

Bacillus thuringiensis (Bt) Toxin Susceptibility and Isolation of Resistance Mutants in the Nematode *Caenorhabditis elegans*

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ABSTRACT

The protein toxins produced by *Bacillus thuringiensis* (Bt) are the most widely used natural insecticides in agriculture. Despite successful and extensive use of these toxins in transgenic crops, little is known about toxicity and resistance pathways in target insects since these organisms are not ideal for molecular genetic studies. To address this limitation and to investigate the potential use of these toxins to control parasitic nematodes, we are studying Bt toxin action and resistance in *Caenorhabditis elegans*. We demonstrate for the first time that a single Bt toxin can target a nematode. When fed Bt toxin, *C. elegans* hermaphrodites undergo extensive damage to the gut, a decrease in fertility, and death, consistent with toxin effects in insects. We have screened for and isolated 10 recessive mutants that resist the toxin's effects on the intestine, on fertility, and on viability. These mutants define five genes, indicating that more components are required for Bt toxicity than previously known. We find that a second, unrelated nematocidal Bt toxin may utilize a different toxicity pathway. Our data indicate that *C. elegans* can be used to undertake detailed molecular genetic analysis of Bt toxin pathways and that Bt toxins hold promise as nematocides.

THE crystal-forming δ -endotoxins produced by *Bacillus thuringiensis* (Bt) are the most widely used biologically produced insecticides (Cannon 1996; Schnepf *et al.* 1998; de Maagd *et al.* 1999). These toxins, used by organic farmers, offer a remarkable alternative to chemical pesticides since they are nontoxic to vertebrates, generally target insects within a single order, are more benign to the environment, and can be genetically engineered into crops to provide constant protection. The tremendous success of this technology is demonstrated by the fact that in 1999, 25% of U.S. corn was grown from plants expressing Bt toxin. Transgenic Bt cotton and Bt potatoes were also widely grown.

Although the physiological mode of Bt toxin action in insects is known, there is little information at the molecular level since the target insects are not readily amenable to molecular genetic studies (Höfte and Whiteley 1989; Knowles and Dow 1993; Schnepf *et al.* 1998). After ingestion by a susceptible insect, Bt toxins are solubilized and proteolytically activated in the gut. Activated toxin binds to the midgut, inserts into the membrane, and forms a pore, resulting in intestinal lysis and insect death. Two candidate receptors for toxin binding and one candidate processing protease have been identified (Knight *et al.* 1994; Sangadala *et al.* 1994; Vadlamudi *et al.* 1995; Oppert *et al.* 1997), although the exact requirement of these proteins for tox-

icity remains unclear. Even less is known about the steps in the toxicological cascade related to pore formation and downstream events.

To complement and address the limitations of Bt toxin studies in insects and to explore the potential of Bt toxins as nematode control agents, we have initiated a study of toxin action and resistance in the nematode *Caenorhabditis elegans*. Here, we demonstrate that a single, cloned Bt toxin can be toxic to *C. elegans*. We have developed an efficient genetic screen for recovering mutants resistant to one of the Bt toxins, Cry5B. These mutants qualitatively and quantitatively resist the toxic effects of Cry5B but not of an unrelated Bt toxin, Cry6A. This work indicates that *C. elegans* can be used to improve our understanding of Bt toxin function, determine molecular mechanisms of resistance, test Bt toxins for nematocidal properties, and develop strategies for improved toxin usage.

MATERIALS AND METHODS

C. elegans strains were cultured using standard techniques (Brenner 1974). The following alleles were used for chromosome mapping: linkage group LGI, *dpy-5(e61)*; LGII, *unc-4(e120)*; LGIII, *unc-32(e189)*; LGIV, *unc-22(e66)* or *dpy-20(e1282)*; LGV, *dpy-11(e224)*; LGX, *dpy-6(e14)* or *dpy-7(e88)*. Other strains included: LGI—*fer-1(ba576)*, *dpy-5(e61) unc-29(e1072)*; LGIII—*unc-32(e189) dpy-18(e364)*; LGIV—*spe-26(hc138)*, *dpy-20(e1282) unc-31(e928)*.

Cry5B and Cry6A crystal-spore toxin lysates were prepared using standard procedures (Borgonie *et al.* 1996b) from a nontoxic host Bt strain (HD-1 *cryB*) transformed with a plasmid encoding either Cry5B or Cry6A (gifts from Mycogen Corp., now Dow AgroSciences). The concentration of toxin was mea-

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sured on protein gels relative to BSA standards. Autoclaved spore-crystal lysates were not toxic; this confirmed the absence of β -exotoxins. Nontoxic lysates were similarly prepared from the Cry⁻ HD-1 host.

For production in *Escherichia coli*, the *cry5B* toxin gene was PCR-amplified with Pfu polymerase and *cry5B*-specific primers (*Bam*HI or *Hind*III restriction sites added: cggctcgatccgcaacaattaatgagttgatcc and cgctcgaagcttttggtattattcactgtattgc), ligated into the expression vector pQE9 (QIAGEN), and transformed into a suitable *E. coli* strain, JM103 (Ge *et al.* 1990). Cry5B protein was induced using 1 mM IPTG. Production of the expected 140-kD toxin protein was verified by SDS-PAGE.

Toxicity assays: A single-well assay was devised to test the effects of known concentrations of toxin on single nematodes. L4 staged nematodes were individually picked into the wells of 96-well microtiter plates including: S medium (Sulston and Hodgkin 1988), 12.5 μ g/ml Cry5B from *Bt* lysates, 3 μ l of a saturated OP50 culture as a standard *E. coli* food source, and tetracycline and chloramphenicol (30 μ g/ml each) to prevent spore germination and bacterial growth (Borgonie *et al.* 1995, 1996b). The final volume in each well was 120 μ l. To score for susceptibility or resistance, the animals were incubated at 25° for 3 days and scored under a dissecting microscope for overall health including color, movement, brood size, and development of progeny. To examine gut morphology, individual L4 hermaphrodites or males were incubated in toxin for 2 days at 20° (before any larvae could hatch internally), pipetted onto an agarose pad with 3 mM sodium azide as an anaesthetic, and visualized with 600 \times DIC optics.

To calculate LC₅₀ values, *fer-1* hermaphrodites grown at 25° were subjected to a standard single-well assay (*fer-1* hermaphrodites grown at 25° are sterile) and incubated in a humid chamber for 6 days, a time comparable to insect assays (Gill *et al.* 1992; Tabashnik *et al.* 1997). Death was assayed by examining hermaphrodites for movement when the well was disturbed with a pipette. To similarly test lethality of *bre* mutants, double mutants were constructed between *fer-1* and each of *bre-1*, *bre-2*, *bre-3*, and *bre-5* (see Table 3 for alleles) and between *spe-26* and *bre-4* (since *bre-4* and *fer-1* are both on chromosome I, we used the sperm-defective *spe-26* mutant for this double). At greater than 420 μ g/ml of lysate toxin, wells become too opaque with bacterial debris to effectively screen.

For *E. coli* toxin assays, single L4 hermaphrodites (wild type or *bre*) were placed in wells containing 5 μ l of Cry5B-induced *E. coli* and 105 μ l of S medium. Assays were performed for 3 days at 25° with ≥ 16 animals per condition. For morphology, hermaphrodites were grown for 1 day at 25° and then visualized as above. As a control, hermaphrodites were fed induced JM103 cells containing the vector without toxin insert.

Genetic screening for resistance mutants: All resistance mutants except *bre-1* (*ye4*) were identified in a single screen. A large population of synchronized L4 worms was mutagenized in 50 mM ethyl methanesulfonate (Sulston and Hodgkin 1988). The next day, eggs were isolated by standard bleaching and allowed to hatch without food. About 80,000 F₁ larvae were grown until gravid adults, and their eggs were collected and hatched. All 450,000 F₂'s were pipetted onto NG plates and grown until the L4 stage. These were washed off and combined into a single tube. Less than 2% (8000) of these F₂'s were pipetted into wells with toxin at an average density of 2.5 animals per well, incubated at 25° for 4 days, and then scored for resistance. Clonal lines were established from candidate wells and retested. Since we assay only a small fraction of the F₂ generation, statistically each mutant is likely to be independent. This conclusion was supported by the finding that almost all alleles of a given gene behave differently (see Table 1). *bre-1* (*ye4*) was isolated in a separate screen of 700 F₂ animals.

The 10 resistant mutants were outcrossed twice to wild type, and double unlinked mutants were made with either *dpy-5* or *unc-4*. To test growth rates, 10 adult hermaphrodites were allowed to lay eggs on a standard NG plate for 2–3 hr. The adults were removed and the growth of the progeny was monitored twice a day until the next generation of eggs appeared. To calculate complete brood sizes, individual L4 wild-type or *bre* hermaphrodites were picked into wells with toxin at 20°. Every 2 days, the mothers were pipetted in a minimal volume to new wells with toxin until the mother either stopped producing offspring or died. The progeny from the old well were counted the next day. The total brood sizes under nontoxic conditions were similarly determined, except no toxin was added and the amount of OP50 was increased to 15 μ l to make up for the food provided in crude *Bt* lysates.

Complementation, mapping, and Cry6A tests: Complementation data were generated by mating homozygous *bre4* males into marked (*dpy-5* or *unc-4*) *breB* hermaphrodites. Eight cross-progeny from each cross were scored in a single-well assay. Mapping was conducted in one of two ways. Homozygous *bre* males were mated into a marker (*dpy* or *unc*) strain and F₁ cross-progeny were picked onto individual plates. In method I, 16–32 F₂ progeny homozygous for the Dpy or Unc were tested for resistance in a single-well assay (expected outcome is 1/4 resistance if unlinked). In method II, 60 F₂ progeny not homozygous for the marker were tested in a single-well assay for resistance. Those wells showing resistance were plated out and then scored for the Dpy or Unc (if unlinked, expected outcome is 2/3 Dpy or Unc). Method II was used to map the weakest mutant, *bre-1*, and for chromosome X markers, which tended to be sicker. For three-factor mapping, *bre* males were mated into doubly marked Dpy Unc strains. Individual F₁ cross-progeny were cloned onto separate plates. In the F₂ generation, either Dpy non-Unc or Unc non-Dpy recombinants (never both from the same plate) were picked, F₃ animals homozygous for the recombinant chromosome were identified, and then the F₄ progeny were tested in wells with the following results:

bre-2(*ye31*)/*unc-32*(*e189*) *dpy-18*(*e364*): 10/12 Unc non-Dpy recombinants segregated Bre (*i.e.*, resistance); 3/12 Dpy non-Unc recombinants segregated Bre.

bre-3(*ye28*)/*unc-32*(*e189*) *dpy-18*(*e364*): 1/29 Unc non-Dpy recombinants segregated Bre; 28/29 Dpy non-Unc recombinants segregated Bre.

bre-4(*ye13*)/*dpy-5*(*e61*) *unc-29*(*e1072*): 7/7 Unc non-Dpy recombinants segregated Bre; 0/7 Dpy non-Unc recombinants segregated Bre.

bre-5(*ye17*)/*dpy-20*(*e1282*) *unc-31*(*e928*): 9/37 Dpy non-Unc recombinants segregated Bre.

Sensitivity to Cry6A was determined by performing single-well and morphology assays replacing Cry5B lysates with Cry6A lysates at a concentration of 29 μ g/ml Cry6A protein.

RESULTS

The *Bt* toxin Cry5B is toxic to wild-type *C. elegans*: Although *Bt* toxins have been studied almost exclusively in insects, a small family of *Bt* toxins has been described in non-peer-reviewed literature as potentially toxic to nematodes (Feitelson *et al.* 1992; Crickmore *et al.* 1998). For our study, we chose the candidate nematicide Cry5B since it shows significant sequence similarity with insecticidal *Bt* toxins. For example, Cry5B and Cry1Ab, the caterpillar-specific *Bt* toxin expressed in *Bt*-corn,

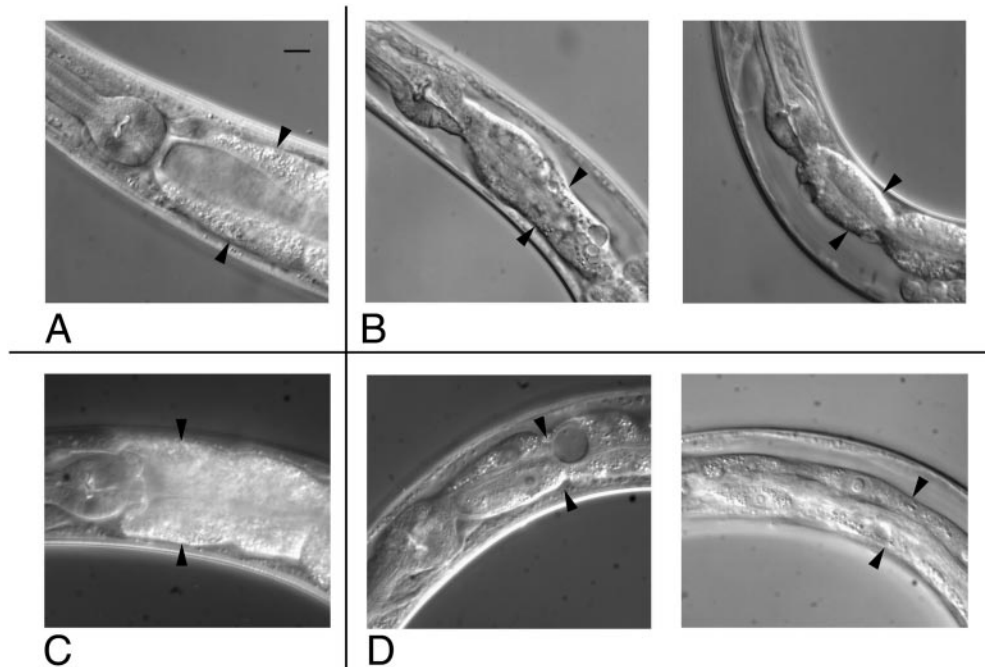


Figure 1.—The *Bt* toxin Cry5B damages the *C. elegans* intestine. Nomarski photomicrographs of wild-type hermaphrodites taken at a magnification of 600 showing the pharynx (to the left) and anterior intestine of hermaphrodites. The width of the anterior intestine is noted by arrowheads. (A) A hermaphrodite fed nontoxic *Bt* lysates. Note the healthy distended intestine with ample gut granules. Bar, 10 μm . (B) Two hermaphrodites fed Cry5B toxic *Bt* lysates. Note formation of vacuole-like structures, pitting, constriction, and degeneration of the intestine after 2 days. (C) A hermaphrodite fed *E. coli* (JM103) transformed with a vector control has a healthy intestine. (D) Two hermaphrodites fed *E. coli* (JM103) transformed with the *cry5B* gene.

Formation of vacuole-like structures, constriction, and degeneration of the intestine are evident. The posterior tip of the pharynx of the second hermaphrodite is just visible to the left.

are 24% identical and 44% similar across their toxin domains (~ 570 amino acids). Cry5B also contains four of five sequence blocks conserved among most Cry toxins (Höfte and Whiteley 1989), suggesting that it folds into a three-domain tertiary structure related to other *Bt* toxins in the main family (Schnepf *et al.* 1998).

We find that *C. elegans* is susceptible to the Cry5B *Bt* toxin. When examined under a dissecting scope, hermaphrodites fed Cry5B toxin for 2–3 days develop decrepit internal morphology, have pale coloration, and move slowly. Moreover, there is a severe reduction in brood size (see below); those progeny that are produced develop slowly. After 3 days, many hermaphrodites contain a few internally hatched larvae. As a control for the presence of *Bt* spores and *Bt* bacteria in the growth media, we verified that *Bt* preparations that lack Cry5B crystals do not adversely affect the growth, morphology, or fertility of wild type. In addition, antibiotics are added in all assays to prevent the germination and growth of bacteria. These data suggest that the toxic effects are due to the Cry5B toxin and not the *Bt* bacterial culture *per se*.

When examined at higher magnification, we found that Cry5B damages the intestine, the target tissue of *Bt* toxins in insects. In contrast to animals fed nontoxic *Bt* preparations (Figure 1A), animals fed Cry5B-containing *Bt* preparations show extensive changes in their gut morphology (Figure 1B). These changes include the formation of vacuole-like structures in gut cells, a shrinking of the gut away from the body wall of the animal, tight constrictions at various points along the gut, and an overall pitted appearance. At this point we

cannot ascertain whether the vacuole-like structures and pits that form are normal compartments that have been enlarged or novel structures induced by the toxin. Other tissues of the animal, including the musculature and nervous system, appear intact and are at least partially functional since the animals can move and respond to stimulus when prodded.

Since the toxin causes wild-type hermaphrodites to accumulate a few internally hatching self-progeny that would kill the mother, we tested whether the toxin was lethal independent of internal progeny. We fed toxin to *fer-1* hermaphrodites that are sterile. Even though these animals cannot produce any internally hatching self-progeny, the toxin is still lethal. We found that the concentration of Cry5B that gives rise to 50% dead animals (LC_{50}) is 12.6 $\mu\text{g}/\text{ml}$ or 90 nm (Figure 2). Consistent with the result that lethality is independent of internal progeny, we confirmed that the toxin can kill first-staged (L1) larvae and males.

To demonstrate conclusively that Cry5B is toxic to *C. elegans* and that other aspects of *Bt* biology were not heavily influencing our results, we heterologously expressed Cry5B in *E. coli*, the standard bacterial food source for *C. elegans*. *C. elegans* fed *E. coli* lacking the Cry5B gene have healthy intestines (Figure 1C). In contrast, *C. elegans* fed *E. coli* expressing the Cry5B gene show extensive intestinal damage very similar to that seen when fed *Bt*-produced Cry5B (Figure 1D). *C. elegans* fed toxin-expressing *E. coli* appear decrepit and have pale coloration, slow movements, and low broods, just as with native toxin produced in *Bt*.

Isolation of mutants resistant to *Bt* toxin: To deter-

mine whether *C. elegans* could be used for studying the molecular genetics of Cry5B toxicity and to identify loci in the target nematode required for *Bt* toxin action, we screened for mutants resistant to the effects of Cry5B. Hermaphrodites were mutagenized with EMS, propagated for two generations on nontoxic *E. coli*, and then plated into wells containing *Bt*-produced Cry5B. After 4 days, the wells were examined for the presence of a healthy mother and good brood, the predicted phenotype of resistant animals. Among 8700 mutagenized F₂ hermaphrodites, we recovered 10 mutants heritably resistant to Cry5B. We designated these mutants as *bre* for **Bacillus-toxin resistant** and refer to animals mutated for a *bre* gene as "*bre* animals."

The *bre* mutants define five genes. All *trans*-heterozygous combinations of these 10 alleles were constructed and assayed for resistance. The mutants fall into five complementation groups based on these results (summarized in Table 1, left column). Given the high frequency with which these alleles were isolated and the fact they are recessive (see below), they are probably loss-of-function or reduction-of-function mutations (Brenner 1974).

Since we isolated multiple alleles in three of five genes, these five loci probably represent most of the genes that can mutate to resistance while the animals remain viable. To confirm this conclusion, we performed a screen for L1 hermaphrodites resistant to *E. coli*-produced Cry5B (L. D. Marroquin and R. V. Aroian, unpublished data). Complementation analyses indicate that we isolated only more alleles of known *bre* genes, namely five additional alleles of *bre-2*, four of *bre-3*, two of *bre-4*, and one of *bre-5*.

***bre* animals qualitatively and quantitatively resist toxicity:** All *bre* mutants resist the effects of the Cry5B toxin

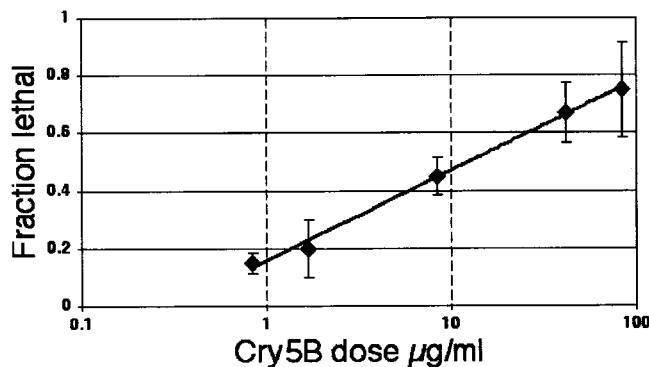


Figure 2.—*C. elegans* displays dose-dependent lethality to Cry5B. A semilog plot of the fraction of animals that died (\pm standard deviation) vs. the concentration of toxin in the well. The data were fit to a line by least-squares ($R^2 = 0.996$). The LC₅₀ of 12.6 $\mu\text{g/ml}$ was calculated from this line fit. The actual data (total number dead/number tested from three to six experiments per dose) are: 7/48 at 0.83 $\mu\text{g/ml}$, 16/82 at 1.7 $\mu\text{g/ml}$, 58/128 at 8.3 $\mu\text{g/ml}$, 61/91 at 42 $\mu\text{g/ml}$, and 36/48 at 83 $\mu\text{g/ml}$.

TABLE 1

Brood size without toxin vs. brood size with toxin

Genotype	Brood size no toxin	Brood size with toxin
Wild type	240 \pm 26	5.2 \pm 3.9
<i>bre-1</i> (<i>ye4</i>)	137 \pm 26	46.3 \pm 9.9
<i>bre-2</i> (<i>ye8</i>)	126 \pm 15	101 \pm 19
<i>bre-2</i> (<i>ye31</i>)	137 \pm 16	103 \pm 22
<i>bre-3</i> (<i>ye9</i>)	256 \pm 34	179 \pm 28
<i>bre-3</i> (<i>ye26</i>)	216 \pm 42	47.8 \pm 19
<i>bre-3</i> (<i>ye28</i>)	163 \pm 3.9	93.7 \pm 50
<i>bre-4</i> (<i>ye12</i>)	134 \pm 47	82.3 \pm 35
<i>bre-4</i> (<i>ye13</i>)	271 \pm 23	180 \pm 37
<i>bre-4</i> (<i>ye27</i>)	263 \pm 23	140 \pm 37
<i>bre-5</i> (<i>ye17</i>)	213 \pm 28	131 \pm 39

Average total progeny counts per hermaphrodite (\pm standard deviation) without and with 12.5 $\mu\text{g/ml}$ Cry5B toxin in wells at 20°. The broods from five to eight hermaphrodites were averaged per data point.

on the intestine. In contrast to the intestines of wild-type animals fed toxin (Figure 3A), the intestines of *bre* animals fed *Bt*-produced toxin have intestinal morphology (Figure 3, B–F) that is often indistinguishable from that of a healthy wild-type intestine unexposed to toxin (Figure 1A).

The *bre* mutants are also highly resistant to Cry5B-induced lethality. Unlike wild type, in which half the animals die in 12.6 $\mu\text{g/ml}$ of toxin (Figure 2), few *bre* animals die even at significantly higher doses. Specifically, in 420 $\mu\text{g/ml}$ Cry5B (33 times the LC₅₀ dose), 4/15 *bre-1* hermaphrodites, 0/16 *bre-2* hermaphrodites, 0/15 *bre-3* hermaphrodites, 0/16 *bre-4* hermaphrodites, and 1/16 *bre-5* hermaphrodites died; the remainder appeared healthy. Our current assay does not allow us to test dose levels higher than 420 $\mu\text{g/ml}$ (see materials and methods).

Apart from their resistance to Cry5B toxin, the mutants behave similarly to wild type. Morphologically they appear normal. Under nontoxic conditions they develop from embryos to adults at the normal rate. This result suggests that their metabolism is not dramatically altered. Five mutant alleles have wild-type brood sizes under nontoxic conditions while the other five have lower brood sizes that are nonetheless robust (Table 1).

We additionally verified that the *bre* mutants resist the detrimental effects of the toxin on fertility. Wild-type animals exposed to toxin show a nearly 50-fold reduction in fertility (Table 1). Our observations indicate that this result is mostly independent of the internal hatching of larvae since the reduction can be seen before internal hatching occurs. When *bre* mutants are exposed to toxin, they show only a modest (4.5- to 1.2-fold) reduction in fertility, with 8 out of 10 showing less than a 2-fold reduction (Table 1).

Although the mutants were isolated based on resis-

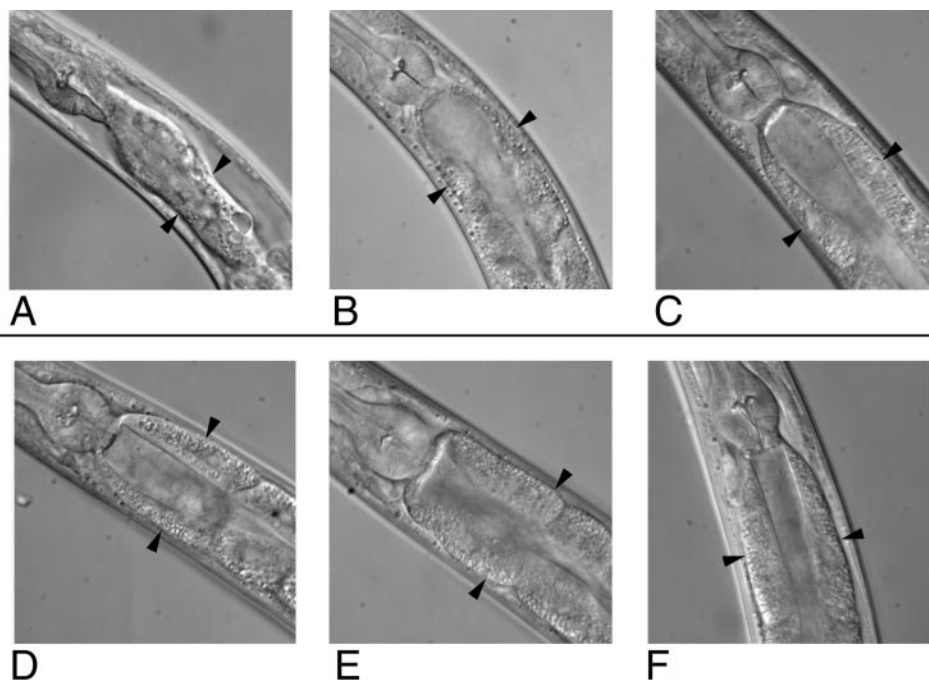


Figure 3.—The *bre* mutants are resistant to toxin-induced damage to the intestine. Animals are at the same orientation and magnification as in Figure 1. (A) A hermaphrodite fed Cry5B from *Bt* lysates (from Figure 1B). (B–F) Respectively, *bre-1*(*ye4*), *bre-2*(*ye31*), *bre-3*(*ye28*), *bre-4*(*ye13*), and *bre-5*(*ye17*) hermaphrodites fed the same dose of Cry5B toxin as in A. The intestines of *bre* animals exposed to toxin fail to develop vacuole-like structures, do not shrink in from the body wall, and have no or minimal constrictions.

tance to Cry5B produced in *Bt*, we have verified that the mutants are resistant to Cry5B produced in *E. coli* as well. In contrast to wild type, all mutants appear healthy, have high broods, and display healthy intestines when fed Cry5B-expressing *E. coli*.

Genetic characterization of resistance genes: Consistent with most insect resistance to *Bt* toxins (Schnepf *et al.* 1998), the *bre* mutant alleles are recessive. Males from all mutants were crossed into visibly marked non-*bre* hermaphrodites (*dpy-5* or *unc-4*), and F₁ cross-progeny were tested in wells with toxin. All heterozygous cross-progeny (hermaphrodite and male) were sensitive. We have also reversed the cross and mated wild-type males into the *bre* mutants. Cross-progeny produced this way are also sensitive.

We have determined the chromosomal location of the *bre* genes (Table 2). Each shows linkage to a single chromosome (LGI: *bre-4*; LGIII: *bre-2* and *bre-3*; LGIV: *bre-1* and *bre-5*). The mapping data also allowed us to deduce that each mutant is likely caused by alteration in a single gene since 1/4 of the progeny from heterozygous mothers are resistant (Method I, Table 2; data for *bre-1* were collected in an independent experiment).

To further refine their locations, we performed three-factor mapping for *bre-2*, *bre-3*, *bre-4*, and *bre-5* (see materials and methods). We found that *bre-2* maps near and to the left of *dpy-18*, *bre-3* maps very close and to the right of *unc-32*, *bre-4* maps to the left arm of chromosome I, and *bre-5* maps near and to the right of *dpy-20*. None of these map to areas with genes similar to those that, on the basis of insect studies, might be expected to mutate to *Bt* resistance (*e.g.*, aminopeptidase N or cadherin-like proteins).

The *bre* mutants are sensitive to an unrelated *Bt* toxin,

Cry6A: To determine whether the *bre* mutants were specifically resistant to Cry5B or to intestinal toxins in general, we tested the mutants for resistance to another *Bt* crystal toxin, Cry6A. Although a candidate nematicide (Feitelson *et al.* 1992), the Cry6A toxin shows no sequence similarity to Cry5B or to other members of the main family of *Bt* toxins, contains none of the five conserved sequence blocks, and is likely to be different in structure than other *Bt* toxins (Feitelson *et al.* 1992; Crickmore *et al.* 1998; Schnepf *et al.* 1998).

As demonstrated in Table 3, wild-type *C. elegans* and all five *bre* mutants are sensitive to Cry6A. In addition, the health and intestinal morphology of wild-type and *bre* mutant hermaphrodites are similarly adversely affected by the Cry6A toxin (not shown).

DISCUSSION

We have demonstrated that a nematode can be the target of a single cloned *Bt* Cry toxin and that *Bt* toxicity can be genetically studied in *C. elegans*. *C. elegans* is susceptible to the *Bt* toxin Cry5B whether the toxin is produced either in the native *Bt* host or in *E. coli*. Ingestion of toxin damages the intestine, as it does in insects, and causes dose-dependent lethality and severe reductions in fertility. The reason for low fertility may simply be that the intestine, which is damaged by the toxin, normally supplies the germ line with nutrients (*e.g.*, vitellogenins). The reduction of fertility and internal hatching of larvae seen upon feeding of toxin to nematodes are reminiscent of what is seen when nematodes starve (in the absence of toxin). Along these lines, we have also noted that the intestines of starved animals display one or two points of constriction and sometimes

TABLE 2
Mapping data for *bre* mutants

	LGI	LGII	LGIII	LGIV	LG V	X
<i>bre-1</i> (<i>ye4</i>)	5/8 ^a	11/20 ^a	10/17 ^a	<u>1/15^a</u>	9/18 ^a	11/14 ^a
<i>bre-2</i> (<i>ye8</i>)	7/40	4/16	<u>0/16</u>	4/22	4/16	11/16 ^a
<i>bre-3</i> (<i>ye28</i>)	4/16	4/16	<u>0/16</u>	7/16	4/16	4/24
<i>bre-4</i> (<i>ye27</i>)	<u>1/32</u>	4/16	5/16	5/24	5/16	8/11 ^a
<i>bre-5</i> (<i>ye17</i>)	7/40	4/32	4/16	<u>0/23</u>	3/16	9/12 ^a

Number of animals that displayed both chromosomal marker and resistance phenotypes. Underlined numbers indicate chromosomal location of each *bre* mutant.

^a Mapped via method II (see materials and methods; 2/3 segregation expected for nonlinkage). Otherwise, mapping was performed via method I (1/4 segregation expected for nonlinkage).

a shrinking in from the body wall, although both to a lesser extent than seen with animals exposed to toxin (J. O'Dell and R. V. Aroian, unpublished observations). Thus, it is possible that part of *Bt* toxicity is to induce a starvation response, perhaps via the destruction of gut. In contrast to the intestines of toxin-fed animals, vacuole-like structures, pitting, and degeneration are not seen in the intestines of starved animals, indicating that these effects are independent of starvation.

Susceptibility to Cry5B in *C. elegans* is attributed to at least five separate genes, *bre-1* through *bre-5*. Mutations in these genes were isolated in a directed screen for resistance. These mutants are resistant to toxin whether made in *Bt* or *E. coli* and confer qualitative and quantitative recessive resistance to all aspects of toxicity that we measure.

There are a scattering of previous studies involving *Bt* toxins and nematodes. In some studies, the nematocidal activity was thermostable (and therefore not protein based); in others, lepidopteran- and dipteran-specific *Bt* strains or uncharacterized *Bt* strains that potentially express multiple toxins were used (Ignoffo and Dropkin 1976; Osman *et al.* 1988; Meadows *et al.* 1989; Zuckerman *et al.* 1993; Borgonie *et al.* 1996b). In addition to our genetic characterization of resistance, our study differs from these others in that our *Bt* strain expresses

a single, cloned *Bt* toxin, and that we showed that toxicity was due to a single protein by transferring the toxin gene from *Bt* to *E. coli*.

Our study of Cry5B toxicity in *C. elegans* has broad implications for *Bt* toxin biology. Since Cry5B has significant and extensive homology with *Bt* toxins commercially in use, some, if not many, of the genes and pathways required for Cry5B toxicity in *C. elegans* are likely to have counterparts in other target organisms, such as insects. Our studies complement work in insect systems and promise to elucidate aspects of the toxicological cascade that are poorly understood, such as pore formation and subsequent events. Knowing the molecular basis of resistance is also important for designing or selecting new toxins and for developing strategies to cope with the evolution of pest resistance that may threaten the long-term use of *Bt* toxins.

That our mutants are still susceptible to the unrelated *Bt* toxin Cry6A also has important implications. First, our data exclude a model whereby the *bre* mutants act nonspecifically to block Cry5B toxicity (*e.g.*, in such a model mutants might line their gut with a mucus that prevents any toxin from binding). Second, our data suggest that mutational events that give rise to resistance tend not to offer general protection against unrelated toxins. These data also indicate that Cry6A uses a different pathway for toxicity than Cry5B and therefore is an excellent candidate to be coexpressed with Cry5B to delay or prevent the development of resistance. The potential value of Cry6A as a combinatorial toxin may extend as well to the commercially important insecticidal *Bt* toxins related to Cry5B since Cry6A is reported to be toxic additionally to lepidoptera and coleoptera (Soares *et al.* 1989; Uyeda *et al.* 1991).

As part of this work, we developed assays for testing potential nematocidal properties of *Bt* toxins. Nematocidal *Bt* toxins, expressed transgenically in appropriate root tissue, might provide an effective strategy to control plant-parasitic nematodes, a major class of agricultural pest that causes billions of dollars in crop damage per year in the United States alone (Sasser and Freckman 1987). The urgency of this goal is underscored by the

TABLE 3
Sensitivity to Cry6A

Genotype	Brood in Cry6A ^a
Wild type	12.5 ± 4.9
<i>bre-1</i> (<i>ye4</i>)	4.1 ± 2.6
<i>bre-2</i> (<i>ye31</i>)	14.1 ± 6.9
<i>bre-3</i> (<i>ye28</i>)	9.8 ± 2.3
<i>bre-4</i> (<i>ye13</i>)	7.6 ± 2.9
<i>bre-5</i> (<i>ye17</i>)	10.0 ± 2.9

^a Average brood (± standard deviation) from eight hermaphrodites in a 3-day single-well assay at 29 µg/ml toxin. In the absence of toxin, wild type give rise to an average of 134 ± 18 progeny in this assay (*n* = 7).

fact that methyl bromide, the most widely used nematocidal agent in agriculture, is mandated by the Montreal Protocol to be phased out within the next few years. Although in a previous study it was suggested that nematocidal *Bt* toxins do not hold promise as biological control agents (Borgonie *et al.* 1996a), our work demonstrates that Cry5B, which was not used in the 1996 or any previous published study, does hold such promise.

In addition to studying *Bt* toxicity and resistance, our *C. elegans* assays are potentially useful for testing various strategies for coping with the development of resistance. For example, large numbers of wild-type nematodes could be mixed with small numbers of marked (*e.g.*, with GFP) resistant nematodes. These artificial populations could then be treated with ultrahigh toxin levels, an alternative toxin, or on/off applications of toxin. The number of wild-type and resistant nematodes could be counted readily at the end of the simulation experiment. In this way, a regimen of toxin application that minimizes the amplification of resistant animals could be developed prior to field testing.

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