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Development of *Bacillus thuringiensis* CryIC Resistance by *Spodoptera exigua* (Hübner) (Lepidoptera: Noctuidae)†

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Selection of resistance in *Spodoptera exigua* (Hübner) to an HD-1 spore-crystal mixture, CryIC (HD-133) inclusion bodies, and trypsinized toxin from *Bacillus thuringiensis* subsp. *aizawai* and *B. thuringiensis* subsp. *entomocidus* was attempted by using laboratory bioassays. No resistance to the HD-1 spore-crystal mixture could be achieved after 20 generations of selection. Significant levels of resistance (11-fold) to CryIC inclusion bodies expressed in *Escherichia coli* were observed after seven generations. Subsequent selection of the CryIC-resistant population with trypsinized CryIC toxin resulted, after 21 generations of CryIC selection, in a population of *S. exigua* that exhibited only 8% mortality at the highest toxin concentration tested (320 μg/g), whereas the 50% lethal concentration was 4.30 μg/g for the susceptible colony. Insects resistant to CryIC toxin from HD-133 also were resistant to trypsinized CryIA(b), CryIC from *B. thuringiensis* subsp. *entomocidus*, CryIE-CryIC fusion protein (G27), CryIH, and CryIIA. In vitro binding experiments with brush border membrane vesicles showed a twofold decrease in maximum CryIC binding, a fivefold difference in K_d, and no difference in the concentration of binding sites for the CryIC-resistant insects compared with those for the susceptible insects. Resistance to CryIC was significantly reduced by the addition of HD-1 spores. Resistance to the CryIC toxin was still observed 12 generations after CryIC selection was removed. These results suggest that, in *S. exigua*, resistance to a single protein is more likely to occur than resistance to spore-crystal mixtures and that once resistance occurs, insects will be resistant to many other Cry proteins. These results have important implications for devising *S. exigua* resistance management strategies in the field.

Use of microbial insecticides based on *Bacillus thuringiensis* is expected to increase considerably in the future because of escalating environmental concerns over the use of synthetic insecticides. Resistance to *B. thuringiensis* in the field is a major concern associated with increased usage (see Tabashnik [28] for a review). *Spodoptera exigua* (Hübner) (Lepidoptera: Noctuidae) is a cosmopolitan pest on many economically important crops. Historically, formulations of *B. thuringiensis* subsp. *kurstaki* HD-1 applied at recommended field rates usually were not efficacious against *S. exigua* (32). Formulations of HD-1 typically contain CryIA(a), CryIA(b), CryIA(c), and CryIIA proteins (terminology after Höfte and Whiteley [9]), all of which have relatively low levels of toxicity to *S. exigua* (15, 17). Therefore, high selection pressure for physiological resistance would not be expected to occur against *S. exigua*. Products containing CryIC (the most active *B. thuringiensis* δ-endotoxin against *Spodoptera* spp.) have recently been developed and targeted for commercial use against *S. exigua* (9, 13, 15, 35). Xentari (Abbott Laboratories, North Chicago, Ill.) is a *B. thuringiensis* subsp. *aizawai*-based product containing CryIC that is more than sevenfold more toxic against *S. exigua* than *B. thuringiensis* subsp. *kurstaki*-type products (e.g., Dipel 2X) (14). As the toxicity and use of new materials formulated from *B. thuringiensis* or transgenic organisms expressing *B. thuringiensis* proteins against *S. exigua* increase, concern for the development of resistance also should increase.

Lepidopteran resistance to *B. thuringiensis* has been known since 1985, but only in a few taxonomic families (28). Resistance to *B. thuringiensis* in *Plodia interpunctella* (Hübner) (Pyralidae) and *Plutella xylostella* (L.) (Plutellidae) has been associated with a reduction in toxin binding (6, 34). Nonetheless, these insects were susceptible to other *B. thuringiensis* toxins (31, 34). Gould et al. (8) reported broad-spectrum resistance to *B. thuringiensis* toxins in *Heliothis virescens* (F.) (Noctuidae). In this insect, resistance to CryIA(c) conferred resistance to CryIA(a), CryIA(b), CryIB, CryIC, and CryIIA. Furthermore, there were no significant changes in toxin binding. Recently, Estada and Ferre (5) reported that a strain of *Trichoplusia ni* (Hübner) (Noctuidae) was resistant to CryIA(b) but was still susceptible to CryIA(c), even though both proteins seemed to share the same high-affinity binding site. Clearly, resistance to Cry proteins cannot be generalized, and additional studies of resistance are needed.

The objective of our studies was to determine if resistance to *B. thuringiensis* could be selected for in *S. exigua* by using spore-crystal mixtures and a single δ-endotoxin. *S. exigua* is in the same taxonomic family (Noctuidae) as *H. virescens* and *T. ni*, so the results from our study can be used to increase our understanding of resistance within a taxonomic family. Information on the likelihood and rate of resistance development, the possibility of cross-resistance, and the rate of reversion to susceptible status after selection pressure has been removed will allow us to develop specific resistance management strategies to reduce the threat of *B. thuringiensis* resistance in *S. exigua*.

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We report here that high levels of resistance to CryIC by *S. exigua* have been achieved in a laboratory experimental system. Resistance cannot be totally explained by toxin binding. Resistance to CryIC results in broad-spectrum resistance to all other *B. thuringiensis* toxins tested. When selection pressure with CryIC is removed, resistance to CryIC is stable for at least 10 generations. Additionally, purified spores from HD-1 act in synergy with CryIC toxin against resistant *S. exigua.*

MATERIALS AND METHODS

Insect colony. An *S. exigua* colony was established at Auburn University with insects collected from cotton prior to the initiation of the study. An additional 10 to 20 *S. exigua* egg masses were collected in 1991 from the Wiregrass Substation, Headland, Henry County, Ala, and were introduced into the laboratory colony just before our study was begun. The colony was reared on an artificial diet (4) at 28 ± 1°C with a photoperiod of 16:8 h (light:dark).

Bacterial strains, plasmids, and culturing. *B. thuringiensis* subsp. *kurstaki* HD-1 was obtained from L. Masson, National Research Council, Ottawa, Ontario, Canada. This strain contains all three *cry*IA genes associated with HD-1 (15). *Escherichia coli* strains containing the *cry*IA(b) gene from *B. thuringiensis* subsp. *kurstaki* HD-1 and the *cry*IC gene from *B. thuringiensis* subsp. *aizawai* HD-133 were obtained from L. Masson, National Research Council, Canada. The *cry*IC gene from *B. thuringiensis* subsp. *entomocidus* 60.5 was inserted into the *Bacillus-E. coli* shuttle vector pHT3101 and was transformed into an acrystalliferous strain of HD-1 [HD-1 (Cry^-)] (10). Trypsinized CryIC from *B. thuringiensis* subsp. *entomocidus* 60.5 and a trypsinized CryIE-CryIC fusion protein (G27) were obtained as described by Bosch et al. (3). Trypsinized CryIH was obtained from Plant Genetic Systems, Ghent, Belgium. CryIIA inclusion bodies were obtained from an NRD-12 *cry*IIA gene expressed in *E. coli* BL21(λDE3) (17).

Cultures of HD-1 were grown in a glucose, yeast, and salts medium (16). All *E. coli* strains containing *cry* genes were grown in Terrific broth in the presence of ampicillin, and inclusion bodies were purified (17). The HD-1 (Cry^-) strain containing the *cry*IC gene from *B. thuringiensis* subsp. *entomocidus* was grown in nutrient broth in the presence of erythromycin, and inclusion bodies were purified (17).

Protein concentrations for CryIC (38.14 mg/ml) and CryIIA (86.65 mg/ml) inclusion bodies were determined by the Micro BCA Protein Assay (Pierce Chemical Co., Rockford, Ill.). The purities of the protoxins were estimated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. For toxin purification, CryIA(b) and CryIC inclusion bodies were digested without previous solubilization by using 50 mM CAPs buffer (pH 10.5) containing 1 mg of bovine pancreas trypsin (Sigma Chemical Co., St. Louis, Mo.) per ml. Trypsinized toxins were purified and isolated by high-pressure liquid chromatography (22).

Spore purification. Purification of HD-1 spores from sporulated cultures was performed as described by Moar et al. (16). Spore preparations were estimated to contain fewer than 1.0% bipyramidal crystals by volume as observed under the stereomicroscope (a minimum of 1,000 bipyramidal crystals were examined).

Insect toxicity assays. Initial bioassays were conducted to determine the relative sensitivity of *S. exigua* to HD-1 spores-crystals and CryIC inclusion bodies. Seven to eight concentrations were assayed against neonate *S. exigua* by diet incorporation assays. Toxin was added to 16 ml of artificial diet (4), and the mixture was poured into a 24-well bioassay tray. One or two neonates were loaded per well. Trays were placed at 28 ± 1°C with a photoperiod of 16:8 h (light:dark). Mortality was recorded after 7 days. Mortality of controls was <10%. Assays were replicated three to four times. Data were analyzed by probit analysis (25).

Large-scale bioassays similar to those described above were conducted for the selection experiments. Surviving individuals from concentrations representing the 5 to 10% lethal concentration (LC_{5-10}) were removed after 5 days on the treated diet and were transferred to an untreated diet to complete development or for use as fifth instars for binding studies (CryIC toxin population only). Every subsequent generation was selected by using either the same or a twofold greater concentration compared with that used with the previous generation. *S. exigua* populations resistant to CryIC inclusion bodies were divided into two groups for subsequent selection with CryIC inclusion bodies and trypsinized protein (subsequently referred to as toxin) beginning at generation 14. Selection with CryIC inclusion bodies was continued until generation 16. Selection with CryIC toxin was maintained exclusively at 320 μg of toxin per g of diet beginning with generation 17. After bioassays determined that the *S. exigua* population resistant to HD-133 CryIC toxin was also resistant to the CryIC toxin from *B. thuringiensis* subsp. *entomocidus* 60.5, populations were selected exclusively with *B. thuringiensis* subsp. *entomocidus* 60.5 beginning at generation 32 because of the increased level of expression and ease of extracting the CryIC from *B. thuringiensis* subsp. *entomocidus*.

At selected generations, susceptible and resistant larvae were bioassayed concurrently to determine the levels of resistance to HD-1, CryIC inclusion bodies, or CryIC toxin. When the reversion (see section on stability of resistance) population was available, susceptible, resistant, and reversion populations were bioassayed with the CryIC toxin. Five concentrations were typically used for susceptible insects, whereas one to nine concentrations were used for resistant and reversion insects. At least three replicates were performed per resistant generation when larvae from all three populations were available; however, toxicity data from one to two replicates also were included for several generations. Otherwise, bioassays were performed as discussed above. Resistance ratios (RRs) were calculated between resistant and susceptible populations or reversion and susceptible populations. The RR was defined as the LC_{50} for the resistant or reversion population divided by the LC_{50} for the susceptible population. When the highest concentration tested for the resistant or reversion colony did not result in at least 50% mortality, the RR was calculated as if the LC_{50} for the resistant or reversion population equaled the highest concentration tested divided by the LC_{50} for the susceptible population.

Bioassays conducted with other Cry toxins (cross-resistance bioassays) usually were performed with two to six and one to six concentrations for susceptible and CryIC-resistant *S. exigua*, respectively. Bioassays were performed concurrently with both populations and were replicated only two or three times because of limited quantities of material and the relatively high concentrations required.

Joint-action bioassays with single concentrations of HD-133 CryIC toxin, CryIIA, and HD-1 spores were conducted concurrently with resistant insects from generation 35 and susceptible insects. Bioassays were replicated three times. Joint-action bioassay data were analyzed by a χ^2 test (23).

Preparation of BBMVs and binding assays. Full-length guts from fifth instar susceptible or CryIC-resistant *S. exigua* larvae were removed in ice-cold MET buffer (17 mM Tris HCl [pH 7.6], 5 mM EGTA [ethylene glycol-bis(*p*-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid], 300 mM mannitol) and were stored at −75°C. Gut tissue was thawed, and brush border membrane vesicles (BBMVs) were prepared as described by Luo and Adang (11). CryIC toxin was purified and iodinated with $Na^{125}I$ (11). The specific activity of CryIC was 11 μCi/μg of input toxin. Two separate binding experiments were performed with tissue from resistant generations 24 and 27, representing RRs of 100 and >500, respectively. Binding assays were performed by the method of Garczynski et al. (7). In the competition experiments, the reaction mixtures contained 80 and 100 μg of BBMV proteins per ml from CryIC-resistant and susceptible *S. exigua*, respectively, and 0.1 nM ^{125}I-labeled CryIC toxin, plus increasing concentrations of unlabeled toxins. Data were analyzed by the LIGAND computer program (19).

Stability of resistance to CryIC. A new population of *S. exigua* larvae was established from the CryIC-resistant survivors of generation 29 on CryIC at 320 μg of toxin per g of diet. These insects were reared on untreated diet as described above. Neonate larvae (reversion 1) from the adult survivors of generation 29 were placed on untreated diet and grown to pupation. This procedure was repeated for 12 generations. Bioassays were conducted at selected generations of the reversion population to determine levels of resistance to CryIC.

TABLE 1. *S. exigua* resistance to the HD-1 spore-crystal mixture of *B. thuringiensis*

Generation[a]	n^b	LC_{50} (95% FL)[c]	Slope (mean ± SE)	RR[d]
10	157	144 (102–190)	1.91 ± 0.29	1.98
Susc.	129	73 (51–94)	2.65 ± 0.48	
11	187	98 (7–306)	0.95 ± 0.28	2.22
Susc.	190	44[e]	0.92 ± 0.46	
20	51	254 (161–446)	2.19 ± 0.54	1.09
Susc.	88	232[e]	1.76 ± 0.58	

[a] Generation of *S. exigua* continuously selected with HD-1. Susc., susceptible.
[b] Total number of insects tested (one replicate with three to six concentrations).
[c] LC_{50}s are in micrograms of HD-1 spore-crystal mixture per gram of diet. FL, fiducial limits.
[d] LC_{50} for the selected generation divided by the LC_{50} for the susceptible colony.
[e] Fiducial limits could not be generated.

RESULTS

Selection for resistance. RRs of *S. exigua* to the HD-1 strain ranged from 1.09 to 2.22 for selected generations 20 and 11, respectively (Table 1). These bioassays were conducted on generations that were continuously selected with the HD-1 spore-crystal mixture. All eggs laid by adults from generation 22 were sterile, so the study was discontinued.

Elevenfold resistance to the *E. coli*-expressed HD-133 CryIC

TABLE 2. *S. exigua* resistance to the CryIC toxin of *B. thuringiensis*

Generation[a]	N^b	LC_{50} (95% FL)[c]	Slope (mean ± SE)	RR[d]
14	662	41.2 (8.3–416)	0.70 ± 0.18	7
Susc.	320	5.9 (1.0–18.8)	1.07 ± 0.23	
17	376	9.0 (4.0–15.3)	2.00 ± 0.45	1.8
Susc.	391	4.9 (0.7–20.3)	1.76 ± 0.56	
22	153	209 (111–1612)	0.91 ± 0.29	54
Susc.	187	3.9 (3.0–5.4)	1.76 ± 0.22	
23	755	165 (108–293)	0.75 ± 0.08	86
Susc.	681	1.92 (0.8–3.6)[e]	1.35 ± 0.25	
25	569	544 (244–1963)	0.57 ± 0.08	850
Susc.	411	0.6 (0.3–1.0)	0.92 ± 0.11	
27	326	21% mortality at 240 μg/g	0.22 ± 0.32	(49)
Susc.	362	4.9 (3.9–6.4)	1.52 ± 0.14	
28	82	29% mortality at 640 μg/g	0.63 ± 0.42	(291)
Susc.	348	2.2 (0.7–4.5)	1.03 ± 0.21	
29	205	25% mortality at 320 μg/g	0.59 ± 0.40	(51)
Susc.	717	6.2 (5.3–7.4)	1.50 ± 0.09	
34	69	53% mortality at 320 μg/g	ND[e]	(330)
Susc.	477	1.0 (0.7–1.2)	2.12 ± 0.22	
35	178	8% mortality at 320 μg/g	ND	(75)
Susc.	515	4.3 (3.8–4.9)	2.36 ± 0.18	

[a] Generation of *S. exigua* continuously selected with CryIC toxin beginning with generation 14. Susc., susceptible.
[b] Total number of insects tested (one to five replicates with one to nine concentrations).
[c] LC_{50}s are in micrograms of CryIC protoxin per gram of diet or percent mortality at the highest concentration tested. FL, fiducial limits.
[d] LC_{50} for the selected generation divided by the LC_{50} for the susceptible colony. Numbers in parentheses represent RRs when LC_{50} was assumed to be at the highest concentration tested.
[e] ND, not determined.

TABLE 3. Resistance of CryIC-resistant *S. exigua* to *B. thuringiensis* CryIC inclusion bodies expressed in *E. coli*

Generation[a]	n^b	LC_{50} (95% FL)[c]	Slope (mean ± SE)	RR[d]
22	320	293 (161–653)	0.84 ± 0.13	11
Susc.	245	27 (13–51)	1.19 ± 0.23	
23	694	493 (292–1034)	0.64 ± 0.07	12
Susc.	675	40 (33–49)	1.37 ± 0.09	

[a] Generation of *S. exigua* continuously selected with CryIC inclusion bodies for generations 1 to 14 and then continuously selected with CryIC toxin. Susc., susceptible.
[b] Total number of insects tested (one to four replicates with four to nine concentrations).
[c] LC_{50}s are in micrograms of CryIC inclusion bodies per gram of diet. FL, fiducial limits.
[d] LC_{50} for the selected generation divided by the LC_{50} for the susceptible colony.

inclusion bodies was achieved after seven generations (LC_{50} for susceptible *S. exigua*, 9.2 μg/g; LC_{50} for resistant *S. exigua*, 100 μg/g). Interestingly, although selection continued, no consistent increase in resistance was observed (data not shown).

Initial bioassays with CryIC toxin conducted with generation 14 of CryIC inclusion body-resistant *S. exigua* showed an RR of 7 (Table 2). However, continuous selection with CryIC toxin resulted in a considerable decrease in mortality beginning with generation 22 (RR = 54). Increased resistance was observed again at generation 25 (RR = 850). Because of the limited availability of CryIC toxin and the large concentrations required to establish LC_{50}s with this highly resistant population, only one concentration of CryIC toxin was used for bioassays against the CryIC-resistant population beginning with generation 27. After 35 generations, only 8% mortality was observed with the CryIC-resistant population at 320 μg of toxin per g of diet, whereas the LC_{50} for susceptible *S. exigua* was 4.3 μg of CryIC toxin per g of diet. Bioassays also were conducted with HD-133 CryIC inclusion bodies against CryIC-resistant larvae from generations 22 and 23. Even though these populations were 54- and 86-fold more resistant to CryIC toxin, respectively, they were only 11- and 12-fold more resistant to the HD-133 CryIC inclusion bodies, respectively (Table 3).

CryIC-resistant *S. exigua* insects were resistant to all other Cry proteins tested, including CryIA(b), CryIC from strain 60.5 of *B. thuringiensis* subsp. *entomocidus*, G27, CryIH, and CryIA (Tables 4 and 5). However, the RRs presented in Tables 4 and 5 are not valid in most cases because (i) mortality of the CryIC resistant insects was <50% at the highest concentration tested and (ii) limited quantities of the available Cry toxins did not allow the generation of reliable LC_{50}s of several of the Cry toxins even for susceptible insects. Because HD-133 CryIC-resistant *S. exigua* also was resistant to CryIC from *B. thuringiensis* subsp. *entomocidus* 60.5, this toxin replaced the HD-133 CryIC toxin for selection bioassays beginning with generation 29.

Binding. Binding experiments were performed with BBMVs isolated from resistant generations 24 and 27 (representing RRs of 100 and >500, respectively) and parallel control populations. The results from both resistant samples were nearly identical, and the data were pooled for analyses. Binding of CryIC to BBMVs was reduced to about 50% of the normal level in both CryIC-resistant populations. At concentrations of about 150 μg of vesicle protein per ml, binding in the resistant strain was 7%, compared with 13% in the susceptible strain (Fig. 1A). Figure 1B shows the results of homologous competition experiments in which the amounts of ^{125}I-labeled CryIC and BBMVs were kept constant and the amount of unlabeled CryIC was increased. The curve for the resistant insects was shifted to the right, indicating a decrease in the affinity of the binding sites for CryIC. Analyses by LIGAND estimated a K_d of 1.5 nM for the susceptible larvae and a K_d of 8.5 nM for the BBMVs from resistant insects. The concentration of high-affinity binding sites does not change, as indicated by similar concentrations of binding sites (B_{max}). Visual inspection of the portion of the curves in Fig. 1B showing data for large excess unlabeled toxin concentrations reveals a difference in binding not subject to analysis by LIGAND. Unlabeled CryIC at 100 nM displaced 87% of the ^{125}I-labeled CryIC in susceptible insects but only 62% in the resistant insects. Together, the data from the two types of binding experiments indicate that CryIC-resistant insects have (i) an overall twofold decrease in total CryIC binding, (ii) a fivefold decrease in the affinity of CryIC for the high-affinity site, (iii) an increase in nonsaturable CryIC binding, and (iv) unchanged binding site concentration.

Joint action. The addition of HD-1 spores (at a concentration causing only 4 to 6% mortality alone against resistant or susceptible insects) resulted in a significant ($P < 0.05$) synergistic interaction with either CryIC or CryIIA (Table 6). Synergism was observed for both CryIC-resistant and CryIC-susceptible *S. exigua*.

TABLE 4. Cross-resistance of CryIC-resistant *S. exigua* to other Cry proteins from *B. thuringiensis*

Generation[a]	Toxin[b]	n[c]	LC_{50} (95% FL)[d]	Slope (mean ± SE)	RR[e]
20	CryIA(b)	92	ND[f]	ND	(20)
Susc.		86	25.3 (7.2–41.7)	1.70 ± 0.47	
22	CryIA(b)	96	5,866[g]	0.46 ± 0.37	93
Susc.		96	63.2 (39.3–112)	1.37 ± 0.34	
34	CryIIA	258	10,731[g]	1.15 ± 0.40	73
Susc.		282	147 (4.9–471)	0.86 ± 0.22	
34	CryIH	53	51% mortality at 80 μg/g		12
Susc.		409	6.6 (3.8–8.9)	2.93 ± 0.45	

[a] Generation of *S. exigua* selected with CryIC toxin beginning with generation 14. Susc., susceptible.
[b] All proteins used were toxins except CryIIA (protoxin).
[c] Total number of insects tested (one to three replicates with one to five concentrations).
[d] LC_{50}s are in micrograms of protein per gram of diet or percent mortality at highest concentration tested. FL, fiducial limits.
[e] LC_{50} for the selected generation divided by the LC_{50} for the susceptible colony. Numbers in parentheses represent the RR when the LC_{50} was assumed to be at the highest concentration tested.
[f] ND, not determined. Random 20 to 25% mortality among all concentrations tested; highest concentration, 500 μg of toxin per g of diet.
[g] Fiducial limits could not be generated.

Stability of resistance. CryIC-resistant *S. exigua* insects were selected from generation 29 and were grown on the untreated diet for subsequent generations. Significant resistance (RR > 10) was observed after 12 generations of culture on untreated diet (Table 7). Because the insects from the 12th generation of culture on untreated diet exhibited resistance (less than 50% mortality) at the highest concentration tested (320 μg of toxin per g of diet), a comparison could not be made to determine if (or at what level) resistance had declined in comparison with that in the CryIC-resistant population. However, results with insects from generations 8 and 12 suggest that resistance was declining in those populations.

DISCUSSION

Resistance to the HD-1 spore-crystal mixture could not be accomplished after 20 generations of selection. Even though HD-1 is relatively nontoxic to *S. exigua*, we conducted this experiment because most *B. thuringiensis* products used against *S. exigua* in the field are based on a *B. thuringiensis* subsp. *kurstaki* strain (i.e., HD-1) and the only documented cases of resistance in the field have been with products containing a *B. thuringiensis* subsp. *kurstaki* strain (29). No development of resistance has been reported with *Spodoptera littoralis* after 8 and 10 generations of laboratory selection with HD-1 (Dipel 2X) and *B. thuringiensis* subsp. *entomocidus* spore-crystal mixtures, respectively (24, 26).

The HD-1 spore-crystal mixture used for our studies contains CryIA and CryIIA protoxins (packaged into crystals) as well as spores. Our results document that the RRs for the CryIC inclusion bodies (containing protoxin) were substantially lower than those for the CryIC toxin, suggesting that development of resistance to protoxin or HD-1 should be less likely to occur. Moar et al. (16) and Moar (13a) have shown that HD-1 spores are highly synergistic against *S. exigua* when the spores are combined with a mixture of CryIA-CryIIA proteins from HD-1 or the *E. coli*-expressed CryIA(b) from HD-1. Our results demonstrate that HD-1 spores and CryIIA also have synergistic activity against susceptible *S. exigua*, suggesting that another mode of action or mechanism is involved in the spore-toxin interaction. Therefore, another mutation or gene(s) may be needed to confer resistance to HD-1 by *S. exigua*. All recent reports of Lepidoptera that have evolved to be resistant to *B. thuringiensis* indicate that resistance occurred either in the field after repeated applications of *B. thuringiensis* or with field-collected insects selected in the laboratory to be

TABLE 5. Cross-resistance of HD-133 CryIC-resistant *S. exigua* to other CryIC-type toxins from *B. thuringiensis*

Population[a]	Toxin	n[b]	LC_{50} (95% FL)[c]	Slope (mean ± SE)	RR[d]
Res.	CryIC	205	25% mortality at 320 μg/g		(12.8)
Susc.	HD-133	717	6.2 (5.3–7.4)	1.50 ± 0.09	
Res.	CryIC	107	68.8 (68.8–ND[e])	0.5 ± 0.21	80
Susc.	60.5	98	0.86 (0.23–1.67)	1.06 ± 0.26	
Res.	G27	122	73.6[f]	0.38 ± 0.28	32
Susc.		113	2.28[f]	0.67 ± 0.27	

[a] Bioassays conducted with HD-133 CryIC-resistant *S. exigua* generation 29. Res., resistant; Susc., susceptible.
[b] Total number of insects tested (one to three replicates with one to five concentrations). HD-133 replicates were not bioassayed concurrently with 60.5 and G-27.
[c] LC_{50}s are in micrograms of toxin per gram of diet or percent mortality at the highest concentration tested. FL, fiducial limits.
[d] LC_{50} for the selected generation divided by the LC_{50} for the susceptible colony. Numbers in parentheses represent the RR when the LC_{50} was assumed to be at the highest concentration tested.
[e] ND, not determined.
[f] The highest concentration tested was 39 μg of G27 per g of diet. Fiducial limits could not be generated.

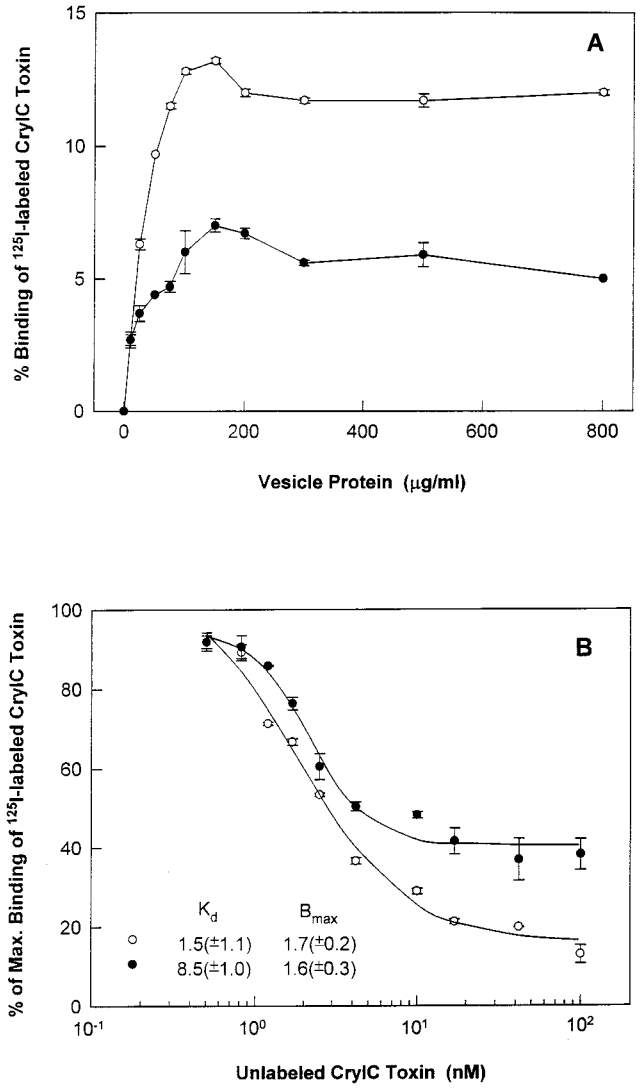

FIG. 1. CryIC binding to *S. exigua* BBMVs. (A) Binding of ^{125}I-labeled CryIC toxin as a function of vesicle concentration. The indicated vesicle concentrations of a susceptible strain (○) or a resistant strain (●) were incubated with 0.1 nM ^{125}I-labeled CryIC toxins. (B) Competition of ^{125}I-labeled CryIC binding by unlabeled CryIC toxins. ^{125}I-labeled CryIC toxin (0.1 nM) was incubated with vesicles from susceptible (○) or resistant (●) strains in the presence of the indicated concentrations of unlabeled CryIC toxins. Binding is expressed as a percentage of the maximum amount of toxin bound upon incubation with labeled toxin alone. The binding parameters (mean ± standard error) estimated by LIGAND analysis are presented in the inset. K_d and B_{max} values are calculated from two homologous competition experiments, performed on two independently prepared batches of vesicles. K_d is expressed in nanomolar concentrations, and B_{max} is expressed in picomoles per milligram of vesicle protein. For both panels, each point is the mean of two binding experiments. Standard errors are shown by bars.

TABLE 6. Interactions of HD-1 spore with CryIC and CryIIA from *B. thuringiensis* against susceptible and CryIC-resistant *S. exigua*

Toxin	% Mortality[a]	
	Resistant[b]	Susceptible
Spore[c]	4 (0–22)	6 (0–12)
CryIC[d]	0 (0–0)	8 (5–15)
CryIIA[e]	2.5 (0–7)	17 (3–37)
Spore + CryIC[c,d]	45 (35–54)[f]	90 (86–94)[f]
Spore + CryIIA[c,e]	55 (50–62)[f]	90 (84–100)[f]

[a] Bioassays were replicated three times; values in parentheses represent ranges. See footnotes *b* to *d* for concentrations.
[b] Resistant were insects taken from generation 35.
[c] A total of 100 μg of HD-1 spore per g of diet.
[d] A total of 1.0 and 20 μg of CryIC toxin per g of diet for susceptible and resistant insects, respectively.
[e] A total of 500 μg of CryIIA protoxin per g of diet.
[f] Synergistic activity ($P < 0.05$).

resistant to *B. thuringiensis*. Therefore, our inability to develop resistance to HD-1 still may be due to limited genetic heterogeneity (often associated with laboratory colonies which are small relative to field populations) or some other field-related factor. Given the fact that we did achieve high levels of resistance with the CryIC toxin in *S. exigua*, this seems surprising.

S. exigua can evolve to be resistant to purified CryIC inclusion bodies (containing protoxin) as well as toxin, although the RRs for the inclusion bodies were generally low. These results are the fourth example of resistance to Cry proteins by insect species belonging to the family Noctuidae. *H. virescens* was selected in the laboratory for resistance to inclusion bodies containing CryIA(c) protoxin (8, 27). Estada and Ferre (5) reported selection of CryIA(b) resistance by *T. ni* in the laboratory. More recently, Müller-Cohn et al. (18) reported the selection of CryIC protoxin resistance by *S. littoralis* in the laboratory. However, parameters associated with resistance such as cross-resistance to other Cry toxins and toxin binding (to BBMVs) are not consistent among all four insect species. Resistance parameters in *S. exigua* are similar to those reported by Gould et al. (8) for *H. virescens* in that both species are cross-resistant to all other Cry toxins tested. *S. littoralis* insects resistant to CryIC also were resistant to CryID and CryIE. However, CryIA(b)-resistant *T. ni* were susceptible to CryIA(c). Furthermore, the dramatic decrease in receptor binding observed for CryI-resistant *P. interpunctella* and *P. xylostella* has not been observed in *H. virescens* and *S. exigua* (6, 8, 12, 34). Although Gould et al. (8) reported no difference in binding affinity between susceptible and CryIA(c)-resistant *H. virescens*, Macintosh et al. (12) did report a two- to fourfold decrease in binding affinity for the same species. Although the results from those two reports are still similar compared with those for *P. interpunctella* and *P. xylostella*, our results would more closely match the observations of Macintosh et al. (12). Estada and Ferre (5) report that, on the basis of binding site models, CryIA(b) and CryIA(c) should have bound to the same high-affinity binding site, even though resistance to CryIA(b) did not confer resistance to CryIA(c).

Binding studies with CryIC toxin indicated changes in the brush border membrane of *S. exigua* larvae which showed 100- to 500-fold resistance to CryIC toxin. Of interest is that CryIC-resistant *S. exigua* insects also were resistant to G27, a fusion protein in which domain III of CryIC is transferred to CryIE (which is nontoxic to *S. exigua*). In *S. exigua*, CryIE and G27 bind to a receptor different from that to which CryIC binds (3). Additionally, BBMVs from *S. exigua* have been shown to contain multiple Cry binding proteins (20). The proteins (molecular mass) that bind CryIC (40 kDa) were different from the proteins that bind CryIA(b) (180 and 200 kDa) and CryIA(c) (110 and 130 kDa). Therefore, while changes in binding correlate with CryIC resistance, the changes are more complex than a simple reduction in binding affinity.

There is a similarity between the characteristics of CryIC binding in resistant *S. exigua* and susceptible *H. virescens*. CryIC has a relatively low level of toxicity to *H. virescens* larvae

TABLE 7. Stability of *S. exigua* resistance to the CryIC toxin of *B. thuringiensis*

Generation[a]	n[b]	LC_{50} (95% FL)[c]	Slope (mean ± SE)	RR[d]
7	68	76% mortality at 320 μg/g	ND[e]	ND
Susc.	477	1.0 (0.7–1.2)	2.12 ± 0.22	
34 (resistant)	69	53% mortality at 320 μg/g	ND	(330)
8	211	252 (150–1037)	1.92 ± 0.52	23
Susc.	135	11.2 (5.1–158)	2.37 ± 0.65	
35 (resistant)	80	8% mortality at 320 μg/g[f]	ND	(29)
12	20	30% at 320 μg/g	ND	ND
3 (resistant)[g]	22	0% at 320 μg/g	ND	ND

[a] Insect generations were reared on an untreated diet after being taken from generation 29 of the CryIC toxin-resistant colony. Susc., susceptible.
[b] Total number of insects tested (one to three replicates with one to eight concentrations).
[c] LC_{50}s are in micrograms of CryIC toxin per gram of diet or percent mortality at the highest concentration tested. FL, fiducial limits.
[d] LC_{50} for the selected generation divided by the LC_{50} for the susceptible colony. Numbers in parentheses represent the RRs when the LC_{50} was assumed to be at the highest concentration tested.
[e] ND, not determined.
[f] Not concurrent.
[g] Resistant colony from generation 10 of reversion colony.

(as observed for our CryIC-resistant larvae) (33). Similar to the present study, total binding to *H. virescens* BBMV is roughly 40% of that to susceptible species *Manduca sexta* and *S. littoralis* (33). A difference between our study and that of Van Rie et al. (33) is that we measured a K_d of 8.5 nM for resistant *S. exigua* and Van Rie et al. (33) reported a K_d of 22 nM for the high-affinity site in *H. virescens*. Therefore, differences in total binding of CryIC (specific and nonspecific) do not adequately explain differences in susceptibility.

Vesicles prepared from resistant insects had an increased level of nonspecific binding compared with those from susceptible insects. This is defined as the inability of unlabeled CryIC to compete with ^{125}I-labeled CryIC for binding sites. The relationship between nonspecific binding and toxicity is not known. Luo and Adang (11) observed an increase in nonspecific CryIC binding when CryIC had adhering subtoxin peptides. While Gould et al. (8) reported no significant differences in CryIA(b) binding to resistant *H. virescens* larvae, an inspection of their CryIA(b) binding curve to cross-resistant *H. virescens* BBMVs shows a twofold increase in nonspecific binding. If nonspecific binding interferes or competes with specific high-affinity binding and reduces pore formation, nonspecific binding could be a factor in resistance to toxins.

Explanations other than receptor binding have been implicated in resistance to *B. thuringiensis* in at least one other insect species. *P. interpunctella* insects that were resistant to *B. thuringiensis* subsp. *entomocidus* and CryIC had a fivefold reduction in proteolytic activity against a synthetic trypsin substrate, α-*N*-benzoyl-DL-arginine *p*-nitroanilide, and activated CryIA(c) and CryIC protoxins at a reduced rate (21). This result suggests that insects should be more resistant to protoxin than to toxin because of a decrease in proteolysis. Our results support just the opposite hypothesis. However, altered proteases that recognize other sites within the polypeptide could cleave critical areas of the protein, rendering it nontoxic.

Bai and Degheele (1) reported that insects which were more tolerant to *B. thuringiensis* subsp. *thuringiensis* crystals (such as *S. littoralis*) had reduced concentrations of regurgitated gut juice protein. They suggest that higher levels of proteinases may facilitate the digestion of the crystal proteins. Bai et al. (2) reported that *S. littoralis* gut juice contained only about 50% of total protease activity compared with that in the gut juice of *P. brassicae* and contained about six times less chymotrypsin-like activity and about five times less trypsin-like activity compared with those in the gut juice of *P. brassicae*. Therefore, *Spodoptera* spp. may be inherently more tolerant to Cry proteins because of their inability to digest Cry protoxins. Although one mechanism of resistance may be reduced proteolysis, other unknown mechanisms or explanations may exist.

Our recent experiments suggest that insects transferred from toxin-treated to untreated diet lose some of their resistance to Cry proteins. Because most *B. thuringiensis* resistance studies select for resistance of insects on a treated diet and then transfer larvae onto an untreated diet after about 5 days to continue development (and for extracting BBMVs), the differences observed in total and high-affinity binding in our study may be due to a relatively quick reversion to the normal physiological status. On-going studies will determine if binding parameters are different between insects selected on a treated diet for the entire larval period compared with the typical approach of selecting on a treated diet for only 5 days.

HD-1 spores have synergistic activity with CryIC and CryIIA against CryIC-resistant and susceptible *S. exigua*. Because only 8% mortality was observed for the CryIC-resistant population at 320 μg/g for generation 35 (a concentration 16-fold greater than that used in the present joint-action test), an increase in mortality to 45% (for the CryIC–HD-1 spore interaction) is probably of more interest than the increase in mortality to 90% for the susceptible population. HD-1 spores have been shown to act synergistically with Cry toxins against *S. exigua*, but spores from an HD-1 (Cry^-) strain do not (13a, 16). This suggests that compounds (or other mechanisms) other than Cry toxins liberated from inclusion bodies are involved and that selection for CryIC resistance does not select for concomitant resistance to the HD-1 spore.

Resistance to CryIC was observed even after 12 generations without selection, although the resistance ratio appears to be decreasing in comparison with that for *S. exigua* raised for consecutive generations with CryIC resistance selection. Our CryIC-resistant population was lost after generation 36 because of a viral epizootic. However, a population of reversion 10 was reselected with 320 μg of CryIC per g of diet. Most larvae survived, suggesting that resistance was easily reestablished, as has been documented for *P. interpunctella* (30). Müller-Cohn et al. (18) reported that fitness parameters such as pupal weight, sex ratio, and developmental time for CryIC-

resistant *S. littoralis* were the same as those for susceptible insects. Preliminary observations with our CryIC-resistant *S. exigua* population agree with those of Müller-Cohn et al. (18), although these observations have not yet been quantified. Low observable fitness costs therefore would increase the likelihood of resistance in the field.

S. exigua is a major pest of many crops and will probably be increasingly exposed to formulations of *B. thuringiensis* or transgenic plants that express *B. thuringiensis* toxin. Our research documents that resistance to an activated toxin may be more easily achieved than resistance to inclusion bodies containing protoxin, although continuous laboratory selection was required for at least seven generations. Furthermore, resistance to a spore-crystal mixture could not be achieved. Resistance to one toxin conferred cross-resistance to most, if not all, available Cry toxins. These results suggest that use of individual protoxins or toxins, especially as expressed in transgenic organisms, could induce resistance in field populations more readily than formulated materials containing multiple Cry proteins and spores. Strategies which exploit mechanisms other than changes in receptor binding will need to be pursued in order to continue the use of *B. thuringiensis*.

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