

CHARACTERIZATION OF SPORE COAT PROTEINS OF *BACILLUS THURINGIENSIS* AND *BACILLUS CEREUS**

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Abstract—1. Spore coat extracts from *Bacillus thuringiensis* subspecies *kurstaki* and *israelensis* and *Bacillus cereus* T and *B. cereus* NRRL 569 were characterized by polyacrylamide gel electrophoresis in sodium dodecyl sulfate and by amino acid analysis.

2. Both *B. cereus* spore coats had similar electrophoretic profiles.

3. The *B. thuringiensis* spore coats contained crystal proteins as major components as well as lower mol. wt proteins.

4. *B. thuringiensis* subsp. *israelensis* had a unique coat protein profile which was different from *B. cereus* and *B. thuringiensis* subsp. *kurstaki* coats.

5. Insecticidal activity of spores against the tobacco hornworm, *Manduca sexta*, and the mosquito, *Aedes aegypti*, also was determined.

6. *B. thuringiensis* subsp. *kurstaki* spores were lethally toxic to the tobacco hornworm (Lepidoptera) larvae, whereas spores of the other subspecies were not.

7. Except for subspecies *israelensis*, none of the spores was effective against the mosquito (Diptera) larvae.

INTRODUCTION

Bacillus thuringiensis and *B. cereus* are often considered as one group of bacteria, and their relatedness has been shown by various classification methods (Buchanan & Gibbons, 1974). For example, the two species are indistinguishable by zone electrophoresis of certain enzymes (Baptist *et al.*, 1978); they have a high degree of DNA sequence homology (54–80%) (Seki *et al.*, 1978; Somerville & Jones, 1972); and bacteriophages have been isolated that carry out generalized transduction in both organisms (Thorne, 1978). The feature that has been used most to distinguish the two species is the presence of a discrete parasporal crystal that is formed along side the endospore in *B. thuringiensis* (Buchanan & Gibbons, 1974). The crystal, which is insecticidal to lepidopteran larvae, contains a single glycoprotein subunit that has an apparent mol. wt of 1.34×10^5 (Bulla *et al.*, 1977b). *B. cereus* does not form such a crystalline inclusion. Interestingly, spore coats of *B. thuringiensis* contain the crystal protein (Bulla *et al.*, 1977a), and spores of this bacterium also are insecticidal (Schesser & Bulla, 1978).

B. cereus spore coats, on the other hand, contain a 13,000-dalton monomer and a 26,000-dalton dimer (Aronson & Pandey, 1978). Spores of *B. cereus* do not contain a 134,000-dalton glycoprotein like those of *B. thuringiensis* subsp. *kurstaki* (Bulla *et al.*, 1977b), they

are not insecticidal (unpublished data), and they germinate more rapidly than do *B. thuringiensis* spores (Aronson & Pandey, 1978).

Recently, a new strain of *B. thuringiensis* was isolated, designated subspecies *israelensis*, that synthesizes parasporal inclusions which are toxic to mosquitos and blackflies (deBarjac, 1978; Tyrell *et al.*, 1979; Undeen & Nagel, 1978). Unlike most of the other subspecies of *B. thuringiensis*, it does not kill lepidopteran insects (moths and butterflies). The major crystal component of this bacterium is a glycoprotein with an apparent mol. wt of 26,000 (Tyrell *et al.*, unpublished data). Because of the apparent similarity in protein composition of crystals of *B. thuringiensis* subsp. *israelensis* to that of spore coats of *B. cereus*, i.e. the reported 26,000-dalton protein (Aronson & Pandey, 1978), we wanted to investigate the spore coat protein composition of *B. thuringiensis* subsp. *israelensis* for comparative analysis. In this study, the spore coat of *B. thuringiensis* subsp. *israelensis* was compared to subspecies *kurstaki* spore coat and to spore coats from *B. cereus* T and *B. cereus* NRRL 569.

MATERIALS AND METHODS

Organisms and cultural conditions

B. thuringiensis subsp. *kurstaki* was isolated from a commercial insecticidal formulation called Dipel® (Abbott Laboratories, North Chicago, IL). *B. thuringiensis* subsp. *israelensis* was a gift from David Rockhold, Stauffer Chemical Co., San Jose, CA. *B. cereus* T was a gift from A.

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I. Aronson, Purdue University, West Lafayette, IN, and *B. cereus* NRRL 569 was given to us by C. B. Thorne, University of Massachusetts, Amherst, MA.

Stock cultures were maintained on MD agar (Bulla *et al.*, 1970) or glucose yeast extract agar (Nickerson *et al.*, 1974) slants. Cells for experimental use were cultured in the manner previously described for *B. thuringiensis* subsp. *kurstaki* (Bulla *et al.*, 1977b).

Isolation of spores and crystals

Spores and parasporal crystals of the *B. thuringiensis* subspecies were isolated by bouyant density centrifugation in Renografin gradients (Sharpe *et al.*, 1975), washed at least three times in water, and lyophilized. Purity of preparations was monitored at each step of the separation procedure by phase-contrast microscopy. Sporulated *B. cereus* cultures were centrifuged in a Sorval SS3 centrifuge at 10,000 rpm for 10 min. The pellets were washed three times with distilled water and centrifuged at 3000 rpm for 10 min after each wash. Purity of these preparations was monitored also by phase-contrast microscopy. The washed spores were then lyophilized.

Insect bioassays

Bioassays against the tobacco hornworm, *Manduca sexta*, and the mosquito, *Aedes aegypti*, were done as previously described (Schesser & Bulla, 1978; Tyrell *et al.*, 1979).

Spore coat extractions

All spores were extracted with three aliquots of 6 M guanidinium chloride, 0.1 M β -mercaptoethanol, pH 8.6, (extraction buffer A). The material was subsequently dialyzed against 0.1 M Tris-HCl and 9 M urea (pH 8.6) for 24 hr and against water (40–44 l) for 48 hr and then lyophilized.

B. cereus T spores also were extracted with three aliquots of the extraction buffer described by Cheng & Aronson (1977) containing 8 M urea, 0.07 M DTT*, 1% SDS, 5 mM CHES, pH 9.6 (extraction buffer B). The combined extracts were either used immediately for polyacrylamide gel electrophoresis, or refrigerated overnight and then electrophoresed, or dialyzed and lyophilized as described above. The exact same results were obtained with all of these preparations.

B. cereus T and *B. thuringiensis* subsp. *kurstaki* spores were extracted in a third way using buffer B without SDS and containing 2 mM PMSF (extraction buffer C) and dialyzed and lyophilized as described for the buffer A extracts.

Polyacrylamide gel electrophoresis

Spore extracts were solubilized by incubating the lyophilized material in 1% (wt/vol) SDS, 1% (vol/vol) β -mercaptoethanol, 6 M urea, 0.01 M $\text{NaH}_2\text{PO}_4:\text{Na}_2\text{HPO}_4$ at pH 7.2 for 1 hr at room temperature. These solubilized extracts as well as the undialyzed buffer B extracts of *B. cereus* T were subjected to electrophoresis on SDS polyacrylamide gradient slab gels (1.5 \times 140 \times 170 cm; Buchler Polyslab polyacrylamide gel electrophoresis system) using the method of Weber & Osborn (1972). Gradient gels contained 5–20% or 10–20% acrylamide. Electrophoresis was carried out at 40–90 v. Gels were stained 3–12 hr in 0.25% (wt/vol) Coomassie brilliant blue (Eastman R250) in methanol:acetic acid:water (25:7.5:62.5, vol/vol/vol) and destained for 16–48 hr in methanol:acetic acid:water (25:7.5:62.5, vol/vol/vol). Molecular weight standards were cross-linked bovine serum albumin (68,000, 136,000, etc.) from Sigma; bovine albumin (65,000), ovalbumin (45,000), beef pancreas chymotrypsinogen A (25,000) and sperm

whale myoglobin (17,000) from Schwarz/Mann; ribonuclease A (13,000) from Pharmacia; and bovine insulin (A chain, 2384) from Sigma. Destained gels were photographed on 35 mm high contrast copy film which was developed in Kodak D-19. Gels were dried using a Bio-Rad Model 224 slab gel dryer.

Amino acid analysis

Parasporal crystals of *B. thuringiensis* subsp. *kurstaki* and *israelensis* and buffer A spore coat extracts were analyzed on a Beckman 120C analyzer after hydrolysis in 3 M p-toluenesulfonic acid containing a small amount of tryptamine. The hydrolysis was carried out for 24 hr at 110°C in vacuo (Liu & Chang, 1971). Labile amino acids were quantitated by extrapolation to zero time. Cystine and cysteine were determined as cysteic acid and methionine as the sulfone after performic acid oxidation (Hirs, 1967).

RESULTS AND DISCUSSION

Insect toxicity

Spores of both *B. cereus* strains caused no mortality of *M. sexta* larvae at concentrations as high as 6.8 μg per cm^2 of diet surface. Similarly, *B. thuringiensis* subsp. *israelensis* spores caused no mortality of this insect at concentrations as high as 20.5 μg per cm^2 of diet surface. Crystals from these bacteria also are non-toxic to *M. sexta* (Tyrell *et al.*, 1979). Spores of *B. thuringiensis* subsp. *kurstaki* did kill tobacco hornworms. The lethal concentrations (50% endpoints) or LC_{50} of *B. thuringiensis* subsp. *kurstaki* spores were determined previously (Schesser & Bulla, 1978), and the spore protein concentration at the LC_{50} value (9.4 mg/cm^2) was 24% less than that of the crystal protein (7.6 mg/cm^2).

Both *B. cereus* subspecies and *B. thuringiensis* subsp. *kurstaki* caused no mortality of the mosquito, *A. aegypti*, at spore concentrations as high as 100 $\mu\text{g}/\text{ml}$ (dry wt). *B. thuringiensis* subsp. *israelensis* spores had an LC_{50} value of 4×10^{-2} $\mu\text{g}/\text{ml}$ against this mosquito, two hundred times more than that of the crystal ($\text{LC}_{50} = 1.9 \times 10^{-4}$ $\mu\text{g}/\text{ml}$). The relatively low toxicity of *israelensis* spores, compared to that of crystals, may be due to inefficient extraction of the protein in the mosquito larval gut, to modification of the protein during coat assembly so that it loses toxicity, or to the differences in the crystal and coat proteins.

Spore coat protein extraction and gradient gel electrophoresis

Various spore extraction methods were tried using *B. cereus* T and *B. thuringiensis* subsp. *kurstaki* spores. The *B. cereus* T extracts are shown in Fig. 1. Figure 1A shows a comparison of buffer B (tracks 2–4) and buffer A (tracks 5–7) extracts. Both methods produced the same protein profiles with buffer B extracting slightly more of a 12,500-dalton polypeptide (band a). Buffer A, containing guanidine and β -mercaptoethanol, is the buffer we routinely use for extracting *B. thuringiensis* spores, whereas buffer B containing urea, SDS, CHES, and DTT, is the buffer used by Aronson & Pandey (1978) and Cheng & Aronson (1977) to extract *B. cereus* T spores. The SDS gels used by these investigators contained urea and were buffered in Tris-glycine whereas ours were phosphate-buffered. These investigators (Aronson & Pandey, 1978; Cheng

* Abbreviations used: SDS—sodium dodecyl sulfate; CHES—cyclohexylaminoethane sulfonic acid; PMSF—phenylmethylsulfonyl fluoride; DTT—dithiothreitol.

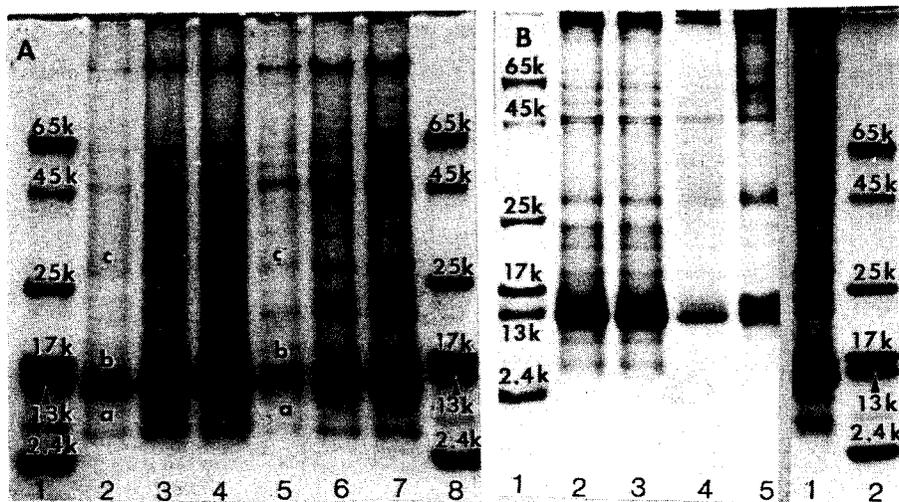


Fig. 1. SDS polyacrylamide gels of *B. cereus* T spore extracts. (A) 5–20% acrylamide gradient. Tracks 1 and 8: standards: bovine serum albumin (65,000), ovalbumin (45,000), chymotrypsinogen A (25,000), myoglobin (17,000), ribonuclease A (13,000), and bovine insulin (A chain, 2,400); tracks 2, 3 and 4: buffer B extract, dialyzed and lyophilized; tracks 5, 6, and 7: buffer A extract. (B) 10–20% acrylamide gel. Track 1: standards same as tracks 1 and 8 (A); tracks 2 and 3: buffer B extract, fresh; track 4: buffer B extract, dialyzed; track 5: buffer C extract. (C) 5–20% acrylamide gel. Track 1: buffer B extract refrigerated overnight; track 2: standards, same as tracks 1 and 8 (A).

& Aronson, 1977) reported a 13,000-dalton coat protein and a 26,000-dalton dimer as the major extractable proteins. Our 5–20% acrylamide gradient gel (Fig. 1A) resolved a 13,000-dalton protein into two bands with mol. wt_{app} of 12,500 (band a) and 14,000 (band b). We found no 26,000-dalton dimer in buffer A or B under our electrophoresis conditions, although we did observe a 28,000-dalton protein (band c) that could be a dimer of the 14,000-dalton polypeptide (band b). Buffer B extracts of *B. cereus* T, which were not dialyzed or lyophilized, are shown in Fig. 1B and C. Nondialyzed buffer B extracts (tracks 2 and 3, Fig. 1B) produced electrophoretic profiles identical to dialyzed and lyophilized protein (track 4, Fig. 1B). Buffer C extract (track 5) also is shown in Fig. 1B. This buffer contained urea, DTT, and PMSF but no SDS. The profile was identical to buffer A extracts (tracks 5–7, Fig. 1A) which also contained no SDS.

In contrast to *B. cereus*, *B. thuringiensis* subsp. *kurstaki* spore extracts gave different profiles after extraction with buffers A and C. These are shown in Fig. 2. The buffer A extract (track 2) contained the 134,000-dalton (a) crystal protein and 68,000-dalton (b) conversion product (Bulla *et al.*, 1979) as its major spore coat proteins along with a 13,000-dalton polypeptide (c). However, when buffer C was used to extract the *B. thuringiensis* subsp. *kurstaki* spores (tracks 3 and 4) most of the 134,000 (a) and 68,000 (b) mol wt_{app} proteins were degraded to polypeptides with mol. wt_{app} of 11,000 (d) and 13,000 (c). The serine protease inhibitor PMSF did not prevent this degradation.

Buffer A, which contains guanidine and mercaptoethanol, but no SDS, was chosen as the extraction buffer for the comparison of coat proteins because it prevented degradation of *B. thuringiensis* coat proteins without having them bound by SDS, and it

extracted the same proteins as buffer B from *B. cereus* T. The coat profiles of the *B. cereus* strains and the coat and crystal protein profiles of the *B. thuringiensis* strains are shown in Fig. 3. As can be seen in Fig. 3, tracks 7 and 8, the *B. cereus* coat profiles were very similar. The coats of both strains contained many proteins with the same mol. wt. Coats of both *B. cereus* T and 569 contained major components with mol. wt_{app} of 14,000 and 12,500. *B. cereus* 569 coats

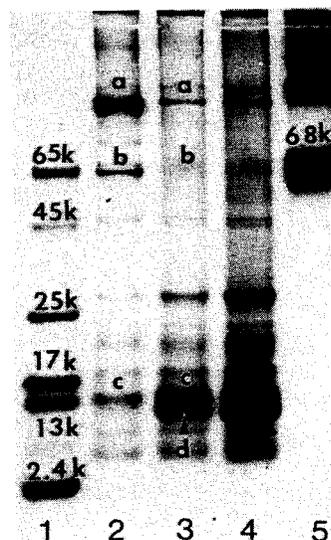


Fig. 2. SDS 5–20% polyacrylamide gel of *B. thuringiensis* subsp. *kurstaki* spore extracts. Track 1: standards same as Fig. 1; track 2: buffer A extract; tracks 3 and 4: buffer C extracts; track 5: cross-linked bovine serum albumin (68,000, 136,000, etc.).

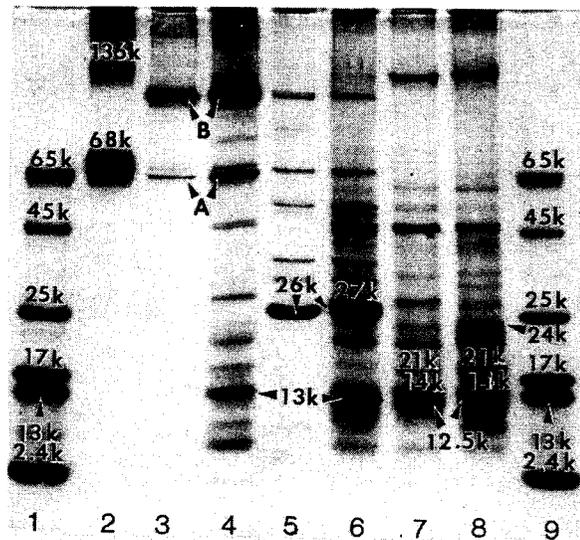


Fig. 3. SDS 5–20% polyacrylamide gel of *B. cereus* and *B. thuringiensis* spore coats extracted with buffer A, and of solubilized *B. thuringiensis* crystals. Tracks 1 and 9: standards same as Fig. 1; track 2: crosslinked bovine serum albumin (68,000, 136,000, etc.); (3) *B. thuringiensis* subsp. *kurstaki* crystal, (4) *B. thuringiensis* subsp. *kurstaki* spore coat, (5) *B. thuringiensis* subsp. *israelensis* crystal, (6) *B. thuringiensis* subsp. *israelensis* spore coat, (7) *B. cereus* T spore coat, (8) *B. cereus* 569 spore coat.

also contained major proteins with mol. wt_{app} of 21,000–24,000 (track 8). The proteins were present in smaller amounts in *B. cereus* T coats (track 7).

The *B. thuringiensis* coats were different from each other as well as from the *B. cereus* coats. The *B. thuringiensis* subsp. *kurstaki* coat protein composition was similar to those of other *B. thuringiensis* subspecies toxic to Lepidoptera (Tyrell *et al.*, 1981 press). It contained large amounts of the crystal protoxin (B) monomer (mol. wt_{app} = 134,000) and of the toxin (A)

which is a conversion product (mol. wt_{app} = 68,000) of the monomer (Bulla *et al.*, 1977b; see tracks 3 and 4, Fig. 3). The major low mol. wt coat protein (not a crystal coat component) had an mol. wt_{app} of 13,000 (see arrow track 4). *B. thuringiensis* subsp. *israelensis* spore coat contained crystal proteins, the major band having an mol. wt_{app} of 26,000 (see arrow, track 6, Fig. 3). The non-crystal coat proteins were a 27,000-dalton protein (possibly, a modified crystal protein) and a 13,000-dalton polypeptide. Furthermore, coats

Table 1. Amino acid compositions of parasporal crystals and spore coat extracts*†

	<i>B. thuringiensis</i> subsp. <i>kurstaki</i>		<i>B. thuringiensis</i> subsp. <i>israelensis</i>		<i>B. cereus</i> T	<i>B. cereus</i> 569
	crystals	spore coats	crystals	spore coats	spore coats	spore coats
Trp	0	5.3	0	5.2	6.3	5.2
Lys	2.8	3.5	4.2	3.9	3.5	4.6
His	1.8	1.8	1.3	2.3	1.9	3.7
Arg	6.7	3.0	2.3	2.8	2.4	2.8
Asp	13.5	10.5	14.2	11.9	10.2	10.3
Thr	6.2	8.9	8.0	8.2	9.1	8.0
Ser	8.3	7.3	6.9	6.7	7.8	7.0
Glu	12.2	9.9	10.7	10.3	9.5	8.3
Pro	4.4	4.8	5.0	5.0	7.0	8.1
Cys/2‡	2.0	2.6	2.1	2.0	1.0	1.9
Gly	7.2	11.4	5.4	10.3	11.3	12.1
Ala	5.4	7.6	7.3	6.9	9.3	8.9
Val	6.2	4.4	7.7	4.9	4.1	3.8
Met‡	1.0	0.5	1.9	0.7	0.4	0.3
Ile	4.9	3.7	6.1	3.8	3.4	2.6
Leu	8.2	7.5	8.2	7.8	7.5	6.8
Tyr	4.9	2.9	3.8	3.1	2.0	1.5
Phe	5.2	4.6	4.8	4.0	3.4	4.1

* Results in mol %.

† Determined by hydrolysis in p-toluenesulfonic acid (Hirs, 1967).

‡ Determined as cysteic acid and methionine sulfone after performic acid oxidation (Liu & Chang, 1971).

of *B. thuringiensis* subsp. *israelensis* contained several minor components not found in the *B. cereus* or *B. thuringiensis* subsp. *kurstaki* coats (compare tracks 4, 6, 7, and 8, Fig. 3).

Amino acid analyses were done using buffer A spore coat extracts and *B. thuringiensis* crystals. The data are presented in Table 1. The amino acid contents of the *B. cereus* coats were very similar. The only major difference was that *B. cereus* 569 had almost twice as much histidine as *B. cereus* T. Differences of only 1 mol/100 mol or less were observed in the other amino acids.

Likewise, *B. thuringiensis* coat extracts were similar. Approximately 1.4 mol/100 mol or less variance occurred between the amounts of each amino acid in each coat. There also was much similarity to both the *B. cereus* coat amino acid compositions, although several small differences were evident. Major differences occurred between the *B. thuringiensis* crystal and coat amino acid profiles, particularly in the amounts of tryptophan, arginine, and glycine. Probably, this was due to the presence of noncrystal coat components rich in certain amino acids.

The results of this investigation indicate that the new *B. thuringiensis* subsp. *israelensis* has a unique spore coat protein profile unlike those of lepidoteran toxic *B. thuringiensis* coats, such as subspecies *kurstaki*, and *B. cereus* coats. Previously, we had shown that spores of acrySTALLIFEROUS mutants of *B. thuringiensis* subsp. *kurstaki* germinate more rapidly than spores of *B. cereus* (Stahly *et al.*, 1978). Interestingly, the rapidly germinating spores lacked the 134,000-dalton crystal protein in their coats, and the spores were not toxic to *M. sexta*. What particular function(s), besides insecticidal activity, that the high mol. wt coat proteins have is not known. Perhaps, survival in nature is dependent upon spore germinability, which, in turn may be determined by certain coat proteins. Certainly, the differences in coat proteins among the organisms that we studied could affect the stability of their spores in certain environments. If so, the unique spore coat protein profile of *B. thuringiensis* subsp. *israelensis* may afford that organism a distinct survival advantage over the other bacilli in mosquito breeding sites.

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