Bacteriophage resistance evolution in a honey bee pathogen

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ABSTRACT
Honey bee (*Apis mellifera*) larvae are susceptible to the bacterial pathogen *Paenibacillus larvae*, which causes severe damage to bee colonies. Antibiotic treatment requires veterinary supervision in the United States, is not used in many parts of the world, perpetuates problems associated with antibiotic resistance, and can necessitate residual testing in bee products. There is interest in using bacteriophages to treat infected colonies (bacteriophage therapy) and several trials are promising. Nevertheless, the safety of using biological agents in the environment must be scrutinized. In this study we analyzed the ability of *P. larvae* to evolve resistance to several different bacteriophages. We found that bacteriophage resistance is rapidly developed in culture but often results in growth defects. Mutations in the bacteriophage-resistant isolates are concentrated in genes encoding potential surface receptors. Testing one of these isolates in bee larvae, we found it to have reduced virulence compared to the parental *P. larvae* strain. We also found that bacteriophages are likely able to counteract resistance evolution. This work suggests that while bacteriophage-resistance may arise, its impact will likely be mitigated by reduced pathogenicity and secondary bacteriophage mutations that overcome resistance.

INTRODUCTION
Managed honey bees (mainly *Apis mellifera)* pollinate around one-third of the world's pollinator-dependent crops, making their health critical to our food security and integral to food prices. In total, managed honey bees provide $182-577 billion USD/year in global crop pollination services (1, 2). Unfortunately, beekeepers regularly loose around one-third of their colonies every year to a combination of disease and other stressors (3, 4). Efforts to prevent these loses and replace colonies are costly. Among the diseases that contribute to the operational costs of beekeepers is American Foulbrood (AFB). AFB is caused by the gram-negative, spore-forming bacteria *Paenibacillus larvae*. This disease stands out as particularly devastating because of its limited treatment options. In the United States, veterinarian-prescribed antibiotics can be used to clear *P. larvae* infections but the emergence of antibiotic-resistant strains of *P. larvae* raises concerns over AFB management (5). Moreover, antibiotics do not kill *P. larvae* spores, which can remain in the hive for decades. In many European Union countries, regulations over antibiotic use and the level of antibiotic residues in bee products curtails their use. As a response to this crisis, researchers have begun to investigate the use of bacteriophages—viruses that specifically

target and infect bacteria—as potential allies in the fight against AFB. These bacterial predators hold immense promise due to their precision, efficacy, and eco-friendly nature (6, 7). Bacteriophages (phages) present an attractive solution to the problem of antibiotic resistance.

Table 1. List of phages used in this study. See (9, 17) for details.

Phage Strain	Accession Number	Clade
Vegas	KT361654	A-A1
Fern	KT361649	B-B1
Willow	KT361650	B-B1 (99% ANI to Fern)
Xenia	KT361652	B-B2
Heath	MH460826	C-C1
Scottie	MH460825	C-C1(99% ANI to Heath)
Unity	MH460824	C-C2

Many phages of *P. larvae* have been isolated [Table 1 and (8–15)]. These phages can kill nearly all known *P. larvae* genotypes in culture (8, 9) and reduce disease burden when tested on bee hives (10, 16). However, all discovered *P. larvae* phages are temperate, a potentially problematic property for their use as therapeutics because of the ability of temperate phages to transfer genetic information between hosts by transduction.

The host range (breadth of susceptible host genotypes) of *P. larvae* phages is very broad—a positive characteristic for their utilization in treating infected hives. Thus, phage cocktails comprised of only a few phages can be designed to treat all or nearly all *P. larvae* genotypes that currently infect *A. mellifera*. The genetic diversity of *P. larvae* genotypes is organized by clustering variants into five subgroups based on enterobacterial repetitive intergenic consensus (ERIC) amplification patterns (18, 19). These subgroups differ in their geographic distribution, prevalence, and infection characteristics. ERIC-1 and 2 are currently are the most widespread and problematic (20–22). In this study, we assessed the evolutionary capacity of an ERIC-1 *P. larvae* isolate NRRL B-3650 to gain resistance against phages that could be used for phage therapy. In addition, we analyzed how the acquisition of resistance to one phage affects an isolate's susceptibility to other phages. These analyses will help us design effective phage cocktails and understand potential problems when using phage therapy to treat infected animals.

RESULTS

Rapid resistance evolution

We used seven different phages (Table 1) to evolve resistance in *P. larvae* strain B-3650 (ERIC I) by infecting lawns of *P. larvae* with phages at an MOI around 5. After 24-48 hours, phage resistant colonies were visible in all seven challenges. We picked 3 to 5 colonies from each phage challenge, confirmed their resistance to the phage initially used, and sequenced the genomes of 26 isolates. In these 26 isolates, a total of 18 unique mutations (11 unique genes) were observed (Table 2, SI Table 1). Eight samples had mutations in the prsA gene, which encodes for the foldase protein PrsA. This membrane-bound lipoprotein assists in the folding of secreted proteins (23). The surface exposed region of this protein would be accessible as a phage receptor. Five unique mutations were observed in this gene; two small deletions, one deletion of 138 bp, an insertion of a single thymine, and an insertion of a transposase at base 563,273. This insertion disrupted the coding sequence of prsA. One only isolate with a prsA mutation had other mutations present in its genome. Four isolates had a mutation in dnaJ, which encodes for a co-chaperone protein that increases the activity of the heatshock protein DnaK (Hsp70) (24).

Table 2. Mutations in phage-resistant *P. larvae* isolates. Whole genome sequencing of phage-resistant isolates show mutations in several genes. *isolates resistant to these phages were used to study growth defects.

Resistant to phage		# isolates	Genes with mutations
99%	**Fern***	3	lepA, mannitol operon activator
	Willow	3	dnaJ, prsA
	Xenia*	3	dnaJ, ylzA, hypothetical protein, intergenic
99%	**Heath**	5	prsA, transcriptional regulator of GlcNAc
	Scottie*	4	prsA, transcriptional regulator of GlcNAc
	Unity*	5	prsA, cwlJ, transcriptional regulator of GlcNAc
	Vegas*	3	gerE, deletion of 5 genes

This protein complex is essential for protein folding and is required for the replication of λ phage in *Escherichia coli* (25). These four isolates all had the same 51 bp deletion near the N-terminal of the dnaK gene. This in-frame deletion resulted in the removal of 17 amino acids. Seven samples had an intergenic mutation (genome position 302,778) in a N-Acetylglucosamine (GlcNAc) biosynthesis gene cluster, just downstream of a predicted transcriptional regulator. GlcNAc is component of the peptidoglycan layer. These genes are essential in peptidoglycan synthesis and recycling. Isolate Fern-r3650-yb had a nonsynonymous mutation in a mannitol operon activator, BglG family CDS. Isolate Xenia-r3650-z had a nonsynonymous mutation in gene ylzA, which is a regulator of extracellular matrix formation in *Bacillus subtilis*. This ylzA mutation was accompanied by mutations in a hypothetical protein and an intergenic region. One Unity-resistant isolate contained a mutation in cwlJ, a gene encoding a spore cortex-lytic enzyme. This enzyme is involved in peptidoglycan remodeling during spore formation. Two isolates that were resistant to phage Vegas contained a large deletion of five genes (MurR/RpiR-family regulator, mutT/nudix-family protein, emrE, acrR-family regulator, and a hypothetical protein). MurR/RpiR is a transcriptional regulator of sugar metabolism, including MurNAc synthesis. MutT/nudix-family proteins are hydrolases with broad functions. *P. larvae* has four genes annotated as mutT/nudix-family CDS. EmrE is likely a transporter that can provide resistance to ethidium bromide and methyl viologen. The acrR-family gene is likely a transcriptional regulator of efflux pump proteins, possibly involved in biofilm signaling and formation. Asides from the MurNAc regulation, which may impact peptidoglycan structure, it is unclear how these mutations would provide resistance from bacteriophages. Apparent phage integration events were detected in all the Fern- and Vegas-resistant isolates. Evidence for this was first noticed in the breseq (see methods) output as new junctions between the B-3650 and either Fern or Vegas phage genomes when breseq was run on a multifasta file containing both genomes. Subsequently, we undertook a careful investigation of potential recombination events by remapping reads and analyzing read coverage. We found elevated (near host depth) coverage for Fern and Vegas genomes only in isolates challenged with Fern or Vegas. Reads mapping to these phage reference genomes matched at 100% identity, suggesting that they were not misaligned. There are regions in B-3650 with high identity to Fern and Vegas. However, in both cases, large regions of the genome with dissimilarity make it possible to distinguish the phage from the temperate phage regions in B-3650. Using the breseq junction coordinates and read mapping coordinates for soft-clipped reads aligned to the junctions, we identified the integration sites. For Fern-challenged isolates, the Fern genome is inserted in B-3650 between bases 1,359,652 and 1,359,687, in the coding sequence for translation elongation factor LepA. These 35 bases are identical to a 35-base region in Fern at genome coordinates 19,500-19,535. This 35 bp region is now duplicated, with copies flanking the now integrated Fern genome. The Fern genome is annotated with a 68 amino acid hypothetical protein in this location, which is directly 5' to Fern's integrase gene. The Vegas-resistant isolates

now have a phage integrated between genome positions 1,285,348 and 1,285,358. These 9 bases are now duplicated, flanking the now integrated Vegas genome, which was disrupted at base 38,418. Integration occurred in the major transcriptional regulator of spore coat formation GerE gene in B-3650 and an intergenic region in Vegas. We aligned reads to these new sequences and in both cases, read coverage is even with no breaks, suggesting that these new junctions are correct. No breaks in coverage or soft-clipped reads were found in any isolates other than those challenged with Fern and Vegas.

Growth defects in resistant isolates

We compared the growth rate of 14 of the phage-resistant isolates of *P. larvae* to the parental B-3650 strain and found that most of the isolates had growth defects (Fig 1). Lag time, doubling time, and maximum density were calculated and used as response variables. All phages except for Xenia caused a significant reduction in maximum density ($p<0.05$, linear regression). At the level of individual isolates, maximum density differed from the B-3650 for 7 of 14 isolates ($p<0.05$, linear regression). One isolate (Xenia-resistant isolate Bb) had an increase in maximum density compared to B-3650. All other significantly different isolates (Fern resistant isolate yb, Scottie resistant isolate b, Unity resistant isolate y, Unity resistant isolate z, Vegas resistant isolate x, and Vegas resistant isolate y) had reduced maximum growth density. In all cases, there was variation between isolates within an exposure group—some isolates grew significantly different than the ancestor while some did not, even if they were all evolved on the same phage. These differences suggest that the outcome of resistance evolution can be highly variable, which could impact the competitiveness of phage-resistant isolates in the bee larvae gut microbiome.

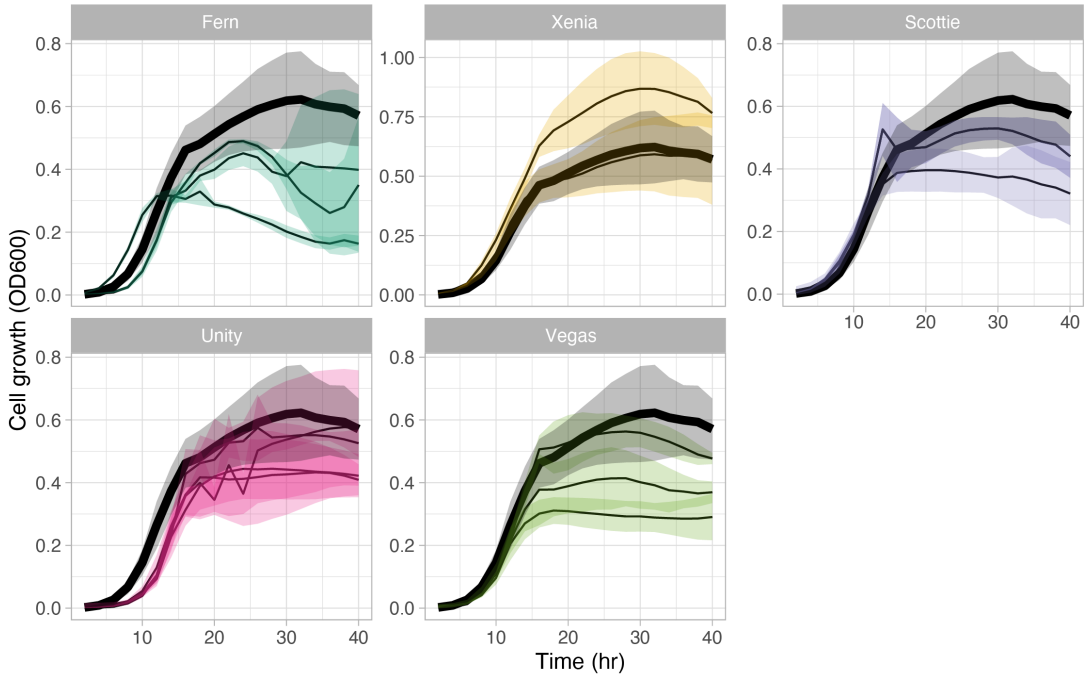

Figure 1. Phage-resistant variants have growth defects. Growth curves of phage-resistant isolates (thin black lines) compared to parental strain B-3650 (thick black line). The standard deviation for at least three replicates is shown by the shaded region.

While verifying the resistance of phage-resistance colonies, we discovered plaques on 18 of the 26 phage-resistant isolates, suggesting that either resistance is partial or a portion of the phage population is able to overcome resistance. We further investigated this by measuring

the relative efficiency of plating (EOP) of these phages on B-3650 and phage-resistant isolates. EOP is a commonly used phenotype that is calculated by measuring the number of viruses that can form plaques. In our case, we compared how many plaques form on the ancestor versus resistant hosts. The mean EOP of the initial phage lysates on resistant hosts was 4.5E-5 $\pm$ 8.4E-5 (Fig 2). We were unsure if the plaques on resistant hosts were genetic variants with the ability to plaque on resistant hosts or if resistance was incomplete, allowing some phage growth. To investigate this, we picked one plaque off every phage-resistant strain and measure EOP of these picked plaques (titer on resistant host / titer on B-3650). The mean EOP of these plaques was 9.6 $\pm$ 24.5 (Fig 2). The minimum EOP for these "survivor" phages was 0.4, meaning that just under half of the phages could plaque on their resistant host after just one round of selection. The EOP for this phage before this round of selection was 6.9E-8, meaning that only about 1 in every 100,000,000 phages formed plaques on the original host. Therefore, we conclude that rare genetic variants likely exist in the original phage stock that facilitate growth on evolved hosts. These "survivor" phages grew slightly worse on the parental host, showing that there is a trade-off associated with good growth on phage-resistant hosts.

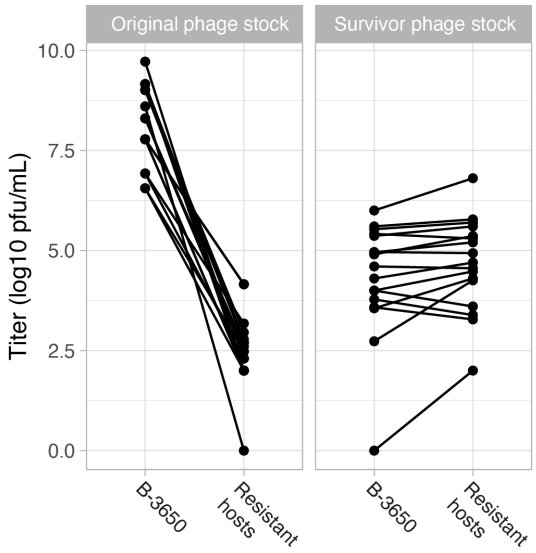

Figure 2. A small subset of the phages could overcome resistant bacteria. A fraction (18/28) of the phages types could form plaques on resistant hosts with very reduced plating efficiency (left panel). When cultivated on resistant hosts, these "survivor" phage isolates had higher plating efficiency on the resistant hosts than the parental host (right panel).

Resistance changes susceptibility to other phages

To understand how the evolution of resistance will impact treatment with phage cocktails, we characterized the susceptibility of 27 phage-resistant bacterial isolates to the seven phages used in this study. There were no instances of *P. larvae* evolving resistance to only the phage that it was exposed to (Fig 3). Resistant isolates usually showed resistance to at least one other phage, often, closely related phages. Isolates resistant to Heath, Scottie, or Unity (phages from one cluster) almost always (14/15 isolates) showed some resistance to the other two phages in the cluster. However, many of these isolates were at least partially susceptible to the other two phages. One isolate that evolved resistance to Scottie was completely susceptible to Unity. More variation in cross-resistance patterns occurred in hosts that evolved resistance to phages the cluster of Fern-like phages (Fern, Willow, Xenia) (17). Phages Fern and Willow, which have 99% average nucleotide identity (ANI), usually infected the same host isolates, with three exceptions. The most distantly related phage in the Fern cluster (Xenia, 67% ANI) could usually infect Fern and Willow resistant hosts. The opposite was not true. Hosts that evolved resistance to Xenia usually also gained resistance to Fern and Willow. Xenia also differed from Fern and Willow in its ability to infect other phage-resistant isolates. For example, Vegas-resistant hosts were resistant to Xenia, but not Willow and Fern. There were many exceptions to this expected outcome. For example, hosts resistant to phage Xenia could also resist infections from phages Fern and Willow, which have around 67% nucleotide sequence identity to Xenia. While the general pattern is that resistance evolution includes closely related phages, there were many sporadic instances of cross-resistance. For example, Heath-resistant isolate B was resistant to closely related phages Scottie and Unity as expected, but also to phages Fern and Willow,

which are distantly related. Isolates that evolved resistance to phage Vegas gained broad resistance to many of the phages. Interestingly, all three Vegas-resistant isolates were resistant to phages Xenia and Heath, which are from different phylogenetic clusters.

For hosts that gained resistance to phages other than the challenge phage, we measured the EOP of the starting phage stock on all partially resistant, permissive hosts. We found that when resistance is only partial, the plating efficiency of the challenge phage (Fig 2) and other phages is greatly reduced (Fig 3). However, resistance in the form of reduced plating efficiency is equivalent for phages closely related to the one used as a selective pressure for evolving resistant hosts (p=0.632, Shapiro-Wilk normality test). This is easily visualized in Fig 3 by comparing the transparency of dots in each row to the dot in the box. The transparency of dots in the box are very similar to dots outside the box. We also wanted to see if resistance could be as easily overcome by these related phages as it is by the challenge phage. Following what we did to measure the EOP for "survivor" phages (Fig 2, right panel), we titered one survivor phage of each type that would plaque on every resistant. We expected that these phages might overcome host resistance more readily than the phage that the host evolved resistance to. The EOP relative to strain B-3650 was greatly increased for all survivor phages, even when they are picked from a host that evolved resistance to a different phage.

Figure 3. Phage resistance evolution protects hosts against closely-related phages. We tested the susceptibility of phage-resistant isolates (y-axis) to seven different phages. Bacterial grow (resistance) is shown by the colored circles, with the shading indicating the level of resistance. The absence of a circle means that the isolate was killed by the phage named along the top (x-axis) label. The boxes indicate the results of testing a host isolate against the phage that was used to evolve resistance. These should all be filled in circles, but there are some exceptions. Nucleotide sequence identity between phages is shown along the top. *Indicates subtle differences in host range among the three methods used.

Reduced virulence in bee larvae

To determine if the fitness effects of phage resistance that we observed in culture are relevant to bees, we performed a small larvae infection trial with four treatments. Newly hatch larvae were grafted into queen cell cups treated with phage Fern, phage Fern + *P. larvae* strain B-3650, *P. larvae* strain B-3650 alone, or Fern-resistant *P. larvae* strain B-3650 (isolate yB). This mutant has one non-synonymous mutation (F109I) in a mannitol operon activator gene. Because phage Fern has been tested for its ability to protect larvae from *P. larvae* in the past (see (26)), we only did one replicate (48 larvae) with these treatments. The other treatments were replicated at least twice with 12-32 larvae. Nearly all the mock-infected larvae survived. Larvae infected with *P. larvae* and treated with Fern phage survived at ~75%, which is less than control (p=5E-5, Log-Rank test with BH correction). Larvae infected with *P. larvae* strain B-3650 had the worst survival, with only 3% of them surviving 3 days post infection. 18% of the larvae infected with Fern-resistant strain B-3650 survived, which is better than the wildtype B-3650 infections (p=0.001, Log-Rank test with BH correction).

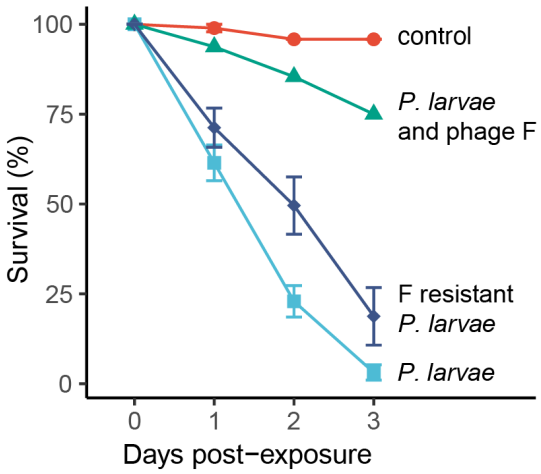

Figure 4. A phage resistant *P. larvae* isolate is less virulent than susceptible *P. larvae*. Recently hatched larvae were reared with diets containing *P. larvae* B-3650, F-resistant *P. larvae* B-3650, *P. larvae* B-3650 and Fern phage, or media only. 12, 16, or 48 larvae were used in replicate experiments. Standard error bars are show. No replicates were performed for the *P. larvae* and phage Fern treatment (n=48 larvae).

Identification of anti-phage systems

In addition to evolving mutations as a resistance strategy, bacterial hosts are armed with an array of defense mechanisms that protect against plasmid replication and predation by bacteriophages. An analysis of the parental strain B-3650 for host defense mechanisms was conducted using DefenseFinder (27). Eight individual defense systems wre identified: Wadjet III, Mokosh Type II, Gao let, Restriction-Modification (RM) Type I and II, CRISPR-Cas Class I subtypes I and III, and MazEF systems. These systems encompass various resistance strategies such as plasmid resistance via circular DNA cleavage (Wadjet III), recognition and cleavage of foreign DNA (RM Type I and II), RNA helicase and nuclease activity targeting phage RNA (Mokosh Type II), programmed cell death toxin and antitoxin (MazEF), and ATPase and protease-mediated mechanisms (Gao let).

DISCUSSION

Using bacteriophages to treat bacterial infections (phage therapy) has remained a promising approach for over a hundred years. However, much about the basic biology of phage-bacteria interactions in animal microbiomes needs to be understood to overcome understandable doubt in using biological entities as drugs to treat infections. Two key questions are addressed in this work; how does phage resistance evolve and how does that resistance alter pathogenicity? Honey bees offer a useful system to study phage-host dynamics in animal microbiomes because of the tractability of working with them in the laboratory environment. Honey bees are susceptible to two bacterial infections that can be treated with antibiotics (usually Terramycin or

Tylosin). However, antibiotics are not always easily accessible, they promote the evolution of resistance, and can require testing for residuals in bee products. The primary goal of this work was to determine if resistance to bacteriophages can arise through evolutionary changes in *Paenibacillus larvae*, the pathogen that causes American Foulbrood. In addition, this work addresses an important issue in phage therapy more broadly—characterizing the side effects of phage resistance evolution.

Phage resistance readily arose in an ERIC-I strain of *P. larvae*, a group of pathogens commonly observed in honey bee colonies (20). Even though *P. larvae* has several anti-phage systems (28), it is susceptible to many previously isolated phages. When we challenged *P. larvae* with seven different phages, resistant isolates were observable within two days. This type of experimental evolution has been done with many phage-host combinations and is commonly used to identify potential phage receptors (29–33). Each of the 26 resistant isolates that were sequenced had between one and three mutations compared to the ancestor, but only 18 unique mutations were found. Many (9/18) of these mutations were indels, one being a large deletion of five genes. The annotation of many of the affected genes suggest that the mutations have some plausible role in phage receptor presentation; either the genes encode surface proteins or they are involved in sugar or peptidoglycan biosynthesis. It is worth mentioning that all 48 *P. larvae* phages sequenced to date contain a N-acetylmuramoyl-L-alanine amidase (15). These endolysins are likely involved in the lysis of host cells. Thus, is it conceivable that the peptidoglycan mutations we observed somehow alter the cell wall structure, preventing lysis rather than entry. The three phylogenetic groups of phages that we worked with had unique sets of mutations within each group. The Fern cluster (Fern, Willow, Xenia) had mutations impacting dnaJ, a mannitol operon activator, and set of three mutations (hypo, ylzA, intergenic). One isolate (Willow resistant isolate z) had a mutation in prsA. However, we are inclined to think that this isolate may have been inadvertently switched because the cross-resistance patterns changed for this isolate after the initial cross-streaking test. The cross-resistance spot plating for this Willow isolate are identical to the two of the Heath isolates. We have no way to confirm this suspicion. The cluster containing Heath, Scottie, and Unity were impacted by five different mutations impacting prsA. Isolates without prsA mutations had a mutation in a transcriptional regulator of N-acetylgalactosamine. It is unclear if the impact of this mutation affects metabolism or cell surface sugar molecule presentation because the genes under control of this regulator region could impact both (34, 35). The conversion of B-3650 into lysogens by Fern and Vegas likely provided resistance against these phages and provides support for the careful use of temperate phages in phage therapy. It is worth noting that B-3650 has large genome regions almost identical to Fern and Vegas, thus these phages would the best choices for treating *P. larvae* infections in honey bee colonies. It is unclear how if the other mutations also contribute to resistance.

Phage cross-resistance was largely clustered by phylogenic relatedness of the challenge phages. Evolving resistance to one phage often confers resistance to phages in the same phylogenetic cluster. There are some interesting exceptions to this rule that are not easily reconciled with the dogma that phage are generally highly host specific. Hosts that evolved resistance to Vegas acquired broad protection from phages in all three clusters. Isolates that evolved resistance to Willow and Xenia were also commonly resistant to Vegas. No resistance mutations were found in common between Vegas and these other two phages, so it is unclear how resistance against Vegas is gained. Several instances of asymmetric resistance acquisition was observed. For example, isolates that gained resistance to Willow were not resistant to Xenia, but those resistant to Xenia were resistant to Willow. Isolates resistant to Vegas were not resistant to Willow, but Willow-resistant isolates were resistant to Vegas. The Scottie/Heath/Unity-resistant isolates were not resistant to Xenia, but Xenia-resistant isolates

gained partial resistance to these three phages. Others have also reported asymmetric cross-resistance. Among 263 phage resistant isolates of *P. aeruginosa*, Wright et al. (2018) found isolates to be cross-resistant to 10-80% of the other 27 phages used in the study (36). Cross-resistance is also common when phage receptors are transporters involved in antibiotic resistance (37–39). Gao et al. (2022) reported both asymmetric cross-resistance and somewhat sporadic patterns of cross-resistance in Salmonella enterica (40). Another surprising result is that cross-resistance is strong against the non-challenge phage. We expected to see more effective resistance to the phage that was used during evolution than cross-resistant phages but observed the same EOP values for challenge and cross-resistant phages (p=0.632, Shapiro-Wilk normality test). These results suggest that even when combinations of phylogenetically divergent phages are used to treat bacterial infections, there is some likelihood that universal resistance will evolve.

In this study we found evidence that phage resistance can come at a cost in terms of growth rate and pathogenicity. However, like other studies have found (41, 42), these costs are not universal—we observed growth effects in culture for only half of the isolates that we tested. The one isolate that we tested in bee larvae was significantly slower at killing larvae than B-3650, but more isolates should be tested, particularly those that do not have reduced maximum density in culture. Many other studies have found that evolution to resist phage infection is accompanied by a fitness costs, particularly in the context of a natural microbial community (reviewed in (43)) and thus phage therapy is unlikely to result in widespread resistance against phages (44). However, resistance evolution may likely change the microbial community dynamics as phage resistant and phage susceptible subpopulations compete in the changing ecosystem of an animal digestive tract (or other environment). Phages that have been preadapted (e.g., “trained”) to overcome bacterial resistance have been shown to reduce the emergence and rise of phage resistant genotypes (45, 46). I our case, phages that plaqued on phage-resistant isolates grew worse on the starting, non-resistant host. This results suggests that perhaps a cocktail of preadapted and non-preadapted phages may best work to treat infections.

METHODS

Microbiology – P. larvae strain NRRL B-3650 (10.60712/SI-ID10709.1) and all eight phages were obtained from Dr. Penny Amy (University of Nevada Las Vegas). B-3650 was resequenced to confirm its identity. One single nucleotide polymorphism and one single base indel compared to CP019651 were identified. Glycerol stocks of B-3650 was streaked on Brain Heart Infusion (BHI) agar plates for the isolation of a single genotype (2X re-streaks). A single colony was cultured in 3 mL BHI liquid media for ~30 hrs to an OD600 of ~0.7. Glycerol stocks of the eight phages were revived by mixing freezer stock with 100 μL of OD600 ~0.7 cells in warm mBHI top agar (BHI, 1mM CaCl2, 1 mM MgCl2, 0.7% agar). A single colony was picked and amplified to high titer overnight in 3 mL of mBHI with B-3650. New phage stocks were generated by pelleting cellular debris from spent cultures (1000 x g) then chloroform treating the supernatant (50 μL of chloroform added to 500 μL supernatant). Residual chloroform was removed by centrifuging for 4 minutes at 13000 x g and pipetting off the aqueous layer. These stocks were titered and stored at 4° C for immediate use. For the resistance evolution experiment, 100 μL of OD600 ~0.7 cells were well-mixed with phage stocks at an MOI of 5 in mBHI top agar (0.7% agar). Plates were incubated for 24 h at 37° C in 5% CO_2. Cleared plates with phage-resistant colonies were visible between 24-72 h. To confirm resistance, individual colonies were picked, grown overnight in mBHI broth, Cross-resistance testing was performed by streaking overnight cultures of B-3650 ancestor and phage-resistant isolates on mBHI agar

plates across phage stock that was dibbled perpendicular to the colony streak (for example see Fig SI 1). 20-50 μL of phage stock was dribbled across the agar plate and allowed to soak into the agar for ~2 minutes. A 10 μL loop-full of overnight culture from putatively resistant hosts was streaked perpendicular to the phage. The B-3650 ancestor was always included as a control. This same cross-streak method was used to determine cross resistance shown in Fig 3. Two additional tests were used for cross-resistance testing. First, spot plating 5 μL of phage stock on lawns of B-3650 and its derivative phage-resistant isolates. Phage stock titers for the spot plating are shown in SI Table 2. Lastly, phage stocks were tittered on every host using the top-agar method described above. The full matrix of titers (including survivor phage isolates) on every host isolate are provided in SI Table 3. The growth rates of phage-resistant isolates of *P. larvae* were measured in 24-well shaking plate assays in an incubating plate reader (BioTek® Instrument, Inc. USA). Control wells included BHI broth control, bacteria control, and phage control. The plates were incubated while shaking for 48 hours with the reading of optical density (OD600) recorded from each well at the interval of every 15 minutes (SI Table 4).

Sequencing and analysis – Overnight cultures of phage-resistant isolates were harvested by centrifugation and resuspension in lysis buffer followed by DNA isolation (ZymoBIOMICS). Illumina sequencing (150 bp PE) was performed at SeqCoast Genomics (Portsmouth, NH). Illumina reads were quality filtered and trimmed using fastp (v0.23.4). Nanopore reads were obtained for the starting P. *larvae* strain (B-3650) using a Minion device (r10 chemistry). This genome was assembled using Unicycler and annotated with RAST. Illumina reads from resistant isolates were mapped to this new reference using BREseq v0.38.1. Custom R scripts (available on Github) were used for data processing and plotting. Additional mapping was performed using bwa mem (0.7.18-r1243-dirty) and visualized in Tablet (v1.21.02.08).

Larval grafting – Age-matched 1-3 day old larvae were transported from our apiary to our laboratory in a pre-heated foam nuc box containing a bottle of hot water and were covered with a damp paper towel. Within 30-60 minutes from being removed from the hive, larvae were grafted using a Chinese grafting tool into brown queen cups in 48 well tissue culture plates containing 20 µL of preheated artificial food “A” (44.25% royal jelly, 5.3% glucose, 5.3% fructose, 0.9% yeast extract, 44.25% water). Larvae were incubated for 48 hours, then 20 µL of artificial food “B” (42.95% royal jelly, 6.4% glucose, 6.4% fructose, 1.3% yeast extract, 42.95% water) was added to cells. Plates were photographed daily and death was measured by observing larval discoloration and the absence of diet consumption. Larvae were reared according to (47). Temperature and humidity were monitored constantly and remained at 35° C and 90-100% humidity. On day zero, 10, 50, 100, or 1000 colony forming units in 1 µL of mBHI growth media was added to the artificial food. Larval survival was compared in R using the “survival” and “survminer” packages. The Cox proportional hazards regression model was fit according to the following formula, (days post infection, survival) ~ treatment. P values were calculated for all pairwise comparisons using Log-Rank tests with Benjamini-Hochberg multiple test correction.

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DATA AVAILABILITY

Analysis scripts and processed data were published on Github at https://github.com/jtvanleuven/plarvae_resistance. Raw sequencing reads were deposited at NCBI under BioProject number PRJNA1130131.

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