APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Apr. 2002, p. 1509–1515 Vol. 68, No. 4
0099-2240/02/$04.00+0 DOI: 10.1128/AEM.68.4.1509–1515.2002

Role of *Bacillus thuringiensis* Cry1 δ Endotoxin Binding in Determining Potency during Lepidopteran Larval Development

Androulla Gilliland,* Catherine E. Chambers, Eileen J. Bone, and David J. Ellar

Department of Biochemistry, University of Cambridge, Cambridge CB2 1GA, United Kingdom

Received 15 October 2001/Accepted 10 January 2002

Five economically important crop pests, *Manduca sexta*, *Pieris brassicae*, *Mamestra brassicae*, *Spodoptera exigua*, and *Agrotis ipsilon*, were tested at two stages of larval development for susceptibility to *Bacillus thuringiensis* toxins Cry1Ac, Cry1Ca, Cry1J, and Cry1Ba. Bioassay results for *M. sexta* showed that resistance to all four Cry toxins increased from the neonate stage to the third-instar stage; the increase in resistance was most dramatic for Cry1Ac, the potency of which decreased 37-fold. More subtle increases in resistance during larval development were seen in *M. brassicae* for Cry1Ca and in *P. brassicae* for Cry1Ac and Cry1J. By contrast, the sensitivity of *S. exigua* did not change during development. At both larval stages, *A. ipsilon* was resistant to all four toxins. Because aminopeptidase N (APN) is a putative Cry1 toxin binding protein, APN activity was measured in neonate and third-instar brush border membrane vesicles (BBMV). With the exception of *S. exigua*, APN activity was found to be significantly lower in neonates than in third-instar larvae and thus inversely correlated with increased resistance during larval development. The binding characteristics of iodinated Cry1 toxins were determined for neonate and third-instar BBMV. In *M. sexta*, the increased resistance to Cry1Ac and Cry1Ba during larval development was positively correlated with fewer binding sites in third-instar BBMV than in neonate BBMV. The other species-instar-toxin combinations did not reveal positive correlations between potency and binding characteristics. The correlation between binding and potency was inconsistent for the species-instar-toxin combinations used in this study, reaffirming the complex mode of action of Cry1 toxins.

The δ endotoxins are a family of insecticidal proteins produced by *Bacillus thuringiensis* during sporulation. These toxins are typically found in parasporal crystals that are released into the environment with the bacterial spores. Numerous δ endotoxins produced by *B. thuringiensis* have been identified and are grouped on the basis of sequence homology and insect specificity (40). The Cry1 toxins are a group of δ endotoxins that principally target lepidopteran species, including several important crop pests.

The mechanism of action of the Cry1 δ endotoxins begins with solubilization of the protoxin in the alkaline larval midgut, followed by proteolytic processing by midgut proteases (40). The stable 60- to 65-kDa toxins then bind to midgut receptors and insert into the apical membrane of brush border epithelial cells to form pores. These pores disrupt functional membrane processes and are ultimately responsible for larval death (40).

Each type of Cry1 toxin has a unique spectrum of activity and targets only a small range of lepidopteran species. Within the small target ranges there are dramatic differences in potency between species that are often closely related (12, 15, 31). Indeed, the potency of a Cry1 toxin can significantly decrease as the larvae age (1, 38). Variations in the potencies of Cry1 toxins for different lepidopteran species and different larval stages may reflect differences in any one of the prebinding, binding, and pore-forming events required for full toxicity. By discovering why Cry1 toxins exhibit such diverse potencies during larval development or for different species, it may be possible to engineer more potent toxins.

Early binding studies with purified radiolabeled Cry1 δ endotoxins and midgut brush border membrane vesicles (BBMV) showed that toxin-specific binding proteins are present and essential for toxicity (14, 48). The majority of Cry1 toxin binding proteins have been identified as aminopeptidases. Aminopeptidase N (APN) has been identified as a binding protein for the Cry1Ac toxin in *Manduca sexta*, *Heliothis virescens*, *Lymantria dispar*, and *Plutella xylostella* (9, 20, 33, 45). APN has also been identified as a Cry1Aa binding protein in *Bombyx mori* (18) and as a Cry1C binding protein in *M. sexta* (32). Other toxin binding proteins have been identified as cadherin-like glycoproteins that bind Cry1Aa and Cry1Ab in *B. mori* and *M. sexta*, respectively (36, 43, 44).

In this study, we were principally interested in assessing if Cry1 toxin binding is a key factor in determining differences in potency during larval aging. Five economically important crop pests, *M. sexta*, *Pieris brassicae*, *Mamestra brassicae*, *Spodoptera exigua*, and *Agrotis ipsilon*, were tested to determine their susceptibilities and characteristics of binding to Cry1Ac, Cry1Ca, Cry1J, and Cry1Ba at the neonate and third-instar stages of larval development. Our data show that for some, but not all, species-instar-toxin combinations, Cry1 toxin binding can indeed be a very strong indicator of potency.

MATERIALS AND METHODS

Bacterial species and plasmids. All toxin genes used in this study were cloned into the mutagenic shuttle vector pMSV1. This vector is a modified version of pAlter (Promega) in which the tetracycline resistance gene has been replaced by the chloramphenicol resistance gene and the *Staphylococcus aureus* origin of replication from pC194 in a manner similar to that used for the construction of

* Corresponding author. Present address: Department of Plant Sciences, University of Cambridge, Downing Street, Cambridge CB2 3EA, United Kingdom. Phone: 44 (0) 1223 330215 or 44 (0) 1223 330224. Fax: 44 (0) 1223 333900. E-mail: ak10017@cus.cam.ac.uk.

pSV1 (7). The Cry1J gene was cloned from *B. thuringiensis* subsp. *morrisoni* II808 by introducing two *Hin*dIII sites just before and after the Cry1J gene (46). The Cry1Ba gene was previously cloned from *B. thuringiensis* YBT-226 (52). The 8.4-kb *Sal*I-*Bam*HI fragment encoding the gene was subcloned into pMSV1 to create pMSV1Ba (this study). Cry1Ac was subcloned from pSVJWKa (22) into pMSV1 by introducing a *Hin*cII site downstream of the coding region. The 4-kb *Hin*cII fragment was then inserted into the multiple cloning site of pMSV1 (41). Cry1Ca was cloned from *B. thuringiensis* subsp. *aizawai* HD-229 using a 5.8-kb *Eco*RI fragment encoding the full-length gene.

Plasmids pMSV1Ac, pMSV1Ba, pMSV1Ca, and pMSV1J were introduced by electroporation into the acrystalliferous host *B. thuringiensis* subsp. *israelensis* 78/11 (49), and crystals containing toxin were recovered using sucrose density gradients by the method of Thomas and Ellar (42). Toxin yield was quantified by the method of Lowry et al. (29) using bovine serum albumin (BSA) as the standard.

Insects. Insects were supplied as eggs by various laboratories as described below. Larvae were reared at 21 to 25°C by using cycles consisting of 16 h of light and 8 h of darkness and artificial diets. *M. sexta* (3), *S. exigua* (46), *M. brassicae* (8), and *A. ipsilon* (8) were raised on previously described diets. *P. brassicae* was raised on the *M. brassicae* diet supplemented with 16.2 g of dried organic cabbage powder per liter. *M. sexta* eggs were purchased from S. Reynolds (School of Biological Sciences, University of Bath, Bath, United Kingdom). *M. brassicae* and *S. exigua* eggs were purchased from T. Carty (N.E.R.C. Institute of Virology & Environmental Microbiology, Oxford, United Kingdom). *S. exigua* eggs were also kindly donated by DuPont. *P. brassicae* eggs were a gift from P. Jarrett (Horticultural Research International). *A. ipsilon* eggs were purchased from French Agricultural Research Inc., Lamberton, Minn.

Bioassays. Bioassays were performed with neonate and third-instar larvae. For the bioassays, protoxin crystals were added to molten (55°C) diet and vortexed to mix the preparations thoroughly. The diet was then added to 1.5-ml Eppendorf tubes in 100-μl aliquots (neonate *M. brassicae* larvae and neonate *S. exigua* larvae), to 1.5-ml Eppendorf tubes in 500-μl aliquots (third-instar *S. exigua* larvae), to petri dishes in 5- to 10-ml aliquots (neonate *A. ipsilon* larvae and neonate and third-instar *P. brassicae* larvae), to 48-well culture plates in 300-μl aliquots (neonate *M. sexta* larvae), to individual wells of insect trays in 4- to 5-ml aliquots (third-instar *M. sexta* larvae and *A. ipsilon* larvae), and in 1-ml aliquots to 7-ml Greiner tubes (third-instar *M. brassicae* larvae). To prevent cannibalism, one larva of *M. sexta, M. brassicae*, or *S. exigua* or one third-instar larva of *A. ipsilon* was added to each aliquot of medium. *P. brassicae* and neonate *A. ipsilon* larvae were typically assayed in groups consisting of 20 individuals. Larval mortality and weight loss were assessed after 6 days, and 50% lethal concentrations (LC_{50}) were determined by using the program of Lieberman (26) for probit analysis (11) and the results of at least three separate experiments in which there was less than 10% mortality of the controls.

Isolation of BBMV. To determine whether differences in toxicity during larval development could be attributed to changes in binding characteristics, BBMV were prepared from neonate, third-instar, and fifth-instar larvae by differential centrifugation using the method of Carroll and Ellar (6). Escriche et al. (10) produced BBMV from whole and dissected midguts of *Phthorimaea operculella* and *P. xylostella* and found no significant differences in ^{125}I binding characteristics, irrespective of the insect starting material, for either species. With this in mind, whole larvae were used to prepare BBMV from neonate larvae, since they were too small for dissection. So that data would be directly comparable, BBMV from third-instar larvae were also prepared from whole larvae. Dissected midguts were used to prepare BBMV from fifth-instar larvae. Whole larvae and dissected guts were collected over a period of several weeks, frozen in buffer A (250 mM sucrose, 5 mM Tris-HCl; pH 8) in liquid nitrogen, and stored at −70°C. The small biomass of the neonates limited the size of the BBMV preparation. Consequently, the preparations for all larval stages were scaled down to ensure that comparisons were relevant. The starting material was 0.5 g of material for all species except *M. sexta*, for which 2 g was used. The small working volumes were handled in 2-ml Eppendorf tubes and were centrifuged by using a fixed-angle rotor (Jouan MR22I). The final BBMV pellets were resuspended in 100 μl of buffer A and stored in aliquots at −20°C. Protein concentrations of the BBMV preparations were determined by the method of Bradford (4) with a Bio-Rad protein assay solution. Before BBMV were used in binding experiments, they were rapidly thawed at 37°C and placed on ice.

APN assays. BBMV preparations were diluted (10 to 100 μg/ml) with buffer containing 250 mM sucrose and 5 mM Tris-HCl (pH 8) and were assayed for APN activity at 25°C by monitoring the change in absorbance at 405 nm using the chromogenic substrate L-leucine-*p*-nitroanilide (Sigma) (13). Standard concentrations of *p*-nitroanilide (Sigma) were used to calculate specific enzyme activities, and the absorption coefficient of *p*-nitroanilide was taken to be 9.9 liters·mmol^{-1}·cm^{-1}. Assays were done in the presence and in the absence of 0.1% (vol/vol) Triton X-100 to reveal any latent activity.

Toxin activation and purification. Unlabeled protoxins used for competition assays were solubilized at room temperature in 50 mM Na_2CO_3 (pH 10)–10 mM dithiothreitol (DTT) for 1 h. Insoluble material was removed by centrifugation at 12,000 × *g*. Cry1Ac, Cry1Ca, and Cry1J (0.5 to 1 mg/ml) were digested for 2 h at room temperature with *N*α-*p*-tosyl-L-lysine chloromethyl ketone (TLCK)-treated trypsin (Sigma) at a concentration of 0.2 mg/ml. Cry1Ba was digested with 2% (vol/vol) *P. brassicae* gut extract (21) for 2 h at room temperature. Insoluble precipitate was removed by centrifugation at 12,000 × *g*, and the activated toxins were quantified by the method of Bradford (4) using Bio-Rad protein assay solution with BSA as the standard. Quantification and activation were confirmed by resolving the toxins and BSA standards by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Activated toxins were further purified by column chromatography before they were labeled with Na^{125}I.

Column purification of Cry1 toxins. Activated Cry1Ac was purified by an established protocol (5) that involves anion-exchange fast protein liquid chromatography with an HR5/5 column (Pharmacia) packed with 1 ml of Source 15Q (Pharmacia). This protocol was found to be unsuitable for purification of the other three toxins; in particular, the nonionic buffer required for the column resulted in significant precipitation of Cry1Ca, Cry1J, and Cry1Ba. A new purification protocol was, therefore, developed during this study.

Eighty milligrams of each protoxin was activated in Na_2CO_3–10 mM DTT–1% (vol/vol) *P. brassicae* gut extract at pH 10 for 1 h, and this was followed by treatment with DNase I (2 mg) for 30 min. For Cry1Ca a prototype method in which low-pressure adsorption chromatography was used was developed. The toxin was applied to a 10-ml column of hydroxyapatite resin (HA-ultragel; Sigma), which was washed with water to elute nonadsorbing protein. Basic proteins were then eluted with a high-salt buffer (1 mM Na_2CO_3, 1 mM Na_2HPO_4, 0.9 M NaCl; pH 8.6). The protein was further eluted with a low-phosphate buffer (10 mM Na_2CO_3, 10 mM Na_2HPO_4; pH 8.6), followed by a high-phosphate buffer (500 mM Na_2HPO_4, 10 mM Na_2CO_3; pH 8.6), using a one-step gradient. Cry1Ca eluted with the low-phosphate buffer.

To further improve the purification procedure, fast protein liquid chromatography with a Waters 650E system was employed. Prior to adsorption chromatography a size fractionation step with a Superose 12 HR 10/30 column (Pharmacia) was used to remove any small peptides that might have interfered with purification. Toxins were eluted from this column with 1 mM Na_2CO_3–1 mM Na_2HPO_4–5 mM DTT (pH 10). Adsorption chromatography was performed with an XK 16/20 column (Pharmacia) packed with 15 ml of 40-μm hydroxyapatite. Activated toxins were loaded directly from the Superose 12 column, and the low-phosphate buffer was applied until all the unbound protein was eluted. Basic proteins were then eluted with a high-salt buffer before a gradient starting with low-phosphate buffer and ending with high-phosphate buffer was established. The toxin eluted several minutes after the gradient had finished.

Iodination of toxins. Column-purified toxins were iodinated by using the IODO-BEAD (Pierce) method according to the manufacturer's instructions. For each toxin two iodobeads were preincubated in 200 μl of binding buffer (phosphate-buffered saline [PBS]) (8 mM Na_2HPO_4, 2 mM KH_2PO_4, 150 mM NaCl; pH 7.4) containing 1 mCi of Na^{125}I (Amersham) for 5 min. Toxins were added at the following concentrations with the following buffers, depending on the method of purification: 25 μg of Cry1J in 800 μl of 10 mM Na_2CO_3–500 mM Na_2HPO_4 (pH 8.6) and 120 μg of Cry1Ba in 400 μl of the same buffer; 100 μg of Cry1Ac in 400 μl of 20 mM piperazine–0.6 M NaCl (pH 9.5); and 25 μg of Cry1Ca in 800 μl of 10 mM Na_2CO_3–10 mM Na_2HPO_4 (pH 8). Toxins were incubated with the Na^{125}I for 15 min, and free iodine was removed by using PD-10 columns (Pharmacia) equilibrated with PBS. The specific activities of the radiolabeled preparations were as follows: Cry1Ac, 53 Ci/mmol; Cry1Ca, 23 Ci/mmol; Cry1Ba, 20 Ci/mmol; and Cry1J, 157 Ci/mmol.

Saturation binding experiments. Saturation binding experiments were done to determine the percentage of radiolabeled toxin capable of binding if toxin binding was specific and to identify a suitable concentration of BBMV to use in subsequent homologous competition experiments. ^{125}I-labeled toxins were added to duplicate samples at fixed concentrations (Cry1Ac, 0.9 nM; Cry1Ca, 0.39 nM; Cry1Ba, 1.08 nM; and Cry1J, 0.12 nM) and were equilibrated at room temperature with and without 1,000 nM unlabeled toxin. Various concentrations of BBMV (0 to 0.5 mg/ml) were added to initiate the reaction. Pellets were collected and radioactivity was quantified as described above for the homologous competition experiments. The percentages of radioligands capable of total binding were as follows: Cry1Ac, 10%; Cry1Ca, 1.5%; Cry1Ba, 11%; and Cry1J, 14%.

Homologous competition. BBMV were incubated with ^{125}I-labeled toxin in 200 μl (final volume) of PBS containing 0.1% (wt/vol) BSA (Sigma). Prior to the addition of BBMV, ^{125}I-labeled toxins were added at fixed concentrations

TABLE 1. LC_{50} values of Cry1 δ endotoxins administered to neonate and third-instar lepidoptera

Toxin	Larvae	*M. sexta*			LC_{50} (μg/ml) for *P. brassicae*	*M. brassicae*			*S. exigua*			*A. ipsilon*		
		LC_{50} (μg/ml)	% Mortality	% Weight loss		LC_{50} (μg/ml)	% Mortality	% Weight loss	LC_{50} (μg/ml)	% Mortality	% Weight loss	LC_{50} (μg/ml)	% Mortality	% Weight loss
Cry1J	Neonate[a]	2.15 (1.85–2.58)[c]			5.23 (3.38–8.27)	>150	5	83	34.99 (21.86–59.12)			>200	12	48
	Third instar[b]	5.09 (3.98–6.63)			76.42 (66.06–91.48)	>150	0	3	52.94 (10.65–84.62)			>200	0	4
Cry1Ca	Neonate	0.889 (0.732–1.07)			12.97 (9.87–18.98)	3.5 (2.83–4.50)			23.84 (19.29–29.23)			>200	0	3
	Third instar	5.56 (4.15–7.61)			14.63 (9.71–21.62)	>50	3	90	20.32 (5.88–36.46)			>200	0	8
Cry1Ac	Neonate	0.033 (0.022–0.049)			0.165 (0.12–0.27)	>150	20	94	105.79 (82.63–160.8)			>200	40	90
	Third instar	1.24 (0.95–1.65)			0.872 (0.53–2.25)	>150	20	34	116.16 (84.14–214.2)			>200	0	16
Cry1Ba	Neonate	42.40 (32.26–53.39)			0.20 (0.146–.273)	>150	0	20	>150	0–10	0–40	>200	0	0
	Third instar	>150	0	84	0.306 (0.212–0.391)	>150	0	0	>150	0–10	0–20	>200	0	0

[a] BBMV were prepared from whole neonate larvae.
[b] BBMV were prepared from whole third-instar larvae.
[c] The values in parentheses are 95% confidence intervals.

(Cry1Ac, 0.9 nM; Cry1Ca, 0.39 nM; Cry1Ba, 1.08 nM; and Cry1J, 0.12 nM) together with different concentrations of unlabeled toxin as the homologous competitor (0 to 2,000 nM). Most reactions were initiated by adding BBMV to a final concentration of 0.1 mg/ml; the exceptions were the *S. exigua* reaction mixtures, in which the final concentration was 0.05 mg/ml. The preparations were equilibrated at room temperature for 45 min without shaking, and unbound toxin was separated from bound toxin by centrifugation at 13,500 × *g* for 10 min. Each pellet containing bound toxin was washed twice with PBS containing 0.1% (wt/vol) BSA. The radioactivity in the pellet was measured with a gamma counter (Cobra II; Packard). Eight concentrations of unlabeled toxin were prepared in duplicate, and the experiments were repeated twice.

Analysis of binding experiments. Purification protocols designed to remove contaminants from activated toxins have been described previously (14, 30, 31); however, radiolabeling of Cry toxins remains problematic. Typically, small amounts of contaminating peptides are labeled with disproportionate ease, resulting in mixtures in which the labeled ligand accounts for a fraction of the total radioactivity (2, 16, 30, 47). Under these circumstances the specific activity of the tracer is overestimated, which results in underestimation of the total binding site concentration (B_{max}). This artifact can be partially corrected by using saturation binding experiments to determine the percentage of radiolabeled mixture that is capable of receptor binding (17) and adjusting the specific activity of the toxin accordingly.

Competition binding data were analyzed using the software package KELL for Windows (version 6; Biosoft, Cambridge, United Kingdom). The data were first processed using RADLIG (34), which estimated the competition constant (K_{com}) (nanomolar) and the B_{max} (picomoles per milligram). The results of repeated experiments were then coanalyzed by using LIGAND (35) to determine the final binding constants, K_{com}, and B_{max}. Liang et al. (25) proposed a kinetic analysis for Cry1 binding that considered both reversible and irreversible binding. It was suggested that the K_d value commonly calculated from homologous binding experiments should be designated K_{com} to indicate the contribution of both the equilibrium constant for reversible binding (K_d) and the rate of conversion to irreversible binding. We used the term K_{com} instead of K_d in recognition of the irreversible binding component that is intrinsic to competition binding assays for Cry toxins.

The LIGAND program also compares single- and multiple-binding-site models, comparing the goodness of fit for the models entered into the program. Whenever possible, binding data were fitted to both one- and two-receptor-site models. These models were then statistically tested by LIGAND for the significantly best fit by using an extra sum of squares *F* test.

RESULTS

Potency of Cry1 toxins against lepidopteran larvae. Table 1 shows the marked variations in potency that underpin this study. It shows not only the differences in potency of the same toxin for different species but also the dramatic differences in potency of the same toxin for a species during larval development.

Table 1 shows that for lepidopteran larvae there are distinct decreases in susceptibility to Cry1 protoxins as the larvae age. For *M. sexta*, this was observed with all four toxins; the most pronounced developmental changes were with Cry1Ac (37-fold decrease in potency) and Cry1Ba (larvae resistant at the third-instar stage). The LC_{50}s for *P. brassicae* increased during development for Cry1J (15-fold) and Cry1Ac (5-fold). *M. brassicae* was susceptible only to Cry1Ca (neonate stage), and it was resistant to this toxin at the third-instar stage. *S. exigua* was susceptible to Cry1J and Cry1Ca, and there were no changes in the potency of the protoxins during development. *A. ipsilon* exhibited significant resistance to all four protoxins at both developmental stages.

A comparison of the LC_{50}s of the same protoxin for different insects revealed many insights concerning the target range of the toxin. The following potency data (Table 1) are discussed because we have neonate and third-instar binding data (see Table 3) to compare them to. *S. exigua* was significantly less susceptible to Cry1J than *M. sexta* and *P. brassicae* were (Table 1) at the neonate stage. *M. sexta* was more susceptible to Cry1Ca than *M. brassicae* (Table 1) at both the neonate stage (fourfold) and the third-instar stage (ninefold). Cry1Ac was an effective toxin only for *M. sexta* and *P. brassicae. M. sexta* neonates were more susceptible to Cry1Ac than *P. brassicae* neonates (fivefold), but this was not the case for third-instar larvae (Table 1). Cry1Ba was dramatically more potent for *P. brassicae* than for *M. sexta* (Table 1) at both larval stages (210-fold for neonates and 490-fold for third-instar larvae). Another noteworthy result is that although Cry1Ba was more potent for *P. brassicae* than for the other species studied, there were no differences in the LC_{50}s during larval aging (Table 1).

Isolation of BBMV and assay of APN activity. APN activity was monitored because it is a marker for the brush border membrane and because APN is a putative receptor for Cry1 toxins. Neonate larvae are too small to obtain sufficient dissected midgut biomass for BBMV production; therefore, whole larvae were used. BBMV from whole third-instar larvae were also prepared so that data would be directly comparable.

TABLE 2. APN activity and enrichment in microscale preparations of BBMV from whole neonate and third-instar larvae

Larvae	*M. sexta*		*P. brassicae*		*M. brassicae*		*S. exigua*	
	APN sp act (μmol min^{-1} mg^{-1})[a]	Enrichment factor[b]	APN sp act (μmol min^{-1} mg^{-1})	Enrichment factor	APN sp act (μmol min^{-1} mg^{-1})	Enrichment factor	APN sp act (μmol min^{-1} mg^{-1})	Enrichment factor
Neonate[c]	0.59 ± 0.1	2.5 ± 0.5	0.2 ± 0.11	1.33 ± 0.33	0.4 ± 0.11	5.82 ± 1.28	2.2 ± 0.2	15.71 ± 1.43
Third instar[d]	1.7 ± 0.9	3.25 ± 1.75	0.66 ± 0.13	3.73 ± 0.48	0.86 ± 0.24	8.0 ± 2.0	1.3 ± 0.3	6.77 ± 1.22

[a] APN specific activity in purified BBMV preparation. The values are means ± standard errors of the means ($n = 3$).
[b] Enrichment of APN specific activity from the crude insect homogenate to the final purified BBMV preparation. The values are means ± standard errors of the means ($n = 3$).
[c] BBMV prepared from whole neonate larvae. The starting biomass of insect material was 0.5 g for all species except *M. sexta*, for which the starting biomass was 2 g.
[d] BBMV prepared from whole third-instar larvae. The starting biomass of insect material was the same as that described in footnote *c*.

Despite the low starting biomass used for BBMV production, enrichment of APN activity was observed for all species and developmental stages (Table 2). With the exception of *S. exigua* BBMV, BBMV from neonate larvae had significantly lower APN activity than BBMV from third-instar larvae (Table 2); thus, APN activity was inversely correlated with increased resistance to some Cry1 toxins during larval development.

Homologous competition binding: comparisons during larval development. We were interested in determining whether differences in binding characteristics could explain the increased resistance observed with some insect-toxin combinations as larvae age. *M. sexta* third-instar larvae had significantly fewer binding sites (B_{max}) for Cry1Ac and Cry1Ba than neonates had (Table 3). Thus, these data clearly correlate with the increased resistance to these two toxins that occurs during *M. sexta* larval development (Table 1).

For *P. brassicae* a two-site fit for Cry1Ba was found in neonate BBMV but not in third-instar BBMV (Table 3); however, no changes in the potency of Cry1Ba during development of *P. brassicae* were observed (Table 1).

For *M. brassicae* there was a significant increase in Cry1Ca binding site concentration from the neonate stage to the third-instar stage (Table 3); this increase was apparently inversely correlated with resistance to Cry1Ca during larval development (Table 1).

Binding experiments performed with *S. exigua* and Cry1J revealed no significant difference in either K_{com} or B_{max} during development (Table 3); this finding agrees well with the bioassay data which revealed no significant differences in LC_{50}s between larval stages (Table 1).

Since *A. ipsilon* was highly resistant to all four toxins and there were no differences in potency for different developmental stages, BBMV from fifth-instar larvae were used for binding assays. Binding was not observed with Cry1Ba, which correlated with resistance to this toxin. Despite the lack of toxicity, binding was observed with the other three Cry1 toxins; indeed, for Cry1Ca, a two-site fit was determined (K_{com1}, 0.21 ± 0.29 nM; K_{com2}, 64 ± 27 nM; B_{max1}, 0.83 ± 0.9 pmol/mg; B_{max2}, 293 ± 107 pmol/mg). For Cry1J the binding values were as follows: K_{com}, 121 ± 132 nM; and B_{max}, 137 ± 163 pmol/mg. For Cry1Ac K_{com} was 310 ± 85.6 nM and B_{max} was 2,409 ± 837 pmol/mg.

In addition to neonate and third-instar larvae, binding assays were also performed with fifth-instar *M. sexta* larvae. LIGAND analysis of fifth-instar data detected two binding sites for Cry1Ac (K_{com1}, 0.93 ± 1.6 nM; K_{com2}, 74.4 ± 18.4 nM; B_{max1},

TABLE 3. Binding characteristics of ^{125}I-labeled Cry1 toxins as determined by homologous competition assays using BBMV from whole neonate and third-instar larvae[a]

Toxin	Larvae	*M. sexta*		*P. brassicae*		*M. brassicae*		*S. exigua*	
		K_{com} (nM)	B_{max} (pmol/mg)	K_{com} (nM)	B_{max} (pmol/mg)	K_{com} (nM)	B_{max} (pmol/mg)	K_{com} (nM)	B_{max} (pmol/mg)
Cry1J	Neonate[b]	80.3 ± 24.6	159 ± 47.3	58 ± 25	94 ± 33			231 ± 109	680 ± 326
	Third instar[c]	61.3 ± 17.16	109 ± 28	39 ± 10	58 ± 14	ND[d]	ND	438 ± 96	1,130 ± 287
Cry1Ca	Neonate	7.2 ± 2.6	114 ± 32			2.2 ± 1.9	53 ± 33		
	Third instar	7.6 ± 2.3	72 ± 18	ND	ND	3.4 ± 2.3	172 ± 62	ND	ND
Cry1Ac	Neonate	103 ± 45	2,988 ± 1,055	415 ± 113	2,556 ± 860				
	Third instar	25 ± 23	211 ± 189	406 ± 361	1,558 ± 1760	ND	ND	ND	ND
Cry1Ba	Neonate	202 ± 85	1,873 ± 812	2.8 ± 2.3, 424 ± 189[e]	8.1 ± 5.8, 1,320 ± 608[e]	ND	ND	ND	ND
	Third instar	105 ± 31.5	738 ± 214	174 ± 46.3	726 ± 207				

[a] K_{com} and B_{max} were calculated from a LIGAND analysis of the binding experiment results. The values are means ± standard errors of the means.
[b] BBMV were prepared from whole neonate larvae.
[c] BBMV were prepared from whole third-instar larvae.
[d] ND, not determined.
[e] The first value is the value for a high-affinity binding site, and the second value is the value for a low-affinity binding site.

5.52 ± 8.9 pmol/mg; B_{max2}, 909 ± 146 pmol/mg). However, these binding sites were not found when experiments were performed with *M. sexta* neonate and third-instar larvae (Table 3). This discrepancy may have resulted from the small quantities of whole larvae used in the third-instar larva binding experiments (see Materials and Methods). However, it may also have been a true reflection of the Cry1Ac binding characteristics of the younger larvae (see below).

Homologous competition binding: comparisons between species treated with the same toxin. Interestingly, *S. exigua* neonates were less susceptible to, and had a correspondingly lower binding affinity for, Cry1J than *M. sexta* and *P. brassicae* neonates (Tables 1 and 3). *M. sexta* was more susceptible to Cry1Ca than *M. brassicae* at both the neonate and third-instar stages, but this was not reflected in the binding data. *M. sexta* neonates were more susceptible to, and had a correspondingly higher affinity for, Cry1Ac than *P. brassicae* neonates (Tables 1 and 3). Although Cry1Ba was dramatically more potent for *P. brassicae* than for *M. sexta* at both larval stages studied (Table 1), only the binding data for *P. brassicae* neonates, in which two binding sites were found, correlated with the bioassay results.

DISCUSSION

Understanding the reasons for the marked differences in the potencies of Cry1 toxins for economically important lepidopteran species, both within a species and during larval development, is crucial for future improvement of these biopesticides and for successful implementation of resistance prevention strategies. In this study, homologous competition binding assays were performed in an effort to correlate Cry1 potency, at two stages of larval development (neonate and third instar), with Cry1 binding on BBMV isolated from larvae at the same stage of development.

Aminopeptidase activity was detected in all BBMV studied here, although no clear relationship could be established between APN activity and the potency of Cry1 toxins or the binding of toxins. This finding is supported by the findings of other workers. Lorence et al. (28) showed that the action of Cry1Ac was dependent on its presence but not on its enzymatic activity, and Jenkins et al. (19) showed that APN activity was not directly correlated with toxin binding.

The increased resistance of *M. sexta* third-instar larvae to Cry1Ac and Cry1Ba may be due to loss of the neonate binding sites (B_{max}) for these toxins. There has been one other report correlating the loss of a binding site during larval development with resistance. Rausell et al. (38) showed that one of two Cry1Ab binding sites for *Thaumetopoea pityocampa* was lost between the first- and second-instar stages, which correlated with 10% mortality for second-instar larvae at the neonate LC_{50}. A complete lack of binding has also been implicated in development of field resistance, most notably in *P. xylostella* (39).

In this study, *P. brassicae* was the most susceptible species to Cry1Ba and, correspondingly, the only insect (at the neonate stage) to exhibit a high-affinity binding site for Cry1Ba. Hofmann et al. (14) previously described a correlation between Cry1Ba binding and toxicity for *P. brassicae* fifth-instar larvae . In this study, these observations were extended to show the significant changes in the binding characteristics of Cry1Ba during larval development in *P. brassicae.*

Binding data for Cry1Ac and *M. sexta* show that the binding affinity (K_{com}) in neonates may be lower than that in third-instar larvae, which inversely correlates with toxicity. The other inverse correlation between binding and toxicity detected in this study was that observed with Cry1Ca and *M. brassicae*, which exhibited a significant increase in binding site concentration during larval development despite the resistance seen in the third-instar larvae. Other workers have also had difficulty correlating binding characteristics with toxicity. Luo et al. (31) investigated the relationship between binding and toxicity for *S. exigua* and *Spodoptera frugiperda*. High-affinity binding sites were identified for Cry1Bb, Cry1Ca, and Cry1Ac, but the binding constants did not correlate with toxicity. Ballester et al. (2) demonstrated that the loss of a low-affinity binding site for Cry1Ab in *P. xylostella* resulted in resistance in this organism even though a high-affinity binding site was still available. Liu et al. (27) showed there were no significant differences in Cry1C binding to resistant and susceptible strains of *P. xylostella*. Van Rie et al. (47) observed that Cry1E had two binding sites in BBMV prepared from *M. sexta* and *Spodoptera littoralis*. Although these two insects had similar susceptibilities to Cry1E and similar affinities for the low-affinity binding sites (correlating with toxicity), the high-affinity binding site for *M. sexta* had a K_{com} at least 10 times lower than the K_{com} for *S. littoralis* and was therefore not positively correlated with potency.

The conflicting results mentioned above could be due in part to interpretation of the binding data. For example, most bioassays have been done with neonate larvae, whereas binding assays are usually done with midguts dissected from fifth-instar larvae (12, 31, 50). However, as we have clearly shown in this study, there are many instances in which toxicity decreases dramatically during larval development. Therefore, using binding data for a larval stage different from the stage used for a bioassay could be problematic and in some cases could lead to misinterpretation of data (e.g., the data for *M. sexta* fifth-instar larvae in this study). In studies in which the workers have compared toxicity and binding data for corresponding larval stages, positive correlations have often, although not always, been found (25, 37, 38; this study).

Interpretation of binding data is further complicated by the irreversible associations, as well as the reversible associations, of the toxins with BBMV; perhaps the more subtle differences in potency may go undetected unless these two components are measured separately. Liang et al. (25) showed the importance of obtaining such measurements by demonstrating that irreversible binding was directly correlated with toxicity but not with B_{max} and K_d. Wu and Dean (51) found that there was a direct correlation between potency and irreversible binding of the loop III block mutant but no correlation with reversible binding. Thus, even distinguishing between irreversible binding and reversible binding can make data difficult to interpret, as concluded by Luo et al. (31), who found that Cry1Ac, Cry1Ca, and Cry1Bb bound irreversibly to *S. exigua* BBMV, even though the toxicities of the molecules were different. In this study, the homologous competition assay was a useful screening technique; it highlighted where binding was or was not

clearly related to potency, and indeed, Liang et al. (25) supported the use of this type of assay for comparative purposes.

Cry1 toxin binding is necessary, although not sufficient, for toxicity (12, 37, 50). Although positive correlations between toxicity and binding have been found in this and other studies, Lee et al. (23, 24) have pointed out that nonfunctional receptors may be present on the surface of BBMV, which would further complicate interpretation of binding data. The presence of nonfunctional receptors may go some way in explaining the binding observed in *A. ipsilon* with Cry1Ac, Cry1Ca, and Cry1J and in *M. sexta* with Cry1Ba despite the resistance of these insects to these toxins.

In this study we investigated the possibility that changes in binding properties may influence changes in toxin potency during larval development using five lepidopteran species and four Cry1 toxins. Binding characteristics did not positively correlate with potency in every case in this study. The difficulties in correlating APN activity and binding parameters with potency underscore the complexity of the mode of action of these toxins, and further study of the toxins is warranted.

ACKNOWLEDGMENTS

This work was funded by the Biotechnology and Biological Sciences Research Council.

We thank T. Sawyer for technical assistance.

REFERENCES

1. **Ali, A., and S. Y. Young.** 1996. Activity of *Bacillus thuringiensis* berliner against different ages and stages of *Helicoverpa zea* (Lepidoptera: Noctuidae) on cotton. J. Entomol. Sci. **31:**1–8.
2. **Ballester, V., F. Granero, B. E. Tabashnik, T. Malvar, and J. Ferre.** 1999. Integrative model for binding of *Bacillus thuringiensis* toxins in susceptible and resistant larvae of the diamondback moth (*Plutella xylostella*). Appl. Environ. Microbiol. **65:**1413–1419.
3. **Bell, R. A., and F. G. Joachim.** 1976. Techniques for rearing laboratory colonies of tobacco hornworm and pink bollworm. Ann. Entomol. Soc. Am. **69:**365–373.
4. **Bradford, M. M.** 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. **72:**248–254.
5. **Burton, S. L., D. J. Ellar, J. Li, and D. J. Derbyshire.** 1999. *N*-Acetylgalactosamine on the putative insect receptor aminopeptidase N is recognised by a site on the domain III lectin-like fold of a *Bacillus thuringiensis* insecticidal toxin. J. Mol. Biol. **287:**1011–1022.
6. **Carroll, J., and D. J. Ellar.** 1993. An analysis of *Bacillus thuringiensis* δ-endotoxin action on insect-midgut-membrane permeability using a light-scattering assay. Eur. J. Biochem. **214:**771–778.
7. **Crickmore, N., C. Nicholls, D. J. Earp, T. C. Hodgman, and D. J. Ellar.** 1990. The construction of *Bacillus thuringiensis* strains expressing novel entomocidal δ-endotoxin combinations. Biochem. J. **270:**133–136.
8. **David, W. A. E., and B. O. C. Gardiner.** 1965. Rearing *Pieris brassicae* L. on a semisynthetic diet. Nature **207:**882.
9. **Denolf, P., K. Hendrickx, J. Van Damme, S. Jansens, M. Pereroen, D. Degheele, and J. Van Rie.** 1997. Cloning and characterization of *Manduca sexta* and *Plutella xylostella* midgut aminopeptidase N enzymes related to *Bacillus thuringiensis* toxin-binding proteins. Eur. J. Biochem. **248:**748–761.
10. **Escriche, B., F. Silva, and J. Ferre.** 1995. Testing the suitability of brush border membrane vesicles prepared from whole larvae from small insects for binding studies with *Bacillus thuringiensis* Cry1A(b) crystal protein. J. Invertebr. Pathol. **65:**318–320.
11. **Finney, D. J.** 1952. Probit analysis, 2nd ed. Cambridge University Press, Cambridge, United Kingdom.
12. **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.
13. **Hafkensheid, J. C. M.** 1984. Aminopeptidases and amino acid arylamidases, p. 2–34. *In* H. U. Bergmeyer (ed.), Methods of enzyme analysis, vol. V. Enzymes 3: peptidases, proteinases and their inhibitors. Verlag Chemie, Weinheim, Germany.
14. **Hofmann, C., H. Vanderbruggen, H. Hofte, J. Van Rie, S. Jansens, and H. Van Mellaert.** 1988. Specificity of *Bacillus thuringiensis* δ-endotoxins is correlated with the presence of high-affinity binding sites in the brush border membrane of target insect midguts. Proc. Natl. Acad. Sci. USA **85:**7844–7848.
15. **Hofte, H., and H. R. Whiteley.** 1989. Insecticidal crystal proteins of *Bacillus thuringiensis*. Microbiol. Rev. **53:**242–255.
16. **Hofte, H., H. De Greve, J. Seurinck, S. Jansens, J. Mahillon, C. Ampe, J. Vandekerckhove, H. Vanderbruggen, M. Van Montagu, M. Zabeau, and M. Vaeck.** 1986. Structural and functional analysis of a cloned delta endotoxin of *Bacillus thuringiensis berliner* 1715. Eur. J. Biochem. **161:**273–280.
17. **Hulme, E. C., and N. J. M. Birdsall.** 1992. Strategy and tactics in receptor-binding studies, p. 63–176. *In* E. C. Hulme (ed.), Receptor-ligand interactions, a practical approach. IRL Press, Oxford, United Kingdom.
18. **Ihara, H., T. Uemura, M. Mashura, S. Ikawa, K. Sugimoto, A. Wadano, and M. Himeno.** 1998. Purification and partial amino acid sequences of the binding protein from *Bombyx mori* for Cry1Aa δ-endotoxin *of Bacillus thuringiensis*. Comp. Biochem. Physiol. **120:**197–204.
19. **Jenkins, J. L., M. K. Lee, S. Sangadala, M. J. Adang, and D. H. Dean.** 1999. Binding of *Bacillus thuringiensis* Cry1Ac toxin to *Manduca sexta* aminopeptidase-N receptor is not directly related to toxicity. FEBS Lett. **462:**373–376.
20. **Knight, P. J. K., N. Crickmore, and D. J. Ellar.** 1994. The receptor for *Bacillus thuringiensis* Cry1Ac δ-endotoxin in the brush border membrane of the lepidopteran *Manduca sexta* is aminopeptidase N. Mol. Microbiol. **11:**429–436.
21. **Knowles, B. H., W. E. Thomas, and D. J. Ellar.** 1984. Lectin-like binding of *Bacillus thuringiensis* var. *kurstaki* lepidopteran-specific toxin is an initial step in insecticidal action. FEBS Lett. **168:**197–202.
22. **Kronstad, J. W., and H. R. Whiteley.** 1986. Three classes of homologous *Bacillus thuringiensis* crystal-protein genes. Gene **43:**29–40.
23. **Lee, M. K., F. Rajamohan, F. Gould, and D. H. Dean.** 1995. Resistance to *Bacillus thuringiensis* Cry1A delta-endotoxins in a laboratory-selected *Heliothis virescens* strain is related to receptor alteration. Appl. Environ. Microbiol. **61:**3836–3842.
24. **Lee, M. K., T. H. You, F. L. Gould, and D. H. Dean.** 1999. Identification of residues in domain III of *Bacillus thuringiensis* Cry1Ac toxin that affect binding and toxicity. Appl. Environ. Microbiol. **65:**4513–4520.
25. **Liang, Y., S. S. Patel, and D. H. Dean.** 1995. Irreversible binding kinetics of *Bacillus thuringiensis* CryIA δ-endotoxins to gypsy moth brush border membrane vesicles is directly correlated to toxicity. J. Biol. Chem. **270:**24719–24724.
26. **Lieberman, H. R.** 1983. Estimating LD_{50} using the probit technique. A. BASIC computer program. Drug Chem. Toxicol. **6:**111–116.
27. **Liu, Y.-B., B. E. Tabashnik, L. Masson, B. Escriche, and J. Ferre.** 2000. Binding and toxicity of *Bacillus thuringiensis* protein Cry1C to susceptible and resistant diamondback moth (Lepidoptera: Plutellidae). Insect. Resist. Resist. Man. **93:**1–6.
28. **Lorence, A., A. Darszon, and A. Bravo.** 1997. Aminopeptidase dependent pore formation of *Bacillus thuringiensis* Cry1Ac toxin on *Trichoplusia ni* membranes. FEBS Lett. **414:**303–307.
29. **Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall.** 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. **193:**265–275.
30. **Luo, K., and M. J. Adang.** 1994. Removal of adsorbed toxin fragments that modify *Bacillus thuringiensis* Cry1C δ-endotoxin iodination and binding by sodium dodecyl sulfate treatment and renaturation. Appl. Environ. Microbiol. **60:**2905–2910.
31. **Luo, K., D. Banks, and M. J. Adang.** 1999. Toxicity, binding, and permeability analyses of four *Bacillus thuringiensis* Cry1 δ-endotoxins using brush border membrane vesicles of *Spodoptera exigua* and *Spodoptera frugiperda*. Appl. Environ. Microbiol. **65:**457–464.
32. **Luo, K., Y.-J. Lu, and M. J. Adang.** 1996. A 106 kDa form of aminopeptidase is a receptor for *Bacillus thuringiensis* Cry1C δ-endotoxin in the brush border membrane of *Manduca sexta*. Insect Biochem. Mol. Biol. **26:**783–791.
33. **Luo, K., S. Sangadala, L. Masson, A. Mazza, R. Brousseau, and M. J. Adang.** 1997. The *Heliothis virescens* 170kDa aminopeptidase functions as 'receptor A' by mediating specific *Bacillus thuringiensis* Cry1A δ-endotoxin binding and pore formation. Insect Biochem. Mol. Biol. **27:**735–743.
34. **McPherson, G. A.** 1983. A practical computer based approach to the analysis of radioligand binding experiments. Comput. Programs Biomed. **17:**107–114.
35. **Mundson, P., and D. Rodbard.** 1980. LIGAND: a versatile computerized approach for characterisation of ligand-binding systems. Anal. Biochem. **107:**220–239.
36. **Nagamatsu, Y., S. Toda, F. Yamaguchi, M. Ogo, M. Kogure, M. Nakamura, Y. Shibata, and T. Katsumoto.** 1998. Identification of *Bombyx mori* midgut receptor for *Bacillus thuringiensis* insecticidal Cry1A(a) toxin. Biosci. Biotechnol. Biochem. **62:**718–726.
37. **Rajamohan, F., O. Alzate, J. A. Cotrill, A. Curtiss, and D. H. Dean.** 1996. Protein engineering of *Bacillus thuringiensis* δ-endotoxin**:** mutations at domain II of Cry1Ab enhance receptor affinity and toxicity toward gypsy moth larvae. Proc. Natl. Acad. Sci. USA **93:**14338–14343.
38. **Rausell, C., A. C. Martinez-Ramirez, I. Garcia-Robles, and M. D. Real.** 2000. A binding site for *Bacillus thuringiensis* Cry1Ab toxin is lost during larval development in two forest pests. Appl. Environ. Microbiol. **66:**1553–1558.
39. **Sayyed, A. H., R. Hayward, S. Herrero, J. Ferre, and D. J. Wright.** 2000.

Genetic and biochemical approach for characterization of resistance to *Bacillus thuringiensis* toxin Cry1Ac in a field population of the diamondback moth, *Plutella xylostella*. Appl. Environ. Microbiol. **66:**1509–1516.

40. **Schnepf, E., N. Crickmore, J. Van Rie, D. Lereclus, J. Baum, J. Feitelson, D. R. Zeigler, and D. H. Dean.** 1998. *Bacillus thuringiensis* and its pesticidal crystal proteins. Microbiol. Mol. Biol. Rev. **62:**775–806.
41. **Smedley, D. P.** 1996. Mutagenesis of a *Bacillus thuringiensis* entomocidal toxin. Ph.D. thesis. University of Cambridge, Cambridge, United Kingdom.
42. **Thomas, W. E., and D. J. Ellar.** 1983. *Bacillus thuringiensis* var. *israelensis* crystal δ-endotoxin: effects on insect and mammalian cells *in vitro* and *in vivo*. J. Cell Sci. **60:**181–197.
43. **Vadlamundi, R. K., H. Ji, and L. A. Bulla, Jr.** 1993. A specific binding protein from *Manduca sexta* for the insecticidal toxin of *Bacillus thuringiensis* subsp. *berliner*. J. Biol. Chem. **268:**12334–12340.
44. **Vadlamundi, R. K., E. Weber, I. Ji, H. Ji, and L. A. Bulla, Jr.** 1995. Cloning and expression of a receptor for an insecticidal toxin of *Bacillus thuringiensis*. J. Biol. Chem. **270:**5490–5494.
45. **Valiatis, A. P., A. Mazza, R. Brousseau, and L. Masson.** 1997. Interaction analyses of *Bacillus thuringiensis* Cry1A toxins with two aminopeptidases from gypsy moth midgut brush border membranes. Insect Biochem. Mol. Biol. **27:**529–539.
46. **Van-Nguyen, A.** 1995. Biochemistry and molecular genetics of a novel entomocidal toxin from *Bacillus thuringiensis*. Ph.D. thesis. University of Cambridge, Cambridge, United Kingdom.
47. **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* delta-endotoxins. Appl. Environ. Microbiol. **56:**1378–1385.
48. **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 mid-gut of target insects. Eur. J. Biochem. **186:**239–247.
49. **Ward, E. S., and D. J. Ellar.** 1983. Assignment of the δ-endotoxin gene of *Bacillus thuringiensis* var. *israelensis* to a specific plasmid by curing analysis. FEBS Lett. **158:**45–49.
50. **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 membranes for the toxins. Experientia **46:**475–477.
51. **Wu, S.-H., and D. H. Dean.** 1996. Functional significance of loops in the receptor binding domain of *Bacillus thuringiensis* Cry IIIA δ-endotoxin. J. Mol. Biol. **255:**628–640.
52. **Zhong, C.** 1996. Characterisation of a *Bacillus thuringiensis* δ-endotoxin toxic to three insect orders. Ph.D. thesis. University of Cambridge, Cambridge, United Kingdom.