

October 1996

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## *Bacillus thuringiensis* HD-73 Spores Have Surface-Localized Cry1Ac Toxin: Physiological and Pathogenic Consequences

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Received 13 June 1995/Accepted 20 July 1996

**Spores from Cry<sup>+</sup> strains of *Bacillus thuringiensis* bound fluorescein isothiocyanate-labeled antibodies specific for the 65-kDa activated Cry 1Ac toxin, whereas spores from *Bacillus cereus* and Cry<sup>-</sup> strains of *B. thuringiensis* did not. The Cry<sup>+</sup> spores could be activated for germination by alkaline conditions (pH 10.3), whereas Cry<sup>-</sup> spores could not. Once the surrounding exosporia had been removed or permeabilized, Cry<sup>+</sup> spores were able to bind the toxin receptor(s) from insect gut brush border membrane vesicle preparations, and their germination rates were increased ca. threefold in the presence of brush border membrane vesicles. A model is presented whereby in the soil the Cry toxins on the spore surface are protected by the exosporium while in the gut they are exposed and available for binding to the insect receptors. This model explains why the disulfide-rich C terminus of the cry genes is so highly conserved even though it is removed during the processing of the protoxin to the activated toxin. It also highlights the trade-off resulting from having Cry toxins located on the spore surface, i.e., decreased spore resistance versus enhanced insect pathogenesis.**

*Bacillus thuringiensis* is an aerobic spore-forming bacterium that produces parasporal protein inclusions concomitant with sporulation. These inclusions are called insecticidal crystal proteins (ICPs) because they are often crystalline and often insecticidal. During insect pathogenesis, the crystals are ingested by susceptible larvae and then solubilized and activated (2, 14), whereupon the active toxin binds to one or more membrane receptors located on the cells lining the gut (17, 28, 35). A single crystal may contain one million protein subunits (10) held together by interchain disulfide bonds, and thus the cleavage of those disulfide bonds is a critical step in crystal solubilization (10).

Many thousands of *B. thuringiensis* strains have been isolated (22), and they exhibit a great diversity in the spectrum of their insect toxicities (15, 21). Höfte and Whiteley (15) analyzed the nucleotide sequences of 42 crystal (*cry*) genes. Four major classes of *cry* genes and Cry proteins were recognized and designated CryI, CryII, CryIII, and CryIV depending on host specificity and the degree of amino acid sequence homology (15). Many *B. thuringiensis* strains contain multiple *cry* genes. *B. thuringiensis* HD-73 is comparatively unusual in that it produces crystals from only a single Cry1Ac gene.

For the CryI toxins, the C-terminal portion of the 133-kDa protoxin is removed by proteolysis, leaving an active toxin of ca. 65 kDa. The active toxin, located in the N-terminal half of the protoxin, binds to the insect receptors (17, 28). The function of the C-terminal half is less well understood, even though its amino acid sequences are more highly conserved than those in the N-terminal half, which contains the active toxin (15). What is the functional significance of the C terminus? One explanation is that all of the cysteine residues of the protoxin are located in the C-terminal half and the disulfide bonds formed by these cysteines are essential for crystal formation and the distinctive alkaline solubility of insecticidal crystals (10). A second explanation is that during spore formation, some of the ICPs are incorporated on the spore surface rather

than being packaged into crystals (2). Presumably, this attachment occurs by disulfide interchange between ICPs and the disulfide-rich spore coat proteins. Thus, the C terminus also serves as a spore attachment structure. The present paper provides evidence for ICP-spore attachment and then shows how these Cry<sup>+</sup> spores differ physiologically from Cry<sup>-</sup> spores. In particular, Cry<sup>+</sup> spores recognize specific insect receptors and probably play a key role in insect pathogenesis. In the process, we also provide an attractive *raison d'être* for the exosporia often observed surrounding *B. cereus* and *B. thuringiensis* spores.

### MATERIALS AND METHODS

**Organisms and culture conditions.** *B. thuringiensis* HD-1 was isolated from a concentrated spore-crystal slurry (Abbott Laboratories, North Chicago, Ill.), while HD-73 and SHP 2-17 were obtained from Howard Dulmage and Phyllis Martin, respectively. *Bacillus cereus* HWT was obtained from Harlyn Halvorson. All strains were grown to sporulation (3 days) in a glucose-yeast extract-salts medium (26) at room temperature, whereupon the spores and crystals were purified by NaBr density gradient centrifugation (1).

Cry<sup>-</sup> strains of HD-1 and HD-73 were selected on the basis that their spores were no longer activated at pH 10.3. Purified spores were incubated with 0.1 M Na<sub>2</sub>CO<sub>3</sub> and 50 mM 2-mercaptoethanol (pH 10.3) at room temperature for 30 min, washed three times with 50 mM Tris (pH 7.6), and resuspended in the same Tris buffer. L-Alanine and adenosine were added at final concentrations of 10 and 1 mM, respectively, whereupon the spores were incubated at room temperature for 30 min, heated at 80°C for 10 min, and plated on tryptic soy agar (TSA) and TSA containing 5% NaCl. On TSA, wild-type (crystal plus) strains of *B. thuringiensis* form large colonies with smeared margins while the crystal-free mutants appear as smaller colonies with sharp borders. On TSA with 5% NaCl, the first visible colonies are highly enriched for Cry<sup>-</sup> mutants. Individual colonies were picked and transferred to fresh glucose-yeast extract-salts medium for testing. After sporulation, the cultures were checked for spore and crystal production by phase-contrast microscopy. The Cry<sup>-</sup> spores did not react with anti-Cry fluorescein isothiocyanate (FITC)-labeled antibodies, whole cultures were nontoxic to *Manduca sexta* larvae, and when analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), the spores did not have detectable bands at 130 to 135 kDa.

Crystal violet stain (0.1% crystal violet dissolved in ethanol and then diluted 10-fold with water) was used to help distinguish the crystals from other spore-related particles such as poly-β-hydroxybutyrate. In phase-contrast microscopy, the crystals and vegetative cells stained orange, the exosporia stained violet (12), and phase-bright spores and poly-β-hydroxybutyrate granules were unstained.

**Preparation of HD-73 activated toxin.** Purified crystals were dissolved in 0.1 M Na<sub>2</sub>CO<sub>3</sub>-50 mM 2-mercaptoethanol (pH 10.3) for 30 min at room temperature. After centrifugation, trypsin was added to the supernatant at 1 mg/ml for digestion overnight at room temperature with shaking. The 60- to 65-kDa fragment was purified on a Sephadex G-100 column with phosphate-buffered saline (PBS;

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100 mM NaCl, 20 mM phosphate [pH 7.5]) as the eluant. Toxin purity was checked by SDS-PAGE, and toxin activity (50% lethal concentration) was measured with *M. sexta* larvae (10). Fractions with the same molecular weight were pooled, adjusted to a protein concentration of ca. 400  $\mu$ g/ml, aliquoted, and stored at  $-70^{\circ}\text{C}$ .

**Preparation of anti-HD-73 active toxin serum and fluorescence-labeled antibodies.** Rabbit polyclonal antiserum to the HD-73 activated toxin was prepared by the University of Nebraska Antibody Core Research Facility. The antibody fraction was purified by ammonium sulfate precipitation. Saturated ammonium sulfate was added to 1 volume of the serum to bring the final ammonium sulfate concentration to 33% saturation. After 1 h on ice, the serum was centrifuged for 10 min at  $3,000 \times g$ , whereupon the supernatant was removed, adjusted to an ammonium sulfate concentration of 50% saturation, and incubated overnight at  $4^{\circ}\text{C}$ . After centrifugation, the pellet was resuspended in 0.5 volume of PBS, dialyzed overnight against three changes of PBS, and clarified by centrifugation for 15 min at  $27,000 \times g$ . This supernatant was our antiserum preparation. It was then diluted ninefold with 30 mM sodium carbonate (pH 9.3), and 50  $\mu$ l of 20 mM fluorescein isothiocyanate (FITC, isomer I; Sigma, St. Louis, Mo.) in carbonate buffer was added. After 1 h at room temperature, excess FITC was removed by dialysis against PBS.

**Immunofluorescence microscopy.** We used the methods described by Lam and Mutharia (18) for immunofluorescence microscopy. Briefly, spores were heat fixed on glass slides and incubated with 5  $\mu$ l of 1:100 FITC-antibody for 5 min in the dark. Free antibodies were washed away with PBS. A mounting fluid consisting of glycerol and 0.5 M carbonate (pH 9.6) (10:90) was used to enhance the fluorescence. Photographs were taken with a Leitz DM RB phase and fluorescence microscope at a magnification of  $\times 1,000$ .

**Exosporium removal.** Exosporia were removed or permeabilized by treatment in a Braun homogenizer. An ice-cold suspension (10 ml) of NaBr-purified spores was mixed with 10 g of 0.10-mm-diameter glass beads and homogenized twice for 30 s at 4,000 rpm, with cooling on ice for 5 min between homogenizations. This procedure causes some spore breakage, and, accordingly, the treated spores were repurified on NaBr gradients prior to use. The extent of exosporium removal was monitored by phase-contrast microscopy following staining with crystal violet.

**Spore germination.** For heat activation, we generally used the methods of Aronson and Fitz-James (3). Concentrated spores in 50 mM Tris (pH 7.6) were heated at  $65^{\circ}\text{C}$  for 25 min and then adjusted to an optical density at 660 nm of 0.6 with the same buffer. For alkali activation, spores were incubated with 0.1 M sodium carbonate–50 mM 2-mercaptoethanol (pH 10.3) for 30 min at room temperature, washed three times with 50 mM Tris buffer (pH 7.6), and resuspended in the same buffer. To start the germination, 3 ml of spore suspension was mixed in a cuvette with L-alanine and adenosine at final concentrations of 0.5 and 0.05 mM, respectively. The drop in the optical density at 660 nm (27) was recorded every 4 min. In some experiments, the spore suspensions (3 ml) were also supplemented with 30  $\mu$ l of brush border membrane vesicles (BBMVs) (ca. 8 mg of protein per ml) or 30  $\mu$ l of activated toxin (2 mg/ml in PBS).

**Purification of toxin receptors from BBMVs by spore affinity.** BBMVs were prepared from *M. sexta* larvae as described by Wolfersberger et al. (38) and stored frozen. Toxin receptors were purified from BBMVs on the basis of their ability to bind purified, alkali-activated HD-73 spores. After alkali treatment as described above, the spores were repurified on NaBr gradients (1). The spore pellet was washed and resuspended in buffer 1 (17), containing 10 mM Tris (pH 8), 150 mM KCl, and 1 mM phenylmethylsulfonyl fluoride. BBMVs (5 mg/ml) were solubilized in buffer 1 containing 50 mM 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS) detergent (17), whereupon the CHAPS concentration was reduced to  $\leq 8$  mM by dialysis against buffer 1 containing 0.2 mM phenylmethylsulfonyl fluoride (17). The alkali-treated spores and solubilized BBMVs were mixed on ice for 40 min, whereupon the spores were collected by centrifugation, washed four times in buffer 1, resuspended in 0.2 M *N*-acetylgalactosamine on ice for 30 min, and centrifuged again. The supernatants were then analyzed by SDS-PAGE (7.5% polyacrylamide). After electrophoresis, the proteins were transferred to polyvinylidene difluoride membranes, blocked with 5% dry milk in TBST buffer (10 mM Tris, 150 mM NaCl, 0.25% Tween 20 [pH 7.4]), and incubated in sequence with the activated HD73 toxin, primary antibody against the activated toxin, and secondary antibody conjugated with alkaline phosphatase. Color was developed with the AP substrate kit II (Vector Laboratories, Burlingame, Calif.). As a control, duplicate membranes were run with and without incubation with activated toxin. As an additional control, spores from a Cry<sup>-</sup> strain of HD-73 were used instead of the wild-type Cry<sup>+</sup> HD-73 spores.

## RESULTS

**Fluorescent detection of spore toxin.** Fluorescent antibodies produced in response to the 65-kDa activated toxin from *B. thuringiensis* HD-73 reacted with purified spores of the same strain (Fig. 1). Juxtaposition of the phase-contrast (Fig. 1A) and fluorescent (Fig. 1B) micrographs from the same field of view shows that the antibodies reacted with all the spores present, including both phase-bright and phase-dark spores.

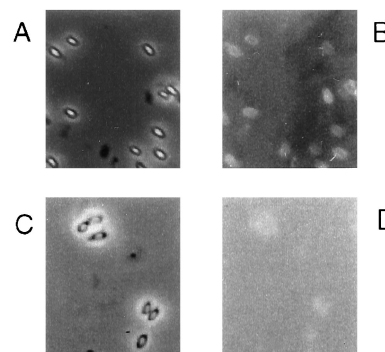


FIG. 1. Binding of crystal-specific FITC-labeled antibodies to alkali-activated HD-73 spores (A and B) and spores from Cry<sup>-</sup> HD-73 (C and D). (A and C) Phase-contrast microscopy; (B and D) fluorescence microscopy. Magnification,  $\times 1,000$  for all panels.

The toxin-specific antibodies also reacted with spores from HD-1 and SHP2-17. They did not react with vegetative cells or with spores from *B. cereus* HWT or a Cry<sup>-</sup> strain of HD-73 (Fig. 1D).

The strong antibody binding shown in Fig. 1B used alkali-treated Cry<sup>+</sup> spores. Spores which had been pretreated in a Braun homogenizer also exhibited strong antibody binding (data not shown). Pretreatment either with alkali or by mechanical shear was necessary because untreated native spores bound antibody infrequently and with a greatly reduced fluorescent intensity. Braun homogenization, like passage through a French press, uses mechanical shear to remove the exosporium surrounding some *Bacillus* spores (23). Presumably, an intact exosporium prevents antibody access to toxin located on the spore surface.

**Alkaline activation of spore germination.** Reversible heat activation is a common characteristic of *Bacillus* spores (16). However, *B. thuringiensis* spores can also be activated by alkaline conditions. Wilson and Benoit (37) showed that HD-1 spores were activated by 0.1 M potassium carbonate (pH 10). We have confirmed their observations with spores from HD-73 (Table 1) and SHP 2-17 (data not shown). Alkali activation was found only in spores produced by Cry<sup>+</sup> strains of *B. thuringiensis*. With wild-type Cry<sup>+</sup> spores (Table 1), germination rates

TABLE 1. Germination efficiency of heat- and alkali-activated spores

Spore type	% Germination <sup>a</sup> of:		
	Heat-activated spores	Alkali-activated spores	Untreated spores
<i>B. thuringiensis</i>			
HD-73 Cry <sup>+</sup>	52	53	10
HD-73 Cry <sup>-</sup>	72	20	2
HD-73 Cry <sup>+</sup> (0.5% ethanol)	52	13	— <sup>b</sup>
HD-73 Cry <sup>+</sup> (BBMV)	—	83	—
<i>B. cereus</i>			
Cry <sup>-</sup>	65	<1	2
Cry <sup>-</sup> (BBMV)	66	—	—

<sup>a</sup> Percent germination after 20 min. Germination was triggered by adding L-alanine and adenosine at  $T_0$  and measured as the percent drop in optical density at 660 nm (27) at 4-min intervals. Values reported are the means of three to five experiments.

<sup>b</sup> —, not done.

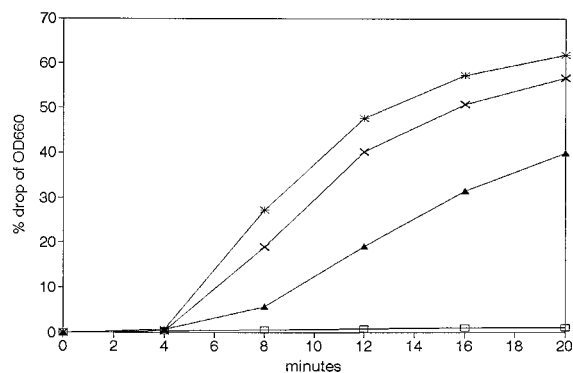


FIG. 2. Effect of insect BBMVs on germination rates of alkali-activated  $Cry^+$  HD-73 spores. The germinants (L-alanine and adenosine) were added at  $T_0$ . Symbols: ▲, germinants only; ★, germinants plus BBMVs; ×, germinants plus both BBMVs and 65-kDa activated toxin; □, no germinants. OD660, optical density at 660 nm.

following heat activation and those following alkaline activation were roughly equivalent. However, alkali activation was irreversible. The spores were still activated 3 to 4 weeks later (irreversible), whereas the heat-activated spores had returned to their native, nonactivated form within 24 h (reversible). We observed no alkali activation of *B. cereus* spores and little alkali activation of spores from  $Cry^-$  strains of HD-73 (Table 1). However, as observed by others (2, 4), spores from  $Cry^+$  strains of *B. thuringiensis* appeared to germinate more slowly than those from their  $Cry^-$  counterparts following heat activation.

**Ethanol inhibition of alkali-activated spore germination.** Evidence that activation by heat and activation by alkali constitute distinct pathways is provided by their different sensitivities toward ethanol (Table 1). The germination of heat-activated spores was not influenced by ethanol (up to 2%), whereas the germination of alkali-activated spores was retarded fourfold by 0.5% ethanol (Table 1) and prevented entirely by 1% ethanol. Note that the ethanol was added with the L-alanine and adenosine germinants; it was not present during activation.

**BBMVs and spores.** Our evidence for specific binding between spores and insect receptors is twofold. The first part comes from the effect of insect BBMVs on the germination of  $Cry^+$  spores. For alkali-activated  $Cry^+$  spores, BBMVs from *M. sexta* increased both the rate (Fig. 2) and completeness (Table 1) of germination. When triggered by L-alanine and adenosine, germination rates were ca. three times faster in the presence of BBMVs than in their absence. BBMVs had no effect on heat-activated *B. cereus* spores (Table 1).

**Purification of toxin receptor by spore binding.** The stimulatory effect of BBMVs on spore germination suggested that HD-73 spores recognized one or more sites on the BBMVs, possibly coinciding with the  $Cry1Ac$ -binding sites on the BBMVs. The second part of our evidence for a spore-insect recognition system comes from the usefulness of spores in purifying a known (17, 28) toxin receptor from solubilized BBMVs. Figure 3 shows that purified HD-73 spores bound a protein of 115 to 120 kDa from solubilized BBMVs (Fig. 3, lanes 2 and 3). The protein was able to bind  $Cry^+$  spores but not  $Cry^-$  spores (compare lanes 2 and 5). The toxin-binding ability of this 115-kDa protein was shown by its presence in lane 2 (with toxin) and absence whenever the 65-kDa toxin was omitted in the blotting sequence. Also, as a step in the purification process, this protein was released from the spores by 0.2

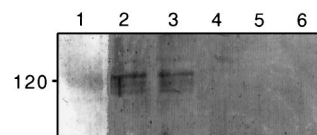


FIG. 3. Purification of toxin receptors from BBMVs by spore affinity. The Western blots (immunoblots) were incubated with 65-kDa activated toxin and the indicated primary and secondary antibodies. Lanes: 1, CHAPS-solubilized BBMVs, no spores; 2, solubilized BBMVs mixed with alkali-treated HD-73 spores; 3, solubilized BBMVs premixed with activated HD-73 toxin and then mixed with alkali-treated HD-73 spores; 4, HD-73 spores alone, no BBMVs; 5, solubilized BBMVs mixed with alkali-treated  $Cry^-$  HD-73 spores; 6,  $Cry^-$  HD-73 spores alone, no BBMVs. Molecular mass marker indicates 120 kDa.

*M* *N*-acetylgalactosamine. It could also be removed from the spores by extraction with 1.0% SDS. Therefore, we presume that the 115- to 120-kDa protein is identical to the aminopeptidase N receptor identified from *M. sexta* (17, 28).

We also detected a weak protein band at ca. 133 kDa that reacted with toxin-specific antibodies (Fig. 3). It was present (Fig. 3, lanes 2 to 4) in samples containing  $Cry^+$  spores and absent (lanes 5 and 6) in samples containing  $Cry^-$  spores. Presumably, this band is the 133-kDa protoxin which has been extracted from the  $Cry^+$  spores.

**Physiological differences between  $Cry^+$  and  $Cry^-$  spores.** While the precise nature of toxin attachment in  $Cry^+$  spores has not been determined, it is probably covalent. The spores were harvested from 35% (wt/wt) sodium bromide, washed four times, and then given two 1-min pulses in a Braun homogenizer prior to use. Because disulfide bonding is such an important characteristic of both *B. thuringiensis* crystals (10) and spore coat proteins (39), we think it likely that the C terminus of the  $Cry1Ac$  toxin is anchored to the spore via heterologous disulfide bonding. An intact coat is considered critical for the exceptional resistance properties of bacterial spores (2), and, not surprisingly, the presence of crystal toxin embedded in the spore coat alters the physiology and resistance properties of the spore in many ways. Table 2 summarizes the differences between  $Cry^+$  and  $Cry^-$  spores. These differences are extensive. Thus, in terms of the distinction between *B. cereus* and *B. thuringiensis*, *B. thuringiensis* has acquired a plasmid and a crystal and also a modified spore.

TABLE 2. Physiological differences between spores formed in the presence and absence of insecticidal crystals<sup>a</sup>

Spore characteristic	Presence and/or value in:		Reference(s)
	$Cry^+$ spores	$Cry^-$ spores	
Protoxin on surface	Yes	No	4, 31, 32 and this study
Heat resistance	Lower <sup>b</sup>	High	9, 33
UV resistance	Lower	High	6, 8, 13, 25
Osmotic resistance	Lower <sup>b</sup>	High	9
Germination in 0.25 M acetate	No	Yes	34
Heat activation	Yes	Yes	16, 26
Alkali (pH 10) activation	Yes	No	5, 37 and this study
Binding of insect BBMVs	Yes	No	This study
Spore coat dimensions	Thin	Thick	4

<sup>a</sup>  $Cry^-$  spore data include data from *B. thuringiensis* and *B. cereus* spores.

<sup>b</sup>  $Cry^+$  spore data are from *B. thuringiensis* subsp. *israelensis*.

## DISCUSSION

We have shown that spores from Cry<sup>+</sup> strains of *B. thuringiensis* (i) can be alkali activated, (ii) have crystal toxin attached to their surface, (iii) have the N-terminal portion of that toxin available for functional interactions, (iv) bind specific toxin receptors in insect BBMV, and (v) are stimulated ca. threefold in their rate of germination by binding to insect BBMV. The first two findings are not novel. The existence of alkali activation (37) and its positive correlation with crystal production (5) have been reported by Benoit and colleagues. Similarly, the presence of crystal antigens on Cry<sup>+</sup> spores has been reported many times previously (4, 19, 31, 32). In particular, Aronson's laboratory has worked extensively on the surface localization of toxin (4) as it pertains to the differences observed between Cry<sup>+</sup> and Cry<sup>-</sup> spores (2). With regard to the significance of having Cry toxin on the spores, one study emphasized the evolutionary origins of crystals (arising from overproduction of spore coat proteins [33]) while another emphasized bacterial survival in a soil environment (4).

However, the rationale for alkali activation and surface localization of toxin has never before focused on the trade-off between spore resistance and insect pathogenesis. In this paper, we have shown that the N-terminal "business end" of the toxin is exposed on the spore surface and oriented toward the exterior. As a consequence, Cry<sup>+</sup> spores can interact with both BBMV (Fig. 2 and 3) and anti-65-kDa antibodies (Fig. 1). The survival value of such an arrangement explains why the cysteine-rich C terminus of Cry toxins is so highly conserved (15) even though it does not contribute to the actual 65-kDa insecticidal toxin. In terms of trade-offs, an improved capacity for insect pathogenesis would compensate for a spore which had multiple defects (Table 2) in coping with environmental extremes (30).

With regard to insect pathogenesis, the implications of our findings are fivefold. (i) Lepidopteran larvae fall into three types depending on their response to the active components of *B. thuringiensis* (7). Larvae of types 1 and 2 are rapidly killed by crystals alone, whereas larvae of type 3 require both spores and crystals for pathogenesis (7, 20). The pH in the midgut of most healthy Lepidopteran larvae is 10 to 10.5 (14, 36), and *B. thuringiensis* is not an alkalophile (36). However, the dormant spores would be activated at the alkaline gut pH (36, 37) and then triggered for germination by binding to the gut receptors. Thus, the spores could contribute to the overall toxin load (29) as well as providing invasive vegetative cells (24) ready to participate in pathogenesis as soon as the gut pH drops sufficiently (14, 36). This mechanism would ensure that the spores germinate in the right place at the right time. Increasing the rate of spore germination may be critical because of the comparatively short transit time for food passing through the larval gut.

(ii) In comparison with *B. cereus*, most *B. thuringiensis* spores germinate slowly (4). Aronson et al. (2) analyzed these differences between *B. cereus* and *B. thuringiensis* and concluded that "for some reason the germination mechanism is defective" in Cry<sup>+</sup> spores. They further suggested that the correct germinants had not yet been found for *B. thuringiensis*. We suggest that those germinants are located in the insect gut receptors. Thus, the host specificity of the insecticidal crystals would correspond directly to the host specificity for spore germination.

(iii) If the Cry proteins on the spore surface are needed to trigger spore germination in the insect gut, they would need to be protected while the spore remains dormant in the soil. The exosporium would serve this purpose. It is a complex mem-

brane loosely surrounding the spore. Its presence is species specific (23), but it is commonly found on spores from *B. cereus*, *B. anthracis*, and *B. thuringiensis* (11, 12, 23). The exosporium from *B. cereus* (23) contains 52% protein, 20% polysaccharide, 12.5% neutral lipid, and 5.5% phospholipid. Its function is currently unknown. We suggest that one function of exosporia is to protect the Cry proteins on the spore surface. To achieve reproducible binding to either BBMV (Fig. 2 or 3) or toxin-directed antibodies (Fig. 1), we had to use spores which had been homogenized or alkali activated. In the soil, the Cry proteins would be protected within the exosporium. However, once the exosporium has been removed or permeabilized, as in the highly alkaline, reducing, and proteolytic larval midgut, the newly exposed Cry toxins would be available to participate in pathogenesis. Thus, the presence of an exosporium would be correlated with toxin production or a recent history of toxin production. Of course, this suggestion regarding the survival value of exosporia is not exclusive. There may be other advantages to a spore having an exosporium.

(iv) A great many "nontoxic" strains of *B. thuringiensis* exist. In one study (22), 8,916 isolates were obtained from soil samples, with fully 40% of them being nontoxic. SHP2-17 was one of those nontoxic strains (22), and we confirmed that purified SHP2-17 crystals were completely nontoxic to *M. sexta* larvae at the highest concentration tested (10). Why would bacteria produce noninsecticidal protein crystals (10, 21)? Our present data show that SHP2-17 spores can be alkali activated and that they do contain cross-reacting Cry protein antigens on their surface. Thus, in these cases, the role of the Cry proteins in triggering spore germination may be more important than the production of an insecticidal crystal.

(v) It is likely that many millions of *B. thuringiensis* strains actually exist (21). If we assume that each of these strains resembles HD-1 and HD-73 in having Cry proteins embedded on the spore surface, we can visualize this continuum of millions of spore types as the bacterial equivalent of clonal selection in the immune system. Memory B cells have surface antibodies and are triggered for cell division by binding the corresponding antigen; *B. thuringiensis* spores have surface Cry toxins and are triggered for spore germination by binding the corresponding insect receptor.

## ACKNOWLEDGMENTS

This research was supported by grants from the Center for Biotechnology, University of Nebraska—Lincoln; Entotech, Inc., Davis, Calif.; CIBA-GEIGY Corp., Research Triangle Park, N.C.; and the Consortium for Plant Biotechnology Research (grant 593-0009-04).

We thank James Buckner and Carlee O'Dell (USDA, Fargo, N.D.) and Lynda Liska (USDA, Beltsville, Md.) for providing *M. sexta* eggs and Sandie Franks (University of Nebraska—Lincoln) for preparing the polyclonal antibodies.

## REFERENCES

1. Ang, B. J., and K. W. Nickerson. 1978. Purification of the protein crystal from *Bacillus thuringiensis* by zonal gradient centrifugation. *Appl. Environ. Microbiol.* **36**:625-626.
2. Aronson, A. I., W. Beckman, and P. Dunn. 1986. *Bacillus thuringiensis* and related insect pathogens. *Microbiol. Rev.* **50**:1-24.
3. Aronson, A. I., and P. C. Fitz-James. 1975. Properties of *Bacillus cereus* spore coat mutants. *J. Bacteriol.* **123**:354-365.
4. Aronson, A. I., D. J. Tyrell, P. C. Fitz-James, and L. A. Bulla, Jr. 1982. Relationship of the syntheses of spore coat protein and parasporal crystal protein in *Bacillus thuringiensis*. *J. Bacteriol.* **151**:399-410.
5. Benoit, T. G., K. A. Newman, and G. R. Wilson. 1995. Correlation between alkaline activation of *Bacillus thuringiensis* var. *kurstaki* spores and crystal production. *Curr. Microbiol.* **31**:301-303.
6. Benoit, T. G., G. R. Wilson, D. L. Bull, and A. I. Aronson. 1990. Plasmid-associated sensitivity of *Bacillus thuringiensis* to UV light. *Appl. Environ. Microbiol.* **56**:2282-2286.

7. **Burges, H. D., E. M. Thomson, and R. A. Latchford.** 1976. Importance of spores and  $\delta$ -endotoxin protein crystals of *Bacillus thuringiensis* in *Galleria mellonella*. *J. Invertebr. Pathol.* **27**:87–94.
8. **Cantwell, G. E., and B. A. Franklin.** 1966. Inactivation by irradiation of spores of *Bacillus thuringiensis* var. *thuringiensis*. *J. Invertebr. Pathol.* **8**:256–258.
9. **Cucchi, A., and C. S. Rivas.** 1994. Sensitivity of spores and growing cells of *Bacillus thuringiensis* var. *israelensis* and *Bacillus sphaericus* to osmotic variations. *Curr. Microbiol.* **28**:123–127.
10. **Du, C., P. A. W. Martin, and K. W. Nickerson.** 1994. Comparison of disulfide contents and solubility at alkaline pH of insecticidal and noninsecticidal *Bacillus thuringiensis* protein crystals. *Appl. Environ. Microbiol.* **60**:3847–3853.
11. **Gerhardt, P., H. S. Pankratz, and R. Scherrer.** 1976. Fine structure of the *Bacillus thuringiensis* spore. *Appl. Environ. Microbiol.* **32**:438–440.
12. **Gerhardt, P., and E. Ribl.** 1964. Ultrastructure of the exosporium enveloping spores of *Bacillus cereus*. *J. Bacteriol.* **88**:1774–1789.
13. **Griego, V. M., and K. D. Spence.** 1978. Inactivation of *Bacillus thuringiensis* spores by ultraviolet and visible light. *Appl. Environ. Microbiol.* **35**:906–910.
14. **Heimpel, A. M., and T. A. Angus.** 1960. Bacterial insecticides. *Bacteriol. Rev.* **24**:266–288.
15. **Höfte, H., and H. R. Whiteley.** 1989. Insecticidal crystal proteins of *Bacillus thuringiensis*. *Microbiol. Rev.* **53**:242–255.
16. **Keynan, A., and Z. Evenchik.** 1969. Activation, p. 359–396. In G. W. Gould and A. Hurst (ed.), *The bacterial spore*. Academic Press Ltd., London.
17. **Knight, P. J. K., N. Crickmore, and D. J. Ellar.** 1994. The receptor for *Bacillus thuringiensis* Cry 1A(c) delta-endotoxin in the brush border membrane of the lepidopteran *Manduca sexta* is aminopeptidase N. *Mol. Microbiol.* **11**:429–436.
18. **Lam, J. S., and L. M. Mutharia.** 1994. Antigen-antibody reactions, p. 104–132. In P. Gerhardt, R. G. E. Murray, W. A. Wood, and N. R. Krieg (ed.), *Methods for general and molecular bacteriology*. American Society for Microbiology, Washington, D.C.
19. **Lecadet, M.-M., G. Chevrier, and R. Dedonder.** 1972. Analysis of a protein fraction in the spore coats of *Bacillus thuringiensis*—comparison with crystal protein. *Eur. J. Biochem.* **25**:349–358.
20. **Li, R. S., P. Jarrett, and H. D. Burges.** 1987. Importance of spores, crystals, and  $\delta$ -endotoxins in the pathogenicity of different varieties of *Bacillus thuringiensis* in *Galleria mellonella* and *Pieris brassicae*. *J. Invertebr. Pathol.* **50**:277–284.
21. **Martin, P. A. W.** 1994. An iconoclastic view of *Bacillus thuringiensis* ecology. *Am. Entomol.* **40**:85–90.
22. **Martin, P. A. W., and R. S. Travers.** 1989. Worldwide abundance and distribution of *Bacillus thuringiensis* isolates. *Appl. Environ. Microbiol.* **55**:2437–2442.
23. **Matz, L. L., T. C. Beaman, and P. Gerhardt.** 1970. Chemical composition of exosporium from spores of *Bacillus cereus*. *J. Bacteriol.* **101**:196–201.
24. **Miyasono, M., S. Inagaki, M. Yamamoto, K. Ohba, T. Ishiguro, R. Takeda, and Y. Hayashi.** 1994. Enhancement of  $\delta$ -endotoxin activity by toxin-free spore of *Bacillus thuringiensis* against the diamondback moth, *Plutella xylostella*. *J. Invertebr. Pathol.* **63**:111–112.
25. **Nicholson, W. L.** 1995. Photoreactivation in the genus *Bacillus*. *Curr. Microbiol.* **31**:361–364.
26. **Nickerson, K. W., G. St. Julian, and L. A. Bulla, Jr.** 1974. Physiology of spore-forming bacteria associated with insects. VI. A radiorespirometric survey of carbohydrate metabolism in 12 serotypes of *Bacillus thuringiensis*. *Appl. Microbiol.* **28**:129–132.
27. **Powell, J. F.** 1950. Factors affecting the germination of thick suspensions of *Bacillus subtilis* spores in L-alanine solution. *J. Gen. Microbiol.* **4**:330–338.
28. **Sangadala, S., F. S. Walters, L. H. English, and M. J. Adang.** 1994. A mixture of *Manduca sexta* aminopeptidase and phosphatase enhances *Bacillus thuringiensis* insecticidal Cry 1A(c) toxin binding and  $^{86}\text{Rb}^+$ - $\text{K}^+$  efflux in vitro. *J. Biol. Chem.* **269**:10088–10092.
29. **Schesser, J. H., and L. A. Bulla, Jr.** 1978. Toxicity of *Bacillus thuringiensis* spores to the tobacco hornworm, *Manduca sexta*. *Appl. Environ. Microbiol.* **53**:121–123.
30. **Setlow, P.** 1992. I will survive: protecting and repairing spore DNA. *J. Bacteriol.* **174**:2737–2741.
31. **Short, J. A., P. D. Walker, R. O. Thomson, and H. J. Somerville.** 1974. The fine structure of *Bacillus finitimus* and *Bacillus thuringiensis* spores with special reference to the location of crystal antigen. *J. Gen. Microbiol.* **84**:261–276.
32. **Somerville, H. J., and H. V. Pockett.** 1975. An insect toxin from spores of *Bacillus thuringiensis* and *Bacillus cereus*. *J. Gen. Microbiol.* **87**:359–369.
33. **Stahly, D. P., D. W. Dingman, L. A. Bulla, Jr., and A. I. Aronson.** 1978. Possible origin and function of the parasporal crystals in *Bacillus thuringiensis*. *Biochem. Biophys. Res. Commun.* **84**:581–588.
34. **Travers, R. S., P. A. W. Martin, and C. F. Reichelderfer.** 1987. Selective process for efficient isolation of soil *Bacillus* spp. *Appl. Environ. Microbiol.* **53**:1263–1266.
35. **Vadlamudi, R. K., E. Weber, I. Ji, T. H. Ji, and L. A. Bulla, Jr.** 1995. Cloning and expression of a receptor for an insecticidal toxin of *Bacillus thuringiensis*. *J. Biol. Chem.* **270**:5490–5494.
36. **Walther, C. J., G. A. Couche, M. A. Pfannenstiel, S. E. Egan, L. A. Bivin, and K. W. Nickerson.** 1986. Analysis of mosquito larvicidal potential exhibited by vegetative cells of *Bacillus thuringiensis* subsp. *israelensis*. *Appl. Environ. Microbiol.* **52**:650–653.
37. **Wilson, G. R., and T. G. Benoit.** 1993. Alkaline pH activates *Bacillus thuringiensis* spores. *J. Invertebr. Pathol.* **62**:87–89.
38. **Wolfersberger, M., P. Lüthy, A. Maurer, P. Parenti, F. V. Sacchi, B. Giordana, and G. M. Hanozet.** 1987. Preparation and partial characterization of amino acid transporting brush border membrane vesicles from the larval midgut of the cabbage butterfly (*Pieris brassicae*). *Comp. Biochem. Physiol.* **86A**:301–308.
39. **Zhang, J., P. C. Fitz-James, and A. I. Aronson.** 1993. Cloning and characterization of a cluster of genes encoding polypeptides present in the insoluble fraction of the spore coat of *Bacillus subtilis*. *J. Bacteriol.* **175**:3757–3766.