



A mid-gut microbiota is not required for the pathogenicity of *Bacillus thuringiensis* to diamondback moth larvae.

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2 A mid-gut microbiota is not required for the pathogenicity of *Bacillus*
3 *thuringiensis* to diamondback moth larvae.

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1 Summary

2 The mode of action of the entomopathogenic bacterium *Bacillus thuringiensis* is
3 still a matter of debate. Recent reports have claimed that aseptic lepidopteran
4 hosts were not susceptible to *B. thuringiensis* (*Bt*) and that inoculation with mid-
5 gut bacteria restores pathogenicity. These claims are controversial because
6 larvae were rendered aseptic by consuming antibiotics, although the effect of
7 these antibiotics on *Bt* was not examined. We tested the generality of the mid-gut
8 bacteria hypothesis in the diamondback moth, *Plutella xylostella* using properly
9 controlled experiments that investigated the effect of antibiotic consumption and
10 absence of gut microbiota separately. We found that purified *Bt* toxin and
11 spore/toxin mixtures were fully pathogenic to larvae reared aseptically.
12 Persistence of antibiotics in larval tissues was implicated in reducing host
13 mortality since larval consumption of the antibiotic rifampicin reduced the
14 pathogenicity of rifampicin sensitive *Bt* strains but not rifampicin resistant
15 strains. Inoculating larvae with *Enterobacter* sp. Mn2 reduced the mortality of
16 larvae feeding on *Bt* HD-1 and the presence of a culturable gut microbiota also
17 reduced the pathogenicity of the *Bt* toxin Cry1Ac, in agreement with other
18 studies indicating that an intestinal microbiota can protect taxonomically diverse
19 hosts from pathogen attack. Since ingestion of antibiotics suppresses host
20 mortality the vegetative growth of *Bt* in the host must be important for its
21 pathogenicity. Furthermore, claims that aseptic larvae are not susceptible to *Bt*
22 must be supported by experiments that control for the effect of administering
23 antibiotics.

24

25

1 **Introduction**

2 *Bacillus thuringiensis* (*Bt*) is a gram positive spore forming invertebrate pathogen that
3 is widely exploited in pest management. Its utility derives from the large quantities of
4 proteinaceous toxins that form crystalline parasporal inclusions, termed the Cry and
5 Cyt proteins (Schnepf et al., 1998). *Bt* toxin genes have been widely incorporated into
6 genetically modified insect resistant crops (ISAAA, 2007) but are also widely
7 exploited as biopesticides with target hosts ranging from mosquitoes to lepidopteran
8 pests of horticulture and forestry (Glare and O'Callaghan, 2000). Although the
9 structure and mode of action of *Bt* Cry toxins has been intensively researched, the
10 biology and ecology of the bacterium is not fully characterized (Jensen et al., 2003;
11 Raymond et al., 2008a). Disagreement on the biology of *Bt* extends to the exact
12 mechanism responsible for the death of the host. A purely toxin based hypothesis
13 suggests that the action of predominantly Cry toxins paralyses the mid-gut and leads
14 to eventual death by starvation (Knowles, 1994). Alternatively, Cry toxins cause
15 extensive cell lysis in the mid-gut that allows bacteria access to the haemolymph, thus
16 rapid vegetative growth of *Bt* within the host (septicaemia) is proposed as an
17 alternative cause of death (Schnepf et al., 1998).

18 Recently, a novel pathogenic mechanism was suggested by the claim that *Bt*
19 and its toxins are incapable of killing aseptically reared gypsy moth, *Lymantria*
20 *dispar*, larvae but that pathogenicity can be restored by inoculating hosts with a gut-
21 associated strain of *Enterobacter* (Broderick et al., 2006). More recent work has
22 extended this claim to a wide range of insect hosts (Broderick et al., 2009). An
23 obligate association with the gut microbiota challenges previous models of the
24 pathogenicity of *Bt* toxins, and has considerable implications for the ecology of *Bt* and
25 the evolution of novel resistance mechanisms in invertebrate pests. The gut

1 microbiota hypothesis remains highly controversial, given previous evidence of the
2 lethality *Bt* in aseptically-reared insects (Takatsuka and Kunimi, 2000) and the
3 synergistic action of the *Bt* spore on the toxicity of crystal toxins in some hosts
4 (Heimpel and Angus, 1959; Li et al., 1987; Liu et al., 1998).

5 Moreover, the experimental findings of Broderick et al. (2006, 2009) suffer
6 from two issues that may confound their results. First, the authors administered high
7 doses of four broad-spectrum antibiotics to hosts in order to eliminate their gut
8 microbiota, but they did not directly control for the effects of antibiotics on *Bt* itself.
9 Second the work exclusively used a strain of *Bt* (*B. thuringiensis* subsp. *kurstaki* HD-
10 1) that was isolated from a commercial biopesticide (DiPel DF). This strain grows
11 poorly in some hosts (Raymond et al., 2008a) and while the precise evolutionary
12 background of this strain is unknown, biopesticide strains are typically selected for
13 increased toxin production in artificial media and may have undergone little recent
14 selection in live hosts.

15 Here we aimed to test the applicability of the claim that a culturable gut
16 microbiota is required for the pathogenicity of *Bt* and its Cry toxins in an alternative
17 host, the diamondback moth, *Plutella xylostella*, and by using protocols which
18 produced aseptic larvae without directly administering antibiotics. More specifically,
19 we tested two hypotheses: (1) that strain attenuation in biopesticides leads to a
20 dependence on culturable gut microbiota for pathogenicity and (2) that the carry-over
21 of ingested antibiotics in host larvae can reduce the pathogenicity of *Bt*.

22

23 **Results**

24 *Toxicity of purified Bt CryIAC protoxin in aseptic and conventionally reared larvae*

1 Cry1Ac toxicity bioassays showed that a culturable midgut microbiota was not
2 required to induce host mortality. Moreover, purified protoxin was approximately
3 twice as lethal in aseptically reared larvae in comparison to larvae with a well-
4 developed gut microbiota (Table 2; effect of rearing treatment $\chi^2 = 52.0$, $df = 1$, P
5 $\ll 0.0001$; log-transformed assay dose $\chi^2 = 467.0$, $df = 1$, $P \ll 0.0001$). We found that
6 16S rRNA genes could not be amplified from aseptically reared DBM larvae (15/15
7 larvae) whereas amplicons were consistently produced from non-sterile larvae reared
8 in the absence of antibiotics (15/15 larvae). Similarly, culturable bacteria were only
9 found within the non-sterile larvae. Note that these non-sterile larvae were derived
10 from non-sterile parents in contrast to the bacterial bioassays below. Sequencing of a
11 subset of gut bacteria from this population showed that this population is typically
12 colonized by *Enterococcus* spp. and that *Enterobacter* sp. isolates were relatively rare.

13

14 *Testing the pathogenicity of biopesticidal, wild-type and host passaged Bt strains in*
15 *aseptically-reared and Enterobacter inoculated larvae*

16 We investigated whether attenuation in strains derived from biopesticides would
17 affect their ability to kill aseptic hosts. To this end we compared the pathogenicity of
18 three biopesticidal strains; three wild type isolates of *Bt* and three passaged strains
19 (R1-K, R2-K and R3-K) in aseptically-reared rifampicin fed *P. xylostella* larva. All
20 passaged strains were originally isolated from a spontaneous rif^R mutant of the
21 biopesticide DiPel WP (*B. thuringiensis kurstaki* HD-1) and subsequently infected
22 and re-isolated three times in *P. xylostella* larvae.

23 If strain attenuation had affected the pathogenicity of *Bt* biopesticides in
24 aseptic hosts then WT and host passaged strains should incur higher mortality rates
25 than the biopesticide strains in the absence of any gut microbiota. In addition, if the

1 gut microbiota hypothesis is general none of the nine isolates in this experiment
2 should be pathogenic to these hosts. We found neither of these patterns. The passaged
3 rifampicin resistant (rif^{R}) strains were highly pathogenic to aseptic, rifampicin treated
4 *P. xylostella* and there were clear differences in the mortality imposed by the three
5 groups of stains (binomial glm $F_{2,23} = 77.1$, $P < 0.0001$; Figure 1). Comparisons
6 (using *post hoc* treatment contrasts) between these groups of strains showed that the
7 rif^{R} strains were more pathogenic than either the WT ($t = 8.68$, $P < 0.0001$) or the
8 biopesticide strains ($t = 5.26$, $P < 0.0001$) (Figure 1). Overall, strains isolated from
9 similar sources had similar pathogenicity, *i.e.* there was no significant effect of
10 pooling strains into their three groups (biopesticides, wild-type, and passaged strains)
11 ($F_{9,21} = 1.08$, $P = 0.47$). Bioassay dose had a strong effect on larval mortality ($F_{1,25} =$
12 9.72 , $P < 0.0001$) and increasing bioassay doses had less effect on the pathogenicity
13 of WT and biopesticide strains (dose X strain interaction $F_{2,21} = 7.20$, $P = 0.004$).
14 Dose effects have not been plotted for graphical simplicity (Figure 1).

15

16 *Distinguishing between the effect of antibiotic treatment and absence of gut*
17 *microbiota on the pathogenicity of Bt*

18 There were two possible explanations for the results of the previous experiment,
19 either rifampicin in larval diet was persisting in larval hosts and inhibiting *Bt*
20 pathogenicity or culturable mid-gut microbiota are required for the pathogenicity of
21 both WT and biopesticide strains but not the recently passaged rif^{R} strains. We
22 conducted two full factorial experiments with different isolates of *Bt* to tease apart
23 these explanations and to test whether the absence of gut microbiota or treatment with
24 antibiotics was responsible for inhibiting the pathogenicity of a proportion of *Bt*
25 strains. These experiments used both antibiotic sensitive and rif^{R} strains of *Bt* HD-73

1 and *Bt* HD-1 strains. In these experiments the majority of larvae died by the end of
2 the five-day observation period and survival data was analysed using a Cox
3 proportional hazard model. As above, all treatments were replicated at three doses of
4 *Bt* with approximately 30 larvae per dose. The experiments with HD1 and HD73
5 used the same generation of insects, however, the HD1 experiment was initiated a
6 week earlier with second instar larvae; the HD73 experiment used third instar larvae.

7

8 In both experiments antibiotic sensitive strains of *Bt* were pathogenic to larvae reared
9 aseptically without antibiotics (Fig. 2, Fig. 3), and there was no evidence that absence
10 of a culturable gut microbiota was inhibiting the pathogenicity of this bacterium. In
11 contrast, we did find evidence that carry-over of antibiotics in hosts could affect the
12 pathogenicity of *Bt*: larval consumption of rifampicin substantially reduced the
13 pathogenicity of all antibiotic sensitive strains, but did not reduce the mortality of the
14 rif^R strains (HD-1 experiment: strain*diet interaction $df = 1$, residual $df = 824$, $\chi^2 =$
15 250, $P \ll 0.001$; HD-73 experiment: strain*diet interaction $df = 1$, residual $df = 761$,
16 $\chi^2 = 37$, $P \ll 0.001$).

17 The effect of inoculation with *Enterobacter* sp. Mn2 was different in the two
18 experiments. In the HD-1 experiment the presence of *Enterobacter* sp. Mn 2 had a
19 small but significant effect on reducing the pathogenicity of both the rifampicin
20 susceptible and the rif^R strain ($df = 1$, residual $df = 824$, $\chi^2 = 6.0$, $P = 0.014$; Fig 2).
21 Note that the survivorship analysis can only differentiate between treatments when
22 mortality is below 100%. However, inoculation with *Enterobacter* sp. Mn2 did not
23 alter the pathogenicity of *Bt* HD-73 ($df = 1$, residual $df = 763$, $\chi^2 = 0.1$, $P = 0.8$, Fig.
24 3). Bioassay dose affected mortality rates in the HD-1 experiment and interacted with
25 pathogen strain ($df = 1$, residual $df = 824$, $\chi^2 = 156.2$, $P < 0.0001$) but not

1 *Enterobacter* sp. Mn2 inoculation ($df = 1$, residual $df = 823$, $\chi^2 = 1.9$, $P = 0.2$), or the
2 application of rifampicin ($df = 1$, residual $df = 822$, $\chi^2 = 1.54$, $P = 0.12$). In the HD-
3 73 experiment dose also significantly affected mortality ($\chi^2 = 8.5$, $P < 0.0001$) but did
4 not interact with bacterial strain ($df = 1$, residual $df = 759$, $\chi^2 = 1.7$, $P = 0.2$) or
5 *Enterobacter* sp. Mn2 inoculation ($df = 1$, residual $df = 760$, $\chi^2 = 1.1$, $P = 0.3$) but did
6 interact with rifampicin consumption ($df = 1$, residual $df = 761$, $\chi^2 = 7.2$, $P = 0.007$).

7

8 We examined control insects from the first two bacterial bioassays for the presence of
9 culturable strains (using high-nutrient LB agar) and unculturable bacteria (using PCR
10 amplification of 16S rRNA genes). Larvae were removed at the end of the
11 experiment for DNA extraction, in order to maximize the probability of recovering
12 contaminants. Homogenized whole larvae from both the antibiotic-free and
13 rifampicin-diet rearing treatments failed to produce any bacterial colonies when plated
14 on LB media ($n=10$ per treatment). In contrast, 60% of whole insect homogenates
15 from larvae inoculated with *Enterobacter* sp. Mn2 contained detectable culturable
16 levels of this bacterium ranging 0 - 3×10^3 CFU per insect on antibiotic free diet and 0
17 - 4×10^1 CFU per insect on rifampicin diet, although there was no significant
18 difference between diet treatments ($df = 13$, $t = 1.46$, $P = 0.17$). PCR conditions that
19 were sufficiently sensitive to amplify a product from 40 pg of *Enterobacter* sp.
20 template DNA, roughly equivalent to 10^4 cells of *Escherichia coli* (Kubitschek and
21 Friedman, 1971), failed to amplify a product from larvae reared-aseptically on
22 antibiotic-free diet. We could, however, recover visible PCR products from 5/8
23 aseptically-reared larvae that were experimentally inoculated with *Enterobacter* sp.
24 Mn2.

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1

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3 **Discussion**

4 We have shown that both *Bt* toxins and spore/toxin mixtures are pathogenic in
5 the absence of midgut bacteria when carry-over effects of antibiotic are excluded by
6 using aseptically reared hosts. In addition we have shown that antibiotic sensitive *Bt*
7 strains are much less pathogenic to hosts that have ingested a single antibiotic
8 (rifampicin) but antibiotic resistant strains of *Bt* are not affected in this way.

9 Therefore in *P. xylostella* larvae, pathogenicity of *Bt* or one of its toxins does not
10 depend upon the culturable gut microbiota. For one strain of *Bt* (HD-1), and for pure
11 toxin preparations, the presence of culturable gut microbiota led to a small but
12 significant reduction in host mortality, implying that the gut microbiota confers some
13 protection against pathogen attack, as found in other vertebrates and invertebrate hosts
14 (Jarosz, 1979; Dillon and Charnley, 1995; Filho-Lima et al., 2000; Servin, 2004). A
15 similar protective role of gut microbiota against the toxicity of pure Cry1Ac was found
16 in a subsequent paper by Broderick et al (2009) in which antibiotic treatment
17 increased the susceptibility of *Pectinophora gossypiella* towards this toxin. These
18 results also imply that inhibiting the growth of *Bt* within the host with antibiotics
19 reduces pathogenicity, indicating that septicaemic proliferation of *Bt* contributes to
20 the pathogenicity of this bacterium. Our demonstration of the pathogenicity of
21 purified Cry toxins in aseptic hosts does not contradict our interpretation of the role of
22 septicaemia since living bacterial spores have long been known to substantially
23 increase toxin-induced mortality in *P. xylostella* and other hosts (Li et al., 1987;
24 Dubois and Dean, 1995; Liu et al., 1998).

25

1 Broderick et al. (2006, 2009) reported the importance of culturable gut
2 microbiota for the pathogenicity of *Bt* and/or its Cry toxins in a wide range of
3 Lepidopteran hosts, but our findings do not support this interpretation in diamondback
4 moth. Although Broderick et al (2006, 2009) found that treating larvae with
5 antibiotics inhibited the pathogenicity of *Bt* in 5 different Lepidopteran species and
6 that inoculation with *Enterobacter* sp. NAB3 restored the pathogenicity of *Bt* in 4 of
7 these. Their interpretation was that the midgut microbiota commonly contribute to the
8 pathogenicity of *Bt*. Our results suggest that there may be an alternative explanation
9 for their data. Just one of the antibiotics they used to produce aseptic larvae
10 (rifampicin) was sufficient to drastically reduce the pathogenicity of *Bt* in this study.
11 Given our results and the fact that orally fed antibiotics can persist in the tissues and
12 haemolymph of lepidopteran larvae (Sikorowski and Thompson, 1985); it is plausible
13 that the direct effects of antibiotics, and not the absence of the gut microbiota, account
14 for the low pathogenicity of *Bt* in antibiotic-fed Lepidopteran larvae (Broderick et al.,
15 2006; Broderick et al., 2009). Broderick et al (2009) found that administration of
16 antibiotics to *Heliothis virescens* larvae greatly reduced subsequent pathogenicity of
17 *Bt*. This result is noteworthy as the authors did not detect gut microbiota in *H.*
18 *virescens* larvae prior to antibiotic administration. Thus, in agreement with the results
19 presented here, the observed attenuation of *Bt* pathogenicity in antibiotic-treated *H.*
20 *virescens* larvae is likely to result from past antibiotic exposure and not from the
21 removal of gut microbiota. Broderick et al (2009) inferred that antibiotics were
22 unlikely to affect *Bt* because (1) they found similar levels of colony forming units of
23 *Bt* in the guts of antibiotic free and antibiotic fed larvae and (2) because *E. coli*
24 (engineered to express *Bt* toxins) and *Enterobacter* sp. NAB3 could both proliferate in
25 antibiotic treated larvae. However, colony counts from gut material may be derived

1 from spores and do not imply that the intestine is a suitable site for growth (Raymond
2 et al., 2008a). Moreover, gram negative *E. coli* and *Enterobacter* spp. are poor
3 controls for the effects of antibiotics on a gram positive *Bacillus*; both *E. coli* and
4 *Enterobacter/Klebsiella* are approximately 400 times less susceptible to rifampicin
5 than gram positive Staphylococci, for example (McCabe and Lorian, 1968).

6 Broderick et al. (2006) also found that *Bt* was incapable of growing in filtered
7 gypsy moth haemolymph. This, however, is not a typical result. Many studies have
8 used intra-haemocoelic inoculation to demonstrate that *Bt* is capable of rapid growth
9 within the haemocoel of lepidopterans including *Callosamia promethean* (Edlund et
10 al., 1976), *Cecropia hyalophora* (Heierson et al., 1986), *Trichoplusia ni* (Zhang et al.,
11 1993), *Bombyx mori* (Fedhila et al., 2004) and *Galleria mellonella* (Salamitou et al.,
12 2000). Where investigated, it was found that lethal infections were characterised by
13 rapid growth of *Bt* within the haemocoel (Heierson et al., 1986). In *P. xylostella*,
14 toxin-free spores of *B. thuringiensis kurstaki* HD1 were found to synergise the
15 toxicity of purified crystals from the same strain (Miyasono et al., 1994). Moreover,
16 synergism was characterised by spore germination and invasion of the larval
17 haemocoel where *B. thuringiensis* densities increased to over 10^6 cells per μ l
18 haemolymph after 72 hours (Miyasono et al., 1994).

19 We do not contest the possibility that different insect hosts may harbour
20 facultative pathogens within the mid-gut that could potentially synergize the toxicity
21 of Cry toxins. Nevertheless, there are a number of studies suggesting that
22 antagonistic interactions between the gut microbiota and *Bt* are probably the norm.
23 For example, intestinal microbiota invading the cadaver directly compete with *Bt* for
24 the limited resources offered by the insect host (Takatsuka and Kunimi, 2000). It
25 would therefore make poor adaptive logic for a pathogen to rely upon its competitors

1 for full pathogenicity and the action of its highly costly Cry toxins. Diverse bacterial
2 antagonists are produced by *Bt* (Pendleton, 1968; Dong et al., 2002; Nair et al., 2004),
3 and some of these, for example the quorum quenching N-acyl homoserine lactonases
4 are clearly targeted at gram negative competitors (Dong et al., 2002). Finally, mixed
5 infections with antibiotic producing relatives can increase the pathogenicity of *Bt*
6 infections in *P. xylostella* and the gypsy moth (Broderick et al., 2000; Raymond et al.,
7 2008b). In one of these studies antibiotic production was associated with the
8 suppression of natural gut microbiota and domination of the gut community by
9 *Bacillus cereus* (Raymond et al., 2008b). Gut microbiota suppression via in vivo
10 antibiotic production was therefore implicated in the *increased* pathogenicity of *Bt*.
11 This pattern ties in neatly with the results presented here on the protective role of the
12 culturable gut microbiota. In other words suppression of the intestinal microbiota
13 would seem to be a sound evolutionary strategy for *Bt*: it may increase the mortality
14 rates of hosts and also reduce the number of competitors within any resulting cadaver.

15

16 **Experimental procedures**

17 *Bacterial strains.*

18 *Bacillus thuringiensis* subsp. *kurstaki* HD 1 was isolated from a commercial DiPel
19 stock (DiPel WP, Valent Biosciences Corporation) using *Bacillus cereus* selective
20 agar (BcSA) (Oxoid, UK). Wild-type *Bt* isolates were collected from a native,
21 perennial crucifer, *Brassica oleracea* in semi-natural habitats on the Dorset coast in
22 the UK and from the Silwood Park campus, Imperial College, UK, using previously
23 described methods (Collier et al., 2005). Both habitats adjoin parkland or arable and
24 pasture crops that are not typically sprayed with *Bt* biopesticides. Three strains of *Bt*
25 (Dt 6.2.y, Dt 7.1.o and C3o20) were shown to be genotypically identical to other *Btk*

1 strains by multilocus-sequence typing of isolates at seven chromosomal genes (ST8,
2 primers and sequence data available at <http://pubmlst.org/bcereus>). All strains were
3 polymyxin resistant, expressed parasporal inclusions, are pathogenic to *P. xylostella*,
4 and carry the genes for Cry1 and Cry2 toxins (B. Raymond unpubl. dat.). They can
5 be differentiated from Dipel derived strains by phenotypic antibiotic assays (Nair et
6 al., 2004) which reveal the improved ability of WT isolates in suppressing the growth
7 of *Pantoea agglomerans* (B. Raymond unpubl. dat.) One of these strains (Dt 6.2.y)
8 also has improved reproduction in hosts relative to DiPel WP. A spontaneous
9 rifampicin resistant mutant derived from DiPel WP, *Btk rif^R*, was described previously
10 (Raymond et al., 2007). A spontaneous rifampicin resistant strain of *Bt* HD-73 was
11 isolated by plating 10^8 - 10^9 cells of overnight culture onto LB agar containing $100 \mu\text{g}$
12 ml^{-1} rifampicin, and sub-culturing strongly growing colonies. A description of all
13 strains used is given in Table 1. For all bioassays fresh spores and crystals of *Bt* were
14 prepared by growth on BcSA for 5 days, purification and assessment of spore density
15 followed established techniques (Raymond et al., 2007). Bacterial stocks were
16 pasteurized (30 min at 65°C) and stored at 10°C for no more than 5 days.

17 *Enterobacter sp.* Mn2 was isolated from the dissected mid-gut of fourth instar DBM
18 larvae, reared on Chinese cabbage, *Brassica pekinensis*, in insect culture rooms at the
19 Department of Zoology, University of Oxford and identified by sequencing of 16S
20 rRNA genes using the universal primers 63f (5'- CAG GCC TAA CAC ATG CAA
21 GTC-3') and 518r (5'- GTA TTA CCG CGG CTG CTG-3') (Genbank accession
22 number FJ668636).

23

24 *Rearing and bioassay of insect larvae*

1 The Geneva *P. xylostella* strain was used in all artificial diet experiments (purchased
2 from CEH Oxford UK). The parents of all aseptically reared larvae were reared on a
3 wheat-germ based diet (Hunter et al., 1984) containing aureomycin (2.25 g l⁻¹) and
4 streptomycin (0.25 g l⁻¹). Thereafter, aseptic larvae were prepared by surface-
5 sterilizing eggs (2% sodium hypochlorite with 100 µl l⁻¹ Triton X100 and three
6 washes of sterile MilliQ water) and rearing on autoclaved diet in 50mm Petri dishes.
7 *P. xylostella* larvae were either reared on rifampicin diet (containing 500 µg ml⁻¹
8 rifampicin) or antibiotic-free diet. Experimental inoculation of insects (second or
9 third instar larvae) with *Bt* always took place on ≈ 330 mm² antibiotic-free diet, in
10 fresh Petri dishes. Diet was inoculated with 75µl of pasteurized *Bt* spore/crystal
11 suspension and three *Bt* concentrations (200, 400 and 800 spores µl⁻¹) were applied
12 with 30 larvae tested per dose. In the *Enterobacter* sp. Mn2 inoculation treatments *Bt*
13 spore/crystal suspensions were prepared in a 1000-fold dilution of an overnight
14 culture of *Enterobacter* sp. Mn2 (LB broth 30°C) isolated as described above. Diet
15 was allowed to dry before larvae were added. For determination of the toxicity of
16 purified protoxin, parents of the aseptically-reared larval progeny to be used were
17 reared on sterile artificial diet containing the antibiotics rifampicin and streptomycin
18 (100 µg g⁻¹ diet for each). Eggs derived from these parents were surface sterilized as
19 described above and larvae were reared on sterile artificial diet containing rifampicin
20 and streptomycin (100 µg g⁻¹ diet for each) Parents of non-sterile larvae, and these
21 larvae prior to exposure to protoxins, were reared on artificial diet without antibiotics
22 and with no attempt to disrupt vertical transmission of microbes.
23
24 *Passage of DiPel derived strains in diamondback moth.*

1 We selected for improved reproduction within hosts by passaging *Btk* rif^R in *P.*
2 *xylostella* larvae in four independent evolutionary lineages. For each lineage we
3 inoculated ten *Brassica pekinensis* leaf discs by dipping in suspensions of 2000 spores
4 μl^{-1} . Leaf discs were air-dried and placed in 50mm Petri dishes with moistened filter
5 paper and five third instar larvae that had previously been reared on diet containing
6 $500 \mu\text{g ml}^{-1}$ rifampicin. After seven days cadavers were homogenized in 500 μl
7 saline and plated onto BcSA containing $100 \mu\text{g ml}^{-1}$ rifampicin. Selection was
8 imposed by pooling cadavers from each Petri dish at homogenization, so that cadavers
9 with improved reproduction were better represented in the subsequent strain isolation.
10 We also selected between Petri dishes within a lineage, by propagating bacteria for
11 the next passage with approximately equal proportions of bacteria from the five plates
12 within highest *Bt* counts. Glycerol stocks were stored at -80°C after each passage.

13

14 *Crystal toxin bioassays*

15 Cry1Ac protoxins were expressed as crystals in *E. coli*, the crystals were purified
16 away from disrupted cells and stored at -80°C as described previously (Sayyed et al.,
17 2005). Bioassays were conducted with five concentrations and third-instar *P.*
18 *xylostella* larvae using discs of sterile artificial diet containing rifampicin and
19 streptomycin ($100 \mu\text{g g}^{-1}$ diet for each). Streaking out of purified crystals revealed no
20 contamination with culturable bacteria. Each diet disc ($r=24 \text{ mm}$, $h=10 \text{ mm}$) was
21 immersed in a Cry1Ac protoxin suspension for 30 s and allowed to dry at room
22 temperature for 30 min. Suspensions were prepared using sterile distilled water with
23 Triton X-100 ($50 \mu\text{g ml}^{-1}$). Control diet discs were immersed in sterile distilled water
24 with Triton X-100. Each diet disc was added to a sterile 50 ml sample bottle. Five
25 larvae were sealed in each bottle, and each treatment was repeated six times (i.e. $N =$

1 30 larvae per dose). Mortality was determined after 120 h at 25°C. Larvae were
2 considered dead if unable to move when prodded with a blunt, sterile probe.

3 Bioassays were repeated twice.

4

5 *DNA extraction and PCR amplification of DNA from larvae and their gut microbiota*

6 DNA was extracted from whole insects or from over-night cultures of *Enterobacter*
7 sp. Mn2 using the CTAB method (Ausubel et al., 1999) and DNA re-suspended in
8 50µl of 10mM Tris pH 8.0 and stored at -20°C. PCR amplification with the universal
9 bacterial 16S rRNA gene primers, 63f and 1492r (Marchesi et al., 1998), was used to
10 test for the presence of bacteria in larvae. Amplifications were carried out in 25 µl
11 reactions containing 0.25 µl *Taq* polymerase (Qiagen), 1µl dNTPs (10mM stock), 5
12 pico-moles of each primer, and 2µl of DNA extraction in a Techne Flexigene thermal
13 cycler. The reaction conditions were 94°C for 4min followed by 30 cycles of 94°C
14 for 45s, 57°C for 45s and 72°C for 1m 20s. In order to establish the sensitivity of the
15 amplifications to bacterial DNA we ran positive controls with a 10-fold dilution series
16 of *Enterobacter* sp. Mn2 DNA. Control larvae that had been reared on antibiotic-free
17 diet, and fed on with *Enterobacter* sp. Mn2 -inoculated diet, were also used as
18 positive controls to test whether insect DNA or material was an inhibitor of the PCRs.
19 We did not attempt to amplify 16S rRNA genes from toxin-killed larvae as
20 chromosomal DNA fragments from Cry toxin-producing bacteria are known to
21 associate with toxin crystals (Sun et al., 2007) and these fragments could lead to false-
22 positive identification of unculturable mid-gut bacteria.

23

24 *Statistical Analysis*

1 Statistical analysis was carried out in R (<http://www.r-project.org>) using analysis of
2 variance, generalized linear modelling, and survival analysis. Cumulative insect
3 mortality was analysed with survival analysis using Cox proportional hazard models,
4 where possible. These models are more powerful than simple analyses of
5 proportional mortality but rely upon an assumption of a constant probability of death
6 (Tableman and Sung Kim, 2004). When this assumption was violated or when
7 mortality data were available from a single time point proportional mortality was
8 analysed with generalized linear modelling, a logit-link function and binomial errors
9 (Crawley, 2005). The function “quasibinomial” was used to compensate for over-
10 dispersion where appropriate, and estimates of LC₅₀ values with 95% confidence
11 limits (95% CL) were calculated according to (Collett, 1991). Model assumptions
12 (normality, homoscedasticity, error distribution) were confirmed with graphical
13 analyses.

14

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19

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2

3 Table 1. *Bacillus thuringiensis* strains used in this study.

4

Name	Description	Source
DiPel WP, DiPel DF	biopesticidal strains of <i>Bt kurstaki</i> HD-1	commercial spore preparations
HD-1 1971	biopesticidal isolate of HD-1 released by Institut Pasteur in 1971	Christina Nielsen-LeRoux
<i>Btk</i> rif ^R	spontaneous rifampicin resistant mutant of DiPel WP	(Raymond et al. 2007)
R1-K, R2-K, R3-K	strains of <i>Btk</i> rif ^R independently passaged three times in <i>P. xylostella</i>	this study
HD-73	<i>Bt kurstaki</i> HD-73	BGSC† 4D4
HD-73 rif ^R	spontaneous rifampicin resistant mutant of HD-73	this study
Dt 7.1.o, Dt 6.2.y	wild type isolates of <i>B. thuringiensis</i>	plant material, Dorset, UK
C3 o20	wild type isolate of <i>B. thuringiensis</i>	plant material, Ascot, UK, (Collier et al. 2005)

5 † BGSC- Bacillus Genetic Stock Centre, Ohio State University

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Table 2. Effect of intestinal bacteria on the toxicity of Cry1Ac protoxin to third-instar *P. xylostella* larvae.

Rearing	Culturable bacteria	16S rRNA	LC₅₀ (ng/ml)	95% CL
Normal	+	+	69.3	56.4 - 85.0
Aseptic§	-	-	26.7	21.6 - 32.9

7

8 § Larvae were rendered aseptic by treating the parental generation with antibiotics
9 (100 µg g⁻¹ diet) and by surface sterilization of their eggs.

1

2 **Fig. 1.** Contrasting pathogenicity of different strains of *Bt* in aseptic hosts treated
3 with rifampicin. The passaged rif^R strains (black lines- filled diamonds) had been
4 independently cultured three times in *P. xylostella* larvae; the HD-1 strains were all
5 derived from biopesticidal sources (dashed lines – open squares); wild-type isolates
6 (grey lines - asterisks) were collected from plant material in the UK. Each data point
7 represents the mean mortality for three strains of each category; bioassays were
8 replicated three times at different doses for each strain ($n = 796$ insects overall)
9 bioassay dose effects have been pooled for graphical simplicity. The data are mean
10 cumulative proportional mortality + SE.

11

12 **Fig. 2.** The effect of the addition of rifampicin and the gut bacterium *Enterobacter*
13 sp. Mn2 on the mortality of aseptically reared second instar *P. xylostella* exposed to
14 rifampicin susceptible and resistant *B. thuringiensis kurstaki* HD-1. Solid symbols
15 indicate that hosts were reared on rifampicin-amended diet, open symbols indicate
16 that hosts were reared in the absence of antibiotics. Circular symbols and grey lines
17 indicate that *Bt* was coinfecting with *Enterobacter* sp. Mn2, triangular symbols and
18 dashed lines indicate that insects were infected with *Bt* only. In this experiment HD-1
19 rif^R refers to a host- passaged rifampicin resistant derivative of this strain (R1-K).
20 Bioassays were replicated three times at different doses for each strain ($n = 829$
21 insects overall) but dose effects have been pooled for graphical simplicity. The data
22 are mean cumulative proportional mortality + SE.

23

24 **Fig. 3.** The effect of the addition of rifampicin and the gut bacterium *Enterobacter*
25 sp. Mn2 on the mortality of aseptically reared third instar *P. xylostella* exposed to

1 rifampicin susceptible and resistant *B. thuringiensis kurstaki* HD-73. Solid symbols
2 indicate that hosts were reared on rifampicin-amended diet, open symbols indicate
3 that hosts were reared in the absence of antibiotics. Circular symbols and grey lines
4 indicate that *Bt* was coinfecting with *Enterobacter* sp. Mn2, triangular symbols and
5 dashed lines indicate that insects were infected with *Bt* only. In this experiment HD-
6 73 rif^R refers to a spontaneous rifampicin resistant derivative of HD-73. Bioassays
7 were replicated three times at different doses for each strain ($n = 767$ insects overall)
8 dose effects did not interact with *Enterobacter* or diet treatments so dose effects have
9 been pooled for graphical simplicity. The data are mean cumulative proportional
10 mortality + SE.

11

12





