Bacillus thuringiensis isolates from the Philippines: habitat distribution, δ-endotoxin diversity, and toxicity to rice stem borers (Lepidoptera: Pyralidae)

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Abstract

Bacillus thuringiensis Berliner isolates were detected in 57% of 801 samples of rice grain dust, soil, rice field arthropods, and miscellaneous habitats (rice straw compost and mammal faeces) collected at 100 sites in the Philippines. The collection yielded 3950 isolates of *B. thuringiensis* (8.7 isolates/positive sample). Grain dust from rice mills was the richest source (63% of the samples were positive, with 10.2 isolates/positive sample), followed by rice field arthropods, soil, and miscellaneous habitats. Polyclonal antibodies to six δ-endotoxin groups (Cry1A, Cry1B, Cry1C, Cry1D, Cry1E, and Cry3A) were used in enzyme-linked immunosorbent assays (ELISA) to characterize the toxins produced by each isolate. Subsamples of isolates representing the diversity of isolate sources and δ-endotoxin profiles were bioassayed against the yellow stem borer, *Scirpophaga incertulas* (Walker) and striped stem borer, *Chilo suppressalis* (Walker). Eighteen isolates highly toxic to both species were selected for characterization of δ-endotoxin genes by polymerase chain reaction (PCR) with primers specific to 14 genes or gene subfamilies, and Western blotting with Cry2A antibodies. At least two novel δ-endotoxin genes, related to *cry1B* and *cry1F*, were detected by DNA sequencing of PCR products.

Introduction

Bacillus thuringiensis Berliner is a rod-shaped, gram-positive bacterium abundant in soil and other habitats throughout the world. During sporulation, *B. thuringiensis* produces a parasporal crystal composed of proteins known as δ-endotoxins or insecticidal crystal proteins (ICPs). Following ingestion, solubilization, and, in some cases, proteolytic activation, these toxins bind to receptors and form pores in the midgut epithelium of susceptible insects (Gill *et al.*, 1992). This process results in disruption of membrane integrity, starvation, and ultimately death. Because δ-endotoxins are generally safe to vertebrates (Siegel & Shadduck, 1989) and beneficial arthropods (Flexner *et al.*, 1986) and are often highly toxic to insect pests at relatively low doses, genes encoding these proteins were among the first to be used in genetic engineering of plants for enhanced insect resistance (Vaeck *et al.*, 1987).

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Genetic engineering of rice with δ-endotoxin genes is a promising approach for improved management of stem boring caterpillar pests of rice, including the yellow stem borer, *Scirpophaga incertulas* (Walker) (Lepidoptera: Pyralidae), and striped stem borer, *Chilo suppressalis* (Walker) (Lepidoptera: Pyralidae). These insects bore into the rice plant shortly after emerging from eggs and cannot be controlled by topically-applied *B. thuringiensis* sprays. In addition, high levels of stem borer resistance have not been achieved through conventional plant breeding, despite many years of extensive effort (Chaudhary *et al.*, 1984). Rice lines transformed with *cry1Ab* genes, truncated and codon-optimized for improved expression in plants, have shown greatly increased resistance to *S. incertulas* and *C. suppressalis* (Fujimoto *et al.*, 1993; Wünn *et al.*, 1996; Ghareyazie *et al.*, 1997).

In 1988, the International Rice Research Institute (IRRI) and Plant Genetic Systems, N.V. (PGS) established a collaboration to collect *B. thuringiensis* isolates in the Philippines and identify new δ-endotoxins for control of insect pests of rice and other crops. Descriptions of two new genes isolated from the collection have been published (Lambert *et al.*, 1992, 1996). In this paper, we describe the habitat distribution and preliminary immunological characterization of the collection as a whole, and provide a more detailed characterization of 18 isolates highly toxic to *S. incertulas* and *C. suppressalis*.

Materials and methods

Habitat sampling and B. thuringiensis *identification*

A total of 801 samples was collected from 100 sites distributed widely between 6–18.5 N° and 117.5–125.5 E° in the Philippine archipelago. The samples included grain dust from rice mills (503); soil (2 cm below the surface) from rice fields and associated agricultural land (195); arthropods collected in rice fields (82); and rice straw compost and mammal faeces (21). None of the areas sampled had received applications of *B. thuringiensis* insecticide.

Bacillus thuringiensis was isolated by selective sample enrichment (Travers *et al.*, 1987) and subsequent plating on an agar medium. Samples (0.5 g) were incubated in a water bath shaker at 150 rpm and 30°C for 4 h in 10 ml 2 M Luria broth–sodium acetate medium (50% Luria broth 2×: 2% tryptone, 1% yeast extract, 2% sodium chloride; 12.5% sodium acetate 2 M, pH 6.8). Small volumes (2 ml) of the sample suspension were incubated at 60°C for 1 h. Aliquots of serial dilutions were then each plated on 10 petri plates containing T3 medium (0.3% tryptone, 0.2% tryptose, 0.15% yeast extract, 0.0005% $MnCl_2$, 0.05 M sodium phosphate, 1.5% agar, pH 6.8) and incubated at 26°C for 2–3 days.

Bacillus thuringiensis-like colonies were checked for the presence of crystals under a phase contrast microscope and isolated by subculturing on T3 agar plates at 26°C for 2–3 days. Spore-crystal mixtures were harvested in phosphate buffered saline for further analysis or stored in 25% glycerol at −70°C. Small volumes (1 ml) of the spore-crystal suspensions were centrifuged and washed in phosphate buffered saline. Crystals were solubilized by overnight incubation at 37°C in 50 mM Na_2CO_3 (pH 10) with 10 mM dithiothreitol. The production of ICPs was confirmed by the presence of typical protein bands after sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) of trypsinated ICP solutions.

Preliminary immunological characterization

Enzyme-linked immunosorbent assay (ELISA) was used to determine the ICP composition, as described by Höfte *et al.* (1988). Standardized amounts of ELISA plates were coated overnight at 4°C with 100 µl of solubilized ICPs (0.5 µg/100 µl). Polyclonal antibodies for Cry1A, Cry1B, Cry1C, Cry1D, Cry1E, and Cry3A were used. (We use the δ-endotoxin nomenclature developed by Crickmore *et al.* (1995), in which Roman numerals have been replaced by Arabic numerals. See also: Crickmore, N., Zeigler, D.R., Feitelson, J., Schnepf, E., Lereclus, D., Baum, J., Van Rie, J. & Dean, D.H. *Bacillus thuringiensis* delta-endotoxin nomenclature. WWW site: http://epunix.biols.susx.ac.uk/Home/Neil_Crickmore/Bt/index. html)

Bioassays of representative isolates

Scirpophaga incertulas and *C. suppressalis* females were collected by hand from the canopy of numerous rice fields in the vicinity of IRRI and caged on rice plants in a greenhouse until they produced egg masses. We used neonate larvae emerging from these egg masses in our bioassays. To obtain δ-endotoxin samples for bioassays, isolates were cultured and δ-endotoxins were solubilized as described above, and the solubilized protein was quantified by the Bradford method (Bradford, 1976). The ICPs were activated by treatment with trypsin (0.05 mg trypsin/mg ICP overnight at 37°C) and incorporated into artificial diet at six to nine different doses. Bioassay conditions were as described in Lee *et al.* (1997). Briefly, *c.* 5 ml of artificial diet was poured into 15 ml scintillation vials and infested with six neonate *S. incertulas* or ten *C. suppressalis* neonate larvae per vial. In *S. incertulas* bioassays, there were eight replicate vials per dose and 24 control vials (containing toxin-free diet); for *C. suppressalis* we used five vials per dose and ten control vials. Mortality was recorded after incubation at 27°C for 96 h.

Following the preliminary immunological characterization of the collection, a representative sample of 976 isolates was selected for bioassays against *S. incertulas*. This sample included isolates of each toxin profile identified in the collection. Selected isolates that produced greater than 90% mortality at 2 µg/ml artificial diet in two consecutive bioassays were examined in preliminary tests against *C. suppressalis*. We then conducted a series of detailed bioassays against *C. suppressalis* with a selection of 18 isolates that showed high toxicity to both species. All 18 isolates were tested simultaneously using larvae that emerged from the same pool of egg masses, and the entire experiment was repeated on each of four days between August 6 and 26, 1996. The egg masses for each day were obtained from separate collections of *C. suppressalis* females, each made from several rice fields within the vacinity of IRRI. Cry1Ac was included in the bioassays as a positive control.

Bioassay data were analysed by probit analysis using the POLO-PC package (LeOra Software, 1987). LC_{50} values were log-transformed (because variances were proportional to means) and subjected to analysis of variance by use of the SAS package (SAS Institute, 1985).

PCR analysis and sequencing of PCR products

Bacillus thuringiensis isolates were grown in 2×YT broth (16 g tryptone, 10 g yeast extract and 5 g NaCl per litre) at 28°C with shaking (200 rpm) for 5–6 h. The cells were harvested by centrifugation and total DNA was isolated following the method of Kalman *et al.* (1993). We analysed total DNA from 20 isolates with PCR primers for eleven genes in the *cry1*, *cry2*, and *cry9* gene families. We selected these genes because our interest was in toxins active against Lepidoptera. Sequences and annealing temperatures for primer pairs for *cry2A*, *cry5*, and *cry9C* are presented in table 1. We designed the *cry1I* and *cry9C* primers; the *cry2A* primers were designed by D. Dean, Ohio State University. For eight additional *cry1* genes, we used the primers described by Kalman *et al.* (1993), but found that results were more reliable when these primers were used pairwise for individual genes instead of in combination for 'multiplex PCR' as intended. The annealing temperatures we used were 55°C (*cry1B* and *cry1F*), 60°C (*cry1E*), 65°C (*cry1C* and *cry1Ab*), 68°C (*cry1D*), and 70°C (*cry1Aa* and *cry1Ac*).

The PCR reactions contained 1 µl DNA template (20 ng), 0.5 mM each primer, 200 mM deoxyribonucleoside triphosphates, 1×PCR buffer (10 mM Tris-HCl, pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$) and 1 U Taq DNA polymerase in a 25 µl volume. PCR amplifications were carried out in a DNA thermal cycler with an initial denaturing step of 1 min at 94°C followed by 35 cycles of 1 min at 94°C, 1 min at the specified annealing temperature (see table 1) and 1 min at 72°C. A final extension step of 5 min at 72°C was added at the end of the cycles. PCR products were separated by electrophoresis in 1% agarose gels, stained with ethidium bromide and visualized with UV illumination.

Amplified PCR products were excised from the agarose gel and the DNA isolated and purified using the GeneClean kit (Bio101, USA). Purified PCR products were cloned into the pMOSBlue T vector and the construct used to transform MOSBlue cells (pMOSBlue T vector kit, Amersham Life Science, UK). DNA sequence from cloned PCR products was determined using the Sequenase Version II DNA sequencing kit (Amersham Life Science, UK) with the vector-specific T7 promoter and U-19 primers.

Immunoblotting

Bacillus thuringiensis isolates were cultured in liquid media (8 g nutrient broth, 0.25 g $MgSO_4 \cdot 7H_2O$ and 1 g KCl per litre) at 28°C with shaking at 200 rpm for three days. The cells were harvested by centrifugation and washed at least three times with PBS (140 mM NaCl, 3 mM KCl, 10 mM Na_2HPO_4, 2 mM KH_2PO_4, pH 7.4). The spore-crystal pellet was suspended in 50 mM Na_2CO_3, 10 mM dithiothreitol (pH 10) containing protease inhibitors (Complete protease inhibitor cocktail tablet, Boehringer Mannheim, Germany) and incubated overnight at 37°C. The supernatant containing the solubilized ICPs was separated by centrifugation. Solubilized ICPs were separated by SDS-PAGE. Gels were either stained with 0.25% Coomassie blue R250 or analysed by Western blotting (Burnette, 1981) with anti-Cry2A antibody (a gift of D.H. Dean, Ohio State University).

Results and Discussion

Incidence of B. thuringiensis *in different habitats in the Philippines*

Of the 801 samples collected, 457 (57%) tested positive for *B. thuringiensis* (table 2). These yielded 3950 isolates, an average of 8.6 isolates per sample containing *B. thuringiensis*. The richest source for *B. thuringiensis* isolates was grain dust from rice mills, which yielded 10.2 isolates per positive sample, followed by rice field arthropods, soil, and miscellaneous habitats. The grain dust samples also had the highest number of isolates producing identical ELISA profiles (four identical isolates per positive sample). The actual number of isolates in these grain dust samples was even higher than indicated in table 2, since the high incidence of *B. thuringiensis* forced us to sample colonies randomly instead of culturing of isolates. By contrast, we were able to culture all isolates from the other habitat samples.

We found *B. thuringiensis* in most of the 82 arthropod specimens that we processed. Most of the predators (39 of 61) and parasites (4 of 5) caught alive and half of the 16 other insects (cadavers) were found to carry isolates. The average number of isolates from these dead insects was higher than in the ones caught alive (6.6 vs. 4.4 per positive sample). The dead insects also had a higher number of isolates with identical ELISA profiles (3.3 vs. 2.3 per positive sample) (table 2). It is known that *B. thuringiensis* proliferates in arthropod cadavers under some conditions (Prasertphon *et al.*, 1973; Aly *et al.*, 1985).

Numerous studies have found that grain dust and soils are environments in which *B. thuringiensis* is abundant (Meadows, 1993). Grain dust and soil were the richest habitats surveyed for *B. thuringiensis* in Pakistan (Khan *et al.*, 1995) and in a collection of stored product, soil, insect, plant, and other samples assembled from 80 countries (Bernhard *et al.*, 1997). Martin & Travers (1989) isolated *B. thuringiensis* from 70% of soil samples collected from 30 countries, including in 94% of samples from Asia, and Padua *et al.*

Table 1. Properties of polymerase chain reaction primers for *cry1I*, *cry2A*, and *cry9C*.

Gene(s) recognized	GenBank accession no.	Position[a]	Predicted size of PCR product (bp)	Annealing temp. (°C)	Sequence (5'–3')[a]
cryII	X62821	F: 617–636	919	55	F: TTA TCT TAG GIG AGC TIT GG
		R: 1516–1535			R: GTG AAC IGT AAT GTT ACI GG
cry2A	M31738	F: 1297–1318	625	68	F: TAC AGA TCG AGA GGG CGT TGC
		R: 1886–1912			R: CGA GCT CCA TTA TCA TTA ACT CCA TCG
cry9C	Z22513	F: 678–697	990	60	F: AGT CAC TTC TCC CTG GTG CG
		R: 1644–1667			R: ACC TTA CTC CCT CCA CTA CTC TCG

[a]F: forward primer; R: reverse primer.

Table 2. Incidence of *Bacillus thuringiensis* in different habitats in the Philippines.

	Habitat						
	Grain	Arthropods[a]			Soil	Misc.[b]	Total
		living	dead	total			
Samples	503	66	16	82	195	21	801
Samples containing *Bt*	319	43	8	51	80	7	457
% Positive samples	63	65	50	62	41	33	57
B. thuringiensis isolates	3261	189	53	242	425	22	3950
Isolates/sample (mean)	10.2	4.4	6.6	4.7	5.3	3.1	8.6
Identical isolates/sample[c] (mean)	4	2.3	3.3	2.5	2.8	1.6	3.7

[a]Sixty-one living predator specimens, of the following species: *Agriocnemis pygmaea* (Rambur) (Odonata: Coenagrionidae), *Agriope catenulata* (Doleschall) (Araneae: Araneidae), *Anaxipha longipennis* (Serville) (Orthoptera: Gryllidae), *Conocephalus longipennis* (de Haan) (Orthoptera: Tettigoniidae), *Cyrtorhinus lividipennis* Reuter (Hemiptera: Miridae), *Pardosa pseudoannulata* (Bösenberg & Strand) (Araneae: Lycosidae), *Metioche vittaticollis* Stål (Orthoptera: Gryllidae), *Micraspis* sp. (Coleoptera: Coccinellidae), *Ophionea nigrofasciata* Schmidt-Goebel (Coleoptera: Carabidae), *Phidippus* sp. (Araneae: Salticidae), and *Tetragnatha maxillosa* Thorell (Araneae: Tetragnathidae); five living parasitoid specimens: *Cotesia* sp. (Hymenoptera: Braconidae), *Stenobracon nicevillei* (Bingham) (Hymenoptera: Braconidae), *Temelucha philippinensis* Ashmead (Hymenoptera: Ichneumonidae), *Trichomma cnaphalocrocis* Uchida (Hymenoptera: Ichneumonidae), and *Xanthopimpla flavolineata* Cameron (Hymenoptera: Ichneumonidae); and sixteen dead rice pest specimens of the following species: *Cnaphalocrocis medinalis* (Guenée) (Lepidoptera: Pyralidae), *Marasmia patnalis* Bradley (Lepidoptera: Pyralidae), *Melanitis leda ismene* (Cramer) (Lepidoptera: Satyridae), *Rivula* nr. *atimeta* (Swinhoe) (Lepidoptera: Noctuidae) and *Naranga aenescens* Moore (Lepidoptera: Noctuidae).

[b]Rice straw compost (one sample); faeces of water buffalo, *Bubalus bubalis*, (seven samples); faeces of cave bat, *Eonycteris spelaea glandifera*, (ten samples); and faeces of cattle, *Bos indicus*, (three samples).

[c]Number of isolates per positive sample producing identical insecticidal crystal proteins.

(1982) found *B. thuringiensis* in 18% of soil samples analysed from the Philippines. Our data on miscellaneous habitats are consistent with other reports that organic material is not favourable for the survival of *B. thuringiensis* (Saleh *et al.*, 1970; West & Burges, 1985). Of the 21 samples from rice straw compost and mammal faeces only 33% contained *B. thuringiensis*, and the number of isolates was lower than in any other habitat (3.1 per positive sample).

Preliminary immunological characterization

Altogether, 48 δ-endotoxin profiles, consisting of single toxins or combinations, were found by ELISA with the polyclonal antisera (table 3). Only one toxin type or subtype was detected in 56% of the isolates. Combinations of two (31%), three (12%) or four (1%) toxins were detected in the remaining isolates. Eight percent of the isolates produced δ-endotoxin(s) detected by SDS-PAGE but not recognized by the polyclonal antisera used in ELISA. (These are referred to as 'unknown' in table 3.) A further 20% produced only a protein that was immunologically related to both Cry1 and Cry3 proteins. A gene encoding this protein, designated *cry1Ha1*, was later isolated and sequenced at PGS (GenBank accession no. Z22513). (The protein encoded by *cry1Ha1*, i.e. Cry1H, should not be confused with the protein now designated Cry9C (Lambert *et al.*, 1996), which was formerly known as CryIH).

Large percentages of isolates produced only Cry1A (17%) or Cry1B (11%), while few isolates produced only Cry1C, Cry1D, Cry1E or Cry3A (<2%). Cry1A and Cry1B not only occurred frequently by themselves but almost all of the isolates producing combinations of proteins produced one or both of these proteins. Isolates producing Cry1A in different combinations made up 37% of the total collection, and accounted for 86% of the combinations observed. Cry1B occurred in 20% of the collection or 46% of the combinations observed. It was therefore not surprising that the most frequent combination was Cry1A/Cry1B (11%). There was

Table 3. Frequency of δ-endotoxin profiles in the Philippine collection of *Bacillus thuringiensis* isolates as determined by enzyme-linked immunosorbent assay.

δ-endotoxin(s)	Isolates producing only one toxin (%)	Isolates producing indicated toxin in combination with others (%)
Cry1A	17	37
Cry1B	11	20
Cry1C	<1	14
Cry1D	<1	6
Cry1E	1	11
Cry1H	18	2
Cry3A	1	10
Unknown	8	

also a high number of isolates producing Cry1A/Cry1E (7%) and Cry1A/Cry1C (4%). Cry3A was detected in a total of 11% of the isolates. In contrast, *cry3* genes were not detected in any of 225 *B. thuringiensis* isolates collected in Taiwan (Chak *et al.*, 1994).

Bioassays and molecular analysis of representative isolates

Of 976 isolates that we selected to represent the diversity of δ-endotoxin profiles in the collection, 100 produced more than 90% mortality of *S. incertulas* larvae at the highest dose tested, 2 μg/ml artificial diet, in two consecutive bioassays. These 100 highly toxic isolates contained a diversity of ELISA profiles, although none of the isolates produced Cry1H. Cry1H was also not toxic to several additional species of Lepidoptera and Coleoptera tested in Europe (B. Lambert, unpublished data).

We identified 18 of the 100 isolates that also showed high toxicity to *C. suppressalis* and selected them for more extensive bioassays with this species (table 4). The mean estimated LC_{50}s of the trypsinated ICPs from these 18 isolates, based on four days of bioassays, ranged from 0.10 to 160 μg/ml artificial diet (table 4). The mean estimated LC_{50} for purified Cry1Ac was 5.5 ± 5.1 μg/ml. There was a high degree of variability of LC_{50} estimates within toxin preparations (i.e. purified Cry1Ac or ICPs from each isolate), as was also observed with four purified δ-endotoxins assayed against *S. incertulas* and *C. suppressalis* (Lee *et al.*, 1997). The effect of toxin preparation on LC_{50} was not significant ($F = 1.67$; df = 18, 54; $P = 0.075$), while the effect of day of bioassay on LC_{50} was highly significant ($F = 5.70$; df = 3,54; $P = 0.0018$). The high day-to-day variability of LC_{50} estimates in our stem borer bioassays may be attributable to our use of F_1 progeny of separate field collections of females on each day. Experiments to test this hypothesis are in progress.

Analysis of the 18 representative isolates by PCR indicated that most contained several *cry1* genes, as well as *cry2A* (table 4). However, isolates 93 and 898 contained only *cry1Ac* and *cry2A*, respectively. *cry1Ab*, *cry1Ac*, *cry1B*, *cry1I*, and *cry2A* were found in 11 or more isolates.

For all 20 isolates except 93 and 898, there were discrepancies between the profiles of δ-endotoxin genes detected by PCR and the proteins detected by ELISA or Western blotting (table 4). In most cases, the diversity of genes detected was greater than that of proteins. For example, although we detected the *cry2A* gene by PCR in 15 isolates, on Western blots we detected Cry2A protein in only ten of the isolates. In only one case was a protein detected by ELISA for which no gene was detected by PCR: Cry1B in isolate 144. The detection of many more genes than proteins may be due to a lack of gene expression or low levels of expression of some of the amplified genes. Other possible explanations are contamination of genomic DNA samples with DNA from PCR products or other isolates, or amplification of non-target genes that yield fragments of similar size but that do not encode proteins that cross-react with antibodies to the target genes.

There was no obvious correspondence between δ-endotoxin protein or gene profiles and toxicity to *C. suppressalis* (table 4). Any such relationship may have been obscured by variation in the levels of gene expression among isolates containing the same gene(s) and variation in the LC_{50} estimates for each isolate.

We obtained DNA sequence from 12 PCR products. Several of them differ to varying degrees from expected nucleotide sequences and appear to be fragments of novel *cry* genes. The PCR product generated with *cry1B* primers from isolates 222 and 734 is 807 base pairs (bp) in length, instead of the expected 830 bp. Its nucleotide sequence most closely matches that of *cry1Ba2* but is only 65% identical (excluding the primer sequences). The PCR product from isolate 734 generated with *cryIF* primers has the expected length of 368 bp but is only 70% identical to *cry1Fa2*. We obtained *c.* 600 bp of sequence of *cry1I* products from isolates 222 and 734. They are 84 and 95% identical to *cry1Ia1*, respectively. The *cry1Ab* sequence from isolates 734 and 222 is 90% identical to *cry1Ab9*. The *cry1Ac* products from isolates 93 and 222, the *cry2A* product from isolate BTP898, and the *cry9C* product from isolates 734 and 222 were 95–100% identical with nucleotide sequences for these genes.

Use of the collection for rice stem borer management

When this project was initiated in 1988, the objective of the International Rice Research Institute was to isolate novel genes for use in genetic engineering to enhance resistance to rice stem borers, most importantly *S. incertulas* and *C. suppressalis*. Following the preliminary characterization of the entire collection by ELISA, and bioassays of 976 isolates representing the diversity of source habitats and immunological profiles, we selected a further representative sample of 18 isolates out of the 100 that showed high toxicity to *S. incertulas*. Nine of these 18 isolates (85, 105, 182, 222, 252, 261, 271, 364, and 898) appeared particularly interesting because, despite their high toxicity, the ELISA screening did not detect any δ-endotoxins of known toxicity to Lepidoptera (table 4). However, Western blotting with antiserum to Cry2A, which was not available at the time of the ELISA, revealed that seven of these isolates produced Cry2A (table 4), a protein with high toxicity to *S. incertulas* and *C. suppressalis* (Lee *et al.*, 1997). Subsequently, PCR analysis indicated that the other two isolates, 222 and 271, contain genes encoding δ-endotoxins of known toxicity to *S. incertulas* and *C. suppressalis* (*cry1Ab*, *cry1Ac*, *cry1B*, and *cry2A* in the case of isolate 222; and *cry1Ab* in that of isolate 271), although we do not know if these genes are expressed. Polymerase chain reaction analysis also revealed that each of the remaining nine isolates selected for detailed characterization contained several genes for δ-endotoxins known to be effective against *S. incertulas* and *C. suppressalis* (with the exception of isolate 93, in which we detected only one gene, *cry1Ac*).

Thus, the strategy that we designed to efficiently identify new genes for use against *S. incertulas* and *C. suppressalis* (i.e. first identifying isolates that were highly toxic but that did not produce toxins of known efficacy against *S. incertulas* and *C. suppressalis*, then focusing cloning efforts on those isolates), was ultimately not successful. Upon extended examination, we eventually found that all the promising isolates in fact contained genes for δ-endotoxins that we already knew to be toxic. Unexpectedly, DNA sequencing of PCR products from some of these isolates did reveal that we had amplified fragments of novel genes related to *cry1B* and *cry1F*. However, we do not have evidence that these genes are expressed or that their products are effective against *S. incertulas* or *C. suppressalis*. This will require isolation of full length clones of the genes, production of

Table 4. δ-endotoxin profiles of selected *Bacillus thuringiensis* isolates as determined by polymerase chain reaction, enzyme-linked immunosorbent assay, and Western blotting; and toxicity to *Chilo suppressalis*[a].

	B. thuringiensis isolate																	
	29	33	64	85	93	105	144	182	222	252	261	271	364	523	526	734	898	966
Gene(s) detected																		
cry1Aa	+		+	+		+					+		+	+	+			
cry1Ab	+	+	+			+	+	+	+	+	+	+	+	+	+	+		+
cry1Ac	+	+	+		+	+	+		+				+	+	+			+
cry1B	+		+	+		+		+	+		+		+	+	+	+		
cry1C		(+)					(+)											(+)
cry1D		+					+											(+)
cry1E		+					(+)											+
cry1F	(+)							+	+	+		(+)		+	+	+		
cry1I	(+)	+	+	+		+	+	+	+	+	+		+	+	+	+		+
cry2A	(+)	(+)	+	+		+	(+)	+	+	+	+	+	+	+	+		+	(+)
cry9C	+							+	+			+				+		
Protein(s) detected[b]																		
CryIA	+	+	+		+													
CryIB							+							+	+	+		
CryIC		+																+
CrylD																		+
Cry2A		+	+	+		+				+	+		+	+	+		+	
Cry3A	+	+					+					+						
Unknown δ-endotoxin(s)				+				+	+	+							+	
Toxicity[c] to *C. suppressalis*	160 ± 160	10 ± 7.3	0.10 ± 0.07	6.4 ± 6.1	1.1 ± 0.47	0.60 ± 0.16	1.6 ± 1.5	5.9 ± 3.1	100 ± 98	6.0 ± 2.4	3.4 ± 1.2	12 ± 8.9	1.4 ± 0.92	3.5 ± 3.5	0.19 ± 0.09	4.4 ± 2.5	37 ± 25	1.2 ± 0.59

[a]+, PCR product of expected size or protein detected; (+) faint PCR product of expected size. Blank cells indicate that no PCR products of expect size or proteins were detected.

[b]Cry1 and Cry3 proteins detected by ELISA. Cry2A proteins detected by Western blotting. Cry1E and CrylH were not detected in any of the isolates. The 'unknown δ-endotoxins' were detected by SDS-PAGE but did not cross-react with antisera.

[c]LC_{50} ± SE, μg/ml artificial diet. n = 4 bioassay dates.

purified proteins in an expression system, and insect bioassays.

Several developments since the initiation of this project have reduced the importance of cloning additional δ-endotoxin genes for use against *S. incertulas* and *C. suppressalis*. First, through bioassays with purified toxins, we have determined that numerous known toxins (Cry1Aa, Cry1Ab, Cry1Ac, Cry1C, Cry1D, Cry1E, Cry1F, and Cry 2A) are effective against these pests, at levels that can be achieved in transgenic plants (Lee *et al.*, 1997; Aguda *et al.*, unpublished data). In particular, the LC_{50}s of these toxins to *S. incertulas* are extremely low, in the range of 0.01–1 ng/ml artificial diet. Second, it was determined that some of these toxins bind to different brush border membrane receptors (Lee *et al.*, 1997). For example, in both *S. incertulas* and *C. suppressalis* Cry1Aa and Cry1Ac recognize the same binding site, but one that is different from those recognized by Cry1C and Cry2A. This indicates that receptor mutations conferring resistance to Cry1Aa and Cry1Ac will not affect susceptibility to Cry1C and Cry2A. Thus, δ-endotoxin combinations that might produce more durable resistance to stem borers, e.g. Cry1Ac with Cry1C, can be achieved with genes that are already available. Third, Gould *et al.* (1992) found that some insect mutations conferring resistance to δ-endotoxins are not receptor-based, and may confer cross resistance to toxins that are not closely related and/or do not recognize the same brush border membrane receptors. This finding indicates that it may be more successful to combine δ-endotoxins with unrelated toxins, having different modes of action, than to combine δ-endotoxins alone. Ultimately, our collection of *B. thuringiensis* isolates may be most useful as a source of novel insecticidal proteins unrelated to δ-endotoxins, such as the recently discovered vegetative insecticidal proteins or VIPs (Estruch *et al.*, 1996).

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