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Relationship of the Syntheses of Spore Coat Protein and Parasporal Crystal Protein in *Bacillus thuringiensis*

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Two major classes of polypeptides were extracted from the spore surface of *Bacillus thuringiensis* subsp. *kurstaki*: the 134,000-dalton protoxin that is the major component of the crystalline inclusion and spore coat polypeptides very similar to those found on *Bacillus cereus* spores. The quantity of spore coat polypeptides produced was reduced when compared with that produced by certain acrystalliferous mutants or by *B. thuringiensis* subsp. *israelensis*. The latter organism produced an inclusion toxic to mosquito larvae, but deposited very little of the inclusion protein on the spore surface. The reduction in spore coat protein in *B. thuringiensis* subsp. *kurstaki* was also seen in freeze-etched electron micrographs of spores. *B. thuringiensis* subsp. *kurstaki* spores germinated rather slowly when compared with related species, a property previously correlated with a deficiency or defect of the spore coat. Many mutants of *B. thuringiensis* subsp. *kurstaki* unable to form a crystalline inclusion were nontoxic and lacked a well-defined spore coat. Other mutants isolated either directly from the wild type or from coat-deficient mutants produced spores that were identical to those produced by the closely related species, *Bacillus cereus*, on the basis of morphology, germination rate, and the size and antigenicity of the spore coat polypeptides. Most of the protein extractable from the inclusion produced by *B. thuringiensis* subsp. *israelensis* was about 26,000 daltons, considerably smaller than the major polypeptide extractable from other inclusions. Some of the *B. thuringiensis* subsp. *israelensis* inclusion protein was found on the spore surface, but the majority of the extractable spore coat protein was the same size and antigenicity as that found on *B. cereus* spores. The *B. thuringiensis* subsp. *israelensis* spores germinated at a rate close to that of *B. cereus*, especially when the spores were formed at 37°C, and the morphology of the spore surface was very similar to that of *B. cereus*.

Certain varieties of sporulating bacilli are unique in their capacity to form a parasporal inclusion. These inclusions consist predominantly of a single species of polypeptide that is a protoxin for the larvae of specific insect species (6). In most cases, this structure is formed during sporulation, but in at least one case (*Bacillus medusa*), the structure may be seen 2 h before the end of exponential growth (4). There are also variations in the location of the parasporal body, i.e., whether it lies within or outside the exosporium (4, 21). In the latter case, the inclusion can be readily separated from the spore by density gradient centrifugation (20). The purified inclusion consists primarily of protein, probably a single species of about 134,000 daltons (6–8, 14), plus a netlike covering that may be the same protein in an altered conformation (P. C. Fitz-James, unpublished data). The capacity to form this protein is correlated with the presence of one or more plasmids found in these strains (12, 25). Of particular interest is the evidence that the protoxin or a closely related polypeptide may be a major constituent of the spore coat, at least in the case of *Bacillus thuringiensis* subsp. *kurstaki* (5, 16, 18, 24, 29).

Aside from the production of a parasporal body, these bacilli resemble in many respects the sporeforming *Bacillus cereus*. The similarity includes DNA homology (23) as well as morphological and nutritional properties (22). The coats of *B. cereus* spores are composed of unique low-molecular-weight polypeptides (3, 4), and the absence or alteration of these polypeptides results in slower germination of the spores, implying a direct or indirect role for these surface

components in the germination process (3, 26). Spores of *B. thuringiensis* subsp. *kurstaki* germinate much slower than those of *B. cereus* (5), again implying that the spore surface is altered.

This interrelationship between spore surface components and formation of a parasporal body has been further studied in *B. thuringiensis* subsp. *kurstaki* and *B. thuringiensis* subsp. *israelensis* as well as in acrystalliferous mutants of the former species. *B. thuringiensis* subsp. *israelensis* forms an inclusion that is toxic to mosquito larvae rather than to *Lepidopteran* larvae (6, 11, 29). In the present study, the morphology of the spores, their germination rates, and the pattern of surface proteins were compared in an attempt to better define the relationship between formation of proteins unique to spore coats and those of parasporal crystals.

MATERIALS AND METHODS

Organisms and culture conditions. *B. cereus* T, *B. cereus* NRRL 569, *B. thuringiensis* subsp. *kurstaki*, and *B. thuringiensis* subsp. *israelensis* were grown in G-Tris medium (2) at 30 and 37°C or in a low sulfur G-Tris medium (3) at 30°C for labeling with [^{35}S]methionine. For the latter experiments, cultures were monitored in a phase-contrast microscope to determine the percentage of phase-white endospores present. A mutant of *B. thuringiensis* subsp. *kurstaki* resistant to 50 μg of D-cycloserine per ml was used for the isolation of acrystalliferous mutants. Acrystalliferous mutants were isolated as previously described (25), except for mutant Mit6, which was isolated after treatment of exponentially growing *B. thuringiensis* subsp. *kurstaki* cells with 0.2 μg of mitomycin C per ml. The resulting spores were enriched for fast germinators as previously described (25).

Isolation of spores and crystals. Spores and parasporal crystals of *B. thuringiensis* subsp. *kurstaki* and *B. thuringiensis* subsp. *israelensis* were isolated by buoyant density centrifugation in Renografin (66% diatrizoate meglumine plus 10% sodium diatrizoate; E. R. Squibb & Sons) gradients (20), washed at least three times with water, and lyophilized. The purity of the preparations was monitored at each step of the separation procedure by phase-contrast microscopy. Sporulated *B. cereus* cultures were centrifuged at 4°C in a Sorvall SS34 rotor at 10,000 rpm for 10 min. The pellets were washed once with 1 M KCl and three times with distilled water and were centrifuged at 6,000 rpm for 10 min after each wash. The purity of these preparations was monitored by phase-contrast microscopy. The washed spores were then lyophilized or extracted directly (see figure legends). Germination studies were done as previously described (3, 26).

Spore coat extraction. Spores were extracted three times by incubation at 37°C for 1 h each either with 6 M guanidinium chloride–0.1 M beta-mercaptoethanol, pH 8.6 (buffer A), or with an extraction buffer (9) containing 8 M urea, 0.07 M dithiothreitol, 1% sodium dodecyl sulfate (SDS), and 5 mM cyclohexylaminoethane sulfonic acid, pH 9.6 (buffer B). The combined extracts were either used immediately for polyacrylamide gel electrophoresis, refrigerated overnight and then electrophoresed, or dialyzed against 0.1 M Tris-hydrochloride–9 M urea (pH 8.6) for 24 h and against water for 48 h and then lyophilized.

Polyacrylamide gel electrophoresis. Spore extracts were solubilized by incubating the lyophilized material in 1% (wt/vol) SDS–1% (vol/vol) beta-mercaptoethanol–6 M urea–0.01 M NaH_2PO_4-Na_2HPO_4 (pH 7.2) for 1 h at 27°C. These solubilized extracts as well as the undialyzed buffer B extracts were subjected to electrophoresis in SDS-polyacrylamide (10 to 20%) gradient slab gels by the method of Weber et al. (30), in 5 to 20% gradients, or in 15% gels, using the buffer system of Laemmli (15). Electrophoresis was carried out at 45 V (see Fig. 9) or at 35 mA (constant values) (see Fig. 1–3, 10). Gels were stained for 3 to 12 h in 0.25% (wt/vol) Coomassie brilliant blue (Eastman R250; Eastman Chemical Products, Inc.) in methanol-acetic acid-water (25:7.5:62.5, vol/vol/vol) and destained for 16 to 48 h in methanol-acetic acid-water (25:7.5:62.5, vol/vol/vol). Molecular weight standards were cross-linked bovine serum albumin (68,000 for the monomer), ovalbumin (45,000), beef pancreas chymotrypsinogen A (25,000), and sperm whale myoglobin (17,000) from Schwarz/Mann; ribonuclease A (13,700) from Pharmacia Fine Chemicals, Inc.; and cytochrome *c* (12,700) and bovine insulin (A chain, 2,384) from Sigma Chemical Co.

Antibody experiments. Cells (5 ml) in low-sulfur G-Tris, grown as described above, were incubated with 70 μCi of [^{35}S]methionine per ml. After 4 min, 1.5 ml was pipetted onto frozen crushed antibody buffer (0.05 M sodium phosphate, 0.05 M NaCl, 2 mM phenylmethylsulfonyl fluoride, 0.05% sodium deoxycholate, 0.005 M disodium EDTA [pH 8.0]) plus 300 μg of L-methionine and 200 μg of chloramphenicol. L-Methionine (100 μg/ml) was added to the remainder of the culture, and an additional sample was removed at 7 min. Four milliliters of unlabeled cells was added to each sample as carrier, and the cells were pelleted in an RC2B Sorvall centrifuge at 10,000 × *g* for 3 min. Each sample was washed three times with 8 ml each of antibody buffer plus 100 μg of L-methionine per ml. The samples were finally suspended in 0.1 ml of antibody buffer and sonicated (on ice) for 5 min, using a Branson model 200 with a microtip and 50% pulses. Each sample was cooled on ice for 1 to 2 min after each 1 min of sonication. The extracts were centrifuged at 12,000 × *g* for 20 min, the supernatants were stored on ice, and the pellets were suspended in 0.1 ml of buffer B. After being incubated in a boiling water bath for 90 s, the samples were kept at 37°C for 1 h. The tubes were centrifuged as described above, and the supernatants were pooled with the initial ones. These extracts were then dialyzed against 1,000 volumes of antibody buffer at 4°C for 12 to 16 h.

Each sample was divided into thirds, and various quantities of *B. cereus* spore coat antibody or preimmune serum were added. Antibody was prepared (13) against purified total spore coat isolated from a mutant forming cytoplasmic coat deposits (27). By Ouchterlony tests, this antibody was found to be as specific as one prepared against purified 13,000-dalton spore coat protein, but the total coat antibody had a much higher titer. Antibody reactivity with spore coat extracts from *B. cereus*, *B. thuringiensis* subsp. *kurstaki*, and *B. thuringiensis* subsp. *israelensis* was compared by

spotting various concentrations of extracts on Whatman no. 1 filter paper disks and incubating with antibody and then ^{125}I-labeled protein A (28). The intensity of the spots in autoradiograms was found to be comparable (unpublished results). There was no reaction with extracts from exponentially growing cells. The antiserum was fractionated with 50% saturation (0°C) ammonium sulfate (13), and the pellet was dissolved in 0.05 M sodium phosphate (pH 7.0) to the original volume and dialyzed against the resuspension buffer at 4°C for 16 h. Concentrations of antibody used varied between 10 and 30% of the extract volume.

After incubation at 37°C for 1 h and then at 4°C for 8 to 12 h, Sepharose-linked goat anti-rabbit serum (Bio-Rad Laboratories) was added (50 μl per 100 μl of antibody plus extract volume). Incubation was continued at 37°C for 90 min, and the tubes were then centrifuged at 10,000 × *g* for 5 min. The pellets were washed twice by suspension in 0.5 ml of antibody buffer, centrifuged, and finally suspended in buffer B as described above. Samples (0.2 ml) in duplicate were added to 2 ml of 10% trichloroacetic acid and incubated at 0°C for 40 min, and the precipitates were collected on glass fiber filters (Whatman GF/A) for radioactivity measurements in Omnifluor cocktail (New England Nuclear Corp.).

Cross-linking of spore surface polypeptides. Tartryl di(glycylazide) (about 13 Å [1.3 nm]) was prepared as described by Lutter et al. (17), using a generous gift of tartryl di(glycylhydrazide) provided by H. Fasold. Washed spores were treated with the reagent for 1 h at 27°C and then purified by centrifugation through a 25 to 60% continuous Renografin gradient. Spores were then extracted with buffer B and fractionated on a 5 to 20% gradient acrylamide gel.

Electron microscopy. Samples for electron microscopy were chilled on ice and fixed with cold 3% glutaraldehyde for 4 h, washed four times with ice-cold sodium phosphate buffer (0.1 M, pH 6.7), suspended in 1% osmium in acetate-Veronal buffer (10), and stored at 2 to 4°C for 4 to 12 h or until dark brown. After being washed in acetate-Veronal buffer, the fixed samples were suspended into agar, stained, dehydrated, embedded in Vestopal (Polysciences), and then sectioned and stained by procedures already detailed (10). Grids were examined in a Phillips electron microscope (model EM300) at 60 kV and photographed at different magnifications (×11,000 to ×19,000).

Freeze-cleave etching was carried out as already described in detail elsewhere (3) and examined with a Phillips EM300 as described above.

RESULTS

***B. thuringiensis* subsp. *kurstaki* spore surface and crystal components.** Two buffers were used to solubilize parasporal crystal and spore coat components. Buffer A proved useful for the former, especially in preserving toxicity; buffer B was found to be most effective for solubilizing virtually all of the coat proteins of *B. cereus* (4). The SDS gel profiles of *B. cereus* spore proteins solubilized by these buffers were virtually identical, although there could be differences in the efficiency of extraction.

A comparative spore coat extraction profile for *B. cereus* T and *B. thuringiensis* subsp. *kurstaki* is shown in Fig. 1. In this case, comparable numbers of spores were extensively extracted to ensure solubilization of most of the coat protein. The extracted spores were rendered lysozyme sensitive, implying extensive if not complete removal of coat proteins (confirmed by examination of sections with the electron microscope). The *B. thuringiensis* subsp. *kurstaki* coat protein profile contained major species of polypeptides (the same size as those extractable from the crystal; see Fig. 9) with a predominant band of about 134,000 daltons, another band at about 60,000 daltons, and a small amount of protein at about 13,000 daltons. The latter is generally missing from crystal extracts, implying that it is spore specific (see Fig. 9). The very prominent high-molecular-weight band is not always present in spore extracts (Fig. 1e and g). In this latter case, spores were prepared by centrifuging to equilibrium twice in

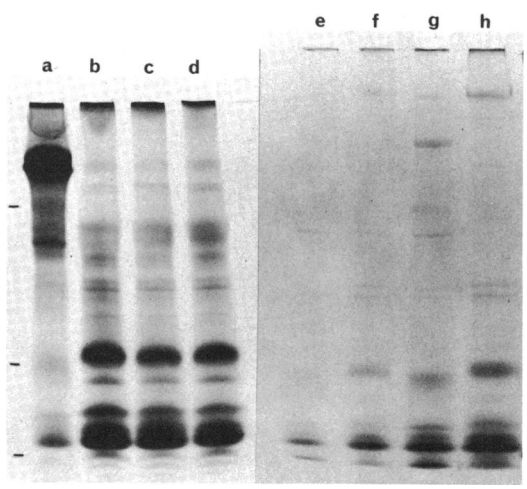

FIG. 1. Polyacrylamide gel profiles of polypeptides in spore extracts fractionated on a 5 to 20% gradient gel. An equal number of spores (2×10^9) was exhaustively extracted with buffer B, and equivalent portions were placed on the gels. For lanes a through d, the spores were purified by pelleting through a 25 to 50% Renografin step gradient. Lanes: (a) *B. thuringiensis* subsp. *kurstaki*, (b) *B. thuringiensis* mutant R6 (acrystalliferous), (c) *B. thuringiensis* mutant Mit6 (acrystalliferous), and (d) *B. cereus* T. In lanes e through h, spores were purified by banding twice in 20 to 60% Renografin continuous gradients. (e and g) *B. thuringiensis* subsp. *kurstaki*, (f and h) *B. cereus* T. Twice as much extract was used for (g) and (h) as compared with (e) and (f). Markers on the left indicate positions of migration; those from top to bottom indicate positions of migration of bovine serum albumin (molecular weight, 68,000), chymotrypsinogen (25,000), and cytochrome *c* (12,700).

25 to 60% linear Renografin gradients before extraction.

The loss of the high-molecular-weight proteins may be due to degradation or their loose association with spores. In some subspecies, a small beaded structure that is toxic for silkworm larvae is produced in addition to the major parasporal body, and it may be a contaminant of the spore fraction (Fitz-James, unpublished data). At least some of the 134,000-dalton polypeptides were cross-linked to the 13,000-dalton spore coat protein, however, implying close spacial association (Fig. 2). This cross-linking may be reversed by treatment with periodate (17), and both protein species were shown to be present in the complex (data not shown). Other data supporting the presence of protoxin polypeptides on the spore surface will be discussed later.

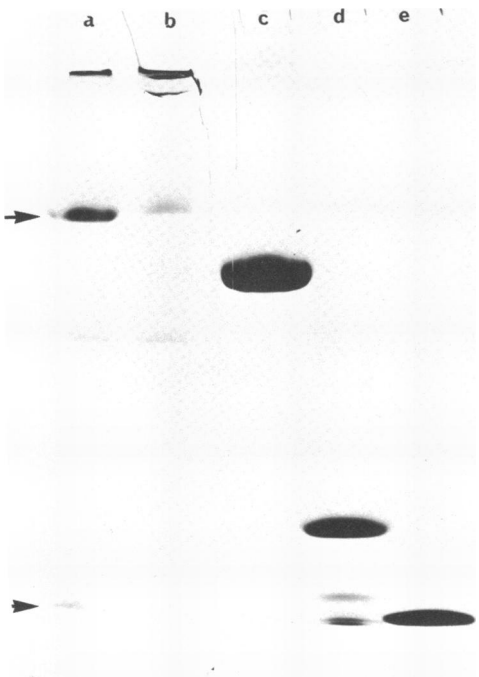

FIG. 2. Gel electrophoresis profile of extracts of spores of *B. thuringiensis* subsp. *kurstaki* treated with the protein cross-linking reagent tartryl di(glycylazide) as described in the text. After purification, the spores were extracted with buffer B, and the extracts were fractionated on a 5 to 20% acrylamide gel. Lanes: (a) Control, (b) treated spores, (c) bovine serum albumin, (d) chymotrypsinogen, and (e) cytochrome *c*. The lower arrow indicates the position of ca. 13,000-dalton spore coat proteins (virtually absent in [b]); the upper arrow indicates the position of 134,000-dalton protoxin. Note the slightly slower migration of the band in (b).

In all cases, the quantity of a major group of spore coat polypeptides of about 13,000 daltons was reduced in extracts of *B. thuringiensis* subsp. *kurstaki* spores as compared with extracts of *B. cereus* spores (Fig. 1). Since the spores were extensively extracted and virtually devoid of coat, the deficiency reflects either an inability to deposit low-molecular-weight spore coat polypeptides on the spore surface or a depression of synthesis of these polypeptides or both.

A second major difference was the absence of a 26,000-dalton component in extracts of *B. thuringiensis* subsp. *kurstaki* spores. As previously reported (4), *B. cereus* T contains predominant species of about 13,000 daltons and variable quantities of a 26,000-dalton polypeptide (Fig. 1). The latter is most likely a dimer of one or more of the 13,000-dalton proteins (1) and, in fact, was not detected in some cases (see Fig. 9). Differences in spore extraction conditions (volume of buffer relative to spore number, pH, etc.) influence the relative amount of the 26,000-dalton protein present (1), but the protein was invariably absent from *B. thuringiensis* subsp. *kurstaki* spore extracts.

The deficiency of *B. cereus*-like spore coat proteins on *B. thuringiensis* subsp. *kurstaki* spores could be due to some defect in the deposition of coat proteins on the forespore outer membrane rather than a lack of synthesis. Extracts of sporulating cells were examined, therefore, for the presence of antigens cross-reacting with *B. cereus* spore coat antibody (Table 1). There was detectable labeled protein in antibody precipitates of *B. thuringiensis* subsp. *kurstaki*, but considerably less than in extracts from a comparable number of *B. cereus* cells. These cells were labeled at other stages of sporulation (stage II and stage V) with similar results, indicating a decreased synthesis of *B. cereus*-like spore coat antigen in *B. thuringiensis* subsp. *kurstaki* cells throughout sporulation.

A second, more indirect, confirmation of the lack of spore coat polypeptides similar to those found in *B. cereus* was obtained by examining the spore coat profiles of acrystalliferous mutants (25; Fig. 3, lanes A through C). These mutants were isolated after heating the spores. They were found to lack all of the plasmids present in the wild type and the 134,000-dalton protoxin and were nontoxic to tobacco hornworm larvae (19, 25). The spore coat profiles were identical even though the spores produced by one mutant, S1, were sensitive to lysozyme. There were no major spore coat proteins; especially notable was the lack of the 13,000- and 26,000-dalton polypeptides prevalent in *B. cereus* (Fig. 3, lane E). It was also possible to obtain mutants forming spores with *B. cereus*-like coat

TABLE 1. Immunoprecipitation of coat protein

Cells[a]	Time of labeling	Coat antibody (cpm)[b]	Control serum (cpm)[b]	Δ Value
B. cereus	4 min	3,800	1,100	2,700
	4 min plus 3-min chase	2,800	700	2,100
B. thuringiensis subsp. *kurstaki*	4 min	850	650	200[c]
	4 min plus 3-min chase	750	600	150[c]
B. thuringiensis subsp. *israelensis*	4 min	2,100	800	1,300
	4 min plus 3-min chase	2,050	850	1,200

[a] Cells were incubated with [^{35}S]methionine as described in the text. Cells were grown until about 5 h after the end of exponential growth (early phase white endospores, stage IV). Conditions of extraction, antibody precipitation, and sampling were as described in the text.

[b] The specific activities of the extracts were similar (3×10^4 to 4×10^4 cpm/100 μg of protein), and constant amounts of the extracts (2×10^4 to 3×10^4 cpm) were treated with various concentrations of antibody to isolated *B. cereus* spore coats or preimmune serum. Values for precipitates (two of 20 μl) with the highest counts per minute are reported.

[c] A range of Δ values of 150 to 350 was obtained in four experiments.

proteins (Fig. 1, lane b; Fig. 3). Mutant R6 was selected by enriching for rapid germination (25) from an acrystalliferous mutant forming lysozyme-sensitive spores (S1). Mutant Mit6 was also selected by enriching for rapid germinating spores, but directly from the wild type after treatment of cells with mitomycin C (see Materials and Methods). In addition to having different origins, the strains differed in that mutant R6 was totally devoid of plasmids (25), whereas mutant Mit6 had lost only 1 to 2 plasmids of 30 to 50 megadaltons (unpublished data). As expected, neither cell extracts nor spores of these mutants were toxic for neonate larvae of the tobacco hornworm. In both cases, the coat profiles were identical to those of *B. cereus* T (Fig. 1 and 3), indicating that the capacity to synthesize or deposit (or both) these low-molecular-weight spore coat proteins is inhibited, but not absent, in acrystalliferous coat-deficient mutants.

A further indication of differences in spore surfaces between *B. cereus* and *B. thuringiensis* subsp. *kurstaki* was obtained by comparing the rates of germination. As mentioned previously, spores with deficient or defective coats respond poorly to germinants (3, 26). Spores formed by wild type and mutant *B. thuringiensis* subsp. *kurstaki* responded relatively slowly to *B. cereus* germinants (Fig. 4). Mutants that regained spore coat, however (i.e., R6 and Mit6 in Fig. 1, lanes b and c), responded as well as *B. cereus* to these germinants (data not shown).

The spores of wild-type *B. thuringiensis* subsp. *kurstaki* formed by aeration in a fluid medium possessed coats with the same layering found in the coats of typical *B. cereus* spores (Fig. 5). The surface cross-patch profile, although present, was not as prominent as that on spores of non-crystal-forming *B. cereus* (4). The freeze-etch micrographs confirmed the presence of a thinner deposit of organized cross-patch layer, with a resulting visualization of considerable underlying pitted layer (Fig. 6). In both sections and freeze-etch replicas, occasional segments of redundant coat deposition were also seen.

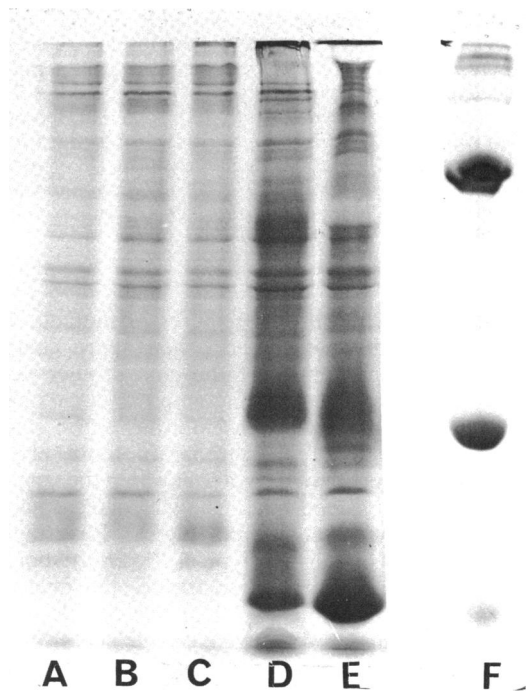

FIG. 3. Gel electrophoresis profile of extracts of spores of acrystalliferous *B. thuringiensis* subsp. *kurstaki* mutants. Spore purification and extraction with buffer B were as described in the text and in the legend to Fig. 1. Electrophoresis was in a 15% acrylamide-urea-SDS gel at 35 mA for 2 h. Lanes: (A) spore extract of mutant S1 (lysozyme-sensitive spores), (B) spore extract of mutant Cry$^-$ B (lysozyme-resistant spores), (C) spore extract of mutant Cry$^-$ C (lysozyme-resistant spores), (D) spore extract of mutant R6 (fast-germinating, acrystalliferous mutant derived from S1), (E) spore extract of *B. cereus* T, and (F) protein standards as described in the legend to Fig. 1.

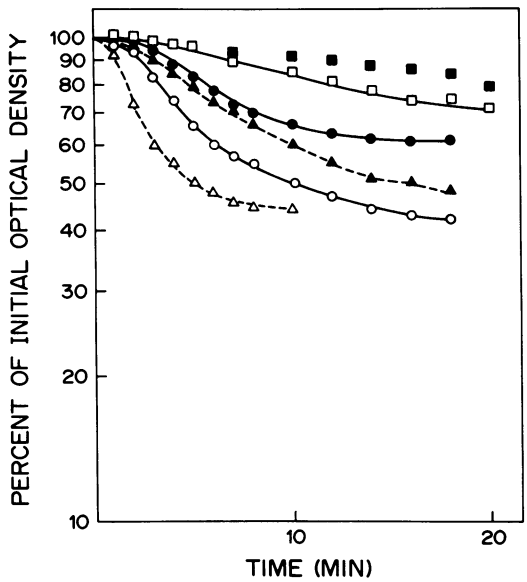

FIG. 4. Germination of *B. cereus*, *B. thuringiensis* subsp. *kurstaki*, and *B. thuringiensis* subsp. *israelensis* spores. Spores were prepared, activated, and germinated as described previously (3, 26). Symbols: (○) *B. cereus* spores formed at 30°C, (△) *B. cereus* spores formed at 37°C, (●) *B. thuringiensis* subsp. *israelensis* formed at 30°C, (▲) *B. thuringiensis* subsp. *israelensis* formed at 37°C, (□) *B. thuringiensis* subsp. *kurstaki* formed at 30°C, and (■) *B. thuringiensis* subsp. *kurstaki* mutant S1 formed at 30°C.

The lysozyme-sensitive spores of mutant S1 were markedly defective in their spore coats (Fig. 7). Discontinuity of the coat profile was characteristic of most sections; cross-patch and undercoat layers were absent from either surface of the basic coat profile. In freeze-etch replicas, the plane of cleavage exposed the cortical surface; cleaved coats showing typical patterns were not encountered (Fig. 8). Revertant R6 was very similar to a typical *B. cereus* spore both in thin section and in freeze-etch micrographs (not illustrated). All of the coat layers were prominent and intact.

***B. thuringiensis* subsp. *israelensis* spore coat and parasporal crystal proteins.** In contrast to those of *B. thuringiensis* subsp. *kurstaki*, the extracts of *B. thuringiensis* subsp. *israelensis* parasporal crystals contained a predominant protein of about 26,000 daltons and some higher-molecular-weight species, including one of about 134,000 (Fig. 9, lane 5). Spore extracts of *B. thuringiensis* subsp. *israelensis* contained the parasporal body protein plus *B. cereus*-like spore coat proteins of 13,000 and 26,000 daltons (Fig. 9, 10). There seemed to be a doublet in the 26,000-dalton region of spore extracts (Fig. 9, lane 6, and Fig. 10, lane C), indicating that the 26,000-dalton polypeptides from the parasporal body and spore coats were different. When growth and spore formation occurred at 37°C, rather than at 30°C, there was a decreased deposition of crystal protein both in the spore fraction and as an inclusion (Fig. 10). Spore coat proteins identical in size to those extracted from *B. cereus* spores were prevalent in extracts from spores formed at either temperature. When the parasporal crystals were extracted with buffer B rather than buffer A, there appeared to be relatively more higher-molecular-weight polypeptide (Fig. 10, lane E, versus Fig. 9, lane 5). To date, a 26,000-dalton polypeptide has been found only in parasporal crystal extracts of *B. thuringiensis* subsp. *israelensis* (14, 29).

Consistent with the presence of *B. cereus*-like polypeptides in spore extracts was the presence of *B. cereus* spore coat antigens in extracts of sporulating cells (Table 1). Coat antigens were detected in amounts somewhat less than were found in *B. cereus* extracts, but considerably more than those found in *B. thuringiensis* subsp. *kurstaki* extracts. In addition, *B. thuringiensis* subsp. *israelensis* spores germinated almost as rapidly as *B. cereus* spores (Fig. 4) and considerably faster than *B. thuringiensis* subsp. *kurstaki* spores.

The spore coat profile in section and freeze etchings of *B. thuringiensis* subsp. *israelensis* was similar to that of *B. cereus* (Fig. 11A). However, some of the spores in the culture, both free and during formation, had coats with an unstructured thickness, which in sharp profile appeared as a wide external deposit (Fig. 11B). A robust cross-patch layer was seen in most cleavages of these spores (Fig. 12). Some granular deposition of additional material appeared to be contaminating the cross-patch layer (Fig. 12). In addition, fibrous strands of material identical to those which appear to form a surface layer on the parasporal inclusions of *B. thuringiensis* subsp. *israelensis* (A. R. Tam and P. C. Fitz-James, unpublished data) were visible on the surface of the exosporium (Fig. 12).

DISCUSSION

Whereas both *B. thuringiensis* subsp. *kurstaki* and *B. thuringiensis* subsp. *israelensis* produced parasporal inclusions, they differed in their specificity as toxins for insect larvae and in some properties of their spore surfaces. We assumed that these species were very similar to *B. cereus* on the basis of considerable morphological and biochemical data and thus expected to find a *B. cereus*-type spore coat (4). This assumption was supported by the cross-reactivity of antibody to *B. cereus* spore coat with *B. thuringiensis* coat proteins, the presence of a spore coat indistinguishable (morphologically

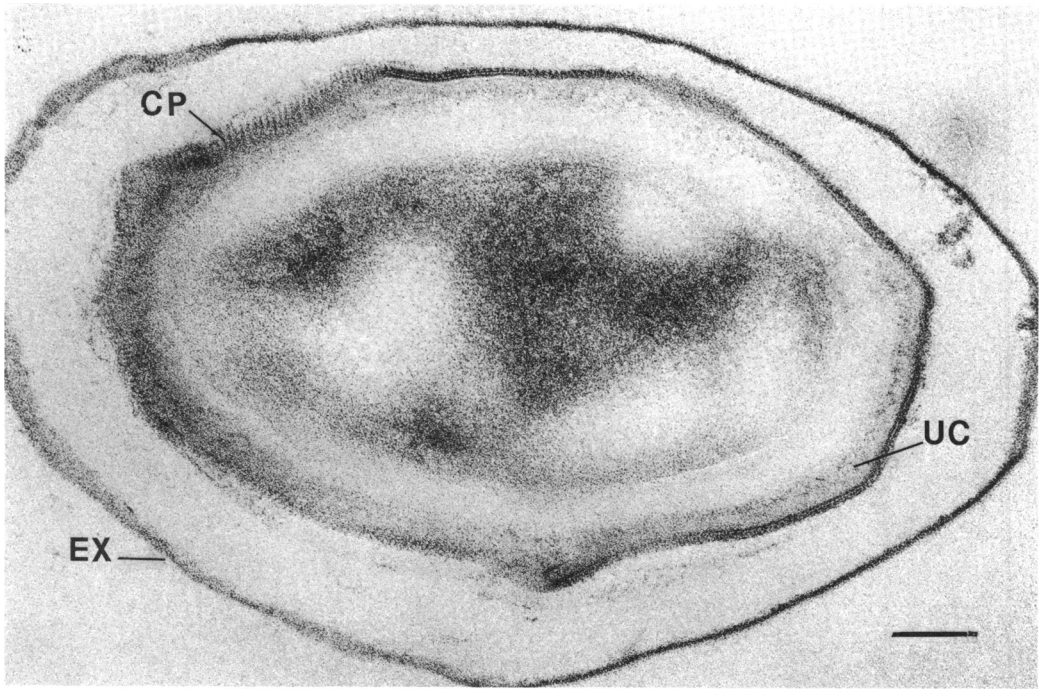

FIG. 5. Thin-section electron micrograph of a free spore of *B. thuringiensis* subsp. *kurstaki* showing the typical *B. cereus* coat layers under the exosporium (EX). An oblique cut at one end revealed the 9-mm repeating pattern of surface cross-patch rodlets (CP) on the basic "double-track" coat. The less-defined undercoat (UC) layer may also be seen. The complete coat was closely applied to the surface of the cortex (magnification, ×104,000); the bar marker is 100 nm in this and subsequent micrographs.

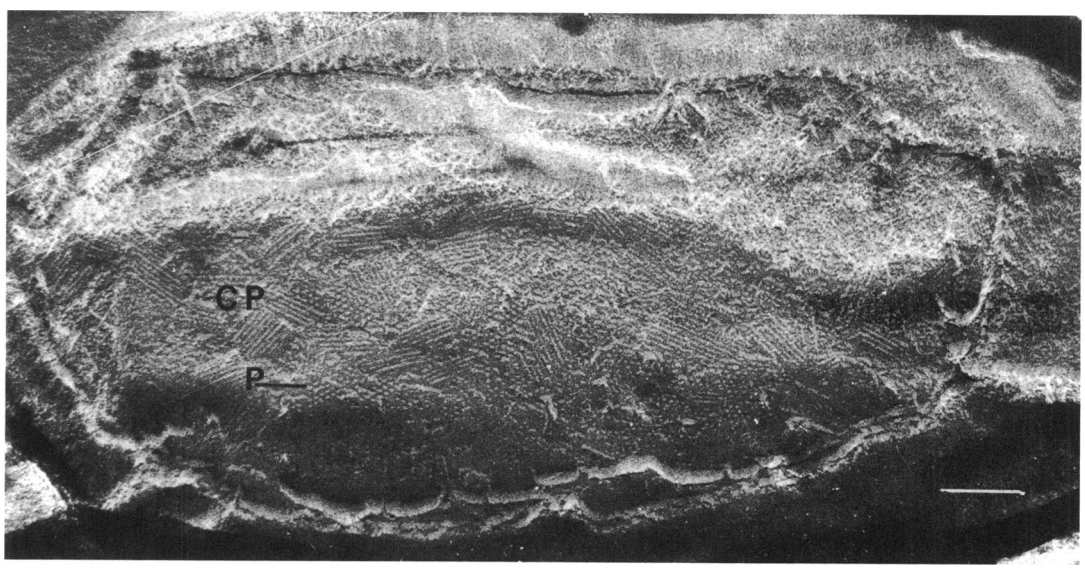

FIG. 6. Freeze-etch replica of *B. thuringiensis* subsp. *kurstaki* showing the rather scant cross-patch (CP) deposit on the pitted (P) layer of the basic coat. A redundant coat layer, perhaps the protoxin protein, appeared to lie under part of the exosporium. Magnification, ×115,500.

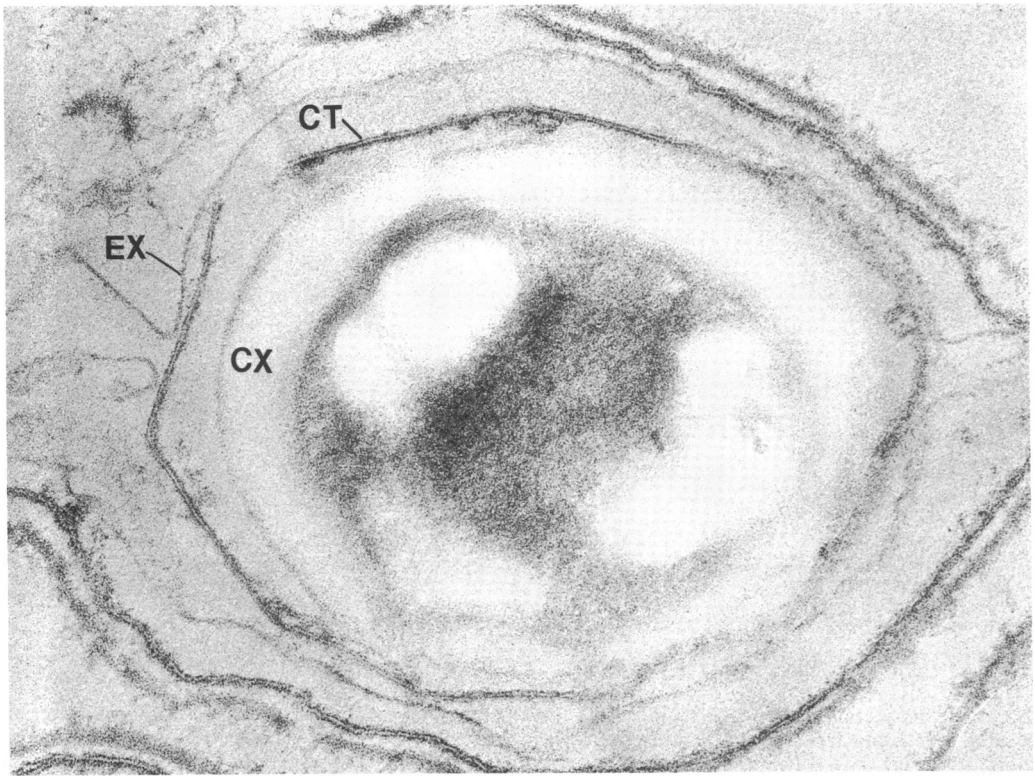

FIG. 7. Spore of mutant S1 (lysozyme sensitive) undergoing liberation from sporangial remnants. The exosporium (EX) appears intact, but the basic coat (CT) was incomplete in its coverage of the cortex (CX) and deficient in both outer cross-patch and undercoat layers. Magnification, ×104,000 as indicated in the legend to Fig. 5.

FIG. 8. The lysozyme-sensitive mutant, S1, invariably showed the cortical surface (CX) in freeze-cleave-etch replicas. Magnification, ×79,750.

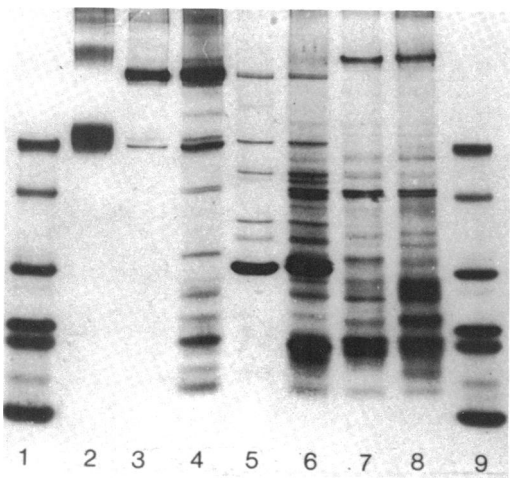

FIG. 9. Polyacrylamide gel profiles of *B. cereus* and *B. thuringiensis* spore coats and of *B. thuringiensis* crystals solubilized with buffer A and electrophoresed on a 5 to 20% gradient gel. (Lanes 1 and 9) Molecular weight standards of bovine serum albumin (68,000), ovalbumin (45,000), chymotrypsinogen A (26,000), myoglobin (17,000), ribonuclease A (13,700), and bovine insulin (A chain, 2,400); (lane 2) cross-linked bovine serum albumin (68,000, 136,000, etc.); (lane 3) *B. thuringiensis* subsp. *kurstaki* crystal; (lane 4) *B. thuringiensis* subsp. *kurstaki* spore coat; (lane 5) *B. thuringiensis* subsp. *israelensis* crystal; (lane 6) *B. thuringiensis* subsp. *israelensis* spore coat; (lane 7) *B. cereus* T spore coat; (lane 8) *B. cereus* NRRL 569 spore coat extract (included to show the extent of variation among *B. cereus* strains). Fresh extracts containing 60 μg of protein were added to each slot.

and biochemically) from *B. cereus* in *B. thuringiensis* subsp. *israelensis* (Fig. 12), and in some acrystalliferous isolates of *B. thuringiensis* subsp. *kurstaki* such as R6 (Fitz-James, unpublished data).

B. thuringiensis subsp. *kurstaki* contained a 134,000-dalton protein not only as a crystalline inclusion but in most cases as a major component of the coat proteins extracted from purified spores. In some cases, purification of spores by washing and then centrifugation to equilibrium in Renografin gradients once or twice resulted in spores that contained extractable low-molecular-weight spore coat proteins, but were devoid of high-molecular-weight protoxin components (Fig. 1, lane e), raising the possibility that the latter was a contaminant and not part of the spore surface. In fact some *B. thuringiensis* subsp. *kurstaki* cells produced a beadlike inclusion in addition to the crystal (Fitz-James, unpublished results). The beaded inclusion was toxic for larvae and was sufficiently dense to be present in the spore fraction after centrifugation through Renografin gradients. It is more likely, however, that the protoxin protein extracted from purified spores was present as a surface component rather than as a contaminant for the following reasons. (i) Short et al. (21) found crystal antigen in sections of *B. thuringiensis* spores, specifically in the coat layers; (ii) the protoxin could be chemically cross-linked to low-molecular-weight spore coat polypeptides (Fig. 2); and (iii) the beaded inclusion contained polypeptides of 68,000 to 70,000 daltons (Fitz-James, unpublished results), whereas much of the protoxin extracted from purified spores had a molecular weight of 134,000 (Fig. 2, 9). Protoxin protein probably forms a loose association with the coat and may be readily removed or degraded during spore purification.

A deficiency of low-molecular-weight, *B. cereus*-like spore coat polypeptides as in *B. thuringiensis* subsp. *kurstaki* (Fig. 1) has also been found in *B. thuringiensis* subspecies *berliner*,

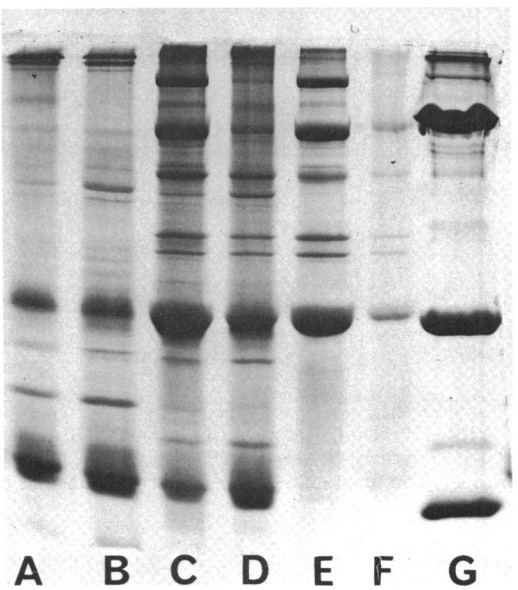

FIG. 10. Gel electrophoresis profiles of *B. cereus* T spore extracts and *B. thuringiensis* subsp. *israelensis* spore and parasporal body extracts. Spores and inclusions were prepared and extracted with buffer B as described in the text. Electrophoresis was in a 15% acrylamide-urea-SDS gel at 35 mA for 2 h. Lanes: (A) extract of *B. cereus* spores formed at 30°C, (B) extract of *B. cereus* spores formed at 37°C, (C) extract of *B. thuringiensis* subsp. *israelensis* spores formed at 30°C, (D) extract of *B. thuringiensis* subsp. *israelensis* spores formed at 37°C, (E) extract of *B. thuringiensis* subsp. *israelensis* crystalline inclusion from 30°C culture, (F) extract of *B. thuringiensis* subsp. *israelensis* crystalline inclusion from 37°C culture, (G) protein molecular weight standards, from top to bottom: bovine serum albumin (68,0000), chymotrypsinogen (26,000), and cytochrome *c* (12,700).

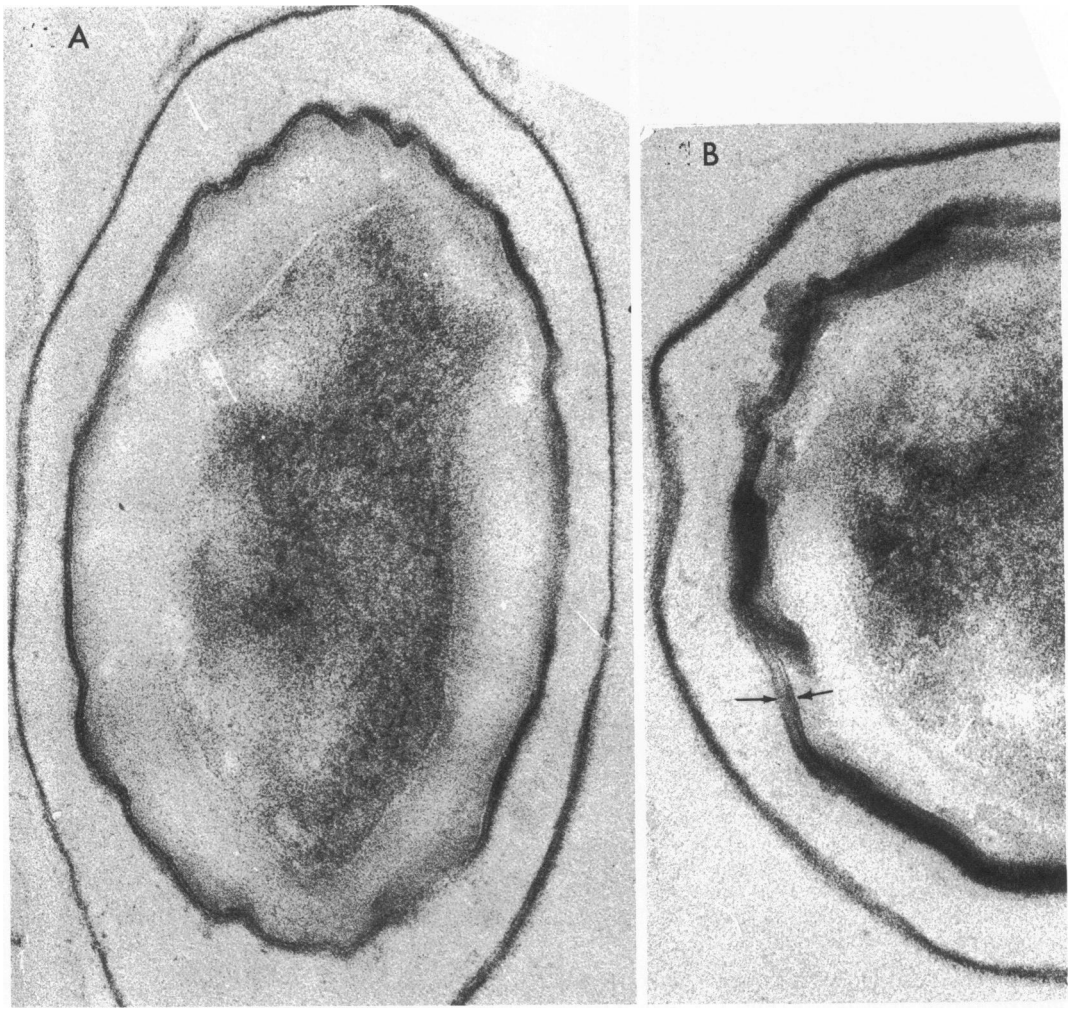

FIG. 11. Thin section of free spores of *B. thuringiensis* subsp. *israelensis*. Many of the spores had coat profiles typical of *B. cereus* (A). Some had an additional deposit with thin coats which, in clear sections (arrows), presented an unstructured outer layer (B). Magnification, ×115,000.

alesti, *fowler*, and *finitimus* (29, unpublished results). In fact, the only exception to date is *B. thuringiensis* subsp. *israelensis*. In all of the above cases, high-molecular-weight polypeptides, presumably the same as those present in the parasporal inclusion, could be extracted from purified spores. A correlation of inhibition of spore coat synthesis and deposition of parasporal protein thus seems to exist among these particular organisms.

The presence of protoxin on the spore surface would help to account for the frequent isolation of mutants almost totally devoid of coat (S1 and Cry^- B in Fig. 3). These mutants were acrystalliferous and had lost the capacity to produce a protoxin, and thus perhaps a secondary spore coating that could help to sustain the integrity of the small amount of spore coat produced. In addition, a deficiency of *B. cereus*-like coat protein in many *B. thuringiensis* subspecies could provide space for protoxin deposition on the spore surface and, thus, formation of toxic spores (18, 24). Larval ingestion of spores would not only result in toxicity, but would permit the spore to proliferate in the larval hemolymph (6, 18, 19).

In either case, a regulatory element controlling spore coat protein synthesis should be present in many strains that produce parasporal bodies. An apparent role for a plasmid in protoxin formation (12, 25) implies that the regulatory element may have its origin there. Since some plasmid-cured strains retained the inability to make complete spore coats, the regulatory

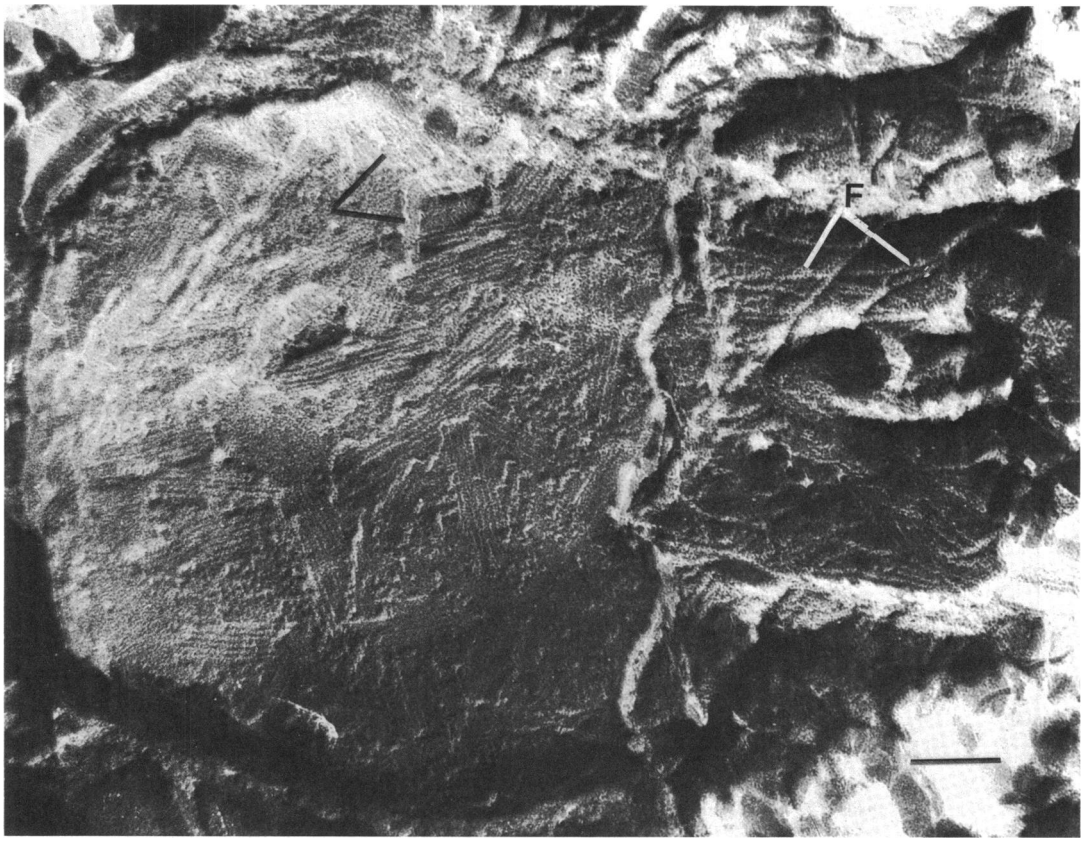

FIG. 12. A freeze-etch replica of a free spore of *B. thuringiensis* subsp. *israelensis* showing a fairly dense deposit of cross-patch coat layer associated with other small granular deposits (pointers). The surface of the exosporium is heavily covered with long fibrils (F). Magnification, ×115,500.

element may be a transposon or an insertion sequence. These could have been lost from or altered in strains that regained the ability to produce complete *B. cereus*-like spore coats (i.e., R6 in Fig. 1).

B. thuringiensis subsp. *israelensis* differed from *B. thuringiensis* subsp. *kurstaki* in that there was little, if any, deficiency of low-molecular-weight spore coat proteins, and the predominant polypeptide extractable from the inclusion had a molecular weight of about 26,000, although there was some protein of 134,000 daltons. The smaller protein may be a degradation product since a polypeptide of 134,000 daltons is a predominant component extractable from parasporal crystals of several other subspecies (14, 29; unpublished results). The spore surface of *B. thuringiensis* subsp. *israelensis* contained some parasporal protein (especially when spores were produced at 30 rather than at 37°C) in addition to substantial amounts of coat polypeptides analogous to those found in extracts of *B. cereus* spores. The latter were also detected in antibody precipitates of sporulating cells, and the presence of a *B. cereus*-like spore coat was evident in electron micrographs (Fig. 11 and 12). Consistent with the chemical analysis and morphology was the relatively low toxicity of the spores and a germination rate intermediate between that of *B. thuringiensis* subsp. *kurstaki* and *B. cereus* (Fig. 4).

The capacity of *B. thuringiensis* subsp. *israelensis* to form a *B. cereus*-like spore coat and of *B. thuringiensis* subsp. *kurstaki* to do so in certain mutants lacking the ability to form a crystalline inclusion raises again the relatedness of these organisms to *B. cereus* (22). On the basis of numerous criteria (nutrition, morphology, cross-hybridization of DNA) these bacilli seem to be virtually identical. The major difference appears to be the ability to form a crystalline inclusion, a phenotype that can be readily lost, resulting in many cases in a spore indistinguishable from that formed by *B. cereus*. It may be more meaningful to consider *B. thuringiensis* subspecies as *B. cereus* varieties that have gained the ability to produce an inclusion, perhaps involving plasmid-mediated genes.

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