Bacillus thuringiensis crystal proteins that target nematodes

Jun-Zhi Wei[†‡§], Kristina Hale[†‡], Lynn Carta[¶], Edward Platzer[‖], Cynthie Wong[†], Su-Chiung Fang[†], and Raffi V. Aroian[*,**]

[†]Section of Cell and Developmental Biology, University of California, San Diego, CA 92093-0349; [¶]Nematology Laboratory, U.S. Department of Agriculture–Agricultural Research Service, Beltsville Agricultural Research Center, Beltsville, MD 20705; and [‖]Department of Nematology and Biology, University of California, Riverside, CA 92521

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***Bacillus thuringiensis* (*Bt*) crystal proteins are pore-forming toxins used as insecticides around the world. Previously, the extent to which these proteins might also target the invertebrate phylum Nematoda has been mostly ignored. We have expressed seven different crystal toxin proteins from two largely unstudied *Bt* crystal protein subfamilies. By assaying their toxicity on diverse free-living nematode species, we demonstrate that four of these crystal proteins are active against multiple nematode species and that each nematode species tested is susceptible to at least one toxin. We also demonstrate that a rat intestinal nematode is susceptible to some of the nematicidal crystal proteins, indicating these may hold promise in controlling vertebrate-parasitic nematodes. Toxicity in nematodes correlates with damage to the intestine, consistent with the mechanism of crystal toxin action in insects. Structure–function analyses indicate that one novel nematicidal crystal protein can be engineered to a small 43-kDa active core. These data demonstrate that at least two *Bt* crystal protein subfamilies contain nematicidal toxins.**

Crystal toxin proteins from the Gram-positive soil bacterium, *Bacillus thuringiensis* (*Bt*), are used extensively to control insect pests. These range from caterpillars (Lepidoptera) and beetles (Coleoptera) that infest crops to black flies and mosquitoes (Diptera) that transmit human diseases (1, 2). The success of these toxins is caused in large part by their high toxicity toward insects but no/low toxicity toward other animals. They have an excellent track record in >50 years of use by organic and conventional farmers and are very effective when expressed directly in transgenic plants.

It is puzzling why a bacterium that is so ubiquitously found in the soil might have evolved ingestible toxins to target insects that may spend little time feeding in the soil. On the other hand, there are estimated to be >100,000 species of nematodes (3), many of which live in the soil and ingest bacteria. Could nematodes be a prime target for *Bt* and its crystal proteins? In support of this hypothesis, we have previously characterized the toxic effects and toxicity pathways of the *Bt* crystal protein Cry5B on the nematode *Caenorhabditis elegans* and also demonstrated that two other crystal proteins, Cry6A and Cry14A, can lower its brood size (4, 5). However, to our knowledge, these are the only published studies that address the effects of specific *Bt* crystal proteins on a nematode.

A more complete understanding of the relationship between *Bt* crystal proteins and nematodes is important to address the question of how generally *Bt* toxins might target nematodes and how these potential hosts and the pathogen might have coevolved. In addition, some nematodes are important parasites of animals and plants, and *Bt* toxins might have the potential to control them safely. The extent to which *Bt* toxins generally affect nematodes also has repercussions for environmental impact from their widespread use in insect abatement programs.

Here, we study the effects of two largely unstudied classes of crystal proteins on five phylogenetically diverse free-living nematode species and one parasitic species. We find that some of these *Bt* crystal proteins intoxicate multiple nematode species, whereas other related *Bt* crystal proteins do not. These results demonstrate that *Bt* crystal proteins can target nematodes. Furthermore, we show that *C. elegans* provides a superb system for structure–function studies on *Bt* crystal proteins.

Materials and Methods

Nematodes and Their Maintenance. *C. elegans* (strain N2), *Pristionchus pacificus* (strain PS312), *Panagrellus redivivus* (strain PS1163), *Acrobeloides* (strain PS1146), and *Distolabrellus veechi* (strain LKC10) were maintained on standard NG plates at room temperature and fed with *Escherichia coli* strain OP50 (6). *Nippostrongylus brasiliensis* was maintained in rats at the University of California at Riverside by using procedures approved by the Chancellor's Committee on Laboratory Animal Care (AM 0107053-1).

Phylogeny. Evolutionary trees were constructed with a neighbor-joining absolute distance algorithm bootstrapped for 3,000 replicates as implemented in PAUP* 4.0b4 by Swofford (Sinauer Associates, Sunderland, MA). The evolutionary tree of *Bt* Cry proteins was generated by using their predicted toxin domains representing 717 CLUSTALW (at the European Bioinformatics Institute; ref. 7) alignment positions. We also estimated the phylogeny by using a Bayesian analysis, which essentially shows the same relationships among the Cry proteins as constructed with the neighbor-joining method (see *Supporting Text*, which is published as supporting information on the PNAS web site, www.pnas.org). The neighbor-joining nematode phylogram was constructed from 1,413 nucleotide positions in a CLUSTAL alignment of 18S rDNA gene sequences from the taxa used in this article.

***Bt* Toxin Gene Isolation, Subcloning, and Expression.** All *Bt* crystal genes were obtained from the U.S. Department of Agriculture-Agricultural Research Service Culture Collection as *E. coli* transformants in an unknown vector. The genes were PCR-amplified by using *Pfu* DNA polymerase (Promega) or a 1:1 mix of *Pfu*/*Taq* and primers at the very 5′ and 3′ ends of each gene that also included restriction sites. The PCR products were ligated into expression vector pQE9 (Qiagen, Chatsworth, CA) and transformed first into *E. coli* strain XL-1 blue for verification and plasmid purification and then into *E. coli* strain JM103, a strain favorable for *Bt* crystal protein expression (8). PCR-generated point mutations were found in Cry5Aa, Cry5B, Cry6B, Cry12A, Cry13A, and Cry14A. In the cases of Cry5Aa, Cry5B, Cry6B, and Cry12A, the point mutations were corrected by using the QuikChange kit (Stratagene). We were unable to obtain corrected Cry13A or Cry14A genes in expression vectors, presumably because the corrected proteins are toxic to *E. coli*. Because the mutated Cry14A gene (Thr-164 is altered to Ile) was found to be toxic to some nematodes, we continued our analysis by using this clone. Mutated Cry13A was not toxic to nematodes

Abbreviation: *Bt*, *Bacillus thuringiensis*.

[‡]J.-Z.W. and K.H. contributed equally to this work.

[§]Present address: Maxygen, Inc., 515 Galveston Drive, Redwood City, CA 94063.

**To whom correspondence should be addressed. E-mail: raroian@ucsd.edu.

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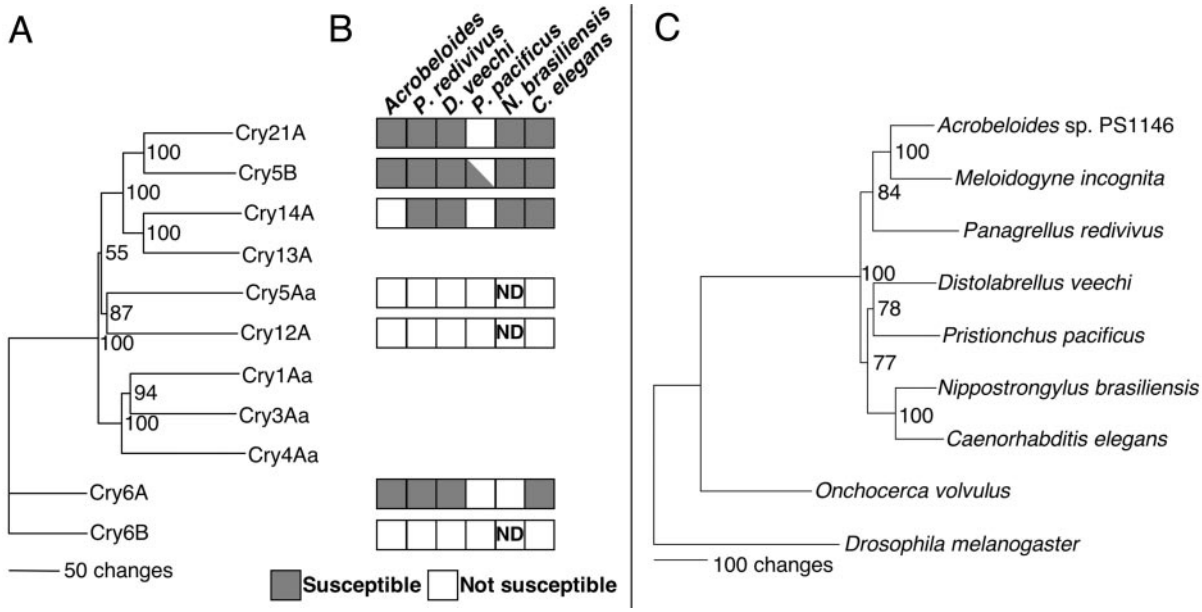

Fig. 1. Crystal proteins and nematodes used in this study. (*A*) Evolutionary tree of *Bt* crystal proteins with three insecticidal proteins (Cry1Aa, Cry3Aa, and Cry4Aa) included for comparison. (*B*) Summary of results from this article. A filled box indicates that the nematode listed above was susceptible to the toxin to the left, and an open box indicates that the nematode was not affected by the toxin. The half-shaded box indicates *P. pacificus* was susceptible to exogenously added Cry5B but not Cry5B encased in *E. coli*. ND, not determined. (*C*) Evolutionary tree for nematode taxa. The plant parasitic nematode *Meloidogyne incognita*, the distantly related animal parasite, *Onchocerca volvulus*, and the Dipteran *Drosophila melanogaster* are added for comparison.

and continued attempts to clone it into other *E. coli* vector systems with tight expression control failed. Hence, Cry13A is not analyzed here. Crystal protein concentrations were determined relative to BSA standards on Coomassie-stained SDS acrylamide gels. All crystal proteins were produced at their predicted full-length sizes.

Toxicity Assays. ***Plate assay.*** Thirty microliters of overnight culture of JM103 transformed with pQE9 with or without a crystal gene insert was spread on nematode growth plates containing 0.25 mM isopropyl β-D-thiogalactoside and carbenicillin and cultured at room temperature or 37°C overnight. Thirty L4 stage nematodes of a single species were put on each plate and assayed for intoxication/death after the time indicated at room temperature. Animals that crawled off the plate were not included in the analysis.

Developmental assay. Embryos from each species were allowed to hatch in liquid medium in the absence of food and the larvae were gently placed into wells of a 96-well microtiter plate (one to four animals per well) containing 115 μl of S medium, 5 μl of induced bacterial culture (resuspended in S medium), 30 μM tetracycline, and 30 μM chloramphenicol. For Cry5B, twice the volume of bacterial culture was used to compensate for poor expression (in this case, twice the bacteria was also used for vector-only control with no significant change in normal development). The number of animals scored for pQE9, Cry5A, Cry5B, Cry6A, Cry6B, Cry12A, Cry14A, and Cry21A, respectively are as follows: *C. elegans* 412, 77, 37, 63, 27, 39, 59, 61; *P. pacificus* 54, 45, 46, 19, 19, 20, 20, 19; *D. veechi* 37, 10, 66, 30, 22, 25, 25, 25; *P. redivivus* 25, 16, 50, 20, 18, 17, 26, 22; and *Acrobeloides* 20, 20, 46, 55, 23, 20, 29, 52. Intoxication in more than two-thirds of the larva was arbitrarily chosen as the threshold for scoring a positive interaction between a toxin and nematode.

Morphology. L4 stage nematodes were individually picked into the wells of 96-well microtiter plates including 115 μl of S medium and 5 μl of isopropyl β-D-thiogalactoside-induced cells. Anterior intestines were examined at ×400 differential interference contrast optics after 2 days, except for *Acrobeloides* (3 days).

Quantitative brood size. These were determined as described (4). Each data point represents the brood sizes from a total of 15–30 hermaphrodites in three independent experiments. IC_{50} values were calculated by performing regression analysis on the linear portion of the response curves shown in Fig. 3*C*.

Soluble toxin assays. *P. pacificus* was fed purified Cry5B toxin as described for *C. elegans* (4).

Cry6A Structure–Function Analyses. The truncated Cry6A DNA sequences were synthesized by PCR (Pfu) using a series of primers located at different 5′ and/or 3′ sites. *Bam*HI and *Pst*I sites were incorporated in the 5′ and 3′ primers, respectively. PCR products were restriction-digested and subcloned into expression vector pQE9, which was then transformed into *E. coli* strain JM103. The expression of all Cry6A-truncated variants was verified by SDS/PAGE. Toxicity was determined by using our standard plate assay described above.

Results

The Effects of Seven *Bt* Crystal Proteins on Five Nematode Species. The *Bt* crystal protein sequences cluster in phylogenetic subfamilies that target similar classes of invertebrates (2). Of the >80 crystal protein sequences currently published, we chose to focus on the members of the Cry5 and Cry6 subfamilies because at least one protein from each of these families has been demonstrated to affect *C. elegans* (5). The Cry5 subfamily contains six proteins that share a common ancestry (Fig. 1*A*): Cry5Aa (and the >95% identical Cry5Ab and Cry5Ac), Cry5B, Cry12A, Cry13A, Cry14A, and Cry21A (2, 9). These six proteins are clearly related to the commercially important Cry1A, Cry3A, and Cry4A insecticidal toxins and contain four of the five protein motifs conserved among the main family of Cry proteins. The Cry6 subfamily contains two proteins, Cry6A and Cry6B (Fig. 1*A*). These are ≈50% identical to each other, but they are unrelated by protein sequence to the main family of *Bt* crystal toxins. All eight Cry5 and Cry6 family toxins have been described in patents on nematicidal *Bt* toxins, but only sparse data were presented (10–12). We set out to rigorously and more completely test the efficacy of these individual crystal proteins

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Table 1. Crystal protein concentrations used in assays

Cry protein	Concentration on IPTG plates, ng/cm^2	Concentration in L1 assays, ng/ml
Cry5A	1,000	1,200
Cry5B	100	250
Cry6A	4,000	2,500
Cry6B	2,000	1,500
Cry12A	800	500
Cry14A	300	500
Cry21A	1,000	1,500

IPTG, isopropyl β-D-thiogalactoside.

on nematodes. Because Cry5Aa is almost identical to Cry5Ab and Cry5Ac, we selected Cry5Aa as representative of these proteins (referred to below as Cry5A). We have subcloned and expressed versions of all of these crystal proteins in *E. coli* (Table 1), except for Cry13A (see *Materials and Methods*).

We selected five free-living, bacterial-feeding nematode species, *C. elegans*, *P. pacificus*, *D. veechi*, *P. redivivus*, and *Acrobeloides* sp., as hosts for crystal protein studies. These nematodes were chosen because they represent somewhat diverse phylogeny, especially within the two clades that harbor many of the free-living terrestrial species (13). They also can be readily propagated in monoaxenic culture with *E. coli*. A phylogenetic comparison of the nematodes used here is presented in Fig. 1*C*.

We developed a plate assay for efficiently evaluating the intoxicating effects of a *Bt* toxin on bacterial-feeding nematodes. Fourth larval (L4)-stage nematodes were transferred onto plates spread with *E. coli* cells that either did not express a crystal protein (negative control) or expressed one of the seven crystal proteins (Table 1). All nematodes fed *E. coli* JM103 transformed with plasmid without an insert (negative control) were healthy and vigorous (Fig. 2*A Left*). *E. coli* strains producing some crystal proteins had no adverse affects on nematodes (Fig. 2*A Center*) whereas *E. coli* strains producing other crystal proteins intoxicated some nematodes (Fig. 2*A Right*). Intoxication was readily judged by lack of pumping, inhibition of growth (i.e., small size), pale coloration, and lethargy. In almost all cases where intoxication was seen, some of the animals also were killed within this time frame. Quantitative results of this plate assay are graphically represented in Fig. 3*A*.

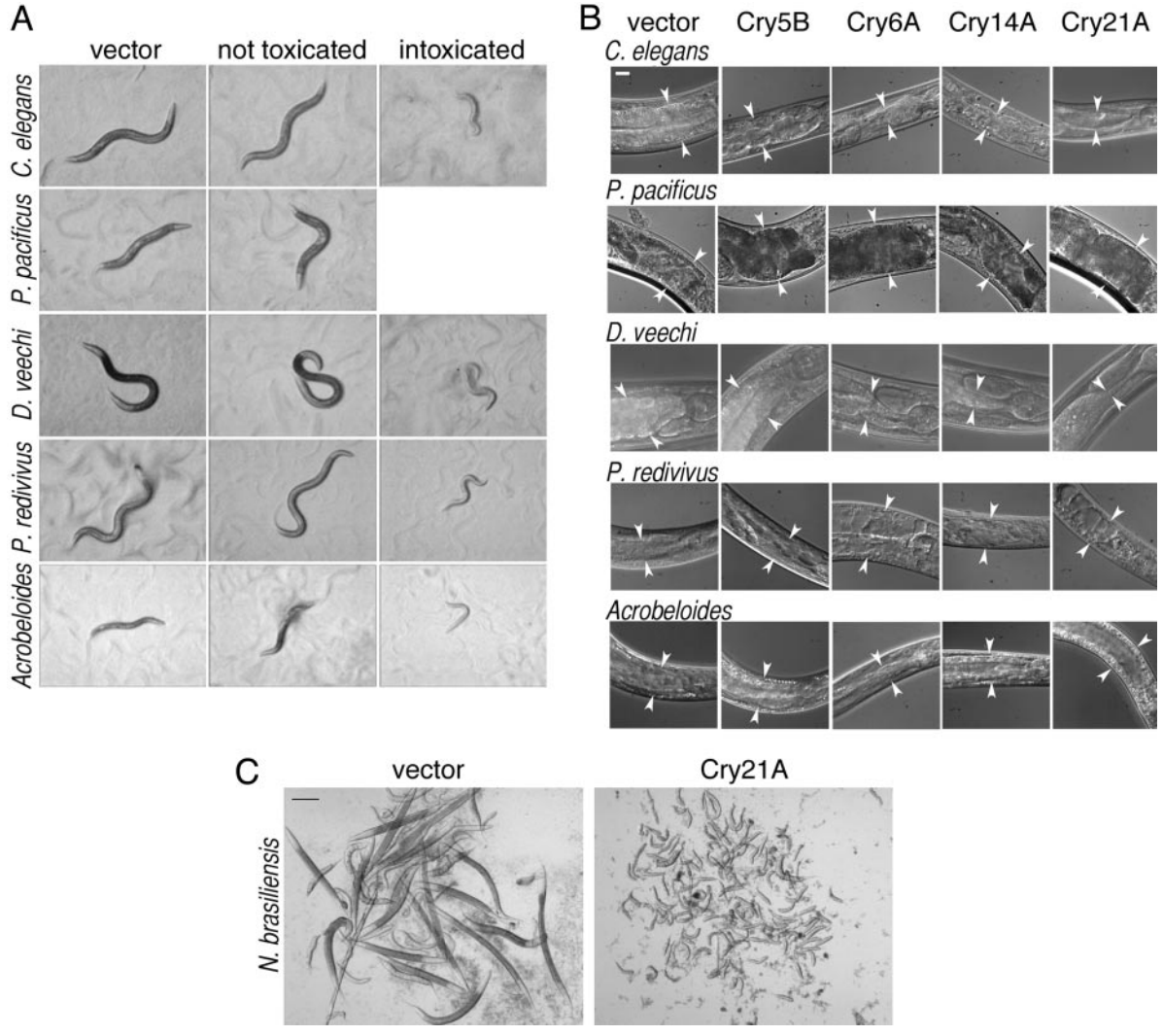

Fig. 2. Morphological effects of crystal proteins on nematodes. (*A*) Typical morphology (at ×60 magnification) seen in the L4 plate assay after feeding nematode species *E. coli* transformed with empty vector (*Left*), vector plus nontoxic Cry protein insert (*Center*), or vector plus toxic Cry protein insert (*Right*). (*B*) Photographs of the anterior intestine of nematodes fed the four toxic crystal proteins in *E. coli*. Arrowheads delineate the width of the intestine at one position near the anterior. (Bar = 20 μm.) (*C*) *N. brasiliensis* larvae photographed at ×75 magnification 3 days after eggs were plated on NG-isopropyl β-D-thiogalactoside plates spread with JM103 *E. coli* transformed with empty vector (*Left*) or Cry21A insert (*Right*). (Bar = 100 μm.)

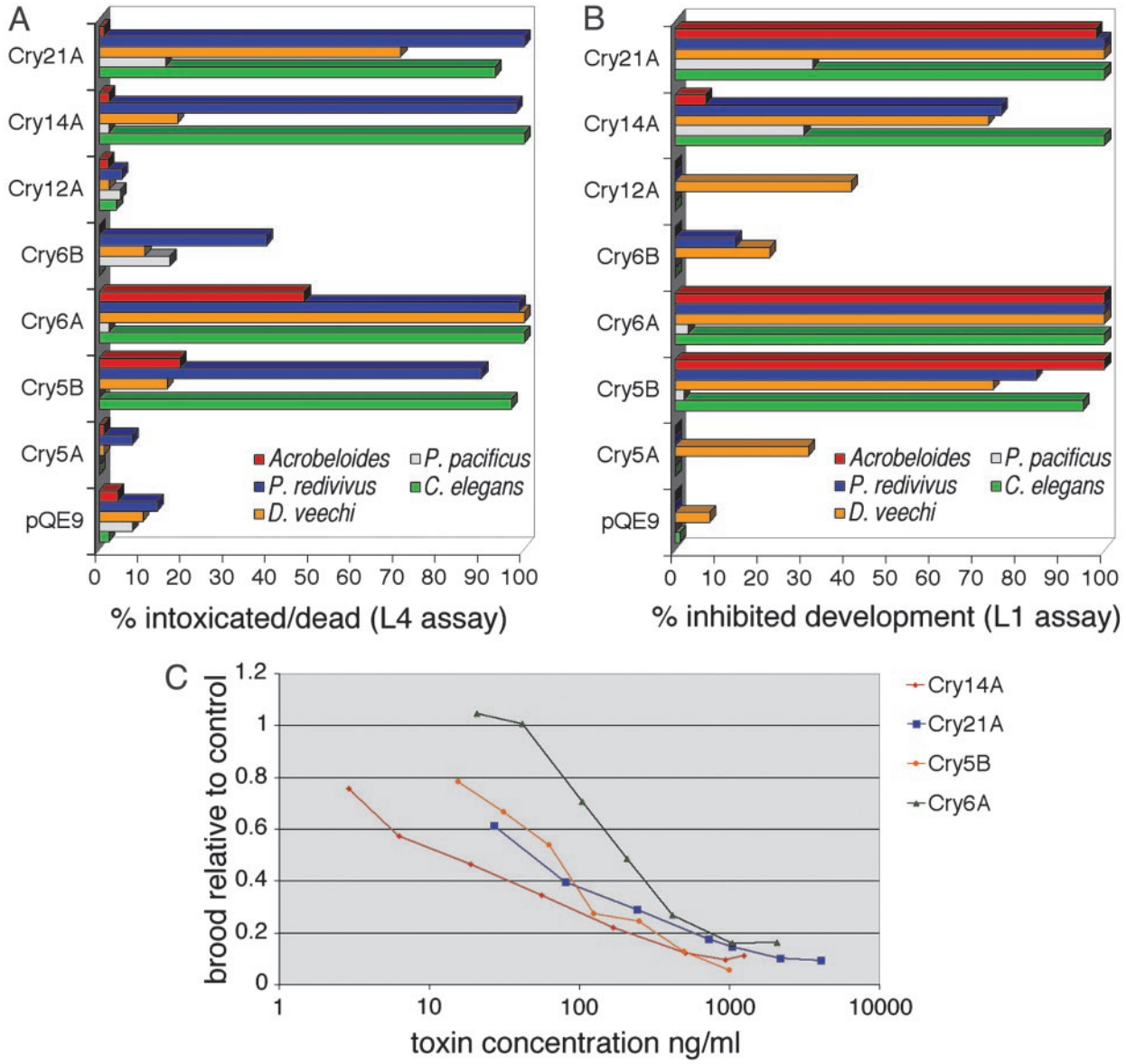

Fig. 3. Quantitation of the effects of crystal proteins fed to nematodes in intact *E. coli* bacteria. (*A*) Plate assay results showing the percentage of animals that were intoxicated or dead when fed crystal protein produced by *E. coli* on a plate. Each bar represents the average from three independent trials with ≈30 animals per trial. pQE9, vector no insert. (*B*) Developmental assay results showing the percentage of animals that were inhibited in development. (*C*) Three-day brood sizes of *C. elegans* hermaphrodites in increasing doses of four different crystal proteins normalized to that of hermaphrodites fed vector-expressing bacteria alone.

To extend these data, the five species of nematodes were subjected to a developmental inhibition assay (Fig. 3*B*). First-stage larval (L1) animals from each species were fed JM103 *E. coli* in microtiter wells in the presence of antibiotics. When fed JM103 transformed with vector alone, virtually all nematodes are able to grow to adults in 2 days (*C. elegans* and *P. pacificus*), 3 days (*D. veechi* and *P. redivivus*), or 8 days (*Acrobeloides* sp.) (the timing reflects natural differences in generation times of these nematode species). The five nematode species were then fed *E. coli* producing each of the seven crystal proteins (doses in Table 1), and each individual animal was scored for the ability to grow up to an adult as above. Animals that failed to fully develop were scored as intoxicated. In many cases where toxicity was seen, the nematodes failed to develop past the first or second larval stage. The data generally agree well with results from the plate health assay above; Cry5A, Cry6B, and Cry12A crystal proteins were not toxic, whereas Cry5B, Cry6A, Cry14A, and Cry21A were toxic to many of the nematode species.

***Bt* Toxins Appear to Act Via the Intestine of Affected Nematodes.** The mechanism of toxicity of *Bt* crystal proteins toward insects is accepted to be damage to the gut. To establish whether the same is true of nematodes, we fed each of the four toxic crystal proteins (Cry5B, Cry6A, Cry14A, and Cry21A) to L4-stage larvae of all five of the nematode species and then examined anterior gut morphology as described (5). We found good correlation between the nematode susceptibility as ascertained by the L4 plate assay with an altered appearance to the nematode gut (Fig. 2*B*). For example, the intestine of *C. elegans* was damaged by all four crystal proteins whereas the intestine of *Acrobeloides* appeared to be significantly damaged only by Cry6A. Indications of intoxication include constriction and thinning of intestine cells, shrinkage of the gut from the body wall, vacuolization, and a degenerative appearance.

***P. pacificus* Is Not Resistant to Crystal Protein.** In the assays above, *P. pacificus* appeared resistant to all crystal proteins. However, it

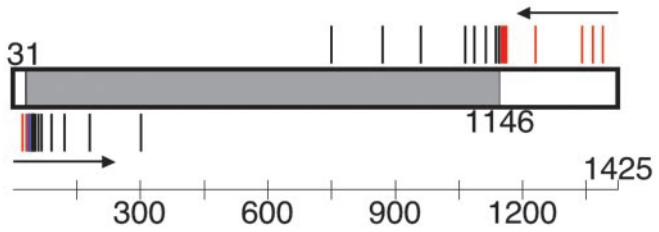

Fig. 4. Structure–function analysis of Cry6A. The Cry6A gene is schematized as a horizontal rectangle (full length = 1–1,425 nt; scale in nt shown at bottom). Bars above the gene show the locations of 17 C-terminal truncations tested; bars below the gene show the locations of 14 N-terminal truncations tested. Red bar, a truncation that is toxic; black bar, a truncation that is not toxic; blue bar, a truncation that is weakly toxic. Thicker bars indicate a cluster of truncations made in that region. The minimal toxic fragment from nucleotides 31–1146 is indicated by gray box. Exact locations of the truncations made are given in Table 3, which is published as supporting information on the PNAS web site.

was pointed out to us (Leon Avery, University of Texas Southwestern Medical Center, Dallas) that this nematode lacks a pharyngeal grinder. The "grinder" is located in the posterior bulb of the pharynx (anterior of the gut) and physically grinds bacteria open so that nutrients can subsequently be extracted in the gut. We verified that *P. pacificus*, alone among the nematodes tested, lacks a grinder. This physiology raised the possibility that *P. pacificus* appeared to be resistant to crystal proteins because of differences in the way it digests *E. coli* (e.g., during the process of breaking open and digesting bacteria without a grinder, toxin is destroyed). We therefore fed *P. pacificus* purified Cry5B crystal protein in microtiter wells so that the animals would have access to toxin without having to break open the bacteria. Although we occasionally noticed a slight change in the health of the animals, we did notice a significant reduction in brood size. In the absence of crystal protein, *P. pacificus* hermaphrodites have an average 3-day brood of 115 ± 19 (n = 18) whereas in 50 μg/ml Cry5B they have an average 3-day brood of 16 ± 14 (n = 13). Purified Cry5B also inhibited the development of *P. pacificus* L1 larvae (data not shown). Thus, *P. pacificus* is susceptible to Cry5B crystal protein.

Cry14A Is the Most Potent Crystal Protein on *C. elegans*. These assays indicate which toxins are able to affect nematodes but do not indicate their relative toxicities. For this information, we measured the brood size of *C. elegans* at different concentrations of the four nematicidal crystal proteins. Brood size appears to be an indirect measure of the health of the mother's intestine, and we have used this assay in the past to quantitate relative toxicities (4). The advantages of this method for quantitation are that it can be directly carried out by feeding the nematodes intact *E. coli* without crystal protein purification and that it is dose dependent over the range of crystal protein made by *E. coli*, which is less than that made by *Bt*. We find that all four toxins produce a dose-dependent reduction in progeny production (Fig. 3*C*). Cry14A is the most toxic of these crystal proteins on *C. elegans* (50% inhibition or IC_{50} = 16 ng/μl), followed by Cry21A and Cry5B (IC_{50} = 47 ng/μl and 66 ng/μl, respectively), and finally Cry6A (IC_{50} = 230 ng/μl).

Cry6A Contains a Small Active Core. Main family crystal proteins are often synthesized as large protoxins that contain smaller active toxin cores. Because the sequence of Cry6A (1,425 nt) is completely unrelated to main family crystal proteins, it is not clear whether it, too, contains a smaller active core, and, if so, how small it might be. To address this question, we took advantage of the rapid plate assay system developed above. More than 30 C-terminal and N-terminal Cry6A truncations were made and tested for toxicity against *C. elegans* (Fig. 4). We found that C-terminal truncations that retain 1,146 nt are toxic but truncations that retain 1,143 nt are not. N-terminal truncations that begin at nucleotide 31 are toxic but truncations that begin at nucleotide 34 are much less active. Cry6A truncated at both ends (nucleotides 31–1146) is also toxic, although qualitatively weaker than full length. In summary, we have found Cry6A contains an unusually small active toxin core with a predicted molecular mass of 43 kDa.

Table 2. Inhibition of animal parasite growth

Insert*	% Developed†
None	75.9 (336)
Cry5B	5.1 (295)
Cry6A	83.2 (309)
Cry14A	0 (139)
Cry21A	0 (290)

**E. coli* JM103 was transformed with pQE9 plus the insert indicated and then fed to *N. brasiliensis.*

†Percent of animals (total number) that developed past the early L1 stage in 2 days at room temperature.

Intoxication of an Animal Parasitic Nematode. The phylum Nematoda contains, in addition to beneficial, free-living species, parasitic species that infect animals and plants. Because all of the nematodes tested above respond to some crystal proteins, they must possess the processing enzymes, receptors, and gut environment necessary for the action of crystal proteins. We asked whether the same might be true of a parasitic nematode. To address this question, we selected the nematode *N. brasiliensis*, a worldwide intestinal parasite of rats and a member of the largest nematode group parasitic in domestic animals (superfamily Trichostrongylidae) that also contains *Haemonchus contortus*, *Cooperia*, and *Ostertagia* (14, 15). Its life cycle is as follows: eggs are passed out of the infected host into the environment and develop to the L1 stage in 24 h at 25°C. L1 larvae emerge, feed on bacteria in host feces, and after 4–5 days develop to an infectious third larva stage (L3). These enter the host via penetration of the skin, eventually making their way first to the lungs and then to the gastrointestinal tract.

We found that larvae hatched from *N. brasiliensis* embryos can feed and develop from small L1 to large (presumably infectious L3) larvae over the course of 3 days on standard *C. elegans* growth plates spread with *E. coli* that is not expressing crystal protein (Fig. 2*C*; Table 2). These larvae are healthy, motile, and display darkened guts characteristic of feeding nematodes. Conversely, we found that *N. brasiliensis* development and health are severely compromised when fed *E. coli* expressing Cry5B, Cry14A, or Cry21A crystal protein (Fig. 2*C*; Table 2). Virtually all of the parasites fed these crystal proteins were small and intoxicated or dead. *N. brasiliensis* did not appear to be affected by Cry6A (Table 2).

Discussion

We have demonstrated that six phylogenetically diverse nematodes are susceptible to *Bt* crystal proteins, thus demonstrating that the phylum Nematoda is a target of *Bt* crystal proteins. Three of the crystal proteins that belong to the Cry5 family, namely Cry5B, Cry14A, and Cry21A, and one of the proteins that belongs to the Cry6 family, namely Cry6A, are each toxic to at least four nematode species (summarized in Fig. 1*B*). Toxicity was demonstrated by using intoxication, developmental, fecundity, and gut morphology assays. In addition, our experiments that delineate the minimal toxic Cry6A fragment point out the utility of using *C. elegans* to perform structure–function analysis of *E. coli*-expressed *Bt* crystal proteins.

Previous studies on *Bt*–nematode interactions (e.g., ref. 16) have revealed toxic effects on *C. elegans* of the native *Bt* bacterial isolate B-18247, which makes Cry6B (17). However, conclusions from

these studies are complicated by the fact that three crystal proteins (two uncharacterized) are made by this strain and that the native bacterium can also produce noncrystal protein toxins. Moreover, in these earlier studies the nematodes were assayed under growth conditions where the animals may have been stressed (200 animals per well in PBS in a 96-well plate format). There are also a few older studies on the effects of insecticidal *Bt* preparations on nematodes (e.g., ref. 18). These either showed low activity or (the vast majority) included toxicity toward nematode eggs. Because it is highly unlikely that crystal proteins can penetrate the impervious eggshell of nematodes, these studies were probably evaluating the effects of non-Cry toxins made by *Bt*.

We found that the two Cry5 family proteins, Cry5A and Cry12A, and one Cry6 family member, Cry6B, are not toxic to the nematodes studied. Negative results must be considered with care because, for example, *E. coli* might not fold these (but not the other) crystal proteins correctly, these Cry proteins might be active against other nematodes, or they might be nematicidal at higher doses. Nonetheless, it has recently been shown that Cry5A is active against Hymenoptera (19), consistent with our observations that this toxin does not target nematodes. In addition, we note that our assays are very sensitive (e.g., dilution of some toxins by 10-fold still showed strong developmental inhibition of *C. elegans*) and that *E. coli* has been successfully used in many studies to produce active insecticidal crystal proteins (e.g., ref. 20). We therefore interpret our Cry5A and Cry12A results to indicate that main family *Bt* crystal proteins do not nonspecifically target nematodes. An important extension of this interpretation is that commonly used insecticidal crystal proteins, which are even more distantly related to the nematicidal Cry5B, Cry14A, and Cry21A than Cry5A and Cry12A are, should not significantly target nematodes. Indeed, we have confirmed that *Bt kurstaki* and Cry1Ac are not toxic toward *C. elegans* and other free-living nematodes (unpublished observations), and a recent study indicates that Cry1Ab released from root exudates and biomass of *Bt* corn has no effect on nematodes (21).

The relationship between nematode susceptibility and toxin phylogeny is shown in Fig. 1*B*. The phylogeny of main family Cry5 crystal proteins shows excellent correlation with nematicidal properties; Cry5A and Cry12A are most closely related to each other and neither is nematicidal whereas Cry5B and Cry21A are most closely related and both are nematicidal. In the case of Cry6A and Cry6B (which are more similar to each other than other pairs of crystal proteins in this study), this correlation breaks down, suggesting that evolution of the Cry6 family may be different from that of main family crystal proteins. Conversely, all nematodes tested were susceptible to at least one crystal protein in at least one assay. These results are encouraging for the use of these crystal proteins in nematode control. There are, however, some idiosyncrasies. *N. brasiliensis* is unexpectedly resistant to Cry6A. We also would have predicted that, based on nematode phylogeny, *P. pacificus* should respond to the *E. coli*-produced Cry5B, Cry6A, Cry14A, and Cry21A. We speculate that physiological adaptations independent of phylogeny (e.g., *P. pacificus*' lack of a grinder) may play important roles in some cases.

Our work has potential implications for understanding the natural ecology of *Bt* (reviewed in ref. 22). The role of *Bt* and its insecticidal crystal proteins in nature remains enigmatic, e.g., *Bt* rarely causes natural epizootics in insects, recycles poorly in insect populations, and can be found in environments with no insects or in locales where it is not toxic to insects found there. We speculate that soil-dwelling nematodes might have contributed to the evolution and/or spreading of this bacterium and its crystal proteins. It seems plausible that a soil bacterium might take advantage of the fact that soil nematodes use bacteria as a food source and evolve crystal proteins to help it propagate inside the host nematode once its spores and crystals are ingested. In addition, because many nematodes associate with insects in parasitic and especially phoretic relationships, nematodes might have promoted interactions between the bacterium and insects.

The most important conclusion of these studies is that *Bt* crystal proteins have potential in controlling nematode pests that infect animals and plants. In particular, the effectiveness of Cry5B, Cry14A, and Cry21A against the free-living stage of an animal parasite, *N. brasiliensis*, is promising. Although it remains to be seen whether these toxins are effective against other parasites or against nematodes in parasitic stages, these results demonstrate that a parasitic nematode can express the proper molecular elements (e.g., processing proteases and receptors) for responding to *Bt* toxins. Given the very low toxicity of *Bt* crystal proteins in general toward vertebrates (22), *Bt* crystal proteins may one day provide safe, cost-effective control of nematode parasites, such as those that infect more than one-fourth of the human population (23).

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