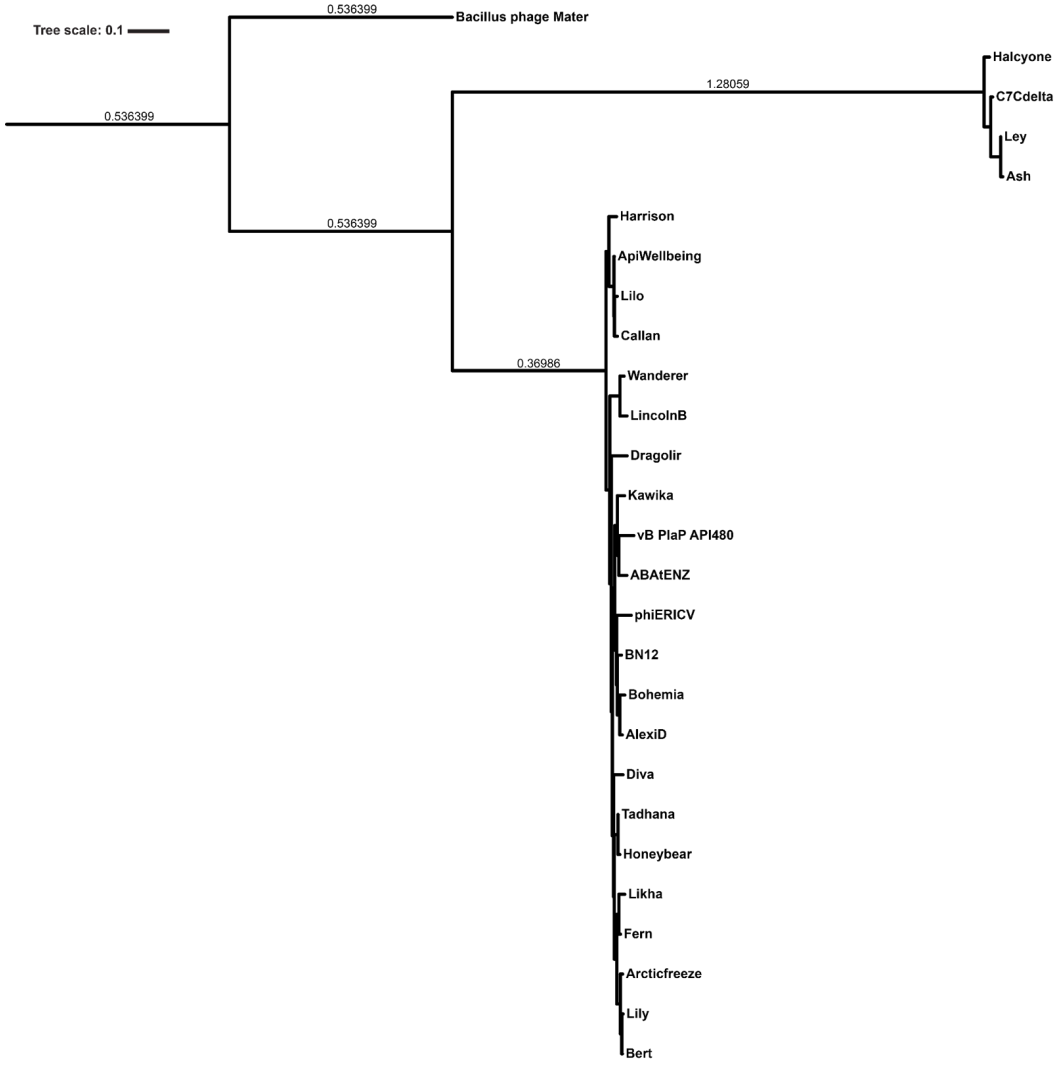


Figure 6.11 Gene tree of the 26 different representative types of N-acetylmuramoyl-L-alanine amidase using iTOL (https://itol.embl.de)

Rooted using a N-acetylmuramoyl-L-alanine amidase domain from Bacillus phage Mater (A0A0A0RNZ5). Branch lengths smaller than 0.1 were deleted for clarity.

6.3.7 CRISPR Protospacer Sequences in the 26 New Zealand P. larvae Phages

As previously described CRISPR systems were found in the *P. larvae* bacterial strains used to isolate our phages (Chapter 3). We used CRISPRFinder to detect 29 unique spacers in our eight bacterial genomes (Figure 6.12). The sequences of all spacers can be found in Supplemental Table S6.3. To ascertain if our phages contained the 29 spacers identified in our bacterial genomes, we searched our 26 phages using the scan function in DNA Master. All 23 New Zealand phages from the Vegas cluster, represented in Figure 6.12 by ABAtENZ, contained a pseudomatch (of 91.7%) to spacer 17 within a DNA Polymerase gene.

Harrison cluster phages Callan and Dash each had one protospacer (Spacer 6) within the ERF Superfamily gene (GP53 and 55 respectively) (Figure 6.12). Callan, Dash, and Lilo contained a pseudomatch to spacer 10 of 81.3% - 84.4% identity within an intergenic region (Figure 6.12).

Interestingly, protospacer 6, found in Callan and Dash was also found in seven out of eight bacterial strains; these seven isolates are all lysed by Callan and Dash.

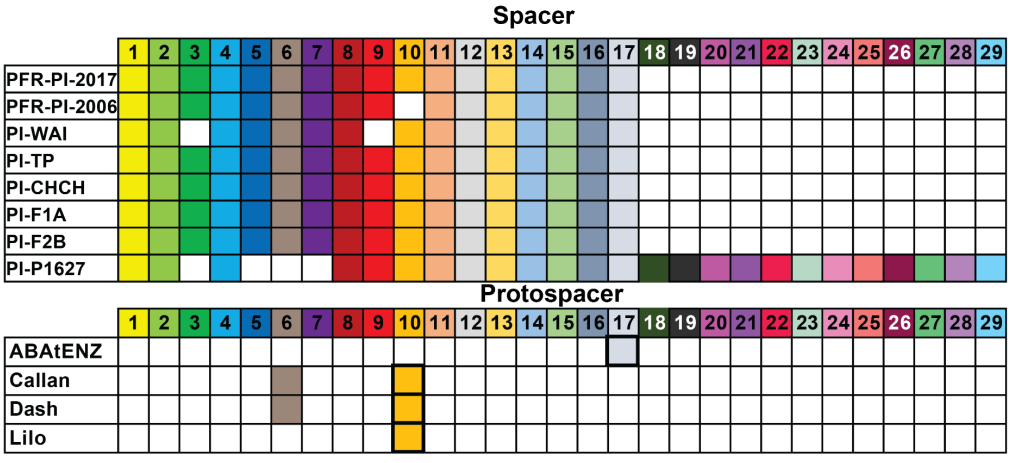


Figure 6.12 Spacers and protospacers found in eight New Zealand *P. larvae* bacterial strains and 26 New Zealand phages
ABAtENZ represents all 23 phages within the New Zealand Vegas sub-cluster. Thick black boxes indicate the protospacer was not a 100% match in the phage genome.

6.4. Discussion

In this chapter, we have expanded on the 48 previously analysed *P. larvae* phage genomes and included an additional 47 *P. larvae* phages that have been isolated and sequenced since 2018 (Stamereilers et al., 2018). The phages that have been isolated to date have come from a variety of locations around the world.

According to our analyses, the 26 New Zealand phages are members of two distinct pre-existing clusters (Harrison and Vegas). The New Zealand phages in the Harrison cluster form two sub-clusters, while those in the Vegas cluster are highly similar to one another and form a single subcluster. This suggests that *P. larvae* phages have entered into New Zealand on at least two separate occasions, either alongside the pathogen or as a prophage within the pathogen. Recently 55 *P. larvae* prophages were discovered in the genomes of 11 isolated *P. larvae* strains from AFB outbreaks around the world (Ribeiro et al., 2022). This taken together with the multiple occasions we have seen *P. larvae* phages isolated from lysogens (Dingman et al., 1984; Bryan D. Merrill et al., 2018; Oliveira et al., 2013; Tsourkas et al., 2015; Walker et al., 2018), shows it is possible the New Zealand phages could have arisen via excision from the bacterial genome.

Our clustering of all 95 sequenced phages adds to the previous study performed on 48 *P. larvae* phages (Stamereilers et al., 2018). Consistent with the previous work, we used three clustering methods which completely agree on the appropriate clustering of the 95 *P. larvae* phages. Two additional singletons resulted from newly discovered phages: phiERICV a prophage published by a laboratory in Germany (Beims et al., 2020) and vB_PlaP_API480 a

phage isolated in Spain by a laboratory in Portugal (Ribeiro et al., 2019). The other newly isolated phages fit within existing clusters, but in some cases, new sub-clusters were formed.

In the Harrison cluster, two additional sub-clusters were formed, one containing Callan and Dash, and a second sub-cluster containing Lilo, all three of which were isolated in New Zealand. The Vegas cluster acquired one new sub-cluster that contained the 23 phages from the larger cluster isolated in New Zealand, this quadrupled the size of the known Vegas cluster phages.

The Halcyone cluster contained no new additions. The cluster with the most changes to the sub-clusters was the large Fern cluster, which went from four sub-clusters and six singletons to seven sub-clusters and nine singletons. The addition of four phages isolated in Poland to the Xenia sub-cluster, which previously only contained phages from the US, showed that even though phages have been isolated from geographically distinct regions, they still have high similarity at the nucleotide level. Again, this suggests that the phages and the *P. larvae* bacterium itself are likely transcontinental. The fact that the distribution of *P. larvae* phages hasn't fundamentally changed despite the near-doubling of the number of sequenced *P. larvae* phage genomes suggests that the majority of the genomic diversity of these phages has been discovered.

A toxin was identified in two of our Harrison cluster New Zealand phages and a total of six *P. larvae* phages. This toxin has already been described, as it is also found in the bacterial strain *P. larvae* ATCC 9545 ERIC I (Ebeling et al., 2021). It is highly conserved between phages and the bacterial strain, with > 99% ANI. It has previously been suggested that the presence

of Plx1 in the *P. larvae* ERIC I genome is a consequence of lysogenic conversion from temperate phages and while the six phages that contain the toxin are temperate (Ebeling et al., 2021), the host in which they are able to genetically integrate is not currently known. Ebeling and colleagues have suggested that Plx1 may be present in distantly related *P. larvae* phages due to an ancestral contribution by the bacterial strain via an abnormal excision event. They further suggest that the presence of the toxin in the prophage region of the bacterial genome is due to lysogenic conversion.

We discovered a complete set of either the Harrison or Dash repeat sequences in four of the six phages as well as the bacterial strain. The set of Harrison repeat sequences were found more frequently and there was only one complete set of the Dash repeat sequences found. Tandem repeat sequences of this kind, with internal inverted homologous repeat structure have the potential to form hairpins. These flanking repeat sequences may be part of some mobile element. The heterogeneous distribution of this toxin and its repeats in phage clusters suggests that it has been introduced on multiple occasions, possibly from the bacterial strain.

Yerffej and philBB_P123 (Fern cluster phages) no longer contain a complete set of Left and Right repeat elements, which may suggest that this is an older event than that in Dash, Lilo, Harrison, and Paisley (Harrison cluster phages). If these repeat elements do mobilise the toxin, the other four phages may still actively exchange this element with the bacterial strain via recombination. If a phage genome carries a mobile genetic element, then phage infection but not lysogenic conversion would be necessary for this selfish genetic element to take up

residence in the bacterial genome. This means the question of whether these phages can form lysogens with the *P. larvae* host is still an open one.

The capacity for this putative selfish genetic element for continued mobility requires further investigation, but the presence of a potentially highly mobile toxin gene in six *P. larvae* phages with lysogenic potential means that these phages are not good candidates for applied use.

Comparative analysis was also undertaken on N-acetylmuramoyl-L-alanine amidase, which has been found in every *P. larvae* phage genome isolated to date. Previously, 12 distinct N-acetylmuramoyl-L-alanine amidases in two groups were found within *P. larvae* phage genomes (Stamereilers et al., 2018). In this study, we show that there are now 26 distinct N-acetylmuramoyl-L-alanine amidases within the same two highly divergent groups. The longer of the two amidases is found exclusively in the Halcyone cluster phages, which have longer genomes and use a different DNA packaging strategy than all other *P. larvae* phages. The shorter amidase is found in all other *P. larvae* phages, including highly divergent singletons, indicative of a high degree of conservation. The N-acetylmuramoyl-L-alanine amidase is a type of endolysin and is the only *P. larvae* phage protein whose function has been experimentally verified. Endolysins are important as they allow phages to destroy the bacterial cell wall and lead to the rupture of the bacterial host, ultimately resulting in its demise (Davies et al., 2021; Young, 2014). Without endolysins phages would be incapable of lysing bacterial cells and would be ineffective biocontrol agents. It is currently not known what are the functional differences between the two different amidases, if any, and this would be an interesting future direction.

The work contained within this study has updated our knowledge of *P. larvae* phages by including recently isolated and sequenced genomes from New Zealand and around the world. It has also expanded our understanding of the putative mobile element that contains a toxin that contributes to the virulence of *P. larvae* ERIC I. Some key areas of further studies would be: to ascertain, in the laboratory, if the putative mobile element is still functional, what are the differences between the two main classes of amidases and identifying the functions of more *P. larvae* phage proteins.

P. larvae phages are capable of infecting and destroying the causative agent of the destructive honey bee disease AFB (Kok & Hendrickson, 2023).The increase in the number of sequenced and annotated P*. larvae* phages provides a better understanding of the genetic diversity of a potentially important biocontrol agent.

6.5 References

Abraham, J., Bousquet, A.-C., Bruff, E., Carson, N., Clark, A., Connell, A., Davis, Z., Dums, J., Everington, C., Groth, A., Hawes, N., McArthur, N., McKenney, C., Oufkir, A., Pearce, B., Rampal, S., Rozier, H., Schaff, J., Slehria, T., … Miller, E. S. (2016). *Paenibacillus larvae* Phage Tripp Genome Has 378-Base-Pair Terminal Repeats. *Genome Announcements*, *4*(1). https://doi.org/10.1128/genomeA.01498-15

Alippi, A. M., López, A. C., Reynaldi, F. J., Grasso, D. H., & Aguilar, O. M. (2007). Evidence for plasmid-mediated tetracycline resistance in *Paenibacillus larvae*, the causal agent of American Foulbrood (AFB) disease in honeybees. *Veterinary Microbiology*, *125*(3–4), 290–303.

Beims, H., Bunk, B., Erler, S., Mohr, K. I., Spröer, C., Pradella, S., Günther, G., Rohde, M., von der Ohe, W., & Steinert, M. (2020). Discovery of *Paenibacillus larvae* ERIC V: Phenotypic and genomic comparison to genotypes ERIC I-IV reveal different inventories of virulence factors which correlate with epidemiological prevalences of American Foulbrood. *International Journal of Medical Microbiology: IJMM*, *310*(2), 151394.

Beims, H., Wittmann, J., Bunk, B., Spröer, C., Rohde, C., Günther, G., Rohde, M., von der Ohe, W., & Steinert, M. (2015). *Paenibacillus larvae*-Directed Bacteriophage HB10c2 and Its Application in American Foulbrood-Affected Honey Bee Larvae. *Applied and Environmental Microbiology*, *81*(16), 5411–5419.

Biosecurity Act 1993 No 95 (as at 01 July 2022), Public Act Contents – New Zealand Legislation. (2022, July 5). New Zealand Parliamentary Counsel Office. https://www.legislation.govt.nz/act/public/1993/0095/latest/DLM314623.html

Boncristiani, H., Ellis, J. D., Bustamante, T., Graham, J., Jack, C., Kimmel, C. B., Mortensen, A., & Schmehl, D. R. (2021). World Honey Bee Health: The Global Distribution of Western Honey Bee (Apis mellifera L.) Pests and Pathogens. *Bee World*, *98*(1), 2–6.

Brady, T. S., Merrill, B. D., Hilton, J. A., Payne, A. M., Stephenson, M. B., & Hope, S. (2017). Bacteriophages as an alternative to conventional antibiotic use for the prevention or treatment of *Paenibacillus larvae* in honeybee hives. *Journal of Invertebrate Pathology*, *150*, 94–100.

Bryant, D., & Moulton, V. (2004). Neighbor-net: an agglomerative method for the construction of phylogenetic networks. *Molecular Biology and Evolution*, *21*(2), 255–265.

Carson, S., Bruff, E., DeFoor, W., Dums, J., Groth, A., Hatfield, T., Iyer, A., Joshi, K., McAdams, S., Miles, D., Miller, D., Oufkir, A., Raynor, B., Riley, S., Roland, S., Rozier, H., Talley, S., & Miller, E. S. (2015). Genome Sequences of Six *Paenibacillus larvae* Siphoviridae Phages. *Genome Announcements*, *3*(3). https://doi.org/10.1128/genomeA.00101-15

Cresawn, S. G., Bogel, M., Day, N., Jacobs-Sera, D., Hendrix, R. W., & Hatfull, G. F. (2011). Phamerator: a bioinformatic tool for comparative bacteriophage genomics. *BMC Bioinformatics*, *12*, 395.

Davies, C. G., Reilly, K., Altermann, E., & Hendrickson, H. L. (2021). PLAN-M; Mycobacteriophage Endolysins Fused to Biodegradable Nanobeads Mitigate Mycobacterial Growth in Liquid and on Surfaces. *Frontiers in Microbiology*, *12*, 562748.

Dingman, D. W., Bakhiet, N., Field, C. C., & Stahly, D. P. (1984). Isolation of two bacteriophages from *Bacillus larvae*, PBL1 and PBL0.5, and partial characterization of PBL1. *The Journal of General Virology*, *65 (Pt 6)*, 1101–1105.

Ebeling, J., Fünfhaus, A., & Genersch, E. (2021). The Buzz about ADP-Ribosylation Toxins from *Paenibacillus larvae*, the Causative Agent of American Foulbrood in Honey Bees. *Toxins*, *13*(2). https://doi.org/10.3390/toxins13020151

Elke Genersch. (2010). American Foulbrood in honeybees and its causative agent, *Paenibacillus larvae*. *Journal of Invertebrate Pathology*, *103*(S), S10–S19.

Fünfhaus, A., Poppinga, L., & Genersch, E. (2013). Identification and characterization of two novel toxins expressed by the lethal honey bee pathogen *Paenibacillus larvae*, the causative agent of American foulbrood. *Environmental Microbiology*, *15*(11), 2951–2965.

Ghorbani-Nezami, S., LeBlanc, L., Yost, D. G., & Amy, P. S. (2015). Phage Therapy is Effective in Protecting Honeybee Larvae from American Foulbrood Disease. *Journal of Insect Science* , *15*(1), 84–85.

Gillingham, A. (n.d.). Beekeeping. *Te Ara—The Encyclopedia of New Zealand. Updated*.

Gosselin, S., Fullmer, M. S., Feng, Y., & Gogarten, J. P. (2022). Improving Phylogenies Based on Average Nucleotide Identity, Incorporating Saturation Correction and Nonparametric Bootstrap Support. *Systematic Biology*, *71*(2), 396–409.

Haseman. (1961). How long can spores of American foulbrood live. *American Bee Journal*.

Hatfull, G. F., Jacobs-Sera, D., Lawrence, J. G., Pope, W. H., Russell, D. A., Ko, C.-C., Weber, R. J., Patel, M. C., Germane, K. L., Edgar, R. H., Hoyte, N. N., Bowman, C. A., Tantoco, A. T., Paladin, E. C., Myers, M. S., Smith, A. L., Grace, M. S., Pham, T. T., O'Brien, M. B., … Hendrix, R. W. (2010). Comparative genomic analysis of 60 Mycobacteriophage genomes: genome clustering, gene acquisition, and gene size. *Journal of Molecular Biology*, *397*(1), 119–143.

Huson, D. H., & Bryant, D. (2006). Application of phylogenetic networks in evolutionary

studies. *Molecular Biology and Evolution*, *23*(2), 254–267.

Jończyk-Matysiak, E., Owczarek, B., Popiela, E., Świtała-Jeleń, K., Migdał, P., Cieślik, M., Łodej, N., Kula, D., Neuberg, J., Hodyra-Stefaniak, K., Kaszowska, M., Orwat, F., Bagińska, N., Mucha, A., Belter, A., Skupińska, M., Bubak, B., Fortuna, W., Letkiewicz, S., … Górski, A. (2021). Isolation and Characterization of Phages Active against *Paenibacillus larvae* Causing American Foulbrood in Honeybees in Poland. *Viruses*, *13*(7). https://doi.org/10.3390/v13071217

Jończyk-Matysiak, E., Popiela, E., Owczarek, B., Hodyra-Stefaniak, K., Świtała-Jeleń, K., Łodej, N., Kula, D., Neuberg, J., Migdał, P., Bagińska, N., Orwat, F., Weber-Dąbrowska, B., Roman, A., & Górski, A. (2020). Phages in Therapy and Prophylaxis of American Foulbrood - Recent Implications From Practical Applications. *Frontiers in Microbiology*, *11*, 1913.

Katoh, K., Misawa, K., Kuma, K.-I., & Miyata, T. (2002). MAFFT: a novel method for rapid multiple sequence alignment based on fast Fourier transform. *Nucleic Acids Research*, *30*(14), 3059–3066.

Katoh, K., & Standley, D. M. (2013). MAFFT multiple sequence alignment software version 7: improvements in performance and usability. *Molecular Biology and Evolution*, *30*(4), 772–780.

Kearse, M., Moir, R., Wilson, A., Stones-Havas, S., Cheung, M., Sturrock, S., Buxton, S., Cooper, A., Markowitz, S., Duran, C., Thierer, T., Ashton, B., Meintjes, P., & Drummond, A. (2012). Geneious Basic: an integrated and extendable desktop software platform for the organization and analysis of sequence data. *Bioinformatics* , *28*(12), 1647–1649.

Kok, D. N., & Hendrickson, H. L. (2023). Save our bees: bacteriophages to protect honey bees

against the pathogen causing American foulbrood in New Zealand. *New Zealand Journal of Zoology*, 1–16.

Krumsiek, J., Arnold, R., & Rattei, T. (2007). Gepard: a rapid and sensitive tool for creating dotplots on genome scale. *Bioinformatics* , *23*(8), 1026–1028.

Lazeroff, M., Ryder, G., Harris, S. L., & Tsourkas, P. K. (2021). Phage Commander, an Application for Rapid Gene Identification in Bacteriophage Genomes Using Multiple Programs. *PHAGE (New Rochelle, N.Y.)*, *2*(4), 204–213.

LeBlanc, L., Nezami, S., Yost, D., Tsourkas, P., & Amy, P. S. (2015). Isolation and characterization of a novel phage lysin active against *Paenibacillus larvae,* a honeybee pathogen. *Bacteriophage*, *5*(4), e1080787-16.

Lester, P. (2021). *Healthy Bee, Sick Bee: The Influence of Parasites, Pathogens, Predators and Pesticides on Honey Bees*. Victoria University of Wellington Press.

Letunic, I., & Bork, P. (2021). Interactive Tree Of Life (iTOL) v5: an online tool for phylogenetic tree display and annotation. *Nucleic Acids Research*, *49*(W1), W293–W296.

Merrill, Bryan D., Fajardo, C. P., Hilton, J. A., Payne, A. M., Ward, A. T., Walker, J. K., Dhalai, A., Imahara, C., Mangohig, J., Monk, J., Pascacio, C., Rai, P., Salisbury, A., Velez, K., Bloomfield, T. J., Buhler, B., Duncan, S. G., Fuhriman, D. A., George, J., … Tsourkas, P. K. (2018). Complete Genome Sequences of 18 *Paenibacillus larvae* Phages from the Western United States. *Microbiology Resource Announcements*, *7*(13). https://doi.org/10.1128/MRA.00966-18

Miyagi, T., Peng, C. Y., Chuang, R. Y., Mussen, E. C., Spivak, M. S., & Doi, R. H. (2000). Verification of oxytetracycline-resistant American foulbrood pathogen *Paenibacillus larvae* in the United States. *Journal of Invertebrate Pathology*, *75*(1), 95–96.

Murray, D., & Aronstein, K. A. (2006). Oxytetracycline-resistance in the honey bee pathogen *Paenibacillus larvae* is encoded on novel plasmid pMA67. In *Journal of Apicultural Research* (Vol. 47, Issue 3, pp. 207–214). https://doi.org/10.3896/ibra.1.45.4.07

Oliveira, A., Leite, M., Kluskens, L. D., Santos, S. B., Melo, L. D. R., & Azeredo, J. (2015). The First *Paenibacillus larvae* Bacteriophage Endolysin (PlyPl23) with High Potential to Control American Foulbrood. *PloS One*, *10*(7), e0132095-16.

Oliveira, A., Melo, L. D. R., Kropinski, A. M., & Azeredo, J. (2013). Complete Genome Sequence of the Broad-Host-Range *Paenibacillus larvae* Phage phiIBB_Pl23. *Genome Announcements*, *1*(5). https://doi.org/10.1128/genomeA.00438-13

Philipson, C. W., Voegtly, L. J., Lueder, M. R., Long, K. A., Rice, G. K., Frey, K. G., Biswas, B., Cer, R. Z., Hamilton, T., & Bishop-Lilly, K. A. (2018). Characterizing Phage Genomes for Therapeutic Applications. *Viruses*, *10*(4). https://doi.org/10.3390/v10040188

Pope, W. H., & Jacobs-Sera, D. (2018). Annotation of Bacteriophage Genome Sequences Using DNA Master: An Overview. *Methods in Molecular Biology* , *1681*, 217–229.

Ribeiro, H. G., Melo, L. D. R., Oliveira, H., Boon, M., Lavigne, R., Noben, J.-P., Azeredo, J., & Oliveira, A. (2019). Characterization of a new podovirus infecting *Paenibacillus larvae*. *Scientific Reports*, *9*(1), 20355.

Ribeiro, H. G., Nilsson, A., Melo, L. D. R., & Oliveira, A. (2022). Analysis of intact prophages in genomes of *Paenibacillus larvae:* An important pathogen for bees. *Frontiers in Microbiology*, *13*, 903861.

Salisbury, A., & Tsourkas, P. K. (2019). A Method for Improving the Accuracy and Efficiency of Bacteriophage Genome Annotation. *International Journal of Molecular Sciences*, *20*(14). https://doi.org/10.3390/ijms20143391

Santos, S. B., Oliveira, A., Melo, L. D. R., & Azeredo, J. (2019). Identification of the first

endolysin Cell Binding Domain (CBD) targeting *Paenibacillus larvae*. *Scientific Reports*, *9*(1), 2568.

Siguier, P., Perochon, J., Lestrade, L., Mahillon, J., & Chandler, M. (2006). ISfinder: the reference centre for bacterial insertion sequences. *Nucleic Acids Research*, *34*(Database issue), D32-6.

Stamereilers, C., Fajardo, C., Walker, J., Mendez, K., Castro-Nallar, E., Grose, J., Hope, S., & Tsourkas, P. (2018). Genomic Analysis of 48 *Paenibacillus larvae* Bacteriophages. *Viruses*, *10*(7), 377–329.

Stamereilers, C., LeBlanc, L., Yost, D., Amy, P. S., & Tsourkas, P. K. (2016). Comparative genomics of 9 novel *Paenibacillus larvae* bacteriophages. *Bacteriophage*, *6*(3), e1220349.

Stamereilers, C., Wong, S., & Tsourkas, P. K. (2021). Characterization of CRISPR Spacer and Protospacer Sequences in *Paenibacillus larvae* and Its Bacteriophages. *Viruses*, *13*(3). https://doi.org/10.3390/v13030459

Tsourkas, P. K. (2020). *Paenibacillus larvae* bacteriophages: obscure past, promising future. *Microbial Genomics*, *6*(2). https://doi.org/10.1099/mgen.0.000329

Tsourkas, P. K., Yost, D. G., Krohn, A., LeBlanc, L., Zhang, A., Stamereilers, C., & Amy, P. S. (2015). Complete Genome Sequences of Nine Phages Capable of Infecting *Paenibacillus larvae,* the Causative Agent of American Foulbrood Disease in Honeybees. *Genome Announcements*, *3*(5). https://doi.org/10.1128/genomeA.01120-15

Walker, J. K., Merrill, B. D., Berg, J. A., Dhalai, A., Dingman, D. W., Fajardo, C. P., Graves, K., Hill, H. L., Hilton, J. A., Imahara, C., Knabe, B. K., Mangohig, J., Monk, J., Mun, H., Payne, A. M., Salisbury, A., Stamereilers, C., Velez, K., Ward, A. T., … Tsourkas, P. K.

(2018). Complete Genome Sequences of *Paenibacillus larvae* Phages BN12, Dragolir, Kiel007, Leyra, Likha, Pagassa, PBL1c, and Tadhana. *Genome Announcements*, *6*(24). https://doi.org/10.1128/genomeA.01602-17

Yost, D. G., Chang, C., LeBlanc, L., Cassin, E., Peterman, C., Rai, P., Salisbury, A., Barroga, N., Cisneros, R., Fersini, J., Juste, J., Ines, J., Leyva, G., Macalinao, D., Muscelli, S., Reyes, G. S., Rhoden, H., Tan, R., Torres, E., … Tsourkas, P. K. (2018). Complete Genome Sequences of *Paenibacillus larvae* Phages Halcyone, Heath, Scottie, and Unity from Las Vegas, Nevada. In *Microbiology Resource Announcements* (Vol. 7, Issue 12). https://doi.org/10.1128/mra.00977-18

Yost, D. G., Tsourkas, P., & Amy, P. S. (2016). Experimental bacteriophage treatment of honeybees \(Apis mellifera\) infected with *Paenibacillus larvae*, the causative agent of American Foulbrood Disease. *Bacteriophage*, *6*(1), 1–15.

Young, R. (2014). Phage lysis: three steps, three choices, one outcome. *Journal of Microbiology* , *52*(3), 243–258.

6.6 Supplementary Data

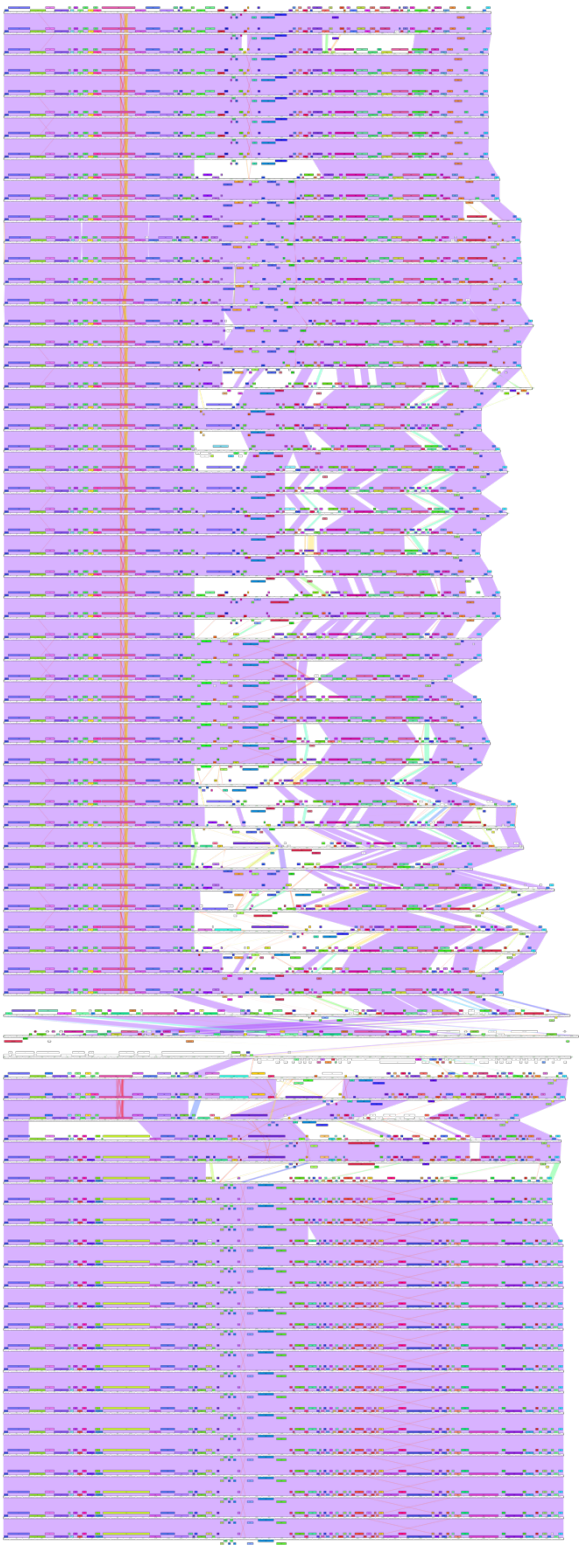


Figure S6.1 Genome maps of 95 *P. larvae* phages displayed using Phamerator
Coloured boxes represent genes and genes of the same colour indicate they belong to the same pham. Shading between the genomes indicates how similar an aligned region is at the nucleotide level according to the E-value, with purple depicting an E-value of zero, white indicating no recognisable similarity, and red indicating similarity at the cut-off threshold of 1E-4.

Table S6.1. 95 *P. larvae* phages isolated from around the world

Phage Name	Institution	Country	Isolation Source	Cluster	GenBank Accession No.	Reference
vB_PlaP_API480	University of Minho	Portugal	Hive soil	Singleton	MK533143	(Ribeiro et al., 2019)
phiERICV	Technical University of Braunschweig	Germany	Prophage	Singleton	CP019719	(Beims et al., 2020)
Mock2	North Carolina State University	USA	Infected frame	Fern	MW927518	
Bert	North Carolina State University	USA	Prophage	Fern	MW927512	
Bloom	Brigham Young University	USA	Bee debris	Fern	MH454077	(Bryan D. Merrill et al., 2018)
DevRi	Brigham Young University	USA	Bee debris	Fern	MH431933	(Bryan D. Merrill et al., 2018)
Genki	Brigham Young University	USA	Bee debris	Fern	MH454082	(Bryan D. Merrill et al., 2018)
Gryphonian	Brigham Young University	USA	Bee debris	Fern	MH431934	(Bryan D. Merrill et al., 2018)
Arcticfreeze	Brigham Young University	USA	Bee sample	Fern	MH431932	(Bryan D. Merrill et al., 2018)
Jacopo	Brigham Young University	USA	Infected hive	Fern	MH454079	(Bryan D. Merrill et al., 2018)
vB_PlaS-3/A	Polish Academy of Sciences	Poland	Bees	Fern	OP503994	(Jończyk-Matysiak et al., 2021)
Shelly	North Carolina State University	USA	Honeycomb	Fern	KP296795	(Carson et al., 2015)
Hobie	North Carolina State University	USA	Infected frame	Fern	MW927516	
Gohan	North Carolina State University	USA	Infected frame	Fern	MW927515	
Fitz	North Carolina State University	USA	Infected frame	Fern	MW927514	
Xenia	University of Nevada Las Vegas	USA	Infected larva	Fern	KT361652	(Tsourkas et al., 2015)
Picard	North Carolina State University	USA	Prophage	Fern	MW927523	
Newport	North Carolina State University	USA	Prophage	Fern	MW927520	
vB_PlaS-1/A	Polish Academy of Sciences	Poland	Wax comb	Fern	OP503992	(Jończyk-Matysiak et al., 2021)
vB_PlaS-2/A	Polish Academy of Sciences	Poland	Wax comb	Fern	OP503993	(Jończyk-Matysiak et al., 2021)
vB_PlaS-5/A	Polish Academy of Sciences	Poland	Wax comb	Fern	OP503995	(Jończyk-Matysiak et al., 2021)
BN12	Brigham Young University	USA	Bee debris	Fern	MG727695	(Walker et al., 2018).

Phage Name	Institution	Country	Isolation Source	Cluster	GenBank Accession No.	Reference
Saudage	Brigham Young University	USA	Bee debris	Fern	MH454083	(Bryan D. Merrill et al., 2018)
Toothless	Brigham Young University	USA	Bee debris	Fern	MH454084	(Bryan D. Merrill et al., 2018)
Lucielle	Brigham Young University	USA	Dead bees	Fern	MH431937	(Bryan D. Merrill et al., 2018)
Honeybear	Brigham Young University	USA	Feral bees	Fern	MH431935	(Bryan D. Merrill et al., 2018)
Pahemo	North Carolina State University	USA	Infected frame	Fern	MW927522	
Pagassa	Brigham Young University	USA	Prophage	Fern	MG727699	(Walker et al., 2018).
Tadhana	Brigham Young University	USA	Prophage	Fern	MG727700	(Walker et al., 2018)..
Fern	University of Nevada Las Vegas	USA	Prophage	Fern	KT361649	(Tsourkas et al., 2015)
Willow	University of Nevada Las Vegas	USA	Soil	Fern	KT361650	(Tsourkas et al., 2015)
Norbert	North Carolina State University	USA	Infected frame	Fern	MW927521	
Riker	North Carolina State University	USA	Prophage	Fern	MW927524	
Eltigre	Brigham Young University	USA	Bee debris	Fern	MH454078	(Bryan D. Merrill et al., 2018)
Kiel007	Brigham Young University	USA	Bee debris	Fern	MG727696	(Walker et al., 2018).
HB10c2	Berlin Institute of Technology	Germany	Dead larva	Fern	KP202972	(Beims et al., 2015)
Redbud	North Carolina State University	USA	Honeycomb	Fern	KP296794	(Carson et al., 2015)
Rani	North Carolina State University	USA	Honeycomb	Fern	KP296793	(Carson et al., 2015)
JackPlaque	North Carolina State University	USA	Infected frame	Fern	MW927517	
AlexiD	North Carolina State University	USA	Infected frame	Fern	MW927511	
Yerffej	Brigham Young University	USA	Bee debris	Fern	MH431931	(Bryan D. Merrill et al., 2018)
Leyra	Brigham Young University	USA	Bee debris	Fern	MG727701	(Walker et al., 2018).
Kawika	Brigham Young University	USA	Dead bees	Fern	MH431936	(Bryan D. Merrill et al., 2018)
Likha	Brigham Young University	USA	Honeycomb	Fern	MG727702	(Walker et al., 2018).
Diva	North Carolina State University	USA	Honeycomb	Fern	KP296791	(Carson et al., 2015)
Sitara	North Carolina State University	USA	Honeycomb	Fern	KP296796	(Carson et al., 2015)
Neutron	North Carolina State University	USA	Infected frame	Fern	MW927519	

Phage Name	Institution	Country	Isolation Source	Cluster	GenBank Accession No.	Reference
PBL1c	University of Iowa	USA	Prophage	Fern	MG727698	(Walker et al., 2018).
phiIBB_Pl23	University of Minho	Portugal	Prophage	Fern	KF010834	(Oliveira et al., 2013)
Bohemia	North Carolina State University	USA	Infected frame	Fern	MW927513	
Sneaky	North Carolina State University	USA	Prophage	Fern	MW927525	
Callan	University of Canterbury	New Zealand	Soil	Harrison	OP503989	Chapter 3
Dash	University of Canterbury	New Zealand	Soil	Harrison	OP503990	Chapter 3
Harrison	University of Nevada Las Vegas	USA	Soil	Harrison	KT361651	(Tsourkas et al., 2015)
Paisley	University of Nevada Las Vegas	USA	Soil	Harrison	KT361653	(Tsourkas et al., 2015)
Lilo	University of Canterbury	New Zealand	Soil	Harrison	OP503991	NZ
C7Cdelta	Brigham Young University	USA	Bee sample	Halcyone	MH431938	(Bryan D. Merrill et al., 2018)
Ash	Brigham Young University	USA	Prophage	Halcyone	MH454076	(Bryan D. Merrill et al., 2018)
Ley	Brigham Young University	USA	Prophage	Halcyone	MH454080	(Bryan D. Merrill et al., 2018)
Scottie	University of Nevada Las Vegas	USA	Hand cream	Halcyone	MH460825	(Yost et al., 2018)
Halcyone	University of Nevada Las Vegas	USA	Soil	Halcyone	MH460827	(Yost et al., 2018)
Heath	University of Nevada Las Vegas	USA	Soil	Halcyone	MH460826	(Yost et al., 2018)
Tripp	North Carolina State University	USA	Honeycomb	Halcyone	KT755656	
Unity	University of Nevada Las Vegas	USA	Beehive	Halcyone	MH460824	(Yost et al., 2018)
Lily	North Carolina State University	USA	Honeycomb	Singleton	KP296792	(Carson et al., 2015)
Bob	University of Canterbury	New Zealand	Soil	Vegas	OP503972	Chapter 3
GIW2016	University of Canterbury	New Zealand	Soil	Vegas	OP503977	Chapter 3
Rosalind	University of Canterbury	New Zealand	Soil	Vegas	OP503984	Chapter 3
AJGG	University of Canterbury	New Zealand	Soil	Vegas	OP503969	Chapter 3
ABAtENZ	University of Canterbury	New Zealand	Soil	Vegas	OP503968	Chapter 3
ApiWellbeing	University of Canterbury	New Zealand	Soil	Vegas	OP503970	Chapter 3
BarryFoster_Benicio	University of Canterbury	New Zealand	Soil	Vegas	OP503543	Chapter 3

Phage Name	Institution	Country	Isolation Source	Cluster	GenBank Accession No.	Reference
Bloomfield	University of Canterbury	New Zealand	Soil	Vegas	OP503971	Chapter 3
Dante	University of Canterbury	New Zealand	Soil	Vegas	OP503974	Chapter 3
GaryLarson	University of Canterbury	New Zealand	Soil	Vegas	OP503976	Chapter 3
Jacinda	University of Canterbury	New Zealand	Soil	Vegas	OP503978	Chapter 3
Lena	University of Canterbury	New Zealand	Soil	Vegas	OP503979	Chapter 3
Logan	University of Canterbury	New Zealand	Soil	Vegas	OP503980	Chapter 3
LunBun	University of Canterbury	New Zealand	Soil	Vegas	OP494865	Chapter 3
NHScienceFair	University of Canterbury	New Zealand	Soil	Vegas	OP503981	Chapter 3
Rae.2Bee1	University of Canterbury	New Zealand	Soil	Vegas	OP503983	Chapter 3
Ted	University of Canterbury	New Zealand	Soil	Vegas	OP503985	Chapter 3
TonyLawson	University of Canterbury	New Zealand	Soil	Vegas	OP503986	Chapter 3
UtuhinaGold_Zacery	University of Canterbury	New Zealand	Soil	Vegas	OP503987	Chapter 3
Wildcape	University of Canterbury	New Zealand	Soil	Vegas	OP503988	Chapter 3
Carlos	University of Canterbury	New Zealand	Soil	Vegas	OP503973	Chapter 3
FutureBees	University of Canterbury	New Zealand	Soil	Vegas	OP503975	Chapter 3
Ollie	University of Canterbury	New Zealand	Soil	Vegas	OP503982	Chapter 3
Dragolir	Brigham Young University	USA	Bee debris	Vegas	MG727697	(Walker et al., 2018).
LincolnB	Brigham Young University	USA	Bee debris	Vegas	MH454081	(Bryan D. Merrill et al., 2018)
Wanderer	Brigham Young University	USA	Bee debris	Vegas	MH431930	(Bryan D. Merrill et al., 2018)
Vadim	University of Nevada Las Vegas	USA	Lip balm	Vegas	KT361656	(Tsourkas et al., 2015)
Vegas	University of Nevada Las Vegas	USA	Lip balm	Vegas	KT361654	(Tsourkas et al., 2015)
Diane	University of Nevada Las Vegas	USA	Prophage	Vegas	KT361657	(Tsourkas et al., 2015)
Hayley	University of Nevada Las Vegas	USA	Soil	Vegas	KT361655	(Tsourkas et al., 2015)

Table S6.2. Clustering of all 95 *P. larvae* phages based on ANI presented as a percentage (%)

	ABAtENZ	Rae2Bee1	Lena	LunBun	TonyLawson77	Jacinda	BarryFoster_Benicio	GaryLarson	UtuhinaGold_Zacery	NHScienceFair	Bloomfield	Ollie
ABAtENZ		99.99	99.99	99.97	99.97	99.97	99.94	99.95	99.95	99.95	99.96	99.96
Rae2Bee1	99.99		99.98	99.98	99.97	99.96	99.95	99.95	99.96	99.95	99.96	99.96
Lena	99.99	99.98		99.97	99.97	99.96	99.94	99.94	99.95	99.94	99.97	99.96
LunBun	99.97	99.98	99.97		99.99	99.97	99.96	99.96	99.97	99.95	99.95	99.95
TonyLawson77	99.97	99.97	99.97	99.99		99.98	99.95	99.95	99.96	99.95	99.94	99.94
Jacinda	99.97	99.96	99.96	99.97	99.98		99.94	99.95	99.95	99.95	99.94	99.94
BarryFoster_Benicio	99.94	99.95	99.94	99.96	99.95	99.94		99.99	99.98	99.99	99.91	99.91
GaryLarson	99.95	99.95	99.94	99.96	99.95	99.95	99.99		99.99	99.99	99.92	99.92
UtuhinaGold_Zacery	99.95	99.96	99.95	99.97	99.96	99.95	99.98	99.99		99.98	99.93	99.93
NHScienceFair	99.95	99.95	99.94	99.95	99.95	99.95	99.99	99.99	99.98		99.92	99.92
Bloomfield	99.96	99.96	99.97	99.95	99.94	99.94	99.91	99.92	99.93	99.92		99.99
Ollie	99.96	99.96	99.96	99.95	99.94	99.94	99.91	99.92	99.93	99.92	99.99	
FutureBee	99.96	99.96	99.96	99.94	99.94	99.94	99.91	99.91	99.93	99.91	99.99	99.99
Dante	99.96	99.96	99.96	99.95	99.94	99.94	99.91	99.92	99.93	99.92	99.99	99.99
AJG77	99.92	99.92	99.92	99.91	99.9	99.89	99.88	99.88	99.89	99.88	99.94	99.94
Logan	99.83	99.82	99.82	99.83	99.84	99.84	99.86	99.87	99.86	99.87	99.8	99.8
Ted	99.81	99.81	99.81	99.82	99.82	99.82	99.85	99.85	99.84	99.85	99.79	99.79
ApiWellbeing	99.65	99.65	99.65	99.67	99.67	99.68	99.7	99.7	99.69	99.7	99.63	99.63
Carlos	99.83	99.83	99.83	99.82	99.81	99.83	99.79	99.79	99.8	99.79	99.81	99.82
Wildcape	99.79	99.79	99.79	99.81	99.8	99.81	99.84	99.84	99.83	99.83	99.76	99.76
GIW2016	97.68	97.68	97.67	97.68	97.68	97.68	97.72	97.72	97.71	97.72	97.65	97.65
Rosalind	97.69	97.69	97.69	97.7	97.71	97.7	97.73	97.74	97.73	97.73	97.67	97.67
Bob	97.41	97.41	97.41	97.43	97.43	97.42	97.46	97.46	97.45	97.46	97.39	97.39
Hayley	76.28	76.28	76.29	76.27	76.26	76.26	76.24	76.25	76.25	76.24	76.31	76.31
Vadim	73.96	73.96	73.97	73.95	73.94	73.94	73.92	73.93	73.93	73.92	73.99	73.99
Diane	73.96	73.96	73.97	73.95	73.94	73.94	73.92	73.93	73.93	73.92	73.99	73.99
Vegas	73.96	73.96	73.96	73.95	73.94	73.94	73.92	73.93	73.93	73.92	73.98	73.98
Dragolir	45.56	45.56	45.57	45.56	45.56	45.56	45.55	45.56	45.56	45.56	45.57	45.57
LincolnB	49.71	49.71	49.71	49.71	49.71	49.71	49.72	49.72	49.72	49.72	49.72	49.72
Wanderer	49.65	49.65	49.65	49.66	49.66	49.67	49.66	49.67	49.67	49.66	49.67	49.67
Callan	28.6	28.6	28.6	28.6	28.6	28.61	28.6	28.6	28.6	28.61	28.59	28.59
Dash	28.84	28.84	28.84	28.84	28.84	28.85	28.84	28.85	28.85	28.85	28.84	28.84
Lilo	29.86	29.86	29.86	29.86	29.86	29.87	29.86	29.86	29.86	29.87	29.85	29.85
Redbud	36.87	36.86	36.87	36.87	36.86	36.87	36.86	36.86	36.86	36.87	36.85	36.85
Kiel007	36.89	36.89	36.89	36.9	36.89	36.9	36.89	36.89	36.89	36.89	36.88	36.88
Rani	36.87	36.87	36.87	36.87	36.87	36.88	36.86	36.87	36.87	36.87	36.86	36.86
AlexiD	36.8	36.79	36.8	36.8	36.79	36.8	36.79	36.79	36.79	36.8	36.78	36.78
JackPlaque	36.81	36.81	36.81	36.81	36.81	36.82	36.81	36.81	36.81	36.81	36.8	36.8
HB10c2	34.84	34.84	34.83	34.84	34.83	34.84	34.83	34.83	34.83	34.84	34.83	34.83
Sitara	34.54	34.54	34.54	34.54	34.54	34.54	34.53	34.54	34.54	34.54	34.53	34.53
Eltigre	36.02	36.02	36.02	36.02	36.02	36.02	36.01	36.02	36.02	36.02	36.01	36.01
Fitz	34.43	34.43	34.43	34.43	34.43	34.43	34.42	34.42	34.43	34.43	34.42	34.42
Newport	34.43	34.43	34.43	34.43	34.43	34.43	34.42	34.42	34.43	34.43	34.42	34.42
Gohan	33.83	33.83	33.83	33.83	33.83	33.83	33.82	33.83	33.83	33.83	33.82	33.82
Shelly	34.42	34.43	34.42	34.43	34.42	34.43	34.42	34.42	34.42	34.42	34.42	34.42
vB-PlaS-2A	34.41	34.41	34.41	34.41	34.41	34.41	34.4	34.41	34.41	34.41	34.4	34.4
vB_PlaS-3A	34.39	34.39	34.39	34.39	34.38	34.39	34.38	34.38	34.39	34.39	34.38	34.38
vB_PlaS-1A	34.28	34.28	34.28	34.28	34.27	34.28	34.27	34.27	34.28	34.28	34.27	34.27
vB_PlaS-5A	34.21	34.21	34.21	34.21	34.21	34.21	34.2	34.21	34.21	34.21	34.2	34.2
Xenia	34.39	34.4	34.39	34.39	34.39	34.4	34.39	34.39	34.39	34.39	34.38	34.39
Hobie	34.37	34.37	34.37	34.37	34.36	34.37	34.36	34.36	34.37	34.37	34.36	34.36
Picard	34.38	34.38	34.37	34.38	34.37	34.38	34.37	34.37	34.37	34.38	34.37	34.37
Diva	33.15	33.15	33.15	33.15	33.14	33.15	33.14	33.14	33.15	33.15	33.14	33.14
Leyra	36.58	36.58	36.58	36.58	36.57	36.58	36.57	36.57	36.57	36.58	36.56	36.57
PBL1c	34.75	34.75	34.75	34.75	34.75	34.75	34.74	34.74	34.75	34.75	34.74	34.74
Neutron	38.4	38.4	38.4	38.4	38.4	38.4	38.39	38.4	38.4	38.4	38.4	38.39
Yerffej	35.19	35.18	35.19	35.18	35.18	35.19	35.18	35.18	35.18	35.19	35.18	35.18
philBB_Pl23	34.76	34.76	34.76	34.77	34.76	34.78	34.76	34.76	34.76	34.76	34.76	34.76
Likha	37.46	37.45	37.45	37.45	37.45	37.46	37.45	37.45	37.45	37.45	37.44	37.44
HoneyBear	36.25	36.25	36.25	36.25	36.25	36.26	36.25	36.25	36.25	36.25	36.24	36.23
Toothless	37.08	37.08	37.08	37.08	37.07	37.08	37.07	37.07	37.07	37.08	37.07	37.07
Pagassa	36.3	36.3	36.3	36.3	36.3	36.31	36.29	36.3	36.3	36.3	36.28	36.28
Tadhana	37.61	37.6	37.6	37.6	37.6	37.61	37.6	37.6	37.6	37.6	37.59	37.59
Saudage	36.88	36.88	36.88	36.88	36.88	36.88	36.87	36.87	36.87	36.88	36.87	36.87
Lucielle	36.73	36.73	36.73	36.73	36.73	36.73	36.72	36.72	36.72	36.73	36.72	36.72
Fern	37.38	37.38	37.38	37.38	37.38	37.39	37.37	37.38	37.38	37.38	37.37	37.37
Willow	37.38	37.38	37.38	37.38	37.38	37.39	37.37	37.38	37.38	37.38	37.37	37.37
Kawika	35.54	35.54	35.54	35.54	35.53	35.54	35.53	35.53	35.53	35.54	35.53	35.53
Bohemia	36.75	36.75	36.75	36.75	36.74	36.75	36.74	36.74	36.74	36.75	36.74	36.73
Sneaky	36.76	36.76	36.76	36.76	36.76	36.77	36.75	36.75	36.75	36.76	36.75	36.75
Pahemo	36.98	36.97	36.98	36.97	36.97	36.98	36.97	36.97	36.97	36.97	36.96	36.96
BN12	36.64	36.64	36.64	36.64	36.64	36.65	36.63	36.64	36.64	36.64	36.63	36.63
ArcticFreeze	38.52	38.52	38.52	38.51	38.51	38.52	38.52	38.52	38.52	38.52	38.5	38.51
DevRi	38.53	38.53	38.53	38.52	38.52	38.53	38.52	38.53	38.53	38.53	38.51	38.51
Bloom	38.52	38.53	38.52	38.52	38.52	38.53	38.52	38.53	38.53	38.53	38.51	38.51
Jacopo	38.58	38.58	38.58	38.57	38.57	38.58	38.57	38.58	38.58	38.58	38.56	38.56
Gryphonian	38.51	38.51	38.51	38.51	38.51	38.52	38.51	38.51	38.52	38.52	38.5	38.5
Genki	38.49	38.49	38.49	38.48	38.48	38.49	38.49	38.49	38.49	38.49	38.48	38.48
Norbert	37.66	37.66	37.65	37.66	37.65	37.66	37.65	37.65	37.66	37.65	37.64	37.64
Riker	37.66	37.66	37.66	37.66	37.66	37.67	37.66	37.66	37.66	37.66	37.65	37.65
Bert	37.92	37.92	37.92	37.92	37.92	37.92	37.92	37.92	37.92	37.93	37.91	37.91
Mock2	37.98	37.98	37.98	37.98	37.98	37.98	37.98	37.98	37.99	37.99	37.97	37.97
Paisley	42.7	42.7	42.7	42.7	42.7	42.71	42.7	42.7	42.7	42.7	42.7	42.7
Harrison	42.55	42.55	42.55	42.55	42.55	42.57	42.55	42.56	42.56	42.55	42.55	42.55
Lily	25.87	25.87	25.87	25.87	25.87	25.88	25.87	25.87	25.87	25.87	25.86	25.86
phiERICV	15.08	15.08	15.08	15.08	15.08	15.09	15.08	15.08	15.08	15.08	15.08	15.09
Ash	21.71	21.71	21.71	21.71	21.71	21.71	21.71	21.71	21.71	21.71	21.71	21.71
Ley	21.72	21.72	21.72	21.72	21.72	21.72	21.72	21.72	21.72	21.72	21.72	21.72
C7Cdelta	21.93	21.93	21.93	21.93	21.93	21.93	21.93	21.93	21.93	21.93	21.93	21.93
Unity	21.27	21.27	21.28	21.27	21.27	21.27	21.27	21.27	21.27	21.27	21.27	21.27
Heath	20.9	20.9	20.9	20.9	20.9	20.9	20.9	20.9	20.9	20.9	20.9	20.9
Halcyone	20.91	20.91	20.91	20.91	20.91	20.91	20.91	20.91	20.91	20.91	20.91	20.91
Scottie	20.83	20.83	20.84	20.83	20.83	20.83	20.83	20.83	20.83	20.83	20.83	20.84
Tripp	21.9	21.9	21.9	21.9	21.9	21.89	21.89	21.9	21.9	21.9	21.9	21.9
vB_PlaP_API480	23.85	23.86	23.85	23.85	23.85	23.86	23.86	23.86	23.86	23.86	23.84	23.84

	FutureBee	Dante	AJG77	Logan	Ted	ApiWellbeing	Carlos	Wildcape	GIW2016	Rosalind	Bob	Hayley
ABAtENZ	99.96	99.96	99.92	99.83	99.81	99.65	99.83	99.79	97.68	97.69	97.41	76.28
Rae2Bee1	99.96	99.96	99.92	99.82	99.81	99.65	99.83	99.79	97.68	97.69	97.41	76.28
Lena	99.96	99.96	99.92	99.82	99.81	99.65	99.83	99.79	97.67	97.69	97.41	76.29
LunBun	99.94	99.95	99.91	99.83	99.82	99.67	99.82	99.81	97.68	97.7	97.43	76.27
TonyLawson77	99.94	99.94	99.9	99.84	99.82	99.67	99.81	99.8	97.68	97.71	97.43	76.26
Jacinda	99.94	99.94	99.89	99.84	99.82	99.68	99.83	99.81	97.68	97.7	97.42	76.26
BarryFoster_Benicio	99.91	99.91	99.88	99.86	99.85	99.7	99.79	99.84	97.72	97.73	97.46	76.24
GaryLarson	99.91	99.92	99.88	99.87	99.85	99.7	99.79	99.84	97.72	97.74	97.46	76.25
UtuhinaGold_Zacery	99.93	99.93	99.89	99.86	99.84	99.69	99.8	99.83	97.71	97.73	97.45	76.25
NHScienceFair	99.91	99.92	99.88	99.87	99.85	99.7	99.79	99.83	97.72	97.73	97.46	76.24
Bloomfield	99.99	99.99	99.94	99.8	99.79	99.63	99.81	99.76	97.65	97.67	97.39	76.31
Ollie	99.99	99.99	99.94	99.8	99.79	99.63	99.82	99.76	97.65	97.67	97.39	76.31
FutureBee		99.98	99.94	99.8	99.78	99.62	99.8	99.76	97.64	97.66	97.38	76.3
Dante	99.98		99.94	99.8	99.78	99.63	99.81	99.76	97.64	97.67	97.39	76.31
AJG77	99.94	99.94		99.76	99.74	99.59	99.77	99.72	97.61	97.63	97.35	76.27
Logan	99.8	99.8	99.76		99.98	99.82	99.66	99.71	97.84	97.81	97.58	76.12
Ted	99.78	99.78	99.74	99.98		99.83	99.65	99.7	97.85	97.83	97.59	76.11
ApiWellbeing	99.62	99.63	99.59	99.82	99.83		99.81	99.85	97.7	97.67	97.44	75.97
Carlos	99.8	99.81	99.77	99.66	99.65	99.81		99.94	97.52	97.54	97.26	76.14
Wildcape	99.76	99.76	99.72	99.71	99.7	99.85	99.94		97.56	97.59	97.3	76.1
GIW2016	97.64	97.64	97.61	97.84	97.85	97.7	97.52	97.56		99.96	99.53	74.08
Rosalind	97.66	97.67	97.63	97.81	97.83	97.67	97.54	97.59	99.96		99.5	74.09
Bob	97.38	97.39	97.35	97.58	97.59	97.44	97.26	97.3	99.53	99.5		74.51
Hayley	76.3	76.31	76.27	76.12	76.11	75.97	76.14	76.1	74.08	74.09	74.51	
Vadim	73.98	73.99	73.95	73.81	73.79	73.66	73.83	73.79	71.82	71.84	72.24	96.83
Diane	73.98	73.99	73.95	73.81	73.79	73.66	73.83	73.79	71.82	71.84	72.24	96.82
Vegas	73.98	73.99	73.95	73.81	73.79	73.66	73.83	73.79	71.82	71.84	72.24	96.83
Dragolir	45.57	45.57	45.56	45.56	45.57	45.54	45.53	45.53	46.29	46.3	46.38	57.34
LincolnB	49.72	49.72	49.71	49.7	49.72	49.67	49.66	49.68	50.53	50.53	50.61	46.87
Wanderer	49.67	49.66	49.65	49.65	49.66	49.74	49.73	49.75	50.48	50.47	50.56	46.82
Callan	28.6	28.6	28.58	28.6	28.6	28.72	28.72	28.72	28.99	28.99	29	28.94
Dash	28.84	28.84	28.83	28.84	28.84	28.96	28.96	28.96	29.24	29.23	29.25	29.18
Lilo	29.85	29.85	29.84	29.86	29.86	29.99	29.98	29.99	30.27	30.28	30.3	30.2
Redbud	36.86	36.86	36.85	36.86	36.86	36.9	36.9	36.9	37.49	37.49	37.47	36.98
Kiel007	36.88	36.89	36.87	36.88	36.89	36.92	36.93	36.93	37.52	37.52	37.5	37
Rani	36.86	36.86	36.85	36.86	36.87	36.9	36.91	36.9	37.5	37.5	37.48	36.98
AlexiD	36.79	36.79	36.78	36.79	36.79	36.83	36.83	36.83	37.42	37.42	37.4	36.91
JackPlaque	36.8	36.81	36.79	36.8	36.81	36.84	36.85	36.85	37.44	37.44	37.42	36.92
HB10c2	34.83	34.83	34.82	34.84	34.84	34.87	34.87	34.87	35.45	35.45	35.43	35.21
Sitara	34.53	34.53	34.52	34.53	34.53	34.58	34.59	34.58	35.07	35.07	35.06	34.64
Eltigre	36.01	36.01	36	36.01	36.02	36.05	36.05	36.05	36.61	36.61	36.59	36.11
Fitz	34.43	34.42	34.41	34.42	34.42	34.47	34.48	34.48	34.97	34.98	34.96	34.65
Newport	34.43	34.42	34.41	34.42	34.42	34.47	34.48	34.48	34.97	34.98	34.96	34.65
Gohan	33.83	33.83	33.82	33.82	33.83	33.87	33.88	33.88	34.36	34.36	34.35	34.05
Shelly	34.42	34.42	34.41	34.42	34.42	34.47	34.47	34.47	34.97	34.97	34.96	34.65
vB-PlaS-2A	34.41	34.41	34.4	34.4	34.4	34.45	34.46	34.46	34.96	34.96	34.94	34.63
vB_PlaS-3A	34.38	34.38	34.37	34.38	34.38	34.43	34.43	34.43	34.93	34.93	34.92	34.61
vB_PlaS-1A	34.28	34.27	34.26	34.27	34.27	34.32	34.33	34.32	34.82	34.82	34.81	34.5
vB_PlaS-5A	34.21	34.21	34.19	34.2	34.2	34.25	34.26	34.26	34.75	34.75	34.74	34.43
Xenia	34.39	34.39	34.38	34.38	34.39	34.44	34.44	34.44	34.94	34.94	34.93	34.62
Hobie	34.36	34.36	34.35	34.36	34.36	34.41	34.42	34.41	34.92	34.92	34.91	34.56
Picard	34.37	34.37	34.36	34.37	34.37	34.42	34.42	34.42	34.93	34.93	34.92	34.54
Diva	33.14	33.14	33.13	33.14	33.14	33.19	33.19	33.19	33.7	33.7	33.69	33.2
Leyra	36.57	36.57	36.56	36.57	36.57	36.62	36.63	36.63	37.17	37.17	37.15	36.82
PBL1c	34.74	34.74	34.73	34.74	34.74	34.81	34.82	34.82	35.31	35.31	35.29	34.76
Neutron	38.4	38.4	38.39	38.39	38.4	38.45	38.45	38.45	39.03	39.03	39.04	38.64
Yerffej	35.18	35.18	35.18	35.18	35.18	35.18	35.19	35.19	35.7	35.7	35.71	35.4
phiBB_PI23	34.76	34.76	34.76	34.76	34.76	34.89	34.89	34.89	35.28	35.28	35.3	34.99
Likha	37.45	37.45	37.44	37.45	37.46	37.49	37.48	37.48	38.05	38.05	38.07	37.71
HoneyBear	36.24	36.24	36.23	36.26	36.27	36.27	36.25	36.25	36.83	36.83	36.84	36.27
Toothless	37.07	37.07	37.07	37.07	37.08	37.08	37.08	37.07	37.66	37.67	37.68	37.31
Pagassa	36.28	36.29	36.28	36.31	36.31	36.31	36.3	36.3	36.88	36.88	36.89	36.32
Tadhana	37.59	37.6	37.59	37.6	37.6	37.59	37.59	37.59	38.21	38.21	38.22	37.84
Saudage	36.87	36.87	36.87	36.87	36.87	36.87	36.88	36.87	37.5	37.5	37.5	37.08
Lucielle	36.72	36.72	36.72	36.72	36.72	36.72	36.73	36.72	37.35	37.35	37.35	36.88
Fern	37.37	37.37	37.37	37.38	37.38	37.41	37.41	37.41	37.97	37.97	37.99	37.58
Willow	37.37	37.37	37.37	37.38	37.38	37.41	37.41	37.41	37.97	37.97	37.99	37.58
Kawika	35.53	35.53	35.52	35.53	35.54	35.59	35.59	35.58	36.08	36.08	36.09	35.75
Bohemia	36.74	36.74	36.73	36.74	36.75	36.75	36.76	36.75	37.32	37.32	37.33	36.93
Sneaky	36.75	36.75	36.75	36.76	36.76	36.77	36.77	36.76	37.34	37.34	37.35	36.94
Pahemo	36.97	36.97	36.96	36.97	36.97	36.98	36.98	36.98	37.55	37.56	37.56	37.2
BN12	36.63	36.64	36.63	36.64	36.64	36.68	36.68	36.67	37.22	37.22	37.23	36.87
ArcticFreeze	38.51	38.51	38.5	38.51	38.52	38.56	38.56	38.56	39.14	39.14	39.15	38.8
DevRi	38.52	38.52	38.51	38.52	38.53	38.57	38.56	38.57	39.15	39.15	39.16	38.81
Bloom	38.52	38.52	38.51	38.52	38.53	38.57	38.56	38.57	39.15	39.15	39.16	38.81
Jacopo	38.57	38.57	38.56	38.57	38.58	38.62	38.61	38.62	39.2	39.2	39.21	38.86
Gryphonian	38.51	38.51	38.5	38.51	38.52	38.56	38.55	38.56	39.14	39.14	39.15	38.8
Genki	38.48	38.48	38.48	38.49	38.49	38.53	38.53	38.53	39.11	39.12	39.12	38.77
Norbert	37.65	37.64	37.64	37.66	37.66	37.71	37.7	37.7	38.27	38.26	38.26	37.87
Riker	37.65	37.65	37.65	37.66	37.67	37.71	37.7	37.7	38.27	38.27	38.27	37.88
Bert	37.92	37.91	37.91	37.92	37.92	37.97	37.96	37.97	38.53	38.53	38.54	38.11
Mock2	37.98	37.98	37.97	37.98	37.98	38.03	38.02	38.02	38.59	38.59	38.6	38.17
Paisley	42.7	42.69	42.69	42.7	42.7	42.83	42.82	42.83	43.33	43.32	43.34	43.5
Harrison	42.55	42.54	42.54	42.55	42.55	42.68	42.68	42.68	43.18	43.17	43.19	43.36
Lily	25.86	25.86	25.86	25.86	25.86	25.91	25.91	25.91	26.23	26.23	26.23	25.97
phiERICV	15.09	15.09	15.08	15.08	15.08	15.1	15.11	15.1	15.26	15.26	15.28	15.07
Ash	21.71	21.71	21.71	21.71	21.71	21.71	21.71	21.71	21.86	21.86	21.87	21.62
Ley	21.72	21.72	21.72	21.72	21.72	21.72	21.72	21.72	21.87	21.87	21.88	21.64
C7Cdelta	21.93	21.93	21.93	21.93	21.93	21.92	21.93	21.92	22.09	22.08	22.09	21.88
Unity	21.27	21.27	21.27	21.28	21.28	21.29	21.28	21.28	21.44	21.43	21.45	20.99
Heath	20.9	20.9	20.9	20.91	20.91	20.92	20.91	20.91	21.06	21.05	21.07	20.61
Halcyone	20.91	20.91	20.91	20.92	20.92	20.93	20.92	20.92	21.07	21.06	21.08	20.62
Scottie	20.83	20.84	20.83	20.85	20.85	20.85	20.84	20.84	20.99	20.98	21	20.55
Tripp	21.9	21.9	21.9	21.9	21.9	21.89	21.89	21.89	22.06	22.05	22.06	22.15
vB_PlaP_API480	23.85	23.84	23.83	23.87	23.86	23.89	23.88	23.89	24.18	24.18	24.19	24.06

	Vadim	Diane	Vegas	Dragolir	LincolnB	Wanderer	Callan	Dash	Lilo	Redbud	Kiel007	Rani
ABAtENZ	73.96	73.96	73.96	45.56	49.71	49.65	28.6	28.84	29.86	36.87	36.89	36.87
Rae2Bee1	73.96	73.96	73.96	45.56	49.71	49.65	28.6	28.84	29.86	36.86	36.89	36.87
Lena	73.97	73.97	73.96	45.57	49.71	49.65	28.6	28.84	29.86	36.87	36.89	36.87
LunBun	73.95	73.95	73.95	45.56	49.71	49.66	28.6	28.84	29.86	36.87	36.9	36.87
TonyLawson77	73.94	73.94	73.94	45.56	49.71	49.66	28.6	28.84	29.86	36.86	36.89	36.87
Jacinda	73.94	73.94	73.94	45.56	49.71	49.67	28.61	28.85	29.87	36.87	36.9	36.88
BarryFoster_Benicio	73.92	73.92	73.92	45.55	49.72	49.66	28.6	28.84	29.86	36.86	36.89	36.86
GaryLarson	73.93	73.93	73.93	45.56	49.72	49.67	28.6	28.85	29.86	36.86	36.89	36.87
UtuhinaGold_Zacery	73.93	73.93	73.93	45.56	49.72	49.67	28.6	28.85	29.86	36.86	36.89	36.87
NHScienceFair	73.92	73.92	73.92	45.56	49.72	49.66	28.61	28.85	29.87	36.87	36.89	36.87
Bloomfield	73.99	73.99	73.98	45.57	49.72	49.67	28.59	28.84	29.85	36.85	36.88	36.86
Ollie	73.99	73.99	73.98	45.57	49.72	49.67	28.59	28.84	29.85	36.85	36.88	36.86
FutureBee	73.98	73.98	73.98	45.57	49.72	49.67	28.6	28.84	29.85	36.86	36.88	36.86
Dante	73.99	73.99	73.99	45.57	49.72	49.66	28.6	28.84	29.85	36.86	36.89	36.86
AJG77	73.95	73.95	73.95	45.56	49.71	49.65	28.58	28.83	29.84	36.85	36.87	36.85
Logan	73.81	73.81	73.81	45.56	49.7	49.65	28.6	28.84	29.86	36.86	36.88	36.86
Ted	73.79	73.79	73.79	45.57	49.72	49.66	28.6	28.84	29.86	36.86	36.89	36.87
ApiWellbeing	73.66	73.66	73.66	45.54	49.67	49.74	28.72	28.96	29.99	36.9	36.92	36.9
Carlos	73.83	73.83	73.83	45.53	49.66	49.73	28.72	28.96	29.98	36.9	36.93	36.91
Wildcape	73.79	73.79	73.79	45.53	49.68	49.75	28.72	28.96	29.99	36.9	36.93	36.9
GIW2016	71.82	71.82	71.82	46.29	50.53	50.48	28.99	29.24	30.27	37.49	37.52	37.5
Rosalind	71.84	71.84	71.84	46.3	50.53	50.47	28.99	29.23	30.28	37.49	37.52	37.5
Bob	72.24	72.24	72.24	46.38	50.61	50.56	29	29.25	30.3	37.47	37.5	37.48
Hayley	96.83	96.82	96.83	57.34	46.87	46.82	28.94	29.18	30.2	36.98	37	36.98
Vadim		99.99	99.94	58.56	45.6	45.54	29.4	29.85	30.89	37.13	37.16	37.14
Diane	99.99		99.94	58.55	45.6	45.54	29.39	29.85	30.88	37.13	37.16	37.13
Vegas	99.94	99.94		58.56	45.6	45.54	29.39	29.84	30.88	37.14	37.17	37.18
Dragolir	58.56	58.55	58.56		52.64	52.63	26.45	27.35	28.37	31.79	31.8	31.79
LincolnB	45.6	45.6	45.6	52.64		99.73	25.06	25.26	26.4	30.37	30.38	30.36
Wanderer	45.54	45.54	45.54	52.63	99.73		25.12	25.32	26.47	30.42	30.43	30.42
Callan	29.4	29.39	29.39	26.45	25.06	25.12		91.6	71.37	39.01	39.01	39.02
Dash	29.85	29.85	29.84	27.35	25.26	25.32	91.6		78.37	40.06	40.07	40.06
Lilo	30.89	30.88	30.88	28.37	26.4	26.47	71.37	78.37		41.25	41.25	41.26
Redbud	37.13	37.13	37.14	31.79	30.37	30.42	39.01	40.06	41.25		99.96	99.41
Kiel007	37.16	37.16	37.17	31.8	30.38	30.43	39.01	40.07	41.25	99.96		99.45
Rani	37.14	37.13	37.18	31.79	30.36	30.42	39.02	40.06	41.26	99.41	99.45	
AlexiD	37.06	37.06	37.1	31.76	30.35	30.4	39	40.04	41.24	99.23	99.27	99.1
JackPlaque	37.08	37.08	37.09	31.76	30.35	30.4	38.99	40.04	41.23	99.28	99.32	99.04
HB10c2	35.38	35.38	35.39	30.96	28.66	28.72	36.43	37.47	39.48	91.5	91.54	91.31
Sitara	35.27	35.27	35.32	30.51	27.85	27.89	39.45	41.47	41.94	82.91	82.95	83.31
Eltigre	36.28	36.27	36.29	30.88	29.73	29.78	37.92	38.96	40.38	91.58	91.62	91.82
Fitz	35.3	35.3	35.35	30.43	28.05	28.09	39.91	41.95	42.36	69.05	69.08	69.49
Newport	35.3	35.3	35.35	30.43	28.05	28.09	39.91	41.95	42.36	69.05	69.08	69.49
Gohan	34.69	34.69	34.74	29.87	27.55	27.6	39.22	41.23	41.58	67.63	67.66	68.05
Shelly	35.3	35.3	35.35	30.42	28.05	28.09	39.9	41.95	42.36	69.04	69.07	69.48
vB-PlaS-2A	35.29	35.28	35.33	30.4	28.03	28.08	39.88	41.93	42.33	68.99	69.02	69.43
vB_PlaS-3A	35.26	35.26	35.31	30.38	28.01	28.06	39.83	41.88	42.29	68.94	68.97	69.38
vB_PlaS-1A	35.15	35.15	35.2	30.25	27.87	27.92	39.61	41.64	42.03	68.7	68.73	69.11
vB_PlaS-5A	35.09	35.09	35.13	30.23	27.86	27.91	39.6	41.64	42.03	68.54	68.57	68.97
Xenia	35.31	35.31	35.27	30.42	28.05	28.09	39.89	41.95	42.34	68.82	68.85	69.07
Hobie	35.22	35.22	35.27	30.34	28.32	28.36	39.86	41.96	42.38	71.52	71.55	71.97
Picard	35.2	35.2	35.25	30.34	28.34	28.38	39.94	42.03	42.41	71.4	71.43	71.85
Diva	33.88	33.87	33.89	30.73	26.93	26.98	37.64	39.74	40.78	71.1	71.14	71.36
Leyra	37.03	37.03	37.07	31.94	29.32	29.36	40.79	42.28	42.82	81.83	81.87	82.27
PBL1c	35.21	35.21	35.17	30	28.3	28.35	37.64	39.07	39.13	66.24	66.27	66.12
Neutron	38.2	38.2	38.22	32.38	31.77	31.82	44.51	46.81	46.94	73.1	73.13	73.08
Yerffej	36.1	36.1	36.11	31.44	28.37	28.4	49.41	55.93	54	68.55	68.59	68.54
phiIBB_PI23	35.59	35.59	35.61	30.71	28.58	28.64	44.12	49.35	50.44	69.78	69.81	70.09
Likha	37.67	37.67	37.71	32.09	30.5	30.51	41.97	43.01	42.91	71.68	71.72	71.63
HoneyBear	36.52	36.52	36.53	30.81	29.99	30.02	38.44	39.33	40.06	73.27	73.3	73.06
Toothless	37.55	37.55	37.57	31.6	30.66	30.69	39.39	40.31	41.15	75.2	75.23	74.99
Pagassa	36.57	36.57	36.58	30.8	29.97	30	38.47	39.36	40.1	73.75	73.78	73.35
Tadhana	38.08	38.08	38.1	32.24	31.09	31.12	40.75	41.69	41.95	77.2	77.24	76.98
Saudage	37.33	37.33	37.34	31.67	30.33	30.36	38.48	39.41	40.32	74.82	74.85	74.54
Lucielle	37.14	37.13	37.15	31.6	30.27	30.3	38.46	39.38	40.2	73.72	73.75	73.44
Fern	37.83	37.82	37.87	31.88	30.87	30.88	40.24	41.18	41.35	73.92	73.96	74.33
Willow	37.83	37.82	37.87	31.88	30.87	30.87	40.24	41.17	41.35	73.92	73.96	74.33
Kawika	36.01	36	36.05	30.08	29.2	29.24	37.41	38.27	38.94	67.48	67.51	67.87
Bohemia	37.25	37.25	37.28	31.26	30.18	30.16	39.28	40.36	40.84	72.19	72.22	72.4
Sneaky	37.26	37.26	37.29	31.27	30.18	30.17	39.28	40.37	40.85	72.61	72.64	72.28
Pahemo	37.52	37.52	37.57	31.52	30.38	30.37	39.93	41.01	41.54	75.67	75.71	75.5
BN12	37.08	37.07	37.09	31.02	30.15	30.2	39.23	39.99	40.45	74.02	74.05	74.21
ArcticFreeze	39.82	39.82	39.84	36	31.14	31.2	47.94	49.54	49.95	69.59	69.62	69.58
DevRi	39.83	39.83	39.85	36	31.15	31.21	47.93	49.54	49.95	69.63	69.67	69.63
Bloom	39.87	39.87	39.82	36.01	31.15	31.22	47.93	49.53	49.95	69.6	69.64	69.59
Jacopo	39.88	39.88	39.9	36.03	31.17	31.23	48.01	49.61	50.03	69.81	69.84	69.97
Gryphonian	39.86	39.86	39.81	36	31.14	31.2	47.96	49.57	49.98	69.44	69.47	69.28
Genki	39.83	39.83	39.79	35.99	31.14	31.2	47.97	49.58	49.98	69.46	69.49	69.31
Norbert	38.87	38.87	38.92	35.56	30.26	30.32	40.91	42.13	42.95	66.51	66.54	66.96
Riker	38.88	38.88	38.92	35.56	30.26	30.33	40.92	42.14	42.95	66.51	66.54	66.96
Bert	39.11	39.11	39.15	35.46	30.33	30.4	56.62	58.03	55.76	60.15	60.19	60.25
Mock2	39.17	39.17	39.22	35.46	30.35	30.42	56.62	58.03	55.76	60.3	60.33	60.74
Paisley	44.04	44.03	44.03	36.67	34.68	34.74	56.12	62.07	62.49	41.53	41.56	41.55
Harrison	43.89	43.88	43.89	36.55	34.58	34.65	55.11	61.03	61.41	41.25	41.28	41.27
Lily	26.94	26.94	26.95	27.12	22.11	22.21	27.31	28.21	28.29	39.03	39.05	39.13
phiERICV	14.81	14.81	14.82	14.04	14.44	14.48	14.37	14.44	14.93	17.41	17.42	17.43
Ash	22.13	22.13	22.13	20.99	20.05	20.05	21.3	21.78	22.14	24.15	24.15	24.16
Ley	22.15	22.15	22.15	21.01	20.06	20.06	21.32	21.8	22.16	24.15	24.15	24.16
C7Cdelta	22.4	22.4	22.4	21.24	20.29	20.3	21.59	22.08	22.45	24.42	24.43	24.43
Unity	21.1	21.1	21.1	19.8	20.08	20.08	18.97	19.29	19.93	23.33	23.33	23.35
Heath	21.16	21.16	21.16	20.06	19.28	19.29	20.61	21.16	21.41	23.22	23.22	23.24
Halcyone	21.17	21.17	21.17	20.07	19.29	19.29	20.61	21.17	21.41	23.23	23.23	23.24
Scottie	21.09	21.09	21.09	20.01	19.22	19.22	20.95	21.51	21.6	23.05	23.06	23.07
Tripp	22.65	22.65	22.66	21.2	19.34	19.35	22.05	22.73	22.67	23.75	23.75	23.77
vB_PlaP_API480	24.4	24.4	24.41	23.05	22.53	22.57	24.47	25.03	24.52	26.66	26.67	26.72

	AlexiD	JackPlaque	HB10c2	Sitara	Eltigre	Fitz	Newport	Gohan	Shelly	vB-PlaS-2A	vB_PlaS-3A	vB_PlaS-1A
ABAtENZ	36.8	36.81	34.84	34.54	36.02	34.43	34.43	33.83	34.42	34.41	34.39	34.28
Rae2Bee1	36.79	36.81	34.84	34.54	36.02	34.43	34.43	33.83	34.43	34.41	34.39	34.28
Lena	36.8	36.81	34.83	34.54	36.02	34.43	34.43	33.83	34.42	34.41	34.39	34.28
LunBun	36.8	36.81	34.84	34.54	36.02	34.43	34.43	33.83	34.43	34.41	34.39	34.28
TonyLawson77	36.79	36.81	34.83	34.54	36.02	34.43	34.43	33.83	34.42	34.41	34.38	34.27
Jacinda	36.8	36.82	34.84	34.54	36.02	34.43	34.43	33.83	34.43	34.41	34.39	34.28
BarryFoster_Benicio	36.79	36.81	34.83	34.53	36.01	34.42	34.42	33.82	34.42	34.4	34.38	34.27
GaryLarson	36.79	36.81	34.83	34.54	36.02	34.42	34.42	33.83	34.42	34.41	34.38	34.27
UtuhinaGold_Zacery	36.79	36.81	34.83	34.54	36.02	34.43	34.43	33.83	34.42	34.41	34.39	34.28
NHScienceFair	36.8	36.81	34.84	34.54	36.02	34.43	34.43	33.83	34.42	34.41	34.39	34.28
Bloomfield	36.78	36.8	34.83	34.53	36.01	34.42	34.42	33.82	34.42	34.4	34.38	34.27
Ollie	36.78	36.8	34.83	34.53	36.01	34.42	34.42	33.82	34.42	34.4	34.38	34.27
FutureBee	36.79	36.8	34.83	34.53	36.01	34.43	34.43	33.83	34.42	34.41	34.38	34.28
Dante	36.79	36.81	34.83	34.53	36.01	34.42	34.42	33.83	34.42	34.41	34.38	34.27
AJG77	36.78	36.79	34.82	34.52	36	34.41	34.41	33.82	34.41	34.4	34.37	34.26
Logan	36.79	36.8	34.84	34.53	36.01	34.42	34.42	33.82	34.42	34.4	34.38	34.27
Ted	36.79	36.81	34.84	34.53	36.02	34.42	34.42	33.83	34.42	34.4	34.38	34.27
ApiWellbeing	36.83	36.84	34.87	34.58	36.05	34.47	34.47	33.87	34.47	34.45	34.43	34.32
Carlos	36.83	36.85	34.87	34.59	36.05	34.48	34.48	33.88	34.47	34.46	34.43	34.33
Wildcape	36.83	36.85	34.87	34.58	36.05	34.48	34.48	33.88	34.47	34.46	34.43	34.32
GIW2016	37.42	37.44	35.45	35.07	36.61	34.97	34.97	34.36	34.97	34.96	34.93	34.82
Rosalind	37.42	37.44	35.45	35.07	36.61	34.98	34.98	34.36	34.97	34.96	34.93	34.82
Bob	37.4	37.42	35.43	35.06	36.59	34.96	34.96	34.35	34.96	34.94	34.92	34.81
Hayley	36.91	36.92	35.21	34.64	36.11	34.65	34.65	34.05	34.65	34.63	34.61	34.5
Vadim	37.06	37.08	35.38	35.27	36.28	35.3	35.3	34.69	35.3	35.29	35.26	35.15
Diane	37.06	37.08	35.38	35.27	36.27	35.3	35.3	34.69	35.3	35.28	35.26	35.15
Vegas	37.1	37.09	35.39	35.32	36.29	35.35	35.35	34.74	35.35	35.33	35.31	35.2
Dragolir	31.76	31.76	30.96	30.51	30.88	30.43	30.43	29.87	30.42	30.4	30.38	30.25
LincolnB	30.35	30.35	28.66	27.85	29.73	28.05	28.05	27.55	28.05	28.03	28.01	27.87
Wanderer	30.4	30.4	28.72	27.89	29.78	28.09	28.09	27.6	28.09	28.08	28.06	27.92
Callan	39	38.99	36.43	39.45	37.92	39.91	39.91	39.22	39.9	39.88	39.83	39.61
Dash	40.04	40.04	37.47	41.47	38.96	41.95	41.95	41.23	41.95	41.93	41.88	41.64
Lilo	41.24	41.23	39.48	41.94	40.38	42.36	42.36	41.58	42.36	42.33	42.29	42.03
Redbud	99.23	99.28	91.5	82.91	91.58	69.05	69.05	67.63	69.04	68.99	68.94	68.7
Kiel007	99.27	99.32	91.54	82.95	91.62	69.08	69.08	67.66	69.07	69.02	68.97	68.73
Rani	99.1	99.04	91.31	83.31	91.82	69.49	69.49	68.05	69.48	69.43	69.38	69.11
AlexiD		99.94	92.15	82.78	91.18	68.9	68.9	67.48	68.89	68.84	68.79	68.55
JackPlaque	99.94		92.2	82.72	91.23	68.85	68.85	67.42	68.83	68.79	68.74	68.5
HB10c2	92.15	92.2		75.99	85.23	63.45	63.45	62.14	63.45	63.4	63.35	63.1
Sitara	82.78	82.72	75.99		76.75	79.06	79.06	77.47	79.05	78.98	78.81	78.68
Eltigre	91.18	91.23	85.23	76.75		64.7	64.7	63.39	64.69	64.64	64.6	64.33
Fitz	68.9	68.85	63.45	79.06	64.7		100	97.77	99.97	99.89	99.66	99.56
Newport	68.9	68.85	63.45	79.06	64.7	100		97.77	99.97	99.89	99.66	99.56
Gohan	67.48	67.42	62.14	77.47	63.39	97.77	97.77		97.74	97.66	97.44	97.34
Shelly	68.89	68.83	63.45	79.05	64.69	99.97	99.97	97.74		99.87	99.64	99.54
vB-PlaS-2A	68.84	68.79	63.4	78.98	64.64	99.89	99.89	97.66	99.87		99.61	99.51
vB_PlaS-3A	68.79	68.74	63.35	78.81	64.6	99.66	99.66	97.44	99.64	99.61		99.28
vB_PlaS-1A	68.55	68.5	63.1	78.68	64.33	99.56	99.56	97.34	99.54	99.51	99.28	
vB_PlaS-5A	68.39	68.33	62.95	78.53	64.2	99.33	99.33	97.12	99.31	99.28	99.06	98.95
Xenia	69.13	69.11	63.71	78.65	64.32	99.53	99.53	97.31	99.51	99.44	99.21	99.12
Hobie	71.36	71.31	65.72	81.72	66.9	93.76	93.76	91.67	93.73	93.65	93.44	93.32
Picard	71.24	71.18	65.56	81.6	66.78	93.21	93.21	91.13	93.18	93.11	92.89	92.77
Diva	70.75	70.8	69.41	80.48	66.86	86.19	86.19	84.29	86.17	86.1	85.9	85.77
Leyra	81.67	81.62	74.75	84.4	76.97	86.33	86.33	84.52	86.31	86.24	86.05	85.93
PBL1c	66.5	66.54	61.18	70.7	63.73	87.81	87.81	85.92	87.79	87.73	87.66	87.43
Neutron	73.5	73.5	67.77	66.16	74.34	60.85	60.85	59.6	60.84	60.8	60.75	60.5
Yerffej	68.17	68.21	63.06	68.23	69.88	62.63	62.63	61.45	62.62	62.59	62.52	62.28
phiBB_Pl23	69.61	69.6	64.07	69.68	70.94	64.7	64.7	63.44	64.69	64.65	64.57	64.33
Likha	71.84	71.79	66.17	66.96	68.72	76.27	76.27	74.65	76.25	76.21	76.11	75.91
HoneyBear	73.14	73.19	67.84	69.25	69.59	64.17	64.17	62.88	64.16	64.13	64.07	63.83
Toothless	75.07	75.12	69.86	70.92	71.41	66.05	66.05	64.69	66.04	66	65.94	65.7
Pagassa	73.21	73.26	67.95	69.42	69.89	64.36	64.36	63.07	64.35	64.32	64.26	64.02
Tadhana	77.07	77.12	71.59	72.66	72.87	67.71	67.71	66.3	67.7	67.67	67.6	67.36
Saudage	74.84	74.87	68.53	70.42	73	66.71	66.71	65.32	66.7	66.67	66.6	66.36
Lucielle	73.74	73.78	67.44	69.41	71.92	67.19	67.19	65.79	67.18	67.15	67.09	66.84
Fern	73.58	73.6	68.12	70.1	73.84	66.37	66.37	64.98	66.35	66.32	66.26	65.99
Willow	73.58	73.6	68.12	70.1	73.84	66.36	66.36	64.98	66.35	66.32	66.26	65.98
Kawika	67.33	67.28	62.28	64.99	67.44	62.53	62.53	61.31	62.53	62.5	62.44	62.18
Bohemia	72.16	72.2	67.02	71.42	72.59	67.8	67.8	66.42	67.79	67.75	67.7	67.43
Sneaky	72.16	72.19	67.02	71.21	72.49	67.62	67.62	66.24	67.61	67.57	67.52	67.27
Pahemo	75.73	75.74	70.65	74.28	71.91	69.45	69.45	68.03	69.44	69.4	69.34	69.1
BN12	74	74.05	68.84	69.75	70.7	64.9	64.9	63.58	64.89	64.86	64.79	64.54
ArcticFreeze	69.22	69.27	63.87	64.93	66.09	67.87	67.87	66.48	67.86	67.83	67.77	67.51
DevRi	69.27	69.32	63.92	64.97	66.11	67.81	67.81	66.42	67.8	67.76	67.71	67.45
Bloom	69.24	69.29	63.88	64.93	66.07	67.77	67.77	66.38	67.76	67.73	67.67	67.41
Jacopo	69.47	69.5	64.11	65.28	66.43	68.13	68.13	66.73	68.12	68.09	68.03	67.77
Gryphonian	69.59	69.64	64.26	64.65	65.77	67.48	67.48	66.09	67.47	67.43	67.38	67.14
Genki	69.62	69.67	64.28	64.68	65.8	67.51	67.51	66.12	67.5	67.47	67.41	67.17
Norbert	66.35	66.29	62.73	62.72	63.52	65.85	65.85	64.53	65.83	65.8	65.75	65.49
Riker	66.35	66.29	62.73	62.72	63.52	65.85	65.85	64.53	65.84	65.81	65.76	65.49
Bert	60.53	60.47	57.5	56.58	57.57	58	58	56.83	57.99	57.96	57.92	57.67
Mock2	60.14	60.08	57.12	56.98	58.05	58.41	58.41	57.23	58.4	58.37	58.33	58.05
Paisley	41.47	41.48	38.84	42.42	40.36	42.45	42.45	41.71	42.45	42.42	42.37	42.28
Harrison	41.19	41.2	38.56	42.16	40.09	42.16	42.16	41.43	42.15	42.13	42.08	41.99
Lily	39.07	39.07	36.18	37.65	36.88	43.19	43.19	42.42	43.18	43.17	43.14	43.04
phiERICV	17.44	17.44	16.9	16.06	17.08	16.68	16.68	16.45	16.68	16.68	16.67	16.5
Ash	24.12	24.13	22.44	24.3	23.63	23.97	23.97	23.62	23.96	23.96	23.94	23.88
Ley	24.12	24.13	22.44	24.29	23.63	23.97	23.97	23.62	23.96	23.96	23.94	23.88
C7Cdelta	24.4	24.41	22.7	24.55	23.9	24.23	24.23	23.87	24.22	24.22	24.2	24.14
Unity	23.31	23.32	21.88	22.08	22.85	22.4	22.4	22.05	22.4	22.39	22.37	22.3
Heath	23.2	23.21	21.69	23.54	22.76	23.91	23.91	23.56	23.91	23.9	23.88	23.82
Halcyone	23.21	23.22	21.7	23.55	22.77	23.92	23.92	23.57	23.92	23.91	23.88	23.82
Scottie	23.03	23.04	21.54	23.47	22.6	23.82	23.82	23.47	23.82	23.81	23.79	23.73
Tripp	23.76	23.77	22.04	24.29	23.43	24.45	24.45	24.09	24.45	24.44	24.42	24.36
vB_PlaP_API480	26.67	26.67	25.04	25.87	26.25	25.98	25.98	25.53	25.97	25.96	25.94	25.85

	vB_PlaS-5A	Xenia	Hobie	Picard	Diva	Leyra	PBL1c	Neutron	Yerffej	phiBB_PI23	Likha	HoneyBear
ABAtENZ	34.21	34.39	34.37	34.38	33.15	36.58	34.75	38.4	35.19	34.76	37.46	36.25
Rae2Bee1	34.21	34.4	34.37	34.38	33.15	36.58	34.75	38.4	35.18	34.76	37.45	36.25
Lena	34.21	34.39	34.37	34.37	33.15	36.58	34.75	38.4	35.19	34.76	37.45	36.25
LunBun	34.21	34.39	34.37	34.38	33.15	36.58	34.75	38.4	35.18	34.77	37.45	36.25
TonyLawson77	34.21	34.39	34.36	34.37	33.14	36.57	34.75	38.4	35.18	34.76	37.45	36.25
Jacinda	34.21	34.4	34.37	34.38	33.15	36.58	34.75	38.4	35.19	34.78	37.46	36.26
BarryFoster_Benicio	34.2	34.39	34.36	34.37	33.14	36.57	34.74	38.39	35.18	34.76	37.45	36.25
GaryLarson	34.21	34.39	34.36	34.37	33.14	36.57	34.74	38.4	35.18	34.76	37.45	36.25
UtuhinaGold_Zacery	34.21	34.39	34.37	34.37	33.15	36.57	34.75	38.4	35.18	34.76	37.45	36.25
NHScienceFair	34.21	34.39	34.37	34.38	33.15	36.58	34.75	38.4	35.19	34.76	37.45	36.25
Bloomfield	34.2	34.38	34.36	34.37	33.14	36.56	34.74	38.4	35.18	34.76	37.44	36.24
Ollie	34.2	34.39	34.36	34.37	33.14	36.57	34.74	38.39	35.18	34.76	37.44	36.23
FutureBee	34.21	34.39	34.36	34.37	33.14	36.57	34.74	38.4	35.18	34.76	37.45	36.24
Dante	34.21	34.39	34.36	34.37	33.14	36.57	34.74	38.4	35.18	34.76	37.45	36.24
AJG77	34.19	34.38	34.35	34.36	33.13	36.56	34.73	38.39	35.18	34.76	37.44	36.23
Logan	34.2	34.38	34.36	34.37	33.14	36.57	34.74	38.39	35.18	34.76	37.45	36.26
Ted	34.2	34.39	34.36	34.37	33.14	36.57	34.74	38.4	35.18	34.76	37.46	36.27
ApiWellbeing	34.25	34.44	34.41	34.42	33.19	36.62	34.81	38.45	35.18	34.89	37.49	36.27
Carlos	34.26	34.44	34.42	34.42	33.19	36.63	34.82	38.45	35.19	34.89	37.48	36.25
Wildcape	34.26	34.44	34.41	34.42	33.19	36.63	34.82	38.45	35.19	34.89	37.48	36.25
GIW2016	34.75	34.94	34.92	34.93	33.7	37.17	35.31	39.03	35.7	35.28	38.05	36.83
Rosalind	34.75	34.94	34.92	34.93	33.7	37.17	35.31	39.03	35.7	35.28	38.05	36.83
Bob	34.74	34.93	34.91	34.92	33.69	37.15	35.29	39.04	35.71	35.3	38.07	36.84
Hayley	34.43	34.62	34.56	34.54	33.2	36.82	34.76	38.64	35.4	34.99	37.71	36.27
Vadim	35.09	35.31	35.22	35.2	33.88	37.03	35.21	38.2	36.1	35.59	37.67	36.52
Diane	35.09	35.31	35.22	35.2	33.87	37.03	35.21	38.2	36.1	35.59	37.67	36.52
Vegas	35.13	35.27	35.27	35.25	33.89	37.07	35.17	38.22	36.11	35.61	37.71	36.53
Dragolir	30.23	30.42	30.34	30.34	30.73	31.94	30	32.38	31.44	30.71	32.09	30.81
LincolnB	27.86	28.05	28.32	28.34	26.93	29.32	28.3	31.77	28.37	28.58	30.5	29.99
Wanderer	27.91	28.09	28.36	28.38	26.98	29.36	28.35	31.82	28.4	28.64	30.51	30.02
Callan	39.6	39.89	39.86	39.94	37.64	40.79	37.64	44.51	49.41	44.12	41.97	38.44
Dash	41.64	41.95	41.96	42.03	39.74	42.28	39.07	46.81	55.93	49.35	43.01	39.33
Lilo	42.03	42.34	42.38	42.41	40.78	42.82	39.13	46.94	54	50.44	42.91	40.06
Redbud	68.54	68.82	71.52	71.4	71.1	81.83	66.24	73.1	68.55	69.78	71.68	73.27
Kiel007	68.57	68.85	71.55	71.43	71.14	81.87	66.27	73.13	68.59	69.81	71.72	73.3
Rani	68.97	69.07	71.97	71.85	71.36	82.27	66.12	73.08	68.54	70.09	71.63	73.06
AlexiD	68.39	69.13	71.36	71.24	70.75	81.67	66.5	73.5	68.17	69.61	71.84	73.14
JackPlaque	68.33	69.11	71.31	71.18	70.8	81.62	66.54	73.5	68.21	69.6	71.79	73.19
HB10c2	62.95	63.71	65.72	65.56	69.41	74.75	61.18	67.77	63.06	64.07	66.17	67.84
Sitara	78.53	78.65	81.72	81.6	80.48	84.4	70.7	66.16	68.23	69.68	66.96	69.25
Eltigre	64.2	64.32	66.9	66.78	66.86	76.97	63.73	74.34	69.88	70.94	68.72	69.59
Fitz	99.33	99.53	93.76	93.21	86.19	86.33	87.81	60.85	62.63	64.7	76.27	64.17
Newport	99.33	99.53	93.76	93.21	86.19	86.33	87.81	60.85	62.63	64.7	76.27	64.17
Gohan	97.12	97.31	91.67	91.13	84.29	84.52	85.92	59.6	61.45	63.44	74.65	62.88
Shelly	99.31	99.51	93.73	93.18	86.17	86.31	87.79	60.84	62.62	64.69	76.25	64.16
vB-PlaS-2A	99.28	99.44	93.65	93.11	86.1	86.24	87.73	60.8	62.59	64.65	76.21	64.13
vB_PlaS-3A	99.06	99.21	93.44	92.89	85.9	86.05	87.66	60.75	62.52	64.57	76.11	64.07
vB_PlaS-1A	98.95	99.12	93.32	92.77	85.77	85.93	87.43	60.5	62.28	64.33	75.91	63.83
vB_PlaS-5A		98.89	93.11	92.56	85.58	85.75	87.22	60.36	62.17	64.21	75.73	63.69
Xenia	98.89		93.29	92.74	85.78	85.9	88.15	61.13	62.37	64.35	76.39	64
Hobie	93.11	93.29		99.42	89.21	80.5	82.15	63.05	64.69	66.91	71.26	66.38
Picard	92.56	92.74	99.42		88.65	80.38	81.64	62.92	64.58	66.79	71.33	66.27
Diva	85.58	85.78	89.21	88.65		73.69	77.04	62.95	65.04	66.66	66.02	68.42
Leyra	85.75	85.9	80.5	80.38	73.69		75.43	64.81	65.18	66.38	80.15	65.96
PBL1c	87.22	88.15	82.15	81.64	77.04	75.43		62.89	60.95	62.45	71.66	62.68
Neutron	60.36	61.13	63.05	62.92	62.95	64.81	62.89		81.65	74.17	68.06	68.31
Yerffej	62.17	62.37	64.69	64.58	65.04	65.18	60.95	81.65		82.14	64.97	65.66
phiBB_PI23	64.21	64.35	66.91	66.79	66.66	66.38	62.45	74.17	82.14		65.54	65.83
Likha	75.73	76.39	71.26	71.33	66.02	80.15	71.66	68.06	64.97	65.54		67.71
HoneyBear	63.69	64	66.38	66.27	68.42	65.96	62.68	68.31	65.66	65.83	67.71	
Toothless	65.56	65.88	68.4	68.27	70.5	67.55	64.54	70.35	67.45	67.67	69.56	96.53
Pagassa	63.88	64.15	66.58	66.47	68.69	66.15	62.79	68.46	66.09	66	67.87	99.27
Tadhana	67.21	67.54	70.17	70.05	72.18	69.24	66.14	72.74	69.53	69.81	71.78	93.27
Saudage	66.2	66.97	69.23	69.09	69.07	68.22	68.45	71.53	68.08	69.11	68.14	86.51
Lucielle	66.68	67.45	69.73	69.91	69.58	67.21	69.4	70.43	67.08	68.01	67.09	85.31
Fern	65.86	65.95	68.87	68.75	69.55	67.91	66.95	73.57	70.4	70.86	70.18	88.39
Willow	65.85	65.95	68.87	68.75	69.55	67.91	66.95	73.56	70.4	70.86	70.18	88.38
Kawika	62.06	62.14	64.77	64.66	64.5	63.99	61.74	66.61	63.59	64.23	65.27	86.31
Bohemia	67.29	67.46	70.31	70.17	71.22	69.2	67.97	69.94	68.86	69.12	68.32	85.79
Sneaky	67.11	67.39	70.12	69.98	71.13	69.02	68.02	69.97	68.94	68.92	68.46	85.66
Pahemo	68.94	69.24	71.92	71.8	74.07	70.89	67.29	69.5	68.28	68.52	70.32	89.33
BN12	64.41	64.58	67.19	67.07	69.24	66.88	62.93	69.29	66.71	66.98	68.35	87.88
ArcticFreeze	67.37	67.65	71.87	72.38	65.06	68.9	64.61	75.56	69.95	66.06	70.7	65.92
DevRi	67.31	67.59	71.93	72.45	65.1	68.84	64.64	75.61	69.97	66.08	70.76	65.97
Bloom	67.27	67.64	71.89	72.41	65.05	68.8	64.69	75.55	69.93	66.02	70.72	65.94
Jacopo	67.63	67.81	72.27	72.78	65.42	69.17	64.83	75.79	70.19	66.39	70.99	66.1
Gryphonian	66.98	67.78	71.59	72.11	64.76	68.51	65.53	75.86	69.69	65.73	70.94	65.68
Genki	67.01	67.8	71.62	72.14	64.79	68.53	65.56	75.89	69.72	65.76	70.96	65.71
Norbert	65.36	65.44	69.79	70.29	65.1	66.39	62.35	67.8	63.11	61.88	67.86	63.43
Riker	65.37	65.45	69.79	70.29	65.1	66.39	62.36	67.81	63.11	61.88	67.86	63.44
Bert	57.53	58.25	61.42	61.53	55.74	59.69	55.37	67.61	62.41	58.06	62.88	58.29
Mock2	57.94	58	61.85	61.97	56.16	60.11	54.99	67.15	62.66	58.47	62.62	58.42
Paisley	42.17	42.41	42.47	42.54	40.22	43.28	39.24	43.85	50.83	50.63	43.38	40.5
Harrison	41.88	42.12	42.17	42.22	39.92	42.98	38.96	43.56	50.53	50.33	43.06	40.23
Lily	42.96	43.16	38.87	38.89	36.4	44.48	42.25	39.35	37.75	37.16	45.47	37.84
phiERICV	16.54	16.69	17	17	16.5	16.58	16.67	17.73	16.13	16.67	17.27	16.92
Ash	23.86	23.95	23.75	23.74	22.52	24.99	23.13	23.67	24.86	24.34	24.48	23.73
Ley	23.86	23.95	23.74	23.74	22.51	24.99	23.12	23.66	24.86	24.34	24.48	23.72
C7Cdelta	24.11	24.22	24.01	24	22.76	25.26	23.37	23.94	25.13	24.61	24.75	23.97
Unity	22.28	22.39	21.96	21.96	20.71	23.34	22.52	23.9	22.37	22.01	23.51	22.5
Heath	23.79	23.91	23.5	23.5	22.26	24.39	22.99	22.54	23.98	23.53	23.99	22.97
Halcyone	23.8	23.91	23.51	23.5	22.26	24.4	23	22.55	23.99	23.54	24	22.98
Scottie	23.7	23.82	23.42	23.41	22.18	24.31	23.08	22.35	24.11	23.62	23.96	22.86
Tripp	24.33	24.45	23.97	23.97	22.46	25.44	23.57	23.8	25.1	24.49	24.86	23.45
vB_PlaP_API480	25.84	25.94	26.02	26.01	24.61	26.96	25.4	26.98	26.76	26.94	27.15	26.09

	Toothless	Pagassa	Tadhana	Saudage	Lucielle	Fern	Willow	Kawika	Bohemia	Sneaky	Pahemo	BN12
ABAtENZ	37.08	36.3	37.61	36.88	36.73	37.38	37.38	35.54	36.75	36.76	36.98	36.64
Rae2Bee1	37.08	36.3	37.6	36.88	36.73	37.38	37.38	35.54	36.75	36.76	36.97	36.64
Lena	37.08	36.3	37.6	36.88	36.73	37.38	37.38	35.54	36.75	36.76	36.98	36.64
LunBun	37.08	36.3	37.6	36.88	36.73	37.38	37.38	35.54	36.75	36.76	36.97	36.64
TonyLawson77	37.07	36.3	37.6	36.88	36.73	37.38	37.38	35.53	36.74	36.76	36.97	36.64
Jacinda	37.08	36.31	37.61	36.88	36.73	37.39	37.39	35.54	36.75	36.77	36.98	36.65
BarryFoster_Benicio	37.07	36.29	37.6	36.87	36.72	37.37	37.37	35.53	36.74	36.75	36.97	36.63
GaryLarson	37.07	36.3	37.6	36.87	36.72	37.38	37.38	35.53	36.74	36.75	36.97	36.64
UtuhinaGold_Zacery	37.07	36.3	37.6	36.87	36.72	37.38	37.38	35.53	36.74	36.75	36.97	36.64
NHScienceFair	37.08	36.3	37.6	36.88	36.73	37.38	37.38	35.54	36.75	36.76	36.97	36.64
Bloomfield	37.07	36.28	37.59	36.87	36.72	37.37	37.37	35.53	36.74	36.75	36.96	36.63
Ollie	37.07	36.28	37.59	36.87	36.72	37.37	37.37	35.53	36.73	36.75	36.96	36.63
FutureBee	37.07	36.28	37.59	36.87	36.72	37.37	37.37	35.53	36.74	36.75	36.97	36.63
Dante	37.07	36.29	37.6	36.87	36.72	37.37	37.37	35.53	36.74	36.75	36.97	36.64
AJG77	37.07	36.28	37.59	36.87	36.72	37.37	37.37	35.52	36.73	36.75	36.96	36.63
Logan	37.07	36.31	37.6	36.87	36.72	37.38	37.38	35.53	36.74	36.76	36.97	36.64
Ted	37.08	36.31	37.6	36.87	36.72	37.38	37.38	35.54	36.75	36.76	36.97	36.64
ApiWellbeing	37.08	36.31	37.59	36.87	36.72	37.41	37.41	35.59	36.75	36.77	36.98	36.68
Carlos	37.08	36.3	37.59	36.88	36.73	37.41	37.41	35.59	36.76	36.77	36.98	36.68
Wildcape	37.07	36.3	37.59	36.87	36.72	37.41	37.41	35.58	36.75	36.76	36.98	36.67
GIW2016	37.66	36.88	38.21	37.5	37.35	37.97	37.97	36.08	37.32	37.34	37.55	37.22
Rosalind	37.67	36.88	38.21	37.5	37.35	37.97	37.97	36.08	37.32	37.34	37.56	37.22
Bob	37.68	36.89	38.22	37.5	37.35	37.99	37.99	36.09	37.33	37.35	37.56	37.23
Hayley	37.31	36.32	37.84	37.08	36.88	37.58	37.58	35.75	36.93	36.94	37.2	36.87
Vadim	37.55	36.57	38.08	37.33	37.14	37.83	37.83	36.01	37.25	37.26	37.52	37.08
Diane	37.55	36.57	38.08	37.33	37.13	37.82	37.82	36	37.25	37.26	37.52	37.07
Vegas	37.57	36.58	38.1	37.34	37.15	37.87	37.87	36.05	37.28	37.29	37.57	37.09
Dragolir	31.6	30.8	32.24	31.67	31.6	31.88	31.88	30.08	31.26	31.27	31.52	31.02
LincolnB	30.66	29.97	31.09	30.33	30.27	30.87	30.87	29.2	30.18	30.18	30.38	30.15
Wanderer	30.69	30	31.12	30.36	30.3	30.88	30.87	29.24	30.16	30.17	30.37	30.2
Callan	39.39	38.47	40.75	38.48	38.46	40.24	40.24	37.41	39.28	39.28	39.93	39.23
Dash	40.31	39.36	41.69	39.41	39.38	41.18	41.17	38.27	40.36	40.37	41.01	39.99
Lilo	41.15	40.1	41.95	40.32	40.2	41.35	41.35	38.94	40.84	40.85	41.54	40.45
Redbud	75.2	73.75	77.2	74.82	73.72	73.92	73.92	67.48	72.19	72.61	75.67	74.02
Kiel007	75.23	73.78	77.24	74.85	73.75	73.96	73.96	67.51	72.22	72.64	75.71	74.05
Rani	74.99	73.35	76.98	74.54	73.44	74.33	74.33	67.87	72.4	72.28	75.5	74.21
AlexiD	75.07	73.21	77.07	74.84	73.74	73.58	73.58	67.33	72.16	72.16	75.73	74
JackPlaque	75.12	73.26	77.12	74.87	73.78	73.6	73.6	67.28	72.2	72.19	75.74	74.05
HB10c2	69.86	67.95	71.59	68.53	67.44	68.12	68.12	62.28	67.02	67.02	70.65	68.84
Sitara	70.92	69.42	72.66	70.42	69.41	70.1	70.1	64.99	71.42	71.21	74.28	69.75
Eltigre	71.41	69.89	72.87	73	71.92	73.84	73.84	67.44	72.59	72.49	71.91	70.7
Fitz	66.05	64.36	67.71	66.71	67.19	66.37	66.36	62.53	67.8	67.62	69.45	64.9
Newport	66.05	64.36	67.71	66.71	67.19	66.37	66.36	62.53	67.8	67.62	69.45	64.9
Gohan	64.69	63.07	66.3	65.32	65.79	64.98	64.98	61.31	66.42	66.24	68.03	63.58
Shelly	66.04	64.35	67.7	66.7	67.18	66.35	66.35	62.53	67.79	67.61	69.44	64.89
vB-PlaS-2A	66	64.32	67.67	66.67	67.15	66.32	66.32	62.5	67.75	67.57	69.4	64.86
vB_PlaS-3A	65.94	64.26	67.6	66.6	67.09	66.26	66.26	62.44	67.7	67.52	69.34	64.79
vB_PlaS-1A	65.7	64.02	67.36	66.36	66.84	65.99	65.98	62.18	67.43	67.27	69.1	64.54
vB_PlaS-5A	65.56	63.88	67.21	66.2	66.68	65.86	65.85	62.06	67.29	67.11	68.94	64.41
Xenia	65.88	64.15	67.54	66.97	67.45	65.95	65.95	62.14	67.46	67.39	69.24	64.58
Hobie	68.4	66.58	70.17	69.23	69.73	68.87	68.87	64.77	70.31	70.12	71.92	67.19
Picard	68.27	66.47	70.05	69.09	69.91	68.75	68.75	64.66	70.17	69.98	71.8	67.07
Diva	70.5	68.69	72.18	69.07	69.58	69.55	69.55	64.5	71.22	71.13	74.07	69.24
Leyra	67.55	66.15	69.24	68.22	67.21	67.91	67.91	63.99	69.2	69.02	70.89	66.88
PBL1c	64.54	62.79	66.14	68.45	69.4	66.95	66.95	61.74	67.97	68.02	67.29	62.93
Neutron	70.35	68.46	72.74	71.53	70.43	73.57	73.56	66.61	69.94	69.97	69.5	69.29
Yerffej	67.45	66.09	69.53	68.08	67.08	70.4	70.4	63.59	68.86	68.94	68.28	66.71
philBB_PI23	67.67	66	69.81	69.11	68.01	70.86	70.86	64.23	69.12	68.92	68.52	66.98
Likha	69.56	67.87	71.78	68.14	67.09	70.18	70.18	65.27	68.32	68.46	70.32	68.35
HoneyBear	96.53	99.27	93.27	86.51	85.31	88.39	88.38	86.31	85.79	85.66	89.33	87.88
Toothless		95.85	96.53	89.45	88.21	91.44	91.43	89.06	88.73	88.59	92.4	90.91
Pagassa	95.85		92.55	86.79	85.65	88.71	88.7	86.51	85.77	86.17	89.41	87.81
Tadhana	96.53	92.55		92.07	90.8	94.72	94.72	85.92	85.47	85.34	89.12	87.65
Saudage	89.45	86.79	92.07		98.5	96.07	96.07	84.98	86.92	87.23	83.29	81.52
Lucielle	88.21	85.65	90.8	98.5		94.88	94.88	83.88	85.79	86.09	82.16	80.39
Fern	91.44	88.71	94.72	96.07	94.88		99.99	87.65	89.3	89.19	85.28	84.03
Willow	91.43	88.7	94.72	96.07	94.88	99.99		87.64	89.29	89.18	85.28	84.02
Kawika	89.06	86.51	85.92	84.98	83.88	87.65	87.64		84.67	84.46	78.87	82.04
Bohemia	88.73	85.77	85.47	86.92	85.79	89.3	89.29	84.67		99.52	90.27	86.71
Sneaky	88.59	86.17	85.34	87.23	86.09	89.19	89.18	84.46	99.52		90.27	86.28
Pahemo	92.4	89.41	89.12	83.29	82.16	85.28	85.28	78.87	90.27	90.27		90.02
BN12	90.91	87.81	87.65	81.52	80.39	84.03	84.02	82.04	86.71	86.28	90.02	
ArcticFreeze	67.82	66.04	69.83	67.34	68.18	68.12	68.12	63.59	65.92	65.97	67.71	66.81
DevRi	67.87	66.08	69.88	67.36	68.2	68.14	68.14	63.6	65.94	65.99	67.75	66.85
Bloom	67.84	66.05	69.84	67.33	68.17	68.09	68.09	63.56	65.9	65.95	67.71	66.83
Jacopo	68	66.28	70.02	67.55	68.39	68.47	68.46	63.93	66.24	66.16	67.95	67.16
Gryphonian	67.57	65.93	69.58	67.72	69.04	67.79	67.79	63.27	65.6	65.8	67.61	66.6
Genki	67.6	65.96	69.6	67.74	69.07	67.83	67.82	63.3	65.63	65.84	67.64	66.57
Norbert	65.1	63.64	66.91	64.72	65.53	65.62	65.62	61.58	63.66	63.47	65.23	64.5
Riker	65.11	63.64	66.91	64.72	65.53	65.63	65.63	61.59	63.66	63.47	65.24	64.51
Bert	59.94	58.57	62	58.67	58.77	60.59	60.59	56.1	58.35	58.46	60.22	59.13
Mock2	60.07	58.64	62.14	58.25	58.35	61.05	61.05	56.52	58.74	58.55	60.27	59.57
Paisley	41.41	40.53	42.83	40.53	40.4	42.31	42.31	39.27	41.22	41.24	41.88	40.93
Harrison	41.13	40.27	42.55	40.26	40.13	42.03	42.03	38.99	40.96	40.97	41.6	40.66
Lily	38.77	37.82	39.8	38.24	37.65	38.61	38.61	36.66	37.74	37.71	38.94	38.29
phiERICV	17.28	16.93	17.57	17.35	17.3	17.49	17.49	16.68	16.96	16.97	16.47	17.1
Ash	24.21	23.74	24.48	23.96	23.88	24.24	24.24	23.4	24.09	24.09	24.4	23.94
Ley	24.21	23.74	24.48	23.95	23.88	24.24	24.24	23.39	24.08	24.09	24.39	23.94
C7Cdelta	24.47	23.99	24.75	24.22	24.15	24.51	24.51	23.64	24.35	24.35	24.66	24.2
Unity	22.97	22.51	23.24	22.7	22.63	22.98	22.98	22.1	22.56	22.57	22.9	22.8
Heath	23.42	22.97	23.68	23.17	23.11	23.43	23.43	22.58	23.3	23.3	23.62	23.22
Halcyone	23.43	22.98	23.69	23.18	23.12	23.44	23.44	22.59	23.31	23.31	23.63	23.23
Scottie	23.31	22.86	23.56	23.05	22.99	23.3	23.3	22.47	23.24	23.24	23.56	23.21
Tripp	23.94	23.45	24.21	23.85	23.78	24.11	24.11	22.86	24.06	24.05	23.67	23.82
vB_PlaP_API480	26.72	26.09	27.22	26.74	26.69	27.09	27.09	25.63	26.61	26.57	26.82	26.44

	ArcticFreeze	DevRi	Bloom	Jacopo	Gryphonian	Genki	Norbert	Riker	Bert	Mock2	Paisley	Harrison
ABAtENZ	38.52	38.53	38.52	38.58	38.51	38.49	37.66	37.66	37.92	37.98	42.7	42.55
Rae2Bee1	38.52	38.53	38.53	38.58	38.51	38.49	37.66	37.66	37.92	37.98	42.7	42.55
Lena	38.52	38.53	38.52	38.58	38.51	38.49	37.65	37.66	37.92	37.98	42.7	42.55
LunBun	38.51	38.52	38.52	38.57	38.51	38.48	37.66	37.66	37.92	37.98	42.7	42.55
TonyLawson77	38.51	38.52	38.52	38.57	38.51	38.48	37.65	37.66	37.92	37.98	42.7	42.55
Jacinda	38.52	38.53	38.53	38.58	38.52	38.49	37.66	37.67	37.92	37.98	42.71	42.57
BarryFoster_Benicio	38.52	38.52	38.52	38.57	38.51	38.49	37.65	37.66	37.92	37.98	42.7	42.55
GaryLarson	38.52	38.53	38.53	38.58	38.51	38.49	37.65	37.66	37.92	37.98	42.7	42.56
UtuhinaGold_Zacery	38.52	38.53	38.53	38.58	38.52	38.49	37.66	37.66	37.92	37.99	42.7	42.56
NHScienceFair	38.52	38.53	38.53	38.58	38.52	38.49	37.65	37.66	37.93	37.99	42.7	42.55
Bloomfield	38.5	38.51	38.51	38.56	38.5	38.48	37.64	37.65	37.91	37.97	42.7	42.55
Ollie	38.51	38.51	38.51	38.56	38.5	38.48	37.64	37.65	37.91	37.97	42.7	42.55
FutureBee	38.51	38.52	38.52	38.57	38.51	38.48	37.65	37.65	37.92	37.98	42.7	42.55
Dante	38.51	38.52	38.52	38.57	38.51	38.48	37.64	37.65	37.91	37.98	42.69	42.54
AJG77	38.5	38.51	38.51	38.56	38.5	38.48	37.64	37.65	37.91	37.97	42.69	42.54
Logan	38.51	38.52	38.52	38.57	38.51	38.49	37.66	37.66	37.92	37.98	42.7	42.55
Ted	38.52	38.53	38.53	38.58	38.52	38.49	37.66	37.67	37.92	37.98	42.7	42.55
ApiWellbeing	38.56	38.57	38.57	38.62	38.56	38.53	37.71	37.71	37.97	38.03	42.83	42.68
Carlos	38.56	38.56	38.56	38.61	38.55	38.53	37.7	37.7	37.96	38.02	42.82	42.68
Wildcape	38.56	38.57	38.57	38.62	38.56	38.53	37.7	37.7	37.97	38.02	42.83	42.68
GIW2016	39.14	39.15	39.15	39.2	39.14	39.11	38.27	38.27	38.53	38.59	43.33	43.18
Rosalind	39.14	39.15	39.15	39.2	39.14	39.12	38.26	38.27	38.53	38.59	43.32	43.17
Bob	39.15	39.16	39.16	39.21	39.15	39.12	38.26	38.27	38.54	38.6	43.34	43.19
Hayley	38.8	38.81	38.81	38.86	38.8	38.77	37.87	37.88	38.11	38.17	43.5	43.36
Vadim	39.82	39.83	39.87	39.88	39.86	39.83	38.87	38.88	39.11	39.17	44.04	43.89
Diane	39.82	39.83	39.87	39.88	39.86	39.83	38.87	38.88	39.11	39.17	44.03	43.88
Vegas	39.84	39.85	39.82	39.9	39.81	39.79	38.92	38.92	39.15	39.22	44.03	43.89
Dragolir	36	36	36.01	36.03	36	35.99	35.56	35.56	35.46	35.46	36.67	36.55
LincolnB	31.14	31.15	31.15	31.17	31.14	31.14	30.26	30.26	30.33	30.35	34.68	34.58
Wanderer	31.2	31.21	31.22	31.23	31.2	31.2	30.32	30.33	30.4	30.42	34.74	34.65
Callan	47.94	47.93	47.93	48.01	47.96	47.97	40.91	40.92	56.62	56.62	56.12	55.11
Dash	49.54	49.54	49.53	49.61	49.57	49.58	42.13	42.14	58.03	58.03	62.07	61.03
Lilo	49.95	49.95	49.95	50.03	49.98	49.98	42.95	42.95	55.76	55.76	62.49	61.41
Redbud	69.59	69.63	69.6	69.81	69.44	69.46	66.51	66.51	60.15	60.3	41.53	41.25
Kiel007	69.62	69.67	69.64	69.84	69.47	69.49	66.54	66.54	60.19	60.33	41.56	41.28
Rani	69.58	69.63	69.59	69.97	69.28	69.31	66.96	66.96	60.25	60.74	41.55	41.27
AlexiD	69.22	69.27	69.24	69.47	69.59	69.62	66.35	66.35	60.53	60.14	41.47	41.19
JackPlaque	69.27	69.32	69.29	69.5	69.64	69.67	66.29	66.29	60.47	60.08	41.48	41.2
HB10c2	63.87	63.92	63.88	64.11	64.26	64.28	62.73	62.73	57.5	57.12	38.84	38.56
Sitara	64.93	64.97	64.93	65.28	64.65	64.68	62.72	62.72	56.58	56.98	42.42	42.16
Eltigre	66.09	66.11	66.07	66.43	65.77	65.8	63.52	63.52	57.57	58.05	40.36	40.09
Fitz	67.87	67.81	67.77	68.13	67.48	67.51	65.85	65.85	58	58.41	42.45	42.16
Newport	67.87	67.81	67.77	68.13	67.48	67.51	65.85	65.85	58	58.41	42.45	42.16
Gohan	66.48	66.42	66.38	66.73	66.09	66.12	64.53	64.53	56.83	57.23	41.71	41.43
Shelly	67.86	67.8	67.76	68.12	67.47	67.5	65.83	65.84	57.99	58.4	42.45	42.15
vB-PlaS-2A	67.83	67.76	67.73	68.09	67.43	67.47	65.8	65.81	57.96	58.37	42.42	42.13
vB_PlaS-3A	67.77	67.71	67.67	68.03	67.38	67.41	65.75	65.76	57.92	58.33	42.37	42.08
vB_PlaS-1A	67.51	67.45	67.41	67.77	67.14	67.17	65.49	65.49	57.67	58.05	42.28	41.99
vB_PlaS-5A	67.37	67.31	67.27	67.63	66.98	67.01	65.36	65.37	57.53	57.94	42.17	41.88
Xenia	67.65	67.59	67.64	67.81	67.78	67.8	65.44	65.45	58.25	58	42.41	42.12
Hobie	71.87	71.93	71.89	72.27	71.59	71.62	69.79	69.79	61.42	61.85	42.47	42.17
Picard	72.38	72.45	72.41	72.78	72.11	72.14	70.29	70.29	61.53	61.97	42.54	42.22
Diva	65.06	65.1	65.05	65.42	64.76	64.79	65.1	65.1	55.74	56.16	40.22	39.92
Leyra	68.9	68.84	68.8	69.17	68.51	68.53	66.39	66.39	59.69	60.11	43.28	42.98
PBL1c	64.61	64.64	64.69	64.83	65.53	65.56	62.35	62.36	55.37	54.99	39.24	38.96
Neutron	75.56	75.61	75.55	75.79	75.86	75.89	67.8	67.81	67.61	67.15	43.85	43.56
Yerffej	69.95	69.97	69.93	70.19	69.69	69.72	63.11	63.11	62.41	62.66	50.83	50.53
philBB_PI23	66.06	66.08	66.02	66.39	65.73	65.76	61.88	61.88	58.06	58.47	50.63	50.33
Likha	70.7	70.76	70.72	70.99	70.94	70.96	67.86	67.86	62.88	62.62	43.38	43.06
HoneyBear	65.92	65.97	65.94	66.1	65.68	65.71	63.43	63.44	58.29	58.42	40.5	40.23
Toothless	67.82	67.87	67.84	68	67.57	67.6	65.1	65.11	59.94	60.07	41.41	41.13
Pagassa	66.04	66.08	66.05	66.28	65.93	65.96	63.64	63.64	58.57	58.64	40.53	40.27
Tadhana	69.83	69.88	69.84	70.02	69.58	69.6	66.91	66.91	62	62.14	42.83	42.55
Saudage	67.34	67.36	67.33	67.55	67.72	67.74	64.72	64.72	58.67	58.25	40.53	40.26
Lucielle	68.18	68.2	68.17	68.39	69.04	69.07	65.53	65.53	58.77	58.35	40.4	40.13
Fern	68.12	68.14	68.09	68.47	67.79	67.83	65.62	65.63	60.59	61.05	42.31	42.03
Willow	68.12	68.14	68.09	68.46	67.79	67.82	65.62	65.63	60.59	61.05	42.31	42.03
Kawika	63.59	63.6	63.56	63.93	63.27	63.3	61.58	61.59	56.1	56.52	39.27	38.99
Bohemia	65.92	65.94	65.9	66.24	65.6	65.63	63.66	63.66	58.35	58.74	41.22	40.96
Sneaky	65.97	65.99	65.95	66.16	65.8	65.84	63.47	63.47	58.46	58.55	41.24	40.97
Pahemo	67.71	67.75	67.71	67.95	67.61	67.64	65.23	65.24	60.22	60.27	41.88	41.6
BN12	66.81	66.85	66.83	67.16	66.6	66.57	64.5	64.51	59.13	59.57	40.93	40.66
ArcticFreeze		99.93	99.86	99.55	98.78	98.8	86.99	87	85.05	85.39	44.77	44.43
DevRi	99.93		99.93	99.62	98.85	98.87	87.06	87.07	85.12	85.46	44.76	44.43
Bloom	99.86	99.93		99.55	98.89	98.91	87.02	87.02	85.08	85.4	44.75	44.42
Jacopo	99.55	99.62	99.55		99.07	99.09	87.42	87.43	85.34	85.83	44.81	44.48
Gryphonian	98.78	98.85	98.89	99.07		99.95	86.69	86.7	85.42	85.09	44.74	44.41
Genki	98.8	98.87	98.91	99.09	99.95		86.72	86.73	85.45	85.12	44.72	44.38
Norbert	86.99	87.06	87.02	87.42	86.69	86.72		99.99	76.28	76.73	42.14	41.83
Riker	87	87.07	87.02	87.43	86.7	86.73	99.99		76.29	76.74	42.15	41.83
Bert	85.05	85.12	85.08	85.34	85.42	85.45	76.28	76.29		99.43	53.62	52.57
Mock2	85.39	85.46	85.4	85.83	85.09	85.12	76.73	76.74	99.43		53.68	52.63
Paisley	44.77	44.76	44.75	44.81	44.74	44.72	42.14	42.15	53.62	53.68		98.71
Harrison	44.43	44.43	44.42	44.48	44.41	44.38	41.83	41.83	52.57	52.63	98.71	
Lily	47.99	48	47.99	48.06	48.02	48	48.82	48.81	42.16	42.15	28.85	28.6
phiERICV	17.6	17.6	17.6	17.63	17.62	17.62	17.06	17.06	17.29	17.28	15.43	15.35
Ash	25.24	25.25	25.23	25.25	25.2	25.2	24.45	24.45	24.53	24.55	23.21	23.13
Ley	25.24	25.24	25.23	25.25	25.2	25.2	24.45	24.45	24.53	24.55	23.22	23.15
C7Cdelta	25.53	25.54	25.52	25.55	25.49	25.49	24.72	24.73	24.82	24.83	23.53	23.45
Unity	23.96	23.97	23.95	23.98	23.93	23.93	23.12	23.12	23.27	23.28	20.58	20.53
Heath	24.14	24.15	24.13	24.16	24.11	24.11	23.32	23.32	23.46	23.47	22.15	22.11
Halcyone	24.15	24.15	24.14	24.17	24.12	24.12	23.33	23.33	23.47	23.48	22.16	22.12
Scottie	24.06	24.07	24.06	24.08	24.03	24.03	23.25	23.25	23.39	23.4	22.3	22.25
Tripp	25.53	25.54	25.52	25.57	25.52	25.52	24.71	24.71	24.99	25	23.72	23.65
vB_PlaP_API480	28.71	28.72	28.71	28.78	28.74	28.74	27.67	27.66	27.89	27.93	25.9	25.79

	Lily	phiERICV	Ash	Ley	C7Cdelta	Unity	Heath	Halcyone	Scottie	Tripp	vB_PlaP_API480
ABAtENZ	25.87	15.08	21.71	21.72	21.93	21.27	20.9	20.91	20.83	21.9	23.85
Rae2Bee1	25.87	15.08	21.71	21.72	21.93	21.27	20.9	20.91	20.83	21.9	23.86
Lena	25.87	15.08	21.71	21.72	21.93	21.28	20.9	20.91	20.84	21.9	23.85
LunBun	25.87	15.08	21.71	21.72	21.93	21.27	20.9	20.91	20.83	21.9	23.85
TonyLawson77	25.87	15.08	21.71	21.72	21.93	21.27	20.9	20.91	20.83	21.9	23.85
Jacinda	25.88	15.09	21.71	21.72	21.93	21.27	20.9	20.91	20.83	21.89	23.86
BarryFoster_Benicio	25.87	15.08	21.71	21.72	21.93	21.27	20.9	20.91	20.83	21.89	23.86
GaryLarson	25.87	15.08	21.71	21.72	21.93	21.27	20.9	20.91	20.83	21.9	23.86
UtuhinaGold_Zacery	25.87	15.08	21.71	21.72	21.93	21.27	20.9	20.91	20.83	21.9	23.86
NHScienceFair	25.87	15.08	21.71	21.72	21.93	21.27	20.9	20.91	20.83	21.9	23.86
Bloomfield	25.86	15.08	21.71	21.72	21.93	21.27	20.9	20.91	20.83	21.9	23.84
Ollie	25.86	15.09	21.71	21.72	21.93	21.27	20.9	20.91	20.84	21.9	23.84
FutureBee	25.86	15.09	21.71	21.72	21.93	21.27	20.9	20.91	20.83	21.9	23.85
Dante	25.86	15.09	21.71	21.72	21.93	21.27	20.9	20.91	20.84	21.9	23.84
AJG77	25.86	15.08	21.71	21.72	21.93	21.27	20.9	20.91	20.83	21.9	23.83
Logan	25.86	15.08	21.71	21.72	21.93	21.28	20.91	20.92	20.85	21.9	23.87
Ted	25.86	15.08	21.71	21.72	21.93	21.28	20.91	20.92	20.85	21.9	23.86
ApiWellbeing	25.91	15.1	21.71	21.72	21.92	21.29	20.92	20.93	20.85	21.89	23.89
Carlos	25.91	15.11	21.71	21.72	21.93	21.28	20.91	20.92	20.84	21.89	23.88
Wildcape	25.91	15.1	21.71	21.72	21.92	21.28	20.91	20.92	20.84	21.89	23.89
GIW2016	26.23	15.26	21.86	21.87	22.09	21.44	21.06	21.07	20.99	22.06	24.18
Rosalind	26.23	15.26	21.86	21.87	22.08	21.43	21.05	21.06	20.98	22.05	24.18
Bob	26.23	15.28	21.87	21.88	22.09	21.45	21.07	21.08	21	22.06	24.19
Hayley	25.97	15.07	21.62	21.64	21.88	20.99	20.61	20.62	20.55	22.15	24.06
Vadim	26.94	14.81	22.13	22.15	22.4	21.1	21.16	21.17	21.09	22.65	24.4
Diane	26.94	14.81	22.13	22.15	22.4	21.1	21.16	21.17	21.09	22.65	24.4
Vegas	26.95	14.82	22.13	22.15	22.4	21.1	21.16	21.17	21.09	22.66	24.41
Dragolir	27.12	14.04	20.99	21.01	21.24	19.8	20.06	20.07	20.01	21.2	23.05
LincolnB	22.11	14.44	20.05	20.06	20.29	20.08	19.28	19.29	19.22	19.34	22.53
Wanderer	22.21	14.48	20.05	20.06	20.3	20.08	19.29	19.29	19.22	19.35	22.57
Callan	27.31	14.37	21.3	21.32	21.59	18.97	20.61	20.61	20.95	22.05	24.47
Dash	28.21	14.44	21.78	21.8	22.08	19.29	21.16	21.17	21.51	22.73	25.03
Lilo	28.29	14.93	22.14	22.16	22.45	19.93	21.41	21.41	21.6	22.67	24.52
Redbud	39.03	17.41	24.15	24.15	24.42	23.33	23.22	23.23	23.05	23.75	26.66
Kiel007	39.05	17.42	24.15	24.15	24.43	23.33	23.22	23.23	23.06	23.75	26.67
Rani	39.13	17.43	24.16	24.16	24.43	23.35	23.24	23.24	23.07	23.77	26.72
AlexiD	39.07	17.44	24.12	24.12	24.4	23.31	23.2	23.21	23.03	23.76	26.67
JackPlaque	39.07	17.44	24.13	24.13	24.41	23.32	23.21	23.22	23.04	23.77	26.67
HB10c2	36.18	16.9	22.44	22.44	22.7	21.88	21.69	21.7	21.54	22.04	25.04
Sitara	37.65	16.06	24.3	24.29	24.55	22.08	23.54	23.55	23.47	24.29	25.87
Eltigre	36.88	17.08	23.63	23.63	23.9	22.85	22.76	22.77	22.6	23.43	26.25
Fitz	43.19	16.68	23.97	23.97	24.23	22.4	23.91	23.92	23.82	24.45	25.98
Newport	43.19	16.68	23.97	23.97	24.23	22.4	23.91	23.92	23.82	24.45	25.98
Gohan	42.42	16.45	23.62	23.62	23.87	22.05	23.56	23.57	23.47	24.09	25.53
Shelly	43.18	16.68	23.96	23.96	24.22	22.4	23.91	23.92	23.82	24.45	25.97
vB-PlaS-2A	43.17	16.68	23.96	23.96	24.22	22.39	23.9	23.91	23.81	24.44	25.96
vB_PlaS-3A	43.14	16.67	23.94	23.94	24.2	22.37	23.88	23.88	23.79	24.42	25.94
vB_PlaS-1A	43.04	16.5	23.88	23.88	24.14	22.3	23.82	23.82	23.73	24.36	25.85
vB_PlaS-5A	42.96	16.54	23.86	23.86	24.11	22.28	23.79	23.8	23.7	24.33	25.84
Xenia	43.16	16.69	23.95	23.95	24.22	22.39	23.91	23.91	23.82	24.45	25.94
Hobie	38.87	17	23.75	23.74	24.01	21.96	23.5	23.51	23.42	23.97	26.02
Picard	38.89	17	23.74	23.74	24	21.96	23.5	23.5	23.41	23.97	26.01
Diva	36.4	16.5	22.52	22.51	22.76	20.71	22.26	22.26	22.18	22.46	24.61
Leyra	44.48	16.58	24.99	24.99	25.26	23.34	24.39	24.4	24.31	25.44	26.96
PBL1c	42.25	16.67	23.13	23.12	23.37	22.52	22.99	23	23.08	23.57	25.4
Neutron	39.35	17.73	23.67	23.66	23.94	23.9	22.54	22.55	22.35	23.8	26.98
Yerffej	37.75	16.13	24.86	24.86	25.13	22.37	23.98	23.99	24.11	25.1	26.76
philBB_Pl23	37.16	16.67	24.34	24.34	24.61	22.01	23.53	23.54	23.62	24.49	26.94
Likha	45.47	17.27	24.48	24.48	24.75	23.51	23.99	24	23.96	24.86	27.15
HoneyBear	37.84	16.92	23.73	23.72	23.97	22.5	22.97	22.98	22.86	23.45	26.09
Toothless	38.77	17.28	24.21	24.21	24.47	22.97	23.42	23.43	23.31	23.94	26.72
Pagassa	37.82	16.93	23.74	23.74	23.99	22.51	22.97	22.98	22.86	23.45	26.09
Tadhana	39.8	17.57	24.48	24.48	24.75	23.24	23.68	23.69	23.56	24.21	27.22
Saudage	38.24	17.35	23.96	23.95	24.22	22.7	23.17	23.18	23.05	23.85	26.74
Lucielle	37.65	17.3	23.88	23.88	24.15	22.63	23.11	23.12	22.99	23.78	26.69
Fern	38.61	17.49	24.24	24.24	24.51	22.98	23.43	23.44	23.3	24.11	27.09
Willow	38.61	17.49	24.24	24.24	24.51	22.98	23.43	23.44	23.3	24.11	27.09
Kawika	36.66	16.68	23.4	23.39	23.64	22.1	22.58	22.59	22.47	22.86	25.63
Bohemia	37.74	16.96	24.09	24.08	24.35	22.56	23.3	23.31	23.24	24.06	26.61
Sneaky	37.71	16.97	24.09	24.09	24.35	22.57	23.3	23.31	23.24	24.05	26.57
Pahemo	38.94	16.47	24.4	24.39	24.66	22.9	23.62	23.63	23.56	23.67	26.82
BN12	38.29	17.1	23.94	23.94	24.2	22.8	23.22	23.23	23.21	23.82	26.44
ArcticFreeze	47.99	17.6	25.24	25.24	25.53	23.96	24.14	24.15	24.06	25.53	28.71
DevRi	48	17.6	25.25	25.24	25.54	23.97	24.15	24.15	24.07	25.54	28.72
Bloom	47.99	17.6	25.23	25.23	25.52	23.95	24.13	24.14	24.06	25.52	28.71
Jacopo	48.06	17.63	25.25	25.25	25.55	23.98	24.16	24.17	24.08	25.57	28.78
Gryphonian	48.02	17.62	25.2	25.2	25.49	23.93	24.11	24.12	24.03	25.52	28.74
Genki	48	17.62	25.2	25.2	25.49	23.93	24.11	24.12	24.03	25.52	28.74
Norbert	48.82	17.06	24.45	24.45	24.72	23.12	23.32	23.33	23.25	24.71	27.67
Riker	48.81	17.06	24.45	24.45	24.73	23.12	23.32	23.33	23.25	24.71	27.66
Bert	42.16	17.29	24.53	24.53	24.82	23.27	23.46	23.47	23.39	24.99	27.89
Mock2	42.15	17.28	24.55	24.55	24.83	23.28	23.47	23.48	23.4	25	27.93
Paisley	28.85	15.43	23.21	23.22	23.53	20.58	22.15	22.16	22.3	23.72	25.9
Harrison	28.6	15.35	23.13	23.15	23.45	20.53	22.11	22.12	22.25	23.65	25.79
Lily		25.5	21.73	21.73	21.96	21.02	21.29	21.29	21.25	21.38	23.36
phiERICV	25.5		11.67	11.67	11.81	12.16	11.72	11.72	11.66	11.84	14.47
Ash	21.73	11.67		99.83	96.19	73.29	75.47	75.48	75.09	72.38	20.74
Ley	21.73	11.67	99.83		96.18	73.28	75.46	75.47	75.08	72.44	20.75
C7Cdelta	21.96	11.81	96.19	96.18		71.47	73.69	73.71	73.32	75.07	20.95
Unity	21.02	12.16	73.29	73.28	71.47		87.98	87.99	84.09	60.44	19.6
Heath	21.29	11.72	75.47	75.46	73.69	87.98		99.97	94	62.93	20.2
Halcyone	21.29	11.72	75.48	75.47	73.71	87.99	99.97		94.03	62.94	20.2
Scottie	21.25	11.66	75.09	75.08	73.32	84.09	94	94.03		63.06	20.14
Tripp	21.38	11.84	72.38	72.44	75.07	60.44	62.93	62.94	63.06		20.95
vB_PlaP_API480	23.36	14.47	20.74	20.75	20.95	19.6	20.2	20.2	20.14	20.95	

Table S6.3. Spacer sequences found in eight NZ *P. larvae* bacterial strains

	Spacer Sequence	Spacer Length (bp)	Strains containing Spacer	Phages containing Protospacer	Gene containing Protospacer
1	TATGACCGGGTATCTGCTGTTGCAGAAGCCGAAGGGT	37	PFR-Pl-2017 PFR-Pl-2006 Pl-WAI Pl-TP Pl-CHCH Pl-P1627 Pl-F1A Pl-F2B	-	
2	ACGGTATGAATCGTAAGTCGCCTGACTCACGATATG	36	PFR-Pl-2017 PFR-Pl-2006 Pl-WAI Pl-TP Pl-CHCH Pl-P1627 Pl-F1A Pl-F2B	-	
3	CGTTCTGTGGCCAGTGTCATATGGCGACAGGCGAAT	36	PFR-Pl-2017 PFR-Pl-2006 Pl-TP Pl-CHCH Pl-F1A Pl-F2B	-	
4	AAAAACCCCGATTATGTGGCGGCCGTCCGCACGC	34	PFR-Pl-2017 PFR-Pl-2006 Pl-WAI Pl-TP Pl-CHCH Pl-P1627 Pl-F1A Pl-F2B	-	
5	ACATCATCTGAAACACCCTCAGGAAACAATTCCT	34	PFR-Pl-2017 PFR-Pl-2006 Pl-WAI Pl-TP Pl-CHCH Pl-F1A Pl-F2B	-	

	Spacer Sequence	Spacer Length (bp)	Strains containing Spacer	Phages containing Protospacer	Gene containing Protospacer
6	AACAATTACAAATATGCAACTGAAGCAGATGTAAAT	36	PFR-Pl-2017 PFR-Pl-2006 Pl-WAI Pl-TP Pl-CHCH Pl-F1A Pl-F2B	Callan, Dash 100% match	ERF superfamily
7	CATCATCAAAATACAAACCGAATTGATTGCTATTCCC	37	PFR-Pl-2017 PFR-Pl-2006 Pl-WAI Pl-TP Pl-CHCH Pl-F1A Pl-F2B	-	
8	ACCATGGAAGCGTTGAGACATGGGCCAGAAGATCCA	36	PFR-Pl-2017 PFR-Pl-2006 Pl-WAI Pl-TP Pl-CHCH Pl-P1627 Pl-F1A Pl-F2B	-	
9	GGATAATTTCCGAAAGGTTATTTTGGTTTTCAAT	34	PFR-Pl-2017 PFR-Pl-2006 Pl-TP Pl-CHCH Pl-P1627 Pl-F1A Pl-F2B	-	
10	CAATTAAGCCGACCGCCATATAGCGCGGCTAT	32	PFR-Pl-2017 Pl-WAI Pl-TP Pl-CHCH Pl-P1627 Pl-F1A Pl-F2B	Callan, Dash, Lilo 81.3-84.4% match	Intergenic

	Spacer Sequence	Spacer Length (bp)	Strains containing Spacer	Phages containing Protospacer	Gene containing Protospacer
11	CCAATTCTTTTTGTTCACAAAGTGCTTTCATCTCC	35	PFR-Pl-2017 PFR-Pl-2006 Pl-WAI Pl-TP Pl-CHCH Pl-P1627 Pl-F1A Pl-F2B	-	
12	GCCAAAGGGCGGATAATTTGAGGAGGGCTGTGTGA	35	PFR-Pl-2017 PFR-Pl-2006 Pl-WAI Pl-TP Pl-CHCH Pl-P1627 Pl-F1A Pl-F2B	-	
13	CTACTTATAACTGCTGAATACACTGTCGCTACTGC	35	PFR-Pl-2017 PFR-Pl-2006 Pl-WAI Pl-TP Pl-CHCH Pl-P1627 Pl-F1A Pl-F2B	-	
14	AAGGGCAGGTACGACGCCCACGCGGCGGGCAAT	33	PFR-Pl-2017 PFR-Pl-2006 Pl-WAI Pl-TP Pl-CHCH Pl-P1627 Pl-F1A Pl-F2B	-	
15	ATACGGAAACGGAAGAGTATTGGGAAATGGAAGAACT	37	PFR-Pl-2017 PFR-Pl-2006 Pl-WAI Pl-TP Pl-CHCH Pl-P1627 Pl-F1A Pl-F2B	-	

	Spacer Sequence	Spacer Length (bp)	Strains containing Spacer	Phages containing Protospacer	Gene containing Protospacer
16	GGAATGATTCGGTATTCGTCTGCCTGATACTTTTCC	36	PFR-Pl-2017 PFR-Pl-2006 Pl-WAI Pl-TP Pl-CHCH Pl-P1627 Pl-F1A Pl-F2B	-	
17	GGCAAATGGTGGACGGACACGGAATCACCCACACCA	36	PFR-Pl-2017 PFR-Pl-2006 Pl-WAI Pl-TP Pl-CHCH Pl-P1627 Pl-F1A Pl-F2B	All NZ Vegas Cluster 91.7% match	DNA Polymerase
18	CATGAAGTAATTGCAGTTTCGAACAACGCTAAAG	34	Pl-P1627	-	
19	ATTGATCGTTTCTTTAAAGACTGCCCAGGCAAA	33	Pl-P1627	-	
20	AAAACACAGTTAAATGGTTTACGCACATAATTTTTTA	37	Pl-P1627	-	
21	ACTAGAGCAATGAGCATTAACGGGATTCCAATCA	34	Pl-P1627	-	
22	CACATCATATGAAGGTGTATGGGAGTGTGAAACA	34	Pl-P1627	-	
23	CCTGAGTGAGTGAAATCAAAGGAGCGATACTGG	33	Pl-P1627	-	
24	GGATGTTAAAAAAGCAACCTTTGACATGGATAT	33	Pl-P1627	-	
25	TCAACGGACGCAAGGTCTATATTGTTTACGACAA	34	Pl-P1627	-	
26	CAATTGCGTTCGGGTTGTGCTTTATACAGTAATT	34	Pl-P1627	-	
27	TGCATACATGTTCTTTTTGCGGCTCCATTGTAACA	35	Pl-P1627	-	
28	GGCTTGGGGCCCTTGTCGCTGCACCGATCAATGCTG	36	Pl-P1627	-	
29	TACTTGGAGTACAGTGGCGTACAAAGTCTACCAGGA	36	Pl-P1627	-	

Chapter 7

General Discussion/Conclusion

7.1 Introduction

The services and products of the European honey bee are worth more than NZD $5 billion annually to the New Zealand economy (Ministry for Primary Industries, 2021; Newstrom-Lloyd, 2013). A key goal of the AFB Pest Management Plan is to eliminate American Foulbrood (AFB) in New Zealand. It is estimated that AFB will cost New Zealand beekeepers $3.6 million in hive losses in 2022/23 (Nimmo-Bell & Associates, 2022).

The use of antibiotics to mask or treat AFB infection is strictly prohibited in New Zealand and many other countries. (AFBPMP, 2017; Alippi, 2014; *Biosecurity Act 1993 No 95 (as at 01 July 2022), Public Act Contents – New Zealand Legislation*, 2022). Using antibiotics in beehives has several disadvantages, including contamination of honey with antibiotic residues, increase in antibiotic resistance, and the disruption of the natural microbiota of beehives (Alippi, 2014; Alippi et al., 2007).

An alternative to antibiotics is to prophylactically treat beehives with bacteriophages (phages) to protect against AFB infection. Phages are highly specific and abundant viruses that infect and kill bacteria (Hendrix et al., 1999). Research undertaken in other laboratories has shown this alternative solution has the potential to protect beehives from AFB (Brady et al., 2017; Ghorbani-Nezami et al., 2015). Owing to New Zealand's strict biosecurity laws and the need to apply these phages to beehives in the field, it is important to isolate a suite of native phages from New Zealand. These phages needed to be discovered locally to enable them to be concentrated and reapplied to beehives domestically.

The aims of this thesis were to isolate a collection of *P. larvae* bacterial strains from around New Zealand to isolate and test a novel and robust cocktail of *P. larvae* phages, native to New Zealand to act as a prophylactic against AFB infection in beehives.

Briefly, Chapter 2 reviewed the literature on beekeeping in New Zealand with an emphasis on AFB and the potential of phages to control *P. larvae*. Chapter 3 outlined the discovery of eight novel *P. larvae* strains from New Zealand and the 26 phages discovered that were able to infect these isolates, including *in-vitro* cocktail testing of four distinct phage cocktails on four *P. larvae* isolates. Chapter 4 continued with the *in-vivo* testing of the New Zealand phage cocktails on honeybee larvae. Chapter 5 describes a new technique, adapted from a previously described protocol to evolve phages to have a higher titer for downstream use. In Chapter 6, we discuss the genomes of the 26 New Zealand phages and compare them with the 69 *P. larvae* phages already discovered from around the world. Finally, in this chapter, the general outcome of the experiments undertaken during this thesis are discussed.

7.2 Discovery of *P. larvae* bacterial strains and phages

When this research began, there were no known collections of the *P. larvae* pathogens or phages capable of lysing these bacteria in New Zealand. Therefore, our first step was to discover a suite of native *P. larvae* bacteria on which to initiate our phage hunt. Thanks to help from AsureQuality we were provided with infected hive material to isolate *P. larvae* bacterial strains. These eight strains were bolstered to 30 strains with a generous contribution from ApiWellbeing. The ApiWellbeing project was an MPI led initiative that started in July 2019 to isolate and sequence *P. larvae* strains in New Zealand (Ministry for Primary Industries, n.d.). From the initial eight bacterial isolates, we were able to discover 26 novel *P. larvae* phages

upon which we undertook host range assays, TEM, cocktail formulation, *in-vitro,* and *in-vivo* testing. This is the first known collection of *P. larvae* specific phages from New Zealand.

7.3 *In-vitro* and *in-vivo* testing of our phage cocktails

In-vitro and *in-vivo* testing needed to be conducted with the phage cocktails formulated in Chapter 3. *In-vitro* testing showed promising results, with two cocktails capable of lysing three out of four of the bacterial strains tested. The fourth strain, *P. larvae* Pl-P1627 is resistant to all phages discovered to date. We then performed *in-vivo* testing based on the results of the *in-vitro* testing. Due to the COVID-19 lockdowns, we were unable to undertake these experiments in the timeframe originally planned. We had to redesign our experiments to incorporate fewer cocktails tested on fewer bacterial strains. Ultimately, the experiments failed, with the median survival of the larvae being three days across all treatment groups. One interesting observation from these experiments was that larvae exposed to the phage cocktail experienced more death than those in the control and that this was a statistically significant difference.

We hypothesised that the reason for these results could have been one of two things. 1) An inadvertent exposure to residual chloroform, which was used to remove bacteria from the phage lysates. 2) The presence of a toxin, which was subsequently discovered in one the genome of one of the phage in the cocktail tested and which contributes to the virulence of the bacteria. Future work should include further testing of these hypotheses. Removal of the toxin to ensure that the phage cocktail does not have a negative impact on bee larvae is crucial.

7.4 A novel technique to increase phage titers

During the discovery of the phages, we noticed that our phages had tiny pin-prick plaques and we were unable to produce high-titer lysates, sometimes only achieving titers as high as 1×10^2 mL^{-1}. We tried several different techniques to increase titers, including webbed plates, liquid infections, large volume infections, and enhanced centrifugation. When these protocols did not work, we modified an established protocol known as the Appelmans Method to evolve our phages. This modified Appelmans method was very successful and resulted in an increase in titer ranging from 100 - 185,000 fold in as little as four days.

To better understand how these phages could have gained beneficial mutations in such a short time, a mathematical model was assembled. The model eloquently showed that it was possible for phages to gain a positive mutation within four days of experimental evolution. A mutation that caused an increase in phage fitness of only 5 fold resulted in as many as 50% of simulations with at least 70% mutant phages.

We also tested the host range of the modified phages and noted an increase in the host range of 11 phages. Remarkably, there was no evidence of host range reduction in any of the phages. As we were unable to extract DNA from the ancestral phages, we could not compare the genomes to establish the reasons for the increase in titer or host range. We hypothesise that a beneficial mutation that could result in phage evading bacterial defence mechanisms or making the phage better at infecting the bacteria could explain this increase in titer.

7.5 Genomic analysis of *95 P. larvae* phages

After we experimentally evolved our phages and extracted quality DNA, we sequenced all 26 phages. We assembled and annotated the genomes and used three different techniques to cluster the New Zealand phages. We also clustered our phages with the 69 *P. larvae* phages

contained in NCBI GenBank. New Zealand phages formed two distinct clusters, with a large, very closely related cluster, and a smaller cluster containing three phages.

Of particular interest in the genomes of two of our phages (Dash and Lilo) is the Plx1 toxin. This toxin has already been identified in four *P. larvae* phage genomes and in the *P. larvae* ERIC I bacterial strain. After further investigation, the toxin was found to be surrounded by repetitive elements, suggesting that the toxin is part of a putative mobile element. This discovery has significant implications for the cocktails tested during the *in-vitro* and *in-vivo* experiments. The two cocktails that were most effective during *in-vitro* testing contained Dash, and one of these cocktails was used during *in-vivo* testing. As previously mentioned, we observed negative effects to bee larvae after the application of phage cocktails and hypothesised that this could be due to the toxin. We repeated some of the *in-vitro* testing and swapped Dash in the two cocktails for Callan (Appendix I). Callan has the same host range and is genetically similar to Dash with the exception of a region of approximately five genes that includes the toxin.

Further investigation and testing is required to establish the key differences between using Callan and Dash. The most obvious explanation would seem to be that the presence of the toxin has some negative effect on the *P. larvae* cells. We consider this to be unlikely because the Plx1 toxin causes apoptosis in Eukaryotic cells and should not have a similar effect on bacterial cells.

7.6 Recommendations for future work

The experiments undertaken in this thesis focused on the discovery and initial testing of phage cocktails on *P. larvae*. There is still a lot of work to be done to get this type of product to market. My first recommendation would be to reformulate phage cocktails based on newly

acquired information regarding the toxin present in some phages. Cocktails could also be reformulated with the expanded host range in mind, ensuring that the breadth of activity is as high as possible and that phages within cocktails do not show antagonistic relationships.

In-vivo experiments would need to be repeated at an appropriate time in the queen bee laying cycle to ensure a higher survival rate of larvae. It would also be prudent to undertake a protocol to remove any protein toxins present in phage lysates. Several of the newly formulated cocktails would need to be tested on a wider range of bacterial isolates. Once the most appropriate cocktail has been formulated and tested, field trials would need to be undertaken to test the cocktails suitability and stability in a natural environment.

7.7 Overall Conclusions

Previous studies and *in-vitro* testing have shown promising results for the prophylactic application of phage cocktails in honeybee hives. The work undertaken in this thesis shows the importance of genome sequencing and *in-vivo* testing of phages to ensure that they are appropriate for use in phage therapy. Further work is needed to formulate and test the most appropriate combination of phages for prophylactic use in New Zealand.

7.8 References

AFBPMP. (2017). *AFBPMP BRIEFING DOCUMENT - MPI Government 01112017*. 1–23.

Alippi, A. M. (2014). Bee diseases (pathogenesis, epidemiology, diagnosis, therapy and prophylaxis). Bacterial diseases of honey bees. *Bee Health and Veterinarians*, 117–124.

Alippi, A. M., López, A. C., Reynaldi, F. J., Grasso, D. H., & Aguilar, O. M. (2007). Evidence for plasmid-mediated tetracycline resistance in Paenibacillus larvae, the causal agent of American Foulbrood (AFB) disease in honeybees. *Veterinary Microbiology*, *125*(3–4), 290–303.

Biosecurity Act 1993 No 95 (as at 01 July 2022), Public Act Contents – New Zealand Legislation. (2022, July 5). New Zealand Parliamentary Counsel Office. https://www.legislation.govt.nz/act/public/1993/0095/latest/DLM314623.html

Brady, T. S., Merrill, B. D., Hilton, J. A., Payne, A. M., Stephenson, M. B., & Hope, S. (2017). Bacteriophages as an alternative to conventional antibiotic use for the prevention or treatment of Paenibacillus larvae in honeybee hives. *Journal of Invertebrate Pathology*, *150*, 94–100.

Ghorbani-Nezami, S., LeBlanc, L., Yost, D. G., & Amy, P. S. (2015). Phage Therapy is Effective in Protecting Honeybee Larvae from American Foulbrood Disease. *Journal of Insect Science* , *15*(1), 84–85.

Hendrix, R. W., Smith, M. C. M., Neil Burns, R., Ford, M. E., & Hatfull, G. F. (1999). Evolutionary relationships among diverse bacteriophages and prophages: All the world's a phage. In *Proceedings of the National Academy of Sciences* (Vol. 96, Issue 5, pp. 2192–2197). https://doi.org/10.1073/pnas.96.5.2192

Ministry for Primary Industries. (2021). *2021 Apiculture Monitoring Data*. https://www.mpi.govt.nz/dmsdocument/48793-2021-Apiculture-monitoring-report-data

Ministry for Primary Industries. (n.d.). *ApiWellbeing*. Retrieved November 29, 2022, from https://www.mpi.govt.nz/biosecurity/how-to-find-report-and-prevent-pests-and-diseases/bee-biosecurity/apiwellbeing/

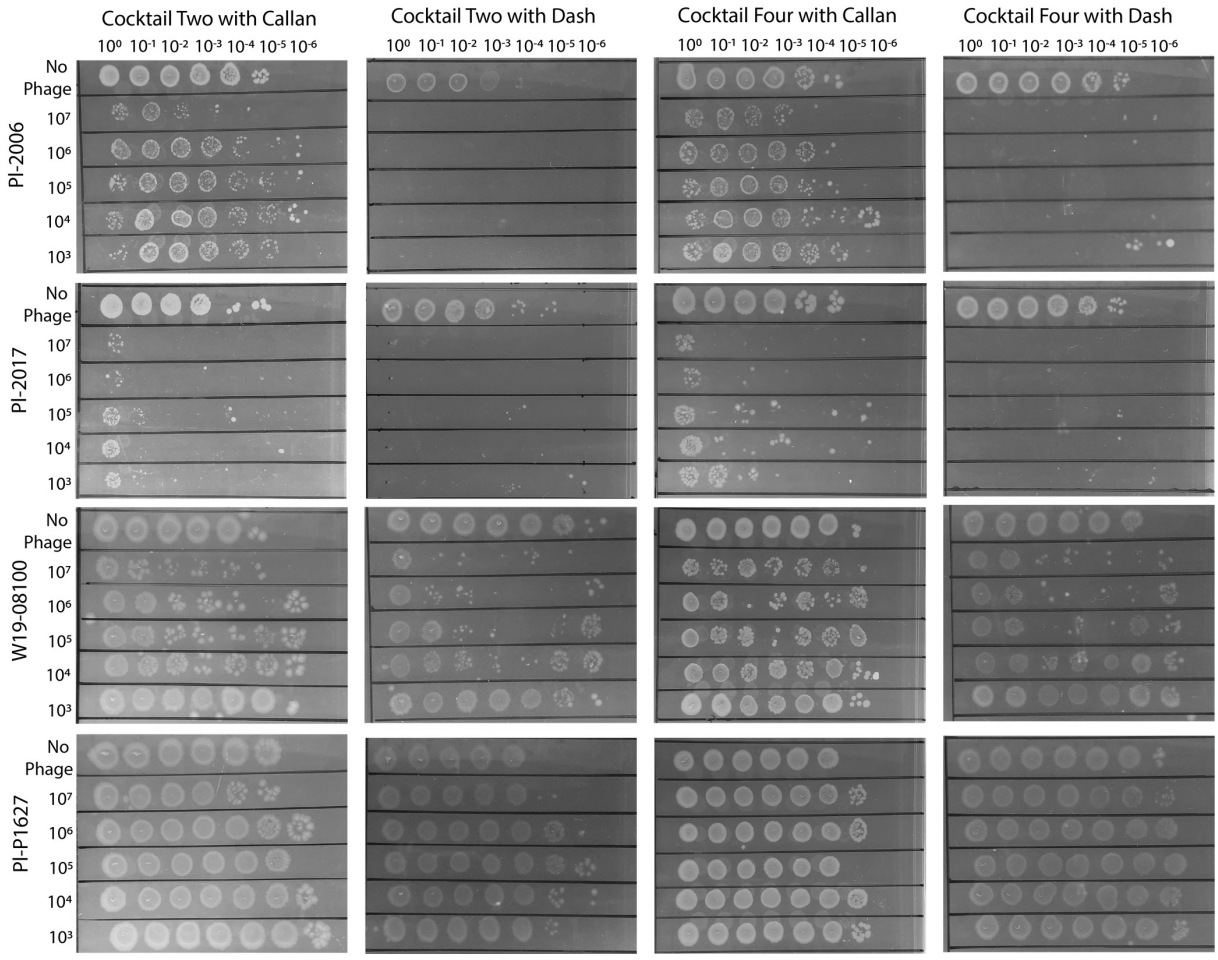
Newstrom-Lloyd, L. E. (2013). Pollination in New Zealand. In J. R. Dymond (Ed.), *Ecosystem services in New Zealand : conditions and trends* (pp. 408–431). Manaaki Whenua Press.

Nimmo-Bell & Associates. (2022). *Cost Benefit Analysis of the National American Foulbrood Pest Management Plan*.

Appendices

I Cocktail Two Redesign

After discovery of the toxin in Dash, we redid the *in-vitro* experiments (as described in Chapter 3). Instead of using Dash, we incorporated Callan into cocktails Two and Four. Unfortunately we saw a reduction in the activity of the cocktails when Callan was used instead of Dash in the three bacterial strains able to be infected. This suggested the success of cocktails Two and Four was linked to Dash and not the other phages contained within these cocktails.



II. Statement of Contribution

DRC 16

MASSEY UNIVERSITY
TE KUNENGA KI PŪREHUROA
UNIVERSITY OF NEW ZEALAND

GRADUATE RESEARCH SCHOOL

STATEMENT OF CONTRIBUTION
DOCTORATE WITH PUBLICATIONS/MANUSCRIPTS

We, the candidate and the candidate's Primary Supervisor, certify that all co-authors have consented to their work being included in the thesis and they have accepted the candidate's contribution as indicated below in the *Statement of Originality*.

Name of candidate:	Danielle Kok
Name/title of Primary Supervisor:	Dr. Heather Hendrickson
In which chapter is the manuscript /published work:	Chapter 2

Please select one of the following three options:

(●) The manuscript/published work is published or in press

- Please provide the full reference of the Research Output:

Kok DN. and Hendrickson HL. (2022) Save our bees: bacteriophages to protect honey bees against the pathogen causing American foulbrood in New Zealand. New Zealand Journal of Zoology

( ) The manuscript is currently under review for publication – please indicate:

- The name of the journal:

- The percentage of the manuscript/published work that was contributed by the candidate: 100%

- Describe the contribution that the candidate has made to the manuscript/published work:

The candidate reviewed the literature and write the manuscript.

( ) It is intended that the manuscript will be published, but it has not yet been submitted to a journal

Candidate's Signature:	Kok, Danielle Digitally signed by Kok, Danielle Date: 2023.03.14 11:44:00 +13'00'
Date:	14.03.2023
Primary Supervisor's Signature:	Heather Hendrickson Digitally signed by Heather Hendrickson Date: 2023.03.14 17:18:56 +13'00'
Date:	14.03.2023

This form should appear at the end of each thesis chapter/section/appendix submitted as a manuscript/ publication or collected as an appendix at the end of the thesis.

GRS Version 5 – 13 December 2019
DRC 19/09/10

MASSEY UNIVERSITY
TE KUNENGA KI PŪREHUROA
UNIVERSITY OF NEW ZEALAND

GRADUATE RESEARCH SCHOOL

STATEMENT OF CONTRIBUTION
DOCTORATE WITH PUBLICATIONS/MANUSCRIPTS

We, the candidate and the candidate's Primary Supervisor, certify that all co-authors have consented to their work being included in the thesis and they have accepted the candidate's contribution as indicated below in the *Statement of Originality*.

Name of candidate:	Danielle Kok
Name/title of Primary Supervisor:	Dr. Heather Hendrickson
In which chapter is the manuscript /published work:	Chapter 3

Please select one of the following three options:

○ The manuscript/published work is published or in press

- Please provide the full reference of the Research Output:

○ The manuscript is currently under review for publication – please indicate:

- The name of the journal:

- The percentage of the manuscript/published work that was contributed by the candidate: 90%

- Describe the contribution that the candidate has made to the manuscript/published work:

The candidate designed and conducted the experiments with assistance from Diana Zhou for the host range experiments, analysed the data and wrote the manuscript.

◉ It is intended that the manuscript will be published, but it has not yet been submitted to a journal

Candidate's Signature:	Kok, Danielle Digitally signed by Kok, Danielle Date: 2023.03.14 11:45:03 +13'00'
Date:	14.03.2023
Primary Supervisor's Signature:	Heather Hendrickson Digitally signed by Heather Hendrickson Date: 2023.03.14 17:18:30 +13'00'
Date:	14.03.2023

This form should appear at the end of each thesis chapter/section/appendix submitted as a manuscript/ publication or collected as an appendix at the end of the thesis.

MASSEY UNIVERSITY
TE KUNENGA KI PŪREHUROA
UNIVERSITY OF NEW ZEALAND

GRADUATE RESEARCH SCHOOL

STATEMENT OF CONTRIBUTION
DOCTORATE WITH PUBLICATIONS/MANUSCRIPTS

We, the candidate and the candidate's Primary Supervisor, certify that all co-authors have consented to their work being included in the thesis and they have accepted the candidate's contribution as indicated below in the *Statement of Originality*.

Name of candidate:	Danielle Kok
Name/title of Primary Supervisor:	Dr. Heather Hendrickson
In which chapter is the manuscript /published work:	Chapter 5

Please select one of the following three options:

○ The manuscript/published work is published or in press

- Please provide the full reference of the Research Output:

◉ The manuscript is currently under review for publication – please indicate:

- The name of the journal:

PHAGE

- The percentage of the manuscript/published work that was contributed by the candidate: 70%

- Describe the contribution that the candidate has made to the manuscript/published work:

The candidate designed and conducted experiments. Jo Turnbull undertook the agar-overlay experiments. Nobuto Takeuchi created the computer simulation. The candidate also performed analyses and wrote the manuscript.

○ It is intended that the manuscript will be published, but it has not yet been submitted to a journal

Candidate's Signature:	Kok, Danielle Digitally signed by Kok, Danielle Date: 2023.03.14 11:46:16 +13'00'
Date:	14.03.2023
Primary Supervisor's Signature:	Heather Hendrickson Digitally signed by Heather Hendrickson Date: 2023.03.14 17:17:58 +13'00'
Date:	14.03.2023

This form should appear at the end of each thesis chapter/section/appendix submitted as a manuscript/ publication or collected as an appendix at the end of the thesis.

MASSEY UNIVERSITY
TE KUNENGA KI PŪREHUROA
UNIVERSITY OF NEW ZEALAND

GRADUATE RESEARCH SCHOOL

STATEMENT OF CONTRIBUTION
DOCTORATE WITH PUBLICATIONS/MANUSCRIPTS

We, the candidate and the candidate's Primary Supervisor, certify that all co-authors have consented to their work being included in the thesis and they have accepted the candidate's contribution as indicated below in the *Statement of Originality*.

Name of candidate:	Danielle Kok
Name/title of Primary Supervisor:	Dr. Heather Hendrickson
In which chapter is the manuscript /published work:	Chapter 6

Please select one of the following three options:

○ The manuscript/published work is published or in press

- Please provide the full reference of the Research Output:

○ The manuscript is currently under review for publication – please indicate:

- The name of the journal:

- The percentage of the manuscript/published work that was contributed by the candidate: 95%

- Describe the contribution that the candidate has made to the manuscript/published work:

The candidate designed and conducted experiments, analysed the data and wrote the manuscript. Sophia Gosselin performed the tANI analysis.

◉ It is intended that the manuscript will be published, but it has not yet been submitted to a journal

Candidate's Signature:	Kok, Danielle Digitally signed by Kok, Danielle Date: 2023.03.14 11:47:00 +13'00'
Date:	14.03.2023
Primary Supervisor's Signature:	Heather Hendrickson Digitally signed by Heather Hendrickson Date: 2023.03.14 17:17:31 +13'00'
Date:	14.03.2023

This form should appear at the end of each thesis chapter/section/appendix submitted as a manuscript/ publication or collected as an appendix at the end of the thesis.