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Toxicity to *Spodoptera exigua* and *Trichoplusia ni* of Individual P1 Protoxins and Sporulated Cultures of *Bacillus thuringiensis* subsp. *kurstaki* HD-1 and NRD-12

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The toxicities to neonate *Spodoptera exigua* and *Trichoplusia ni* of lyophilized powders obtained from sporulated liquid cultures (referred to as sporulated cultures) and *Escherichia coli*-expressed P1 [*cryIA*(a) *cryIA*(b) *cryIA*(c)] protoxins from three-gene strains of NRD-12 and HD-1 of *Bacillus thuringiensis* subsp. *kurstaki* were determined by using diet incorporation bioassays. Although sporulated cultures from both strains were more toxic to *T. ni* than *S. exigua*, there were no differences in toxicity between NRD-12 and HD-1. Toxicities of the three individual P1 protoxins against *S. exigua* varied by at least fivefold, with the *cryIA*(b) protein being the most toxic. These same protoxins varied in toxicity against *T. ni* by at least 16-fold, with the *cryIA*(c) protein being the most toxic. However, when tested against either *S. exigua* or *T. ni*, there were no differences in toxicity between an NRD-12 P1 protoxin and the corresponding HD-1 P1 protoxin. Comparing the toxicities of individual protoxins with that of sporulated cultures demonstrates that no individual protoxin was as toxic to *S. exigua* as the sporulated cultures. However, this same comparison against *T. ni* shows that both the *cryIA*(b) and *cryIA*(c) proteins are at least as toxic as the sporulated cultures. Results from this study suggest that NRD-12 is not more toxic to *S. exigua* than HD-1, that different protein types have variable host activity, and that other *B. thuringiensis* components are not required for *T. ni* toxicity but that other components such as spores might be required for *S. exigua* toxicity.

Although the insecticidal bacterium *Bacillus thuringiensis* subsp. *kurstaki* (serotype H 3a:3b) HD-1 can effectively control some agriculturally important lepidopterous pests, such as the cabbage looper (*Trichoplusia ni* [Hübner]), many key lepidopterous pests, such as the beet armyworm (*Spodoptera exigua* [Hübner]), are relatively tolerant and cause economic damage (14). Commercial formulations containing a relatively new strain of this *B. thuringiensis* subspecies, NRD-12, were shown to be three to four times more toxic to *S. exigua* than commercial formulations of HD-1 (13). Most of the insecticidal activity against lepidoptera in *B. thuringiensis* subsp. *kurstaki* HD-1 and NRD-12 is attributed to the P1 crystal, which is composed of three proteins belonging to the *cryIA*(a), *cryIA*(b), and *cryIA*(c) protein types (7, 8, 15). Moar et al. (15) reported that the NRD-12 lyophilized powder obtained from sporulated liquid cultures (referred to here as sporulated cultures) containing both proteinaceous crystals and spores was 2.5-fold more toxic against *S. exigua* than was HD-1. Most of this increased activity was found to reside in the P1 crystal. However, subsequent studies revealed that the HD-1 strain used in those tests was missing the *cryIA*(b) gene, which has been shown to be required for maximum toxicity to *S. exigua* (15, 19). Although these results demonstrated that the *cryIA*(b) protein confers some insecticidal activity, other proteins also must be partly responsible. Additionally, it is still unclear whether NRD-12 is in fact more toxic to *S. exigua* than a strain of HD-1 containing all three P1 protoxin genes and, if so, what factor is responsible for this activity.

Recently, the three P1 protoxin proteins of HD-1 and NRD-12 have been cloned and expressed in *Escherichia coli* (L. Masson, G. Prefontaine, L. Peloquin, P. C. K. Lau, and R. Brousseau, Biochem. J., in press). It was demonstrated that in both strains, the quantity of each of the three protein types within the P1 crystal varied, with the *cryIA*(b) type constituting the highest percentage of total protein. Additionally, the HD-1 strain was shown to contain slightly more of the *cryIA*(b) protein than NRD-12 did. The purpose of this study, therefore, was to determine the toxicities of individual P1 proteins of HD-1 and NRD-12 against *S. exigua* and *T. ni* and to compare these results with those obtained with sporulated cultures containing all three P1 proteins, P2 proteins, and spores. Finally, the gene encoding the *cryIA*(c) protein of the HD-73 strain of *B. thuringiensis* subsp. *kurstaki* has also been cloned. Although this strain produces a bipyramidal crystal inclusion, the inclusion is composed of only the insecticidal *cryIA*(c) protein. We therefore decided to test the toxicity to *T. ni* of the *cryIA*(c) protein and to compare this result with those obtained with the *cryIA*(c) proteins of NRD-12 and HD-1.

MATERIALS AND METHODS

Bacterial isolates. The NRD-12, HD-1, and HD-73 strains used for gene isolation were obtained from the Forest Pest Management Institute, Sault Ste. Marie, Ontario, Canada. Both the NRD-12 and HD-1 strains were determined to contain all three P1 protoxin genes by Southern blot analyses of total genomic DNA restricted with *Hin*dIII, which produced the characteristic three-band pattern, as determined by Kronstad et al. (9). Isolates were grown on nutrient agar, and single colonies containing bipyramidal inclusion bodies were isolated and grown for further study. *E. coli* HB101 was used as the host bacterium for the pUC-based expression vectors.

Gene isolation. The cloning and expression of the three P1 *cryIA* genes from strains NRD-12 and HD-1 have been

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described in detail by Masson et al. (in press). Briefly, all *cryIA* genes, including that from HD-73, were cloned into the unique *Nde*I site of the plasmid pMPCV. The pMPCV plasmid is a cassette vector created by *Nde*I digestion and religation of the *Hpa*I-*Pst*I-cloned NRD-12 *cryIA*(b) gene in pUC18 (to remove the coding sequences of the crystal protein gene). Subcloning of all *cryIA* genes into this cassette vector (in the proper orientation as 4-kilobase-pair *Nde*I fragments) followed by transformation into *E. coli* HB101 resulted in high-level expression of the protoxin as an insoluble inclusion body (data not shown). These inclusion bodies were rapidly purified by Renografin low-capacity density gradients (E. R. Squibb & Sons, Montreal, Quebec, Canada). The insoluble protoxin inclusion bodies were harvested, and the white inclusion body pellet was washed extensively with water to remove all traces of Renografin. The resulting pellets then were suspended in water. All protoxin levels in the inclusion body preparations were determined by laser densitometry of Coomassie blue-stained sodium dodecyl sulfate-polyacrylamide gels. The stained protoxin band areas were integrated and normalized to account for the contaminated *E. coli* proteins in the preparation (typically 5 to 10%). Total protein concentrations (wt/vol) were determined by the protein-dye method of Bradford (3), with bovine serum albumin as a standard.

Growth conditions for sporulated cultures. HD-1 and NRD-12 cultures were grown in 500 ml of modified glucose-yeast-salts medium until approximately complete autolysis had occurred (15). The cultures were centrifuged, and the sediment was rinsed with water (double distilled) at least three or four times (15). The resulting pellet was suspended and lyophilized.

Determination of toxicity. The toxicities of various *B. thuringiensis* subsp. *kurstaki* components were determined by bioassays with either neonate *S. exigua* or *T. ni* larvae, as described by Moar et al. (15). In the present experiment, 7 to 15 concentrations were tested per powder or protein suspension [except for the *cryIA*(c) protein against *S. exigua*]. Each suspension was incorporated into artificial diet (containing no antibiotics and 0.1% formaldehyde). At least 24 insects were evaluated per concentration, and each treatment was replicated at least four times.

Data were analyzed by the Proc Probit procedure of SAS Institute, Inc. (18), after correction for control mortality with the formula of Abbott (1). Values from individual replicates were pooled. Control mortality was $\leq$10%.

Joint effects of 1:1:1 (wt/wt/wt) combinations of the three protoxins from both HD-1 and NRD-12 were also tested against *S. exigua* concurrently with the bioassays described above. The bioassay procedure was the same as that described above. Only one concentration (50 μg/ml) was used, and the procedure was replicated four to six times. These data were analyzed by a modified version of the method of Finney (5) as reported by Salama et al. (17), such that $E = O_a + [O_b(1 - O_a)] + [O_c(1 - O_a)(1 - O_b)]$, where E is the percentage of mortality expected from the combination, and O_a, O_b, and O_c are the observed percentages of mortality for the three different treatments. Results from χ^2 tests, where $\chi^2 = (O_t - E)^2/[E(1 - E_p)]$ (O_t is the observed mortality for the combination, E is the expected mortality, and E_p is the expected percentage of mortality), were compared with the χ^2 tabular values (df = 1, $P < 0.05$). Values not exceeding the tabular value were considered to have an additive effect, whereas values exceeding the tabular value were considered to have been either potentiated ($O_t > E$) or antagonized ($O_t < E$).

TABLE 1. Toxicity to *T. ni* and *S. exigua* of recombinant protoxins or sporulated cultures of strains of *B. thuringiensis* subsp. *kurstaki*

Protein type and strain	LC_{50} (95% FL)[a]	
	T. ni	*S. exigua*
cryIA(a)		
HD-1	6.67 (4.87–10.26)	275 (210–443)
NRD-12	6.43 (4.14–14.92)	317 (260–414)
cryIA(b)		
HD-1	1.23 (0.90–1.82)	107 (75–187)
NRD-12	0.72 (0.55–0.91)	83 (54–150)
cryIA(c)		
HD-1	0.12 (0.09–0.16)	>500
NRD-12	0.10 (0.06–0.14)	>500
HD-73	0.31 (0.23–0.61)	ND[b]
Sporulated culture		
HD-1	1.21 (1.10–1.34)	30.6 (24.2–38.0)
NRD-12	1.10 (1.05–1.16)	26.3 (22.6–30.4)

[a] LC_{50} values in micrograms of protoxin per milliliter of diet or micrograms of lyophilized sporulated culture per milliliter of diet. FL, Fiducial limit.
[b] ND, Not determined.

RESULTS

Toxicity against *S. exigua*. When the purified *E. coli*-expressed P1 protoxins of HD-1 and NRD-12 were bioassayed against *S. exigua*, there were no differences in responses to the two strains (Table 1). This result is not surprising because there also was no difference in toxicity between the sporulated cultures of both strains (Table 1). NRD-12 contains all three *Hin*dIII fragments that correspond in size to those present in HD-1 (7). Restriction endonuclease analysis of the three genes encoding the *cryIA* proteins shows no difference between HD-1 and NRD-12 for a given protein type (Masson et al., in press). When these *E. coli*-expressed protoxins are cleaved with CNBr, cleavage products within a protein type are the same for both strains (Masson et al., in press), suggesting a very strong similarity between the comparable proteins of both strains. At the genetic level, only the *cryIA*(b) gene from both strains has been cloned and sequenced (2, 7). The NRD-12 *cryIA*(b) protein shares 100% homology with the first 541 amino acids of the HD-1 *cryIA*(b) protein and differs from the entire protein (1,155 amino acids) by only 8 amino acids. Research is needed to determine what roles these eight amino acid changes might have.

A primary justification for cloning and expressing foreign genes in *E. coli* is to be able to test activity of an expressed protein without possible interactions from other compounds found in the native host. This becomes extremely important in the case of *B. thuringiensis* because of various proteins found in the P1 crystal, as well as other insecticidal compounds such as spores, *cryII*-type proteins, and β-exotoxins (10, 13, 15). If no differences in toxicity are detected between this *E. coli*-expressed protein and the native protein (12, 16; M. Pusztai, unpublished data), these *E. coli*-expressed proteins can then be used to help answer questions regarding possible interactions with other compounds.

There were considerable differences in toxicity to *S. exigua* between protein types, with the *cryIA*(b) protein being the most toxic (Table 1). The concentration required to kill 50% of the population (LC_{50}) for the *cryIA*(b) protein of NRD-12 is similar to the reported LC_{50} of the NaBr-purified P1 crystal of NRD-12 (15). The LC_{50} for the *cryIA*(b) protein of HD-1 is slightly lower than the reported LC_{50} for an HD-1 strain lacking the *cryIA*(b) gene (15). Because the *cryIA*(b)

protein is the most toxic P1 protein type of both strains to *S. exigua* and because it occupies 39 to 50% of the crystal (Masson et al., in press), one would expect that the toxicity of the *cryIA*(b) protein from both strains would be higher than that of the entire P1 crystal, especially when only an additive effect ($P < 0.05$) was recorded with combinations of all three *cryIA* protein types. For HD-1, the expected mortality from the combination was 46.1%, the observed mortality was 46.6%, and the χ^2 value was 0.01. For NRD-12, the expected mortality from the combination was 40.0%, the observed mortality was 38.3%, and the χ^2 value was 0.12. Therefore, a two-gene HD-1 crystal missing the *cryIA*(b) gene would be expected to show much less toxicity than that reported by Moar et al. (15). Interestingly, recent high-pressure liquid chromatography analysis of this two-gene HD-1 crystal has been shown to be composed of 45% *cryIA*(a) protein and 55% *cryIA*(c) protein (Pusztai, unpublished data). Further research is required to determine the total P1 protein content in a P1 crystal lacking the *cryIA*(b) protein and to determine the correlation between individual *E. coli*-expressed *cryIA* protein toxicity with the toxicity of an NRD-12 or HD-1 P1 crystal composed of that same individual *cryIA* protein. Possible protoxin combinations at ratios similar to those reported by Masson et al. (in press) also need to be studied.

As stated above, there also was no difference in toxicity to *S. exigua* between HD-1 and NRD-12 sporulated cultures. The LC_{50} of our three-gene NRD-12 is very similar to that of an isolate of NRD-12 obtained from a commercial formulation tested in 1987 (26.3 versus 20.8 μg/ml) (15), suggesting not only that both NRD-12 isolates contained all three *cryIA* proteins but also that the sensitivity of the *S. exigua* colony used for both studies was relatively stable over time. The LC_{50} of HD-1 is substantially lower than that of the two-gene HD-1 reported by Moar et al. (15) (30.6 versus 49.3 μg/ml) and parallels the results of Wilcox et al. (19), who compared two-gene with three-gene HD-1 against third-instar *S. exigua*. These results again demonstrate the importance of the *cryIA*(b) protein for *S. exigua* activity.

Interestingly, there was no difference in toxicity between HD-1 and NRD-12, even though the NRD-12 strains from two different sources have been shown to produce β-exotoxin in glucose-yeast-salts fermentation medium (15; Moar, unpublished data), which can also potentiate the activity of *B. thuringiensis* containing P1 crystals, P2 crystals, and spores (13). However, repetitive washing of the culture prior to lyophilization removes all of the β-exotoxin, as determined by *Musca domestica* L. bioassays (15). Therefore, any production of β-exotoxin by NRD-12 spores or subsequent vegetative cells within the insect probably is negligible to the overall toxicity observed. Because isolates can vary in quantity of protoxin produced depending on the culture medium used (4), it remains unclear whether any differences in toxicity between the two strains could occur under various fermentation conditions. When the LC_{50}s of the protoxins were compared with the LC_{50}s of the sporulated cultures, the resulting ratios were similar for HD-1 and NRD-12 (Table 2). For all protoxin/sporulated culture ratios, the result was greater than one, suggesting that the toxicity of the sporulated cultures could not be attributed exclusively to the protoxins.

Toxicity against *T. ni*. There were no differences in response to the two strains when the purified *E. coli*-expressed P1 protoxins of HD-1 and NRD-12 were bioassayed against *T. ni* (Table 1). There were substantial differences in toxicities to *T. ni* between protein types, with the *cryIA*(c) protein being the most toxic, contrary to the results found with *S. exigua*. This result is substantiated by a report by Wilcox et al. (19), who found little difference in toxicities between two-gene and three-gene HD-1 cultures against *T. ni*. This suggests that the toxicity of the *cryIA*(b) protein, compared with those of the other *cryIA* proteins in the P1 crystal, for *T. ni* is limited. The HD-73 *cryIA*(c) protoxin was approximately three times less toxic than that of either HD-1 or NRD-12, suggesting possible amino acid differences within this protein (Table 1). Our results from HD-73 and the HD-1 *cryIA*(a) protein are similar to those reported by Ge et al. (6). There also was no difference in toxicity between the sporulated cultures of HD-1 and NRD-12 (Table 1).

TABLE 2. LC_{50} ratios between protoxins and sporulated cultures and between *S. exigua* and *T. ni* for strains of *B. thuringiensis* subsp. *kurstaki*

Protein type and strain	LC_{50} ratio between:		
	Protoxins and sporulated cultures		*S. exigua* and *T. ni*
	T. ni	*S. exigua*	
cryIA(a)			
HD-1	5.5	9.0	41.2
NRD-12	5.9	12.1	49.3
cryIA(b)			
HD-1	1.09	3.50	87.0
NRD-12	0.65	3.16	115
cryIA(c)			
HD-1	0.10	>16	>4,000
NRD-12	0.09	>19	>5,000
Sporulated culture			
HD-1			25.3
NRD-12			23.9

When the LC_{50}s of the protoxins were compared with the LC_{50}s of the sporulated cultures, the resulting ratios were similar for HD-1 and NRD-12 (Table 2). This was expected because all *cryIA* protein types exhibited similar toxicities between strains, because the *cryIA*(c) protein is substantially more toxic than the other protein types, and because the *cryIA*(c) protein occupies between 33 and 37% of the P1 crystal in both strains (Masson et al., in press). However, the protoxin/sporulated culture ratios for *cryIA*(b) and *cryIA*(c) proteins were equal to or less than one, suggesting that other *B. thuringiensis* components were not required for maximum toxicity.

Comparison of protein toxicities between species. Comparison of *S. exigua* LC_{50}s with those of *T. ni* within a protein type or sporulated culture showed similar values between HD-1 and NRD-12 (Table 2). Considerable differences were noticed between the different protein types and sporulated cultures, with the largest variation occurring in the *cryIA*(c) ratios, which had at least a 4,000-fold difference in toxicity between *S. exigua* and *T. ni*. This large difference in toxicity is because the *cryIA*(c) protein is the *cryIA*-type protein most toxic to *T. ni* but the type least toxic to *S. exigua*.

DISCUSSION

We have shown that there were no differences in toxicities to *S. exigua* and *T. ni* between HD-1 and NRD-12, although there were differences in toxicities between the *cryIA*-type proteins. Information on the insect toxicity spectrum for individual proteins could be used either to improve new *B. thuringiensis* strains toxic to a given host range or to

determine which protein(s) should be incorporated into plants to control particular insects. Accurate identification of individual *B. thuringiensis* toxins becomes extremely important when looking for toxins to manage the development of resistance because, as McGaughey and Johnson (11) demonstrated, insects resistant to one *B. thuringiensis* isolate can still be sensitive to another *B. thuringiensis* isolate. These data suggest that expression of multiple *B. thuringiensis* toxins in bacteria or in plants could reduce the occurrence of resistance. Access to purified individual proteins and knowledge of their individual toxicities will allow researchers to determine whether populations of insects that are resistant to one *cryIA*-type protein (such as *S. exigua* or *T. ni*) or spore (such as *S. exigua*) could still be susceptible to other *cryIA* types, or at least other *cry* types. This information will allow proper decisions to be made to determine the most appropriate use of *B. thuringiensis* to control insects and to minimize the potential for resistance development.

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