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Cross-Resistance of the Diamondback Moth Indicates Altered Interactions with Domain II of *Bacillus thuringiensis* Toxins†

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We compared responses to six insecticidal crystal proteins from *Bacillus thuringiensis* by a Cry1A-resistant strain (NO-QA) and a susceptible strain (LAB-P) of the diamondback moth, *Plutella xylostella*. The resistant strain showed >100-fold cross-resistance to Cry1J and to H04, a hybrid with domains I and II of Cry1Ab and domain III of Cry1C. Cross-resistance was sixfold to Cry1Bb and threefold to Cry1D. The potency of Cry1I did not differ significantly between the resistant and susceptible strains. Cry2B did not kill resistant or susceptible larvae. By combining these new data with previously published results, we classified responses to 14 insecticidal crystal proteins by strains NO-QA and LAB-P. NO-QA showed high levels of resistance to Cry1Aa, Cry1Ab, and Cry1Ac and high levels of cross-resistance to Cry1F, Cry1J, and H04. Cross-resistance was low or nil to Cry1Ba, Cry1Bb, Cry1C, Cry1D, Cry1I, and Cry2A. Cry1E and Cry2B showed little or no toxicity to susceptible or resistant larvae. In dendrograms based on levels of amino acid sequence similarity among proteins, Cry1F and Cry1J clustered together with Cry1A proteins for domain II, but not for domain I or III. High levels of cross-resistance to Cry1Ab-Cry1C hybrid H04 show that although Cry1C is toxic to NO-QA, domain III of Cry1C is not sufficient to restore toxicity when it is combined with domains I and II of Cry1Ab. Thus, diamondback moth strain NO-QA cross-resistance extends beyond the Cry1A family of proteins to at least two other families that exhibit high levels of amino sequence similarity with Cry1A in domain II (Cry1F and Cry1J) and to a protein that is identical to Cry1Ab in domain II (H04). The results of this study imply that resistance to Cry1A alters interactions between the insect and domain II.

The soil bacterium *Bacillus thuringiensis* produces insecticidal crystal proteins (ICPs) that are becoming a cornerstone of environmentally benign pest control (9, 11). However, evolution of resistance by pests can shorten the useful life of *B. thuringiensis* (16, 29, 35). Laboratory selection has produced resistance to ICPs in many insects, but so far the diamondback moth (*Plutella xylostella*) is the only insect to evolve substantial resistance in open-field populations in response to commercial treatments with *B. thuringiensis* (35, 44). Widespread deployment of ICP-expressing transgenic varieties of corn, cotton, potato, and other crops, as well as greater use of conventional sprays of *B. thuringiensis*, will increase the risk of resistance in pests (16, 29, 35).

If repeated exposure of an insect population to one ICP caused resistance to only that particular ICP, one might be able to counter the resistance simply by switching to a new ICP. Ideally, resistance to one ICP might even cause increased susceptibility to other ICPs (47). However, the phenomenon of cross-resistance limits the potential for switching ICPs to combat resistance. Cross-resistance occurs when selection with one ICP or set of ICPs reduces susceptibility to other ICPs.

Evaluation of a variety of field- and laboratory-selected strains of moths has revealed a spectrum of outcomes ranging from narrow cross-resistance to broad cross-resistance (13, 17, 18, 28, 30, 39, 41, 42, 45, 47). In general, resistance that is associated with reduced binding of the toxin to midgut receptors appears to have a narrower spectrum of cross-resistance than resistance that is not associated with reduced binding (12). Some evidence suggests that resistance to *B. thuringiensis* in the diamondback moth is associated with reduced binding of Cry1A toxins to midgut receptors (5, 13, 39, 45; but see references 10 and 26). Previous studies of resistance to *B. thuringiensis* in the diamondback moth have shown that resistance to Cry1A ICPs from *B. thuringiensis* subsp. *kurstaki* confers cross-resistance to Cry1F, but not cross-resistance to Cry1B, Cry1C, Cry1D, and Cry9C (12, 20, 39, 41, 42, 45). The binding sites for the latter four ICPs apparently differ from the binding sites for Cry1A ICPs (12, 20, 39, 45).

Cross-resistance is most likely when toxins share key structural features, which allows one resistance mechanism to confer resistance to more than one toxin. Thus, we hypothesized that cross-resistance in the diamondback moth is related to the extent of amino acid sequence similarity among toxins. Furthermore, we expected the strongest relationship between cross-resistance and amino acid sequence similarity in regions of toxins that interact differently with susceptible and resistant insects. In particular, when resistance is caused by reduced binding of the toxin to midgut receptors (12, 47), cross-resistance should be related to amino acid sequence similarity in the binding regions of toxins. The ability to test these hypotheses has been limited by the relatively small number of ICPs against which any one resistant strain has been tested.

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TABLE 1. Toxicity of Cry1Bb, CrylD, Cry1I, Cry1J, and H04 to diamondback moth larvae

ICP	Insect strain[a]	No. of larvae tested	Slope[b]	LC_{50} (mg/liter) at 5 days	95% Fiducial limits of LC_{50} (mg/liter)		Resistance ratio[c]
					Lower	Upper	
Cry1Bb	Resistant	478	2.0 ± 0.5	110	71.9	292	6
	Susceptible	327	3.9 ± 0.7	19.1	15.4	23.6	
Cry1D	Resistant	230	2.4 ± 0.5	6.7	4.0	9.4	3
	Susceptible	235	1.4 ± 0.3	2.1	0.8	3.9	
Cry1I	Resistant	201	NA[d]	32.8	NA	NA	3
	Susceptible	200	2.0 ± 0.6	12.9	4.6	41.8	
Cry1J	Resistant	236	NA	>300[e]	NA	NA	>140
	Susceptible	232	2.5 ± 0.7	2.1	0.8	3.0	
H04	Resistant	229	NA	>100[e]	NA	NA	>500
	Susceptible	237	2.7 ± 0.9	0.2	0.05	0.3	

[a] The resistant strain was strain NO-QA, and the susceptible strain was strain LAB-P.
[b] Estimated slope of the probit regression line ± standard error.
[c] LC_{50} of resistant strain/LC_{50} of susceptible strain.
[d] NA, not available.
[e] The highest concentration tested caused <5% mortality.

In the present study, we used bioassays with a Cry1A-resistant strain (NO-QA) and a control susceptible strain (LAB-P) of the diamondback moth to evaluate the following six ICPs: Cry1Bb (formerly CryET5), Cry1D, Cry1I (formerly $CryV_1$), Cry1J (formerly CryET4), Cry2B, and H04 (a hybrid between Cry1Ab and Cry1C). We used the results obtained in combination with previously reported data to characterize cross-resistance and analyze the relationship of cross-resistance to amino acid sequence similarity among toxins. Because previous studies have identified domains II and III of *B. thuringiensis* toxins as determinants of specificity and binding (4, 14, 15, 22, 27, 32, 34, 49), we focused on the relationship between cross-resistance and amino acid sequence similarity in these regions. Analysis of responses to H04, which contains domains I and II from Cry1Ab and domain III from Cry1C (4), enabled us to directly test the role of these domains in cross-resistance.

MATERIALS AND METHODS

Insects. Larvae were obtained from resistant strain NO-QA and susceptible strain LAB-P colonies of the diamondback moth from Hawaii. Both colonies were reared in the laboratory on cabbage. The resistant colony was started from individuals collected at a watercress farm where exposure in the field had produced about 25-fold resistance to Dipel (36), a commercial formulation of strain HD-1 of *B. thuringiensis* subsp. *kurstaki* that contains Cry1Aa, Cry1Ab, Cry1Ac, Cry2A, Cry2B, spores, and formulation ingredients (2). At the time of this study, the resistant colony had been exposed to Dipel repeatedly in the laboratory and was highly resistant to Dipel. The susceptible colony had been reared in the laboratory for more than 100 generations without exposure to any insecticide. For additional details about the colonies and their maintenance see references 37, 38, and 40.

ICPs. Cry1Bb (GenBank accession number L32020), Cry1D (X54160), and Cry1J (L32019) were obtained from Ecogen strains EG7283, EG7300, and EG7279, respectively. A 500-ml shake flask culture of each strain was harvested by centrifugation, and the spore-crystal pellet was suspended in 100 ml of water and lyophilized with a Virtis Freezemobile 12 apparatus. The concentrations of these ICPs were determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis as described by Baum et al. (3). Cry1I (L36338; full new name, Cry1Ia3; formerly $CryV_1$) was produced in *Escherichia coli* containing the cloned ICP gene, and inclusion bodies were prepared as described by Shin et al. (33). To produce Cry2B (M23724), the Cry2B coding sequences were cloned 3′ to a Cry3A promoter as described by Dankocsik et al. (7). This construct was subcloned into an *E. coli-Bacillus* shuttle vector and transformed into an acrystalliferous strain of *B. thuringiensis*. H04 was expressed in and purified from an acrystalliferous strain of *B. thuringiensis* as described by de Maagd et al. (8).

Bioassays. Larvae were tested for susceptibility to the protoxin form of each ICP by leaf residue bioassays at 28°C (36, 38). In all tests, we added a surfactant (0.2% Triton AG98 [Rohm and Haas]). For each ICP, the susceptible and resistant colonies were tested simultaneously. Each bioassay included a series of at least five concentrations of each ICP. Each concentration was replicated at least three times, with 7 to 11 larvae per replicate.

Data analysis. All analyses of bioassay data were based on mortality at 5 days after initial exposure to ICPs. We used probit analysis (31) to estimate the concentrations expected to kill 50% of the larvae (LC_{50}s) and their 95% fiducial limits, as well as the slopes of the concentration-mortality lines and their standard errors (38). LC_{50}s were considered significantly different if their 95% fiducial limits did not overlap. Resistance ratios were calculated by dividing the LC_{50} of resistant strain NO-QA by the LC_{50} of susceptible strain LAB-P. For the eight ICPs tested previously (39, 41, 42) and the six ICPs tested in the present study, we report the percentages of mortality at each of two concentrations for the resistant and susceptible strains. We did not make statistical comparisons among ICPs because we tested various types of protoxin preparations from various sources and we did not test different ICPs simultaneously. Thus, any observed differences among ICPs must be interpreted cautiously.

Amino acid sequence similarity was analyzed with the CLUSTAL program of the Intelligenetics PC/GENE package by using the following parameters: k-tuple value, 1; gap penalty, 5; window size, 10; filtering level, 2.5; and open gap cost and unit gap cost, 10. The numbers of residues included in each domain were 33 to 253 for domain I, 265 to 461 for domain II, and 463 to 609 for domain III (19). In addition to five of the ICPs tested in bioassays in the present study, the following 12 ICPs were included in the similarity analysis: Cry1Aa (U43605), Cry1Ab (M37263), Cry1Ac (U43606), Cry1Ba (X06711; formerly CryIB), Cry1C (X07518), Cry1E (X53985), Cry1F (M63897), Cry1G (Z22510; formerly PrtA), Cry1H (Z22513; formerly PrtC), Cry2A (M31738), Cry9A (X58120; formerly CryIG), and Cry9C (Z37527; formerly CryIH). We used the revised nomenclature for ICPs (6), but for brevity include below only enough characters in each name to enable identification within the context of this paper (e.g., we use Cry1C instead of Cry1Ca1 and Cry1I instead of Cry1Ia3).

RESULTS

Toxicity of Cry1Bb, Cry1D, Cry1I, Cry1J, Cry2B, and H04. Cry1A-resistant strain NO-QA of the diamondback moth was susceptible to Cry1Bb, Cry1D, and Cry1I but not to Cry1J or H04 (Tables 1 and 2). For Cry1J and H04, the resistance ratios exceeded 100 (Table 1), and 10 or 100 mg of protoxin per liter killed 95 to 100% of the larvae of susceptible strain LAB-P but only 0 to 7% of the resistant strain larvae (Table 2). The resistance ratios were 6 for Cry1Bb, 3 for Cry1D, and 3 for Cry1I (Table 1). The difference in LC_{50}s between strains was significant (as determined by nonoverlap of 95% fiducial limits) for Cry1Bb and Cry1D, but not for Cry1I (Table 1). At the highest concentration tested (300 mg/liter), Cry2B killed only 2% of the LAB-P larvae and no NO-QA larvae (Table 2).

Summary of resistance and cross-resistance. On the basis of the bioassay results obtained in the present study and the results extracted from previous publications (Tables 1 and 2), we classified the responses to 14 *B. thuringiensis* ICPs by diamondback moth strains NO-QA and LAB-P. NO-QA showed high levels of resistance to Cry1Aa, Cry1Ab, and Cry1Ac and high levels of cross-resistance to Cry1F, Cry1J, and H04. For each of these six ICPs, the resistance ratios were greater than

TABLE 2. Responses of diamondback moth larvae to *B. thuringiensis* ICPs

ICP	Concn (mg/liter)	% Mortality[a]	
		Susceptible strain	Resistant strain
Cry1Aa[b,c]	10	100	0
	100	100	6
Cry1Ab[b,c]	10	100	10
	100	100	26
Cry1Ac[b,c]	10	94	0
	100	100	9
Cry1Ba[d]	2.5	86	59
	12.5	100	100
Cry1Bb	10	14	0
	100	100	46
Cry1C[b]	1	49	20
	10	97	100
Cry1D	10	79	66
	100	100	100
Cry1E[d]	10	5	0
	100	5	9
Cry1F[d,e]	1[f]	100	0
	10[f]	99	0
Cry1I	5	12	0
	50	92	95
Cry1J[e]	10	95	0
	100	100	0
Cry2A[b]	10	22	6
	100	82	41
Cry2B	100	2	0
	300	2	0
H04[e]	10	100	7
	100	100	4

[a] Level of mortality 5 days after initial exposure to the toxin, adjusted for mortality in untreated controls (1), based on three to nine replicates of 7 to 11 treated larvae and total sample sizes ranging from 30 to 91 larvae per concentration per strain and 20 to 82 untreated control larvae per strain.
[b] Data from reference 42.
[c] Strain NO-QA is highly resistant (the resistance ratio is >100, and the level of mortality at one or more concentrations in strain NO-QA is at least 90% lower than that in strain LAB-P).
[d] Data from reference 41.
[e] Strain NO-QA is highly cross-resistant (for criteria, see footnote *c*).
[f] Concentration in milliliters of Mycogen formulation MYX837-446 per liter.

100 and mortality at one or more single concentrations in NO-QA was lower by at least 90% than that in LAB-P.

NO-QA showed little or no cross-resistance to Cry1Ba, Cry1Bb, Cry1C, Cry1D, Cry1I, and Cry2A. Although NO-QA was significantly less susceptible than LAB-P to Cry1Bb (Table 1), Cry1D (Table 1), and Cry2A (42), the highest resistance ratio for these three ICPs was 6 and the greatest difference in mortality at any single concentration was 54%.

Cry1E and Cry2B exhibited little or no toxicity to either strain (Table 2). None of the ICPs tested was significantly more lethal to NO-QA than to LAB-P; i.e., no negative cross-resistance occurred.

Amino acid sequence similarity. The pattern of responses from bioassays correlated well with a dendrogram based on levels of amino acid sequence similarity for domain II (Fig. 1), but not with a dendrogram based on levels of amino acid sequence similarity for domain I or III (Fig. 2 and 3). On the basis of the bioassay results summarized above, we expected that the level of amino acid sequence similarity to Cry1A ICPs in specificity-determining regions would be greater for Cry1F and Cry1J than for the other ICPs tested. In the domain II dendrogram, the three Cry1A ICPs cluster tightly (Fig. 1). Also, in the domain II dendrogram, Cry1J and Cry1F are

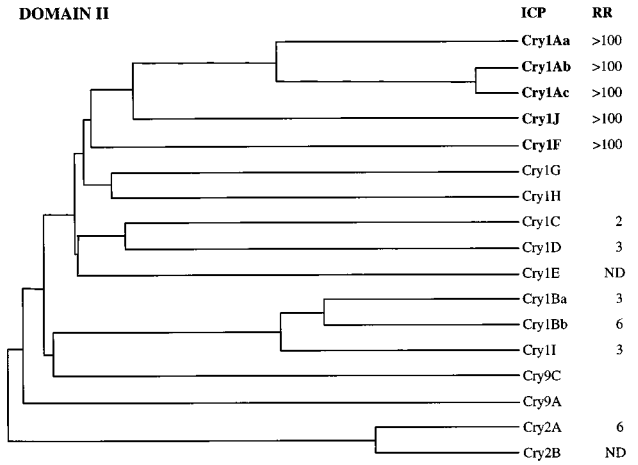

FIG. 1. Amino acid sequence similarity of domain II of *B. thuringiensis* toxins and resistance ratios (RR) of diamondback moth larvae. The dendrogram was produced with the CLUSTAL program of the Intelligenetics PC/GENE package. Each ICP for which the resistance ratio was >100 is shown in boldface type. Resistance ratios were not determined (ND) for Cry1E and Cry2B because these toxins had little or no toxicity to susceptible or resistant larvae. Cry1G, Cry1H, Cry9C, and Cry9A were not tested against diamondback moth strains LAB-P and NO-QA.

immediately adjacent to the Cry1A cluster. In the domain I dendrogram, Cry1D and Cry1E are closer to the Cry1A cluster than Cry1F and Cry1J are (Fig. 2). In the domain III dendrogram, Cry1Ac is widely separated from Cry1Aa and Cry1Ab (Fig. 3). Also, in the domain III dendrogram, Cry1J is close to Cry1Aa and Cry1Ab, but several toxins, including Cry1Bb and Cry1I, intercede between the Cry1Aa-Cry1Ab pair and Cry1F. Clustering of Cry1F and Cry1J with Cry1As for domain II, but not for domain I or III, also occurs in previously published dendrograms (46). These dendrograms for 23 lepidopteran-active ICPs were derived by using a different program (PILE UP from the Genetics Computer Group computer package) and a somewhat different set of ICPs (46) than the program and ICPs used in the present study. Thus, it appears that the domain-specific clustering patterns of Cry1F and Cry1J relative to Cry1A are robust.

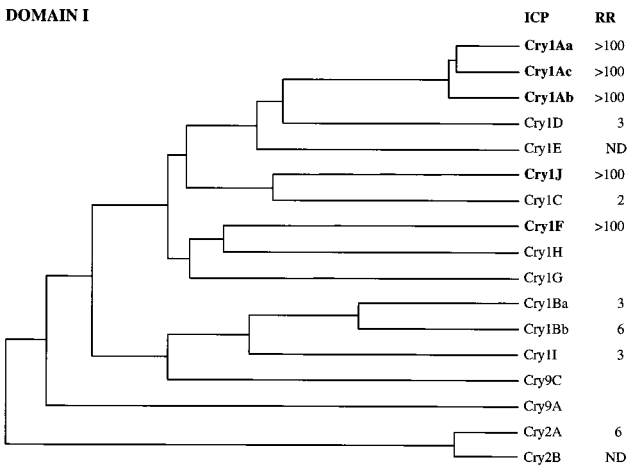

FIG. 2. Amino acid sequence similarity of domain I of *B. thuringiensis* toxins and resistance ratios (RR) of diamondback moth larvae. See the legend to Fig. 1 for details. ND, not determined.

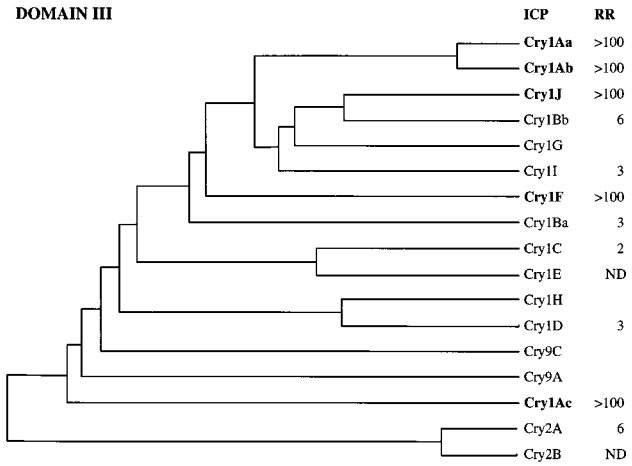

FIG. 3. Amino acid sequence similarity of domain III of *B. thuringiensis* toxins and resistance ratios (RR) of diamondback moth larvae. See the legend to Fig. 1 for details. ND, not determined.

DISCUSSION

The correlation between cross-resistance in strain NO-QA of the diamondback moth and amino acid sequence similarity among ICPs was stronger for domain II than for domain I or III. These results support the hypothesis that domain II contains residues that are important in determining the specificity of ICPs for the diamondback moth. In particular, these results suggest that the mutation or mutations conferring resistance to ICPs in strain NO-QA alter the insect's interactions with domain II.

The responses to H04 also suggest that it is not the interaction with domain III that is altered in NO-QA larvae. NO-QA larvae were highly resistant to this recombinant ICP, which contains domains I and II from Cry1Ab and domain III from Cry1C. If altered interactions with domain III caused the difference in susceptibility between NO-QA and LAB-P, one would expect the toxicity of H04 to resemble that of Cry1C, but it did not.

On the basis of evidence suggesting that resistance in the diamondback moth is associated with reduced binding of Cry1A toxins (5, 13, 39, 45), we suspect that reduced binding of domain II confers resistance in the diamondback moth. Direct tests of this hypothesis will require examination of binding to hybrid ICPs, such as H04.

The data for H04 alone do not exclude a potential role for domain I in determining specificity. However, domain I is thought to be involved primarily in pore formation (19, 23) rather than specificity. Furthermore, the amino acid sequence similarity in domain I shows little correlation with patterns observed in bioassays performed with the diamondback moth (Fig. 1) (see also Fig. 4 in reference 46).

Structural considerations (19, 23) and experimental results obtained with other insects (24, 25, 34, 50) suggest that the loop regions of domain II are involved in binding and specificity. Previously published results obtained with natural variants of Cry1Ac suggest that what is now considered loop 3 of domain II (19) affects toxicity to susceptible diamondback moth larvae (48). An inspection of alignments (Fig. 4) in which Cry1Aa was used as a reference point (19) revealed that loop 2 (Cry1Aa residues 367 to 379) and loop 3 (Cry1Aa residues 438 to 446) vary considerably in size and sequence among the five ICPs that were highly toxic to LAB-P but not to NO-QA (Cry1Aa, Cry1Ab, Cry1Ac, Cry1F, and Cry1J). However, loop

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                                   LOOP 1
Cry1Aa  289 QNIRQPHLMDILNSITIYTDVHR------GFNY--WSGHQITASPVGFS- 329
Cry1Ab  289 GSIRSPHLMDILNSITIYTDAHR------GEYY--WSGHQIMASPVGFS- 329
Cry1Ac  289 RSIRSPHLMDILNSITIYTDAHR------GYYY--WSGHQIMASPVGFS- 329
Cry1J   291 QQIRRPHLMDFFNSMIMYTSDNR------REHY--WSGLEMTAYFTGFA- 331
Cry1F   289 FGVRPPHLMDFMNSLFVTAETVR------SQTV--WGGHLVSSRNT--A- 327
Cry1G   279 NVLRAPHLMDFIDRIVIYTNSVR------STPY--WAGHEVISRRTGQGQ 320
Cry1H   283 NILREPHLMDFLTRLVIYT-GVQ------SGIY-HWAGHEISSRTTGN-L 323
Cry1C   293 SRIRNPHLFDILNNLTIFTDWFS------VGRNFYWGGHRVISSLIGGGN 333
Cry1D   292 AIIRSPHLVDFLNSFTIYTD--S------LARYAYWGGH-LVNSFRTGTT 332
Cry1E   289 SAIRSPHLMDFLNNLTIDTDLIR------GVH--YWAGHRVTSHPTGS-- 328
Cry1Ba  317 AAIRSPHLLDFLEQLTIFSASSRWS-N--TRHMTYWRGHTIQSRPI---- 359
Cry1Bb  324 AIFRPPHLLDFPEQLTIYSASSRWS-S--TQHMNYWVGHRLNFRPI---- 366
Cry1I   328 AVVRNPHLLDFLEQVTIYSLLSRWS-N--TQYMNMWGGHKLEFRTI---- 370
Cry9C   339 AFIRPPHLFDRLNSLTI--SSNRFPVS--SNFMDYWSGHTLRRSYLN--- 381
Cry9A   335 A-IPNPRPSWFLNNMIISTGSLTLPVSPSTDRARVWYGSRDRISPAN--- 381
Cry2A   309 SGISGTRLSITFPNIGGLPGST-------------TTHSLNSARVNYSG 344
Cry2B   309 NGFSGARLSNTFPNIVGLPGST-------------TTHALLAARVNYSG 344

                                   LOOP 2
Cry1Aa  349 -LVS----LTGLGIFRTLSSPL-YRRIILGSGPNNQELFVLDGTEFSFA- 391
Cry1Ab  349 RIVA----QLGQGVYRTLSSTL-YRRP-FNIGINNQQLSVLDGTEFAYG- 391
Cry1Ac  349 RIVA----QLGQGVYRTLSSTF-YRRP-FNIGINNQQLSVLDGTEFAYG- 391
Cry1J   351 TVRS-----VNDGIYRILSAPF-YSAPFLGTIVLGSR-----GEKFDFAL 389
Cry1F   346 WIAD----EDPRPFYRTLSDPV-FVRGGFG---NPHYVLGLRGVAFQ--- 384
Cry1G   340 TIRPTGFTDEQRQWYRARSRVVSFRSS---G----QDFSLVDAVGFLTIF 382
Cry1H   343 NMN----IHHSETIYRTLSAPI-YSVS---GGISPNRTRVVEGVRFLIRD 384
Cry1C   353 SFTFN--G--PV-F-RTLSNPT-LRLLQQPWPAPPFNLRGVEGVEFS--- 392
Cry1D   352 TITAS--PSVPI-F-RTLSYIT-G--LDNSNPVA-----GIEGVEFQ--- 386
Cry1E   348 TIAPSTFPGLNL-FYRTLSNPF-FRRSENITPT--LGINVVQGVGFI--- 390
Cry1Ba  378 SINPVTLRFASRDVYRTESYAGVLLWGIYLEPIHGVPTVRFNFTNPQNIS 423
Cry1Bb  386 SINPVTLQFTSRDVYRTESNAGTNI--LFTTPVNGVPWARFNFINPQNIY 429
Cry1I   389 SINPVTLPFTSRDVYRTESLAGLNL--FLTQPVNGVPRVDFHWKVTHPIA 432
Cry9C   398 R---ATINPGVDGTNRIES-TAVDFRSALIG-IYGVNRASF---VPGGLF 438
Cry9A   400 ILGRNIFRVDSQACNLNDTTYGVNRAVFYHDASEGSQRSVYEGYIRTTGI 449
Cry2A   359 NFNCSTV-------LPPLSTPFVRSWLDSGTDREGVATSTNWQTE---SF 398
Cry2B   359 NFNCSTF-------LPPLLTPFVRSWLDSGSDREGVATVTNWQTE---SF 398

                                   LOOP 3
Cry1Aa  417 QDNSVPPRAGFSHRLSHVTML-SQA--AGAV-YTLRAPTFSWQHRSAE-F 461
Cry1Ab  416 QNNNVPPRQGFSHRLSHVSMF-RSGFSNSSV-SIIRAPMFSWIHRSAE-F 462
Cry1Ac  416 QNNNVPPRQGFSHRLSHVSMF-RSGSSNSSV-SIIRAPMFSWIHRSAE-F 462
Cry1J   415 QDNSVPPHRGSSHRLSHVTM----------RASSPIFHWTHRSAT-- 449
Cry1F   409 QDNSGAPWNDYSHVLNHVTFV-RWPGEISGS-DSWRAPMFSWTHRSAT-P 455
Cry1G   405 EG--TDPFTGYSHRLCHVGFLASSPFI---S-QYARAPIFSWTHRSAT-L 447
Cry1H   409 EDESTPPYIGYSHRLCHARF-ARSPVILEPS-NFARLPVFSWTHRSAS-P 455
Cry1C   415 EDESVPPREGYSHRLCHATFVQR----SGTP-FLTTGVVFSWTDRSAT-L 458
Cry1D   409 QDASVSPAIGYSHRLCHATFLER----ISGP-RIA-GTVFSWTHRSAS-P 451
Cry1E   414 DGEN--SLVGYSHRLSHVTLTRS----LYNT-NITSLPTFVWTHHSAT-N 455
Cry1Ba  451 ETTERPNYESYSHRLSHIGII---------LQSRVNVPVYSWTHRSA--- 488
Cry1Bb  457 ETTERPNYESYSHRLSHIGLI---------IGNTLRAPVYSWTHRSADR- 496
Cry1I   458 EATGQPNYESYSHRLSHIGLI---------SASHVKALVYSWTHRSADRT 410
Cry9C   462 DE----SYGSSTHRLSHVTFFSFQTNQAGSIANAGSVPTYVWTRRDVD-L 506
Cry9A   464 ENSDIPTPEDYTHILS-TTINLTGGLRQVASNRRSSLVMYGWTHKSLAR- 511
Cry2A   414 NSNYFPDY--FIRNISGVPLVIRN-------EDLTRPLHYN-QIRNIESP 453
Cry2B   414 NSNYFPDY--FIRNISGVPLVVRN-------EDLRRPLHYN-EIRNIASP 453
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FIG. 4. Amino acid sequence alignment for loops and adjacent regions of domain II of *B. thuringiensis* toxins. Alignments were produced with the CLUSTAL program of the Intelligenetics PC/GENE package. Potential loops (shown in boldface type) were identified with Cry1Aa as a reference point (19). The order in which the toxins are listed corresponds to the order on the dendrogram based on levels of amino acid sequence similarity of domain II (Fig. 1).

1 (Cry1Aa residues 310 to 313; HRGF) seems to be the same size in all five of these ICPs. Furthermore, each of these five ICPs has an arginine in the second position in loop 1. In contrast, of the six ICPs that were toxic to both strain LAB-P and strain NO-QA (Cry1Ba, Cry1Bb, Cry1C, Cry1D, Cry1I, and Cry2A), only Cry1C has a loop 1 that is the same size as the loop 1 of the three Cry1As, Cry1F, and Cry1J. Serine rather than arginine occurs in the second position of loop 1 of Cry1C. Thus, our data suggest that interactions with loop 1 may be altered in resistant NO-QA larvae. Loop 1 of Cry1E matches loop 1 of Cry1As, Cry1F, and Cry1J in size and also has arginine in the second position. Nonetheless, Cry1E did not bind to diamondback moth brush border membrane vesicles (26) and did not kill susceptible or resistant diamondback moth larvae (13, 41, 45). These data suggest that loop 1 is necessary, but not sufficient, for binding and toxicity of Cry1As, Cry1F, and Cry1J to the diamondback moth.

A comparison with the results of studies of resistance and cross-resistance in other strains of the diamondback moth and other species of moths revealed some intriguing similarities and differences. Resistant strains of the diamondback moth derived from field-selected populations in The Philippines (13), Florida (45), and Hawaii (Tables 1 and 2) all exhibit a relatively narrow spectrum of cross-resistance. In particular,

independent studies of each strain showed that more than 100-fold resistance to one or more Cry1A toxins conferred less than 10-fold cross-resistance to Cry1B or Cry1C (13, 39, 41, 42, 45). The resistant strains from Florida (45) and Hawaii also exhibited less than 10-fold cross-resistance to Cry1D. Cry1D was potent against resistant strains from Florida (45) and Hawaii, as well as susceptible strains from New York (45) and Hawaii. Thus, it appears that the lack of toxicity initially reported for Cry1D against a susceptible strain from The Netherlands and a resistant strain from The Philippines (13) might be indicative of the specific preparation that was tested or the particular bioassays conducted rather than a general trait of Cry1D.

Cry9C from *B. thuringiensis* serovar tolworthi was highly toxic to both susceptible and Cry1A-resistant strains of the diamondback moth (20). The lack of cross-resistance between Cry1A and Cry9C and the low degree of similarity of these toxins in domain II (Fig. 1) are consistent with the patterns obtained for Cry1B, Cry1C, Cry1D, and Cry1I noted above.

The toxicity of Cry1E to three sets of susceptible and resistant diamondback moth strains was low or nil (13, 41, 45). In two independent studies, the potency of Cry2A against susceptible diamondback moth larvae was less than the potency of Cry1A (42, 45). Resistance to Cry2A was either low (42) or nil (45).

Like diamondback moth strain NO-QA, tobacco budworm (*Heliothis virescens*) strain YHD2 selected with Cry1Ac exhibited high levels of cross-resistance to Cry1F and little or no cross-resistance to Cry1C and Cry2A (17). In both of these resistant strains, inheritance of resistance to Cry1A toxins is partly to completely recessive, one or a few major loci are the primary determinants of Cry1A resistance, and some evidence indicates that reduced binding is associated with resistance (12, 17, 21, 43). Thus, data obtained with the diamondback moth and the tobacco budworm show that even in cases of relatively narrow cross-resistance associated with reduced binding of Cry1A toxin, cross-resistance can extend beyond the Cry1A family of toxins. The results of an amino acid sequence similarity analysis suggest that in such cases, cross-resistance is restricted to toxins that are similar to Cry1A in domain II (e.g., Cry1F and Cry1J). Additional tests will be needed to see if this pattern occurs with other strains and other species.

Like patterns seen in resistant strains of the diamondback moth and in strain YHD2 of the tobacco budworm, resistance to Dipel in the Indianmeal moth (*Plodia interpunctella*) is associated with reduced binding of Cry1Ab (47) and exhibits partially recessive inheritance (12). Strains of the Indianmeal moth selected with Dipel also show little or no cross-resistance to Cry1B, Cry1C, and Cry2A (28, 47). Somewhat surprisingly, Cry1Aa was much less toxic than Cry1Ab or Cry1Ac to a susceptible strain of the Indianmeal moth (28). Also, a Dipel-resistant strain of the Indianmeal moth exhibited only 6-fold resistance to Cry1Aa, compared with 260-fold resistance to Cry1Ab and 2,800-fold resistance to Cry1Ac (28). These data suggest that patterns of resistance associated with reduced binding of Cry1A toxins may not be uniform across species of moths. Perhaps more importantly, broad-spectrum cross-resistance that is not associated with greatly reduced binding has been reported in laboratory-selected strains of tobacco budworm and beet armyworm (*Spodoptera exigua*) (18, 30).

The first published data on cross-resistance among *B. thuringiensis* toxins suggested that resistance to Cry1Ab was associated with increased susceptibility to Cry1C in the Indianmeal moth (47). This apparent pattern of negative cross-resistance, which would have been a great boon to resistance management, was not reproducible in the Indianmeal moth (28) and has not been seen in extensive tests with various resistant strains of any other insect. None of 16 *B. thuringiensis* toxins tested against three resistant strains of the diamondback moth (5, 13, 20, 39, 41, 42, 45) was significantly more toxic to a resistant strain than to a susceptible strain. We conclude that negative cross-resistance among *B. thuringiensis* toxins is unlikely. Switching of toxins may have some utility, particularly to combat relatively narrow resistance, such as that observed in the diamondback moth. Any tendency toward restoration of susceptibility to one toxin during selection with another toxin would also enhance the success of this tactic. However, the number of toxins that are effective against any particular resistant strain may be quite limited, which underlines the need to use *B. thuringiensis* judiciously.

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REFERENCES

1. **Abbott, W. S.** 1925. A method of computing the effectiveness of an insecticide. J. Econ. Entomol. **18:**265–267.
2. **Abbott Laboratories.** 1992. B.t. products manual. Abbott Laboratories, North Chicago, Ill.
3. **Baum, J. A., D. M. Coyle, M. P. Gilbert, C. S. Jany, and C. Gawron-Burke.** 1990. Novel cloning vectors for *Bacillus thuringiensis*. Appl. Environ. Microbiol. **56:**3420–3428.
4. **Bosch, D., B. Schipper, H. van der Kleij, R. A. de Maagd, and W. J. Stiekema.** 1994. Recombinant *Bacillus thuringiensis* crystal proteins with new properties: possibilities for resistance management. Bio/Technology **12:**915–919.
5. **Bravo, A., K. Hendrikx, S. Jansens, and M. Peferoen.** 1992. Immunocytochemical localization of *Bacillus thuringiensis* insecticidal crystal proteins in intoxicated insects. J. Invertebr. Pathol. **60:**237–246.
6. **Crickmore, N., D. R. Zeigler, J. Feitelson, E. Schnepf, B. Lambert, D. Lereclus, C. Gawron-Burke, and D. H. Dean.** 1995. Revision of the nomeclature for *Bacillus thuringiensis* cry genes, p. 14. *In* Program and Abstracts of the 28th Annual Meeting of the Society for Invertebrate Pathology. Society for Invertebrate Pathology, Bethesda, Md.
7. **Dankocsik, C., W. P. Donovan, and C. S. Jany.** 1990. Activation of a cryptic crystal protein gene of *Bacillus thuringiensis* subspecies *kurstaki* by gene fusion and determination of the crystal protein insecticidal specificity. Mol. Microbiol. **4:**2087–2094.
8. **de Maagd, R. A., M. S. G. Kwa, H. van der Kleij, T. Yamamoto, B. Schipper, J. M. Vlak, W. J. Stiekema, and D. Bosch.** 1996. Domain III substitution in *Bacillus thuringiensis* delta-endotoxin CryIA(b) results in superior toxicity for *Spodoptera exigua* and altered membrane protein recognition. Appl. Environ. Microbiol. **62:**1537–1543.
9. **Entwistle, P., M. J. Bailey, J. Cory, and S. Higgs (ed.).** 1993. *Bacillus thuringiensis*: an environmental biopesticide. Wiley and Sons, New York.
10. **Escriche, B., B. Tabashnik, N. Finson, and J. Ferré.** 1995. Immunohistochemical detection of binding of CryIA crystal proteins of *Bacillus thuringiensis* in highly resistant strains of *Plutella xylostella* (L.) from Hawaii. Biochem. Biophys. Res. Commun. **212:**388–395.
11. **Feitelson, J. S., J. Payne, and L. Kim.** 1992. *Bacillus thuringiensis*: insects and beyond. Bio/Technology **10:**271–275.
12. **Ferré, J., B. Escriche, Y. Bel, and J. Van Rie.** 1995. Biochemistry and genetics of insect resistance to *Bacillus thuringiensis* insecticidal proteins. FEMS Microbiol. Lett. **132:**1–7.
13. **Ferré, J., M. D. Real, J. Van Rie, S. Jansens, and M. Peferoen.** 1991. Resistance to the *Bacillus thuringiensis* bioinsecticide 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.
14. **Ge, A. Z., D. River, R. Milne, and D. H. Dean.** 1991. Functional domains of *Bacillus thuringiensis* insecticidal crystal proteins: refinement of *Heliothis virescens* and *Trichoplusia ni* specificity domain of CryIA(c). J. Biol. Chem. **266:**17954–17958.
15. **Ge, A. Z., N. I. Shivarova, and D. H. Dean.** 1989. Location of the *Bombyx mori* specificity domain on *Bacillus thuringiensis* δ-endotoxin. Proc. Natl. Acad. Sci. USA **86:**4037–4041.
16. **Gould, F.** 1988. Evolutionary biology and genetically engineered crops. BioScience **38:**26–33.

17. **Gould, F., A. Anderson, A. Reynolds, L. Bumgarner, and W. Moar.** 1995. Selection and genetic analysis of a *Heliothis virescens* (Lepidoptera: Noctuidae) strain with high levels of resistance to *Bacillus thuringiensis* toxins. J. Econ. Entomol. **88:**1545–1559.
18. **Gould, F., A. Martinez-Ramirez, A. Anderson, J. Ferré, F. J. Silva, and W. J. Moar.** 1992. Broad-spectrum resistance to *Bacillus thuringiensis* toxins in *Heliothis virescens*. Proc. Natl. Acad. Sci. USA **89:**7986–7988.
19. **Grochulski, P., L. Masson, S. Borisova, M. Pusztai-Carey, J. L. Schwartz, R. Brousseau, and M. Cygler.** 1995. *Bacillus thuringiensis* CryIA(a) insecticidal toxin: crystal structure and channel formation. J. Mol. Biol. **254:**447–464.
20. **Lambert, B., L. Buysse, C. Decock, S. Jansens, C. Piens, S. Bernadette, J. Seurinck, K. van Audenhove, J. Van Rie, A. van Vliet, and M. Peferoen.** 1996. A *Bacillus thuringiensis* insecticidal crystal protein with a high activity against members of the family Noctuidae. Appl. Environ. Microbiol. **62:**80–86.
21. **Lee, M. K., F. Rajamohan, F. Gould, and D. H. Dean.** 1995. Resistance to *Bacillus thuringiensis* CryIA delta-endotoxins in a laboratory-selected *Heliothis virescens* strain is related to receptor alteration. Appl. Environ. Microbiol. **61:**3836–3842.
22. **Lee, M. K., B. A. Young, and D. H. Dean.** 1995. Domain III exchanges of *Bacillus thuringiensis* CryIA toxins affect binding to different gypsy moth midgut receptors. Biochem. Biophys. Res. Commun. **216:**306–312.
23. **Li, J., J. Carroll, and D. J. Ellar.** 1991. Crystal structure of insecticidal δ-endotoxin from *Bacillus thuringiensis* at 2.5 Å resolution. Nature (London) **353:**815–821.
24. **Lu, H., F. Rajamohan, and D. H. Dean.** 1994. Identification of amino acid residues of *Bacillus thuringiensis* δ-endotoxin CryIAa associated with membrane binding and toxicity to *Bombyx mori*. J. Bacteriol. **176:**5554–5559.
25. **Masson, L., Y. Lu, A. Mazza, R. Brousseau, and M. J. Adang.** 1995. The CryIA(c) receptor purified from *Manduca sexta* displays multiple specificities. J. Biol. Chem. **270:**20309–20315.
26. **Masson, L., A. Mazza, R. Brousseau, and B. Tabashnik.** 1995. Kinetics of *Bacillus thuringiensis* toxin binding with brush border membrane vesicles from susceptible and resistant larvae of *Plutella xylostella*. J. Biol. Chem. **270:**11887–11896.
27. **Masson, L., A. Mazza, J. L. Gringorten, D. Baines, V. Anelunias, and R. Brousseau.** 1994. Specificity domain localization of *Bacillus thuringiensis* insecticidal CryIA toxins is highly dependent on the bioassay system. Mol. Microbiol. **14:**851–860.
28. **McGaughey, W. H., and D. E. Johnson.** 1994. Influence of crystal protein composition of *Bacillus thuringiensis* strains on cross-resistance in Indianmeal moths (Lepidoptera: Pyralidae). J. Econ. Entomol. **87:**535–540.
29. **McGaughey, W. H., and M. E. Whalon.** 1992. Managing insect resistance to *Bacillus thuringiensis* toxins. Science **258:**1451–1455.
30. **Moar, W. J., M. Pusztai-Carey, H. Van Faassen, D. Bosch, R. Frutos, C. Rang, K. Luo, and M. J. Adang.** 1995. Development of *Bacillus thuringiensis* CryIC resistance by *Spodoptera exigua* (Hübner) (Lepidoptera: Noctuidae). Appl. Environ. Microbiol. **61:**2086–2092.
31. **SAS Institute.** 1985. SAS user's guide: statistics, 5th ed. SAS Institute, Cary, N.C.
32. **Schnepf, H. E., K. Tomczak, J. P. Ortega, and H. R. Whiteley.** 1990. Specificity-determining regions of lepidopteran-specific insecticidal proteins produced by *Bacillus thuringiensis*. J. Biol. Chem. **265:**20923–20930.
33. **Shin, B., S. Park, S. Choi, B. Koo, S. Lee, and J. Kim.** 1995. Distribution of *cryV*-type insecticidal protein genes in *Bacillus thuringiensis* and cloning of *cryV*-type genes from *Bacillus thuringiensis* subsp. *kurstaki* and *Bacillus thuringiensis* subsp. *entomicidus*. Appl. Environ. Microbiol. **61:**2402–2407.
34. **Smith, G. P., and D. J. Ellar.** 1994. Mutagenesis of two surface-exposed loops of the *Bacillus thuringiensis* CryIC δ-endotoxin affects insecticidal specificity. Biochem. J. **302:**611–616.
35. **Tabashnik, B. E.** 1994. Evolution of resistance to *Bacillus thuringiensis*. Annu. Rev. Entomol. **39:**47–79.
36. **Tabashnik, B. E., N. L. Cushing, N. Finson, and M. W. Johnson.** 1990. Field development of resistance to *Bacillus thuringiensis* in diamondback moth (Lepidoptera: Plutellidae). J. Econ. Entomol. **83:**1671–1676.
37. **Tabashnik, B. E., N. L. Cushing, and M. W. Johnson.** 1987. Diamondback moth (Lepidoptera: Plutellidae) resistance to insecticides in Hawaii: intra-island variation and cross-resistance. J. Econ. Entomol. **80:**1091–1099.
38. **Tabashnik, B. E., N. Finson, C. F. Chilcutt, N. L. Cushing, and M. W. Johnson.** 1993. Increasing efficiency of bioassays: evaluation of resistance to *Bacillus thuringiensis* in diamondback moth (Lepidoptera: Plutellidae). J. Econ. Entomol. **86:**635–644.
39. **Tabashnik, B. E., N. Finson, F. R. Groeters, W. J. Moar, M. W. Johnson, K. Luo, and M. J. Adang.** 1994. Reversal of resistance to *Bacillus thuringiensis* in *Plutella xylostella*. Proc. Natl. Acad. Sci. USA **91:**4120–4124.
40. **Tabashnik, B. E., N. Finson, and M. W. Johnson.** 1991. Managing resistance to *Bacillus thuringiensis*: lessons from the diamondback moth (Lepidoptera: Plutellidae). J. Econ. Entomol. **84:**49–55.
41. **Tabashnik, B. E., N. Finson, M. W. Johnson, and D. G. Heckel.** 1994. Cross-resistance to *Bacillus thuringiensis* toxin CryIF in the diamondback moth. Appl. Environ. Microbiol. **60:**4627–4629.
42. **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: Plutellidae). Appl. Environ. Microbiol. **59:**1332–1335.
43. **Tabashnik, B. E., J. M. Schwartz, N. Finson, and M. W. Johnson.** 1992. Inheritance of resistance to *Bacillus thuringiensis* in diamondback moth (Lepidoptera: Plutellidae). J. Econ. Entomol. **85:**1046–1055.
44. **Talekar, N. S., and A. M. Shelton.** 1993. Biology, ecology, and management of diamondback moth. Annu. Rev. Entomol. **38:**275–301.
45. **Tang, J. D., A. M. Shelton, J. Van Rie, S. De Roeck, W. J. Moar, R. T. Roush, and M. Peferoen.** 1996. Toxicity of *Bacillus thuringiensis* spore and crystal protein to resistant diamondback moth (*Plutella xylostella*). Appl. Environ. Microbiol. **62:**564–569.
46. **Thompson, M. A., H. E. Schnepf, and J. S. Feitelson.** 1995. Structure, function and engineering of *Bacillus thuringiensis* toxins, p. 99–117. *In* J. K. Setlow (ed.), Genetic engineering, vol. 17. Plenum Press, New York.
47. **Van Rie, J., W. H. McGaughey, D. E. Johnson, B. D. Barnett, and H. Van Mellaert.** 1990. Mechanism of insect resistance to the microbial insecticide *Bacillus thuringiensis*. Science **247:**72–74.
48. **von Tersch, M. A., H. L. Robbins, C. S. Jany, and T. B. Johnson.** 1991. Insecticidal toxins from *Bacillus thuringiensis* subsp. *kenyae*: gene cloning and characterization and comparison with *Bacillus thuringiensis* subsp. *kurstaki* CryIA(c) toxins. Appl. Environ. Microbiol. **57:**349–358.
49. **Widner, W. R., and H. R. Whiteley.** 1989. Two highly related insecticidal crystal proteins of *Bacillus thuringiensis* subsp. *kurstaki* possess different host range specificities. J. Bacteriol. **171:**965–974.
50. **Wu, S. J., and D. H. Dean.** 1996. Functional significance of loops in the receptor binding domain of *Bacillus thuringiensis* CryIIIA δ-endotoxin. J. Mol. Biol. **255:**628–640.