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Toxicity of *Bacillus thuringiensis* Spore and Crystal Protein to Resistant Diamondback Moth (*Plutella xylostella*)

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A colony of *Plutella xylostella* from crucifer fields in Florida was used in mortality bioassays with HD-1 spore, CryIA(a), CryIA(b), CryIA(c), CryIB, CryIC, CryID, CryIE, or CryIIA. The data revealed high levels of field-evolved resistance to HD-1 spore and all CryIA protoxins and no resistance to CryIB, CryIC, or CryID. CryIE and CryIIA were essentially not toxic. When HD-1 spore was combined 1:1 with protoxin and fed to susceptible larvae, spore synergized the activity of CryIA and CryIC 5- to 8-fold and 1.7-fold, respectively, and did not synergize the mortality of CryIIA. When fed to Florida larvae, spore failed to synergize the activity of all three CryIA protoxins, synergized the activity of CryIC 5.3-fold, and did not synergize the mortality for CryIIA. Binding studies with CryIA(b), CryIB, and CryIC were performed to determine possible mechanisms of resistance. The two techniques used were (i) binding of biotinylated toxin to tissue sections of larval midguts and (ii) binding of biotinylated toxin to brush border membrane vesicles prepared from whole larvae. Both showed dramatically reduced binding of CryIA(b) in resistant larvae compared with that in susceptible larvae but no differences in binding of CryIB or CryIC.

The insecticidal activity of *Bacillus thuringiensis* in *Lepidoptera* has been attributed primarily to the crystalline inclusions produced during sporulation (15), although spores have been found to enhance crystal activity in certain species (23, 34). Because toxicity of each crystal protein can depend upon many midgut factors, such as high pH, proteolysis, and the presence of specific binding sites on the surface of microvilli, insecticidal activity is protein specific and should be determined empirically (15). For example, even if a crystal protein binds to midgut vesicle preparations, the protein itself can be nontoxic in diet feeding assays, as was seen for CryIA(c) in *Spodoptera frugiperda* (9). For many *B. thuringiensis* proteins that are toxic, binding rapidly induces histopathological changes (22) as a result of the formation of pores across the midgut epithelium (19). Within minutes of ingesting the crystals, larvae cease to feed and die over the next few days.

Despite the use of *B. thuringiensis* for 20 or so years, cases of field-evolved resistance are relatively few and are restricted to larvae of one species, the diamondback moth, *Plutella xylostella* (L.). Resistant populations have been recorded in the Philippines (17), Hawaii (45), Japan (11), and Florida (41). With the continued use of *B. thuringiensis*, however, resistance is also predicted to evolve in other insects, since repeated selection in the laboratory with either sporulated culture or purified toxin has produced resistant strains in other moth (10, 26, 28, 33, 43) and beetle (37) species.

Studies of resistance to *B. thuringiensis* subsp. *kurstaki* in *Plutella xylostella* have shown that resistance was generally unstable (11, 46), inherited as an autosomal recessive or incompletely recessive trait (11, 24, 50), and controlled primarily by one or few loci (11, 50). One case of stable resistance has occurred after a colony, which was derived from a moderately resistant field population from Hawaii, was selected for 21 of 40 generations in the laboratory (48). When binding activities of CryIA, CryIB, or CryIC in midgut vesicle preparations or tissue sections of susceptible and resistant larvae were compared, only CryIA exhibited reduced binding activity which correlated with the lack of CryIA toxicity in resistant larvae (4, 8, 24, 49). This also corroborated competition binding studies indicating that CryIA did not have common binding sites with CryIB and CryIC (8) and that there was little cross-resistance to non-CryIA toxins (8, 46, 49).

The previously cited studies on CryIA-resistant *Plutella xylostella* used colonies established from the Philippines and Hawaii. In this study, we report on the toxicological and biochemical basis of resistance in *Plutella xylostella* larvae collected from a region in Florida which has the highest documented levels of field resistance to *B. thuringiensis* subsp. *kurstaki* (41). To make our comparisons with Hawaiian (46, 47, 49) and Philippine colonies (8) as comprehensive as possible, we evaluated mortality to CryIA(a), CryIA(b), CryIA(c), CryIB, CryIC, CryID, CryIE, and CryIIA and examined binding activity of CryIA(b), CryIB, and CryIC for both susceptible and resistant larvae. Since there is as yet very little information on spore activity or its possible synergistic effects in susceptible and resistant *Plutella xylostella* larvae, we also included HD-1 spore and HD-1 spore combined 1:1 with CryIA, CryIC, or CryIIA in our mortality studies.

MATERIALS AND METHODS

HD-1 spore and insecticidal crystal proteins (ICPs). HD-1, an isolate of *B. thuringiensis* subsp. *kurstaki*, was obtained from Dipel 2X wettable powder (Abbott Laboratories, North Chicago, Ill.). Cultures were grown in glucose-yeast-salt medium, and spore was isolated by repeatedly extracting and rinsing the foam created by vigorous shaking (34). Spore preparations were determined to be at

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least 99% free of crystal toxins by compound light microscopy. Plating showed that 100 μg of HD-1 spore per ml was equivalent to 7×10^7 to 8×10^7 spores per ml.

All ICPs were produced as recombinant proteins expressed by *Escherichia coli*. Genes for CryIA(a), CryIA(b), CryIA(c), and CryIIA were obtained from *B. thuringiensis* subsp. *kurstaki* (35), the gene for CryIC was obtained from *B. thuringiensis* subsp. *aizawai* (gift from Luke Masson, National Research Council, Canada), the gene for CryIB was obtained from *B. thuringiensis* subsp. *entomocidus* HD-110 (6), the gene for CryID was obtained from *B. thuringiensis* subsp. *aizawai* HD-68 (14), and the gene for CryIE was obtained from *B. thuringiensis* subsp. *darmstadiensis* (53).

The first five proteins listed above were used in protoxin form for mortality bioassays because we wanted the transformation from protoxin to toxin to occur in vivo. Our supply of CryIB, CryID, and CryIE, however, was already in toxin form when received and was used as such in feeding assays. Previous data, however, have shown few differences in protoxin versus toxin activity in feeding assays using several species of moths (5, 53). For all in vitro studies, the protoxin was converted to toxin prior to use (13) because the components needed to process the protoxin to the toxin were not present in the assay. The concentration of protoxin was estimated by measuring total protein by the method of Bradford (2), with bovine serum albumin (BSA) as the standard, and then determining the percent toxin composition after proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (21). The concentrations of purified toxins were calculated from their optical densities at 280 nm.

Insects. The resistant colony was collected from Loxahatchee, Fla., in 1992 and, compared with the susceptible Geneva colony, was found to exhibit >1,500-fold levels of resistance to Javelin WG (6.4% active ingredient; lot no. 7300960 [Sandoz, Des Plaines, Ill.]), a commercial formulation of *B. thuringiensis* subsp. *kurstaki*, in the second generation after the colony was collected from the field (41). This initial level of resistance, however, was unstable and fell within three generations from the time the colony was brought in from the field to 300-fold higher than that of the susceptible Geneva colony. Resistance then stabilized at this level for as many as 19 generations in the absence of selection (50a). It was these larvae, i.e., which were in the stable phase of resistance, that were used for all experiments.

Loxahatchee larvae were maintained on rape plants (*Brassica napus* subsp. *oleifera*) in the greenhouse at 26 to 33°C, with a 16-h-light–8-h-dark photoperiod and 20 to 80% relative humidity. The susceptible colony was collected from Geneva, N.Y., in 1988 and has since been in continuous culture. Eggs produced from the Geneva colony were from larvae reared on an artificial diet (40), but for experiments, both Geneva and Loxahatchee larvae were reared on rape in the greenhouse in separate cages.

Toxicity bioassays. Mortality data for the Loxahatchee and Geneva colonies were generated by a cabbage leaf dip bioassay modified from Shelton et al. (41). For toxic compounds, six to eight concentrations from a 3.16× dilution series plus a control were prepared such that the mortality ranged from 0 to 100%. Where compounds lacked toxicity, the highest concentration tested was 100 or 200 μg/ml in the case of ICP tested with spore in a 1:1 mixture. The replicate for each day included Loxahatchee and Geneva larvae tested against ICP with and without spore. For spore-only tests, the replicate for each day included Loxahatchee and Geneva larvae. Five leaf discs (32-mm diameter) were dipped for 10 s per concentration, allowed to air dry, and inoculated with five larvae per leaf. Mortality was determined after 72 h at 27°C, and tests were replicated on at least two different days. Control mortality typically ranged from 0 to 2%.

Data analysis. Bioassay data from the different-day replicates were pooled and fitted to probit models by use of the POLO program (39). Estimates of the lethal concentration required to kill 50% of the larvae (LC_{50}) were obtained for spore, ICP, and spore-ICP (1:1) mixtures. To estimate the level of synergy, an expected LC_{50} value for the mixture was calculated by a model that assumed similar joint action of the components tested (44). The equation used was

$$LC_{50(ts)} = \left(\frac{0.5}{LC_{50(t)}} + \frac{0.5}{LC_{50(s)}} \right)^{-1} \quad (1)$$

where $LC_{50(ts)}$ was the expected LC_{50} of the toxin-spore mixture, $LC_{50(t)}$ was the observed LC_{50} for toxin, and $LC_{50(s)}$ was the observed LC_{50} for HD-1 spore. Effects were considered synergistic if the expected LC_{50} exceeded the upper limit of the 95% fiducial limit (95% FL) for the observed LC_{50} of the mixture. When probit lines could not be obtained, independent joint action was assumed, and observed and expected mortalities were compared by χ^2 analysis (29). Expected mortality for the mixture (E_{ts}) was calculated at 200 μg/ml as:

$$E_{ts} = O_t + O_s (1 - O_t) \quad (2)$$

where O_t and O_s were the observed mortalities at 100 μg/ml for toxin and spore, respectively. The formula

$$\chi^2 = (O_{ts} - E_{ts})^2 / E_{ts} \quad (3)$$

was used to calculate χ^2 from observed and expected mortalities of the mixture, and χ^2 was compared with tabular values at a *df* of 1. Effects were considered synergistic if the probability of obtaining a larger χ^2 value was ≤0.05.

Biotinylation of ICPs. Toxin samples were dialyzed against $NaHCO_3$ buffer (100 mM $NaHCO_3$, 150 mM NaCl [pH 9]). An appropriate amount of biotinyl-*N*-hydroxysuccinimide ester (BNHS; Amersham) was added to 1 mg of toxin. For CryIA(b) and CryIB, 40 μl of BNHS was used. This represents a molar reaction ratio (number of biotin molecules per ICP molecule used during the reaction) of 37.06. For CryIC, 120 μl of BNHS was used. The mixture was incubated for 1 h at 4°C with constant agitation. Toxin samples were then loaded onto a Sephadex G-25 column to separate biotinylated protein from free BNHS. The A_{260} and A_{280} values of all fractions were determined. Since free BNHS has a stronger A_{260} than A_{280}, it was possible to locate the fractions corresponding to the peak containing biotinylated ICP (B-ICP) from these measurements. The concentration of the fractions from this peak was determined from the A_{280} readings. There were no significant differences in toxicity on *Ostrinia nubilalis* larvae between native and biotinylated CryIA(b) [B-CryIA(b)] (6) or native and B-CryIC (data not shown).

In vitro binding of ICPs to midgut tissue sections. Last-instar *Plutella xylostella* larvae were held without food 2 h prior to dissection. Fixation, embedding, and sectioning of midguts and deparaffination and hydration of midgut sections were performed as described previously (6). Sections were incubated with blocking buffer (100 mM maleic acid, 150 mM NaCl, 1% blocking reagent [pH 7.6; Boehringer Mannheim catalog no. 1096176]) for 30 min to prevent nonspecific binding. Sections were then incubated with 300 μl of toxin solution [20 μg of B-CryIA(b), 10 μg of B-CryIB, or 10 μg of CryIC per ml of TST buffer for 30 min (TST buffer is 10 mM Tris, 150 mM NaCl, 0.1 mM thimerosal, and 0.1% Triton X-100 [pH 7.6]) for 30 min. Bound CryIA(b) and CryIB toxin were detected with a streptavidin-alkaline phosphatase conjugate (6). Detection of bound CryIC toxin was done with monoclonal antibody 4A2 and alkaline phosphatase-conjugated rabbit anti-mouse antibody (6). Sections were subsequently dried on a heating plate and covered with Entellan mounting medium (Merck).

Preparation of BBMVs. Last-instar *Plutella xylostella* larvae were held without food for 1 h and then frozen in liquid nitrogen. Twenty milliliters of MET buffer (300 mM mannitol, 5 mM EGTA, 17 mM Tris-HCl [pH 7.5]) was added to 900 frozen larvae of the Loxahatchee population [EGTA is ethylene glycol-bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid]. Thirty milliliters of MET buffer was added to 1,500 larvae of the Geneva population. Brush border membrane vesicles (BBMVs) were purified from these mixtures by the method of Wolfersberger et al. (55). The pellet resulting from the high-speed centrifugation step had two layers. The bottom layer had a whitish color and probably consisted largely of fatty substances. Only the upper, greenish layer was used for further purification. The protein concentration of vesicle preparations was determined by the method of Bradford (2) with BSA as the standard.

Binding assays of BBMVs. An aliquot of *Plutella xylostella* vesicles was thawed and centrifuged at 13,000 rpm (Heraeus Christ Biofuge A) at 4°C. The resulting pellet was resuspended in phosphate-buffered saline (PBS) containing 0.1% BSA to give a final concentration of 1 mg/ml. Vesicles were added to 20 ng of B-CryIA(b) or 10 ng of B-CryIC in PBS–0.1% BSA (total volume, 100 μl) and incubated at room temperature for 1 h. The mixture was centrifuged at 13,000 rpm (Heraeus Christ Biofuge A) at 4°C for 10 min, and the pellet was washed with 500 μl of PBS–0.1% BSA and finally resuspended in 30 μl of sample buffer mix. The suspension was incubated at 95°C for 10 min. Samples were then separated by SDS-PAGE (21) and blotted onto a nitrocellulose membrane. This membrane was incubated overnight in blocking buffer, washed with Tris-buffered saline (TBS; 20 mM Tris, 137 mM NaCl [pH 7.6]) containing 0.2% Tween 20, and incubated with streptavidin-horseradish peroxidase conjugate, diluted 1/1,000 in TBS containing 0.1% BSA. The membrane was washed for 10 min with TBS–0.2% Tween 20 and for a few minutes with tap water. Washes were repeated three times. Excess fluid was drained from the washed membrane, and equal volumes of detection solution 1 and detection solution 2 (Amersham RPN 2109) were mixed and applied to the membrane. Following a 1-min incubation period, excess detection solution was drained off, and the membrane was placed on a piece of Whatman paper for 1 min, wrapped in SaranWrap, and exposed for 30 s. Depending on the signal obtained, a second exposure was done.

RESULTS

Toxicity of HD-1 spore and ICPs. Leaf dip bioassays demonstrated that despite high levels of toxicity of CryIA(a), CryIA(b), and CryIA(c) to susceptible *Plutella xylostella* larvae (Table 1), these same protoxins were relatively nontoxic to resistant larvae (Table 2). The mortality of resistant larvae to the CryIA protoxins at the highest concentration tested (100 μg/ml) was less than 30% (Table 2), whereas LC_{50} values for susceptible larvae ranged from 0.3 to 1.1 μg/ml (Table 1). Estimating LC_{50} values of CryIA protoxins to be >250 μg/ml, the level of resistance exhibited would be minimally 200-fold higher than that of susceptible larvae. Despite such high levels of resistance to CryIA, there was little evidence for cross-resistance to other CryI proteins. For example, LC_{50} values

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TABLE 1. Toxicity of CryIA, CryIC, and CryIIA when present alone or in combination (1:1) with HD-1 spore on susceptible *Plutella xylostella* larvae

Material	Spore or protoxin alone		Protoxin + spore			
	LC_{50} (μg/ml)[a]	Slope ± SE	LC_{50} (μg/ml)	Slope ± SE	Expected LC_{50} (μg/ml)	Synergism ratio[b]
HD-1 spore	2.8 (2.1–3.4)	2.75 ± 0.37				
CryIA(a)	0.3 (0.2–0.3)	2.14 ± 0.29	0.06 (0.03–0.1)	1.46 ± 0.14	0.5	8.3[c]
CryIA(b)	0.6 (0.4–1.2)	1.27 ± 0.13	0.2 (0.08–0.6)	1.64 ± 0.19	1.0	5.0[c]
CryIA(c)	1.1 (0.5–2.5)	1.27 ± 0.13	0.3 (0.3–0.4)	2.28 ± 0.29	1.6	5.3[c]
CryIC	4.3 (3.3–5.7)	1.64 ± 0.16	1.9 (1.4–2.5)	1.77 ± 0.19	3.2	1.7[c]
CryIIA	777[d]	0.77 ± 0.32	4.3 (2.7–6.1)	1.24 ± 0.16	5.6	1.3

[a] Values in parentheses are 95% FL.
[b] For protoxin plus spore, the synergism ratio equals the expected LC_{50} divided by the observed LC_{50}.
[c] The effect of the combination was synergistic if the expected LC_{50} did not lie within the 95% FL of the observed LC_{50}.
[d] Ninety-five percent fiducial limits were not obtained as a result of low toxicity. Mortality at 100 μg/ml was 30%.

found for CryIB, CryIC, and CryID were at most 3.4-fold higher for resistant larvae than those found for susceptible larvae (Tables 1 to 3). Of the other ICPs tested, neither CryIE nor CryIIA was very toxic even to susceptible larvae (Tables 1 to 3).

Bioassays with HD-1 spore demonstrated that although spore was toxic to susceptible larvae (Table 1), it was relatively nontoxic to resistant larvae (Table 2). Therefore, in addition to exhibiting resistance to CryIA protoxin, Loxahatchee larvae also displayed about 61-fold resistance to HD-1 spore at the LC_{50}.

The effects of adding spore to ICP (1:1) on mortality appeared to depend on insect type and ICP used. For susceptible larvae, the highest level of synergism, 5- to 8.3-fold enhancement of mortality, was observed when spore was added to CryIA(a), CryIA(b), or CryIA(c) (Table 1). A low but significant level of synergism (1.7-fold) was found for CryIC, and no synergism (1.3-fold) was found for CryIIA (Table 1). In resistant larvae, no synergism was produced when spore was combined with CryIA(a), CryIA(b), CryIA(c), or CryIIA (Table 2). Synergy however, was observed (5.3-fold) when spore was combined with CryIC (Table 2). From these data, it appeared that spore could synergize only those ICPs which, when tested alone, already showed high levels of toxicity to the insect type being tested.

TABLE 2. Toxicity of CryIA, CryIC, and CryIIA when present alone or in combination (1:1) with HD-1 spore on resistant *Plutella xylostella* larvae

Material	% Mortality with spore or protoxin alone[a,b]	Protoxin + spore[a,c]		χ^2	$P > \chi^2$[d]
		% Mortality	Expected % mortality		
HD-1 spore[e]	26.0				
CryIA(a)	14.1	38.4	34.6	0.40	0.53
CryIA(b)	9.3	20.0	31.1	2.96	0.09
CryIA(c)	27.0	52.0	44.5	1.26	0.26
CryIC[f]	95.6	100	96.7		
CryIIA	32.0	54.0	48.3	0.32	0.57

[a] *N* ranges from 45 to 100.
[b] The concentration of spore or protoxin was 100 μg/ml.
[c] The concentration of protoxin + spore was 200 μg/ml.
[d] The effect of the combination was synergistic if the probability of obtaining a larger χ^2 value was ≤0.05.
[e] For HD-1 spore, the LC_{50} was 169.9 μg/ml, the 95% FL was 101.4 to 509.3, and the slope was 2.28 ± 0.32.
[f] The mortality of CryIC was too high to permit accurate calculation of a χ^2 value. Instead, LC_{50} values were compared. For CryIC, the LC_{50} was 14.6 μg/ml, the 95% FL was 8.7 to 39.5, and the slope was 1.23 ± 0.26. For CryIC plus spore, the LC_{50} was 5.1, the 95% FL was 2.3 to 26.1, and the slope was 0.95 ± 0.17. The expected LC_{50} for CryIC plus spore was 26.9, which lies outside the observed 95% FL and was 5.3 times greater than the observed LC_{50}. Therefore, the interaction of spore and CryIC was synergistic.

Binding of ICPs to tissue sections. Midgut tissue sections of 11 susceptible larvae and 26 resistant larvae were analyzed for in vitro binding of B-CryIA(b) and B-CryIB (Table 4). A clear difference in staining intensities was observed between both strains for B-CryIA(b) binding. Whereas more than half of the larvae from the resistant population did not bind B-CryIA(b), all larvae of the sensitive population did bind this ICP to some extent. In contrast, only minor differences in staining intensities were observed for B-CryIB binding. Six larvae of both populations were also tested for CryIC binding. Although this sample number was low, similar staining intensities were observed for both strains (data not shown). These data indicate that in the resistant population, the CryIA(b) receptor was altered whereas receptors for CryIB and CryIC were unchanged compared with that in the susceptible population.

Binding of ICPs to BBMVs. To corroborate results from experiments on tissue sections, we studied binding of B-ICPs to BBMVs prepared from whole larvae of both colonies. SDS-PAGE blots clearly showed a band corresponding to B-CryIA(b) in lanes containing BBMVs derived from susceptible larvae, the intensity of which increased with increasing amounts of BBMVs (Fig. 1A, lanes 2 to 5). No such band could be observed in lanes containing BBMVs derived from resistant larvae, even at the highest BBMV concentration tested (Fig. 1A, lanes 6 to 9). The binding patterns of B-CryIC, however, were not significantly different between susceptible (Fig. 1B, lanes 1 to 3) and resistant (Fig. 1B, lanes 4 to 6) larvae. The faint band

TABLE 3. Toxicity of CryIB, CryID, and CryIE to susceptible and resistant *Plutella xylostella* larvae

Toxin	Colony[a]	LC_{50} (μg/ml)	% Mortality[b]	Slope ± SE
CryIB	Sus	0.2 (0.1–0.4)[c]		2.49 ± 0.53
CryIB	Res	0.5 (0.3–1.0)[d]		2.07 ± 0.24
CryID	Sus	0.2 (0.1–0.2)[d]		1.82 ± 0.20
CryID	Res	0.2 (0.1–0.2)[d]		1.71 ± 0.18
CryIE	Sus		13.8	
CryIE	Res		6.1	

[a] Sus, susceptible; Res, resistant.
[b] Because of the low toxicity of CryIE, the percent mortality at 100 μg/ml is reported.
[c] Values in parentheses are 90% FL.
[d] Values in parentheses are 95% FL.

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TABLE 4. In vitro binding of B-CryIA(b) and B-CryIB to tissue sections from midguts of susceptible and resistant *Plutella xylostella* larvae[a]

Toxin	Signal[b]	No. (%) of larvae	
		Susceptible	Resistant
CryIA(b)	−	0	15 (57.7)
	±	3 (27.3)	10 (38.5)
	+	5 (45.5)	1 (3.8)
	++	3 (27.3)	0
CryIB	−	0	1 (3.8)
	±	1 (9.1)	4 (15.4)
	+	5 (45.5)	12 (46.2)
	++	5 (45.5)	9 (34.6)

[a] Bound ICPs were visualized by use of streptavidin-mediated detection.
[b] Signal intensities: −, none; ±, weak; +, strong; ++, very strong.

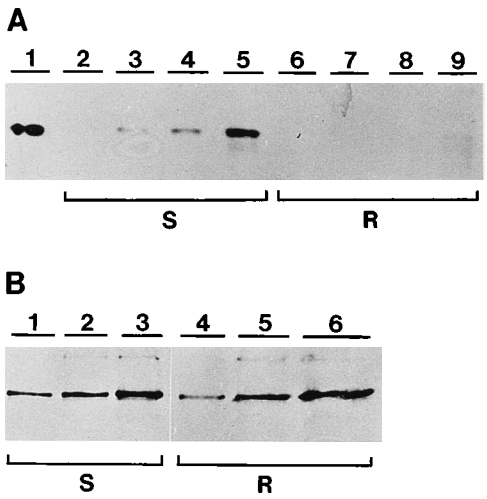

FIG. 1. Binding of CryIA(b) (A) and CryIC (B) to BBMVs from susceptible (S) and resistant (R) *Plutella xylostella* larvae. B-CryIA(b) and B-CryIC were incubated with increasing concentrations of BBMVs from susceptible (A, lanes 2 to 5; B, lanes 1 to 3) and resistant (A, lanes 6 to 9; B, lanes 4 to 6) *Plutella xylostella* larvae. (A) Lanes 2 to 5 and 6 to 9 contain 2.5, 5, 10, and 20 μg of vesicle protein, respectively, and lane 1 contains 5 ng of B-CryIA(b) not incubated with vesicles. (B) Lanes 1 to 3 and 4 to 6 contain 5, 10, and 20 μg of vesicle protein, respectively. Proteins in the pelleted binding mixture were separated by SDS-PAGE and blotted onto a nitrocellulose membrane. Bound ICPs were visualized by use of streptavidin-mediated detection.

in lanes loaded with 10 and 20 μg of BBMV protein (Fig. 1B, lanes 2 to 3 and 5 to 6) was not present in controls (no BBMV protein) (data not shown) and could represent a BBMV protein that had weak affinity for streptavidin or CryIC. Results from experiments with both tissue sections and BBMVs prepared from whole larvae suggest that at least one mechanism of resistance is reduced binding of CryIA(b) to midgut membrane binding site(s) with no changes in the binding sites of CryIB and CryIC.

DISCUSSION

Our investigation of a *Plutella xylostella* colony from Florida showed that field control failures of products containing *B. thuringiensis* subsp. *kurstaki* (41), consisting primarily of spore and CryIA toxins with some CryII protein (16, 20), were due to resistance to spore and the three CryIA ICPs. Screening of available *B. thuringiensis* toxins indicated that resistant larvae were still susceptible to CryIB, CryIC, and CryID and that CryIE and CryIIA were essentially nontoxic to both resistant and susceptible larvae. Similarly, resistant *Plutella xylostella* from Hawaii and the Philippines showed high levels of resistance to one or all three of the CryIA proteins (8, 49), no cross-resistance to CryIB or CryIC (8, 47, 49), and nonsensitive responses to CryIE (8).

Results for CryID are more variable. In terms of our data, CryID was highly toxic to colonies from both Florida and New York (laboratory susceptible), whereas in terms of the data of Ferré et al. (8), CryID showed no toxicity to colonies from either the Philippines or The Netherlands (laboratory susceptible). Because the CryID in both studies came from the same source, differences could be due to the insects themselves or to the different bioassay methods employed, i.e., we used a leaf dip assay, whereas Ferré et al. (8) topically applied toxin to artificial diet.

Smaller discrepancies were observed for CryIIA data. We found CryIIA to be essentially nontoxic to both resistant and susceptible larvae (ca. 30% mortality at 100 μg/ml), whereas Tabashnik et al. (49) saw slightly higher mortality with susceptible Hawaii larvae and low levels of resistance to CryIIA (twofold). Because of the comparatively low toxicity of CryIIA, selection intensity for resistance to this protein would be minimal in the field with commercial formulations of *B. thuringiensis* subsp. *kurstaki*. Although the bulk of these data generally suggest a lack of cross-resistance between CryIA and other toxins, 200-fold resistance was noted for CryIF when tested in leaf disc assays using the Hawaiian colony (47). It remains to be seen, however, whether CryIF and CryIA share or have completely separate high-affinity binding sites in the midgut.

The mechanism of resistance in our *Plutella xylostella* colony from Florida seems to be consistently associated with changes in toxicity and binding of CryIA(a), CryIA(b), and CryIA(c). We found that our Florida larvae were no longer sensitive to each of the three CryIA protoxins and that CryIA(b) exhibited reduced binding in both BBMVs and tissue section experiments. Although we did not examine binding of CryIA(a) and CryIA(c), Escriche et al. (7), using our Florida larvae, observed reduced binding of CryIA(b) and CryIA(c) in midgut tissue sections.

Reduced binding of CryIA in the colonies from Hawaii and the Philippines, however, has been more variable. For example, the Hawaii colony, which initially showed resistance to all three CryIA protoxins (49) and dramatically reduced binding of CryIA(c) in BBMV assays (46), was later found to bind all three CryIA toxins in tissue sections (7) and to bind CryIA(c) in surface plasmon resonance assays (25). At this point, it is unclear whether this variation is due to the different generations of insects used or to the different methods used to measure binding activity.

For the Philippine colony, two collections were made at two different times near Baguio. The first colony described was resistant to CryIA(b) and did not bind CryIA(b) in BBMVs (8) or in tissue sections (3, 4). The second colony described was resistant to CryIA(b) but was determined to be susceptible to CryIA(a) and CryIA(c). Neither of these colonies, however, was found to be resistant to Dipel 2X, a commercial formulation of *B. thuringiensis* subsp. *kurstaki* (1, 8). The authors speculated that this CryIA(b) resistance (1) is possibly a biotype present in the Philippines and not the result of selection with *B. thuringiensis* insecticides.

Binding data for toxins from other CryI families have shown that the lack of cross-resistance with other toxins was correlated with the lack of change in binding of these non-CryIA

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toxins. We found no changes in the binding characteristics of CryIB and CryIC in our Florida larvae, similar to what Ferré et al. (8) observed for CryIB and CryIC in their Philippine colony and what Tabashnik et al. (46) reported for CryIC in their Hawaii colony. It would also be interesting to determine whether the 200-fold cross-resistance observed with CryIF in the Hawaiian colony (47) is correlated with changes in binding and whether CryIF shares binding sites with the CryIA toxins.

Given the identification of an aminopeptidase N (18) and a cadherin-like protein (52) as CryIA toxin receptors in *Manduca sexta*, efforts to determine what types of genetic changes are responsible for reduced binding in each of the resistant colonies of *Plutella xylostella* are under way. Data from work with our Florida colony seem to imply that the evolution of resistance to *B. thuringiensis* in the field may be less complex than might otherwise have been supposed (12). Indeed, it seems reasonable to predict that reduced binding will be a common evolutionary response to intensive use of CryIA toxins in the field in other species of *Lepidoptera*, just as reduced binding has proven to be a major mechanism of resistance to chemical insecticides (42). Whether reduced binding is responsible for all of the resistance to CryIA is still unclear, but on the basis of the survival of our Florida colony on transgenic broccoli expressing the CryIA(c) toxin (30), the mechanism involved clearly protects field-resistant larvae.

Although reduced CryIA binding and lack of cross-resistance to CryIC was also described for *Plodia interpunctella* selected with *B. thuringiensis* subsp. *kurstaki* (54), other moth species, such as *Heliothis virescens* (10) and *Spodoptera exigua* (33), which were laboratory selected, showed only minor changes in receptor binding activity of the selecting toxin and broad-spectrum resistance with toxins from other families. Broad-spectrum resistance to several toxins was also exhibited by lines of *Plodia interpunctella* selected with *B. thuringiensis* subsp. *entomocidus* or *B. thuringiensis* subsp. *aizawai* (28). The responsible mechanisms are unknown, but factors such as altered protoxin activation by midgut proteinases (36) and/or increases in nonspecific binding that compete or interfere with specific binding and pore formation (33) may be involved. It is still not clear, however, whether the mechanism(s) of broad-spectrum resistance selected for in these laboratory strains is likely to occur in the field (38) or likely to provide enough protection for larvae to survive on transgenic plants.

The concomitant evolution of resistance to HD-1 spore, CryIA(a), CryIA(b), and CryIA(c) in our Florida colony of *Plutella xylostella* implies that either separate mechanisms evolved or that one mechanism evolved that conferred resistance to both spore and CryIA because of some shared attribute. Since a primary component of HD-1 spore coat is CryIA or CryIA-like protein (51), it is possible that the 61-fold-resistance to spore we observed was actually due to resistance to the CryIA found in the spore coat.

By comparing the toxicities of the various ICPs with and without spore in susceptible and resistant *Plutella xylostella*, we found that synergy occurred only when spore was combined with those ICPs that when tested alone were toxic. In susceptible larvae, these were CryIA(a), CryIA(b), CryIA(c), or CryIC, and in resistant larvae, it was only CryIC. For susceptible larvae, binding to the midgut epithelium has been shown for CryIA(c) (7), CryIA(b), and CryIC (data presented here). Our data also show binding of CryIC in resistant Florida larvae. This suggests that the synergistic effects of spore may be mediated by the presence of high-affinity binding sites for toxin at the midgut interface. It is possible that toxin bound to the midgut epithelium somehow facilitates spore passage into the hemolymph. This may account for the synergy observed when toxin-denuded HD-1 spore was combined with HD-1 crystal and fed to a laboratory colony of *Plutella xylostella* (31) and why our Florida larvae, which were resistant to CryIA, were also resistant to spore.

Mediation of spore synergy with certain ICPs by the presence of high-affinity binding sites for the ICP may also occur in *Plodia interpunctella* and *S. exigua*. In *Plodia interpunctella*, a strain selected with *B. thuringiensis* subsp. *kurstaki* was, like our Florida colony of *Plutella xylostella*, resistant to HD-1 spore, HD-1 crystals, and the HD-1 spore-crystal combination (27). This selected strain of *Plodia interpunctella* also exhibited reduced binding of CryIA(b) (54). Tests with individual ICPs and actual evaluations of synergy, however, were not done, and so, although parallels with *Plutella xylostella* are evident, further tests will need to be done. For *S. exigua*, synergism was evaluated with HD-1 spore and individual ICPs in a susceptible (32) and a CryIC-selected strain (33). Although CryIC was no longer toxic to the resistant colony, binding data with BBMVs showed that binding sites were still present and that HD-1 spore was still able to synergize CryIC activity in resistant larvae (33). According to our hypothesis, this would be expected since spore-toxin synergism is mediated by the presence of binding sites for the toxin.

From the standpoint of resistance management, it is tempting to assume that resistance in nature will tend to be specific to individual toxin subclasses (e.g., CryIA versus CryIC versus CryIB) and will not extend broadly to other toxin types as has been observed in laboratory selection experiments (10, 28, 33). If monotypic resistance proves to be the rule rather than the exception, even where resistance extends to one or a few other toxin subclasses (47), it will obviously improve the potential for resistance management. Nevertheless, it would be premature to count on this until there has been more intensive and prolonged use of multiple toxins in the field.

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