Experimental evolution of a pathogen confronted with innate immune memory increases variation in virulence

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Abstract

Understanding the drivers and mechanisms of virulence evolution is still a major goal of evolutionary biologists and epidemiologists. Theory predicts that the way virulence evolves depends on the balance between the benefits and costs it provides to pathogen fitness. Additionally, host responses to infections, such as resistance or tolerance, play a critical role in shaping virulence evolution. But, while the evolution of pathogens has been traditionally studied under the selection pressure of host adaptive immunity, less is known about their evolution when confronted to simpler and less effective forms of immunity such as immune priming. In this study, we used a well-established insect model for immune priming – red flour beetles and their bacterial pathogen *Bacillus thuringiensis tenebrionis* – to test whether this form of innate immune memory favors the evolution of higher virulence. Through controlled experimental evolution of the pathogen in primed versus non-primed hosts, we found no change in average virulence after eight selection cycles in primed host. However, we found a significant increase in the variation of virulence (i.e., host-killing ability) among independent pathogen lines evolved in primed host, and bacteria were unable to evolve resistance against host priming. Whole genome sequencing revealed increased activity in the bacterial mobilome (prophages and plasmids). Expression of the Cry toxin – a well-known virulence factor – was linked to evolved differences in copy number variation of the *cry*-carrying plasmid, though this did not correlate directly with virulence. These findings highlight that innate immune memory can drive variability in pathogen traits, which may favor adaptation to variable environments. This underscores the need to consider pathogen evolution in response to innate immune memory when applying these mechanisms in medicine, aquaculture, pest control, and insect mass production.

37 # Introduction

38 Virulence is usually defined as the ability of a pathogen to reduce host fitness (or its components, e.g.
39 survival(1,2)) during the infection process (1,2). The direction in which virulence evolves in an
40 evolutionary arms race often depends on the balance between the benefits and costs it provides to
41 the pathogen. For instance, when the highly virulent *Myxoma* virus was introduced into Australian
42 rabbit populations in the 1950s, it evolved to intermediate levels of virulence while maximizing its
43 fitness (3–5). In this example, the cost associated with high virulence was host death, which limited
44 pathogen transmission. Although transmission of pathogens varies and some pathogens can only be
45 transmitted after host death, several studies have shown that optimal transmission rates happen at
46 intermediate levels of virulence (6–8). Additionally, several experimental evolution studies have
47 revealed the complexity of selection processes acting on both hosts and pathogens during their rapid
48 coadaptation (9,10) in which the coevolution between the host and pathogen favours and maintains
49 intermediate levels of pathogen virulence (10).

50 Host responses and defence mechanisms against pathogens also play a crucial role in the evolution of
51 virulence. Depending on the ability of organisms to resist or tolerate infections, pathogen virulence
52 evolution can take different trajectories (11). The evolution of tolerance, for instance, can strongly
53 influence the evolution of virulence by allowing pathogens to evolve higher transmission rates without
54 increasing the actual level of virulence. As a result, pathogens can become highly virulent in
55 nontolerant hosts and increase the chances of catastrophic epidemics (11). Likewise, epidemiological
56 and mathematical studies propose that "leaky" vaccines, i.e. vaccines that provide incomplete
57 immunity, could enable pathogens to still spread and evolve increased virulence, thus making them
58 more dangerous to unvaccinated individuals (3,12,13) (14). On the other hand, leaky vaccines can also
59 lead to reduced pathogen virulence through the reduction of pathogen load (15). Virulence evolution
60 is a complex trait that may also be influenced by interactions with the hosts' microbiome (16). A recent
61 experimental evolution study in nematodes and the pathogen *Pseudomonas aeruginosa* revealed that

62 incomplete host immunity caused by host microbiota favours the evolution of higher pathogen
63 virulence (17). Additionally, external host immunity has also been shown to drive pathogen evolution
64 towards higher virulence (18). On the other hand, defensive microbes can also reduce pathogen
65 virulence (19). Considering these seemingly contradictory results, more empirical data from
66 experimental evolution studies of different host-pathogen systems are necessary to comprehend how
67 incomplete host immunity influences the virulence of pathogens.

68 Invertebrate innate immune priming has been suggested to be analogous to "leaky" vaccination, as
69 primed individuals can still become infected but also can tolerate elevated pathogen loads, providing
70 an experimental system to assess the effect of innate immune memory on pathogen evolution (20).
71 Immune priming is a form of immune memory provided by the innate immune system, where initial
72 non–lethal pathogen exposure can lead to enhanced protection upon secondary exposure (21–23).
73 Over the last couple of decades, the body of evidence for immune priming in invertebrates has been
74 constantly growing (24–26) and experimental evolution studies revealed the rapid evolution of
75 immune priming (27,28). Innate immune systems of vertebrates show similar phenomena, in the form
76 of trained immunity (29,30). The relevance and potential applications of these forms of innate immune
77 memory are currently intensively discussed (31–33). While it has been observed that priming reduces
78 pathogen transmission (34), we still, however, lack empirical data regarding the effects of these innate
79 forms of immune memory on pathogen evolution and virulence.

80 In this study, we used the red flour beetle *Tribolium castaneum* and its natural pathogen *Bacillus*
81 *thuringiensis tenebrionis* (*Btt*), to investigate whether priming favours the evolution of higher
82 virulence. *T. castaneum* is a well-established model for host-pathogen coevolution and immune
83 priming (35–37) and shows a specific priming response towards the spore-forming pathogen *Btt* after
84 both septic and oral exposure (38–40). *Btt*, widespread and commonly found in diverse environments,
85 produces insecticidal crystal proteins known as Cry toxins, specifically toxic to different insect orders
86 (41). After the spores are ingested orally, *Btt* can infect and reproduce within beetle larvae, ultimately

87 leading to host death (42,43). The oral priming response is triggered by the ingestion of the sterile
88 filtered supernatant of the spore culture (39), which induces the upregulation of immune genes (44).

89 To investigate how host immune priming influences the evolution of pathogen virulence, we
90 experimentally evolved *Btt* in primed and non-primed beetle larvae for eight generations (Fig. 1).
91 During the experiment, beetles were not allowed to evolve by taking them from a stock population
92 (i.e., one-sided evolution of the pathogen). After the eight cycles of experimental evolution, we
93 compared the levels of virulence in *Btt* in primed and non-primed host. We sequenced the genomes
94 of evolved *Btt* to identify whether genetic changes contributed to evolved differences in virulence. To
95 our knowledge, our study is the first to show the influence of immune priming on pathogen evolution,
96 thus broadening our understanding of how host innate immune memory might influence the
97 trajectories of virulence evolution.

98

Results

Does host immune priming affect pathogen virulence evolution?

B. thuringiensis tenebrionis (Btt) bacteria evolved experimentally in either immune primed or control (i.e., non-primed) *T. castaneum* hosts (Fig 1, left side). After this selection treatment for eight cycles, we analysed the response of *Btt* to the selection pressure encountered in primed *vs.* control hosts. Specifically, we compared the virulence (measured as host mortality) of eight primed-evolved *Btt* lines (P1-8) and eight control-evolved *Btt* lines (C1-8) as well as ancestral *Btt* (eight replicates directly taken from stock *Btt*, A1-8). These phenotypic readouts (Fig. 1, right side) were taken from two environments, primed and control host, thereby establishing a 'common-garden' experiment.

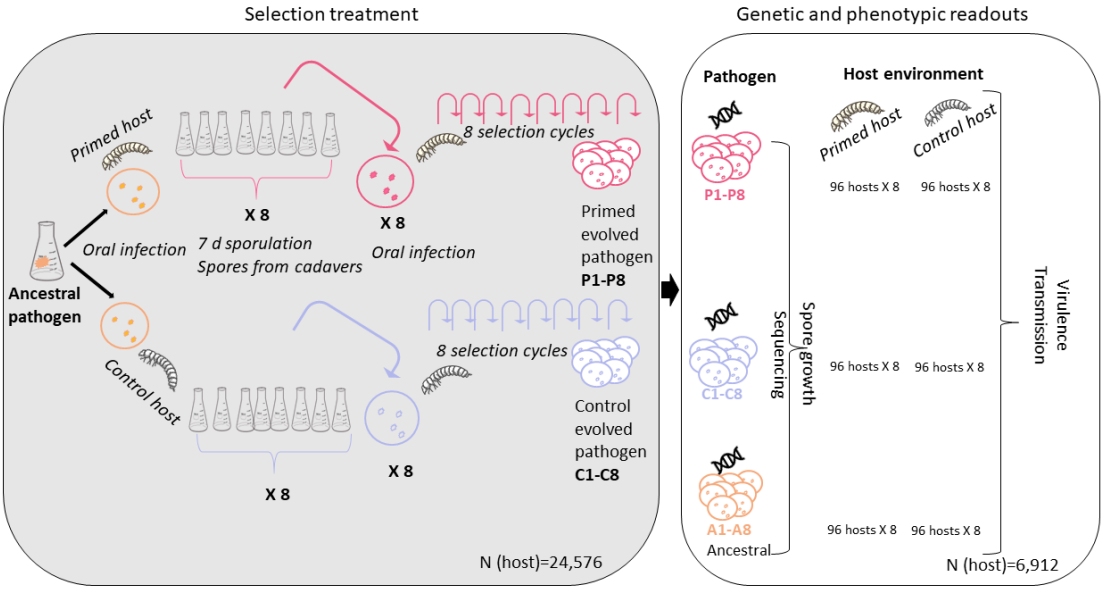


Fig 1. **Experimental design.** We inoculated one colony of the pathogen *Bacillus thuringiensis tenebrionis* (*Btt*) into 100 mL of *Btt* medium. After 7 days of sporulation, we harvested the spores, adjusted the concentration to 5x10^9, and produced an infection diet that was offered to individual primed and control 15 days old beetle larvae (n=192 per replicate line and cycle). For each selection cycle, we used *Btt* spores from cadavers of *T. castaneum* larvae killed by the infection in the previous cycle and produced eight bacterial lines per selection treatment. For each bacterial line, we isolated spores from five cadavers, which were subsequently grown independently in a sporulation medium to achieve high spore numbers for the infection of a sufficient number of beetle larvae for the next infection cycle. In each selection cycle, bacteria reproduced and sporulated within their host followed by amplification in culture medium. We had eight independently evolving pathogen replicate lines each for evolution in control and primed hosts. After eight selection cycles, we phenotyped and genotyped all evolved pathogen lines (P1-8, C1-8) and the ancestral pathogen. For the ancestral pathogen, we produced pseudo-replicates (A1-8), i.e., lines that had not undergone any selection process, but had grown in separate

flasks to control for possible variations from growth in the medium. For phenotyping, we quantified virulence (proportion of host mortality) and transmission (spore growth in cadavers) in two different host environments (primed and control). We also measured spore growth in a liquid medium. For genotyping, we sequenced the whole genomes of the 24 lines and quantified phage and plasmid coverage. We also measured the expression of a key virulence factor, the Cry-encoding gene, with RT-qPCR.

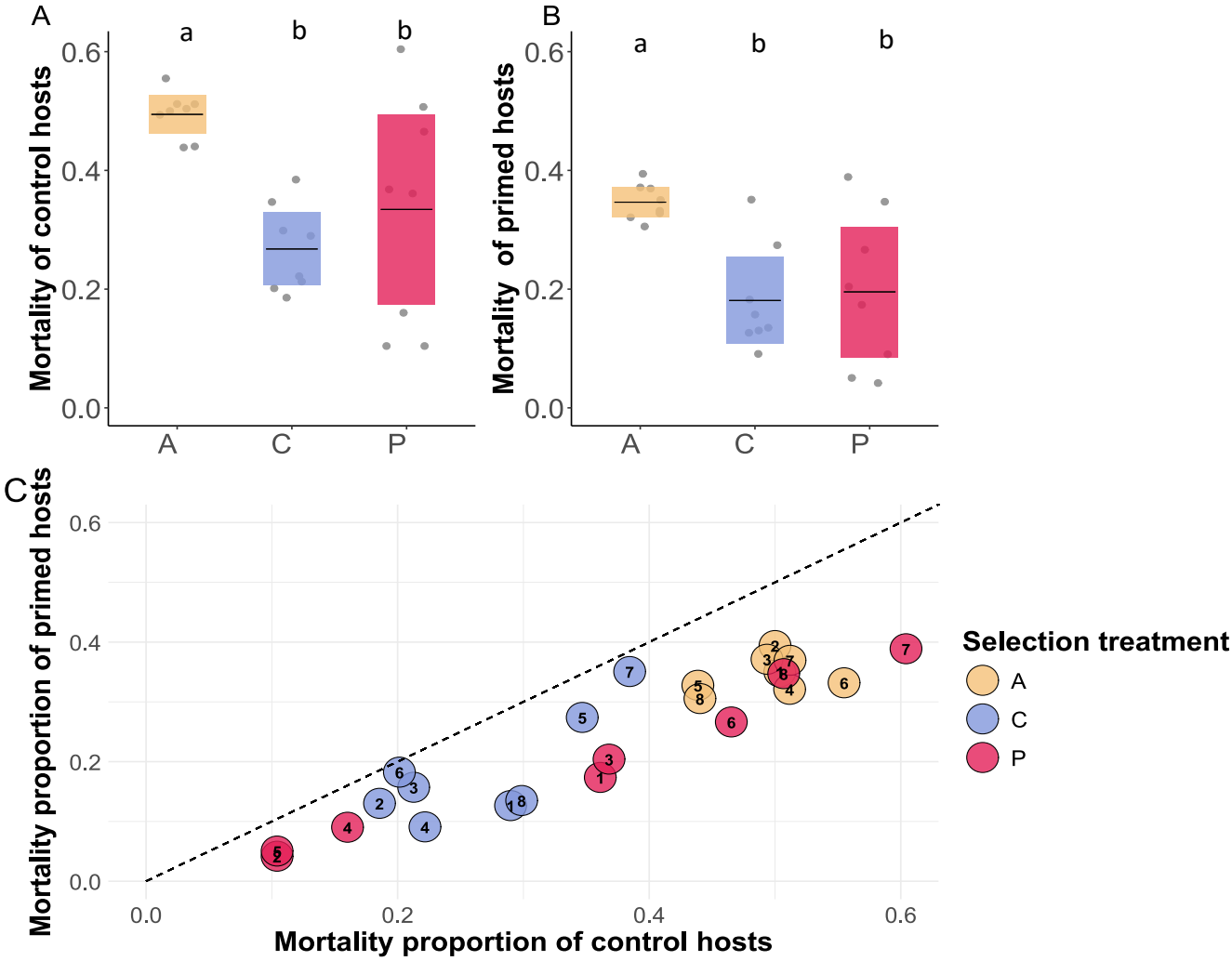


Fig 2. Virulence (proportion of host mortality) of ancestral *Btt* and the evolved lines Letters denote significant differences (A-ancestral, P-primed evolved, and C-control evolved). Both selection treatments showed reduced mortality compared to ancestral in **A** primed host (glmer: X^2 = 10.287, Df = 2, p = 0.006) and **B** control host (glmer: X^2= 10.428, Df = 2, p = 0.005), but there was no difference in virulence between the primed and control evolved lines. The plots show the mean and confidence intervals while each individual dot represents the mean virulence (proportion of host mortality) of each independent replicate line. **C**. Correlation between the virulence in primed and control hosts, showing each independent replicate in primed and control beetle larvae. The dotted line shows where the mortality rates of control hosts and primed hosts would be equal. All pathogen lines are below this line, indicating lower pathogen virulence in primed compared to control hosts (i.e., a 'priming effect').

138 We found that both, the selection treatment (Fig. 1, left side) and the host environment (Fig. 1, right
139 side) significantly affected pathogen virulence (Fig 2; glmer: X^2 = 74.25, Df = 5, p < 0.001). Selection in
140 either primed or control hosts significantly reduced the virulence, compared to the ancestral pathogen
141 (control: p = 0.001; primed: p = 0.006). Regarding the host environment encountered during the
142 readout, i.e. after evolution had happened, the primed host environment reduced virulence compared
143 to the control host environment (p < 0.001; Fig 2A and 2B). This shows that priming was still efficient,
144 also against the lines that had evolved in the primed hosts (Fig. 2 C).

145 We then performed separate analyses for primed and control host environments to see how individual
146 bacterial lines vary in virulence in different host environments. In the primed host environment, we
147 observed a significant effect of the pathogen selection treatment on pathogen virulence (glmer: X^2 =
148 17.054, Df = 2, p < 0.001; Fig. 2B). Overall, all evolved lines displayed lower virulence in the primed
149 host environment than the ancestral lines (primed: p = 0.008; control: p < 0.001), while there was no
150 significant difference between the pathogen lines that had evolved in the control *vs.* primed host (p =
151 0.840). Additionally, primed evolved replicates showed higher variance in virulence (σ^2 = 0.834) than
152 control evolved replicates (σ^2 = 0.166). In the control host environment (Fig. 2A), we obtained similar
153 results, with a significant effect of the pathogen selection treatment on the host mortality with evolved
154 pathogens showing lower virulence than ancestral (glmer: X^2 = 19.267, Df = 2, p <0.001; primed: p =
155 0.032; control: p < 0.001), where no differences were detected between the control and primed
156 selection treatment levels (p = 0.887). As well as in the primed host, we observed higher variance in
157 virulence across primed-evolved replicates (σ^2 = 0.862) than in control evolved replicates (σ^2 = 0.147).

158 To further confirm whether such variation in virulence might be associated with the selection
159 treatment, we modelled the effects of the selection treatments using a Beta distribution in a Bayesian
160 framework, and allowed the selection treatments to influence the precision parameter (φ). We found
161 that the variation in virulence (i.e., dispersion host mortality) was greater in primed-evolved *Btt* than
162 in control-evolved *Btt* with a certainty of 99.06% in the control host environment. This probability
163 slightly decreased to 90.14% in the primed host environment. Overall, both selection treatments

164 exhibited an increase in virulence variation compared with the ancestral *Btt* (Fig S1), with a larger
165 variation observed in the primed-evolved pathogen.

166

167 Do the evolved pathogens differ in spore production, sensitivity to
168 priming and fitness?

169 To evaluate the spore production of the evolved bacteria, we measured the pathogen spore load in
170 cadavers and the spore production in liquid medium. As *Btt* likely transmits through spores produced
171 in cadavers (45), we considered the spore load a good proxy of the transmission rate. There was no
172 difference in spore production between selection treatments in the control host (Fig 3A; lmer: X^2 =
173 0.859, Df = 2, p = 0.650), but in primed hosts, where pathogens that had evolved in primed and control
174 hosts produced fewer spores than the ancestral *Btt* (Fig 3B;3D; lmer: X^2 = 8.807, Df = 2, p = 0.0122;
175 primed-evolved: p = 0.014, control-evolved: p = 0.020). We also examined the correlation between
176 spore load and virulence for all bacteria lines and selection treatments in the primed and control host
177 environments but found no correlation (Fig S2 and S3). Finally, selection treatment had a significant
178 effect on spore production in a liquid medium (F = 40.087, Df = 2, p < 0.001), where both primed-
179 evolved and control-evolved pathogens exhibited lower spore production than the ancestral *Btt* (Fig
180 3C).

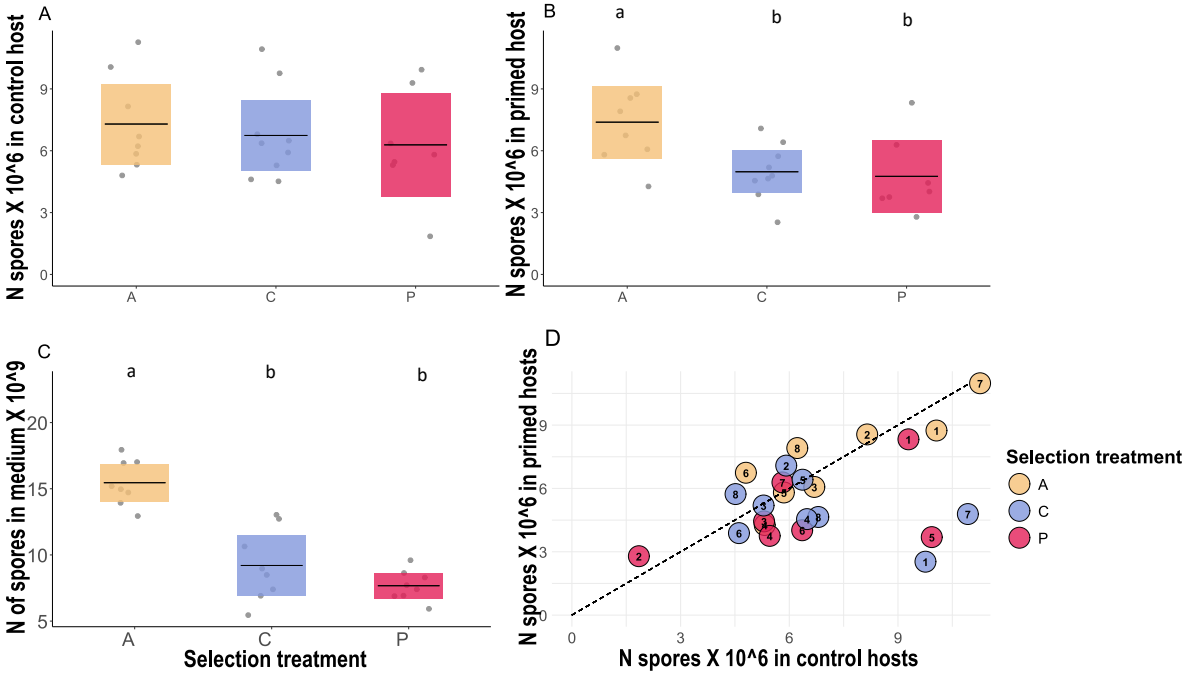
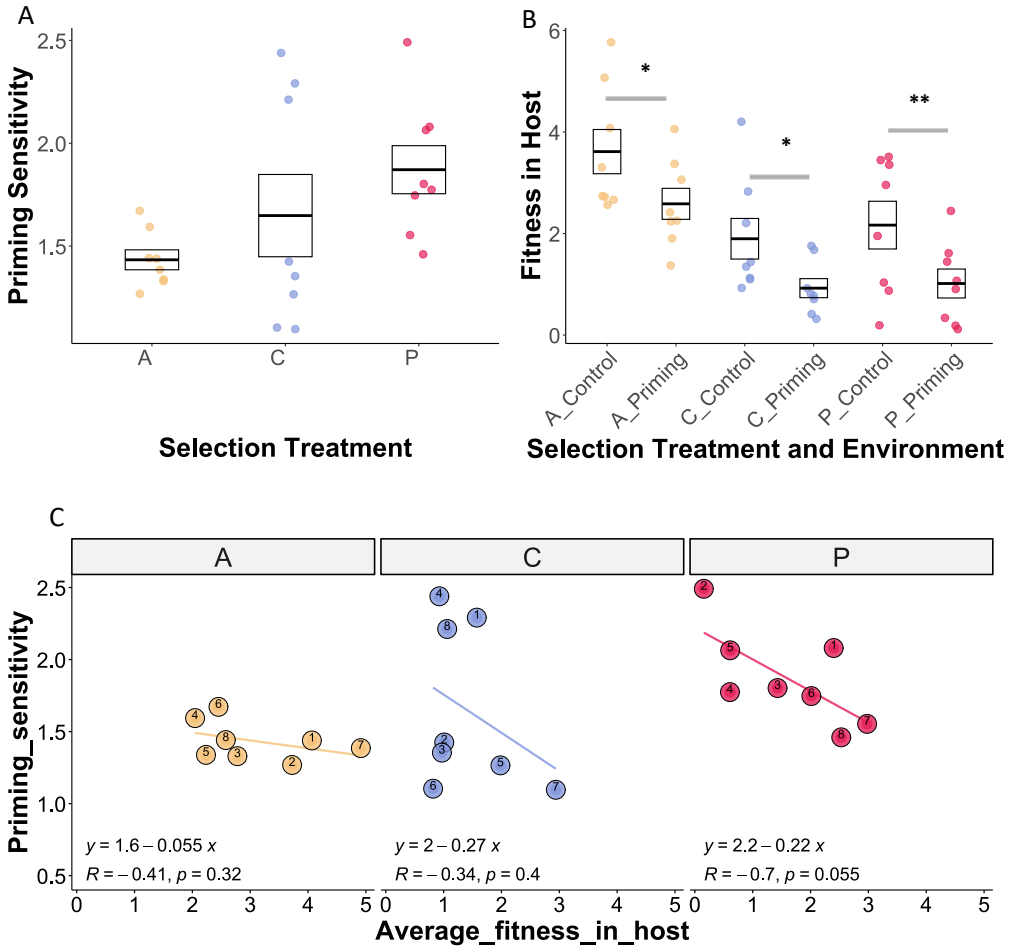


Fig 3. Cadaver spore load in A) primed and B) control hosts. The dots correspond to the mean of ten measurements for each independent pathogen line. The plot shows the mean and the confidence intervals. Ancestral *Btt* produced more spores than both evolved lines, but only in the primed host environment (lmer: X^2= 8.808, Df = 2, p = 0.012, and there seemed to be weak difference in pathogen load between the evolved lines (p = 0.973). Letters denote significant differences between the selection treatments. **C** Spore growth in the medium. Each dot represents the mean of three independent measures of one pathogen line. The graph shows the mean and confidence intervals. Primed- and control-evolved lines produced fewer spores than the ancestral strain (p < 0.001). **D.** Correlation between the spore load in primed and control hosts, showing the number of spores each independent replicate produces in primed and control beetle cadaver. The dotted line shows where the spore load in control hosts and primed hosts would be equal.

Additionally, we calculated the priming sensitivity of bacterial replicates by dividing virulence in control hosts by virulence in primed hosts. This gave us a value indicating how sensitive the pathogen is to the priming response of the host (Fig 4A). The more sensitive to priming the pathogen is, the lower the virulence in the primed hosts would be. Selection treatment affected the priming sensitivity (X^2 = 5.985, Df= 2, p = 0.050), with the primed-evolved pathogen exhibiting the highest priming sensitivity compared to the ancestral pathogen (p = 0.058).

We also assessed the fitness of each bacterial line within the host by combining mortality rates and spore load data (Fig 4B). Our analysis revealed that both selection treatments and host environment significantly influenced pathogen fitness (X^2= 40.385, df = 5, p < 0.001). The primed host environment

202 showed a significant negative effect on pathogen fitness (Estimate = -1.03, p = 0.0012), indicating that
203 the within-host fitness in the priming environment is lower than in the control environment. We finally
204 tested whether priming sensitivity was correlated with pathogen fitness, and found a negative trend
205 for the primed-evolved pathogens (Fig 4C).



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207 **Figure 4. Priming sensitivity and fitness in the host.** Each dot corresponds to bacterial replicate and the box
208 shows mean and confidence intervals. **A** Priming sensitivity of bacterial replicates was calculated by dividing
209 the *proportion of mortality in control/the proportion of mortality in primed hosts*. The higher the value, the more
210 sensitive the pathogen is towards host priming. Selection treatment affected the priming sensitivity (X^2 = 5.985,
211 Df= 2, p = 0.050), with the primed-evolved pathogen exhibiting the highest priming sensitivity compared to
212 the ancestral pathogen (p = 0.058). **B** Fitness in the host of each bacterial line was calculated as *mortality*
213 *proportion X spore load.* Selection treatment and host environment significantly influenced host fitness (X^2=
214 40.385, df = 5, p < 0.001). Pairwise comparisons confirmed that all the selection treatment levels have lower
215 fitness in the priming host environment. **C** Pearson´s correlation between priming sensitivity and average fitness
216 in the host (mean of fitness in primed and control host environment). A, C, and P stand for evolution treatment
217 levels: Ancestral, Control, and Primed evolved pathogen, respectively.

218

219

220 ## Do the evolved pathogens differ in *Cry* gene expression?

221 We tested for potential differences in *cry3a* gene expression, as evolved differences in virulence might
222 be related to this important virulence factor. For this, we performed qPCR on RNA extracted from
223 overnight cultures. In contrast to the negative control (genetically modified *Btt* without *Cry3a*), all
224 tested lines expressed *Cry3a* (Supp. Table 2). Overall, the expression in the evolved lines did not differ
225 significantly from that in the ancestral strain (C: estimate= -0.7565, Df= 14, t = -0.597, p = 0.560; P:
226 estimate= -1.1444, Df= 14, t = -0.903, p = 0.382). Four of the control- and three of the primed-evolved
227 lines showed significantly reduced *cry3a* gene expression compared to the ancestral strain (Suppl.
228 Table 3, Fig S4). However, we found no association between *cry3Aa* expression and virulence of the
229 lines, neither in primed (t = 0.279, Df = 15, p = 0.783) nor in control host environment (t = 0.131, Df =
230 15, p = 0.897)

231 ## Do the evolved pathogens differ in their genomes and mobilome?

232 To investigate whether the observed phenotypes of the evolved *Btt* lines are based on genetic changes,
233 we performed whole genome sequencing of all evolved lines and three replicates of the ancestral
234 pathogen. Compared to the ancestral pathogen, the evolved lines showed a low number of single
235 nucleotide variants (SNVs) and structural variants (SV). Overall, there were 32 identified variants. The
236 primed-evolved lines contained 30 variants and the control-evolved lines contained 28 variants. Only
237 a few of those variants were shared across different replicates and no distinctive pattern could be
238 observed between the primed and control evolution regime, or virulent and non-virulent lines (Fig. 5).
239 Common variants in at least 50% of lines included a G to T substitution at position 2,017,894 in an
240 intergenic region between a hypothetical protein and a putative NTP pyrophosphohydrolase, and a
241 small insertion at position 2,776,026 within a hypothetical protein coding sequence, causing a
242 frameshift (Fig 5). Lines C1 and P8 both had unique variants in the same hypothetical protein DUF4430
243 at different positions: while C1 had a small deletion at position 3,358,458, P8 had an insertion at

position 3,357,970. Both variants caused frameshifts, with the P8 insertion also introducing a premature stop codon.

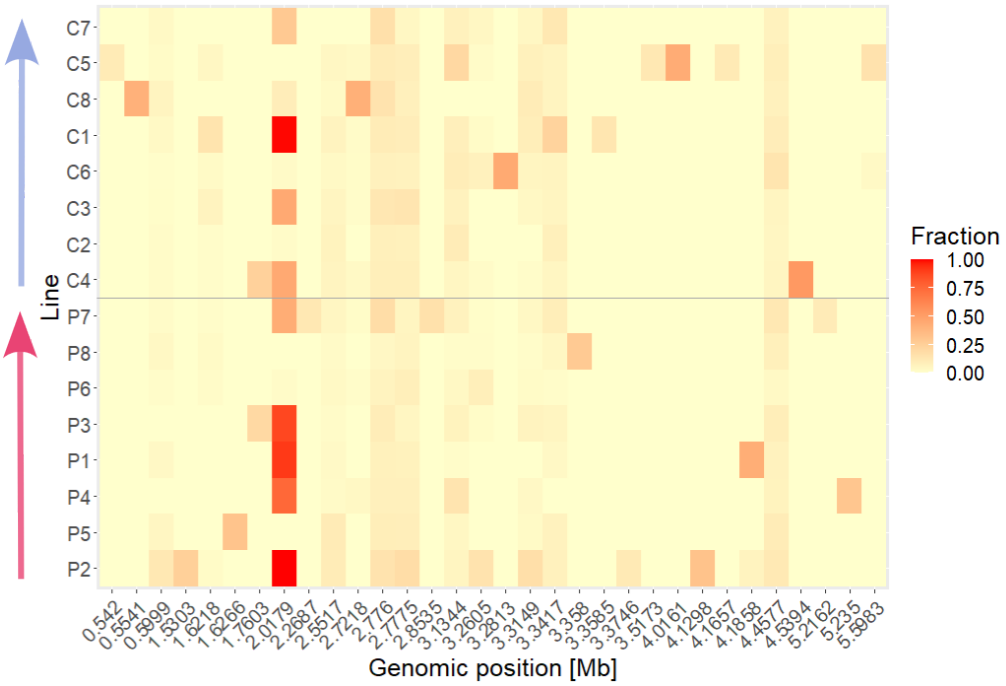
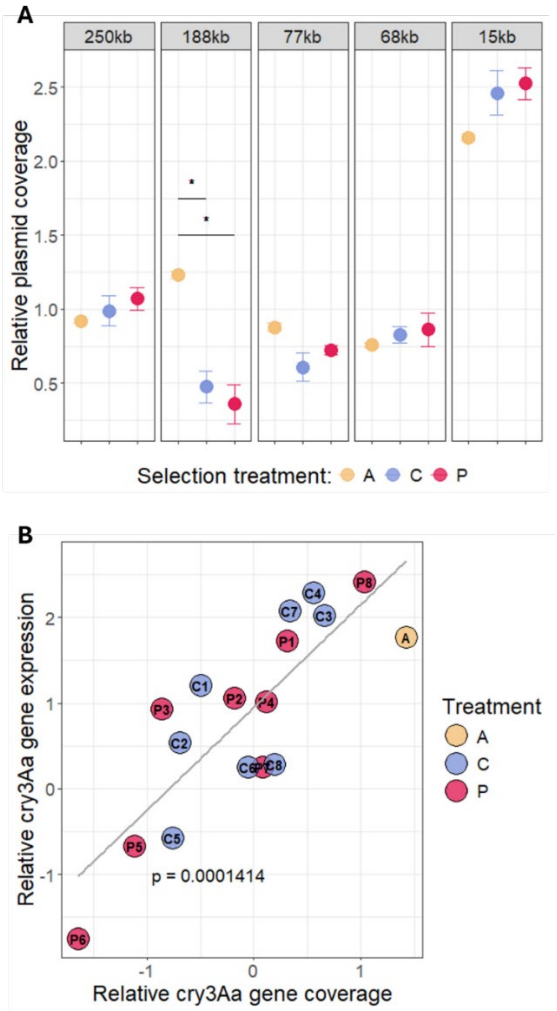


Fig 5. Heatmap displaying the identified single nucleotide variants (SNVs) and structural variants (SVs) at the respective genomic positions. Variants in any line with an alternative allele fraction of >=10% of total read coverage are displayed and alternative allele fraction for each displayed variant was calculated for all remaining lines. Individual lines are ordered within the respective evolution treatment by virulence (*cf.* Fig. 2), from low to high (arrows).

As most bacteria are host to a multitude of mobile genetic elements that can influence the performance in changing environments, we aimed at identifying differences in such elements between the evolution regimes. The NCBI reference sequence (46) contains six plasmid contigs where the two biggest plasmids are 250kb and 188kb, followed by two plasmids of 77kb, 68kb and the smallest 15kb plasmids. The 44kb plasmid is a phagemid, a circularized phage, which additionally is located on the chromosome of the reference sequence and thus was not included in the plasmid coverage analysis. The analysis for plasmid coverage in comparison to chromosome coverage indicated a decrease in the abundance of the 188kb plasmid in the evolved lines compared to the ancestral lines (Fig 6A). However, only line P8 retained a plasmid-to-host ratio similar to the ancestral for this plasmid (Fig S4). This particular plasmid encodes for the important crystal toxin Cry3Aa and other virulence factors (Cry15Aa, Sphingomyelinase C). Additionally, we observed correlation of the log2 transformed Cry3Aa gene-to-

271 chromosome ratio and the relative expression of the *cry3Aa* gene (Fig 6B). However, correlation of
272 other phenotypic traits with active mobile elements showed no statistical significance (Fig S8). The
273 coverage plots across the genome contained peaks in distinct areas of the chromosome (Fig S5) that
274 partially overlapped with annotated prophage areas known as *Bacillus* phages (47). We found that the
275 phages phBC6A52, phi4J1 and phiCM3 had the greatest differences in phage-to-host ratio (Fig 7). We
276 performed double-layer agar assays (DLAs) for two out of 8 lines of each evolution environment and
277 the ancestral strain to verify that phages were active in the evolved lines, but not in the ancestral strain.
278 As expected, we observed visible round plaques in the bacterial lawns of the evolved lines, but not the
279 ancestral lines (Fig S7).



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281 **Fig 6. A Log2 transformed plasmid coverage divided by chromosome coverage (relative plasmid coverage).**
282 Shown are the respective mean values and their standard error. * adjusted $p < 0.05$ **B** Correlation of the Log2
283 transformed *cry3Aa* gene coverage divided by chromosome coverage (relative cry3Aa gene coverage) and delta
284 CT of housekeeping gene expression subtracted by *cry3Aa* gene expression (relative cry3Aa gene expression).

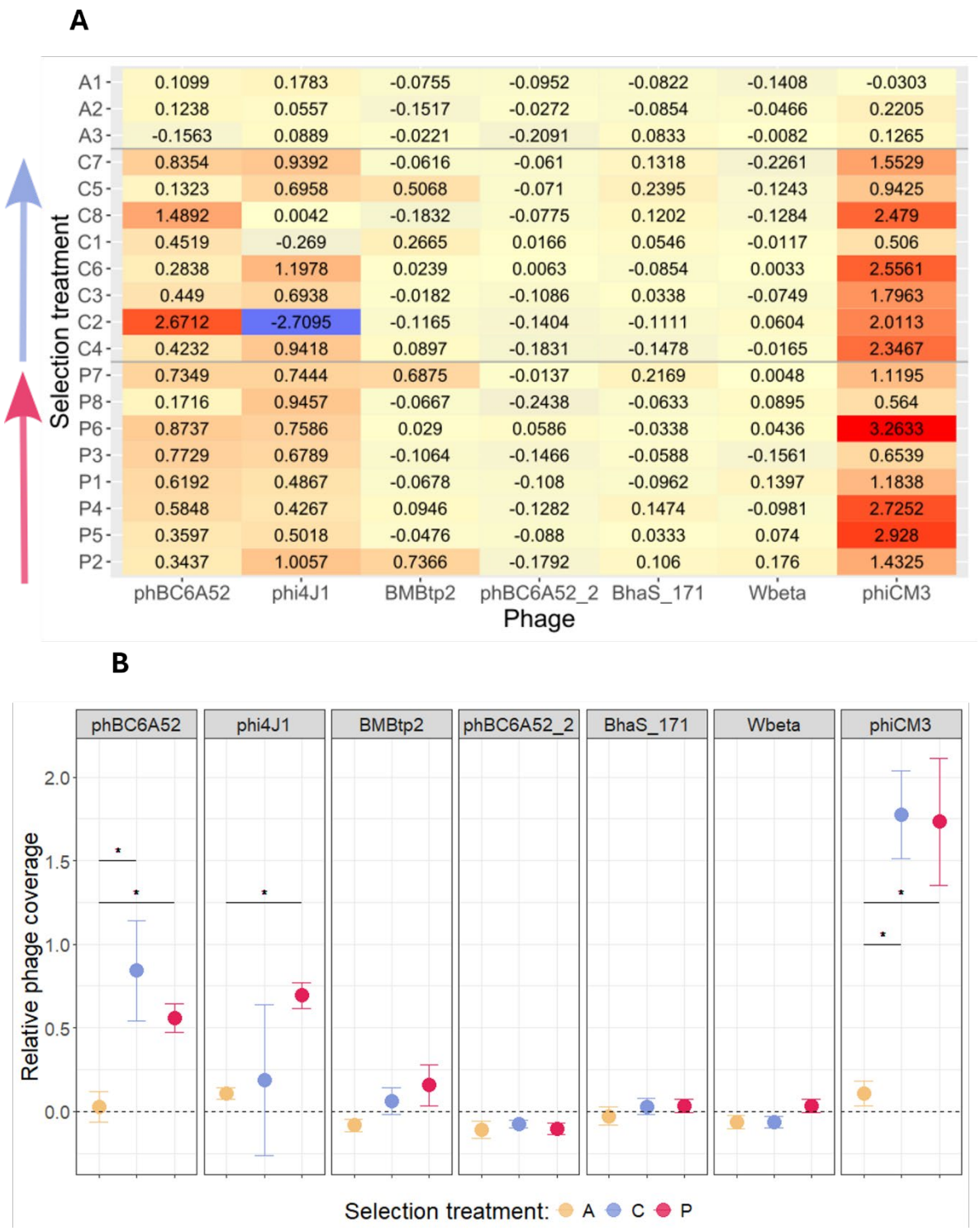

Fig 7. Heatmap for the Log2 transformed values of phage coverage divided by chromosome coverage for each replicate line. A = ancestral, C = control evolved, P = primed evolved. The lines are ordered by evolution treatment and by virulence where the arrow indicates the increase of virulence. **B** Log2 transformed values of phage coverage divided by chromosome coverage (relative phage coverage). Shown are the respective mean values and their standard error. * adjusted $p < 0.05$.

293 # Discussion

294 To investigate the potential effects of immune priming on virulence evolution, we conducted one-
295 sided experimental evolution with *Btt*, using both primed and control *T. castaneum* beetle hosts, and
296 evaluated the phenotype and genotype of the evolved pathogen lines. Although we did not find an
297 increase of virulence in the evolved lines compared to ancestral *Btt* (measured as the proportion of
298 dead hosts), we identified priming as a selective pressure leading to increased variation in virulence
299 (Fig. 2). Some primed-evolved lines showed higher virulence than those of any control-evolved lines
300 and similar to those of ancestral *Btt*, and other primed-evolved lines caused the overall lowest host
301 mortality rates. Another important observation is that the primed-evolved pathogens did not adapt
302 better to the primed host environment. Host priming remained effective and reduced mortality also
303 against the primed-evolved pathogens (Fig. 2C), which were moreover not able to increase spore
304 production in primed hosts (Fig. 3B). This implies that the primed-evolved pathogens failed to develop
305 mechanisms to evade the host's priming immune response. Even the most virulent lines did not
306 overcome the primed immune response, indicating that priming serves as a robust protective measure
307 for the host.

308 Given that our experimental evolution regimes should favour high virulence (extracting the bacteria
309 from larvae that died by infection), the observed lower virulence in the evolved lines than in the
310 ancestral *Btt* isolates was initially unexpected. However, there are a few possible explanations for this
311 outcome. First, we conducted a one-sided adaptation of *Btt*, rather than coevolution. Previous studies
312 have suggested that high virulence may not be favoured due to its costs when the host is not allowed
313 to co-adapt (10). Second, because certain virulence factors are costly and encoded on mobile
314 elements, bacteria may lose them, particularly when propagated in a medium, as demonstrated in
315 previous research (10,48). Indeed, we found a reduction in the overall coverage of the 188kb plasmid
316 encoding for several virulence factors, as well as differences in the expression of the *cry3Aa* gene that
317 correlated with its genomic coverage (Fig. 7). A reduction in Cry toxin production and plasmid carriage

318 during passaging experiments has been shown in other studies using *B. thuringiensis* (49). Such losses
319 can be explained by the advantage of "cheating" cells within a population of *Bt* that do not express
320 *cry3Aa* anymore and thereby gain a growth advantage (49). However, in our experiment, *cry3Aa*
321 expression alone did not explain variation in virulence, and the replicate with the lowest virulence (P2)
322 did not change in *cry3Aa* expression compared to the ancestral strain, whereas the replicate with the
323 highest virulence (P7) showed significantly lower *cry3Aa* expression (Fig. S3). This might be due to the
324 generally less important role of the Cry3Aa toxin for *Btt* virulence in *T. castaneum* (49,50), compared
325 to other insects. This finding also aligns with earlier research demonstrating that purified Cry toxin
326 does not induce beetle mortality, but rather a combination of spores and toxins is required for lethal
327 effects (50,51). Potentially, other virulence factors such as chitinases, metalloproteases or vegetative
328 insecticidal proteins have become more relevant in the evolved lines (41,52). This could be especially
329 relevant in primed-evolved lines, because a recent study indicates that Cry toxins are involved in the
330 induction of the priming response (53). Finally, previous studies have shown that evolution can result
331 in reduced virulence due to the interaction with defensive microbes of the host (19) or due to
332 antagonistic interactions among different pathogen strains (54).

333 Compared to ancestral *Btt*, evolved *Btt* showed reduced sporulation in liquid medium as well as in
334 primed hosts (Fig. 4 and 5). This decline in sporulation may be attributed to the presence of active
335 prophages that we identified in the genomes of the evolved lines (Fig 7). The activation of prophages
336 and the transition from lysogeny (dormant state) to the lytic cycle (active viral replication and bacterial
337 cell lysis) are often triggered by environmental factors (55). Such stress can occur under constant
338 sporulation conditions and is known to cause losses in other *Bacillus* fermentation and to interfere
339 with the evolution of virulence of phages towards their bacterial host (56,57). Interestingly, that a
340 reduction of spore production of evolved lines occurred only in primed but not control hosts (Fig. 4
341 A,B) could suggest that phage induction is primarily promoted in primed hosts. This environment
342 represents a more stressful niche for the pathogen, as priming leads to the damage in the gut of *T.*
343 *castaneum* larvae, triggering an early physiological stress response along with the upregulation of

344 immune related genes (58). Additionally, antagonistic competition may maintain these phages (59).
345 *Bacillus* is a member of the microbiome of *T. castaneum* and immune priming can cause a shift towards
346 higher abundance of *Bacillus* species (60). Releasing lytic phages could provide a competitive
347 advantage by targeting members of the same genus or species. Alternatively, mutations in phage
348 binding sites can prevent lysis by lytic phages (61).

349 While observing changes in phages and plasmids, we could not pinpoint any common chromosomal
350 mutations that are specific to the selection treatments (Fig. 6). This does not exclude that there might
351 be more subtle genetic changes that went undetected because they might be specific to certain
352 replicates. Indeed, the observed increase in variation in virulence indicates that replicates may take
353 different evolutionary routes (62) making it difficult to find common genetic changes.

354 Moreover, the lack of a link between spore load and virulence might suggest that *Btt* virulence and
355 transmission evolve independently. A comparable result was noted in the pathogen *Pseudomonas*
356 *aeruginosa* following evolution in hosts with partial immunity (17). Unlike our findings, the incomplete
357 immunity in that study resulted in increased pathogen virulence. Nevertheless, our results are in line
358 with the mentioned study that revealed that the most virulent pathogens did not develop mechanisms
359 to counteract the protective benefits of priming exposure. It is worth noting, however, that in this case,
360 the priming or partial host immunity was non-specific and triggered by the host's microbiota, while in
361 our study priming is specific and triggered by the pathogen cues (39,53).

362 The increased variance in virulence particularly in primed-evolved hosts might be explained by the
363 more variable niche that a primed host represents for the pathogen (63). *Btt* in primed *T. castaneum*
364 hosts are not only confronted with an increased intensity and diversity of immune defences (44), but
365 moreover with a changed microbiome composition (60). More generally, immune-primed hosts
366 represent a fluctuating environment, known to maintain high levels of phenotypic and genetic
367 variation in microbial populations, e.g. through balancing selection (64) and pleiotropic effects (65).
368 Antagonistic pleiotropy might also stem from active phages that could be beneficial in one, but

369 detrimental to another environment (66). Surprisingly, pathogen lines evolved in primed host showed
370 more priming sensitivity, i.e., lower virulence (Fig 4 A) in the primed host compared to ancestral and
371 control evolved pathogen. However, the assessment of virulence was limited to host mortality. It is
372 possible that the pathogen evolved to prolong the host's survival whilst influencing its development,
373 which we didn't measure in our study. The observed trend of a negative association between the
374 fitness of the primed-evolved pathogen and its priming sensitivity suggests that pathogen strains with
375 higher fitness are less affected by the host's primed immune response (Fig 4). This indicates that fitter
376 pathogens may have developed strategies to more effectively resist or evade the immunological
377 effects of host priming. To gain a more comprehensive understanding, the host's response to the
378 evolved pathogens, particularly in terms of potential downregulation of immune pathways, could be
379 analysed.

380 Our observation of increased virulence variation, rather than increased virulence, may also result from
381 differences in our system compared to models of leaky vaccines, where the pathogen is directly
382 transmitted between vaccinated hosts (12,14,67). In contrast, *B. thuringiensis*, both in our experiment
383 as well as in nature, experiences two distinct environments, as it can thrive outside of its insect hosts
384 (68). In addition to host factors, the longevity of spores and their ability to persist in the environment
385 may also play a role in determining virulence (69).

386 In conclusion, our results are to our knowledge the first to show an effect of specific immune priming
387 on the evolution of bacterial virulence. The observation of increased variance in virulence is
388 evolutionary relevant because selection needs variation to act upon. Therefore, host immune priming
389 and other forms of incomplete, innate immune memory, may boost the evolutionary potential of
390 pathogens and even lead to multiple evolutionary stable states (11). This is important because innate
391 immune memory bears a large potential for applications. Not only the medical relevance of trained
392 immunity in medicine, but also applications of invertebrate immune priming in fields such as
393 aquaculture and insect mass production are gaining momentum (70,71). Studies in further systems

394 with immune priming and trained immunity are needed to better understand and potentially manage
395 the evolution of pathogens when confronted with innate immune memory.

396 Materials and methods

397 Model organisms

398 As pathogen, we used *Bacillus thuringiensis* subspecies *morrisoni* biovar *tenebrionis* (*Btt*) from the
399 Bacillus Genetic Stock Centre (BGSCID), Ohio State University, USA) under code 4AA1. The strain was
400 stored in 500 µL microtubes containing 25% glycerol at -80°C. This strain has been shown to cause
401 mortality in *T. castaneum* larvae upon the oral ingestion of spores and their toxins (42,43).

402 As the host, we used a wild-type *T. castaneum* (Cro1) population collected from Croatia in 2010 (42)
403 and adapted to laboratory conditions for eight years in numerous non-overlapping generations until
404 the start of the experiment in 2018. Beetles were reared on organic wheat flour (Bio Weizenmehl Type
405 550, DM-drogerie Markt GmbH + Co. KG) supplemented with 5% brewer's yeast (the flour mixture was
406 heat-sterilised at 75°C for 24 h). For rearing and infection experiments, beetles were kept at 30°C, 70%
407 relative humidity, and a 12-h/12-h-light–dark cycle.

408

409 Experimental evolution

410 Preparation of bacteria for selection treatment and infections

411 We prepared spore cultures and supernatants as previously (39,40), with minor modifications. To
412 begin the selection experiment, we plated *Btt* vegetative cells from the -80°C glycerol stock on an LB
413 agar plate and incubated it overnight at 30°C. The following day, we inoculated 5 ml of Bt medium
414 (w/V–0.75% Bacto Peptone (Sigma), 0.1% glucose, 0.34% KH2PO4, 0.435% K2HPO4) that was
415 supplemented with 25 µl of a sterile salt solution (0.2 M $MgSO_4$, two mM $MnSO_4$, 17 mM $ZnSO_4$, and

416 26 mM $FeSO_4$) and 6.25 µl of sterile 1 M $CaCl_2 \times 2H_2O$ with a single colony, and incubated it at 30°C and
417 200 rpm overnight. Overnight cultures were moved to 1-L Erlenmeyer flasks containing 300ml of Bt
418 medium supplemented with 1.5 mL of salt solution and 375 µL 1 M $CaCl_2 \times 2H_2O$. Since *Btt* is naturally
419 neomycin-resistant, 15µg/µL of neomycin were added to the sporulation media. This step was taken
420 to guarantee that other spore-forming bacteria that could come from cadavers did not proliferate in
421 the medium. The cultures were kept at 30°C and rotated at 180 rpm for seven days in the dark. On the
422 third day of incubation, 1.5 mL of salt solution and 375 µL of 1 M $CaCl_2 \times 2H_2O$ were added to the
423 cultures. Following seven days of sporulation, the culture was spun for 20 minutes at 4500 rpm. The
424 pellet was washed and redispersed in PBS (Calbiochem). Using means of flow cytometry (FACSCanto
425 II; Becton Dickinson, USA) as described in Milutinović et al. 2015, spore concentration was adjusted to
426 5 x 10^9 /mL, and the diet was prepared by adding heat-sterilized flour with yeast (0.15 g/mL of spore
427 suspension; (42,43). We then pipetted the liquid spore-containing diet (10 µL per well) into the 96 flat
428 bottom microtiter plates (Sarstedt, Germany) under sterile conditions, covered them with a breathable
429 sealing foil for culture plates (Kisker Biotech), and dried them overnight (~17 hours) at 30°C. For all
430 remaining selection cycle experiments, where spores were taken from cadavers, we slightly adjusted
431 the protocol; after processing spores, we inoculated 50 mL of Bt medium supplemented with salts and
432 neomycin in a 100 ml Erlenmeyer flask, followed by seven days incubation at 180 rpm and 30°C.

433

434 Preparation of primed and control host for the selection treatment

435 The primed host environments were performed as previously described (39,40). *Btt* vegetative cells
436 were plated from a -80°C glycerol stock on LB agar plates and incubated overnight at 30°C. Five single
437 colonies were incubated overnight, and sporulation cultures were produced as described above but
438 without adding neomycin. After seven days of sporulation and centrifugation, the collected
439 supernatants were centrifuged twice at 4,500 rpm for 15 min and then filter-sterilized with a 0.45-µm
440 pore-size, followed by a 0.2-µm pore-size cellulose acetate filter (Whatman GmbH). We mixed every

441 millilitre of spore-free supernatant with 0.15 g heat-treated flour (supplemented with 5% yeast). Next,
442 ten microliter of the priming diet mixture was pipetted into each well of a 96-well plate (Sarstedt,
443 Germany), sealed with a breathable foil (Kisker Biotech), and dried overnight at 30°C. For the control
444 treatment, the diet was prepared by mixing 0.15 g heat-treated, yeast-supplemented flour with each
445 millilitre of sterile non-conditioned growth medium.

446 For every selection treatment cycle, we took approximately 2,000 1-month-old adults and left them to
447 lay eggs for 24 hours on the flour. The priming protocol was as follows: 15 days after oviposition, 192
448 larvae per replicate line were individually placed on a previously prepared priming diet in two 96-well
449 flat-bottom plates. The plates were sealed with clear tape and punctured with needles to allow air
450 circulation and were stored under standard rearing conditions. After 24h on a priming diet, we
451 transferred larvae to flour discs consisting of phosphate-buffered saline (PBS) mixed with 0.15 g/mL
452 flour, in which they stayed for four days until we transferred them again to pathogen spore-containing
453 discs.

454 Re-infection for another selection treatment cycle

455 We measured mortality for seven days and then collected larvae that died during the first two days,
456 placed them in the dark, and waited for another seven days to ensure that all *Btt* cells in the cadaver
457 had sporulated. For reinfection, we isolated spores from insect cadavers and propagated them once in
458 liquid sporulation media to obtain a sufficient number of spores for infection. For each bacterial line,
459 we isolated spores from five cadavers, which were subsequently grown independently in a growth
460 medium to achieve high spore numbers for the infection of a sufficient number of beetle larvae for the
461 next infection cycle. Spores from the five cultures derived from the cadavers were combined for
462 infection to reduce the effect of potential clonal interference among rapidly growing clones in culture.
463 We randomly selected five cadavers per individual line and individually homogenized them as
464 described before (43). We then counted spore concentrations using flow cytometry and pasteurized
465 the suspensions at 80°C for 10 minutes (i.e., any possible vegetative cells are killed). Liquid medium

466 was inoculated with 1mL of 1x10^6 spores derived from cadavers for germination, followed by spore
467 production. After seven days of incubation, we pooled all five individually raised spore cultures and
468 prepared the spore-flour diet. Primed and control host larvae were then placed on spore flour discs,
469 and their survival was tracked.

470 Phenotypic readouts

471 Virulence and spore production of evolved bacterial lines

472 All the selection treatment lines were introduced to a primed and control host environment. Host
473 mortality was screened for six days. We performed this experiment in three experimental blocks, with
474 48 larvae per treatment/block, resulting in 144 larvae per treatment combination. For every block, we
475 took approximately 2,000 1-month-old adults and left them to lay eggs on the flour (implemented with
476 5% yeast) for 24 hours, following the priming protocol described above. For each block, we prepared
477 12 96-well plates with a priming diet and 12 96-well plates with control diet. We placed 15-day-old
478 larvae on the plates and, after 24 hours, transferred them to PBS flour discs. After four days, we
479 transferred them to 96-well plates containing spores from 24 bacterial lines (eight Primed evolved,
480 Control evolved and Ancestral). Each 96-well plate contained spores from all 24 bacterial lines to
481 control for the possible plate effect. This resulted in 24 96-well plates for each experimental block.

482 Spores from the bacterial lines were prepared as described above. Frozen spores from the 8th passage
483 and ancestral *Btt* were first plated on an agar plate and after 14 hours 10 colonies were inoculated in
484 50 mL growth medium supplemented with salts. After seven days of bacterial growth and sporulation,
485 spores were washed, and measured via flow cytometry, and the concentration was adjusted to
486 $5x10^9$cells/mL. We used measures of the numbers of spores in a liquid medium from three
487 experimental blocks to test for the differences in spore production between the lines.

488 We quantified the pathogen load of infected larvae that died from the infection. For this, we took ten
489 cadavers per bacterial line, which resulted in a total of 240 cadaver samples. The samples were

490 processed as described before (43). Each cadaver was placed in a 100 µl Eppendorf tube with 50 µL
491 PBS and a sterile metal bead. The cadavers were homogenized using a Mixer Mill MM301 (Retsch) to
492 release spores into the solution. The solution was then washed using a cell strainer to remove larger
493 particles. The solution was diluted 1:1000 and measured via flow cytometry.

494 Cry gene expression in Btt cultures

495 Before RNA extraction, we inoculated two cultures of ancestral and evolved lines in 6 mL LB medium
496 at 30°C, 180 rpm. Following 12 h of incubation, the cultures were centrifuged at 4500 rpm for 10 min
497 and resuspended in 100 µL of PBS. We added 2.2 µl of lysozyme (100 mg/mL) to 100 µL of the bacterial
498 suspension and incubated for 30 min at 37°C with shaking. Subsequently, we implemented an RNA
499 extraction protocol with a combination of TRIzol reagent (Ambion, USA) lysis and extraction with the
500 purification via spin columns from the SV Total RNA Isolation System (Promega) as described before
501 (72)**.** To analyze *cry3a* expression, cDNA synthesis, and qPCR were performed in two identically
502 designed blocks. We conducted reverse transcription of cDNA using the RevertAid First Strand cDNA
503 Synthesis Kit (ThermoScientific) following the manufacturer's instructions. For the reaction, we used
504 2µL of extracted RNA, irrespective of its concentration, along with a random hexamer primer. The
505 resulting cDNA product was then diluted 1:5 with nuclease-free water.

506 Following cDNA synthesis, we performed qPCR. This analysis involved measuring the expression of
507 *cry3a* relative to two housekeeping genes (*yqey* and *rps21*) (Supp. Table 1). **Error! Reference source**
508 **not found.** Prior to the main experiment, we determined the amplification efficiency of the primers by
509 qPCR using a serial 10-fold dilution of the initial cDNA input (Suppl. Table 1).

510

511 # Genome analysis

512 ## Sequencing

513 After culturing samples from all evolved and three ancestral replicate lines on LB agar plates, we
514 extracted DNA using the Monarch Genomic DNA Purification Kit (New England Biolabs GmbH,
515 Frankfurt am Main, Germany). For sequencing on the PacBio Sequel Ile platform (Pacific Biosciences
516 Inc., Menlo Park, CA, USA), we created a library with the SMRTbell Express Template Prep Kit 2.0
517 (Pacific Biosciences Inc.) according to the manufacturer's recommendations, aiming for at least 20x
518 coverage on the HiFi read level. We then mapped the resulting HiFi reads against the sequence of a *Btt*
519 reference strain (NCBI accession no.: GCF_022810725.1) using the SMRT Link software version 11.

520

521 ## Variant detection

522 To call single nucleotide variants (SNVs) we used GATK Haplotypecaller (v4.3.0.0) (73) and Google's
523 DeepVariant (74) on the Galaxy Server (Galaxy Version 1.5.0+galaxy1). We then intersected the
524 resulting vcf files of the evolved lines with those derived from the ancestral lines using the bcftools
525 isec function (version 1.16) (75) to filter out variants that were based on differences between our
526 ancestral lines and the used reference sequence. Of the remaining SNVs we kept those with an
527 alternative allele fraction of >= 10% of the total read coverage at the respective position. To call
528 structural variants (SVs) we used Sniffles2 (Version 2.2) (76), The resulting vcf files were intersected in
529 the same way as the SNV vcf files. Next, we verified all final variants by manually inspecting them in
530 the Integrative Genomics Viewer (IGV) (77). To annotate the SNVs we used SnpEff (version 5.2c (build
531 2024-04-09 12:24)) (78) using the prokaryotic codon table and the reference genbank- and nucleotide
532 fasta files.

533

534 ## Plasmid coverage

535 We calculated the coverage across the chromosome and the different reference plasmids respectively
536 using samtools depth (version 1.16.1) (75) and estimated the abundance of plasmids by dividing each
537 plasmids mean coverage by the chromosomes mean coverage and taking the log2 of those values. We
538 termed the resulting values "Log2(Plasmid-to-Host ratio)".

539 ## Phage analysis

540 To detect potentially complete prophage genomes within our reference genome we used PHASTER
541 (79). Next, we calculated the coverage across the genome using samtools depth and divided the
542 genome into sections of a) outside of prophage regions and b) within prophage regions. We considered
543 b) to be true if the region contained all essential phage genes necessary for its reproduction (from
544 small terminase to lysis cassette). Finally, we divided the means of b) by the means of a) and took the
545 log2 of those ratios. We termed the resulting values "Log2(Phage-to-Host ratio)". The log2 transformed
546 Phage-to-Host ratios were then plotted using ggplot2 in Rstudio. To confirm the presence of active
547 phages in the evolved lines, we performed double-layer agar assays (DLA). In short, we grew bacteria
548 in 20 ml Luria broth (LB) medium overnight. The next morning, we adjusted the overnight culture to
549 an optical density of 1 at 600 nm wavelength (OD_{600} of 1) and inoculated 500 µL into 50 ml fresh LB
550 medium. After four hours, 8 hours, and 12 hours, we took 1 ml of the culture and adjusted it to an
551 OD_{600} of 1.5. We then mixed 400 µl of the culture with 4 ml lukewarm 0.7% LB agar and poured the
552 mix onto a solid 1.5% LB agar plate. After 24 h, we checked the plates for plaques in the bacterial lawn.

553 # Statistics

554 All data were analysed using R (80) and R Studio (81). To test whether the pathogen selection
555 treatment and host environment have an effect on the virulence, we tested the effect of selection
556 treatment (primed-evolved, control-evolved and ancestral) on the host survival by fitting a generalized
557 linear mixed effect model with interaction (GLMM) and binomial error distribution using *the glmer*

558 function in the package *lme4* (82) (mortality~ selection_treatment*host environment)
559 +(1|Replicate_line)+ (1|Block)+(1|Plate). The selection treatment level and host environment were
560 defined as fixed factors, whereas a bacterial line, plate and block were defined as random factor. As
561 interaction was not significant, we then fitted a model with selection treatment as a fixed factor in
562 primed and control host environments separately. To check whether the random effect for Replicate
563 line shows more variation in one selection treatment compared to the other, we fitted a random slope
564 model where the slope of Selection_treatment is allowed to vary across Replicate_line: (mortality ~
565 Selection_treatment + (1 | Replicate_line) + (1 | Block) + (1 | Plate). After fitting a model, we examined
566 the variance estimates for replicate line within selection treatment. To further test of the treatments
567 influence the variation in mortality, we ran a Bayesian Beta regression model in rstan (Stan
568 Development Team 2023) where we allowed the precision parameter (ϕ) to be influenced by the
569 Selection treatment. Then, we used the posterior samples of the model to estimate the probability
570 that the variation in mortality arising from primed, control and ancestral populations are different.

571 For spore load (transmission) analysis, the *lmer* function in the *lme4* package was used with the
572 bacterial line as a random factor (spore_load~ selection_treatment +(1|replicate_line) in primed and
573 control host environment separately. For spore growth analysis we performed linear regression
574 analysis with *lm* function. After fitting the models, the variance between the selection treatment
575 (primed-evolved, control-evolved, ancestral) was assessed using one-way analysis of variance. The
576 means were compared using Tukey's post-hoc analysis with Benjamini-Hochberg correction.

577 Priming sensitivity of each bacterial replicate was determined by dividing proportion of mortality in
578 control host with proportion of mortality in primed host. Kruskal-Wallis test was used to assess
579 differences in priming sensitivity between the selection treatment levels. The means were compared
580 using post-hoc pairwise comparisons using Dunn's test with a Benjamini-Hochberg correction.

581 We determined fitness in the host by combining spore load and proportion of mortality per individual
582 bacterial line. Then we compared it between selection treatment levels using *lmer* function

583 (Fitness_in_host ~ Line * Environment + (1 | Replicate_line). The means were compared using Tukey's
584 post-hoc analysis with Benjamini-Hochberg correction.

585 To analyze gene expression data from qPCRs, we first compared the relative expression of *cry3a* of the
586 primed and control-evolved *Btt* to the expression in the ancestral *Btt* cultures in a linear mixed model
587 with experimental block and evolved replicates as random factors (relative expression ~
588 selection_treatment + (1|block) + (1|replicate)) using the packages *lme4* and lmerTests (83). We also
589 fitted a second linear mixed effects model using the same packages to compare the evolved lines to
590 the ancestral cultures (relative expression ~ selection_treatment + (1|block)).

591 We used various statistical techniques to evaluate the relationships among different variables. The
592 connections between spore load and mortality, mortality and cry expression, and average host fitness
593 with priming sensitivity were analyzed using Pearson's correlation method. We conducted Mann-
594 Whitney tests using the wilcox.test() function to evaluate the significance of differences between log2
595 transformed Plasmid-to-Host and Phage-to-Host ratios. The p.adjust() function with the "holm"
596 adjustment method was applied to address multiple testing concerns. A linear model was constructed
597 to investigate significant interactions between the read coverage for the cry3Aa gene and its
598 expression. The cor() function from the R Stats package (version 4.3.1) was used to determine the
599 correlation strength between different phenotypic readouts and active mobile elements for both
600 primed evolved lines and control evolved lines. To statistically compare the correlation strengths of
601 various readouts between primed and control evolved lines, we utilized the cocor() package (version
602 1.1-4) (84).

603

Data availability

The genomic sequences can be found on NCBI under project number: PRJNA1174726 (https://dataview.ncbi.nlm.nih.gov/object/PRJNA1174726?reviewer=cmkqpsc4dunlettul69l9ta251). It will be made publicly available upon acceptance.

All the data sets, code and RMarkdown file with figures and analysis results can be found: Korša, A., Baur, M., Schulz, N., Anaya-Rojas, J. M., Mellmann, A., & Kurtz, J. (2024). Experimental evolution of a pathogen confronted with innate immune memory increases variation in virulence [Data set]. Zenodo. https://doi.org/10.5281/zenodo.14223031

Authors contribution

AK and JK conceptualisation. AK, MB, NS and AM data curation and investigation. AK, MB, NS, and JAR visualization and formal analysis. AK, MB and JK writing – original draft preparation. AK, MB, NS, JAR, JK, and AM writing – review & editing. JK supervision and funding acquisition.

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