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The Pore-Forming Protein Cry5B Elicits the Pathogenicity of Bacillus sp. against Caenorhabditis elegans

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Abstract

The soil bacterium Bacillus thuringiensis is a pathogen of insects and nematodes and is very closely related to, if not the same species as, Bacillus cereus and Bacillus anthracis. The defining characteristic of B. thuringiensis that sets it apart from B. cereus and B. anthracis is the production of crystal (Cry) proteins, which are pore-forming toxins or pore-forming proteins (PFPs). Although it is known that PFPs are important virulence factors since their elimination results in reduced virulence of many pathogenic bacteria, the functions by which PFPs promote virulence are incompletely understood. Here we study the effect of Cry proteins in B. thuringiensis pathogenesis of the nematode Caenorhabditis elegans. We find that whereas B. thuringiensis on its own is not able to infect C. elegans, the addition of the PFP Cry protein, Cry5B, results in a robust lethal infection that consumes the nematode host in 1–2 days, leading to a “Bob” or bag-of-bacteria phenotype. Unlike other infections of C. elegans characterized to date, the infection by B. thuringiensis shows dose-dependency based on bacterial inoculum size and based on PFP concentration. Although the infection process takes 1–2 days, the PFP-instigated infection process is irreversibly established within 15 minutes of initial exposure. Remarkably, treatment of C. elegans with Cry5B PFP is able to instigate many other Bacillus species, including B. anthracis and even “non-pathogenic” Bacillus subtilis, to become lethal and infectious agents to C. elegans. Co-culturing of Cry5B-expressing B. thuringiensis with B. anthracis can result in lethal infection of C. elegans by B. anthracis. Our data demonstrate that one potential property of PFPs is to sensitize the host to bacterial infection and further that C. elegans and probably other roundworms can be common hosts for B. cereus-group bacteria, findings with important ecological and research implications.

Introduction

The Bacillus cereus group of bacteria comprises six species, including three highly related species, B. cereus sensu stricto, B. anthracis and B. thuringiensis, which are sometimes considered a single species [1,2,3]. Although closely related, these bacteria are associated with very different diseases. B. anthracis is the causative agent of anthrax [4], B. cereus can cause food poisoning and various opportunistic and nosocomial infections [5], and B. thuringiensis is an invertebrate-specific pathogen [6]. The relationship between these three closely related species of Bacillus in the wild, e.g. how they replicate in the soil environment and what environmental niches they occupy relative to one another, has been the subject of much speculation [7,8].

The defining characteristic of B. thuringiensis is the production of Crystal (Cry) proteins, a large family of related proteins that kill insects and nematodes [8,9,10]. Each B. thuringiensis strain found in the wild can produce one or multiple (typically 2–4) Cry proteins. These Cry proteins are pore-forming proteins (PFPs), and thus are members of the single largest class of bacterial virulence factors [11]. Indeed, roughly 25–30% of all protein toxins made by human bacterial pathogens are PFPs, many with proven roles in disease pathogenesis. Purified Cry proteins alone, even in the absence of B. thuringiensis, produce dose-dependent lethality in invertebrates, and as such can be expressed in transgenic crops to control insect pests.

Here for the first time we characterize in detail the effect of Cry protein PFP administration on the outcome of B. thuringiensis infection in the nematode Caenorhabditis elegans, and uncover an unexpected interaction of Cry proteins with other Bacillus species bacteria during C. elegans infection.

Results

Infectivity of C. elegans by Bacillus species in presence of Cry5B pore-forming protein

In our numerous studies of nematicidal Cry proteins in the absence of B. thuringiensis but in the presence of its standard
laboratory food source, *Escherichia coli*, we have found that Cry proteins produce a dose-dependent mortality in nematodes but do not promote an *E. coli* infection. Likewise, non-Cry protein-producing *B. thuringiensis* strains, such as HD1 cry-, do not cause an infection when fed to *C. elegans* in the absence of Cry proteins (Figure 1A; Table 1; [12]). We therefore investigated what would happen if we fed *C. elegans* Cry5B, a Cry protein that forms pores in membranes [13], in the presence of *B. thuringiensis*. Purified Cry5B produces mortality in *C. elegans* over a 6 day time course at 25°C with a calculated LD$_{50}$ of $\sim$8 \mu g/ml [14]. Under these conditions, *E. coli* is used as the food source and no infections (internal multiplication of *E. coli*) are seen, the nematodes die from Cry protein intoxication. Conversely, when we fed Cry5B to *C. elegans* at 100 \mu g/ml in the presence of *B. thuringiensis*, we observed accelerated killing of nematodes in 24–48 h, with the internal structures of the nematodes completely digested by the proliferating bacteria (Figure 1B, Table 1). With *B. thuringiensis*, the majority of the killing takes place in the first 24 h, although we typically let the assays run for 48 h to ensure maximum killing was reached. Nematodes killed by *B. thuringiensis* with Cry5B are filled with vegetative *B. thuringiensis* cells or *B. thuringiensis* spores (Figure 1B), a phenotype we have named “Bob” for Bag of bacteria, where the undigested cuticle of the nematode represents the bag that contains the bacteria.

Quantitation of infected animals in the presence and absence of Cry5B demonstrates that the PFP is absolutely required for the infection process—in the absence of Cry5B no *B. thuringiensis* infections are seen whereas in the presence of Cry5B a very high percentage of the nematodes succumb to accelerated lethal infections (Table 1). To confirm that the infections seen are independent of internal hatching of progeny in hemaphroditic *C. elegans*, we performed similar studies using the *C. elegans* mutant glp-4(bn2), which are sterile at the restrictive temperature and do not produce progeny although they do have a similar response to Cry5B as wild-type *C. elegans* [15]. Similar robust Cry5B-mediated infections by *B. thuringiensis* are found in this genetic background as well (Table 1). In these experiments and other experiments with other *Bacillus* sp., Cry5B purified from *B. thuringiensis* was added exogenously so that all the experiments could be directly compared. We have confirmed that *B. thuringiensis* HD-1 endogenously producing Cry5B also produces a robust number of Bobs (32/33) whereas an isogenic HD-1 strain of *B. thuringiensis* lacking Cry5B production does not (0/31). Thus Cry5B PFP is an essential virulence factor required for lethal infection of *C. elegans* by *B. thuringiensis*.

Cry5B PFP intoxicates and kills *C. elegans* via binding to glycolipid receptors found in the intestine [16]. To confirm that Cry5B-stimulated *B. thuringiensis* infections require the established PFP intoxication pathways, we repeated the challenge experiments in glycolipid-receptor deficient *bre-4(ye13) C. elegans* animals [16,17]. As predicted, nematodes lacking the receptor for Cry5B are not infected by *B. thuringiensis* even in the presence of Cry5B (Table 1).

![Figure 1. Infection of *C. elegans* by *B. thuringiensis* and *B. anthracis*.](M9107371008001)
Dependency of the infection process upon PFP dose and bacterial inoculum size

Our experiments demonstrate that infectivity is fully dependent both upon Cry5B sensitization and the presence of a pathogenic bacterium (B. thuringiensis). We next set out to determine the relationship between each of these elements and their dosage. Infection assays were performed with either highly infective B. thuringiensis or weakly infective B. megaterium (as a negative control; see below) at increasing doses of Cry5B (Figure 2A). As demonstrated above, in the absence of Cry5B, no infections were seen with either bacterium (Figure 2A). As the dose of Cry5B increases, the percent of worms infected by B. thuringiensis HD-1 increases until maximum infectivity is reached at ~10 μg/ml Cry5B (Figure 2A). Thus, B. thuringiensis lethal infection depends upon Cry5B levels in a dose-dependent manner. Because it was the smallest dose that still achieved penetrant infectivity, we utilized 10 μg/ml as our Cry5B dose in all our fixed dose experiments.

Many key epidemiological processes are inoculum-dependent, e.g., the chance of infection in a given host increases with increasing pathogen inoculum (reviewed in [18]). To determine if this relationship between pathogen and host are true for increasing pathogen inoculum (reviewed in [18]). To determine if this relationship is robust to Cry5B sensitization and presence of a pathogenic bacterium (B. thuringiensis) vs. B. megaterium (no Cry5B), we utilized wild type, glp-4(bn2) mutant animals and bre-4(ye13) animals.

Table 1. Infectivity of Bacillus sp. on C. elegans in the presence/absence of pore-forming Cry5B.

| Bacillus species | Total number of animals | Number of Bobs |
|------------------|-------------------------|----------------|
| N2 wild-type animals |                      |                |
| Without Cry5B PFP | 107                     | 0              |
| With Cry5B PFP   | 133                     | 102            |
| B. thuringiensis  | 119                     | 0              |
| B. subtilis PY79  | 122                     | 53             |
| B. subtilis 6051  | 132                     | 0              |
| B. megaterium     | 149                     | 8              |
| glp-4(bn2) mutant animals |              |                |
| Without Cry5B PFP | 80                      | 0              |
| With Cry5B PFP   | 72                      | 68             |
| B. thuringiensis  | 71                      | 0              |
| B. subtilis PY79  | 72                      | 5              |
| B. subtilis 6051  | 90                      | 0              |
| B. megaterium     | 76                      | 1              |
| bre-4(ye13) animals |                       |                |
| Without Cry5B PFP | 115                     | 0              |
| With Cry5B PFP   | 100                     | 0              |
| B. thuringiensis  | 117                     | 0              |
| B. subtilis PY79  | 94                      | 0              |
| B. subtilis 6051  | 103                     | 0              |
| B. megaterium     | 102                     | 0              |

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Temporal requirements of the infection process

Our data demonstrate that incubation of B. thuringiensis and Cry5B continually results in a lethal infection within 48 h. We next set out to study the temporal requirements for the infection process—e.g., does B. thuringiensis and Cry5B need to be continually fed to C. elegans during this entire time period or are shorter incubation periods also effective in establishing an infection? To determine when the infection process was established, a B. thuringiensis pulse-chase experiment was designed (Figure 3A, schematic). Young adult C. elegans hermaphrodites were exposed to pulses of the B. thuringiensis and Cry5B PFP for varying lengths of time that ranged from 5 minutes to 8 hours. Following these pulses, the nematodes were passed through a series of five washes containing B. megaterium (no Cry5B) to dilute out both the infectious bacteria and Cry5B. Animals were incubated in the final wash for a total of 48 hours and then scored for Bobs (Figure 3A, graph). Surprisingly, high levels of lethal infections are established in as little as 15 minutes of exposure to the pathogen plus Cry PFP. Indeed, the high levels of infections seen upon exposure to pathogenic conditions for any of the time points between 15 minutes and 8 hours are not statistically different (ANOVA, P>0.05, Tukey’s post test).

To confirm that the infections seen in the pulse-chase experiment were not the result of residual B. thuringiensis and Cry5B in the final well containing B. megaterium, the experiment
It is known that the intestinal pores formed by a short pulse of Cry5B on *C. elegans* can last for up to 1 d [19]. Thus, it is possible that the effects of Cry5B PFP on the infection process occurs late even though the pulse used in the above experiments was early and brief. To address the issue of whether Cry5B PFP is acting late or early, we first exposed the animals to the infectious bacteria, washed these away, and then added Cry5B (Figure 3B, schematic). If Cry5B were only acting late in the process, we rationalized that there should be no difference between adding Cry5B with the bacteria in the very beginning or adding Cry5B after a slight delay of ~10 min. In fact, we found that adding Cry5B subsequent to the bacteria leads to a significant drop in infection levels. These data indicate that the PFP instigates infection of *B. thuringiensis* in *C. elegans* at the earliest stages.

To see if we could detect visual cues of the beginning of the infection process, green-fluorescent protein (GFP)-expressing *B. thuringiensis* was fed to *C. elegans* along with Cry5B PFP. The earliest and most common phenotype we could see (~3–6 h after initiation of the experiment) was the formation of a cluster of vegetative bacteria in the interior lumen of the intestine in many of the nematodes (Figure 3C). These data suggest that, at least by this time-point, the focus point of the infection process is in the anterior intestine, from which it likely spreads.

Cry5B PFP potentiates the infectivity of many *Bacillus* sp.

Our data demonstrate that exposure to the crystal PFP Cry5B allows *B. thuringiensis* to initiate a lethal infection in *C. elegans*. We tested that the PFP might also potentiate the infectivity of other *Bacillus* sp. First, we tested *Bacillus subtilis*. *B. subtilis* is nonpathogenic towards *C. elegans*, and *C. elegans* have increased longevity when fed on *B. subtilis* than when fed on *E. coli* OP50, the standard laboratory *C. elegans* food source [20,21]. We quantitated the ability of *B. subtilis* (two different strains) to infect *C. elegans* in the absence and presence of Cry5B PFP. Whereas *B. subtilis* alone is unable to infect *C. elegans*, this “nonpathogenic” bacterium is able to infect *C. elegans* in the presence of Cry5B PFP (Table 1). As with *B. thuringiensis*, *B. subtilis* infections occur even in the sterile gfp-4 (bn2) *C. elegans* background (Table 1). Thus, internal hatching of progeny is not required for infection by *B. subtilis*, although some contribution may be reflected by a drop-off in infection rate in gfp-4 (bn2) animals (Table 1). As with *B. thuringiensis*, Cry5B intoxication via the glycolipid receptor is required for subsequent infection by *B. subtilis*, as bre-4(ye13) receptor-less mutants are not infected by *B. subtilis* in the presence of PFP (Table 1).

To look closer at the specificity on the *Bacillus* species, we repeated these Cry5B PFP-mediated infection assays using a broader range of *Bacillus* sp. for these experiments and the *C. elegans* strain gfp-4 (bn2) to avoid any complication in interpretation from internal hatching of larvae (Figure 4). We found that the bacteria from the *B. cereus* group- *B. cereus*, *B. thuringiensis* 407-gfp, *B. thuringiensis* 4Q7 and *B. anthracis* Sterne are the most virulent, each infecting ~25% of the nematodes (Figure 4). As reported in Table 1, *B. subtilis* is able to infect at an appreciable level and *B. megaterium* at a barely detectable level (Figure 4). Interestingly, *B. circulans*, which is phylogenetically quite divergent from the other *Bacillus*, is also capable of infection, but *B. sphaericus*, which is used as an insecticidal bacterium like *B. thuringiensis*, is not (Figure 4). *B. anthracis* infected nematodes look similar to *B. thuringiensis*-infected nematodes under low and high-magnification microscopy (Figure 1C).

It has been previously shown that *B. thuringiensis* is capable of causing lethal infections in insects and that this infectivity is greatly dependent on the master regulator of virulence, PhoR [22,23]. In *Galleria mellonella* larvae, co-infection of the *B. thuringiensis* parental

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**Figure 2. Dose-dependency of lethal infections by Bacillus and Cry5B.**

(A) Infections increase in a dose-dependent manner with increasing Cry5B. Doses from 0 to 20 µg/ml Cry5B were prepared and standard infection assays with *B. thuringiensis* HD-1 carried out. No worms were infected at any of the Cry5B concentrations when exposed to the relatively non-pathogenic *B. megaterium* in the negative control experiment. Error bars indicate standard error. *n* = 3 trials, with an average of 40 worms per condition per trial. The glp-4 (bn2) strain was used to eliminate any concerns of internal hatching of larvae as the source of death. (B) The lethal infection rate induced by Cry5B increases with increasing doses of *B. thuringiensis* or *B. anthracis*. Varying volumes of *B. thuringiensis* or *B. anthracis* were mixed with volumes of the non-pathogenic *B. megaterium* to create different ‘doses’ of the pathogen while presenting a constant total bacterial load to the worms. Error bars indicate the SEM. *n* = 3 trials, with an average of 60 worms per condition per trial. The data for 0% *B. thuringiensis* and 100% *B. anthracis* were collected but cannot be displayed on this log plot; there were no lethal infections at this dose.

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was set up as above but no worms were placed in the initial *B. thuringiensis* well. The same volume as pipetted in the above experiments (without nematodes) was then pasaged from the first well. No infections were ever detected in four repetitions of this experiment. Thus, the amount of *B. thuringiensis* transferred from the first to the final well is not sufficient on its own to cause infection and the infections seen in Figure 3 are the result of interactions between the worms and bacteria exposed to one another in the first well.
strain with a sublethal concentration of Cry1C toxin caused 70% mortality whereas only 7% mortality was recorded if a ΔplcR mutant strain were used [23]. Interestingly, in Cry5B-sensitized C. elegans, loss of PlcR from B. thuringiensis still produces a robust, albeit reduced, percentage of infected dead animals (Figure 4; 44% of Bobs with B. thuringiensis 407ΔplcR versus 73% with the B. thuringiensis-407-gfp parental strain). Moreover, B. anthracis Sterne, which lacks a functional PlcR [22], is able to produce a level of infection in Cry5B-sensitized C. elegans comparable to one of the B. thuringiensis 4Q7 (Figure 4). PlcR is therefore dispensable for infection in C. elegans and much less important for infection in C. elegans than in Galleria. Taken together, Cry5B PFP is able to potentiate the infectivity of many, but not all, Bacillus towards C. elegans, with the tightly knit B. cereus group as the most potentiared.

Figure 3. Temporal aspects of the infection process. (A) Infections are established upon a short exposure to pathogen. Upper: schematic of experiment. Worms are added to the well on the left containing B. thuringiensis and Cry5B. Following either 5 min, 15 min, 30 min, 1 h, 2 h, 4 h, or 8 h, the worms were then moved through a series of five wells lacking Cry5B protein and containing non-infectious B. megaterium instead of B. thuringiensis. Infection outcomes in the final well were scored 48 h later. Lower: results of the experiment depicted in upper schematic. Data shown represent a total of three independent experiments with ~30 animals per time point per experiment. Error bars indicate SEM. Only the infection rate for a 5-minute pulse is significantly different from the other data points (ANOVA, p < 0.05, Tukey’s post test). (B) Cry5B acts early in the infection process as temporal addition of Cry5B protein after, and separate from, pathogen exposure results in a significant drop in infections. Upper: schematic of experiment in which worms are exposed to pathogen first and then to Cry5B. Lower: results in which two sets of experiments were set up simultaneously—a normal 15 minute pulse chase with both Cry5B PFP and pathogen added together (light gray bars; see (A) for set up) and pulse chase in which Cry5B was not added until the end (dark gray bars). Error bars represent SEM. ~30 worms/condition/trial; 3–4 independent trials per condition. (C) The infection process appears to begin with colonization of the anterior intestine. The anterior intestine of a nematode 3 hr after exposure to B. thuringiensis (407-gfp) under pathogenic conditions. The left panel is a DIC image; the right panel deconvolved fluorescent (FITC) of the same animal. Images taken at 600×. Anterior is down.

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**B. anthracis**, which naturally lacks crystal proteins, can infect in the presence of **Cry5B-B. thuringiensis**

By definition **B. thuringiensis** is the bacterium that encodes for and produces Cry proteins whereas none of the other *Bacillus* sp., including the other *B. cereus* family members, do. We therefore wondered whether there might be any instance in which a Cry protein-minus Bacillus bacterium, such as *B. anthracis*, might be able to infect *C. elegans* with the help of *B. thuringiensis*. This question is important because it is still a mystery how *B. anthracis* maintains its life cycle in the soil so that it can be periodically transmitted to herbivore hosts [7,8,24]. We hypothesized that *B. anthracis* might be able to infect *C. elegans* if it was present coincidently with *B. thuringiensis*—i.e. *B. anthracis* could cause a super infection when *B. thuringiensis* was present.

We took a *B. thuringiensis* strain with a Cry5B plasmid, as might be found in the soil, and sporulated it to express Cry5B. We then mixed these *B. thuringiensis* spore-crystal lysates with sporulated *B. anthracis*-GFP spores. In these experiments, the bacteria were mixed at a ratio of about 16 *B. thuringiensis* spores for one *B. anthracis*-GFP spore. After 48 h of incubation at 25°C, we counted the number of worms and looked at them under the fluorescence microscope. We found that some of these infected worms were full of the *gfp*-anthrax (Figure 5; Table 2). Thus, even with an initially much larger load of *B. thuringiensis* being given to the animals and at a temperature (25°C) where *B. thuringiensis* might be expected to perform better, on occasion *B. anthracis* is able to overtake and drive the infection process.

**Discussion**

Here for the first time we characterize in detail the infectivity of *Bacillus* family bacteria in a nematode, *C. elegans*. Beginning with the invertebrate-specific pathogen, *B. thuringiensis*, we find that the infection of *C. elegans* by *B. thuringiensis* is dependent upon the presence of a PFP crystal protein virulence factor, Cry5B. The Cry5B pore-forming protein either can be added exogenously or can be naturally expressed by *B. thuringiensis* in order to elicit infection. The *B. thuringiensis* strains used in this study cannot infect the in the absence of added Cry5B (either exogenous or
endogenous). Infectivity is proportionally dependent upon inoculum size and dose of the pore-forming protein. Although the fact that *B. thuringiensis* (but not other *Bacillus* species) can infect *C. elegans* has been seen before [25,26], the dependence of this infection on crystal protein has not been studied. We furthermore demonstrate that the PFP acts early in the process to instigate infection. The process is likely to be distinct from *B. thuringiensis* infections characterized in insects since the dependence upon the Pfr virulence regulator, which has been shown to be central for infectivity in an insect [23], is minimal in the nematode system.

Surprisingly we find that many *Bacillus* species also possess the ability to infect *C. elegans* in the presence of Cry5B. This list of *Bacillus* sp. that can infect *C. elegans* includes *B. subtilis*, a bacterium considered non-pathogenic to *C. elegans* and even more benign than the standard laboratory *E. coli* fed to it [20,21]. Of all *Bacillus* sp. tested, *B. cereus* group bacteria show the highest level of infectivity in the presence of the Cry5B PFP. The relative role of Cry toxin to various *Bacillus* species has also been investigated in the larvae of the greater wax moth (*G. mellonella*). In this insect Cry1C toxin induced higher mortality to *B. thuringiensis* and *B. cereus* strains and to a much lower level to *B. subtilis* and *B. megaterium* and not at all with *B. anthracis* [23,27], suggesting that *B. anthracis* develops better in nematode than in insects. We speculate that infectivity of nematodes is an ancient hallmark of the *Bacillus* genus and that either many *Bacillus* sp. have subsequently lost the ability to infect nematodes or in the wild factors other than crystal proteins (e.g., environmental stress, starvation of the nematodes, virulence factors not normally expressed in the laboratory) instigate infection.

This work establishes the *Bacillus*-Cry5B-*C. elegans* interaction system as a model for studying specific requirements of the nematode infection process of *Bacillus*, most notably *B. cereus*-family bacteria such as *B. anthracis*, for studying the role of PFPs in promoting bacterial pathogenesis, and for studying host responses to *B. cereus* family bacteria. The fact that both the bacteria and the host are amenable to genetics makes it possible to investigate the interactions of *B. cereus* family bacteria and an animal host at a level not previously attained.

This research also begins to address an important question in the *B. anthracis* field—what are the potential reservoirs of this bacterium in nature? *B. anthracis* causes periodic, large-scale episodes of infection in ruminants. The question has remained: where does it reside and how does it propagate between episodes? One suggestion is that earthworms are reservoirs for *B. anthracis* and *B. cereus* multiplication and persistence [28]. Our data suggest that nematodes are potential targets too, when present with *B. thuringiensis*, *B. anthracis* is able to replicate within a nematode. Therefore, nematodes might provide a key, and heretofore elusive, natural reservoir for *B. cereus* and *B. anthracis* along with earthworms and could play a specific epidemiological role in anthrax outbreaks in the wild. Furthermore, nematodes may provide a key ecological niche linking the three bacteria in the *B. cereus*-group and facilitating genetic exchange, such as the many that have already been observed [29,30] and thus helping shape the common evolution of these bacteria.

Materials and Methods

Bacterial strains

Bacteria were cultured in Brain Heart Infusion (BD, Sparks, MD, USA) at 30°C (37°C for *B. anthracis* Sterne). For sporulation, *B. anthracis* was cultured in Difco Sporulation Medium [31]. The *Bacillus* strains used in this work were: *B. cereus* (ATCC 14579), *B. thuringiensis* (HD-1), *B. thuringiensis* (407-gfp), *B. thuringiensis* (407-APkR-gfp), *B. thuringiensis* (4Q7), *B. anthracis* Sterne, *B. anthracis* 7702 harboring pUTE610 (a gift from Theresa Koehler, University of Texas), *B. subtilis* PY79 (a gift from Richard Losick, Harvard University), *B. subtilis* 3610, *B. subtilis* 6051, *B. subtilis* (ATCC 4325), *B. circulans* (ATCC 4513), and *B. megaterium* (ATCC 14581). *B. thuringiensis* 407-gfp and *B. thuringiensis* (407-APkR-gfp) contain a constitutively expressed Mutll-green fluorescent protein (GFP) carried on a multicopy plasmid (pHT315 papha3::gfp) with an erythromycin selection factor [32].

Nematode strains

*C. elegans* were maintained using standard techniques [33]. Unless otherwise mentioned, *glp-4(me2)* worms were used in all assays to prevent matricidal death due to premature hatching of embryos. At 25°C, this strain lacks germline and does not produce progeny. This strain is often used in *C. elegans* assays involving bacterial pathogens [15,34,35,36,37,38]. We found that many fertile *C. elegans* hermaphrodites can die from the internal hatching of larvae when exposed to *Bacillus* spp. under conditions of the infection model. Invariably, these dead worms would subsequently become infected. Under these circumstances, it was not easy to differentiate between worms that had become infected before dying versus worms that had died from internal hatching of larvae before becoming infected. Thus, we use *glp-4(me2)* in many of our assays to avoid this ambiguity. We do not believe the use of *glp-4(me2)* significantly alters our findings. Similarly robust infections are achieved using wild-type N2 *C. elegans* as with *glp-4(me2)*, infected animals look the same under high power magnification regardless of which strain is used, and a completely different sterile strain, fer-1(me574), shows similar levels of infection to *glp-4(me2)* (data not shown). *glp-4(me2)* worms were maintained at 15°C and shifted at the appropriate times to 25°C when sterility was desired.

Expression and Purification of Cry5B protein

Purified Cry5B protein was prepared using a modified sucrose gradient [39]. Purified Cry5B protein was precipitated, resuspended in double-distilled H2O, frozen into aliquots using liquid nitrogen, and stored at –80°C until needed. On the day of use, aliquots were thawed at room temperature and centrifuged at 16,000 g to pellet the protein and allow aspiration of the supernatant. Pellets were then solubilized in 20 mM citrate buffer (pH 3.0) to a final concentration of 0.4 mg/ml.

| Condition          | Number animals screened | Number Bobs seen | No. GFP-filled Bobs |
|--------------------|-------------------------|------------------|---------------------|
| *B. anthracis-gfp* | 110                     | 0                | 0                   |
| *B. anthracis-gfp* plus Cry5B-Rt | 281                  | 72               | 19                  |

Table 2. Infectivity of *B. anthracis* in the presence of Cry-plus *B. thuringiensis*.
Bacillus infection assays

Unless otherwise specified, infection assays were carried out in 48-well assay plates. The final volume in each assay well was 200 µl. Standard S-medium was used to maintain C. elegans in liquid cultures [33].

Preparation of bacteria. Bacteria were grown overnight in 5 ml liquid BBHI in a standard large test tube (25×150 mm) at 250 rpm, 30°C (37°C for B. anthracis). The overnight culture was then centrifuged in 1.5-ml tubes in a microcentrifuge at 16,000 g for 4 min to pellet cells and the supernatant was aspirated. Bacterial cells were resuspended in S-medium for a final OD$_{600}$ of 0.3+/−0.02. 190 µl of bacteria in S-medium was added to the assay well. This methodology allows for relatively easy normalization amongst the different bacterial strains.

For assays with mixing of B. anthracis and Cry5B-expressing B. thuringensis, B. anthracis-gfp, spores were used since B. thuringensis produces Cry5B during sporulation. Bacteria were grown in 5 ml liquid Difco Sporulation Medium for 48 hours at 37°C produces Cry5B during sporulation. Bacteria were grown in 5 ml liquid Difco Sporulation Medium for 48 hours at 37°C [31]. Cultures were checked using a compound microscope for 48 hours at 37°C for B. anthracis. The overnight culture was then centrifuged in 1.5-ml tubes in a microcentrifuge at 16,000 g for 4 min to pellet cells and the supernatant was aspirated. Bacterial cells were resuspended in S-medium for a final OD$_{600}$ of 0.3+/−0.02. 190 µl of bacteria in S-medium was added to the assay well. This methodology allows for relatively easy normalization amongst the different bacterial strains.

For assays with mixing of B. anthracis and Cry5B-expressing B. thuringensis, B. anthracis-gfp, spores were used since B. thuringensis produces Cry5B during sporulation. Bacteria were grown in 5 ml liquid Difco Sporulation Medium for 48 hours at 37°C for B. anthracis. Cultures were checked using a compound microscope for sporulation and were found to be >99% spores. 0.1 µl of Cry5B spore crystal lysates, containing about 4.0×10$^6$ spores, were added with 10 µl of B. anthracis-gfp spores (containing about 2.5 10$^5$ spores) to 190 µl of S-media in a 48-well plate. gfp-4 (hn2) animals were prepared as described below.

Addition of Cry5B protein. 5 µl of 0.4 mg/ml Cry5B in 20 mM citrate buffer (pH 3.0) was added to appropriate wells for a final well concentration of 10 µg/ml Cry5B. Citrate buffer (pH 3.0) alone was used as a negative control.

Preparation of nematodes. Synchronized populations of gfp-4 (hn2) nematodes were prepared using standard techniques [39]. Two days prior to setting up the infection assay, a synchronous population of L1s was seeded onto standard 3.5-cm M9 plates. The final volume in each assay well was 500 µl. The Bacillus strains, purified Cry5B, and nematodes were prepared in an identical manner to the Bacillus infection assay. The first well of each row was designated the ‘Pulse’ well and the plate was incubated at 25°C for 5 min, 15 min, 30 min, 1 h, 2 h, 4 h, or 8 h. At the end of the exposure time, the worms were serially transferred through the ‘Chase’ wells in a volume of 50 µl (spending less than 1 min in each intermediate well) until the final chase well had a total volume of 500 µl. During the chase transfers, the plates were kept tilted at a 60° angle on the bench top to allow worms to more rapidly settle to the bottom and collect them for pipetting to the next well. Plates were then returned to 25°C. At t$_{ch}$ the final chase wells were scored for dead infected worms. Worms that were not transferred in the 50 µl serial washes and thus did not make it into the final ‘Chase’ well were excluded from analysis.

Microscopy

Images of worms in wells were taken with a Nikon Coolpix digital camera on an Olympus dissecting microscope. Images of the infection process were captured using differential interference contrast (DIC) and fluorescence optics on a Zeiss AxioImager A1 microscope using an AxioCam HRm camera and a 63×1.4 NA PlanApochromat lens. Images of colony formation were taken on an Olympus IX70 DeltaVision microscope (Applied Precision, Issaquah, WA) with a Nikon 60×, 1.4 NA PlanApo oil objective lens. Nematodes were mounted on glass slides with 2% agarose pads with 15 mM sodium azide as an anesthetic prior to imaging. Images were recorded and deconvolved using the SoftWorx program (Applied Precision).

Statistics

Statistical analyses were performed with GraphPad Prism (GraphPad Software, San Diego). For multiple-group comparisons, ANOVA was performed followed by Tukey’s posttest. Values of p<0.05 were considered statistically significant.

Author Contributions

Conceived and designed the experiments: MFK AB RVA. Performed the experiments: MFK AB VB YH. Analyzed the data: MFK AB RVA. Contributed reagents/materials/analysis tools: WH CN-L SMM VN. Wrote the paper: RVA.
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