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Contribution of *Bacillus thuringiensis* Spores to Toxicity of Purified Cry Proteins Towards Indianmeal Moth Larvae

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Abstract. The influence of *Bacillus thuringiensis* subsp. *kurstaki* HD-1 spores upon the toxicity of purified Cry1Ab and Cry1C crystal proteins toward susceptible and BT-resistant Indianmeal moth (IMM, *Plodia interpunctella*) larvae was investigated. With susceptible larvae, HD-1 spores were toxic in the absence of crystal protein and highly synergistic (approximately 35- to 50-fold) with either Cry1Ab or Cry1C protein. With BT-resistant IMM larvae, HD-1 spores were synergistic with Cry1Ab and Cry1C protein in all three resistant strains examined. Synergism was highest (approximately 25- to 44-fold) in insects with primary resistance toward Cry1C (IMM larvae with resistance to *B. thuringiensis* subsp. *aizawai* or *entomocidus*). However, HD-1 spores also synergized either Cry1Ab or Cry1C toxicity toward larvae resistant to *B. thuringiensis* subsp. *kurstaki* at a lower level (approximately five- to sixfold). With susceptible larvae, the presence of spores reduced the time of death when combined with each of the purified Cry proteins. Without spores, the speed of intoxication and eventual death for larvae treated with Cry1C and Cry1Ab proteins was much slower than for the HD-1 preparation containing both spores and crystals together. Neither spores nor toxin dose affected the mean time of death of resistant larvae treated with either Cry1Ab or Cry1C toxins. Both Cry1Ab and Cry1C toxins appeared to reduce feeding and consequently toxin consumption.

The selection of Indianmeal moth (IMM, *Plodia interpunctella*) strains that are resistant to *Bacillus thuringiensis* has provided opportunities for studying insecticidal crystal protein (ICP) mode of action as well as resistance mechanisms and toxin specificity [20, 21, 25]. The crystal proteins of *B. thuringiensis* are many and varied [11], but all appear to cause larval death in similar fashion. That is, they induce gut membrane flux imbalance, causing irregular ionic flow between body cavities, and the intoxicated larva is unable to feed [6, 8, 16]. Each crystal protein type has a different level of toxicity against a specific insect species, thus creating families of appropriate toxin-host relationships, or susceptibilities. The specificity demonstrated between ICP types and various insect groups is fundamental to planning protection strategies for insect control with *B. thuringiensis*.

The traditional role of the bacterial endospore is to ensure continuation of the life cycle during periods of low food levels and/or environmental stress. However, its insecticidal role in combination with the crystal in strains of *B. thuringiensis* is less clear. Most lepidoptera fall into one of three classes of response to *B. thuringiensis*, based upon the rapidity of paralysis and the need for spores in order to achieve larval death [9]. Most highly susceptible lepidopteran larvae will succumb to crystals (δ-endotoxin) alone. However, many will be killed at a faster rate if spores are also present. Spores contribute significantly to Indianmeal moth mortality by *B. thuringiensis* [14, 17]. Consequently, it is difficult to compare mortalities of different toxin preparations that vary in proportion of spores and crystals owing to the contributions from each component.

In this paper, we present evidence that spores in mixtures or with purified δ-endotoxin solutions can act synergistically to increase toxicity in susceptible Indianmeal moth larvae. However, the response of certain strains of BT-resistant Indianmeal moth larvae to the

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addition of spores with Cry1Ab or Cry1C protein depends upon the resistance character of the larvae. The effect of purified HD-1 spores upon the time of death of susceptible and BT-resistant larvae treated with Cry toxins is also reported.

Materials and Methods

Bacterial strains and δ-endotoxins. Several *B. thuringiensis* ICP preparations were used throughout this study. They were: a commercial spore-crystal mixture (Dipel, Abbott Laboratories, Chicago, Illinois) produced from *B. thuringiensis* subsp. *kurstaki* HD-1 and known to contain Cry1Aa, Cry1Ab, Cry1Ac, and Cry2Aa proteins [21]; Cry1Ab protein from *B. thuringiensis* subsp. *berliner*; and Cry1C protein from *B. thuringiensis* subsp. *entomocidus*. Crystals and spores from *B. thuringiensis* subsp. *kurstaki* HD-1 were produced on GYS medium [22] at 30°C with aeration. They were separated and purified by sodium bromide density gradient centrifugation [2]. Purified Cry1Ab and Cry1C proteins were prepared by the method of Höfte et al. [12] and Hofmann et al. [10] from recombinant *Escherichia coli* clones that possessed the appropriate *cry* genes from *B. thuringiensis*. Growth of *E. coli* clones was on LB media at 37°C. The clones were kindly provided by Plant Genetic Systems, Gent, Belgium.

Insect strains. Several colonies of Indianmeal moths with resistance to *B. thuringiensis* were established in 1992 by McGaughey and Johnson [20]. They were selected from the same parent colony (RC688/unt) for resistance to different spore-crystal preparations of *B. thuringiensis*. Selection procedures were described in McGaughey and Johnson [20]. They continue to be maintained under conditions of constant selection pressure. Three of the BT-resistant IMM colonies were used in this study. They were: RC688/112^R (resistant to *B. thuringiensis* subsp. *aizawai* HD-112; approximately 29-fold), RC688/198^R (resistant to *B. thuringiensis* subsp. *entomocidus* HD-198; approximately 21-fold), and RC688/Dip^R (resistant to *B. thuringiensis* subsp. *kurstaki* HD-1; approximately 140-fold).

Bioassay techniques. The *apple slice* bioassay system employed individual larvae in compartmented trays, each treated and contained separately from the others [15]. Small cubes (approximately 2 mm) of semidehydrated apple were placed in separate compartments of bioassay trays (C-D International, Inc., Pitman, New Jersey) and dosed with 2 µl of 1:2 dilutions of toxin suspension (Dipel) or Cry protein solutions in 1% yeast extract. A single late-second to third instar larva (based on head capsule size) [18] was placed in each compartment and incubated at 26°C. The larvae were inspected every 3–4 days for death. Upon complete consumption of the apple cube, any remaining live larvae were refed with complete cracked wheat diet [19], and observation was continued. Typically, 16 or more apple cubes and larvae were used per dose, and at least eight dilutions of toxin were assayed. Each experiment was replicated at least twice, constituting 256 larvae per trial. Mortality was determined from the percentage of survivors (based upon adult emergence), and was corrected for mortality in untreated controls [1]. Mortality data were combined to calculate LD_{50}'s according to the procedure of Finney [7] using a probit analysis program written by G.A. Milliken (Kansas State University, Manhattan, Kansas). In order to judge relative levels of synergism, the expected toxicities of spore/crystal protein combinations were calculated by the method of Tabashnik [24].

Statistical analysis (95% confidence limits, χ^2) was performed with the analytical tools provided by the Sigma Plot for Windows software program (Jandel Scientific Software, San Rafael, California).

Table 1. Toxicity of *Bacillus thuringiensis* subsp. *kurstaki* HD-1 spores, crystals, and purified Cry1Ab and Cry1C protein towards susceptible Indianmeal moth larvae

	IMM Mortality				
Toxin preparation	No. of larvae	LD_{50}[a]	95% CI[b]	Slope	χ^2
Dipel spores/ crystals	256	1.637	1.354–1.991	1.63	13.27
HD-1 spores	256	0.869	0.683–1.086	2.28	5.20
HD-1 crystals	256	0.150	0.118–0.196	4.43	1.25
Cry1Ab	256	0.153	0.106–0.225	1.96	4.68
Cry1C	256	0.354	0.169–0.799	2.01	12.98
Cry1Ab + spores (2)[c]	256	0.012	0.001–0.084	1.68	6.49
Cry1C + spores (2)	256	0.019	0.006–0.036	1.09	9.42

[a] µg dry wt/larva.
[b] 95% confidence interval.
[c] Constant spores (HD-1) = 2 µg dry weight/larva.

Protein determination. The protein content of Cry toxins was measured by the BCA protein assay (Pierce, Rockford, Illinois) at room temperature. Bovine serum albumin (Sigma, St. Louis, Missouri) was used as a protein standard.

Results

Toxicity of HD-1 spores and purified Cry proteins toward IMM larvae. The relative toxicities of *B. thuringiensis* subsp. *kurstaki* spores and crystals, a commercial Dipel® preparation, and purified Cry1Ab and Cry1C toxins toward susceptible Indianmeal moth larvae are shown in Table 1. The toxicity of purified spores, crystals, or Cry protein ranged between 0.15 and 0.87 µg dry weight/larvae. When spores (2 µg dry weight/larva) were applied along with either of the purified Cry proteins, however, the toxicity of the preparation increased approximately 10-fold, to LD_{50} levels ranging from 0.01 to 0.02 µg dry weight/larvae. These values exceed the expected toxicity of spores and crystal protein calculated by the method of Tabashnik [24]. According to this calculation, the expected mortality for Cry1Ab + spores (2 µg/larva) should be 0.637, and 0.714 for Cry1C + spores (2 µg/larva). Thus, spores were clearly synergistic with crystal protein toward susceptible IMM larvae.

When BT-resistant IMM larvae were used, however, the effect of spores on Cry protein toxicity was variable (Table 2). With RC688/112^R (resistant to subsp. *aizawai*) and RC688/198^R (resistant to subsp. *entomocidus*), HD-1 spores increased the toxicity of

Table 2. Toxicity of *Bacillus thuringiensis* subsp. *kurstaki* HD-1 spores and purified Cry1Ab and Cry1C protein toward BT-resistant Indianmeal moth larvae

	IMM mortality					
	RC688/112^R		RC688/198^R		RC688/Dip^R	
Toxin prepration	LD_{50}[a]	95% CI[b]	LD_{50}	95% CI	LD_{50}	95% CI
HD-1 spores	2.32	1.26–3.78	3.76	3.09–4.62	18.65	13.17–43.78
Cry1Ab	1.32	0.98–1.81	2.05	1.23–4.30	1.05	0.82–1.34
Cry1C	0.75	0.43–1.35	1.14	0.93–1.41	1.38	0.97–2.06
Cry1Ab + spores (2)[c]	0.07	0.02–0.06	0.06	0.03–0.10	0.80[d]	0.27–2.02
Cry1C + spores (2)	0.06	0.02–0.14	0.15	0.08–0.27	0.92[d]	0.58–1.65

[a] µg dry weight/larva.
[b] 95% confidence interval.
[c] Spore (HD-1) concentration = 2 µg dry weight/larva.
[d] Spore (HD-1) concentration for RC688/Dip^R larvae = 5 µg dry weight/larva.

both Cry1Ab and Cry1C proteins. In the case of RC688/Dip^R IMM larvae, which are resistant to HD-1 type protein (Cry1A), the insects were highly resistant to HD-1 spores as well and the spores did not appear to significantly increase the toxicity of either Cry1Ab or Cry1C protein. Calculation of the expected toxicities of spore/crystal mixtures toward these resistant larvae resulted in 1.78 (Cry1Ab + spores) and 1.48 (Cry1C + spores) for RC688/112^R larvae; 2.65 and 2.05 respectively for RC688/198^R larvae; and 4.77 and 5.05 respectively for RC688/Dip^R larvae. Accordingly, HD-1 spores are synergistic with both Cry1Ab and Cry1C protein toward all three strains of BT-resistant IMM larvae. The increase in toxicity caused by spores with either toxin amounted to a 25- to 44-fold increase over expected rates toward RC688/112^R and RC688/198^R larvae, but spores resulted in only a five- to six-fold increase for either Cry1Ab or Cry1C toxin toward larvae resistant to BT subsp. *kurstaki* (RC688/Dip^R).

Increasing levels of HD-1 spores in the presence of Cry1Ab or Cry1C protein enhanced toxicity toward both susceptible and BT-resistant IMM larvae. For susceptible larvae, the LD_{50} of Cry1Ab protein was 1.89, 1.26, and 0.06 µg protein/larva in the presence of 0.5, 1.0, and 2.0 µg dry weight spores/larva, respectively (Fig. 1). A similar response was obtained with Cry1C protein toward BT-resistant RC688/198^R larvae supplemented with HD-1 spores. The spore stimulatory response for Cry1Ab and Cry1C proteins was similar toward either of the *entomocidus* or *aizawai*-resistant IMM strains tested, but was less stimulatory with Cry1Ab or Cry1C toxicity toward the Dipel®-resistant strain (data not shown).

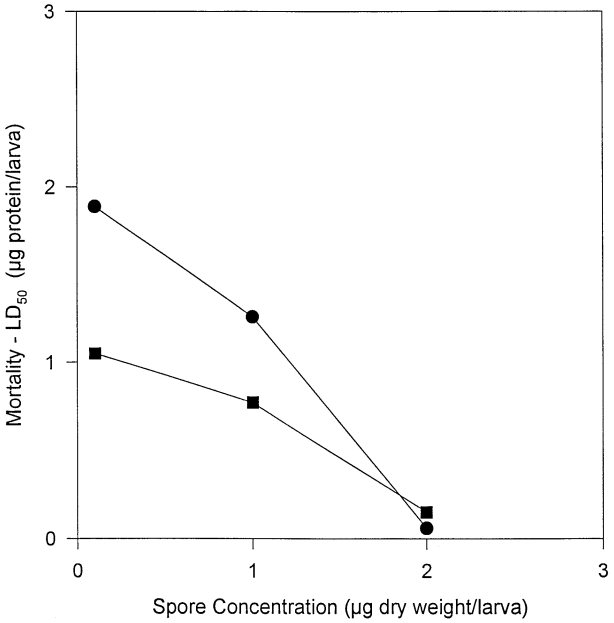

Fig. 1. Effect of increasing concentrations of *B. thuringiensis* subsp. *kurstaki* HD-1 spores on toxicity (LD_{50}) of Cry1Ab and Cry1C proteins toward BT-resistant Indianmeal moth larvae (RC688/198^R). Apple slices were prepared with a graded series of Cry protein and additionally dosed with a uniform concentration of HD-1 spores (either 0.5, 1, or 2 µg dry weight/apple slice). A single larva was allowed to feed on each slice (32 replicates per dose; see Materials and Methods). CryIAb, ●; CryIC, ■.

Effect of HD-1 spores and BT-resistance upon larval time of death. The survival time of larvae treated with various types of toxins also differed between susceptible and BT-resistant IMM strains. For susceptible larvae (RC688/unt), the time of death for larvae was dependent not only on the dose rate of the toxins (Cry1Ab or Cry1C), but also upon the presence of spores in the preparation (HD-1, Dipel; Table 3). At

Table 3. Time of death (days) for Indianmeal moth (*P. interpunctella*) larvae (RC688/unt) treated with varying doses of either HD-1 (Dipel, consisting of spores and crystals), purified Cry1Ab protein, or purified Cry1C protein

	RC688/unt								
	HD-1			Cry1Ab			Cry1C		
Toxin dose[a]	% dead	Time of death (days)	±95% CI[b]	% dead	Time of death (days)	±95% CI	% dead	Time of death (days)	±95% CI
0.02	0.8	29.5	9.3	7.2	29.5	14.6	2.1	34.4	12.1
0.05	2.0	27.0	8.1	12.5	23.8	9.3	3.5	28.4	15.9
0.1	8.4	24.6	10.9	27.3	17.7	12.9	22.6	27.9	11.7
0.2	14.4	21.7	12.2	54.3	19.0	12.5	39.6	30.2	10.1
0.5	21.3	19.6	12.8	72.7	18.6	11.4	64.2	26.3	11.7
1.0	35.6	15.2	11.9	75.8	12.1	8.5	94.0	23.3	13.5
2.0	54.2	10.5	10.1	100.0	9.1	5.3	100.0	18.4	11.7
5.0	81.7	8.5	9.1	100.0	9.3	6.3	100.0	16.3	11.7
10.0	88.5	5.9	5.7	100.0	5.9	4.0	100.0	13.6	8.1
20.0	97.2	4.5	3.0	100.0	4.3	1.1	100.0	13.1	8.0

Each individual larva was examined every 3–4 days for viability after the initial dose was administered. A total of 128 larvae per experiment for each toxin was repeated three times, and the cumulative results were tabulated.

[a] Micrograms dry wt/larva for HD-1 spore/crystal mixture; micrograms protein/larvae for Cry1Ab and Cry1C toxins.

[b] 95% confidence interval.

low doses of toxins, only a few larvae died at approximately 29–34 days, and death was not affected by toxin type. As the dose rate was increased, the time of death for larvae treated with all three types of toxins diminished, but at different rates. At the LD_{50}, the mean time of death for susceptible larvae treated with HD-1 was 10.5 days, compared with 19.0 days for Cry1Ab and 26.3 days for Cry1C. At a high level of HD-1 or Cry1Ab (20 µg toxin/larva), the mean time of death was shortened (approximately 4–5 days, but the mean time of death remained longer with Cry1C (13.1 days).

For resistant larvae (including strains RC688/112^R, RC688/198^R, and RC688/Dip^R), the effect of toxin dose upon larval time of death was eliminated. Also, the presence of spores in the HD-1 spore/crystal mixture had no appreciable effect upon larval time of death for BT-resistant larvae. In Table 4, the mean times of death for Cry1Ab and Cry1C toxins and Dipel toward RC688/Dip^R larvae are shown. Mean time of larval death ranged from 15.6 to 18.7 days at the LD_{50} dose, regardless of the toxin used. Also, the dose effect with higher toxin levels did not lead to a shorter larval time of death in larvae resistant to BT subsp. *kurstaki* as seen with susceptible larvae. The response was similar for RC688/112^R (Table 5), which responded to Cry1Ab and Cry1C toxins and Dipel with a time of death at the LD_{50} dose ranging from 9.6 to 14.6 days. As with the other BT-resistant larvae, the effect of spores upon the time of death of RC688/198^R larvae was negligible (data not shown).

Discussion

Spores are important contributors to the toxicity of *B. thuringiensis* δ-endotoxins towards Indianmeal moth larvae. Purified *B. thuringiensis* HD-1 spores have significant toxicity towards Indianmeal moth larvae in the absence of crystal protein [14]. Spores are known to contain significant amounts of Cry proteins in their proteinaceous spore coat; this contributes significantly to larval toxicity [3, 4]. Also, normal germination and outgrowth in the insect hemolymph leads to a septicemia which can contribute to larval mortality in certain insects [9, 23]. Likewise, we found that the addition of HD-1 spores (2 µg dry weight) to the normal dosage series using either Cry1Ab or Cry1C toxin decreased the LD_{50} for susceptible larvae approximately 35- to 50-fold. Spores (2 µg/larva) were also synergistic with crystal protein for larvae resistant to either *B. thuringiensis* subsp. *aizawai* or *entomocidus*, amounting to approximately a 10- to 25-fold improvement in toxicity over crystal protein. For larvae resistant to Dipel (RC688/Dip^R), however, spores were less synergistic with both Cry1Ab and Cry1C, even at an elevated dose (5 µg/larva). The enhanced syngergism of HD-1 spores for Cry1Ab and Cry1C toxin toward *aizawai* and *entomocidus*-resistant IMM larvae may reflect the Cry toxin composition of the spore coat, which could possess a range of Cry proteins similar to the HD-1 crystal (Cry1Aa, Cry1Ab, Cry1Ac, Cry2Aa, and Cry2Ab) [26]. Primary resistance in these two insect

Table 4. Time of death (days) for BT-resistant Indianmeal moth (*P. interpunctella*) larvae (RC688/Dip^R) treated with varying doses of either HD-1 (Dipel, consisting of spores and crystals), purified Cry1Ab protein, or purified Cry1C protein

	RC688/Dip^R								
	HD-1			Cry1Ab			Cry1C		
Toxin dose[a]	% dead	Time of death (days)	±95% CI[b]	% dead	Time of death (days)	±95% CI	% dead	Time of death (days)	±95% CI
0.1		NT[c]			NT		1.8	14.1	2.86
0.2	2.6	15.5	4.09	2.4	16.9	5.03	7.5	11.9	2.12
0.5	4.1	13.1	4.77	9.2	17.7	5.32	30.6	16.0	3.01
1.0	7.5	15.6	6.10	32.1	15.6	6.81	57.8	18.7	3.32
2.0	14.4	15.7	5.34	92.8	15.5	5.49	84.4	19.4	3.03
5.0	25.0	15.6	6.59	100.0	15.6	6.14	96.9	21.0	3.61
10.0	42.5	14.8	4.32	100.0	13.5	5.62	100.0	17.2	4.87
20.0	87.5	16.2	5.16	100.0	14.4	4.38	100.0	15.7	4.81
40.0	71.9	15.4	6.42		NT			NT	
80.0	87.6	15.6	6.14		NT			NT	

Each individual larva was examined every 3–4 days for viability after the initial dose was administered. A total of 128 larvae per experiment for each toxin was repeated three times and the cumulative results were tabulated.

[a] Micrograms dry wt/larva for HD-1 spore/crystal mixture; micrograms protein/larvae for Cry1Ab and Cry1C toxins.

[b] 95% confidence intervals.

[c] NT, not tested.

Table 5. Time of death (days) for BT-resistant Indianmeal moth (*P. interpunctella*) larvae (RC688/112^R) treated with varying doses of either HD-1 (Dipel, consisting of spores and crystals), purified Cry1Ab protein, or purified Cry1C protein

	RC688/112^R								
	HD-1			Cry1Ab			Cry1C		
Toxin dose[a]	% dead	Time of death (days)	±95% CI[b]	% dead	Time of death (days)	±95% CI	% dead	Time of death (days)	±95% CI
0.1		NT[c]		3.4	9.7	2.36	7.7	13.0	7.42
0.2		NT		10.2	9.6	2.69	8.2	13.9	7.14
0.5	14.1	16.9	6.59	21.3	11.2	3.52	30.0	13.0	5.07
1.0	17.8	14.1	6.55	28.8	12.4	3.38	39.4	14.2	3.64
2.0	43.1	14.6	7.12	56.9	9.6	2.10	92.8	13.2	4.04
5.0	89.4	14.6	5.49	100.0	12.1	3.58	100.0	14.7	2.90
10.0	96.6	11.1	4.18	100.0	11.4	2.86	100.0	14.2	3.08
20.0	100.0	13.0	4.39	100.0	11.4	3.77	100.0	10.7	3.28
40.0	100.0	9.7	3.15		NT			NT	
80.0	100.0	8.8	2.39		NT			NT	

Each individual larva was examined every 3–4 days for viability after the initial dose was administered. A total of 128 larvae per experiment for each toxin was repeated three times, and the cumulative results were tabulated.

[a] Micrograms dry wt/larva for HD-1 spore/crystal mixture; micrograms protein/larvae for Cry1Ab and Cry1C toxins.

[b] 95% confidence intervals.

[c] NT, not tested.

strains was to Cry1C protein, although the insects were also exposed to smaller amounts of Cry1Ab and other Cry toxins [21]. Consequently, any Cry1Ac in the spores could dramatically affect larval mortality, since they were not exposed to this toxin during resistance selection. Likewise, the reduced synergism between HD-1 spores and either Cry1Ab or Cry1C toxin toward Dipel-resistant IMM larvae probably was a result of their previous exposure to the complete range of Cry toxins known to be produced by *B. thuringiensis* subsp. *kurstaki* HD-1.

It is not clear whether synergism between spores and crystal protein arises only from a toxic protein effect or from a condition of septicemia in sick and weakened larvae. The inclusion of spores shortened the time of death of susceptible larvae, apparently

through a combination of synergistic toxin activity and septicemia. When spores were present, larval death was accompanied by a distinctive blackening of the cadaver, characteristic of oxidative degradation of the hemolymph. This character was not so obvious or prevalent in larvae treated with purified Cry toxin as in treatments also containing spores. The lack of larval blackening coupled with loss of variation in the time of death in resistant IMM larvae leads one to speculate on the loss of a partial role for spores in BT-resistance. Even though spores are still synergistic with toxin protein for resistant IMM larvae, the larvae may not be as susceptible to spore germination and resulting septicemia following midgut damage. The absence of a spore-induced septicemia could be the result of an improved immune response in BT-resistant insects, similar to the observed phagocytosis of *Pseudomonas aeruginosa* in larvae of *Manduca sexta* [5, 13]. Indianmeal moth resistance can be established to purified Cry toxins in the absence of spores (unpublished results), however, and these strains will be surveyed for their sensitivity to spores in the presence and absence of Cry protein.

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