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Resistance to *Bacillus thuringiensis* CryIA δ-Endotoxins in a Laboratory-Selected *Heliothis virescens* Strain Is Related to Receptor Alteration

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The *Bacillus thuringiensis* toxin-binding properties of midgut epithelial cells from two strains of *Heliothis virescens* were compared. One *H. virescens* strain (YHD2) which was selected against CryIAc toxin had over 10,000-fold resistance to CryIAc toxin relative to the susceptible strain and was cross-resistant to CryIAa and CryIAb. The second *H. virescens* strain (YDK) was susceptible to these toxins in the order CryIAc > CryIAb > CryIAa. Receptor-binding properties of CryIAa, CryIAb, and CryIAc toxins were compared between the susceptible and resistant strains. Saturation and competition-binding experiments were performed with brush border membrane vesicles prepared from midguts of the susceptible and resistant insects and ^{125}I-labeled toxins. In the susceptible strain, saturable, specific, and high-affinity binding of all three toxins was observed. The relative binding-site concentration was directly correlated with toxicity (CryIAc > CryIAb > CryIAa). In the resistant strains, the binding affinities of CryIAb and CryIAc were similar to that observed with the susceptible strain and only minor differences in binding-site concentration (B_{max}) were observed. The major difference between the two strains was the total lack of binding of CryIAa toxin to the brush border membrane vesicles of the resistant strain. Heterologous competition-binding experiments and ligand blot analysis supported the hypothesis that there were multiple binding sites for the toxins. On the basis of results of the present study, we propose that alterations in binding proteins shared by all three toxins are a major factor in resistance. This suggests that not all receptors of CryIAc might be involved in toxic function.

Bacillus thuringiensis, a gram-positive bacterium, produces crystalline parasporal inclusions containing insecticidal crystal proteins called δ-endotoxins during its sporulation. *B. thuringiensis* δ-endotoxins have been used as an alternative to chemical insecticides for management of insect pests (23). The δ-endotoxin genes have been cloned and expressed in other bacteria and transferred to crop plant genomes (19, 23). These biotechnological developments provide genetic improvement in the potency and use of *B. thuringiensis* toxins. However, development of insect resistance to *B. thuringiensis* toxins is an important concern for the long-term use of these toxins. Recently, high levels of insect resistance have been identified in several lepidopteran insects (6, 11, 12, 16–18). From field selection, resistance has been observed in the diamondback moth, *Plutella xylostella*, in the Philippines and Hawaii (6, 17, 18). High levels of resistance have been also reported from laboratory selection in the Indian meal moth, *Plodia interpunctella*, and in the tobacco budworm, *Heliothis virescens* (3a, 11, 12, 16). Understanding the mechanisms of resistance of these insects to *B. thuringiensis* toxins could be critical for managing rapid development of resistance.

The mode of action of *B. thuringiensis* toxin is a multistep process. When δ-endotoxin crystals are ingested by susceptible insects, the proteins are solubilized in the midgut and then proteolytically activated by midgut proteases. The activated toxin binds to specific receptors on the brush border membrane of midgut epithelial cells (4, 9, 20, 21). The membrane-bound toxins form pores, causing osmotic lysis of midgut epithelial cells and insect death (7). Insect resistance to *B. thuringiensis* toxins may have developed mechanisms that disrupt one or more steps involved in this complex process.

It has been found that the molecular basis of resistance in laboratory-selected strains of *P. interpunctella* and *H. virescens* and in a field-selected strain of *Plutella xylostella* involves an alteration in receptor-binding properties (1, 10, 22). However, in contrast to previously reported studies, Gould et al. (3) observed that resistance in a strain of *H. virescens* was not related to changes in midgut receptors. This observation suggests that the mechanism of resistance in this strain is different from other cases. Recently, Oppert et al. (15) reported that altered protoxin activation by midgut proteases is involved in resistance of a different selected strain of *P. interpunctella* to *B. thuringiensis*.

In the present study, we investigated the possible mechanism(s) of resistance of a laboratory-selected strain of *H. virescens* that had high levels of resistance to CryIA toxin (2a).

MATERIALS AND METHODS

Insect strains. The two *H. virescens* strains used in these experiments are described in detail by Gould et al. (2a). Briefly, the YDK strain was a laboratory strain that was initiated from a field collection of eggs from nine tobacco fields in North Carolina. The YHD2 strain originated from the same sample of eggs but was selected in the laboratory for adaptation to CryIAc toxin.

Preparation of δ-endotoxins. *B. thuringiensis* δ-endotoxin genes, *cryIAa*, *cryIAb*, and *cryIAc*, were cloned and expressed in *Escherichia coli*. The δ-endotoxins were purified from *E. coli* as described by Lee et al. (9). The purified crystal protein was solubilized in solubilization buffer (50 mM Na_2CO_3 [pH 9.5] containing 10 mM dithiothreitol) at 37°C for 2 h. The solubilized protoxin was digested with trypsin at a trypsin/protoxin ratio of 1:25 (by mass) at 37°C for 2 h. Protoxins and trypsin-activated toxins were examined by sodium dodecyl

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sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (10% polyacrylamide) (8).

Midgut isolation and BBMV preparation. Brush border membrane vesicles (BBMV) from last-instar larvae of the susceptible and resistant strain were prepared by the differential magnesium precipitation method of Wolfersberger et al. (24). The final pellet was resuspended in 8 mM $NaHPO_4$–2 mM KH_2PO_4–150 mM NaCl (pH 7.4) buffer. The BBMV were either used immediately or frozen in liquid nitrogen until use. The concentration of BBMV proteins was determined with Coomassie protein assay reagent (Pierce). After the measurement of protein concentration, bovine serum albumin (BSA) was added to the BBMV preparation to a final concentration of 0.1% (wt/vol).

Iodination of toxins. The activated toxins were iodinated with IODO-BEAD (Pierce) as specified by the manufacturer. $Na^{125}I$ (1 mCi; Amersham) was used to label 25 μg of each toxin. The labeled toxins were separated from free iodine on a 2-ml Excellulose column (Pierce). Specific activity was typically 100 to 160 Ci/mmol.

Binding assays with BBMV. For the qualitative competition assay, 1 nM ^{125}I-labeled toxins (CryIAa, CryIAb, and CryIAc) were incubated with 10 μg of BBMV protein from the susceptible and resistant strains in the absence or presence of a 500-fold excess of unlabeled toxins. After 1 h of incubation, reaction mixtures were separated by centrifugation at 13,500 × *g* for 10 min. The pellets were washed three times with binding buffer (8 mM Na_2HPO_4, 2 mM KH_2PO_4, and 150 mM NaCl [pH 7.4], containing 0.1% BSA) and separated by SDS-PAGE (12% polyacrylamide). The dried gel was exposed to Fuji X-ray film for 1 to 3 days.

For the quantitative binding assays, BBMV were incubated with ^{125}I-labeled toxins in 100 μl of binding buffer for 1 h at room temperature. Bound toxins were separated from unbound toxin by centrifugation at 13,500 × *g* for 10 min. The pellet containing the bound toxin was washed with binding buffer three times, and the radioactivity of the resulting pellet was counted in a gamma counter (Beckman). Binding data were analyzed by using the LIGAND computer program (13). Saturation-binding assays were performed with fixed amounts of labeled toxins but varied amounts of BBMV protein. ^{125}I-labeled toxins (1 nM) were incubated with increasing BBMV concentrations (from 10 to 1,000 μg/ml). Nonspecific binding in the presence of excess amounts of unlabeled ligand (500 nM) was subtracted from total binding for each datum point. In homologous and heterologous competition-binding assays, BBMV (20, 10, and 5 μg for CryIAa, CryIAb, and CryIAc, respectively) were incubated with 1 nM labeled CryIAa, CryIAb, and CryIAc toxins for 1 h in the presence of increasing amounts of unlabeled competitors (from 0.25 to 500 nM). For dissociation experiments, 1 nM ^{125}I-labeled CryIAc toxin was incubated with 5 μg of BBMV for 2 h. After incubation, excess (500 nM) unlabeled toxin was added to the incubation mixture, and the reaction was stopped at different time intervals (from 10 to 60 min) by centrifugation.

Identification of CryIA toxin-binding proteins by ligand blotting. BBMV proteins (10 μg) from both strains were separated by SDS-PAGE (7.5% polyacrylamide) and transferred to polyvinylidene difluoride membrane. Biotin-labeled CryIA toxins (5 nM) were incubated with the membrane for 3 h at room temperature. The toxin-binding proteins were visualized with streptavidin-conjugated peroxidase (Boehringer Mannheim) and DAB (3,3′-diaminobenzidine tetrahydrochloride) substrate (Sigma).

RESULTS

Toxicity assays. Bioassay results are described in detail by Gould et al. (2a). The order of toxicity to the susceptible strain (YDK) is CryIAc > CryIAb >> CryIAa. The laboratory-selected strain (YHD2) developed more than 10,000-fold resistance to CryIAc toxin relative to YDK. Additionally, it developed more than 2,000-fold resistance to CryIAb as determined by dose-mortality bioassays. We could not obtain a concentration of CryIAa that caused mortality of the YHD2 strain. However, the growth inhibition of larvae on a diet containing 100 μg of CryIAa per ml was measured. The mean 7-day weights for YDK and YHD2 larvae were 1.02 ± 0.05 and 32.2 ± 1.7 mg, respectively, indicating a high level of resistance.

Binding of ^{125}I-labeled CryIA toxins to BBMV from susceptible and resistant strains. Autoradiography showed that the binding of labeled CryIAb and CryIAc toxins to both resistant and susceptible strains was blocked by the corresponding unlabeled toxins (Fig. 1, lanes 4, 6, 10, and 12). In contrast, unlabeled CryIAa toxin was capable of blocking ^{125}I-labeled CryIAa toxin to some extent in the susceptible strain (lane 2) but not in the resistant strain (lane 8). Furthermore, very weak binding of labeled CryIAa was observed in the resistant strain

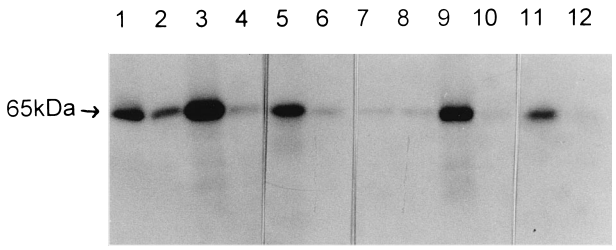

FIG. 1. Binding of ^{125}I-labeled CryIAa, CryIAb, and CryIAc toxins to the BBMV from susceptible and resistant strains. ^{125}I-CryIAa (lanes 1 and 7), CryIAb (lanes 3 and 9), and CryIAc (lanes 5 and 11) toxins (1 nM each) were incubated with 10 mg of BBMV from susceptible (lanes 1 to 6) and resistant (lanes 7 to 12) strains for 1 h in the absence (lanes 1, 3, 5, 7, 9, and 11) or presence (lanes 2, 4, 6, 8, 10, and 12) of 500 nM unlabeled toxins. Unbound toxins were removed by centrifugation. The resulting pellet, containing bound toxins, was washed with binding buffer, and the samples were subjected to SDS-PAGE (12% polyacrylamide). The gel was dried and autoradiographed.

(lane 7). In saturation-binding assays, the binding of CryIAb and CryIAc to the BBMV from both strains was observed to be the same. About 30 to 40% binding was observed at the maximum level of binding (Fig. 2B and C). In contrast, there was a dramatic difference between the binding of CryIAa to the BBMV from the susceptible and resistant strains. In the susceptible strain, maximum binding occurred at 400 μg of vesicle protein per ml, whereas no significant binding was observed in the BBMV from the resistant strain, even at 1 mg/ml (Fig. 2A).

In homologous competition experiments, CryIAc, the most toxic protein to *H. virescens*, showed high-affinity binding to the BBMV from the susceptible and resistant strains (Fig. 3C). CryIAb toxin also showed high binding affinity to BBMV of both strains (Fig. 3B). In the susceptible strain, CryIAa, much less toxic than either CryIAb and CryIAc, exhibited high binding affinity but a smaller number of binding sites. In the resistant strain, the binding of CryIAa to BBMV was negligible and was not inhibited by unlabeled CryIAa even at 500 nM (Fig. 3A). The K_d and B_{max} of labeled toxins were calculated from three separate homologous competition experiments (Table 1).

Heterologous competition assays were performed with each labeled toxin and the unlabeled toxins (Fig. 4). In the susceptible strain, the unlabeled CryIAb and CryIAc toxins competed for the binding of ^{125}I-labeled CryIAa toxin with comparable affinity (Fig. 4A). The binding of ^{125}I-labeled CryIAb toxin was inhibited by unlabeled CryIAc toxin. However, CryIAa toxin only partially displaced the binding of ^{125}I-labeled CryIAb toxin (Fig. 4B). The unlabeled CryIAb toxin competed for the binding of ^{125}I-labeled CryIAc toxin with about the same affinity as the CryIAc toxin did. However, CryIAa could only partially saturate ^{125}I-labeled CryIAc-binding sites (Fig. 4C). Results of heterologous competition assays between CryIAb and CryIAc in the resistant strain were similar to the patterns in the susceptible strain (data not shown). However, CryIAa toxin showed less competition for the binding of ^{125}I-labeled CryIAb and CryIAc in the resistant strain than in the susceptible strain (Fig. 5).

Dissociation experiment to test the binding of toxins to BBMV. We tested the possibility that an alteration in the irreversible binding of the toxin to the BBMV was a major factor for the resistance of insects to the CryIAc toxin. Dissociation experiments showed that the binding of CryIAc toxin to either the susceptible strain or the resistant strain was not reversible (Fig. 6).

Identification of ^{125}I-labeled CryIA-binding proteins by ligand blotting. In the susceptible strain, CryIAa and CryIAb

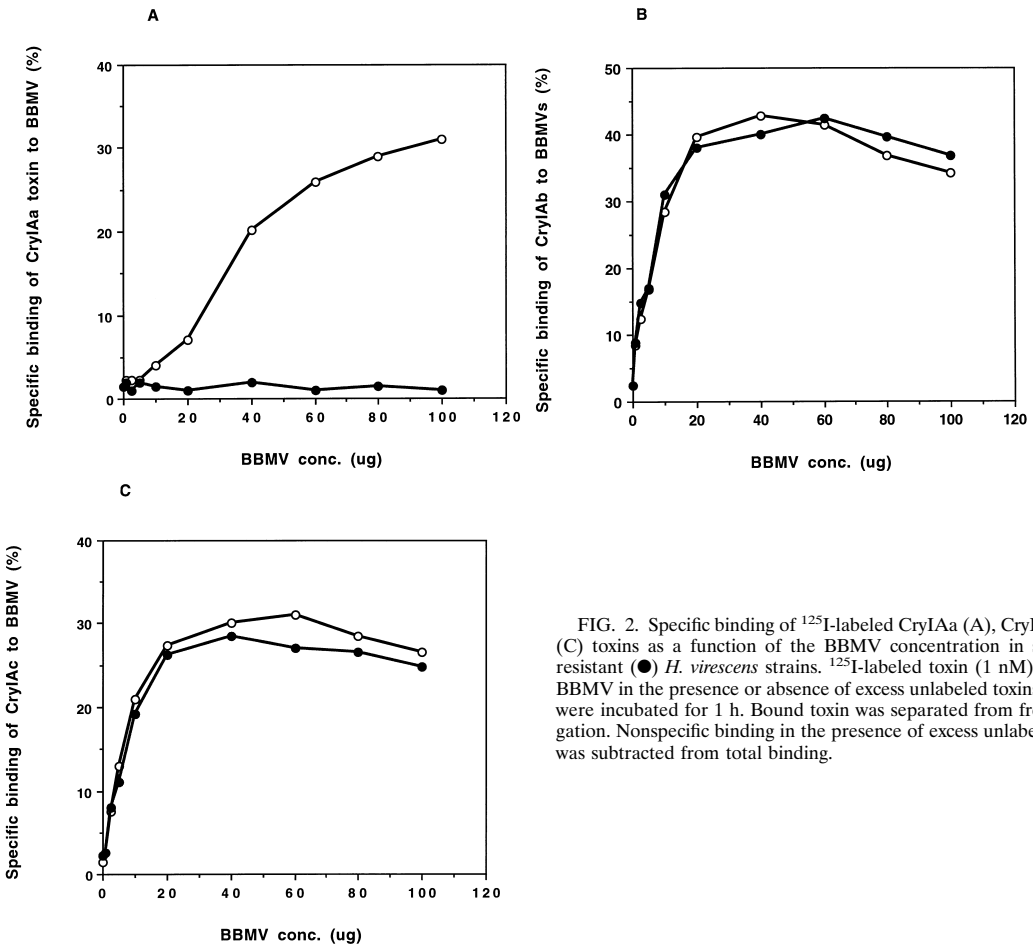

FIG. 2. Specific binding of ^{125}I-labeled CryIAa (A), CryIAb (B), and CryIAc (C) toxins as a function of the BBMV concentration in susceptible (○) and resistant (●) *H. virescens* strains. ^{125}I-labeled toxin (1 nM) was incubated with BBMV in the presence or absence of excess unlabeled toxins (500 nM). Samples were incubated for 1 h. Bound toxin was separated from free toxin by centrifugation. Nonspecific binding in the presence of excess unlabeled toxins (500 nM) was subtracted from total binding.

proteins bound to the same BBMV protein bands of about 170 and 150 kDa. CryIAc protein bound to three major peptides of about 170, 150, and 120 kDa and to a minor 80-kDa peptide (Fig. 7). In the resistant strain, similar protein-binding patterns were observed.

DISCUSSION

The mode of action of *B. thuringiensis* toxins has been suggested to be a multistep process. The mechanism by which insects evolve resistance to *B. thuringiensis* toxins is likely to be related to one or more of the steps involved in the mode of action (1, 10, 15, 22). To evaluate the potential mechanisms of resistance to CryIA toxins in an *H. virescens* strain (YHD2), we have examined receptor-binding properties of ^{125}I-labeled CryIAa, CryIAb, and CryIAc toxins to midgut BBMV from the susceptible and resistant strains.

As a first step, qualitative competition assays with excess amounts of competitor were performed by autoradiography. The binding of ^{125}I-labeled CryIAa, CryIAb, and CryIAc toxins to susceptible-strain BBMV is blocked by the corresponding unlabeled proteins, indicating specific binding. Unlabeled CryIAa blocks less labeled CryIAa toxin than expected, which could be due to higher nonspecific binding for CryIAa than for CryIAb or CryIAc. Interestingly, the binding of ^{125}I-labeled CryIAb and CryIAc to resistant-strain BBMV is also blocked by unlabeled toxins, even though this strain is resistant to these toxins. These qualitative competition experiments suggest that the binding properties of CryIAb and CryIAc toxins are not grossly altered even though the resistant strain was selected with CryIAc toxin. However, the binding of ^{125}I-labeled CryIAa to BBMV of the resistant strain was much weaker than to BBMV of the susceptible strain, as assessed by the lower intensities of toxin bands on the autoradiogram. Additionally, the very weak binding of ^{125}I-labeled CryIAa to the resistant strain was not blocked by unlabeled CryIAa toxin, indicating nonspecific binding (Fig. 1). These results were confirmed by more detailed quantitative ligand-binding assays.

Saturation-binding assays showed that CryIAb and CryIAc toxins bind specifically to BBMV from both strains whereas CryIAa binds only to the susceptible-strain BBMV (Fig. 2). The binding of CryIAa to the resistant-strain BBMV was negligible. Our data from homologous and heterologous competition-binding assays (Fig. 3 and 4) with BBMV from susceptible-insect midguts are in good agreement with earlier observations by Van Rie et al. (20). CryIAa, CryIAb, and CryIAc toxins all show high binding affinities to susceptible-strain BBMV (Table 1). However, the number of binding sites (B_{max}) for each toxin differ and are directly correlated with the level of toxicity to the susceptible strain. Interestingly, we also observed high binding affinities for CryIAb and CryIAc to BBMV from the resistant-insect midguts, even though CryIAb and CryIAc are more than 2,000 times less toxic to this strain (Table 1). These observations correspond to results of Gould et al. (3) with another laboratory-selected *H. virescens* strain

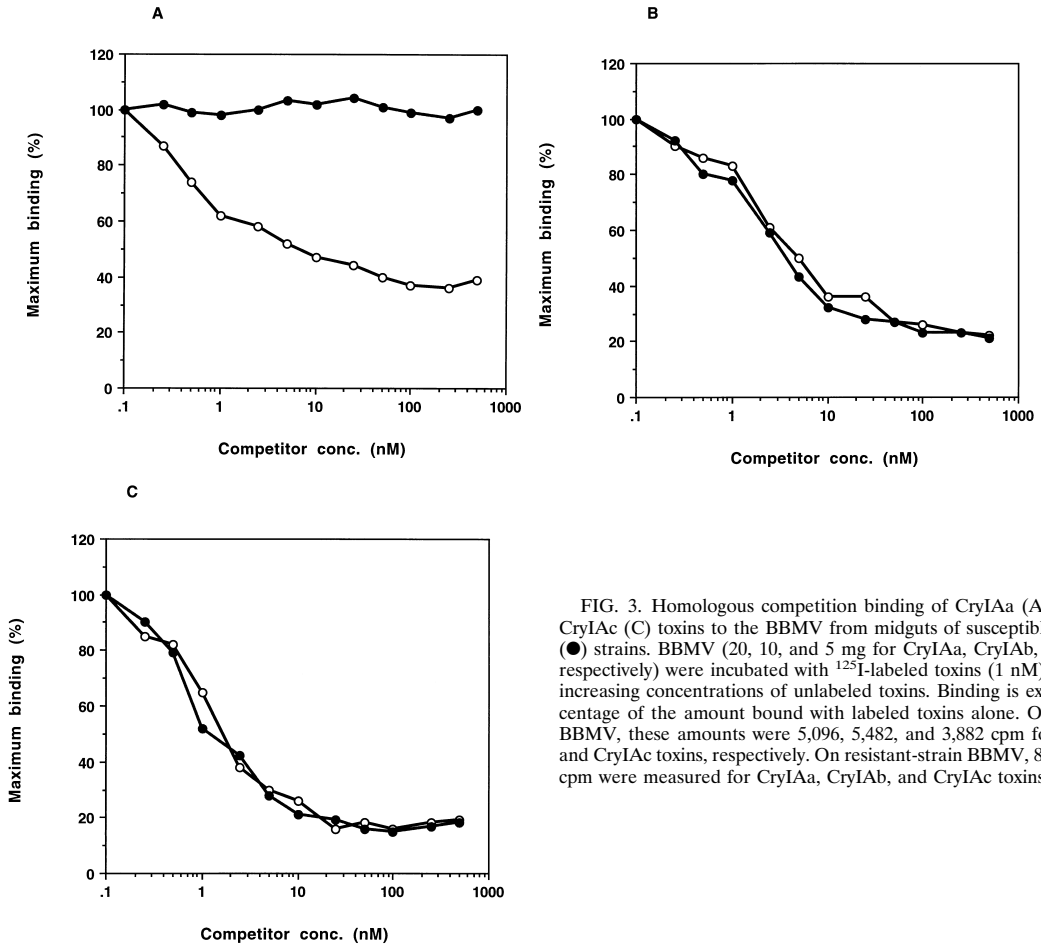

FIG. 3. Homologous competition binding of CryIAa (A), CryIAb (B), and CryIAc (C) toxins to the BBMV from midguts of susceptible (○) and resistant (●) strains. BBMV (20, 10, and 5 mg for CryIAa, CryIAb, and CryIAc toxins, respectively) were incubated with ^{125}I-labeled toxins (1 nM) in the presence of increasing concentrations of unlabeled toxins. Binding is expressed as the percentage of the amount bound with labeled toxins alone. On susceptible-strain BBMV, these amounts were 5,096, 5,482, and 3,882 cpm for CryIAa, CryIAb, and CryIAc toxins, respectively. On resistant-strain BBMV, 813, 4,670, and 3,242 cpm were measured for CryIAa, CryIAb, and CryIAc toxins, respectively.

(CP73). Our competition assay data with CryIAb and CryIAc toxins do not seem to show a direct correlation between toxicity and receptor binding. However, a total lack of binding of CryIAa to the resistant-strain BBMV was observed (Fig. 2A and 3A). To investigate whether the alteration of CryIAa-binding protein could account for the high level of resistance to CryIAb and CryIAc toxins, it is useful to examine the relationship of the binding sites among CryIAa, CryIAb, and CryIAc toxins.

TABLE 1. Toxicity and binding parameters of CryIA toxins on BBMV from midguts of the susceptible (YDK) and resistant (YHD2) strains

H. virescens strain	Toxin	K_d (nM)[a]	B_{max} (pmol/mg of BBMV)[a]	LC_{50} (μg) (95% fiducial limits)[b]
Susceptible (YDK)	CryIAa	0.64 ± 0.11	4.9 ± 1.1	>100
	CryIAb	0.53 ± 0.09	14.1 ± 2.3	0.360 (0.238–0.516)
	CryIAc	0.21 ± 0.05	37.6 ± 3.7	0.050 (0.031–0.071)
Resistant (YHD2)	CryIAa	–	–	>100
	CryIAb	0.61 ± 0.13	10.5 ± 1.5	>1,000
	CryIAc	0.35 ± 0.06	30.1 ± 3.3	506.38 (342.77–725.14)

[a] K_d and B_{max} values are the means from three experiments. K_d and B_{max} for CryIAa on YHD2 BBMV (dashes) could not be calculated because there was no binding.
[b] LC_{50}, 50% lethal concentration.

Van Rie et al. (20) proposed a three-site model to explain the heterogeneity in binding of CryIA toxins to *H. virescens* BBMV. One population of binding sites (receptor A) interacts with all three toxins. A second population (receptor B) binds to CryIAb and CryIAc toxins but not CryIAa toxin, while a third population (receptor C) binds only to the CryIAc toxin. Our heterologous competition experiments with susceptible-strain BBMV support this model. From this hypothesis, it is possible to predict that an *H. virescens* strain selected for resistance to the CryIAc toxin would become cross-resistant to all three of the CryIA toxins if resistance was due to a change in binding "receptor A," which is recognized by all three toxins. Because we observed dramatic changes in the CryIAa binding properties to the resistant strain (Fig. 2A and 3A) and because this strain is resistant to all three toxins, we propose that one of the three receptor populations, receptor A, could be altered in the resistant-strain midgut and that this receptor might be crucial for toxicity.

Heterologous competition assays with the susceptible and resistant strains support this possibility. The unlabeled CryIAa exhibits less competition for the binding of ^{125}I-labeled CryIAb and CryIAc with BBMV from the resistant strain than for the binding with BBMV from the susceptible strain (Fig. 5). This small difference was consistent throughout the repetitions of the experiment. If receptor A of the resistant strain is altered in toxin-binding potential, we would expect a reduction in the number of the total binding sites (B_{max}) for CryIAb and

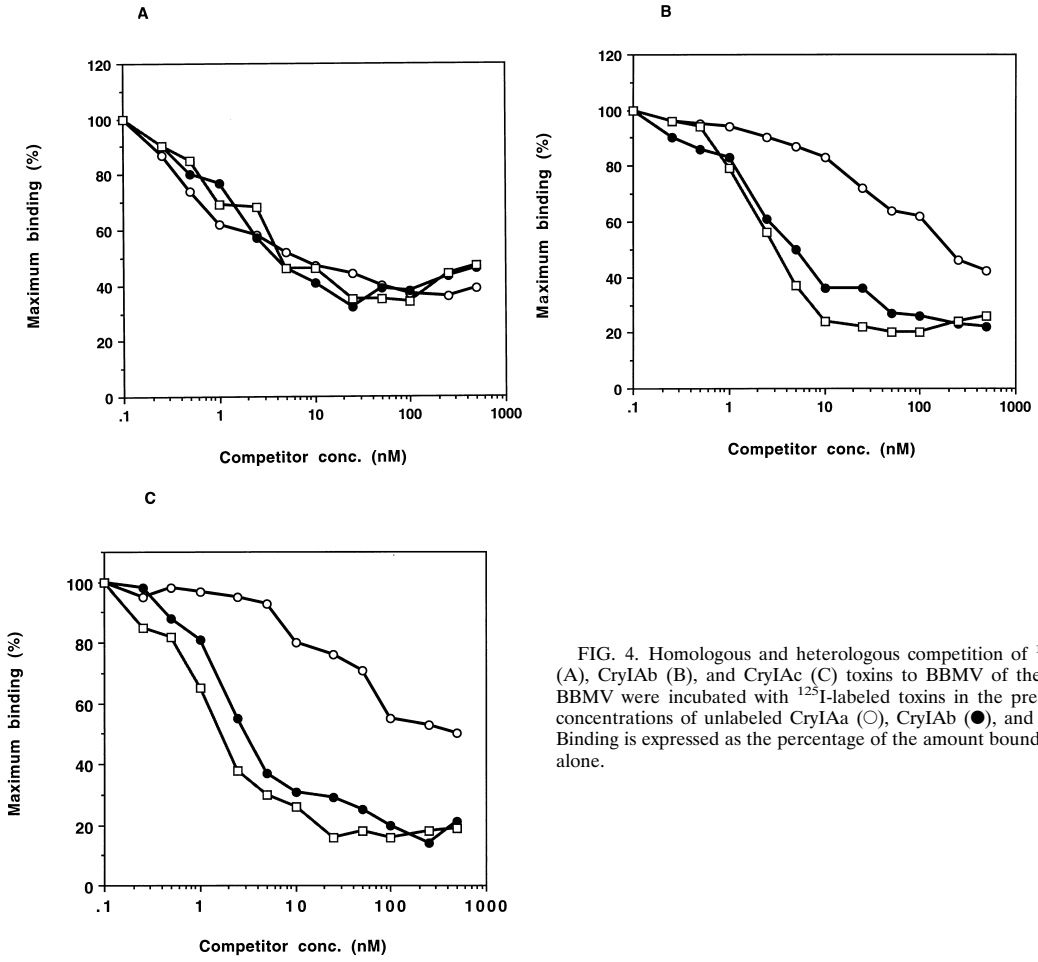

FIG. 4. Homologous and heterologous competition of ^{125}I-labeled CryIAa (A), CryIAb (B), and CryIAc (C) toxins to BBMV of the susceptible strain. BBMV were incubated with ^{125}I-labeled toxins in the presence of increasing concentrations of unlabeled CryIAa (○), CryIAb (●), and CryIAc (□) toxins. Binding is expressed as the percentage of the amount bound with labeled toxins alone.

CryIAc proportional to the change in receptor A. Indeed, our data show a small reduction in the B_{max} for CryIAb and CryIAc in the resistant strain compared with the B_{max} in the susceptible strain (Table 1). Since the majority of CryIAb and CryIAc toxins still bound to the resistant-strain BBMV with high affinity, we may assume that receptor B and receptor C do not play a major role in toxicity. However, if receptor A alone is responsible for toxicity, we would expect equal toxicity among the three toxins. This has not, however, been found in bioassays (Table 1) (2a). The discrepancy may be explained by the following hypothesis. Possibly, the binding of toxins to receptor A is crucial for toxicity, yet binding at receptor B and receptor C is also required to enhance the toxicity. Binding to receptor A, B, or C alone would not be sufficient for full toxicity. Alternatively, it is possible that only receptor A is necessary for toxicity, but the relative pore-forming activity might be CryIAc > CryIAb > CryIAa, while other receptors are not functional.

In previous reports, Oddou et al. (14) identified a 170-kDa peptide for CryIAa and CryIAb toxins and 140-kDa/120-kDa peptides for CryIAc toxin by BBMV ligand blot analysis in *H. virescens*. More complicated CryIAc-binding protein patterns, including 155-, 120-, 103-, 90-, and 63-kDa peptides, have been reported (2). In our ligand blotting, major peptides of 170 and 150 kDa were identified as binding proteins for CryIAa, CryIAb, and CryIAc. Additional peptides of 120 and 80 kDa were identified as CryIAc-binding proteins (Fig. 7).

On the basis of ligand-blotting results, we propose that the 170- and 150-kDa peptides, which are recognized by all three toxins, could be receptor A. The 120- and 80-kDa peptides, which are accessible only to CryIAc toxin, could be receptor C. However, in our assays, we did not observe the peptide which is recognized by only CryIAb and CryIAc toxins (receptor B). These observations are in part consistent with previous reports of Oddou et al. (14). However, we cannot exclude the possibilities that CryIAb and CryIAc recognize an additional receptor protein (receptor B) that comigrates with the 170- or 150-kDa protein (receptor A) in SDS-PAGE or that receptor B in its denatured state does not bind toxin molecules.

Since CryIAa toxin did not bind to the resistant BBMV (Fig. 2A and 3A), we might expect missing bands for CryIAa-binding proteins (170 and 150 kDa) in the ligand-blotting assay with resistant-strain BBMV. However, we observed that CryIAa still binds to two peptides in the resistant-strain BBMV (Fig. 7, lanes 3, 5, and 7). It is possible that the data from in vitro BBMV competition experiments with native BBMV differ from those obtained in BBMV ligand-binding assays in which denatured BBMV are used. In other words, toxins could bind to unaltered sites in the receptor proteins, which are buried or hidden in the native BBMV but exposed in the denatured BBMV. Alternatively, we may assume that the receptors themselves are not genetically altered in the resistant-strain midgut but might be arranged in such a way that toxins cannot reach them. However, under denaturing conditions, all intact receptors could be exposed to the toxins. Interestingly, these two

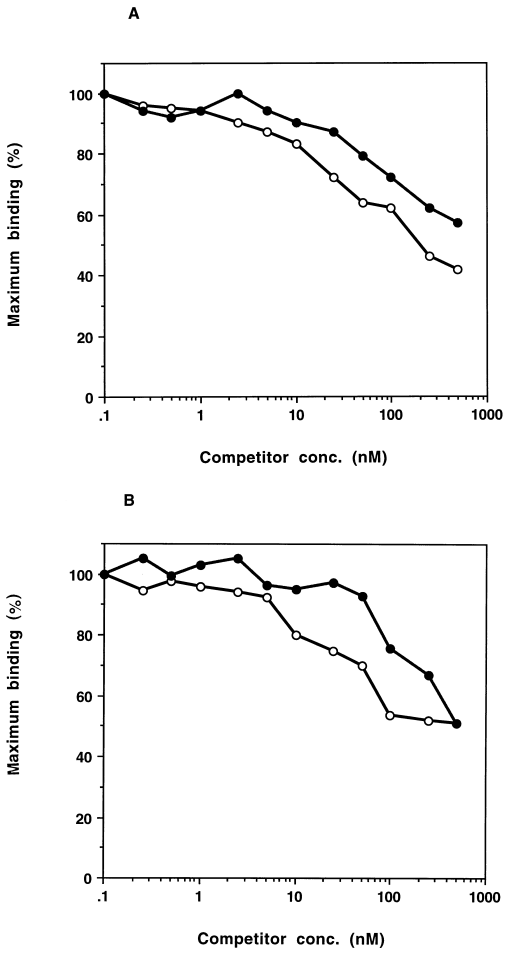

FIG. 5. Heterologous competition between ^{125}I-labeled CryIAb and CryIAc toxins and unlabeled CryIAa toxins for the BBMVs of susceptible (○) and resistant (●) strains. BBMVs from both strains were incubated with ^{125}I-labeled CryIAb (A) or ^{125}I-labeled CryIAc (B) toxins in the presence of increasing concentrations of unlabeled CryIAa toxin.

peptides migrate somewhat more closely on SDS-PAGE when isolated from the resistant-strain BBMV than from the susceptible-strain BBMV (Fig. 7).

Since we have selected insects with activated toxins, we have investigated the stability of CryIA toxins in the resistant-insect midguts. Western blot (immunoblot) assays show that toxin profiles are not altered by incubation with either midgut proteases or BBMV from either strain (data not shown). These data suggest that decreased toxin effects on this resistant strain are not due to the instability of the toxins by midgut proteases. We cannot rule out the possibility that toxins are excluded from passing through the peritrophic membrane in the resistant strain.

Irreversibility of *B. thuringiensis* toxin binding appears to be a general feature of lepidopteran-specific toxins (4, 20). Recently, Ihara et al. (5) have reported that the specific toxicity of CryIAa and CryIAb toxin to *Bombyx mori* depends mainly on the irreversible binding to the midgut. We have investigated the possibility that reversibility is the major factor for the resistance in our resistant strain. Our dissociation experiment shows that the binding of CryIAc toxin is not reversible in either the susceptible or resistant strain (Fig. 6). These findings suggest that reversibility is not a determinant for resistance in this case.

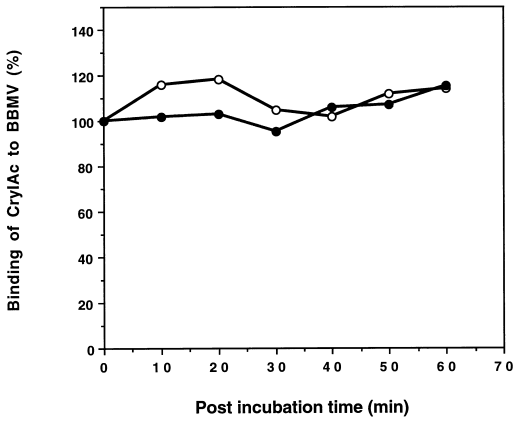

FIG. 6. Dissociation of CryIAc toxin from the BBMVs of susceptible (○) and resistant (●) strains. At 2 h after initiation of the association reaction, the incubation mixture was diluted with 500 nm of unlabeled CryIAc toxin. The time on the *x* axis represents postincubation time after addition of unlabeled toxin.

Several studies have demonstrated that CryIAc shows saturable and specific binding in vitro without showing toxicity in vivo to *Spodoptera frugiperda* (2) and *Lymantria dispar* (25). In another study, no significant changes in receptor-binding properties of CryIAc toxin to the resistant *H. virescens* strain were observed (3). These previous observations suggest the presence of nonfunctional receptors. In this present study, we have observed only a small reduction in binding-site concentrations (B_{max}) for CryIAb and CryIAc toxins in the resistant-strain midgut whereas binding affinities remained the same as in the susceptible strain, despite a high level of resistance to these toxins. These data indicate the presence of nonfunctional receptors. Possibly, the 170- and/or 150-kDa BBMV proteins are the most important receptors in toxin function, while the 120- and 80-kDa proteins are not important for toxicity. These nonfunctional receptors might not be directly involved in toxic function, presumably because of a lack of integration of the toxin into the cell membrane, a failure of pore formation, or the inhibition of toxin oligomerization. More knowledge of the genetics and biochemistry of the resistance mechanism in this *H. virescens* strain could be helpful in developing approaches to prevent the rapid onset of insect resistance to *B. thuringiensis.*

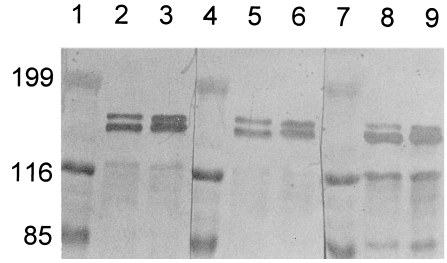

FIG. 7. Binding of biotin-labeled toxins to protein blots of *H. virescens* BBMV. BBMV proteins (10 μg) from the susceptible (lanes 2, 5, and 8) and resistant (lane 3, 6, and 9) strains were separated by SDS-PAGE (7.5% polyacrylamide) and transferred to a polyvinylidene difluoride membrane. Binding proteins were probed with 5 nM biotin-labeled CryIAa (lanes 2 and 3), CryIAb (lanes 5 and 6), and CryIAc (lanes 8 and 9) toxins. Lanes 1, 4, and 7 represent the protein molecular weight standard (molecular weights are given, in thousands, at the left of the figure).

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REFERENCES

1. **Ferre. J., M. D. Real, J. Van Rie, S. Jansens, and M. Peferoen.** 1991. Resistance to the Bacillus thuringiensis in a field population of Plutella xylostella is due to a change in a midgut membrane receptor. Proc. Natl. Acad. Sci. USA **88:**5119–5123.
2. **Garczynski, S. F., J. W. Crim, and M. J. Adang.** 1991. Identification of putative insect brush border membrane-binding molecules specific to *Bacillus thuringiensis* δ-endotoxin by protein blot analysis. Appl. Environ. Microbiol. **57:**2816–2820.

2a. **Gould, F., A. Anderson, A. Reynolds, L. Bumgarner, and W. Moar.** Genetic characterization of a *Heliothis virescens* (Lepidotera: Noctuidae) strain with high levels of resistance to some *Bacillus thuringiensis* toxins. J. Econ. Entomol., in press.

3. **Gould, F., A. Martinez-Ramirez, A. Anderson, J. Ferre, F. J. Silva, and W. J. Moar.** 1992. Broad-spectrum resistance to *Bacillus thuringiensis* toxins in *Heliothis virescens*. Proc. Natl. Acad. Sci. USA **89:**7986–7990.
4. **Hofmann, C., P. Luthy, R. Hutter, and V. Pliska.** 1988. Binding of the delta-endotoxin from *Bacillus thuringiensis* to brush-border membrane vesicles of the cabbage butterfly (*Pieris brassica*). Eur. J. Biochem. **173:**85–91.
5. **Ihara, H., E. Kudora, A. Wadana, and M. Himeno.** 1993. Specific toxicity of δ-endotoxins from *Bacillus thuringiensis* to *Bombyx mori*. BioSci. Biotechnol. Biochem. **57**(2)**:**200–204.
6. **Kirsch, K., and H. Schmutterer.** 1988. Low efficacy of a *Bacillus thuringiensis* (Berl.) formulation in controlling the diamondback moth, *Plutella xylostella* (L.) in the Philippines. J. Appl. Entomol. **105:**249–255.
7. **Knowles, B. H., M. R. Blatt, M. Tester, J. M. Horsnell, J. Carroll, G. Menestrina, and D. J. Ellar.** 1989. A cytolytic δ-endotoxin from *Bacillus thuringiensis* var. *israelensis* forms cation-selective channels in planar lipid bilayers. FEBS Lett. **244:**259–262.
8. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) **227:**680–685.
9. **Lee, M. K., R. E. Milne, A. Ge, and D. H. Dean.** 1992. Location of *Bombyx mori* receptor binding region of a *Bacillus thuringiensis* δ-endotoxin. J. Biol. Chem. **267:**3115–3121.
10. **MacIntosh, S. C., T. B. Stone, R. S. Jokerst, and R. L. Fuchs.** 1991. Binding of *Bacillus thuringiensis* proteins to a laboratory-selected line of *Heliothis virescens*. Proc. Natl. Acad. Sci. USA **88:**8930–8933.
11. **Marrone, P. G., and S. C. MacIntosh.** 1993. Resistance to *Bacillus thuringiensis* and resistance management, p. 221–235. *In* P. F. Entwistle, J. S. Cory, and S. Higgs (ed.), *Bacillus thuringiensis*, an environmental biopesticide: theory and practice. John Wiley & Sons, Chichester, England.
12. **McGaughey, W. H.** 1985. Insect resistance to the biological insecticide *Bacillus thuringiensis*. Science **229:**193–195.
13. **Munson, P. J., and D. Rodbard.** 1980. Data analysis and curve fitting for ligand binding experiments. Anal. Biochem. **107:**220–239.
14. **Oddou, P., H. Harmann, and M. Geiser.** 1991. Identification and characterization of *Heliothis virescens* midgut membrane proteins binding *Bacillus thuringiensis* δ-endotoxins. Eur. J. Biochem. **202:**673–680.
15. **Oppert, B., K. J. Kramer, and D. E. Johnson.** 1994. Altered protoxin activation by midgut enzymes from a *Bacillus thuringiensis* resistant strain of *Plodia interpuctella*. Biochem. Biophys. Res. Commun. **198:**940–947.
16. **Stone, T. B., S. R. Sims, and P. G. Marrone.** 1989. Selection of tobacco budworm for resistance to a genetically engineered *Pseudomonas fluorescens* containing the δ-endotoxin of *Bacillus thuringiensis* subsp. *kurstaki*. J. Invertebr. Pathol. **53:**228–234.
17. **Tabashnik, B. E., N. L. Cushing, N. Finson, and M. W. Johnson.** 1990. Field development of resistance to Bt transgenic plants protected from insect attack J. Econ. Entomol. **83:**1671–1676.
18. **Tabashnik, B. E., N. Finson, M. W. Johnson, and W. J. Moar.** 1993. Resistance to toxins from *Bacillus thuringiensis* subsp. *kurstaki* causes minimal cross-resistance to *B. thuringiensis* subsp. *aizawai* in the diamondback moth (Lepidoptera: Plutelidae) Appl. Environ. Microbiol. **59:**1332–1335.
19. **Vaeck, M., A. Reynaerts, H. Hofte, S. Jansenns, M. De Beuckeleer, C. Dean, M. Zabeau, M. Van Montagu, and J. Leemans.** 1987. Transgenic plants protected from insect attack. Nature (London) **327:**33–37.
20. **Van Rie, J., S. Jansens, H. Hofte, D. Degheele, and H. Van Mellaert.** 1989. Specificity of *Bacillus thuringiensis* δ-endotoxins: importance of specific receptors on the brush border membrane of the midgut of target insects. Eur. J. Biochem. **186:**239–247.
21. **Van Rie, J., S. Jansens, H. Hofte, D. Degheele, and H. Van Mellaert.** 1990. Receptors on the brush border membrane of the insect midgut as determinants of the specificity of *Bacillus thuringiensis* δ-endotoxins. Appl. Environ. Microbiol. **56:**1378–1385.
22. **Van Rie, J., W. H. McGaughey, D. E. Johnson, D. B. Barnett, and H. Van Mellaert.** 1990. Mechanism of insect resistance to the microbial insecticide *Bacillus thuringiensis*. Science **247:**72–74.
23. **Whiteley, H. R., and H. E. Schnepf.** 1986. The molecular biology of parasporal crystal body formation in *B. thuringiensis*. Annu. Rev. Microbiol. **40:**549–576.
24. **Wolfersberger, M., P. Luthy, A. Maurer, P. Parenti, V. F. Sacchi, B. Giordana, and G. M. Hanozet.** 1987. Preparation of brush border membrane vesicles (BBMV) from larval Lepidopteran midgut. Comp. Biochem. Physiol. **A86:**301–308.
25. **Wolfersberger, M. G.** 1990. The toxicity of two *Bacillus thuringiensis* δ-endotoxins to gypsy moth larvae is inversely related to the affinity of binding sites on midgut brush border membrane for the toxins. Experientia **46:**475–477.