

# From Commensal to Pathogen: Translocation of *Enterococcus faecalis* from the Midgut to the Hemocoel of *Manduca sexta*

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**ABSTRACT** A dynamic homeostasis is maintained between the host and native bacteria of the gastrointestinal tract in animals, but migration of bacteria from the gut to other organs can lead to disease or death. *Enterococcus faecalis* is a commensal of the gastrointestinal tract; however, *Enterococcus* spp. are increasingly frequent causes of nosocomial infections with a high mortality rate. We investigated the commensal-to-pathogen switch undergone by *E. faecalis* OG1RF in the lepidopteran model host *Manduca sexta* associated with its location in the host. *E. faecalis* persists in the harsh midgut environment of *M. sexta* larvae without causing apparent illness, but injection of *E. faecalis* directly into the larval hemocoel is followed by rapid death. Additionally, oral ingestion of *E. faecalis* in the presence of *Bacillus thuringiensis* insecticidal toxin, a pore-forming toxin that targets the midgut epithelium, induces an elevated mortality rate. We show that the loss of gut integrity due to *B. thuringiensis* toxin correlates with the translocation of *E. faecalis* from the gastrointestinal tract into the hemolymph. Upon gaining access to the hemolymph, *E. faecalis* induces an innate immune response, illustrated by hemocyte aggregation, in larvae prior to death. The degree of hemocyte aggregation is dependent upon the route of *E. faecalis* entry. Our data demonstrate the efficacy of the *M. sexta* larval model system in investigating *E. faecalis*-induced sepsis and clarifies controversies in the field regarding the events leading to larval death following *B. thuringiensis* toxin exposure.

**IMPORTANCE** This study advances our knowledge of *Enterococcus faecalis*-induced sepsis following translocation from the gut and provides a model for mammalian diseases in which the spatial distribution of bacteria determines disease outcomes. We demonstrate that *E. faecalis* is a commensal in the gut of *Manduca sexta* and a pathogen in the hemocoel, resulting in a robust immune response and rapid death, a process we refer to as the “commensal-to-pathogen” switch. While controversy remains regarding *Bacillus thuringiensis* toxin-induced killing, our laboratory previously found that under some conditions, the midgut microbiota is essential for *B. thuringiensis* toxin killing of *Lymantria dispar* (N. A. Broderick, K. F. Raffa, and J. Handelsman, Proc. Natl. Acad. Sci. U. S. A. 103:15196–15199, 2006; B. Raymond, et al., Environ. Microbiol. 11:2556–2563, 2009; P. R. Johnston, and N. Crickmore, Appl. Environ. Microbiol. 75:5094–5099, 2009). We and others have demonstrated that the role of the midgut microbiota in *B. thuringiensis* toxin killing is dependent upon the lepidopteran species and formulation of *B. thuringiensis* toxin (N. A. Broderick, K. F. Raffa, and J. Handelsman, Proc. Natl. Acad. Sci. U. S. A. 103:15196–15199, 2006; N. A. Broderick, et al., BMC Biol. 7:11, 2009). This work reconciles much of the apparently contradictory previous data and reveals that the *M. sexta*-*E. faecalis* system provides a model for mammalian sepsis.

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*Enterococcus faecalis* is a ubiquitous member of the normal gut microbiota in diverse species, including vertebrates and insects. However, *Enterococcus* species also frequently cause nosocomial infections with a high mortality rate (1–3). Recently, the gastrointestinal tract has been implicated as a reservoir of bacteria that cause serious diseases, including sepsis (4). Therefore, the normal gut microbiota is a significant source of bacteria that have the potential to translocate to the bloodstream and cause septic death. The complexity and diversity of the mammalian indigenous microbiota, coupled with the rapid progress and lethality of the mammalian disease, have hindered previous studies of sepsis

and the mechanisms of bacterial translocation, emphasizing the need for a simple model to advance our understanding of this opportunistic pathogen. We utilized an invertebrate model organism, *Manduca sexta*, to investigate *E. faecalis* OG1RF-induced sepsis and bacterial translocation from the gut.

*M. sexta* represents a desirable model system for studying *E. faecalis* pathogenicity due to the simple gastrointestinal microbiota community, normal presence of *E. faecalis* in the microbiota, rapid larval life cycle, ease of rearing, and absence of adaptive immunity (allowing specific investigation of the innate immune system during the commensal-to-pathogen switch) (5, 6). Al-

though shown to cause sepsis in humans, *E. faecalis* is a normal member of the healthy human gastrointestinal tract. The mechanism of *E. faecalis* translocation from the gut to the bloodstream remains unknown. To investigate bacterial translocation from the midgut, we utilized *Bacillus thuringiensis* toxin (MVPII formulation) to promote loss of gut integrity, which may contribute to sepsis.

*B. thuringiensis* toxins are insecticidal crystal proteins used against lepidopteran pests that bind receptors on the gut epithelium, leading to pore formation and lysis of the midgut epithelial cells (7, 8). A previous study by Broderick et al. demonstrated that following the formation of these pores, native midgut bacteria contribute to the death of *Lymantria dispar* larvae, which respond to infection with activation of the innate immune response (9). However, controversy remains regarding the direct cause of larval death. Some proposed mechanisms attribute death to direct toxin toxicity or *B. thuringiensis* sepsis (7), translocation of indigenous midgut bacteria into the hemocoel (9–11), or developmental arrest and larval starvation (12, 13).

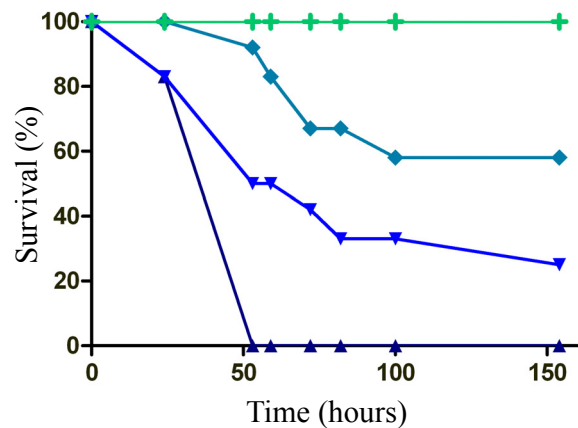
We report here that *E. faecalis* is a commensal in the midguts of *M. sexta* larvae, but when *E. faecalis* is present in the hemolymph, it causes sepsis and rapid death. We present evidence indicating that *B. thuringiensis* toxin (Cry1Ac) mediates the translocation of *E. faecalis* from the gut to the hemolymph, resulting in a commensal-to-pathogen switch and stimulation of the innate immune response.

## RESULTS

***E. faecalis* is a commensal in the gut but a pathogen in the hemocoel.** Although it is found in the gastrointestinal tracts of diverse, healthy animal species, *E. faecalis* has been implicated in translocation from the gut to the bloodstream, causing sepsis (2). Larvae were reared on antibiotic food to clear the midgut microbiota prior to all experiments. *E. faecalis* induced no morbidity or death when early-5th-instar larvae were force fed *E. faecalis* ( $10^8$  CFU), but when injected into the hemocoel, it induced death in a dose-dependent manner (Fig. 1). Prior to death, melanization was observed at the site of injection (first proleg), and over time, melanization progressed through the entire caterpillar in most larvae. *E. faecalis* does not cause morbidity or death when restricted to the gut environment of *M. sexta*, but upon access to the hemocoel, *E. faecalis* leads to rapid, sepsis-like death.

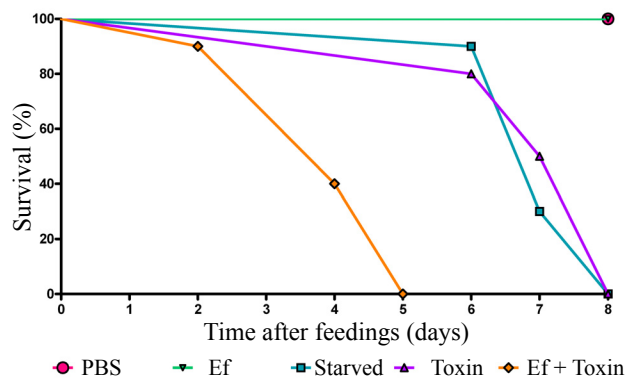
***B. thuringiensis* toxin fed with *E. faecalis* promotes larval death.** Ingested *B. thuringiensis* toxin promotes larval death (14), but the mechanism of killing following pore formation remains under debate. To test the effect of *E. faecalis* in the presence of *B. thuringiensis* toxin, we force fed or injected early-5th-instar larvae. *E. faecalis* and *E. faecalis* plus toxin induced rapid larval death at similar rates when injected (Fig. 2). Phosphate-buffered saline (PBS) induced no death when fed or injected, and *E. faecalis* induced no death when fed to larvae alone. Larvae fed *B. thuringiensis* toxin alone refused all food and died slowly over time from apparent starvation. Larvae fed toxin alone or PBS did not melanize. However, larvae fed *E. faecalis* plus toxin succumbed to a sepsis-like infection that resulted in rapid death. These data support the hypothesis that in the presence of *B. thuringiensis* toxin, *E. faecalis* translocates from the gut to the hemocoel and causes septic death.

***B. thuringiensis* toxin promotes *M. sexta* larval starvation.** To determine whether toxin ingestion promoted weight loss, sim-

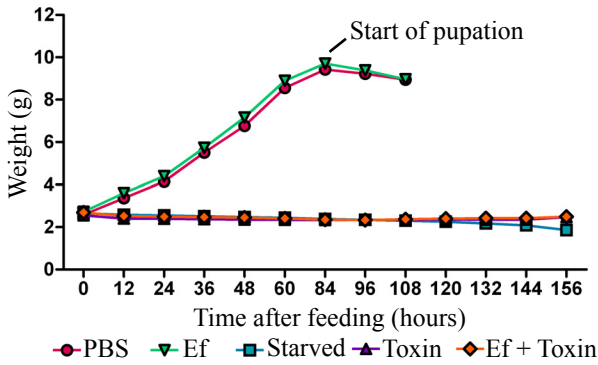


**FIG 1** *E. faecalis* is a commensal in the gut and a pathogen in the hemolymph. Early-5th-instar larvae were fed or injected with PBS or *E. faecalis*, and deaths were recorded over time. The green line (+) represents the following experimental groups, where no deaths were observed: PBS fed, PBS injected,  $10^8$  CFU *E. faecalis* fed,  $10^3$  CFU *E. faecalis* injected, and  $10^4$  CFU *E. faecalis* injected. Symbols: ◆,  $10^5$  CFU *E. faecalis* injected; ▼,  $10^6$  CFU *E. faecalis* injected; ▲,  $10^7$  CFU *E. faecalis* injected ( $n = 20$ /group, 1 representative experiment of 12).

ilar to starvation, the weights of individual 5th-instar larvae were measured every 12 h after feeding (Fig. 3). All larvae except those in the starvation group were fed an unmodified diet *ad libitum* for the duration of the experiment. Larvae force fed *E. faecalis* or PBS continued to gain weight until wandering began at approximately 3 to 4 days. At this point, larvae expelled liquid as they entered the natural pupation phase and were therefore eliminated from the experiment. Larvae fed toxin or *E. faecalis* plus toxin or not fed at all failed to gain weight (Fig. 2). Furthermore, the times to death for toxin-fed and starved larvae were indistinguishable (see Fig. S1 in the supplemental material). Our data suggest that toxin ingestion alone promotes slow death by larval starvation.

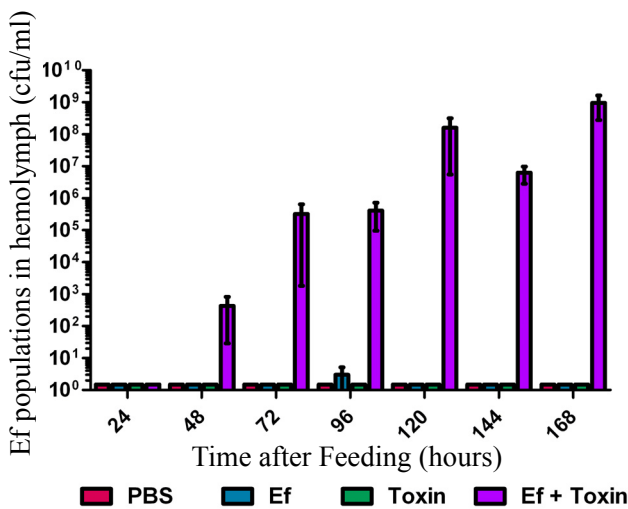


**FIG 2** The presence of *B. thuringiensis* toxin during *E. faecalis* feeding promotes larval death. Early-5th-instar larvae were force fed PBS, *E. faecalis* alone (Ef), *B. thuringiensis* toxin alone (Toxin), or *E. faecalis* and *B. thuringiensis* toxin together (Ef + Toxin). Larvae were given unmodified food *ad libitum* for the duration of the experiment. One experimental group was force fed PBS, and food was subsequently removed for the duration of the experiment (Starved). Larval deaths were recorded over time ( $n = 12$ /group, 1 representative experiment of 12).



**FIG 3** *B. thuringiensis* toxin alone causes larval starvation. Early-5th-instar larvae were force fed PBS, *E. faecalis* (Ef), *B. thuringiensis* toxin (Toxin), or *E. faecalis* and *B. thuringiensis* toxin (Ef + Toxin). Larvae were given unmodified food *ad libitum* for the duration of the experiment. Another experimental group was force fed PBS, and food was subsequently removed for the duration of the experiment (Starved). Larval weight was documented every 12 h, and the mean larval weight over time is depicted ( $n = 20/\text{group}$ , 1 representative experiment of 2).

***B. thuringiensis* toxin results in *E. faecalis* translocation from the gut to the hemolymph.** Larvae fed *E. faecalis* plus toxin died more rapidly than larvae fed toxin alone. To determine whether rapid death was due to *E. faecalis* translocation to the hemolymph, we cultured larval hemolymph for *E. faecalis*. At 24 h after feeding, no *E. faecalis* was detected in the hemolymph of larvae fed PBS, toxin alone, *E. faecalis* alone, or *E. faecalis* plus toxin (Fig. 4). At 48 h after feeding, *E. faecalis* was found in the hemolymph of larvae fed *E. faecalis* plus toxin, and the populations continued to increase over time until larval death (Fig. 4), suggesting that *E. faecalis* escaped from the gut into the hemocoel to cause sepsis-like death. The hemolymph collected from larvae



**FIG 4** *B. thuringiensis* toxin promotes *E. faecalis* translocation from the gut to the hemolymph. Early-5th-instar larvae were force fed, and hemolymph was collected over time. Hemolymph was serially diluted and selectively cultured on BHI agar supplemented with rifampin to look for *E. faecalis* (Ef) translocation to the hemolymph ( $n = 10/\text{group}/\text{time point}$ , 1 representative experiment of 3).

fed PBS, toxin alone, or *E. faecalis* alone contained no detectable *E. faecalis* at any time during the experiment.

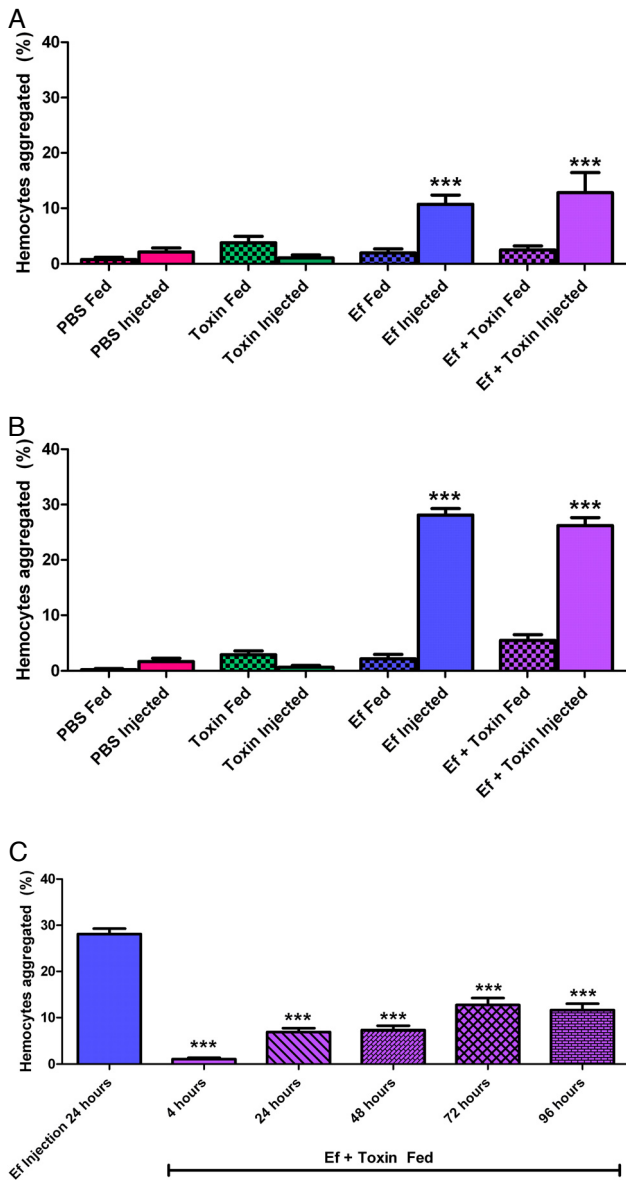
***E. faecalis* persists in the hemolymph of *M. sexta*.** To determine whether *E. faecalis* persisted in the hemolymph of *M. sexta*, early-5th-instar larvae were injected with 10<sup>4</sup> CFU *E. faecalis* and hemolymph was cultured for *E. faecalis* over time. At 6 h after injection, *E. faecalis* was culturable in the hemolymph of larvae (see Fig. S2 in the supplemental material). Interestingly, we consistently observed a slight decrease in *E. faecalis* populations in the hemolymph 24 h after injection. However, *E. faecalis* populations recovered by 48 h after injection and appear to persist over time until larval death.

***E. faecalis* in the hemolymph activates the insect innate immune response.** *M. sexta* larvae succumb to sepsis-like death when *E. faecalis* is present in the hemolymph. To determine whether death was associated with induction of the host innate immune response, hemocyte aggregation was determined after larval feeding or injections. Previous studies have demonstrated that hemocyte aggregation is a reliable indicator of innate immune activation (15, 16). At 4 h after feeding, hemocyte aggregation was at background levels in larvae fed PBS, toxin, *E. faecalis*, or *E. faecalis* plus toxin (Fig. 5A). Furthermore, larvae injected with PBS or toxin alone had minimal hemocyte aggregation, whereas those injected with *E. faecalis* or *E. faecalis* plus toxin had significantly elevated levels of hemocyte aggregation ( $P < 0.001$ ). Hemocyte aggregation did not differ significantly between larvae injected with *E. faecalis* and those injected with *E. faecalis* plus toxin. These data suggest that injection of *E. faecalis* or *E. faecalis* plus toxin results in rapid innate immune recognition and activation.

At 24 h after feeding, hemocyte aggregation remained at background levels in larvae fed or injected with PBS or toxin alone, whereas it was elevated in larvae injected with *E. faecalis* or fed *E. faecalis* plus toxin (Fig. 5B). These data indicate that a rapid innate immune response to *E. faecalis* in the hemolymph increases between 4 and 24 h after injection. To determine whether larvae fed *E. faecalis* plus toxin mount a similar innate immune response but do so more slowly, hemocyte aggregation was determined every day until larval death. Interestingly, despite larval melanization and eventual death, throughout the entire experiment, hemocyte aggregation in larvae fed *E. faecalis* plus toxin did not reach the level found in those injected with *E. faecalis* (Fig. 5C), suggesting that the innate immune response to *E. faecalis* is affected by the route of bacterial entry. Larval hemocytes became activated and aggregated rapidly after *E. faecalis* injection into the hemolymph, regardless of the presence of toxin. However, translocation of *E. faecalis* from the gut to the hemocoel does not activate hemocyte aggregation to a similar level, suggesting an altered response.

## DISCUSSION

The data presented here demonstrate the value of *M. sexta* as a model system for studying the commensal-to-pathogen switch of the opportunistic pathogen *E. faecalis*. We show that *E. faecalis* introduced into the aseptic midgut of early-5th-instar *M. sexta* larvae persisted without apparent harm to the host, but *E. faecalis* induced death when it reached the hemocoel by direct injection or by translocation from the gut, which is enabled by feeding *B. thuringiensis* toxin with *E. faecalis*. *B. thuringiensis* toxin fed alone caused larvae to stop eating; they died of starvation much more slowly than from *B. thuringiensis* and *E. faecalis* cofeeding. Our work establishes conditions that contribute to the commensal-to-



**FIG 5** *E. faecalis* in the hemolymph activates the insect innate immune response. Early-5th-instar larvae were force fed or injected with PBS, *E. faecalis* alone (Ef), *B. thuringiensis* toxin (Toxin), or *E. faecalis* and *B. thuringiensis* toxin together (Ef + Toxin). At each time point, larvae were bled and hemocytes were immediately counted on a hemocytometer. Data depict the percentage of hemocytes that are aggregated out of the total hemocytes. Percentages of hemocytes aggregated were determined at 4 h (A), at 24 h (B), and at later times (C) posttreatment. \*\*\*, *P* value of <0.001 between compared experimental groups. All injections at a single time point were compared to PBS injections at the same time point. Similarly, all feedings at a single time point were compared to PBS feedings at the same time point. Long-term aggregation was statistically significantly different ( $P < 0.0001$ ,  $F = 72.50$ ,  $df = 5$ ) from aggregation for *E. faecalis* injections after 24 h (bottom). Statistical analysis was performed with a one-way ANOVA with a Bonferroni correction for all experimental groups ( $n = 6$  larvae/group/time point, 1 representative experiment of 4).

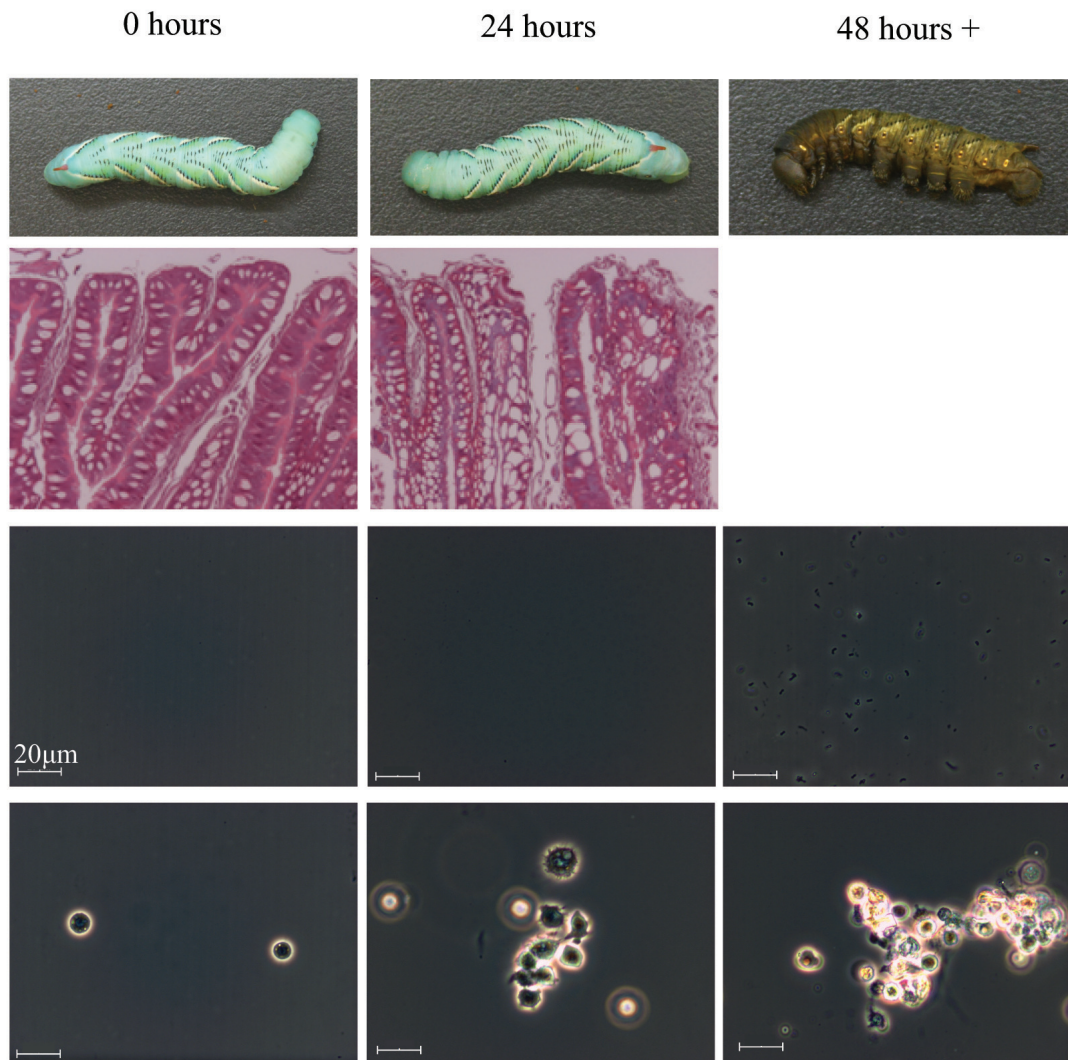
pathogen switch of *E. faecalis* and resolves some of the conflicting results about *B. thuringiensis* toxin (Cry1Ac)-mediated killing.

We propose that *B. thuringiensis* toxin kills *M. sexta* by starva-

tion when fed alone, whereas the insects died of acute infection when *B. thuringiensis* toxin was ingested with *E. faecalis*. These modes of killing are consistent with the three types of killing by *B. thuringiensis* proposed by Heimpel and Angus (14). Type I species are paralyzed by toxin intake and die within hours, type II species suffer a gut-restricted paralysis that includes feeding cessation and death within 2 to 4 days, and type III species require the toxin and *B. thuringiensis* spore for death. This original characterization of toxin mode of action was subsequently developed further by the discovery that many factors altered toxin-induced death, including toxin concentration, host genetics, and larval age (8, 17–20). These factors, which often vary among laboratories, produce conflicting results and controversy in the field. In this study, we standardized many factors. We used the MVPII cell-free formulation of *B. thuringiensis* toxin, which consists of the Cry1Ac protoxin encapsulated in NaCl-killed *Pseudomonas fluorescens*. Previously published work used MVPII, DiPel, or formulations of various toxins, such as Cry1Aa, Cry1Ab, and Cry2A (21–27). MVPII is useful because it circumvents the possible interaction of the host with *B. thuringiensis* cells. Furthermore, to avoid the variation among previous experiments regarding the length of time to host death, we continued all experiments involving larval death for at least 1 week or until death. This time course (Fig. 6) enabled us to determine whether host death was due simply to starvation or to acute bacterial infection and to visualize larval melanization. Our data demonstrate that MVPII *B. thuringiensis* toxin induced larval starvation and eventual death in *M. sexta* and that the indigenous gut microbiota increased the rate of death dramatically.

The effect of *B. thuringiensis* toxin on larval death was previously investigated by our laboratory in six larval species. In *L. dispar*, the indigenous gut microbiota plays a role in *B. thuringiensis* toxin lethality, although this phenomenon varies among labs. The present study highlights the effect of variation in experimental conditions on the mortality rate, including larval species, larval age, experimental duration, rearing conditions, and route of toxin introduction (21, 28). We chose *M. sexta* as our model organism as an alternative to *L. dispar* due to its ease of rearing, numerous previous studies, and ease of altering many experimental variables that may contribute to the conflicting results obtained. To ensure that a consistent dose of toxin or *E. faecalis* was delivered to each larva, we adopted force feeding and injection protocols. We standardized larval size and developmental stage by using exclusively early-5th-instar larvae, which were large enough to be force fed with a gavage needle. While previous studies relied upon the larvae to eat food containing the toxin or bacteria in it, force feeding standardizes the dose and time of ingestion for each larva. In contrast to our previous finding with *L. dispar*, in *M. sexta*, toxin ingestion induced immediate food refusal by *M. sexta* and resulted in slow death from starvation in the absence of gut microbiota (21). *M. sexta* starvation to death takes approximately 1 week, which is the same time to death for larvae fed toxin only. In contrast, *M. sexta* larvae that ingested *E. faecalis* plus toxin died much more rapidly. We propose that *E. faecalis*-induced sepsis was the cause of death of *M. sexta* resulting from *B. thuringiensis* toxin ingestion.

Previously published work suggested that antibiotics alone may confound experimental results. We studied the effect of antibiotic use on larval killing through the use of larvae reared continuously on an unamended diet and larvae reared on an antibiotic diet. We found that the use of antibiotics did not alter the



**FIG 6** Time course of the commensal-to-pathogen switch of *E. faecalis*. The commensal-to-pathogen switch of *E. faecalis* is shown in this representative schematic. Following ingestion of *E. faecalis* and *B. thuringiensis* toxin, there is a loss of gut integrity (second row from top) that allows the translocation of *E. faecalis* into the hemocoel of *M. sexta* (third row from top). The presence of *E. faecalis* in the hemolymph results in hemocyte aggregation/innate immune activation; however, the innate immune response (indicated by hemocyte aggregation; bottom row) is not potent enough to prevent melanization (top row) and sepsis-like death of the larvae.

morbidity and mortality associated with any experimental group (see Fig. S2 in the supplemental material), including those fed *E. faecalis* plus toxin. We concluded that the choice of antibiotics did not appreciably alter the host immune response to systemic infection with *E. faecalis* in *M. sexta*.

Innate immune activation differed between larvae fed or injected with *E. faecalis*. Feeding *E. faecalis* plus toxin heightened the immune response more than feeding of *E. faecalis* alone, but injection of *E. faecalis* directly into the hemolymph resulted in the most hemocyte aggregation, which might be due to the route of entry into the hemolymph. Persistence in the harsh environment of the lepidopteran gut may alter *E. faecalis* gene expression, thereby altering bacterial behavior or initial host immune recognition. During translocation from the midgut, *E. faecalis* levels were lower than those observed during direct injection into the hemocoel, perhaps because it induced a milder host immune re-

sponse. Although ingestion and injection of *E. faecalis* plus toxin and *E. faecalis*, respectively, activated the host's immune response to different degrees, both treatments induced larval death, suggesting that *E. faecalis* might kill by slightly different mechanisms, depending on whether it undergoes translocation from the gut. Further studies are required to identify the specific genes altered during the *M. sexta* innate immune response and the mechanism behind bacterial translocation-induced innate immune alterations.

In this study, we investigated the mode of action of *B. thuringiensis* toxin (Cry1Ac) and resolved some of the contradictions in the literature by standardizing methodologies. Researchers have investigated *B. thuringiensis* toxin toxicity with various protocols that differed in lepidopteran species age, antibiotics in the larval diet, and toxin formulations (14, 27, 29–35), all of which we found to affect results. van Frankenhuyzen et al. also found that small

differences in methodology led to different mortality responses in larvae (27). Here we standardized conditions (antibiotics, toxin, diet, and developmental stage) and report that *E. faecalis* is a commensal in the midguts of *M. sexta* larvae and a pathogen in the hemocoel. *B. thuringiensis* toxin mediates the translocation of *E. faecalis* from the gut to the hemolymph, resulting in a commensal-to-pathogen switch and stimulation of the innate immune response.

## MATERIALS AND METHODS

***M. sexta* larva rearing.** *M. sexta* eggs were obtained from the Carolina Biological Supply Company in Burlington, NC. Eggs were surface sterilized with a solution of Tween 80 (polyoxyethylene sorbitan monooleate), bleach, and distilled water as described previously (36). Eggs were hatched in 100-cm petri plates, and individual larvae were transferred to sterile 2-ounce clear plastic cups with holes for ventilation (SOLO, Lake Forest, IL). Larvae were reared on a sterilized artificial diet (USDA, Hamden Formula) amended with two antibiotics and an antifungal agent (250 mg/liter rifampin, 250 mg/liter gentamicin, or 20 mg/liter nystatin) in an environmental chamber at 26°C and 57% relative humidity on a 16:8 (light/dark) photoperiod.

**Bacterial and toxin strains.** *E. faecalis* OG1RF was provided by Gary Dunny at the University of Minnesota. For bacterial feeding and injection assays, *E. faecalis* was cultured at 37°C with shaking overnight in M9-Casamino Acids-yeast extract medium with 200 mg/liter rifampin (37). Bacterial cultures were washed in phosphate-buffered saline, quantified on a hemacytometer, and diluted as necessary. Bacterial population sizes were confirmed through serial dilutions cultured on brain heart infusion (BHI) agar (supplemented with 200 mg/liter rifampin) and quantified the following day. *B. thuringiensis* toxin was administered via the MVP II formulation (Cry1Ac encapsulated in *P. fluorescens*; Dow AgroSciences, San Diego, CA). MVP II was weighed out and diluted as necessary in PBS.

**Feeding and injection assays.** Bacterial cultures and/or toxin were force fed to early-5th-instar larvae in 10- $\mu$ l doses using a gavage needle with a 1.25-mm tip (Fine Science Tools, Foster City, CA) mounted on a hand-held repetitive Stepper pipette (Tridak, Torrington, CT). Injections of 10- $\mu$ l doses were administered to early-5th-instar larvae using a 30.5-gauge needle mounted on the Tridak Stepper. Larvae were surface sterilized, and the needle was inserted into the first proleg parallel to the epidermis to avoid injury to the alimentary canal.

For all experiments, control larvae were fed or injected with 10  $\mu$ l of PBS. Larvae in mortality assays were placed in clean containers and provided with a sterile, unamended artificial diet for the duration of the assay.

***E. faecalis* population counts in the hemolymph and gut.** Hemolymph was collected from larvae at appropriate time points as previously described (38). Samples were serially diluted in PBS, cultured on BHI agar supplemented with 20 mg/liter rifampin, and incubated overnight at 37°C.

**Hemocytometry aggregate counts.** To assess hemocyte aggregation, larvae were surface sterilized and bled from the first proleg into a 1.5-ml sterile Microfuge tube with 0.02% bromophenol blue. Samples were kept on ice and counted immediately using a hemocytometer. Dead cells were excluded from the analysis, and aggregates were defined as two or more hemocytes in direct contact. Percent hemocyte aggregation was determined as follows: % hemocytes aggregated = [(number of hemocytes in aggregates)/(total number of hemocytes)]  $\times$  100.

**Histological studies.** Gut tissues were fixed in 10% neutral buffered formalin supplemented with 2% dimethyl sulfoxide for 24 h. Tissues were embedded in paraffin, cut, and stained with hematoxylin and eosin for microscopic analysis.

**Statistical analysis.** Statistical analysis of hemocyte aggregation was performed in GraphPad Prism 5. A one-way analysis of variance (ANOVA) with a Bonferroni correction was performed for all experimental groups.

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K.L.M. and T.A.S. contributed equally to the manuscript.

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## SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at <http://mbio.asm.org/lookup/suppl/doi:10.1128/mBio.00065-11/-/DCSupplemental>.

Figure S1, EPS file, 1.891 MB.

Figure S2, EPS file, 1.598 MB.

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