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Gut Bacteria Are Not Required for the Insecticidal Activity of *Bacillus thuringiensis* toward the Tobacco Hornworm, *Manduca sexta*▽†

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It was recently proposed that gut bacteria are required for the insecticidal activity of the *Bacillus thuringiensis*-based insecticide, DiPel, toward the lepidopterans *Manduca sexta*, *Pieris rapae*, *Vanessa cardui*, and *Lymantria dispar*. Using a similar methodology, it was found that gut bacteria were not required for the toxicity of DiPel or Cry1Ac or for the synergism of an otherwise sublethal concentration of Cry1Ac toward *M. sexta*. The toxicities of DiPel and of *B. thuringiensis* HD73 Cry$^-$ spore/Cry1Ac synergism were attenuated by continuously exposing larvae to antibiotics before bioassays. Attenuation could be eliminated by exposing larvae to antibiotics only during the first instar without altering larval sterility. Prior antibiotic exposure did not attenuate Cry1Ac toxicity. The presence of enterococci in larval guts slowed mortality resulting from DiPel exposure and halved Cry1Ac toxicity but had little effect on *B. thuringiensis* HD73 Cry$^-$ spore/Cry1Ac synergism. *B. thuringiensis* Cry$^-$ cells killed larvae after intrahemocoelic inoculation of *M. sexta*, *Galleria mellonella*, and *Spodoptera litura* and grew rapidly in plasma from *M. sexta*, *S. litura*, and *Tenebrio molitor*. These findings suggest that gut bacteria are not required for *B. thuringiensis* insecticidal activity toward *M. sexta* but that *B. thuringiensis* lethality is reduced in larvae that are continuously exposed to antibiotics before bioassay.

Bacillus thuringiensis has long been regarded as a bona fide entomopathogen that can produce an array of virulence factors including insecticidal parasporal crystal (Cry) toxins, vegetative insecticidal proteins, phospholipases, immune inhibitors, and antibiotics (31). *B. thuringiensis* establishes lethal infections in many insect species after intrahemocoelic inoculation (9, 10, 14, 26, 31), and the insecticidal activity of Cry toxins, which lyse the intestinal epithelium, can be synergized by the presence of viable *B. thuringiensis* spores (31). In each instance, synergism has been attributed to hemocoelic infection by *B. thuringiensis*.

A novel hypothesis (6, 7) proposed that *B. thuringiensis* is incapable of killing *Lymantria dispar*, *Manduca sexta*, *Pieris rapae*, or *Vanessa cardui* in the absence of gut bacteria. Prior exposure of *L. dispar* larvae to a combination of four antibiotics severely reduced the subsequent toxicity of the *B. thuringiensis*-based (spores and Cry toxins) bioinsecticide, DiPel (Valent BioSciences) (6). Both larval susceptibility to *B. thuringiensis* and the number of culturable gut bacteria were found to be negatively correlated with the concentration of antibiotics to which larvae were previously exposed. Furthermore, a total reduction in larval susceptibility was coincident with the elimination of any detectable gut bacteria. Experimental reinfection with *Enterobacter* sp. strain NAB3, found in the guts of some populations of *L. dispar* larvae, was found to rescue the toxicity of *B. thuringiensis*, whereas reinfection with *Enterococcus casseliflavus* and *Staphylococcus xylosus* did not. It was also shown that while *Escherichia coli*, *Enterobacter* sp. strain NAB3, and *B. thuringiensis* could all grow in tryptic soy broth, *B. thuringiensis* alone could not grow in filter-sterilized plasma from *L. dispar* larvae. Finally, it was shown that the toxicity of Cry1Aa-expressing *E. coli* JM103 to *L. dispar* larvae was reduced by the prior exposure of larvae to antibiotics and could be eliminated when *E. coli* was also heat killed before use. It was concluded that *B. thuringiensis*-induced mortality results from a mixed infection of the hemocoel that must include bacteria capable of growth within the *L. dispar* larval hemolymph (6).

Using the same methods, it was subsequently reported that prior exposure of *Vanessa cardui*, *M. sexta*, *Pieris rapae*, and *Heliothis virescens* larvae to antibiotics eliminated culturable bacteria and rendered larvae resistant to DiPel (7). Experimental reinfection of larvae with *Enterobacter* sp. strain NAB3 rescued DiPel toxicity in *V. cardui*, *M. sexta*, and *P. rapae* but not in *H. virescens* larvae. Using a continuous-exposure bioassay, the susceptibility of *Pectinophora gossypiella* to the Cry1Ac-based bioinsecticide MVPII was found to be increased by prior exposure to antibiotics. Toxicity from a 48-h exposure of *L. dispar* larvae to MVPII was reduced, but not eliminated, by prior antibiotic exposure and could be rescued by reinfection with *Enterobacter* sp. strain NAB3. It was concluded that "enteric bacteria have important roles in *B. thuringiensis*-induced killing of Lepidoptera across a range of taxonomy, feeding breadth, and relative susceptibility to *B. thuringiensis*" (7).

The present work shows that gut bacteria are not required for the insecticidal activity of *B. thuringiensis* or Cry1Ac toxin toward *M. sexta* but that prior antibiotic exposure reduces larval susceptibility to *B. thuringiensis*.

MATERIALS AND METHODS

Bacterial strains and formulations. DiPel DF with an insecticidal activity of 32 kIU mg^{-1} (Valent BioSciences) was provided by Ben Raymond (University of Oxford, United Kingdom). DiPel solutions were prepared in sterile distilled

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water. A Cry$^-$ *B. thuringiensis* HD73 strain was produced for use in synergism bioassays so as not to inadvertently increase the Cry1Ac concentration. *B. thuringiensis* HD73 was cured of its *cry*-carrying plasmid by culturing in Luria-Bertani (LB) broth at 42°C. *B. thuringiensis* HD73 Cry$^-$ spores were prepared from colonies which had sporulated on Luria agar (LA). Spores were resuspended in sterile distilled water, washed, pasteurized (45 min at 70°C), and quantified by dilution plating.

Insect populations. A field population of *Spodoptera litura* (Noctuidae) was collected from Tamil, India, in March 2007 and reared in the laboratory at 25°C on Hoffman's tobacco hornworm diet (15). Larvae of the greater wax moth, *Galleria mellonella* (Pyralidae), and of the darkling beetles *Tenebrio molitor* (Tenebrionidae) and *Zophobas morio* (Tenebrionidae) were obtained from HPC (West Sussex, United Kingdom). *G. mellonella* larvae were reared in the dark at 25°C on a grain-honey diet described elsewhere previously (11). Tenebrionids were reared at 25°C and fed wheat bran. *M. sexta* (Sphingidae) eggs derived from parents reared on Hoffman's tobacco hornworm diet containing 100 μg ml^{-1} chlortetracycline (pure veterinary-grade Aureomycin; Lederle) were supplied by Stuart Reynolds (University of Bath, United Kingdom). *M. sexta* eggs from a single batch were separated into the three rearing regimens described below and reared at 25°C.

Nonsterile rearing regimen. Nonsterile *M. sexta* larvae were reared individually on a diet that had not been autoclaved and that did not contain antibiotics. The diet components (excluding water) were premixed and frozen at −20°C. Each batch of nonsterile diet was derived from this stock and prepared with sterile distilled water.

Aseptic rearing regimens. Sterile diet was produced by mixing heat-stable components with distilled water followed by autoclaving at 120°C and 20 lb/in^2 for 25 min. Heat-labile components were dissolved in sterile distilled water, sterilized using a 0.22-μm filter, and mixed with the diet base after cooling to 60°C. Molten diet was poured into sterile beakers and allowed to set in a laminar-flow cabinet. *M. sexta* eggs were given three alternating 30-min washes of 3.8% formaldehyde and sterile distilled water and hatched on a sterile diet containing 100 μg ml^{-1} of rifampin and streptomycin. Upon molting to the second instar, larvae were further separated into two regimens. Half of the larvae were transferred to a sterile diet without antibiotics, whereas the remaining larvae continued to be reared on a sterile diet containing 100 μg ml^{-1} of rifampin and streptomycin before being starved 12 h before bioassay. Thus, two groups of aseptic larvae were produced: one group was exposed to antibiotics only during the first instar, whereas the other was continuously exposed to antibiotics prior to bioassay.

***M. sexta* bioassays.** Three different types of feeding bioassays were conducted as described below. Third-instar larvae were used for all bioassays, and larvae were held individually in sterile universal bottles. Mortality was scored daily for 6 days, and larvae were considered dead if they were unable to move when prodded with a blunt sterile probe. All bioassays were repeated twice at least 1 month apart.

DiPel bioassays. DiPel bioassays were conducted as described previously (7). A total of 25 IU of DiPel were applied in 1 μl of sterile distilled water to the surface of a sterile diet disc (r = 1.5 mm; h = 1 mm) without antibiotics. Larvae were starved for 12 h before being fed DiPel-contaminated diet discs for 48 h, after which larvae were fed an uncontaminated sterile artificial diet without antibiotics. Larvae were starved for 12 h only, to avoid a reingestion of antibiotic-containing frass in larvae that had been continuously exposed to antibiotics. Bioassays used at least 30 larvae from all three rearing regimens described above.

Cry1Ac bioassays. Cry1Ac protoxins were produced as inclusion bodies in *E. coli* cultures and purified as previously described (30). Cry1Ac crystals were diluted in sterile distilled water and incorporated into sterile diet at five concentrations as described previously by Gilliland et al. (12). Thirty larvae were assayed against each toxin concentration. Bioassays used nonsterile larvae from the nonsterile rearing regimen and aseptic larvae that had been continuously reared on a sterile diet containing 100 μg ml^{-1} of rifampin and streptomycin.

Synergism bioassays. Cry1Ac protoxins and *B. thuringiensis* HD73 Cry$^-$ spores were diluted in sterile distilled water and incorporated into sterile diet to concentrations of 50 ng ml^{-1} and 10^5 CFU ml^{-1}, respectively. Cry1Ac bioassays described above had previously shown 50 ng ml^{-1} Cry1Ac to be a sublethal concentration that slowed larval growth. Bioassays used at least 30 larvae from all three rearing regimens described above.

Monitoring of larval sterility. Thirty larvae from bioassay control groups representing each rearing regimen were dissected, and their guts were removed. Guts from each rearing regimen were divided into two equal groups. One group was used for dilution plating onto LA followed by incubation at 28°C. The second group of guts was used for cetyltrimethylammonium bromide DNA extraction as described previously by Ausubel et al. (3). Each DNA extract was then used as a template for PCR amplification of 16S rRNA genes using universal primers 27f (5′-GTGCTGCAGAGAGTTTGATCCTGGCTCAG-3′) and 1492r (5′-CACG GATCCTACGGGTACCTTGTTACGACTT-3′) (22). PCR contents for a 50-μl volume were 0.5 μl of 100 pmol μl^{-1} forward (27F) and reverse (1492R) primers, 1 μl of 10 mM deoxynucleoside triphosphates (Promega), 5 μl of 10× *Taq* polymerase buffer (Promega), and 0.5 μl of *Taq* DNA polymerase (2.5 U; Promega). A hot-start protocol was used, with an initial denaturation step at 94°C for 3 min followed by 35 cycles of denaturation at 94°C for 1 min, annealing at 52°C for 1.5 min, and elongation at 72°C for 2 min, followed by a final extension step at 72°C for 10 min. Larvae were considered to be sterile if bacteria could not be detected by culturing or by 16S rRNA gene amplification.

Intrahemocoelic inoculation. *B. thuringiensis* HD73 Cry$^-$ cells were grown to an optical density at 600 nm of 1.0 (approximately 10^8 cells ml^{-1}), washed with sterile water, and diluted to the required densities. Nonsterile larvae (third-instar *M. sexta* and *S. litura* or final-instar *G. mellonella*, *T. molitor*, and *Z. morio* larvae) were chilled on ice, and injection sites (first right proleg for Lepidoptera and third right leg for Coleoptera) were swabbed with 95% ethyl alcohol. A sterile 5-μl Hamilton syringe with a removable 26-gauge needle was used to inject 5 μl of a cell suspension directly into the hemocoel of each larva. Control groups received injections of sterile distilled water only. Larvae were placed into sterile plastic boxes with diet, and mortality was scored each day for 72 h. Larvae were considered dead if they were unable to move when prodded with a blunt sterile probe. Bioassays were repeated twice. Actual doses of cells used were calculated by dilution plating. Bacterial cell densities in dead larvae were calculated by homogenizing cadavers in sterile distilled water followed by dilution plating onto LA. For immunization, larvae were injected with approximately 10^4 *E. coli* JM109 cells or sterile distilled water 24 h before injection of *B. thuringiensis*.

Plasma growth assays. For third-instar *S. litura* and *M. sexta* larvae, the dorsal surface of the antepenultimate body segment was removed with scissors, allowing hemolymph to be collected and flash-frozen on dry ice. Hemolymph from final-instar *T. molitor* larvae was collected from cuts made by slicing across the legs. Hemolymph was collected from >100 larvae of each species, thawed on ice, and prepared as previously described (6). Briefly, four 1-ml plasma pools were constructed for each insect species such that any single larva was represented in only one pool. Pooled hemolymph was centrifuged at 13,000 × *g* for 20 min at 4°C. Glutathione was added to a concentration of 5 mM to inhibit melanization, and each solution was sterilized using a 0.22-μm filter. *B. thuringiensis* HD73 Cry$^-$ or *E. coli* JM109 cells were grown overnight in LB broth and washed, and each culture was added to two separate 1-ml pools of plasma to densities of approximately 5×10^5 CFU ml^{-1}. Actual densities were calculated by dilution plating. The growth of each strain at 28°C was monitored by dilution plating of samples every 1.5 h for 18.5 h.

Hemolymph phenoloxidase (PO) activity assays. Hemolymph from 10 larvae was pooled, diluted with 3 volumes of ice-cold phosphate-buffered saline (PBS), and centrifuged at 13,000 × *g* for 10 min at 4°C. Aliquots (5 μl) of the resulting supernatant were added to 995 μl of 10 mM L-Dopa in PBS, and the absorbance (490 nm) was measured every minute for 60 min. Absorbance was corrected for spontaneous hydrolysis by using a negative control containing only 10 mM L-Dopa and PBS. Experiments were repeated three times.

Statistical analysis. Fifty percent lethal concentration (LC_{50}) or 50% lethal dose (LD_{50}) estimates and their 95% fiducial limits (FLs) were calculated by logit regression using GLIM 3.77 (1985; Numerial Algorithms Group) as previously described (30). Estimates were considered significantly different if their 95% FL values did not overlap.

RESULTS

Aseptic rearing. *M. sexta* eggs were derived from antibiotic-treated parents and harbored spore-forming bacilli at low densities (fewer than 10 CFU per egg). No culturable bacteria were detected after eggs were repeatedly washed in 3.8% formaldehyde. Egg washing delayed hatching by approximately 24 h and decreased total hatching by approximately 10%. Growth rates were severely reduced when *M. sexta* larvae were reared on a combination of antibiotics (500 μg each of gentamicin, penicillin, rifampin, and streptomycin ml^{-1} diet) used in other studies to rear aseptic lepidopteran larvae (6, 7). The use of streptomycin and rifampin (100 μg each ml^{-1} diet) from hatching to the end of the first larval instar was sufficient to maintain sterility without causing deleterious side effects. Bac-

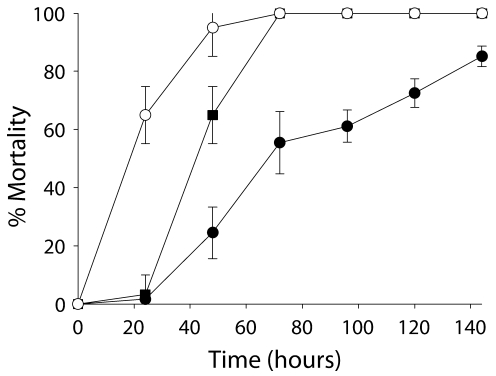

FIG. 1. Effects of different rearing regimens on rates of mortality of third-instar *M. sexta* larvae exposed to DiPel. ■, nonsterile without antibiotic exposure; ○, aseptic with antibiotic exposure during the first instar only; ●, aseptic with continuous antibiotic exposure prior to bioassay. Error bars indicate 95% confidence intervals of the means of data from two repetitions.

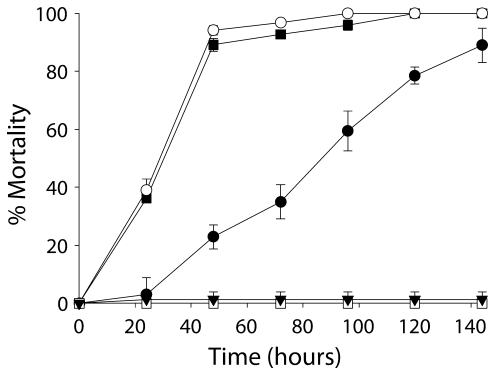

FIG. 2. Effects of different rearing regimens on rates of mortality of third-instar *M. sexta* larvae exposed to *B. thuringiensis* HD73 Cry$^-$ spores and an otherwise sublethal Cry1Ac concentration. ■, nonsterile without antibiotic exposure; ○, aseptic with antibiotic exposure during the first instar only; ●, aseptic with continuous antibiotic exposure prior to bioassay. Error bars indicate 95% confidence intervals of the means of data from two repetitions. Results for Cry1Ac alone (□) and for *B. thuringiensis* HD73 Cry$^-$ spores alone (▼) were combined, as there were no significant differences in mortality rates between larvae from all three rearing regimens.

terial colonization of larval intestines was not detected by culturing or by 16S rRNA gene amplification in either of the aseptic rearing regimens, whereas larvae from the nonsterile rearing regimen harbored a single morphotype of gram-positive cocci (phenotypically identical to an *Enterococcus faecium* group strain isolated from other lepidopterans reared in our laboratory) at an average density of 6.2×10^6 CFU per larva.

Gut bacteria and DiPel toxicity in *M. sexta*. Aseptic larvae that were exposed to antibiotics only during their first instar died more rapidly than did larvae from other rearing regimens, although the final mortality rate was equivalent to that of nonsterile larvae (Fig. 1). Aseptic larvae that were continuously exposed to antibiotics prior to bioassay died more slowly than did larvae from other rearing regimens. Furthermore, the final mortality rate was approximately 20% lower than that of larvae from other rearing regimens. Dead larvae from all rearing regimens were visually indistinguishable and were characterized by progressive melanization extending from the central body segments around the first proleg (see Fig. S1 in the supplemental material). Approximately 24 h after larval death, *B. thuringiensis* dominated cadavers from all rearing regimens and had reached densities of $>10^8$ CFU per cadaver. Enterococcal densities were lower (8×10^3 to 4×10^4 CFU per cadaver) in nonsterile *B. thuringiensis*-killed larvae than in nonsterile control larvae (3×10^6 CFU per larva).

Gut bacteria and Cry1Ac toxicity in *M. sexta*. Cry1Ac was approximately twice as toxic to aseptic larvae that were reared continuously on antibiotics prior to bioassay (LC_{50} = 1.392 μg ml^{-1}; 95% FL = 0.9639 to 1.959) relative to larvae from the nonsterile rearing regimen (LC_{50} = 3.384 μg ml^{-1}; 95% FL = 2.219 to 4.793). Clear differences were observed between larval cadavers of aseptic and nonsterile larvae (see Fig. S2 in the supplemental material). At 24 h after death, aseptic larval cadavers showed no melanization or any other symptoms of septicemia, and cadavers retained structural integrity. In contrast, the cadavers of nonsterile larvae rapidly melanized and were internally liquefied. These cadavers were extremely fragile and oozed fluid from the cuticle. Enterococcal densities were increased (7×10^7 to 2×10^8 CFU per cadaver) in cadavers of nonsterile larvae relative to those of living control larvae (4×10^6 CFU per larva).

Cry1Ac synergism by *B. thuringiensis* spores. The inclusion of *B. thuringiensis* HD73 Cry$^-$ spores within the diet strongly synergized an otherwise sublethal concentration of Cry1Ac (Fig. 2). Spores alone caused no mortality. Mortality was slowed slightly in the presence of gut bacteria and was reduced in aseptically reared larvae that were continuously exposed to antibiotics prior to bioassay. Similar to DiPel bioassays, at approximately 24 h after larval death, *B. thuringiensis* densities reached 8×10^7 to 2×10^8 CFU per cadaver, whereas enterococcal densities decreased to 9×10^2 to 9×10^3 CFU per cadaver.

Intrahemocoelic toxicity of *B. thuringiensis* toward lepidopteran and coleopteran larvae. *B. thuringiensis* HD73 Cry$^-$ cells rapidly killed larvae when injected directly into the hemocoel of *S. litura*, *M. sexta*, or *G. mellonella* larvae, whereas larvae of the tenebrionid beetles *T. molitor* and *Z. morio* were less susceptible to infection, and LD_{50} values could not be calculated (Table 1). Prior immunization with *E. coli* increased immunity to subsequent *B. thuringiensis* infection at ~LD_{85} in *S. litura*,

TABLE 1. Intrahemocoelic toxicity of *B. thuringiensis* HD73 Cry$^-$ cells to lepidopteran and coleopteran larvae

Species	LD_{50} (95% FL) (CFU per larva)	*B. thuringiensis* density (CFU per larva after LD_{50} injection)	
		Cadavers[a]	Survivors[b]
Spodoptera litura	2.5×10^3 (7.8×10^2 – 6.3×10^3)	1×10^9	ND
Galleria mellonella	6.6×10^3 (2.6×10^3 – 1.3×10^4)	4×10^9	ND
Manduca sexta	1.6×10^3 (1.1×10^3 – 3.5×10^3)	1×10^8	ND
Tenebrio molitor	$>10^5$		ND
Zophobas morio	$>10^6$		ND

[a] Cadavers were homogenized and plated approximately 24 h after death.
[b] ND, not detectable.

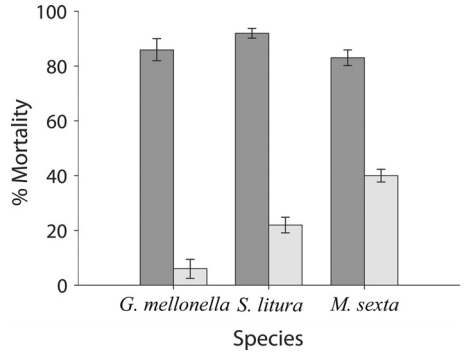

FIG. 3. Effect of immunization on toxicity of intrahemocoelically injected *B. thuringiensis* HD73 Cry$^-$ cells toward larvae of three species of lepidoptera. Dark gray, naïve; light gray, immunized. Error bars indicate 95% confidence intervals of the means of data from two repetitions.

M. sexta, and *G. mellonella* larvae (Fig. 3). In *M. sexta* larvae, prior immunization with *E. coli* elicited an increase in the amount of immunologically detectable hemolymph PO (data not shown) that coincided with increased hemolymph PO activity (slope at V_{max} of 9.45; 95% confidence level of 8.96 to 9.94) relative to that of naïve larvae (slope at V_{max} of 1.65; 95% CL of 0.96 to 2.34). *E. coli* did not grow or cause mortality after injection into any of the species tested.

Growth of *B. thuringiensis* and *E. coli* in plasma. *B. thuringiensis* HD73 Cry$^-$ cells grew rapidly in plasma from *S. litura*, *T. molitor*, and *M. sexta*, whereas *E. coli* grew more slowly, with initial decreases in cell density apparent in plasma from *S. litura* and *T. molitor* (Fig. 4).

DISCUSSION

The results presented here show that the toxicity of *B. thuringiensis* or Cry1Ac toward *M. sexta* does not depend upon the presence of gut bacteria. However, continuous exposure to antibiotics before bioassay reduced mortality when *B. thuringiensis* was subsequently fed to larvae. It is noteworthy that the antibiotic attenuation of *B. thuringiensis* was observed after using two antibiotics at lower concentrations than those used in similar studies (6, 7), which used gentamicin, penicillin, rifampin, and streptomycin each at 500 μg ml^{-1} of diet. It is known that dietary antibiotics can attenuate the toxicity of *B. thuringiensis* spore-crystal mixtures to many lepidopterans (5, 16, 17, 19, 21, 25) and that such attenuation can eliminate spore synergism, leaving only the effect of Cry toxins (25, 32). Therefore, it is proposed that a direct effect of residual antibiotics or an indirect effect such as an induction of a host xenobiotic detoxification response could reduce or eliminate any synergism associated with the germination of *B. thuringiensis* spores, leaving only the effect of Cry toxins. Previous studies (6, 7) exposed larvae to *B. thuringiensis* for 48 h before reverting to uncontaminated sterile diet. This method of bioassay is known to significantly increase the LC_{50} of Cry toxins relative to those reported previously for continuous-exposure bioassays (8). The finding that prior antibiotic exposure, and not an elimination of gut bacteria, attenuates *B. thuringiensis* in lepidopteran hosts is consistent with previously reported results

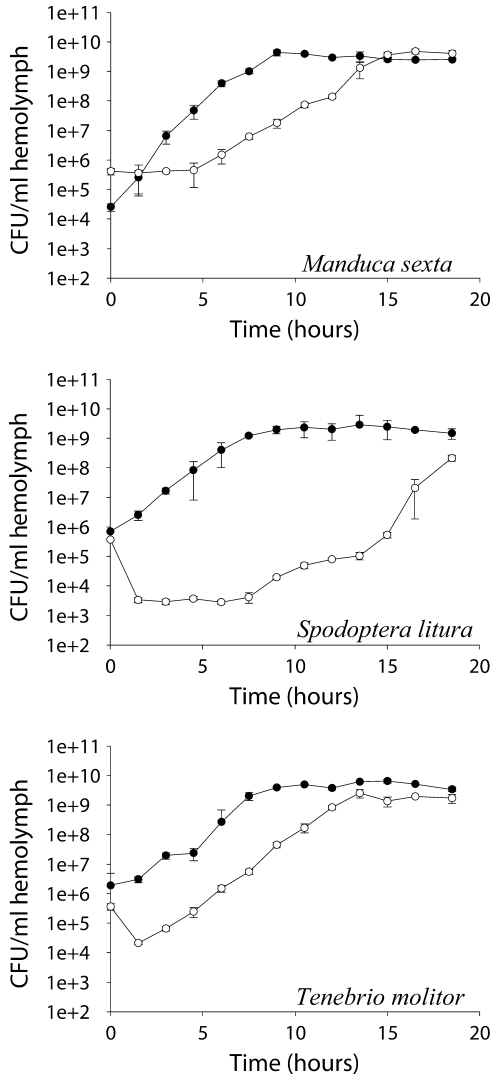

FIG. 4. Growth of *B. thuringiensis* (●) and *E. coli* (○) in plasma from lepidopteran and coleopteran larvae. Error bars indicate 95% confidence intervals of the means of data from two repetitions.

(7), which showed that prior exposure to antibiotics completely attenuates *B. thuringiensis* toxicity toward *H. virescens* larvae. This result cannot be attributed to the removal of gut bacteria, as no bacteria were detected in *H. virescens* larvae before antibiotic treatment (7). Recent work has also implicated antibiotic attenuation in *Plutella xylostella* larvae, where prior rifampin exposure reduced the toxicity of rifampin-sensitive strains of *B. thuringiensis* but not of rifampin-resistant mutants (27).

The observation that *B. thuringiensis* replicates within *M. sexta* cadavers at the expense of gut bacteria is in agreement with data from other studies that demonstrated such competition in lepidoptera (18, 33, 34) and diptera (1). The presence of gut bacteria may afford larvae some protection since aseptic larvae that had been exposed to antibiotics only during first instar were killed more rapidly by DiPel than were nonsterile larvae. The presence of gut bacteria was associated with a slight delay in mortality in synergism bioassays, which may be

due to differences in bioassay methods. Alternatively, the presence of gut bacteria may have different effects on *B. thuringiensis* HD73 and the DiPel strain, HD1. The basis of the protective effect is unclear; however, direct antagonism of *B. thuringiensis* by gut bacteria was previously reported for other species of lepidoptera (20, 34). The level of Cry toxicity may be increased (25), decreased (32), or unaffected (19) by the inclusion of antibiotics in lepidopteran bioassays, although the effects of antibiotics on gut bacteria were not reported. We found aseptic larvae to be approximately twice as susceptible to Cry1Ac compared to nonsterile larvae, which is in agreement with data from recent studies of *P. gossypiella* (7) and *P. xylostella* (27).

B. thuringiensis was found to proliferate in both lepidopteran and coleopteran plasma and to establish lethal infections after injection into the lepidopteran hemocoel. In contrast, *B. thuringiensis* is unable to grow in *L. dispar* plasma (6) and was previously suggested to cooperate with facultatively pathogenic gut bacteria to establish septicemia (6, 7). The work described previously by Zhang et al. (36) was cited in support of the suggestion that the inability of *B. thuringiensis* to infect the hemocoel may be a wider phenomenon (6). It is noteworthy that the work described previously Zhang et al. (36) concerns a pleiotropic avirulent *B. thuringiensis* mutant whose parent strain was able to establish lethal hemocoelic infections in the Lepidoptera (13). Additionally, there is considerable evidence that shows that *B. thuringiensis* is capable of establishing lethal hemocoelic infections in a wide range of insects including many lepidopterans (2, 9, 13, 29, 32, 35, 36). Plasma growth assays do not accurately predict intrahemocoelic growth since *B. thuringiensis* grew in *T. molitor* plasma but not after injection into *T. molitor* larvae. Similarly, *E. coli* grew in lepidopteran plasma but not in living larvae.

Our finding that prior immunization of *M. sexta* larvae with *E. coli* elicits an increase in immunity to subsequent *B. thuringiensis* infection agrees with data from similar studies (9, 13). Immunization is commonly employed in the functional characterization of putative *B. thuringiensis* virulence factors that may be involved in the circumvention of host immune defenses (9, 10, 29). However, it was previously suggested that immunization may promote the growth of *B. thuringiensis* by removing immunological barriers such as defensive enzymes (6). Here it is shown that immunization increases the level of at least one defense enzyme, PO, in larval hemolymph, although there is no evidence of a causal link between increased PO activity and immunity to *B. thuringiensis*.

The present findings suggest that *B. thuringiensis* is an entomopathogen that can kill lepidopteran larvae by breaching the intestinal epithelium and invading the hemocoel. As previously suggested (28), it is probable that the array of antibiotics (24), bacteriocins (4), and quorum quenchers (23) produced by *B. thuringiensis* may confer a competitive advantage over gut microbes that coinvade the hemocoel and compete for the consumption of the host cadaver. Facultatively pathogenic gut bacteria may synergize Cry protein toxicity or the toxicity of *B. thuringiensis* strains which are attenuated versus a particular species of insect. However, the present work does not support an obligate role for gut bacteria in the insecticidal activity of *B. thuringiensis* toward *M. sexta*.

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