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1 # Viral adaptations to alternative hosts in the
2 # honey bee pathogen *Paenibacillus larvae*
3

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14
15 ## ABSTRACT

16 Bacteriophages (phages) that are intended to be used to treat bacterial infections are often
17 improved using genetic engineering or experimental evolution. A protocol called "Appelmans"
18 utilizes evolution in microtiter plates to promote the evolution of phages that can infect
19 nonpermissive hosts. We tested a modification of the Appelmans protocol using the honey bee
20 pathogen, *Paenibacillus larvae*. Three phages evolved together on four *P. larvae* strains
21 following the standard Appelmans protocol and a modified version to ensure high phage
22 diversity throughout ten rounds of passaging. The host range of 360 plaques were characterized
23 and six new phage lysis patterns were identified. These new phage lysis patterns included
24 plaque formation on previously nonpermissive, phage-resistant isolates that were used to
25 identify phage types. The modified protocol did not drastically change the rate or number of new
26 phage types observed but did prevent the phage population from being dominated by one
27 phage that tended to rapidly raise in frequency. These findings showed how a minor
28 modification of the Appelmans protocol influenced the development of phages for phage
29 therapy. The method also provided improved phages for the treatment of bacterial infections in
30 honey bees.
31
32 ## INTRODUCTION

33 Honey bees are considered the most important and effective pollinators in agriculture [1,2]. The
34 pollination performed by managed honey bee hives is essential to meet food production
35 requirements for humans [3]. American Foulbrood Disease (AFB) decreases honey bee (*Apis*
36 *mellifera*) populations, causing economic loss and potential food vulnerabilities. AFB is caused
37 by the bacteria *Paenibacillus larvae* which infects and kills the honey bee larvae of a hive [4–7].

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38 *P. larvae* is a spore forming, gram-positive, rod-shaped bacterium belonging to the class Bacilli.
39 Honey bee larvae become infected with spores when they ingest contaminated food, followed
40 by spore germination, proliferation of bacterial cells, and production of toxins that break down
41 the epithelial lining of the larval digestive tract [8–10].

42 Treatment options for AFB are minimal and destructive, making it difficult to manage.
43 Antibiotics are generally used only by commercial beekeepers in the US and are banned in
44 most European countries. However, using antibiotics is undesirable in most cases because it
45 contributes to the spread of antibiotic resistance [11–15] and necessitates additional testing
46 before human consumption of treated bee products [16]. Currently, the only other treatment
47 available for AFB is extremely destructive and focuses on curtailing spore spread by burning all
48 affected equipment, hives, and bees. Bacteriophages (phages) might be a less destructive
49 solution to treat AFB infections [17–19]. Using phages to kill pathogens—a process called
50 phage therapy—can be safe and effective. Phages can target pathogenic bacteria without
51 harming the natural microbiota. Studies using phage therapy methods to treat *P. larvae*
52 infections have gained recent interest. Currently, there are around 50 characterized *P. larvae*-
53 specific phages [20–25]. Further studies are needed to expand the *P. larvae*-specific phage
54 bank and improve phage therapy efficacy against AFB. The specificity that makes phages ideal
55 at minimizing off-target effects also makes a single phage unlikely to kill all of the strains of a
56 pathogen in circulation. *P. larvae* diversity is currently organized into five phylogenetic groups –
57 ERIC types I-V [26] and no one *P. larvae* phage can infect all types. ERIC types I and II are
58 most commonly isolated, whereas types III and IV have not been isolated for years. Laboratory
59 evolution is commonly used to improve phages. A method that has gained recent interest is
60 called Appelmans protocol (Fig 1) [27]. The primary goal of this method is to adapt phages to
61 new hosts, expanding the host range of a phage or phage cocktail. It has also been used to
62 increase phage titers [28]. These evolutions are generally conducted on microtiter plates where
63 a dilution series of phages are mixed with different hosts and incubated for a set time period
64 (typically until the well appears clear, which indicates complete lysis). Cleared wells are
65 combined and used to start a new round of evolution. Combining phages that grew on different
66 hosts provides the opportunity for recombination to occur between phages, promoting evolution
67 to new hosts. There is however the possibility for one phage to rapidly take over the entire
68 phage population or for one phage in the cocktail to be outcompeted and dropout of the
69 population. Both scenarios reduce the phage diversity. As the standard Appelmans protocol
70 calls for combining all the cleared dilution wells for each host, if one phage grows very well, it
71 can quickly become the numerically dominant phage in the pool. To avoid this, we tested a

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72 modification to the Appelmans
73 protocol, where the original
74 phage cocktail is added to the
75 evolved cocktail between each
76 round of evolution. Our phage
77 cocktail consisted of three
78 previously characterized phages
79 of *P. larvae* (Table 1). These
80 phages are all temperate and
81 favor a lytic lifestyle [17,20,21].
82 Phage Fern and Scottie are
83 known to infect all but one of the
84 host species that we used
85 (Table 2). Recent evidence from
86 a study using *P. larvae* strain
87 3650 suggests that phage Fern
88 may integrate into the host
89 genome [29]. Phage-resistance
90 via genetic mutations can also
91 arise in *P. larvae* strain 3650
92 [29]. Here, we used three
93 phage-resistant strains previously generated by our lab that were resistant to each of the three
94 starting phages to help distinguish parental lysis patterns from phages with new lysis patterns
95 that evolved during Appelmans.

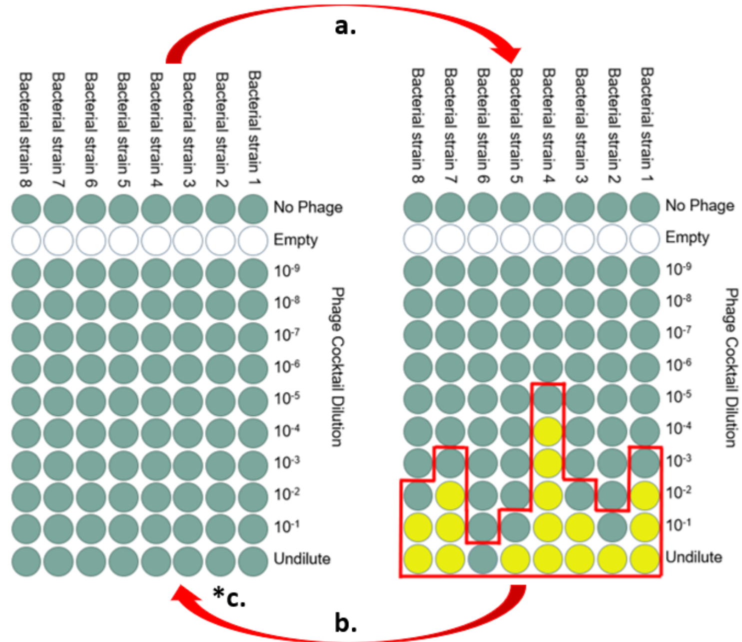

Figure 1. Standard Appelmans protocol to evolve phages on multiple bacterial strains. Each column of the 96-well microtiter plate contains a 200 µL culture of a single bacterial strain. Phages are added in ten-fold dilutions across the rows. (a) Overnight growth results in cleared wells (yellow) and turbid wells (green); (b) the next round of growth is started with a pool (red outline) of successful phages (the cleared wells) and the next turbid well from all columns; (*c) our modification: add 1:10 titer-based ratio of the starting phage cocktail to the pooled cocktail to start the next growth round.

Table 1. The three *P. larvae* phages used in this experiment. Average nucleotide identity (ANI) between the three phages likely impacts the frequency of phage recombination.

Phage Strain	Accession Number	ANI to Fern	ANI to Scottie	ANI to Xenia
Fern	NC_028851	100%	-	-
Scottie	NC_055030	50%	100%	-
Xenia	NC_028837	75%	50%	100%

96
97
98

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Table 2. *P. larvae* strains used for Appelmans phage evolution.

***P. larvae* Strain**	**Strain identifier**	**ERIC Type**	**Phage Susceptibility**
3650	NRRL B-3650	I	Fern, Scottie, Xenia
2605	ATCC-9545	I	Fern, Scottie, Xenia
25747	ATCC-25747	I	Fern, Scottie
368	ATCC-25368	IV	none

RESULTS

Host range expansion

Over the course of ten rounds of passaging we randomly picked 18 plaques per time point for both the regular and modified protocols (18 plaques, 10 time points, 2 treatments = 360 total plaques). Only six new host range patterns were observed among these 360 plaques. On round three of passaging, we observed clearing of host 368 at the highest dilution of phage. This change was interpreted as host range expansion as the concentration of phage stock used to start each round of Appelmans was kept constant at 1E6 plaque forming units per mL (pfu/mL). However, no plaques were observed to form on host 368. Moreover, lysis of 368 was only observed in the well containing the most concentrated dilution of phage, suggesting that productive infection was not occurring, but rather a phenomenon called lysis from without. This indicates that if a phage had evolved to use 368 as a host, it did not improve over the ten

Table 3. Host lysis matrix showing the three initial phages' (Fern, Scottie, Xenia) ability to infect and kill the seven *P. larvae* strains used during evolution (top). Post evolution host lysis matrix showing the six new phage lysis patterns isolated during Appelmans (bottom 6 rows). Grey indicates host lysis, white indicates no host lysis, and "halo" indicates cell death without plaque formation.

Phage strain	*P. larvae* strains				B-3650 phage-resistant isolates		
	3650	**25747**	**2605**	**368**	**Fern-yB**	**Scot-B**	**Xenia-Ba**
Fern							
Scottie							
Xenia							
New 6							
New 8				Halo			
New 9				Halo			Halo
New 10							
New 11							
New 12							

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111 rounds of evolution. Two phage isolates ("New 8" and "New 9") created halos on host 368
112 (Table 3) but did not form plaques. Thus, a phage that can kill, likely by lysis from without, but
113 not replicate on host 368 arose but stayed at relatively low frequency during the experiment. We
114 observed true host range expansion of phage onto the phage-resistant isolates that we were
115 using to identify phage types. A phage-resistant strain of 3650 was developed for each of the
116 three phages used in the Appelmans protocol [29]. Inclusion of these phage resistant strains in
117 the evolution experiment expands our ability to differentiate unique phage lysis patterns. One
118 phage ("New 6") could infect all the hosts except 368. The standard Appelmans protocol in
119 fewer new patterns than our modified protocol. The modified protocol resulted in five of the six
120 new phage lysis patterns, with two patterns unique to this experiment (Fig. 2). The standard
121 protocol had one new lysis pattern, New 9, unique to the experiment, however it was one of the
122 phages unable to amplify on 368 and XIIIβa, so it is only differentiated from phage Fern by its
123 ability to kill but not form plaques on these two hosts.
124
125 **Timing and frequency of new phage types**

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126 For each of the ten rounds of passaging in the control and modified protocols, 18 plaques were isolated, amplified on 3650, and identified based on host-range patterns. New phage lysis patterns were isolated 22 times in the modified protocol experiment compared to the standard protocol, with 15 isolations (Fig 2). The first new phage lysis pattern was isolated in round two of the standard method and new lysis patterns were seen at a relatively consistent rate until the end of 10 rounds of our modified Appelmans protocol (Fig. 3). There were no major differences between the control and modified Appelmans protocols in terms of the rate that new types of phages arose. The new phage types made up a relatively higher proportion of the population in the modified protocol experiment compared to the standard protocol (Fig 4). The modified protocol also maintained higher phage diversity. Whereas phage Xenia was completely lost in the standard protocol experiment, it was present throughout the rounds of evolution in the modified protocol experiment. In both protocols, phage Fern was numerically dominant in the phage population. This highlights one concern of the standard Appelmans protocol. One should pay attention to the relative growth rates and number of permissive hosts for each phage going into Appelmans. In our case, we started with an equal mix of

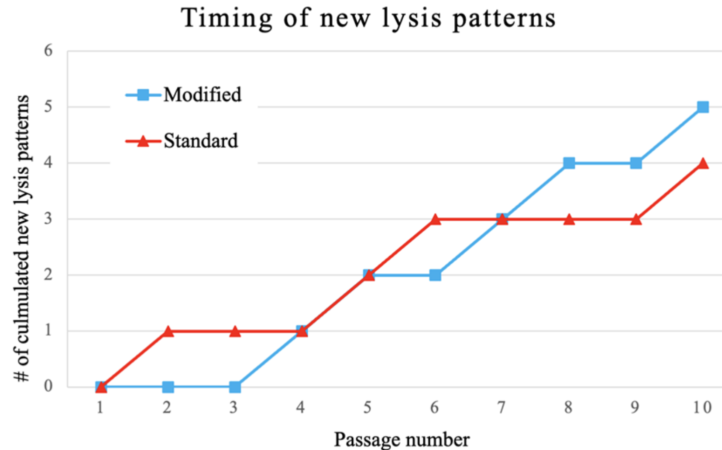

Figure 3. Accumulation of new phage lysis patterns from 360 picked plaques. Host range patterns for each plaque identified by plating on the seven hosts in Table 3.

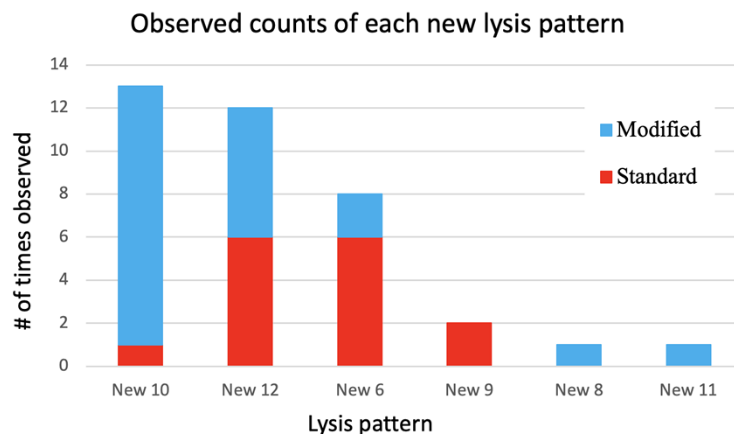

Figure 2. Number of times each new phage lysis pattern was isolated from the standard and modified protocols throughout 10 rounds of Appelmans evolution. These numbers were derived from the 18 randomly selected plaques from each round of evolution (360 total between both experiments).

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160 phage Fern, Scottie, and Xenia. It would be interesting to see the outcome if this starting mix
161 would have been included a much lower titer of Fern.
162
163 **Dynamic phage population**

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164 The Appelmans protocol
165 calls for pooling of all the
166 cleared wells (indicative of
167 lysis) plus the next turbid
168 well (bacteria still growing
169 with the more dilute phage
170 cocktail present) for each
171 host. We measured the
172 phage titer of these lysates
173 in each round and then
174 always started the next
175 round with 1E6 pfu/mL. The
176 titer of the phage population
177 on host 3650 did not change
178 drastically over the ten
179 rounds of evolution, always
180 staying between 5E6 and
181 5E7 pfu/mL. However, we
182 noticed drastic differences
183 between hosts in the dilution
184 of phage that resulted in
185 cleared wells. Comparing
186 the minimum number of
187 phages required to clear a
188 well of each host across the experiment shows these dynamics (Fig. 5). For susceptible hosts, it
189 generally took around one phage to result in a cleared well. That is to say that wells containing
190 susceptible hosts and that we expect to have at least some phage in them, based on the
191 starting titer of 1E6, generally become clear. A decrease in the number of phages it took to clear
192 a well indicated that the phage cocktail increased in efficiency of killing previously unsuceptible
193 *P. larvae* strains. This can be seen clearly in the strain Fγb at round four for the standard and
194 modified experiments (Fig 5). For the other hosts, we observed stochastic variation around one,
195 indicating that the efficiency of killing was not dramatically changing for most hosts. The
196 fluctuations are an unexpected result could indicate fluctuations in populations of phages
197 specific to each of the hosts or that small numbers of phages are unreliably measured at the

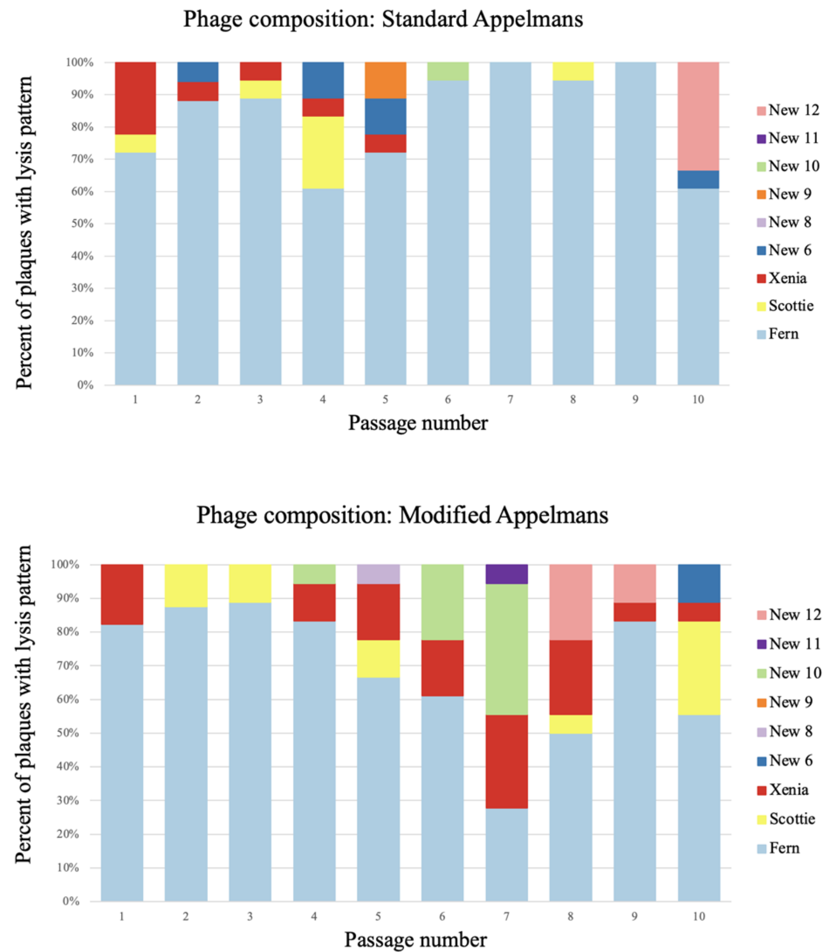

Figure 4. Phage lysis pattern composition for each round in the standard (top panel) and modified protocol (bottom panel) experiments. Each bar represents the composition of 18 plaques that match phages with the lysis patterns shown in the key.

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198 highest dilutions. Either way, we frequently observe wells clearing that were not expected to
199 have phages in them based on the 1E6 starting titer and that this is not consistent across all the
200 hosts. We may see an increase in the highest dilution cleared well for one host, but not the
201 others.
202
203 **DISCUSSION**
204

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205 As phage therapies for American Foulbrood disease are developed in honey bees, an improved understanding of the bacteriophages and how they evolve with their hosts is needed. The complications of phage-resistance and narrow host range are among the issues that would benefit from more research. Appelmans protocol has become a popular method of co-culture that can be used to improve many characteristics of a phage cocktail. As the use of Appelmans expands, several interesting complexities have arisen

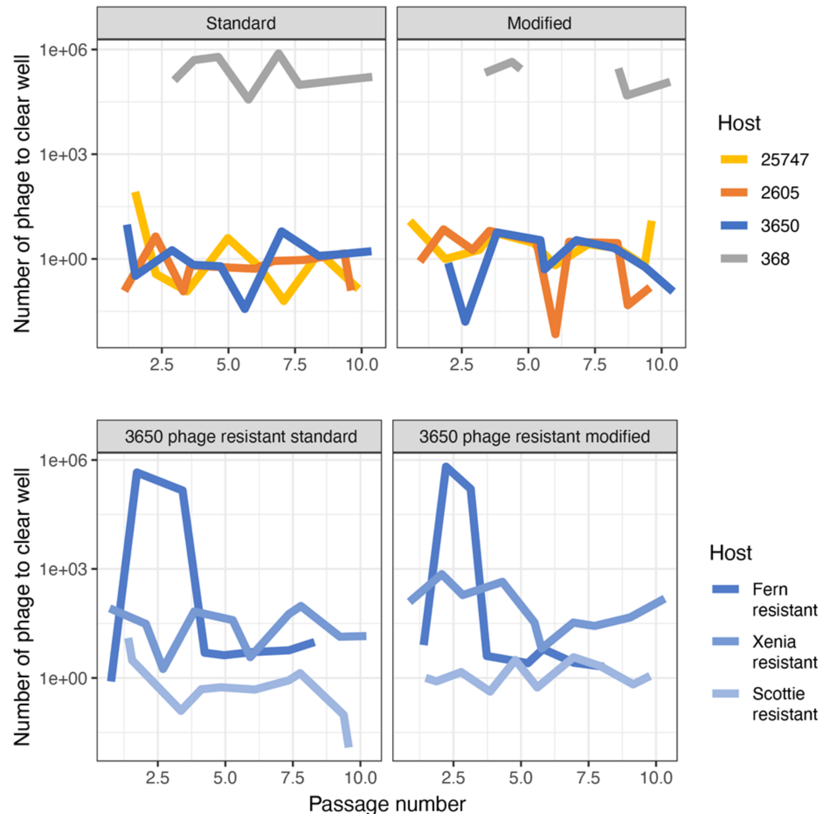

Figure 5. The initial number of phages (1E6 pfu/mL) divided by the phage dilution of the last cleared well for each *P. larvae* strain after every round of growth for both the standard and modified protocols. (a) naïve *P. larvae* strains; (b) phage-resistant *P. larvae* strains.

227 and more detailed studies of the population dynamics in these experiments will lead to an
228 improved understanding of the method and of phage-host evolution in general [31]. We
229 compared two strategies of evolution to determine if improvements could be made that
230 accelerate the development of *P. larvae* phages. Our result show no substantial increase in the
231 number of phages with new host range profiles when we modify the standard Appelmans
232 protocol with the addition of phage each round of passage. This result may have been limited by
233 the small total number of new host range profiles that we observed (only 6 from 360 tested
234 plaques). Most reports using Appelmans (or some variation of it) result in a phage cocktail that
235 is able to infect more hosts than the starting phage mix [27,32–34] but often the exact phage
236 composition and genetic underpinnings of host expansion are not resolved in these studies.
237 When these details are determined the results are often surprising. In a study of *Acinetobacter*
238 *baumannii*, the host range of 56 phage isolates were tested after Appelmans and 12 isolates

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239 with new host range patterns were identified. Our rate (6 of 360 plaques) was substantial lower
240 than this. While several of the *A. baumannii* isolates had gained the ability to grow on a novel
241 host, some actually lost the ability to grown on hosts that they could originally replicate on.
242 Sequencing of these phages showed frequent recombination and the presence of phages not in
243 the starting phage cocktail. Induced prophages contributed to the host range expansion of the
244 phage cocktail. Recombinants of the original phages did not have expanded host range.
245 Temperate phage induction during Applemans has been reported in other studies [34] and
246 highlights an unexpected benefit—or challenge—of using evolution on hosts to improve phages.
247 In the *A. baumannii* study, host expansion was slower than in other applications of Appelmans,
248 with around 70% of the hosts becoming susceptible to the phage cocktail at the end of the
249 experiment. Studies on *Pseudomonas aeruginosa* found 100% of the hosts to be susceptible
250 after a similar experiment [27,33,34]. Since we only had seven hosts, three of which were
251 mutants of the original strains, it is hard to say exactly what this percentage was in our
252 experiment. All seven hosts were killed by the phage cocktail, but 368 never supported phage
253 replication. The modified Appelmans protocol did produce two unique phage lysis patterns,
254 suggesting that perhaps this modification provides some benefit to produce novel phages.
255 A main difference that we observed between the standard and modified Appelmans
256 protocols was the maintenance of a higher amount of phage diversity in the modified protocol.
257 Therefore, addition of the starting phage cocktail to the pool of successful phages each round is
258 a simple step that can benefit the evolution of phages in a cocktail. In our set of phages and
259 hosts, one quickly rose in frequency. In other systems, this may not be the case and the
260 improvement of our modified protocol may not be as necessary.
261 One key to successful phage therapy is the ability for the phages to infect the target
262 bacteria, replicate, and lyse the host, thus killing it. This lytic infection cycle ensures host death
263 along with amplification of the phages. Expansion of our phage cocktail's host range to a
264 nonpermissive *P. larvae* strain with Appelmans evolution was expected to derive phages
265 capable of this lytic infection cycle on new hosts. Due to the clearing observed on host 368 in
266 the undilute wells during Appelmans evolution and partial clearings seen on host range assay
267 plates, new phage lysis patterns New 8 and New 9 were initially described as being able to
268 infect and kill *P. larvae* strain 368, a previously nonpermissive host to phage infection. However,
269 upon further testing no phage replication and amplification was observed on this host. There
270 was clearly death of this *P. larvae* strain though. We predict that there is some mechanism of
271 induced cell death occurring that is triggered by the presence of phages. Situations where a
272 host cell kills itself or stops cellular functions to avoid phage infection is called abortive infection

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273 [11]. Because the killing of 368 did not arise until round two of evolution, we hypothesize that
274 phages evolved to infected 368, but the infection is thwarted at the phage replication step by
275 some phage-defense mechanism. Further genetic and molecular analysis of *P. larvae* strain 368
276 is needed to accurately describe why bacteria cells are dying in the presence of phages rather
277 than directly due to phage infection.

278 Of the seven *P. larvae* strains, only one strain, 368, was nonpermissive to all three
279 phages, Fern, Scottie, and Xenia, initially. Therefore, host range expansion of the phage
280 cocktail with 10 rounds of a modified Appelmans protocol was unfortunately unsuccessful in this
281 case. Increasing the number of evolution rounds may provide more time and opportunity for
282 novel phage mutations to occur and expand the lytic life cycle to nonpermissive *P. larvae*
283 strains.

284 *P. larvae* strains permissive to phage infections will likely evolve to develop resistance to
285 phage infection just as they do to antibiotics [6, 17]. To further characterize these selective
286 pressures between phages and *P. larvae* specifically, developed phage-resistant *P. larvae*
287 strains were used during Appelmans evolution. New phage lysis pattern New 6 is capable of
288 infecting and killing all three of the phage-resistance *P. larvae* strains, a host range pattern not
289 seen in any of the starting phages. These results demonstrate that the inevitable host resistance
290 of *P. larvae* can be overcome by phages evolved together in a cocktail. Therefore, using this
291 evolved phage cocktail will likely benefit the success of phage therapy when treating persistent
292 American Foulbrood infections. Future work will seek to identify the genetic determinants of
293 these evolved phages to identify genes involved in host range expansion.

294 Part of making phage therapy a successful treatment is reducing the amount of time it
295 takes to produce an effective phage cocktail, whether that includes evolution or not. If evolution
296 is needed, our Appelmans protocol can produce new phage lysis patterns in as few as two
297 rounds. Adding the ancestral recombination phage cocktail also showed an increase in
298 individual new phage lysis pattern isolated likely due to more genetic recombination between
299 phages. This addition also produced a diverse phage cocktail with a more proportional
300 composition between the phage lysis patterns observed. This benefits phage therapy
301 applications because a more diverse phage cocktail will likely be able to avoid total host
302 resistance.

303 This research will benefit the pursuit of using phages to treat American Foulbrood
304 disease in honey bees along with defining phage evolution protocols that can develop a more
305 diverse, and therefore effective, phage cocktail.

306

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307 **METHODS**

308 **Bacterial and phage strains, maintenance, and culturing**

309 *Paenibacillus* phages Fern, Scottie, and Zenia and *P. larvae* strains NRRL B-3650 and ATCC-
310 25368 were acquired from Dr. Penny S. Amy at the University of Nevada Las Vegas. Strains
311 ATCC-9545 (B-2605) and ATCC-25747 were ordered directly from the American Type Culture
312 Collection. These bacterials strains will henceforth be referred to as 3650, 368, 2605, and
313 25747, respectively. The phage resistant isolates used in this study were generated in our lab
314 [29] and will be referred to as Fγb (resistant to Fern), Scotβ (resistant to Scottie), and XIIIβa
315 (resistant to Xenia).

316 Frozen stocks were made from overnight cultures grown to 0.7 OD. 1.5 mL of the culture
317 was aliquoted into 2 mL freezer tubes, spun down at 11000 xg for 10 minutes, and the
318 supernatant was discarded. 750 µL of fresh media and 30% glycerol was added to each tube,
319 vortexed thoroughly, and then flash frozen in liquid nitrogen. All frozen stocks were stored at -
320 80℃ and used to start experiments.

321 All *P. larvae* strains were continuously grown in 1 mg/L of thiamine hydrochloride Brain
322 Heart Infusion broth at 37℃ in a shaker incubator at 200 rpm for 24-48 hours [30]. Each shaking
323 broth culture was started from an individual colony picked from streaks made on 1 mg/L of
324 thiamine hydrochloride BHI agar plates. Plates were incubated inverted at 37℃ with 5% CO_2 for
325 24-48 hours. Strains were grown with 5% CO_2.

326

327 **Plaque picking and double isolation**

328 For phage isolation, one clean plaque was picked using a cut pipette tip and put in 750 µL of
329 mBHI broth, 10% v/v chloroform was added, vortexed, and then spun down at 20000 x g for 5
330 minutes. 500 µL of the supernatant was put in a new tube and titered on host strain 3650.
331 Phages were stored at 4℃ and used as working stocks.

332

333 **Bacteriophage titering**

334 Soft mBHI top agar overlays were used to determine phage concentration. 100 µL of *P. larvae*
335 were mixed with varying amounts of a phage stock dilution in 3 mL of warm (50℃) mBHI, 1%
336 agar. The mix was briefly vortexed then poured on top of a BHI agar plate. Plates were
337 incubated upright at 37℃ with 5% CO_2 for 24-48 hours. Plates ranging from 50-250 plaques
338 were counted and then averaged. Phage stock titers were calculated based on the average
339 plaque count and dilution plated.

340

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341 **Bacteriophage host range assays: spot plating**
342 A grid pattern with nine boxes was draw on the bottom of BHI agar plates, one for each phage
343 and one control. Soft mBHI top agar with 100 µL of a *P. larvae* strain was poured on the plate
344 and let sit for an hour. 10 µL of undiluted to 1.0E-2 dilution (depending on titer) of each phage
345 stock was pipetted into its labeled gridded box on the top agar layer and 10 µL of mBHI was
346 pipetted into the control box. Plates sat for another hour to prevent unwanted spreading or
347 mixing of phage and then incubated upright at 37℃ with 5% CO_2 for 24-48 hours. This was
348 repeated for all the *P. larvae* strains. The next day lysis patterns of each phage were observed
349 and recorded.
350
351 **Ancestral bacteriophage recombination mix culturing**
352 To maintain and increase diversity in the phage cocktail, we implemented a modification to the
353 standard Appelmans protocol that involved adding in a mix of the original phages before each
354 new round of evolution. To obtain this mix, we cross streaked phages Fern, Scottie, and Xenia
355 on one plate and obtained phages from the center of the plate where they were co-localized.
356 Briefly, a 100 µL 3650 soft overlay top agar plate was prepared and let sit for an hour at 37℃.
357 10 µL of each phage stock was pipetted on the edges of the plate at equal distances from each
358 other. Using a sterile loop, the drops were streaked straight across the plate crossing in the
359 center. The plate was incubated upright at 37℃ with 5% CO_2 for 24 hours. The next day, a
360 punch from the center clearing was collected in a 5 mL tube with 2 ml mBHI, mashed with a
361 sterile tube mortar, vortexed, chloroformed, and centrifuged at 20000 x g for 10 minutes. The
362 supernatant was collected and titered on 3650. This became the “ancestor recombination mix”
363 fridge stock. This was done to allow growth and recombination between the three phage strains
364 and later be added into each round of the Appelmans recombination series.
365
366 **Appelmans and modified Appelmans protocol**
367 The general methods outlined in Burrows et al. (2019) [27] were followed with some minor
368 modifications. 3 mL BHI broth cultures started from a colony were used to start overnight
369 cultures of all *P. larvae* strains, 3650, 25747, 368, 2605, Fγb, Scotβ, and XIIIβa. The next day
370 bacterial cultures were normalized to 0.3 OD at 600nm. For ease of pipetting, 1.0E-1 dilutions
371 were made of the normalized hosts. The starting phage cocktail, equally comprised of phages
372 Fern, Scottie, and Xenia, had a combined total titer of 1.0E6. The phage cocktail and all ten-fold
373 dilutions of the cocktail (1.0E-1 to 1.0E-9) were in mBHI broth.

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374 A 96-well microtiter plate was used for each round of evolution. Each column (12 wells)
375 was used for one *P. larvae* strain and each row, 8 wells, was used for a dilution of the phage
376 cocktail or control, see Figure 3. Row 1 control was media along with the *P. larvae* to monitor
377 bacterial growth without phage infection. Row 2 control was just media (no bacteria or phage) to
378 monitor media contamination. 100 µL of double strength mBHI broth was added to all 96 wells.
379 100 µL of mBHI was then added to only the row 2 control. For each column, 10 µL of a 1.0E-1
380 dilution of the normalized *P. larvae* strain was added to every well but the second (row 2
381 control). 100 µL of the corresponding phage cocktail dilution was added to each row skipping
382 control rows 1 and 2. The most dilute phage cocktail (1.0E-9) wells were closest to the control
383 rows, whereas the most concentrated phage cocktail (undilute) wells were in the row farthest
384 from the controls (bottom row). For the first round of Appelmans, this set up was done twice to
385 start both the control and recombination series.
386 The microtiter plates were covered with a Breathe-Easy membrane to allow gas
387 exchange while preventing phage and bacterial contamination during growth. Plates were
388 incubated overnight (18-24 hours) at 37℃ in a shaker incubator (120 rpm).
389 The next day, photos were taken of each plate and the cleared wells (lysis) were
390 recorded. 100 µL from each of the cleared wells from all hosts along with the next turbid well
391 (next dilution) were pooled together. This was done separately for each series. The pooled
392 lysate was then chloroformed (10% v/v), vortexed, and spun down at 22000 xg for 10 minutes.
393 The supernatant (normally around 3 mLs) was put into a new tube and stored in the fridge for
394 the next round. 1 mL freezer stocks were made from each round's pooled lysate as well.
395 The pooled lysate from each round was tittered on *P. larvae* strain 3650 to determine the
396 number of phages that will start the next round. For every round besides the first, the
397 recombination series includes the modification where the ancestral recombination phage
398 cocktail was added to the rounds pooled lysate based on a 1:10 titer ratio. Based on the
399 recombination series pooled lysates titer and the known titer of the ancestral recombination
400 phage cocktail, the 1:10 mix was made to start the next round (1 part ancestral recombination
401 phage cocktail titer to 10 parts recombination series pooled lysate titer). The control series did
402 not include this step, however it was also tittered after each round for later data analysis.
403 The set up for the next round of evolution is the same as the first round, but the phage
404 cocktail and dilutions are made from the previous rounds pooled lysate cocktail and pooled
405 lysate cocktail plus the ancestral recombination cocktail for the control and recombination series
406 respectfully. This was repeated for ten rounds of growth.
407

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408 **Appelmans pooled lysates host range determination and isolation**

409 All 18 isolated phages from each round were picked from the 3650 toothpick plate for double
410 isolation using the method described above. Once double isolated stocks were made for all 360
411 picked plaques (180 control series + 180 recombination series), host range assays using the
412 spot plating method were conducted on all 8 hosts (4 naïve *P. larvae* strains, 3 phage resistant
413 *P. larvae* strains, and out outgroup *P. alvei* strain B-383). There was never any lysis on the *P.*
414 *alvei,* so its results were not included in data analysis.

415 Host lysis patterns were characterized and analyzed to predict which phage was present
416 based on the ancestral host lysis patterns. If the observed host lysis pattern did not match any
417 of the ancestral, it was named "new phage lysis pattern x" where x is any number. Spot plating
418 host range assays were repeated to confirm predicted phage lysis patterns.

419

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432
433

434 **REFERENCES**

435

436 1. Matias DMS, Leventon J, Rau A-L, Borgemeister C, von Wehrden H. A review of ecosystem
437 service benefits from wild bees across social contexts. Ambio. 2017;46: 456–467.
438 doi:10.1007/s13280-016-0844-z

439 2. Ferrier PM. Economic Effects and Responses to Changes in Honey Bee Health. 2018; 54.

440 3. Khalifa SAM, Elshafiey EH, Shetaia AA, El-Wahed AAA, Algethami AF, Musharraf SG, et al.
441 Overview of Bee Pollination and Its Economic Value for Crop Production. Insects. 2021;12:
442 688. doi:10.3390/insects12080688

bioRxiv preprint doi: https://doi.org/10.1101/2024.09.01.610711; this version posted September 1, 2024. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY 4.0 International license.

443 4. Poppinga L, Genersch E. Molecular pathogenesis of American Foulbrood: how Paenibacillus
444 larvae kills honey bee larvae. Curr Opin Insect Sci. 2015;10: 29–36.
445 doi:10.1016/j.cois.2015.04.013

446 5. Alippi AM, López AC, Aguilar OM. Differentiation of Paenibacillus larvae subsp. larvae, the
447 Cause of American Foulbrood of Honeybees, by Using PCR and Restriction Fragment
448 Analysis of Genes Encoding 16S rRNA. Appl Environ Microbiol. 2002;68: 3655–3660.
449 doi:10.1128/AEM.68.7.3655-3660.2002

450 6. Fünfhaus A, Ebeling J, Genersch E. Bacterial pathogens of bees. Curr Opin Insect Sci.
451 2018;26: 89–96. doi:10.1016/j.cois.2018.02.008

452 7. Genersch E. American Foulbrood in honeybees and its causative agent, Paenibacillus larvae.
453 J Invertebr Pathol. 2010;103: S10–S19. doi:10.1016/j.jip.2009.06.015

454 8. Alvarado I, Phui A, Elekonich MM, Abel-Santos E. Requirements for In Vitro Germination of
455 Paenibacillus larvae Spores. J Bacteriol. 2013;195: 1005–1011. doi:10.1128/JB.01958-12

456 9. Djukic M, Brzuszkiewicz E, Fünfhaus A, Voss J, Gollnow K, Poppinga L, et al. How to Kill the
457 Honey Bee Larva: Genomic Potential and Virulence Mechanisms of Paenibacillus larvae.
458 PLoS ONE. 2014;9. doi:10.1371/journal.pone.0090914

459 10. Fünfhaus A, Göbel J, Ebeling J, Knispel H, Garcia-Gonzalez E, Genersch E. Swarming
460 motility and biofilm formation of Paenibacillus larvae, the etiological agent of American
461 Foulbrood of honey bees (Apis mellifera). Sci Rep. 2018;8: 8840. doi:10.1038/s41598-018-
462 27193-8

463 11. Masood F, Thebeau JM, Cloet A, Kozii IV, Zabrodski MW, Biganski S, et al. Evaluating
464 approved and alternative treatments against an oxytetracycline-resistant bacterium
465 responsible for European foulbrood disease in honey bees. Sci Rep. 2022;12: 5906.
466 doi:10.1038/s41598-022-09796-4

467 12. Krongdang S, Evans JD, Pettis JS, Chantawannakul P. Multilocus sequence typing,
468 biochemical and antibiotic resistance characterizations reveal diversity of North American
469 strains of the honey bee pathogen Paenibacillus larvae. PLoS ONE. 2017;12: e0176831.
470 doi:10.1371/journal.pone.0176831

471 13. Tian B, Fadhil NH, Powell JE, Kwong WK, Moran NA. Long-Term Exposure to Antibiotics
472 Has Caused Accumulation of Resistance Determinants in the Gut Microbiota of Honeybees.
473 mBio. 2012 [cited 20 Jan 2022]. doi:10.1128/mBio.00377-12

474 14. Evans JD. Diverse origins of tetracycline resistance in the honey bee bacterial pathogen
475 Paenibacillus larvae. J Invertebr Pathol. 2003;83: 46–50. doi:10.1016/S0022-
476 2011(03)00039-9

477 15. Mejias E. American Foulbrood and the Risk in the Use of Antibiotics as a Treatment.
478 Modern Beekeeping - Bases for Sustainable Production. IntechOpen; 2019.
479 doi:10.5772/intechopen.90303

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480 16. Determination of antibiotic residues in honey in relation to different potential sources and
481 relevance for food inspection - ScienceDirect. [cited 24 Jul 2024]. Available:
482 https://www.sciencedirect.com/science/article/abs/pii/S0308814620314370

483 17. Tsourkas PK. Paenibacillus larvae bacteriophages: obscure past, promising future. Microb
484 Genomics. 2020;6. doi:10.1099/mgen.0.000329

485 18. Brady TS, Merrill BD, Hilton JA, Payne AM, Stephenson MB, Hope S. Bacteriophages as an
486 alternative to conventional antibiotic use for the prevention or treatment of Paenibacillus
487 larvae in honeybee hives. J Invertebr Pathol. 2017;150: 94–100.
488 doi:10.1016/j.jip.2017.09.010

489 19. Jończyk-Matysiak E, Popiela E, Owczarek B, Hodyra-Stefaniak K, Świtała-Jeleń K, Łodej N,
490 et al. Phages in Therapy and Prophylaxis of American Foulbrood – Recent Implications
491 From Practical Applications. Front Microbiol. 2020;11: 1913. doi:10.3389/fmicb.2020.01913

492 20. Kok DN, Zhou D, Tsourkas PK, Hendrickson HL. Paenibacillus larvae and their phages; a
493 community science approach to discovery and initial testing of prophylactic phage cocktails
494 against American Foulbrood in New Zealand. Microbiome Res Rep. 2023;2.
495 doi:http://dx.doi.org/10.20517/mrr.2023.16

496 21. Jończyk-Matysiak E, Owczarek B, Popiela E, Świtała-Jeleń K, Migdał P, Cieślik M, et al.
497 Isolation and Characterization of Phages Active against Paenibacillus larvae Causing
498 American Foulbrood in Honeybees in Poland. Viruses. 2021;13: 1217.
499 doi:10.3390/v13071217

500 22. Brady TS, Roll CR, Walker JK, Fajardo CP, Breakwell DP, Eggett DL, et al. Phages Bind to
501 Vegetative and Spore Forms of Paenibacillus larvae and to Vegetative Brevibacillus
502 laterosporus. Front Microbiol. 2021;12. doi:10.3389/fmicb.2021.588035

503 23. Beims H, Wittmann J, Bunk B, Spröer C, Rohde C, Günther G, et al. Paenibacillus larvae-
504 Directed Bacteriophage HB10c2 and Its Application in American Foulbrood-Affected Honey
505 Bee Larvae. Appl Environ Microbiol. 2015;81: 5411–5419. doi:10.1128/AEM.00804-15

506 24. Ribeiro HG, Melo LDR, Oliveira H, Boon M, Lavigne R, Noben J-P, et al. Characterization of
507 a new podovirus infecting Paenibacillus larvae. Sci Rep. 2019;9: 20355.
508 doi:10.1038/s41598-019-56699-y

509 25. Stamereilers C, Fajardo CP, Walker JK, Mendez KN, Castro-Nallar E, Grose JH, et al.
510 Genomic Analysis of 48 Paenibacillus larvae Bacteriophages. Viruses. 2018;10: 377.
511 doi:10.3390/v10070377

512 26. Beims H, Bunk B, Erler S, Mohr KI, Spröer C, Pradella S, et al. Discovery of Paenibacillus
513 larvae ERIC V: Phenotypic and genomic comparison to genotypes ERIC I-IV reveal different
514 inventories of virulence factors which correlate with epidemiological prevalences of
515 American Foulbrood. Int J Med Microbiol. 2020;310: 151394.
516 doi:10.1016/j.ijmm.2020.151394

517 27. Burrowes BH, Molineux IJ, Fralick JA. Directed in Vitro Evolution of Therapeutic
518 Bacteriophages: The Appelmans Protocol. Viruses. 2019;11: 241. doi:10.3390/v11030241

bioRxiv preprint doi: https://doi.org/10.1101/2024.09.01.610711; this version posted September 1, 2024. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY 4.0 International license.

519 28. Kok DN, Turnbull J, Takeuchi N, Tsourkas PK, Hendrickson HL. In Vitro Evolution to
520 Increase the Titers of Difficult Bacteriophages: RAMP-UP Protocol. PHAGE. 2023;4: 68–81.
521 doi:10.1089/phage.2023.0005

522 29. Spencer E, Peters T, Eline Y, Saucedo L, Linzan K, Paull K, et al. Bacteriophage resistance
523 evolution in a honey bee pathogen. bioRxiv; 2024. p. 2024.07.09.602782.
524 doi:10.1101/2024.07.09.602782

525 30. de Graaf DC, Alippi AM, Antúnez K, Aronstein KA, Budge G, De Koker D, et al. Standard
526 methods for American foulbrood research. J Apic Res. 2013;52: 1–28.
527 doi:10.3896/IBRA.1.52.1.11

528 31. Glonti T, Pirnay J-P. In Vitro Techniques and Measurements of Phage Characteristics That
529 Are Important for Phage Therapy Success. Viruses. 2022;14: 1490. doi:10.3390/v14071490

530 32. Vu TN, Clark JR, Jang E, D'Souza R, Nguyen LP, Pinto NA, et al. Appelmans protocol – A
531 directed in vitro evolution enables induction and recombination of prophages with expanded
532 host range. Virus Res. 2023;339: 199272. doi:10.1016/j.virusres.2023.199272

533 33. Mapes AC, Trautner BW, Liao KS, Ramig RF. Development of expanded host range phage
534 active on biofilms of multi-drug resistant Pseudomonas aeruginosa. Bacteriophage. 2016;6:
535 e1096995. doi:10.1080/21597081.2015.1096995

536 34. Peters TL, Schow J, Spencer E, Leuven JV, Wichman H, Miller C. Directed evolution of
537 bacteriophages: impacts of prolific prophage. bioRxiv; 2024. p. 2024.06.28.601269.
538 doi:10.1101/2024.06.28.601269

539