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Spore Coat Protein Synergizes *Bacillus thuringiensis* Crystal Toxicity for the Indianmeal Moth (*Plodia interpunctella*)

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Abstract. Spores from *Bacillus thuringiensis* serovars *kurstaki* and *entomocidus* synergized crystal protein toxicity for larvae of the Indianmeal moth (*Plodia interpunctella*). Preparations of spore-crystal mixtures of either serovar were more toxic for the larvae than either purified spores or crystals alone (based on dry weight). Spores lost 53% of their toxicity for the Indianmeal moth after 2 h of UV-irradiation, but remained partially toxic (28%) even after 4 h of irradiation. Spore coat protein was toxic for the Indianmeal moth and was synergistic with *B. thuringiensis* serovar *kurstaki* HD-1 crystal protein. Enhanced toxicity of the combined spore-crystal preparation was attributed to a combination of crystal and spore coat protein, and included the effects of spore germination and resulting septicemia in the larval hemolymph. Ultraviolet irradiation of spores reduced the toxicity from septicemia but not the synergism caused by spore coat protein. The potencies of spore-crystal preparations must be carefully evaluated on the basis of contributions from all three factors.

Variations between different preparations of laboratory-reared *Bacillus thuringiensis* serovars often result in substantial differences in their toxicity toward susceptible insect larvae [5, 6]. All the components of a *B. thuringiensis* mixture, including spores and crystals and the ratio between them, help to determine the actual toxicity toward the susceptible insect [10, 12, 16, 17, 26]. The entomocidal protein that comprises the inclusion body (crystal) of *B. thuringiensis* is toxic to a wide variety of lepidopteran insect larvae. Some, such as *Bombyx mori* [7] and *Manduca sexta* [22], succumb to the presence of crystal protein alone without any influence or contribution from other bacterial components. Other lepidopteran insects are most sensitive to a combination of *B. thuringiensis* crystals and spores [12, 20]. This is understandable, since the spores of *B. thuringiensis* have long been known to possess spore coat protein that is immunologically similar to crystal protein from the same species [3, 4]. Thus, comparisons of insect toxicity between samples that may contain spores, crystals, or soluble crystal protein from either a *B. thuringiensis* source or transformed *Escherichia coli* clone are difficult with insects that respond differentially to each of these components.

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We compared a number of preparations of *Bacillus thuringiensis* grown under similar conditions for toxicity toward Indianmeal moth (*Plodia interpunctella*) larvae based on several different parameters. These included spore and crystal counts, protein content, and the detection of toxic protein in crude lysates of *B. thuringiensis* HD-198 by Western blots. We also examined the effect of spores and spore coat protein upon the toxicity of crystal protein (including cloned Cry1Aa, Cry1Ab, and Cry1Ac) toward the Indianmeal moth.

Materials and Methods

Bacterial strains and toxins. *Bacillus thuringiensis* serovars *kurstaki* HD-1 and *entomocidus* HD-198 were grown in glucose–yeast extract–salts (GYS) medium [19] at 30°C with agitation for 72 h. Culture growth was in six individual Fernbach flasks, each containing 1 L of growth medium. Inoculation level was 2% from a logarithmic phase seed culture. Spores, crystals, and cellular debris were recovered by centrifugation, washed extensively with 0.5 M salt (either potassium or sodium chloride buffered with 0.05 M Tris-HCl, pH 8.5) solution, dialyzed against 0.02 M Tris-HCl and 0.02 M KCl (pH 8.5), and lyophilized. Spores and crystals were separated by density gradient centrifugation in sodium bromide [2]. Purified spores and crystals were estimated to be at least 95% enriched, as judged by phase contrast microscopy. Cry proteins (Cry1Aa, 1Ab, 1Ac, and 1C) were prepared separately from recombinant genes cloned in *Escherichia coli* or in acrystalliferous *B. thuringiensis*.

Insect strain and bioassays. Indianmeal moths (*Plodia interpunctella* Hübner, IMM) used in this study were from a *B. thuringiensis*-susceptible colony established in 1988 and designated RC688/unt [14]. The insects were reared on an enriched ground wheat diet [13] at 25°C and 60–70% relative humidity. Bioassays were performed by a single larva treatment procedure termed the apple cube bioassay method [11, 15]. 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 [8] with a probit analysis program written by G.A. Milliken (Kansas State University, Manhattan, KS, USA). In order to judge relative levels of synergism, the expected toxicities of spore/crystal protein combinations were calculated by the method of Tabashnik [25]. In this procedure, the expected $LD_{50(m)}$ is the mean of the LD_{50}s of the components of the mixture weighted by their proportions (r_a, r_b, etc.) in the mixture. Thus, the equation used to calculate expected toxicity of two different toxins together becomes:

$$LD_{50(m)} = [r_a/LD_{50(a)} + r_b/LD_{50(b)}]^{-1}$$

Spore, crystal, and protein measurements. Spores were counted by several different methods. Viable spore counts were enumerated by treating a 0.05-g dry weight/ml (in 0.1% yeast extract) suspension of each HD-198 lyophilized powder for 30 min at 80°C, followed by subsequent serial dilutions in 0.1% yeast extract and plating on agar plates containing 1.5% yeast extract, 0.035 M K_2HPO_4, and 0.2% glucose (pH 7.3). Colony-forming units (CFU) were counted after 24-h incubation at 28°C. Total spores and crystals were estimated with serial dilutions of a 0.05-g dry weight/ml suspension, which were counted with the aid of a Petroff-Hausser counting chamber and a phase contrast microscope. Spores were irradiated (ultraviolet shortwave, 254 nm) at an approximate exposure of 200 μW/cm^2 in a shallow vessel (petri plate) in 1% yeast extract solution (approximately 3-mm depth with constant agitation by an orbital platform shaker) within a biosafety cabinet containing a GE G30T8 germicidal lamp. Spore coat protein was extracted by a modification of the method of Somerville and Pockett [24] employing 0.02 M 2-mercaptoethanol in 0.0135 *N* NaOH, pH 11.5 at room temperature. Spore suspensions were subjected to 3-min bursts of ultrasound to accelerate the extraction process. Spore coat protein solutions were dialyzed overnight at 4°C against 50 mM $NaHCO_3$ and 50 mM KCl, pH 9.5. The protein content of each sample was measured with the Pierce BCA protein assay (Pierce Chemical, Rockford, IL, USA).

Western blots. Samples (1 mg dry wt/ml) from each of the HD-198 powders were treated with 2% SDS and 0.1 M 2-mercaptoethanol at 100°C for 3 min. The protein solutions (5 μg/dry wt per lane) were subjected to electrophoresis on 4–15% gradient SDS-PAGE gels (Bio-Rad) and transferred onto PVDF membranes. Membranes were washed extensively in 20 mM Tris-HCl (pH 7.5), 500 mM NaCl, 0.05% Tween-20, and then either stained for total protein with colloidal gold (Bio-Rad, Hercules, CA, USA) or prepared for chemiluminescent detection for the presence of specific Cry proteins by alkaline phosphatase antibody conjugates. The protein content of samples applied to gels intended for chemiluminescent detection was reduced by a factor of 10 (0.5 μg/dry wt per lane). Primary polyclonal antibodies to trypsin-activated Cry1Ab and Cry1C crystal proteins were prepared in adult male outbred rats (Harlan Sprague Dawley) by the procedure of Oppert et al. [21]. Cry1Ab and Cry1C proteins were produced from recombinant genes cloned in *Escherichia coli* and were purified by DEAE Bio-Gel A (Bio-Rad, Hercules, CA, USA) column chromatography. The general protocol followed was that described for the Western-Light system by Tropix (Bedford, MA, USA). The membranes were blocked overnight in blocking buffer (BB) (0.2% I-Block, phosphate-buffered saline, and 0.1% Tween-20) at 4°C. Primary antibody was diluted in BB and the membrane exposed for 1 h. After extensive washing with BB, the membrane was exposed for 1 h to secondary antibody–alkaline phosphatase conjugate in BB that also contained a streptavidin–alkaline phosphatase conjugate for concurrent detection of biotinylated molecular weight markers. Following extensive washing of the membrane in BB, the protein blots were analyzed by the CSPD chemiluminescence detection system as supplied by Tropix.

Table 1. Spore counts (both viable and total count), crystal counts, total protein content, and toxicity of five separate *B. thuringiensis* serovar *entomocidus* HD-198 samples grown under similar conditions (see Materials and Methods)

HD-198 powders	Spore counts		Crystals[c]	Total protein[d]	Toxicity[e]
	Viable[a]	Total[b]			
A	5.01[f,g]	16.7	9.33	0.64	9.28
B	3.25	11.6	4.00	0.88	1.92
C	5.76	14.8	12.80	0.81	3.72
D	4.41	7.6	2.00	0.84	3.65
E	7.39	17.6	5.60	0.78	3.22

[a] Number of viable spores remaining after heat treatment ($\times 10^9$/g dry wt).
[b] Number of total spores counted microscopically ($\times 10^9$/g dry wt).
[c] Number of crystals counted in a Petroff-Hausser counting chamber ($\times 10^9$/g dry wt).
[d] Total protein from spore/crystal mixture (mg protein/g dry wt).
[e] LC_{50}, 50% lethal concentration (μg dry wt/larva); 256 larvae were used for each treatment.
[f] All experimental procedures (spore and crystal counts, protein determinations) were performed at least three times per sample.
[g] Columnar values for each of the first four data sets were not significantly different ($p > 0.05$) from each other by one-way ANOVA.

Results

During the course of experimentation, we noticed significant variability between IMM larval toxicity (LC_{50}) and multiple laboratory-prepared preparations of *B. thuringiensis* serovar *entomocidus* (HD-198). Individual cultures could be fairly wide-ranging in potency, in practice ranging three- to fivefold in toxicity (with an LC_{50} of 1.92–9.28 μg dry wt/larva, Table 1). These measurements were based on final lysate dry weight and were measured with the single larva apple cube bioassay procedure. However, most of the lysate powders were much more consistent with a potency within ±1 SD based on the mean (LC_{50} of 4.36 μg dry wt/larva) of all samples in this study. Because the reason(s) for reduced toxicity in some of these preparations were not immediately apparent, we initially examined five HD-198 powders for spore and crystal content, protein content, spore/crystal ratios, and compared the influence of spores from HD-1 and HD-198 upon cloned crystal protein preparations of Cry1Aa, 1Ab, and 1Ac.

Table 2. Effect of ultraviolet irradiation on toxicity and viability of spores of *B. thuringiensis* serovar *entomocidus* HD-198 to the Indianmeal moth (*Plodia interpunctella*)

Treatment	Toxicity (LC_{50})[a]	95% CL[b]	Viability[c]
No irradiation	0.81	0.59–1.21	58.0
1 h UV	1.17	0.79–1.81	43.2
2 h UV	1.52	1.32–1.83	26.7
3 h UV	1.56	1.31–1.98	18.5
4 h UV	2.87	2.09–4.20	12.3
Crystal protein	1.27	0.84–2.44	—
Crystal protein + spores (2 h UV) (0.2:0.8)	0.38[d]	0.28–0.55	—

[a] 50% lethal concentration (µg dry wt/larva); 256 larvae were used for each treatment.
[b] Confidence limit.
[c] Percentage of viable spores (capable of germination and outgrowth, measured by CFU on agar plates) in total population (by microscopic count).
[d] Expected toxicity = 1.46 (calculated by the method of Tabashnik [25]).

Table 3. Synergism between *B. thuringiensis* serovar *kurstaki* HD-1 spores and *E. coli*-cloned Cry1Aa, Cry1Ab, and Cry1Ac protein in bioassays with the Indianmeal moth (*P. interpunctella*)

Treatment	Toxicity (LC_{50})[a]	95% CL[b]	Expected toxicity[c]
Cry1Aa	66.41	27.95–698.55	—
Cry1Ab	0.33	0.27–0.40	—
Cry1Ac	0.49	0.41–0.59	—
HD-1 spores	0.89	0.68–1.43	—
Cry1Aa + HD-1 spores (1:1)	0.18	0.02–1.13	1.75
Cry1Ab + HD-1 spores (1:1)	0.08	0.05–0.10	0.48
Cry1Ac + HD-1 spores (1:1)	0.08	0.05–0.10	0.63
Cry1Aa + HD-1 spores (9:1)	1.73	1.38–2.17	7.94
Cry1Ab + HD-1 spores (9:1)	0.12	0.08–0.15	0.35
Cry1Ac + HD-1 spores (9:1)	0.29	0.23–0.36	0.51

[a] 50% lethal concentration (µg dry wt/larva); 384 larvae were used for each treatment.
[b] Confidence limit.
[c] Calculated by the method of Tabashnik [25].

Table 4. Synergism between mercaptoethanol-extracted spore coat protein from *B. thuringiensis* serovar *kurstaki* HD-1 spores and *E. coli*-cloned Cry1Aa, Cry1Ab, and Cry1Ac protein against the Indianmeal moth (*P. interpunctella*)

Treatment	Toxicity (LC_{50})[a]	95% CL[b]	Expected toxicity[c]
Cry1Aa	66.41	27.95–698.55	—
Cry1Ab	0.33	0.27–0.40	—
Cry1Ac	0.49	0.41–0.59	—
HD-1 spore coat protein	1.78	1.02–3.73	—
Cry1Aa + HD-1 spore coat (1:1)	0.93	0.81–1.12	3.47
Cry1Ab + HD-1 spore coat (1:1)	0.21	0.17–0.26	0.30
Cry1Ac + HD-1 spore coat (1:1)	0.22	0.18–0.26	0.77

[a] 50% lethal concentration (µg dry wt/larva); 384 larvae were used for each treatment.
[b] Confidence limit.
[c] Calculated by the method of Tabashnik [25].

Spores were counted by two separate methods. A viable count was made by heat shock and subsequent germination of the spores on agar medium. An alternate visual count of all spores present in each sample was done microscopically. Counts from both methods ranged from 3.25×10^9 to 17.6×10^{10} spores/gm dry wt (Table 1). The microscopic count was approximately three- to fourfold higher than the viable count in each instance, but the respective concentrations in each sample were relatively consistent with either method. Even though viable and total spore counts varied somewhat, differences between methods were not significantly different ($p < 0.05$, one-way ANOVA).

Microscopic crystal counts among the five HD-198 powders are also shown in Table 1. Values ranged approximately tenfold from a low of 2×10^9 to 1.3×10^{10} crystals/g dry wt, which was statistically insignificant ($p > 0.05$) due to variation in individual counts. It should be noted that sample A, which possessed a low bioassay toxicity, was still comparable in crystal count to the other preparations.

Total SDS-extractable protein in each of the samples ranged from 0.64–0.88 mg protein/g dry wt (Table 1). Four of the five samples ranged from 0.78–0.88 mg protein/g dry wt. One sample (A) was slightly lower, approximately 0.64 mg protein/g dry wt, but this difference was not statistically significant ($p < 0.05$).

Several different methods were used to determine the effects of spore concentration and viability upon Cry protein toxicity. The first experiment was to examine the contribution from viable spores (and consequent larval septicemia from spore germination and outgrowth in the hemolymph). Table 2 depicts the IMM larval toxicity of purified HD-198 spores that were irradiated for various lengths of time. The toxicity of the spores decreased as irradiation time increased, and spore viability decreased concomitantly with the decrease in potency. When HD-198 crystals were bioassayed with UV-treated (2 h) spores added, the toxicity observed was greater than the expected toxicity calculated from the ratio of toxicity of crystal and nonirradiated spore components measured separately [25].

E. coli-cloned preparations of Cry1Aa, Cry1Ab, and Cry1Ac were bioassayed against the Indianmeal moth. These preparations were completely free of spores, and the toxicities (LC_{50}) of each were 66.41, 0.33, and 0.49 µg dry wt/larva for Cry1Aa, Cry1Ab, and Cry1Ac,

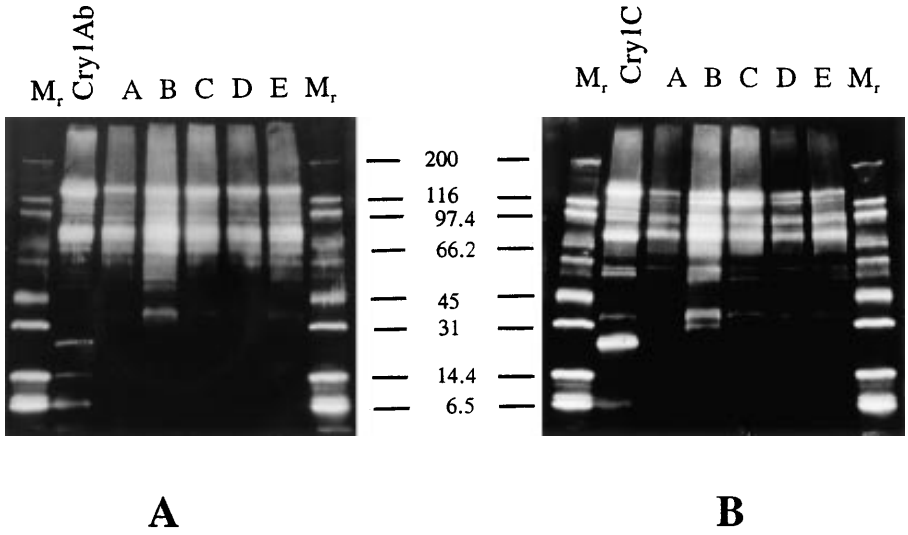

Fig. 1. Western blot analysis of Cry1Ab and Cry1C content of *B. thuringiensis* serovar *entomocidus* HD-198 cultures. Total culture extracts were subjected to electrophoresis on a 4–15% SDS-PAGE gel and transferred to PVDF membranes. Cry1Ab and Cry1C proteins were detected with a rat polyclonal antibody followed by a goat anti-rat alkaline phosphate conjugate and visualized with the chemiluminescent substrate CSPD. Blots exposed to Cry1Ab antiserum (A) or Cry1C antiserum (B). MW markers were biotinylated and detected by the addition of a streptavidin-alkaline phosphatase conjugate. Lanes: M_r, MW markers (kDa); Cry1Ab (A) or Cry1C (B) protoxin; A–E, *B. thuringiensis* HD-198 powders A, B, C, D, and E. Protein: 0.5 µg/lane.

respectively (Table 3). However, when HD-1 spores were added to each of the cloned Cry1Aa, 1Ab, or 1Ac protein, the potency of each preparation increased according to the ratio of Cry1A protein:spore concentration. The resulting toxicity in all cases exceeded the expected potency of each preparation.

It was not known whether the apparent synergism resulted from a septicemia caused by germinating spores and/or contribution from spore coat protein, which is known to be virtually identical to crystal toxin [3, 4]. Consequently, we extracted spore coat protein from HDl spores by alkaline solubilization with mercaptoethanol and combined the dialyzed protein with cloned Cry1A protein (Table 4). Spore coat protein alone had a toxicity of 1.78 µg dry wt/larva, and toxicities of 0.93, 0.21, and 0.22 µg dry wt/larva, respectively, when combined 1:1 with cloned Cry1Aa, 1Ab, or 1Ac protein. These potencies are in excess of expected toxicities and represent synergism between spore coat protein and all three types of Cry1A protein.

Immunoblots of SDS-extracted protein from spore/crystal preparations from each of these samples revealed no differences in protein content in each of the five samples. We used polyclonal antisera to either Cry1Ab or Cry1C to compare variations between crystal protein specificity and type among the five samples. Figure 1 shows the blots of each of the five samples exposed to each Cry protein antiserum. Two major protein species were present in each control, the protoxin at approximately 130,000 daltons and the activated toxin at approximately 70,000 daltons. There was a major, smaller protein species from Cry1C at about 28,000 daltons, which may represent an *E. coli*-immunoreactive protein. No substantial differences among the five samples in their response to either Cry1Ab or Cry1C antisera were detected other than quantity of Cry protein present. Samples B and C contained the largest concentration of Cry protein antigens, and these were also the most toxic in bioassays.

Discussion

Achieving consistency of activity among different preparations of *B. thuringiensis* cultures has always been a problem that can seriously affect reproducibility of results. Activity variations among experimental trials can be as much as fivefold (spore count and International Units) [5, 18]. Depending upon the parameter of interest, whether it is toxicity, component yield, or dry weight, these inconsistencies can alter results or create difficulties when comparing one run with another.

The potency of a *B. thuringiensis* spore/crystal mixture toward the Indianmeal moth cannot be easily judged by a simple measurement of protein, spore count, or dry weight. For insects such as the IMM, approximately equivalent amounts of viable spores and crystals are necessary for optimal toxicity [9, 12]. Thus, a determination of protein level or spore content alone is a poor indicator of the toxicity of a *B. thuringiensis* powder. We found that the spores play a major role in

mixed spore/crystal toxic treatments for the IMM, which includes septicemia from hemocoelic spore germination and outgrowth as well as synergy between spore coat protein and crystal protein toxicity. UV-irradiated spores exhibited decreased toxicity toward IMM larvae, suggesting that reduced viability leads to a diminished septicemia. Because spores possess very efficient UV damage repair mechanisms, we were unable to reduce their viability below 23% under our conditions [23]. However, a synergy was observed between 2-h irradiated HD-198 spores and HD-198 crystals, which may have been due to contribution from the spore coat protein and not from septicemia. Spores from HD-1 were synergistic with Cry1Aa, Cry1Ab, and Cry1Ac cloned protein. Extracted spore coat protein from nonirradiated HD-1 spores also produced a synergy with all three types of Cry1A protein. Thus, three factors must be considered for estimating toxicity toward the IMM. These factors include crystal protein, spore coat protein, and spore germination.

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