

Different Mechanisms of Resistance to *Bacillus thuringiensis* Toxins in the Indianmeal Moth

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Susceptibility to protoxin and toxin forms of Cry1Ab and the binding of ¹²⁵I-labeled Cry1Ab and Cry1Ac has been examined in three *Plodia interpunctella* colonies, one susceptible (688^s) and two resistant (198^r and Dpl^r) to *Bacillus thuringiensis*. Toxicological studies showed that the 198^r colony was 11-fold more resistant to Cry1Ab protoxin than to Cry1Ab activated toxin, whereas the Dpl^r colony was 4-fold more resistant to protoxin versus toxin. Binding results with ¹²⁵I-labeled toxins indicated the occurrence of two different binding sites for Cry1Ab in the susceptible insects, one of them shared with Cry1Ac. Cry1Ab binding was found to be altered in insects from both resistant colonies, though in different ways. Compared with the susceptible colony, insects from the Dpl^r colony showed a drastic reduction in binding affinity (60-fold higher K_d), although they had similar concentrations of binding sites. Insects from the 198^r colony showed a slight reduction in both binding affinity and binding site concentration (five-fold-higher K_d and ca. three-fold-lower R_t compared with the 688^s colony). No major difference in Cry1Ac binding was found among the three colonies. The fact that the 198^r colony also has a protease-mediated mechanism of resistance (B. Oppert, R. Hammel, J. E. Throne, and K. J. Kramer, *J. Biol. Chem.* 272:23473–23476, 1997) is in agreement with our toxicological data in which this colony has a different susceptibility to the protoxin and toxin forms of Cry1Ab. It is noteworthy that the three colonies used in this work derived originally from ca. 100 insects, which reflects the high variability and high frequency of *B. thuringiensis* resistance genes occurring in natural populations.

Bacillus thuringiensis, a gram-positive entomopathogenic bacterium, produces different kinds of crystal inclusions during sporulation (22). These crystal inclusions are composed of one or various Cry proteins (also called δ -endotoxins or ICPs). Some of these proteins are highly toxic to certain insects, but they are harmless to most other organisms, including wildlife and beneficial insects.

The toxicity of *B. thuringiensis* crystal inclusions follows, after ingestion by the insect, a complex process including multiple steps. These include the (i) solubilization of the crystal to release the Cry proteins in their protoxin form, (ii) activation of the protoxins by midgut proteases to their active form, (iii) binding of the toxin to a midgut receptor, and (iv) pore formation. Insects that become resistant to *B. thuringiensis* do so by altering one or more steps of this process. Resistance to *B. thuringiensis* was first reported in *Plodia interpunctella* (11), and it was subsequently described in other insect species that have developed resistance to one or more Cry proteins. Thus, resistance to *B. thuringiensis* was found in field populations of *Plutella xylostella* and in laboratory-selected strains of *Heliothis virescens*, *Spodoptera exigua*, *Trichoplusia ni*, and other species (6, 25).

Knowledge of the mechanism of resistance is important in order to prolong the usefulness of *B. thuringiensis* commercial products, including transgenic plants expressing Cry proteins. The best-characterized mechanism of resistance is the alteration of binding of Cry proteins to their midgut receptors.

Some resistant strains of *P. interpunctella*, *P. xylostella*, and *H. virescens* have been shown to have lost (or have reduced) the capacity of binding Cry1A-type proteins (6, 25). A different mechanism involves alterations in the gut proteinase activities that interact with *B. thuringiensis* toxins and has been described for *P. interpunctella* and in *H. virescens*. Absence of a major gut protease associated with Cry1Ac protoxin activation was demonstrated in the 198^r colony of *P. interpunctella* (17, 18), which had been selected with *B. thuringiensis* subsp. *entomocidus* HD198 and became resistant to Cry1Ab and Cry1Ac (12, 13). Genetic studies in this colony revealed a linkage of the absence of this protease with resistance to *B. thuringiensis* (18). Strain CP73-3 from *H. virescens* showed that, compared to a susceptible control strain, there was slower processing of the Cry1Ac protoxin to the active toxin and faster degradation of the toxin (5); no reduction of Cry1Ac or Cry1Ab binding was detected in this strain (7). Finally, a mechanism involving faster damaged-cell repair has been proposed to be contributing to the resistance in the *H. virescens* CP73-3 strain (10).

In the present work, we examined the binding of Cry1Ab and Cry1Ac in three *P. interpunctella* colonies, one susceptible (688^s) and two resistant to these two Cry proteins (selected with their protoxin form) (13). The resistant colonies (198^r and Dpl^r) had been selected with different *B. thuringiensis* products (the subspecies *entomocidus* and *kurstaki*, respectively) and differed in their levels of resistance (five times higher in the Dpl^r colony) (19). The Dpl^r colony was 59 times more resistant to Cry1Ac than to Cry1Ab, whereas the 198^r colony was 29 times more resistant to Cry1Ac versus Cry1Ab. Additionally, the 198^r colony has a protease-mediated mechanism of resistance and the Dpl^r colony does not (16, 18). Our results indicate that Cry1Ab binding is altered in insects from both resis-

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TABLE 1. Toxicity of Cry1Ab protoxin (inclusion bodies) or chymotrypsin-activated Cry1Ab (soluble toxin) with *B. thuringiensis*-susceptible (688^s) and *B. thuringiensis*-resistant (198^r and Dpl^r) colonies of *P. interpunctella*^a

Cry1Ab	LD ₅₀ (CI) and RR values ^b for colony:				
	688 ^s (LD ₅₀)	Dpl ^r		198 ^r	
		LD ₅₀	RR	LD ₅₀	RR
Protoxin	0.208 (0.072–0.458)	218 (none)	1,049	54.81 (34.19–71.04)	264
Toxin	0.005 (0.002–0.012)	1.45 (none)	290	0.123 (0.062–0.227)	25

^a Tests were performed using a diet cube assay.

^b LD₅₀ values are expressed as micrograms per larvae, with the 95% confidence interval (CI) given in parentheses. The RR value is the LD₅₀ of the resistant colony/LD₅₀ of the 688^s colony.

tant colonies. However, this alteration is substantially different in the two colonies. Furthermore, the fact that the 198^r colony possesses two mechanisms of resistance has also been supported by bioassay tests with protoxin and toxin forms of Cry1Ab.

MATERIALS AND METHODS

Description of colonies. Colonies of *P. interpunctella* included the *B. thuringiensis*-susceptible colony 688^s, collected from farm grain storage in Riley County, Kans., in 1988 (12) and reared continuously in the laboratory on a cracked-wheat diet (14). The *B. thuringiensis*-resistant colonies Dpl^r and 198^r were selected from 688^s with *B. thuringiensis* subsp. *kurstaki* HD-1 (Dipel; Abbott Laboratories, Chicago, Ill.) and *B. thuringiensis* subsp. *entomocidus* HD-198, respectively. Resistant colonies were reared on cracked-wheat diets containing the *B. thuringiensis* formulation used for selection.

Toxin preparation. The Cry1Ab protoxin used for bioassays was an *Escherichia coli* recombinant protein obtained from Plant Genetic Systems (now Aventis, Ghent, Belgium). To obtain the activated form, inclusion bodies of Cry1Ab protoxin were solubilized and then incubated with α -chymotrypsin (bovine pancreas; Sigma Chemical Co., St. Louis, Mo.) 200:1 (wt/wt; protoxin-enzyme) for 2 h, 30°C. Protoxin and toxin forms were verified by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (data not shown). Cry1Ab and Cry1Ac toxins for binding assays were prepared from recombinant *B. thuringiensis* strains EG7077 and EG11070 (Ecogen, Inc.), respectively. Solubilization, activation, and purification of the Cry proteins was performed according to published protocols (21).

Bioassays. The procedure used was a modification of the single-larva bioassay (9). The diet consisted of 2.5 g of semihydrated cereal (Grape Nuts; Post), 2.5 g of wheat germ, 0.2 g of yeast, 9 mg of sorbic acid, 9 mg of methylparaben, 1.25 g of glycerin, and 1.25 g of water. After being mixed, the diet was flattened into a flat, thin "piecrust," and disks were removed with a 4-mm cork borer. The previous bioassay used dehydrated apple cubes and third-instar larvae (9), whereas neonate larvae will readily consume the cereal-based diet used in the present bioassay. Diet cubes were treated with either suspensions of Cry1Ab protoxin inclusions or Cry1Ab toxin solutions placed in 16-well assay trays. Eggs were added to each well. Mortality was calculated from the number of survivors from treated samples compared with untreated controls at 14 days posthatching. For each dose, 16 larvae were used. Statistical analyses were made using the program POLO-PC (20).

Binding assays. Brush border membrane vesicles (BBMV) were prepared from whole last-instar larvae by the differential magnesium precipitation method (4, 28) and then frozen in liquid nitrogen and kept at -80°C until used. The protein concentration in the BBMV was determined by the method of Bradford (3).

Cry1Ab and Cry1Ac were ¹²⁵I labeled by the chloramine-T method (26). Binding assays were performed essentially as described previously (24), in a final volume of 0.1 ml of binding buffer (8 mM Na₂HPO₄; 2 mM KH₂PO₄; 150 mM NaCl, pH 7.4; 0.1% bovine serum albumin) containing various concentrations of BBMV and a concentration of 1.25 nM ¹²⁵I-labeled Cry1Ab or 0.60 nM ¹²⁵I-labeled Cry1Ac. Incubations were carried out at room temperature for 60 min. An excess of unlabeled toxin was used to determine the extent of nonspecific binding, which was ca. 1% of the total radioactivity. For ¹²⁵I-Cry1Ab competition experiments, the reaction mixture contained 7.5, 10, or 15 μ g of BBMV proteins from the 688^s, 198^r, or Dpl^r colonies, respectively. When using ¹²⁵I-Cry1Ac, competitions were performed using 5 μ g of BBMV proteins from either colony. Bound toxins were separated from unbound toxins by filtration through glass-

fiber filters. Cold binding buffer (5 ml/filter) was used to wash the filters, and the radioactivity retained was measured in a model 1282 Compugamma CS gamma counter (LKB Pharmacia). Binding parameters were obtained using the LI-GAND computer program (15).

RESULTS

Susceptibility to Cry1Ab protoxin and toxin. The susceptibilities of larvae from 688^s, Dpl^r, and 198^r colonies to the protoxin and chymotrypsin-activated (toxin) forms of Cry1Ab are shown in Table 1. In the Dpl^r colony the resistant ratio (RR; 50% lethal dose [LD₅₀] of the resistant colony divided by the LD₅₀ of the susceptible colony) was fourfold higher with Cry1Ab protoxin than with toxin. However, larvae from the 198^r colony showed a significantly higher resistance ratio toward the protoxin (RR = 264) than toward the toxin form (RR = 25). The LD₅₀ values were consistently higher than previously reported values (13), presumably due to the difference in assay procedures. The previously obtained LD₅₀ used third-instar larvae, whereas the LD₅₀ in this report were obtained with neonate larvae.

Binding of labeled toxins to BBMV. Specific binding of Cry1Ab and Cry1Ac to BBMV from insects of the 688^s, 198^r, and Dpl^r colonies was tested by incubation of ¹²⁵I-labeled toxins with various concentrations of BBMV. Saturable binding was found with both toxins for all three colonies. Maximum specific binding of ¹²⁵I-Cry1Ab was ca. 2, 1.5, and 1% of the total radioactivity for BBMV from the 688^s, 198^r, and Dpl^r colonies, respectively (Fig. 1A). The maximum specific binding of ¹²⁵I-Cry1Ac to BBMV from all three colonies was ca. 1.3% of the total radioactivity added (Fig. 1B).

Homologous competition experiments. Homologous competition of ¹²⁵I-Cry1Ab was performed to obtain quantitative estimates of the equilibrium dissociation constant (K_d) and the binding site concentration (R_t). Compared with BBMV from the 688^s colony, Dpl^r showed a drastic reduction in binding affinity (60-fold-higher K_d), although a similar R_t value (Table 2 and Fig. 2). The 198^r colony also showed altered Cry1Ab binding compared with the 688^s colony, with a tendency for reduced binding affinity (fivefold-higher K_d , though not significantly different) and a slight significant reduction in the binding site concentration (threefold-lower R_t). The changes in the two parameters add up giving a decrease in overall binding affinity of 16-fold (R_t/K_d). In contrast, homologous competition of ¹²⁵I-Cry1Ac just showed minor differences in binding among BBMV from the three colonies (Table 3 and Fig. 3). K_d and R_t values from 688^s and Dpl^r were not significantly differ-

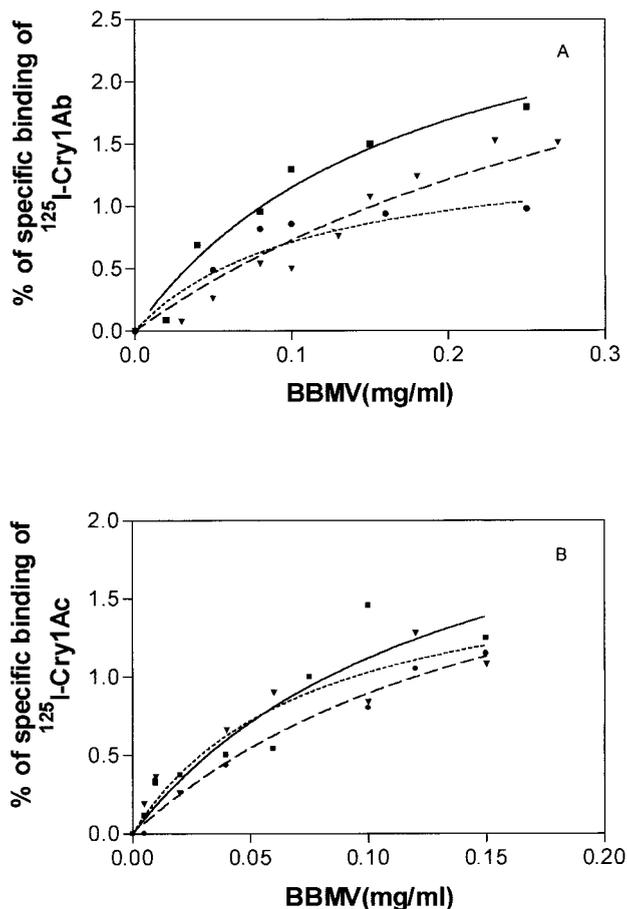


FIG. 1. Specific binding of Cry1Ab (A) and Cry1Ac (B) as a function of *P. interpunctella* BBMV concentration. Nonspecific binding values were subtracted from each datum point. Lines: solid (■), 688^s; dotted (●), Dpl^f; broken (▼), 198^f.

ent, and the decrease in affinity of 198^f, compared to 688^s, is compensated for by the increase in binding site concentration.

Heterologous competition experiments. Incubation of a fixed amount of ¹²⁵I-Cry1Ab with increasing concentrations of unlabeled Cry1Ac showed that both toxins competed for a common binding site in BBMV from the three colonies (Fig. 2). In the 688^s colony, Cry1Ac competed for up to 50% of ¹²⁵I-Cry1Ab specific binding (Fig. 2A), indicating the occurrence of two different binding sites for Cry1Ab, one shared with Cry1Ac and the other not shared. In contrast, total competition of specific binding was obtained when BBMV from the Dpl^f and 198^f colonies were used (Fig. 2B and C).

TABLE 2. Binding parameters from homologous competition experiments with ¹²⁵I-Cry1Ab and BBMV from three *P. interpunctella* colonies

Colony	K _d (nM) (SEM)	R _t (pmol/mg) (SEM)
688 ^s	0.25 (0.12)	2.11 (0.70)
Dpl ^f	14.48 (9.42)	2.25 (0.38)
198 ^f	1.31 (1.00)	0.67 (0.32)

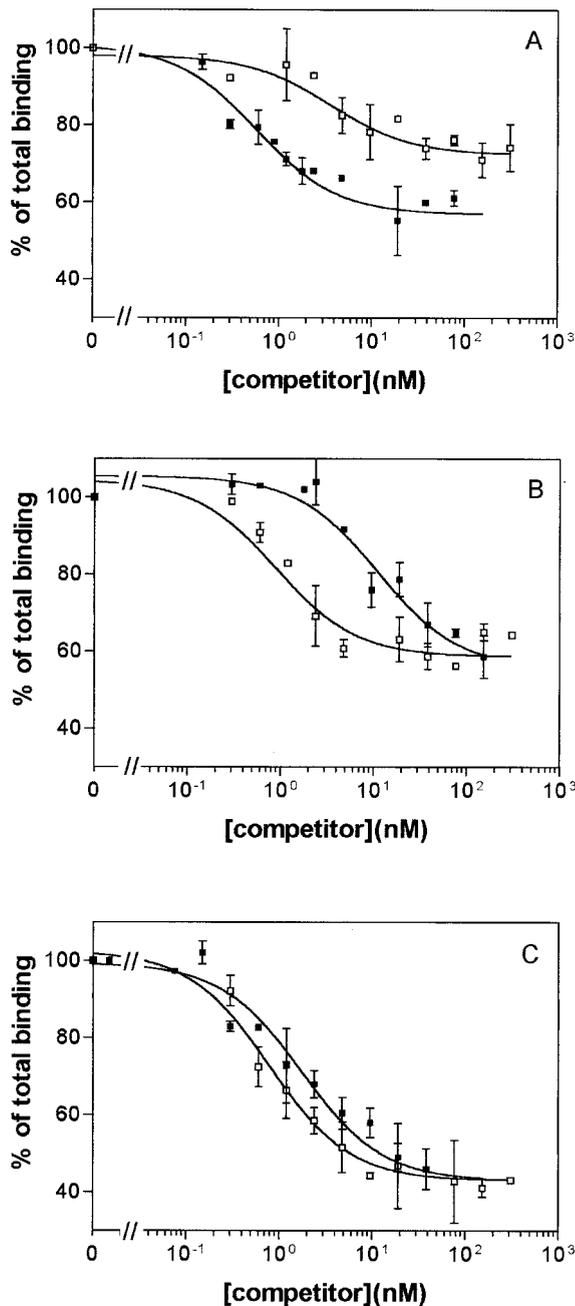


FIG. 2. Binding of ¹²⁵I-Cry1Ab to *P. interpunctella* BBMV at increasing concentrations of unlabeled competitor (Cry1Ab [■] and Cry1Ac [□]). Each point represents the mean of two independent experiments. Panels: A, 688^s; B, Dpl^f; C, 198^f.

DISCUSSION

The three colonies used in the present study were derived from ca. 100 adult insects (colony RC-688) collected from a farm grain storage bin with no known previous exposure to *B. thuringiensis* (12). The 688^s colony was left unselected, Dpl^f was selected with Dipel (*B. thuringiensis* subsp. *kurstaki* HD-1 formulation), and 198^f was selected with *B. thuringiensis* subsp. *entomocidus* HD-198. Previous studies with these colonies re-

TABLE 3. Binding parameters from homologous competition experiments with ^{125}I -Cry1Ac and BBMV from three *P. interpunctella* colonies

Colony	K_d (nM) (SEM)	R_t (pmol/mg) (SEM)
688 ^s	0.21 (0.06)	0.20 (0.09)
Dpl ^r	0.13 (0.02)	0.19 (0.01)
198 ^r	0.48 (0.01)	0.37 (0.16)

vealed that 198^r insects lacked a major gut proteinase that takes part in activation of Cry1Ac protoxin, whereas no difference in proteinase activity was found between Dpl^r and 688^s (18).

Our bioassay results, using protoxin and toxin forms of Cry1Ab, show that levels of resistance in the 198^r colony were different depending on the toxin form employed. Thus, the RR for Cry1Ab protoxin was 11-fold higher than the RR for activated Cry1Ab. This is in agreement with the biochemical feature previously reported for this colony and suggests that both Cry1Ab and Cry1Ac protoxins are processed by the same gut proteinase. In addition, the 198^r colony was also found to be resistant to activated Cry1Ab (25-fold compared to the 688^s colony). The RR of the 198^r colony is 264-fold with the protoxin form and 25-fold with the toxin form of Cry1Ab, an indication that ca. 10% of the total resistance to the Cry1Ab protoxin is due to a mechanism unrelated with the toxin activation. Unexpectedly, the Cry1Ab toxin form used did somewhat influence the resistance levels in the Dpl^r colony. However, the effect of activated toxin was more dramatic with the 198^r colony. Recently, we have found insects from the Dpl^r colony with proteinase patterns corresponding to insects from the 198^r colony. This suggests a slight contamination of the Dpl^r colony with 198^r insects, which would explain the difference in the RR values between protoxin and activated toxin in the Dpl^r colony.

In the present work we have studied differences in binding parameters as a plausible mechanism of resistance in the Dpl^r and 198^r colonies. Although the total absence of binding was not found, quantitative differences in the binding parameters

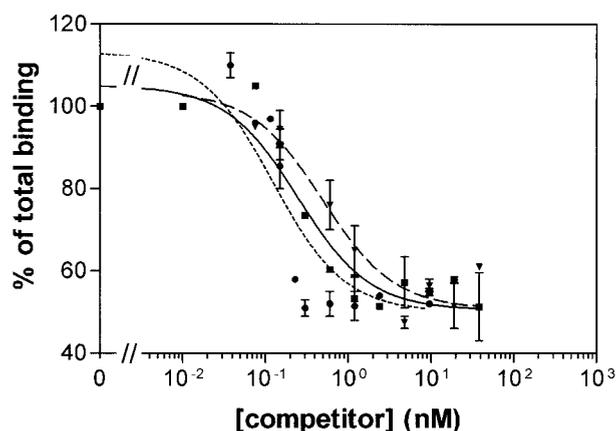


FIG. 3. Binding of ^{125}I -Cry1Ac to *P. interpunctella* BBMV at increasing concentrations of unlabeled Cry1Ac. Each point represents the mean of two independent experiments. Lines: solid (■), 688^s; dotted (●), Dpl^r; broken (▼), 198^r.

of Cry1Ab were detected. Insects from the Dpl^r colony showed a 60-fold reduction in Cry1Ab binding affinity compared to the 688^s colony. This result indicates that the main mechanism of resistance in this colony is due to the alteration of a binding site. It is interesting to note that a similar result was previously reported in a different *P. interpunctella*-resistant colony that had also been selected with Dipel (27). Insects from this colony developed resistance to Cry1Ab and showed a reduction in binding affinity of this toxin of ca. 50-fold. Binding experiments of ^{125}I -Cry1Ab with BBMV from the 198^r colony showed a slightly higher K_d and a lower R_t than the susceptible colony. Compared to the susceptible insects, the 198^r insects show a decrease in the overall binding affinity for Cry1Ab (estimated as the R_t/K_d ratio), which may account for the 25-fold resistance of this colony to the activated form of the Cry1Ab toxin.

Strong reduction or absence of toxin binding has been correlated with resistance to Cry1A toxins in insects from other Lepidoptera species, such as *P. xylostella* and *H. virescens* (25). Tabashnik et al. (23) have called "mode 1" of resistance to *B. thuringiensis* to be characterized by "extremely high resistance to at least one Cry1A toxin, recessive inheritance, little or no cross-resistance to Cry1C, and reduced binding of at least one Cry1A toxin." The Dpl^r colony shows high resistance to Cry1Ab, extremely high resistance to Cry1Ac, and no cross-resistance to Cry1C (13), as well as reduced binding of Cry1Ab. Although no genetic data have been obtained for this colony, the features described above suggest that this colony could also belong to the "mode 1" resistance type.

Heterologous competition of ^{125}I -Cry1Ab with unlabeled Cry1Ac showed that both toxins share a common binding site in *P. interpunctella*. However, the binding of ^{125}I -Cry1Ac was not significantly affected in the Dpl^r and 198^r colonies. It is possible that the same change in the Cry1A binding site had different effects on Cry1Ab and Cry1Ac binding. Whereas Cry1Ab affinity would be reduced, the change could only affect postbinding steps of the Cry1Ac mode of action, such as membrane insertion or pore formation. A similar situation has been found in two resistant strains of *P. xylostella* for which the Cry1A common binding site was altered in such a way that Cry1Ab binding was reduced, whereas the binding of Cry1Ac was not affected (2, 24, 29).

In the susceptible colony, Cry1Ac was unable to completely compete with ^{125}I -Cry1Ab specific binding, indicating that not all Cry1Ab binding sites are accessible to Cry1Ac (Fig. 2A). This result means that Cry1Ab binds at least to two distinct sites in BBMV from the susceptible colony. The fact that the Cry1Ab datum points adjusted better to a one-binding-site model than to a two-binding-site model suggests similar binding parameters for the two sites. This is also in agreement with previous results with this insect species, for which one binding site for Cry1Ab was proposed from homologous competition experiments (27). In the resistant insects, Cry1Ac completely competed with ^{125}I -Cry1Ab specific binding (Fig. 2B and C), which suggests that insects from the two resistant colonies have lost one of the Cry1Ab binding sites. Furthermore, the binding site alteration must be different in these two resistant colonies since Cry1Ab binding parameters and resistance levels differ considerably.

It is important to stress the fact that at least three different resistance alleles and/or genes occurred in the original popu-

lation from which the three colonies in the present study were derived. Moreover, two of the resistance alleles and/or genes were coselected in the 198^r colony. Since the original population consisted of approximately 100 insects (12), the frequency of every resistance allele in the original population should be at least 0.005 (1 in 200 copies of the gene). This frequency is in agreement with estimates obtained in other insect species for *B. thuringiensis*-resistant genes (1, 8, 25). However, in the case of the original population of *P. interpunctella*, this estimate applies to every one of the three different resistance alleles detected.

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