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Urea-Mercaptoethanol-Soluble Protein from Spores of *Bacillus thuringiensis* and Other Species

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Treatment with urea-mercaptoethanol of purified spores of *Bacillus thuringiensis*, other *Bacillus* species, and *Clostridium roseum* solubilizes a protein fraction between 5 and 12% of the dry weight of the spores. This fraction behaves identically to the crystal protein of *B. thuringiensis* on acrylamide-gel electrophoresis. The protein from all of the *Bacillus* species shows partial homology with crystal protein, using the Ouchterlony immunodiffusion technique. A further fraction, similar in amount, can be removed from spores of *B. thuringiensis* by the addition of sodium lauryl sulfate to the urea-mercaptoethanol. Spores of *B. thuringiensis* extracted in these ways show no difference when compared to untreated spores with respect to viability or resistance to heat and ultraviolet-irradiation. The extracted spores do show differences in their germination requirements and their susceptibility to phase-darkening by lysozyme. It is concluded that an urea-mercaptoethanol-soluble protein or class of protein is a widespread component of bacterial spores, possibly located in the spore coat, and that this protein may be related to the crystal protein of *B. thuringiensis*.

It was shown (4, 11) that the protein comprising the crystal inclusion of *Bacillus thuringiensis* and a fraction extracted from its broken spores show extensive immunological and biochemical similarities. Both are insoluble under normal physiological conditions, but dissolve readily in urea-mercaptoethanol or in dilute alkali and remain in solution after dialysis to *p*H 8.3 to 8.5. These solutions show homologous precipitin bands when examined by the Ouchterlony technique by using rabbit antiserum against alkali-dissolved crystals. The urea-spore extract and the urea-crystal solution behave almost identically on electrophoresis in acrylamide gel under two different sets of conditions. Peptide maps of tryptic digests of the two preparations are almost superimposable, and their amino acid analyses are closely similar. Based on these and other data, it was concluded that the crystal protein is closely similar, if not identical, to a fraction of the spore representing a fifth or more of the total spore protein (11).

It seemed reasonable to assume that a protein comprising such a large fraction of the total spore protein should contribute significantly to spore properties. Further, considering the gross similarity of structure and physiology of bacterial endospores, the same or similar proteins might be expected to occur in spores of other species. It was of interest, therefore, to determine whether any of the crystal-like protein could be extracted from intact spores of *B. thuringiensis* and other spore formers. If so, comparison of intact and extracted spores might reveal what physiological properties, if any, this protein confers on the spore. There was a priori reason to expect that extraction of protein from intact spores with urea-mercaptoethanol would be feasible, since Gould and Hitchins (5) reported that unbroken spores of *B. cereus* rapidly lose refractility when incubated in buffered lysozyme after extraction with urea-mercaptoethanol. However, they did not report whether spore material was solubilized by the treatment.

MATERIALS AND METHODS

Organisms. The following cultures were used: (i) a strain of *B. thuringiensis* var. *alesti anduze* doubly resistant to penicillin (1,000 units/ml) and streptomycin (250 μg/ml), which was derived by standard procedures from *B. thuringiensis* var. *alesti anduze* (EAS 0-24-3) originally obtained from E. A. Steinhaus; (ii) a sporulating acrystalliferous mutant of the double-resistant strain selected from cells surviving treatment for 1 hr at 23 C with 0.4% (w/v) *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine in 0.1 M citrate buffer (*p*H 5.0), the parental identity of which was confirmed

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by growth on Difco Nutrient Agar containing penicillin and streptomycin; (iii) *B. thuringiensis* var. *tolworth* obtained from H. de Barjac; (iv) *B. cereus* and (v) *B. megaterium* obtained from the culture collection of this department; (vi) *B. cereus* T obtained from C. Davidson; (vii) *B. subtilis* SB 19 obtained from W. R. Romig; (viii) *Clostridium roseum* kindly donated by R. E. Collier.

Maintenance and cultivation for sporulation. *Bacillus* species were maintained on slants of Difco Nutrient Agar, and *C. roseum* in tubes of Difco Thioglycollate Broth. Organisms identified above (i–vi) were cultured for sporulation on Nutrient Agar supplemented with salts as previously described (4). Spores of *B. subtilis* were obtained from cultures grown on a medium containing 10 g of Difco Tryptone, 10 g of NaCl, 5 g of Difco Yeast Extract, 20 g of Difco Agar, 1.8 mg of $MnCl_2 \cdot 4H_2O$, 8 ml of 1% $CaCl_2$, and 5 ml of 1% $MgSO_4 \cdot 7H_2O$ per liter of distilled water. The calcium and magnesium salt solutions were sterilized separately and added aseptically to the medium. Cultures in Roux bottles were incubated at 30 C for 7 days and harvested with ice-cold 0.05 M potassium phosphate buffer, *p*H 7.0.

C. roseum was cultured for sporulation in a medium containing 7.5 g of trypticase, 2 g of $(NH_4)_2SO_4$, 50 mg of $MnCl_2 \cdot 4H_2O$, 5 mg of $ZnSO_4 \cdot 7H_2O$, 5 mg of $CuSO_4$, 0.4 mg of $FeSO_4$, 20 ml of 10% glucose, 8 ml of 1% $CaCl_2$, and 5 ml of 1% $MgSO_4$ in one liter of 0.01 M potassium phosphate buffer (*p*H 7.0). The glucose solution and the calcium, magnesium, and iron salt solutions were sterilized separately and added aseptically to the medium. The medium (1.5 liters in a 2-liter Erlenmeyer flask) was flushed with nitrogen for 10 min before and after inoculation with 40 ml of an overnight culture in Thioglycollate Broth. After incubation for 7 days at 30 C under nitrogen, cultures were harvested by centrifugation.

Purification of spores and crystals. The purification of spores and crystals from *B. thuringiensis* and the criteria for the cleanliness of the final preparations were described in detail previously (4). Identical procedures were employed to obtain clean spore suspensions of the other organisms except that the final extractions in a two-phase system of sodium dextran sulfate and polyethylene glycol were omitted. The clean "complete" spores were washed at least six times in distilled water, lyophilized, and stored at −20 C. The crystals were washed and stored in distilled water at 4 C.

Fractionation of spores and crystals. "Broken" spores were obtained by ballistic disintegration (10) of complete spores as previously described (4). "Naked" spores were obtained from complete spores by removing exosporia with ultrasonic treatment in an atmosphere of nitrogen by the procedure of Berger and Marr (2).

Solubilization of crystal protein by urea-mercaptoethanol and separation of protein from broken spores into phosphate-soluble and urea-mercaptoethanol-soluble fractions was described (4). Urea-mercaptoethanol extracts of complete spores and naked spores were prepared by suspending such spores [20 to 50 mg (dry weight)/ml] in 8 M urea solution containing 1% (w/v) β-mercaptoethanol, *p*H 8.5. After 3 hr at 23 C, the suspension was centrifuged, and the sedimented spores were briefly extracted twice more with the same reagent. The three extracts were dialyzed separately against several changes of 0.02 M $NaHCO_3$ at 23 C and combined to give the "urea-mercaptoethanol" extract.

The extracted spores were washed four times in 0.05 M potassium phosphate buffer (*p*H 7.0) and stored at −20 C or washed further in distilled water and lyophilized.

In some experiments, extraction with urea-mercaptoethanol reagent was preceded by extraction with 0.02 M potassium phosphate buffer (*p*H 6.8) or with 8 M urea pH 8.5, and followed by two extractions with the urea-mercaptoethanol reagent containing 1.5% (w/v) sodium lauryl sulfate. Extraction in each case was for 1.5 hr at 23 C. Spores were centrifuged from each reagent and washed once with the reagent before extracting with the next. Additionally, spores that were extracted by the complete sequential treatment were washed four times in distilled water, lyophilized, broken, and reextracted successively with the phosphate buffer and the urea-mercaptoethanol reagents. In each case, the urea-containing extracts were dialyzed exhaustively against 0.02 M $NaHCO_3$.

Serology. A solution of purified crystals of *B. thuringiensis* var. *tolworth* was prepared by treatment with 0.1 N NaOH for 3 hr at 23 C, followed by 2 hr of centrifugation at 30,000 × *g* at 2 C and dialysis of the supernatant fluid against 0.02 M $NaHCO_3$. Rabbits were immunized by two subcutaneous injections (spaced 4 weeks apart) of this solution containing 2 to 3 mg of protein, mixed with an equal volume of Freund's adjuvant. The serum was collected after an additional week to ten days.

Precipitation reactions were carried out by using the double-diffusion technique of Ouchterlony (3). The gel contained 1% Difco ion agar and 0.3% NaN_3 in 0.01 M barbital buffer (*p*H 8.6). The agar (4.5 ml) was poured on 5-cm square slides, and after solidification was drilled with 80-μliter holes spaced about 7 to 10 mm apart.

After incubation at 30 C for 48 hr, the slides were washed first for 24 hr in 0.9% NaCl and then three times for 1 hr each in distilled water. They were then dried on blotting paper and stained for 1 to 3 min with a saturated solution of Naphthalene Black in 10% acetic acid-50% methanol. They were destained in the same mixture without the dye.

Viability of spores. Spore suspensions were diluted in 0.05 M potassium phosphate buffer (*p*H 7.0) to roughly 2×10^3 colony forming-units per ml, and 0.1-ml samples were then plated on Nutrient Agar by using standard procedures. Plates were incubated at 30 C for 48 hr and colonies were counted.

The clumping of spores introduced some difficulties, and the number of colony-forming units found for a given lyophilized spore preparation was generally about 50% of the direct microscopic count. However, when the preparation was suspended with the aid of a Sorvall Omnimixer in 0.02 M potassium phosphate buffer (*p*H 6.8) containing 0.01% Triton X-100, the

viable count was in close agreement with the microscopic count.

Heat sensitivity of spores. Spore suspensions, about 5×10^7/ml, were diluted 10-fold in preheated 0.05 M potassium phosphate buffer (pH 7.0) and held at 85 C in a thermostatically controlled water bath. At intervals, samples were withdrawn and immediately chilled in ice. Viable counts were made as described above.

Ultraviolet sensitivity of spores. Spore suspensions, about 5×10^6/ml in 0.05 M potassium phosphate buffer containing 0.01% Triton X-100 (*p*H 7.0) were placed in open petri dishes at 33 cm from a 15-w General Electric germicidal lamp and agitated slowly by rotation during irradiation. Samples were withdrawn at intervals for viability counts.

Lysozyme sensitivity of spores. Spores were treated with crystalline egg white lysozyme (Worthington Biochemical Corp., Freehold, N.J.) in suspensions containing the following in 0.17 ml: 0.1 mg (1×10^8) of spores; 50 μmoles of potassium phosphate buffer (*p*H 8.0); 1.0 μmole of Merthiolate (sodium ethylmercurithiosalicylate); 0.125 mg of lysozyme. Suspensions were incubated at 37 C, and samples were removed at intervals to determine the ratio of refractile to nonrefractile spores by phase-contrast microscopy. The enzyme was dispensed from a stock solution of buffer (2.5 mg/ml), which was stored for no more than 84 hr at 4 C before discarding.

Germination of spores. Suspensions of spores (about 0.4 mg/ml of 0.02 M potassium phosphate buffer, *p*H 7.0) were heated at 75 C for 15 min. They were immediately diluted 1:10 into tubes containing (final concentration in the same buffer) one of the following mixtures: (i) 1 mM glucose, 6 mM L-alanine, and 0.01 mM adenosine; or (ii) 5 mM glucose, 30 mM L-alanine, and 0.05 mM adenosine. Wet mounts were prepared at intervals by mixing a drop of sample and a drop of 0.5% aqueous methylene blue and examined with a phase-contrast microscope. Spores wholly stained were scored as germinated; at least 200 were counted in each sample.

Miscellaneous methods. The methods for determination of alanine racemase and catalase activities, amino acid analysis, and acrylamide-gel electrophoresis were described previously (4, 11). Dipicolinic acid was estimated by the procedure of Janssen et al. (6), protein by the method of Lowry et al. (7), and calcium and magnesium by flame photometry.

RESULTS

Extraction of protein from spores. Table 1 shows the amounts of protein extracted from complete, naked, broken, or from broken preextracted spores of *B. thuringiensis* by successive treatment with various reagents. A comparison of the data for the different spore preparations shows the following pertinent points.

(i) No protein is extracted from unbroken spores by either 8 M urea or 0.02 M potassium phosphate buffer (*p*H 6.8). Either reagent solubilizes about 25 to 30% of the protein of broken spores. This fraction differs markedly from the crystal protein (4) and is not further considered.

(ii) Urea-mercaptoethanol removes some 12% of the total protein of the complete spore, and a comparable quantity is solubilized by subsequent

TABLE 1. *Extraction of protein from spores of Bacillus thuringiensis*

Treatment	Protein solubilized[a]			
	Complete	Broken	Extracted complete and then broken[b]	Naked spores
0.02 M Phosphate (*p*H 6.8) or 8 M urea (*p*H 8.5)	0	15.9	14.3	
8 M Urea 1% mercaptoethanol	6.4	14.4	6.0	6.0
8 M Urea-1% mercaptoethanol-1.5% sodium lauryl sulfate	5.2	14.1		
Residue	40.8	12.5		
Total initial protein	54.0	54.0		51.3
Total protein recovered	52.4	56.9		

[a] The designated spore type (100 mg, dry weight) was sequentially extracted by 5-ml portions of the listed reagents as described in the text.

[b] Complete spores previously extracted sequentially by the three reagents were washed, lyophilized, broken, and reextracted as indicated.

[c] Data given are from an independent experiment with spores of *B. cereus*, with 100 mg (dry weight) of unextracted, complete spores.

extraction by this reagent supplemented with a detergent. These quantities are roughly half of those extracted from broken spores by the same reagents. Prior removal of the exosporia makes no difference in the amounts of protein extracted by urea-mercaptoethanol from otherwise complete spores.

(iii) When complete spores are broken after extraction with the urea-mercaptoethanol reagents and then reextracted with buffer and then with urea-mercaptoethanol, the latter reagent solubilizes a quantity of protein equivalent to the "missing half" (Table 1, column 3). This fraction has a similar amino acid composition to that of the urea-mercaptoethanol fraction obtained from complete spores (Table 3) and behaves identically on electrophoresis under the conditions to be described.

(iv) Dilute phosphate buffer extracts an equivalent quantity of protein from broken spores regardless of whether the spores were preextracted with the mercaptoethanol reagents before breakage. This suggests that the protein removed from unbroken spores by the mercaptoethanol reagents does not include the phosphate-soluble fraction.

Complete spores of an acrystalliferous mutant of *B. thuringiensis*, *B. cereus*, *B. cereus* T, *B. megaterium*, *B. subtilis*, and *C. roseum* were extracted directly with the urea-mercaptoethanol reagent. In each case, 5 to 12% of the total spore protein was removed. Microscopic examination of these extracted spores indicated that in each case they were still refractile.

Effect of extraction of Ca, Mg, and dipicolinic acid content of spores. The calcium, magnesium, and dipicolinic acid contents of untreated and urea-mercaptoethanol-extracted complete spores of *B. thuringiensis* were almost identical (Table 2), showing that these components were not removed by the reagent. Further extraction with urea-mercaptoethanol-detergent did not effect the dipicolinic acid content of the spores; calcium and magnesium were not determined in this case.

Comparison of the protein in urea-mercaptoethanol extracts of spores with crystal protein of *B. thuringiensis*. The urea-mercaptoethanol extracts of intact spores from all cultures, as well as the comparable extract from preextracted and then broken spores of *B. thuringiensis* (Table 1, third column), reacted with anti-crystal antiserum. In the Ouchterlony double-diffusion test, all extracts, except *C. roseum*, showed a type of partial homology with crystal protein similar to that previously described for the urea-mercaptoethanol extract of broken spores of *B. thuringiensis* (4). Figures 1a and b show results with the extracts from *B. megaterium* and *B. cereus* T; they are typical of all the others except *C. roseum*. Similar patterns were obtained with antisera against dissolved crystals of *B. thuringiensis* var. *alesti*.

The urea-mercaptoethanol extracts from all spores tested, including those of *C. roseum*, showed essentially identical behavior to crystal protein in disc electrophoresis by using the split-gel technique. At *p*H 9.5, no protein from the extracts entered 7.5% acrylamide gel, but a single band which co-electrophoresed with crystal protein was obtained with each extract when the acrylamide concentration in the gel was reduced to 5%. The bulk of the protein from each spore extract entered 7.5% gel at *p*H 12 to give a single major band, which also co-electrophoresed with crystal protein under the same conditions. Additional bands were observed in some cases, but these comprised only a small proportion of the stainable material. Figures 2 and 3 show examples of the electrophoresis results.

The bulk of the material in the urea-mercaptoethanol extracts of spores absorbing at 280 nm was excluded from Sephadex G-100 in 0.02 M $NaHCO_3$. Amino acid analyses (Table 3) of each of the excluded fractions are similar both to one another and to the analyses of crystal protein and the urea-mercaptoethanol extract of broken spores of *B. thuringiensis*.

Urea-mercaptoethanol-sodium lauryl sulfate extract of B. thuringiensis spores. As shown (Table 1), complete or broken spores previously extracted with urea-mercaptoethanol release further protein when extracted with the same reagent containing sodium lauryl sulfate. The second fraction is indistinguishable from the first on disc electrophoresis under conditions already described (Fig. 4). Amino acid analyses and immunological tests were not made on this fraction.

Effect of extraction on properties of spores of B. thuringiensis. Viable counts and direct counts of spores of this organism which were extracted with urea-mercaptoethanol or successively extracted with the three reagents shown in Table 1 were in close agreement, when care was taken in obtaining dispersed suspensions. These results show that the extraction procedures had little effect on spore viability. A comparison of urea-mercaptoethanol-extracted spores with untreated spores also showed that this reagent did not appreciably influence their heat resistance or their resistance to ultraviolet irradiation (Tables 4 and 5). Spores additionally extracted with the detergent-containing reagent were not investi-

TABLE 2. *Calcium, magnesium, and dipicolinic acid contents of untreated and of extracted complete spores of B. thuringiensis*

Treatment[a]	Magnesium/100 mg (initial dry wt) of spores		
	Calcium	Magnesium	Dipicolinic acid
None	2.6	0.25	14
8 M Urea-1% mercaptoethanol	3.0	0.33	15
8 M Urea-1% mercaptoethanol-1.5% sodium lauryl sulfate			13

[a] Spores were sequentially extracted by the reagents listed, washed, lyophilized, and analyzed.

gated in detail; however, they retained complete viability after heating at 75 C for 15 min.

A marked difference was noted in the susceptibility of the different spore preparations to phase-darkening by lysozyme (Table 6). The removal of exosporia did not greatly alter the sensitivity of spores to lysozyme. However, after extraction with urea-mercaptoethanol, both complete spores and naked spores showed a marked increase in sensitivity to this enzyme. The effect of a further extraction with the detergent-containing reagent was most striking; after this treatment 100% of the spores were phase-dark within 15 min of exposure to the enzyme.

A similar effect was previously noted by Gould and Hitchins (5) who found that spores of *B. cereus* heated at 70 C at *p*H 3 showed a marked increase in sensitivity to lysozyme. Their reagent was tested on spores of *B. thuringiensis* and gave the same results (Table 6). The treatment at *p*H 3, either at room temperature or at 70 C, solubilized a quantity of protein equivalent to that extracted by the urea-mercaptoethanol reagent, and this fraction behaves similarly to crystal protein on disc electrophoresis (Fig. 4).

Extraction with urea-mercaptoethanol also lowered the concentration of nutrients required for germination of the spores (Table 7). As with sensitivity to lysozyme, the removal of exosporia prior to extraction did not greatly influence the results. In contrast to the results with lysozyme, a further extraction with the detergent reagent did not lead to an increased rate of germination.

DISCUSSION

The data show that endospores of five bacterial species, as distantly related as *B. subtilis* and

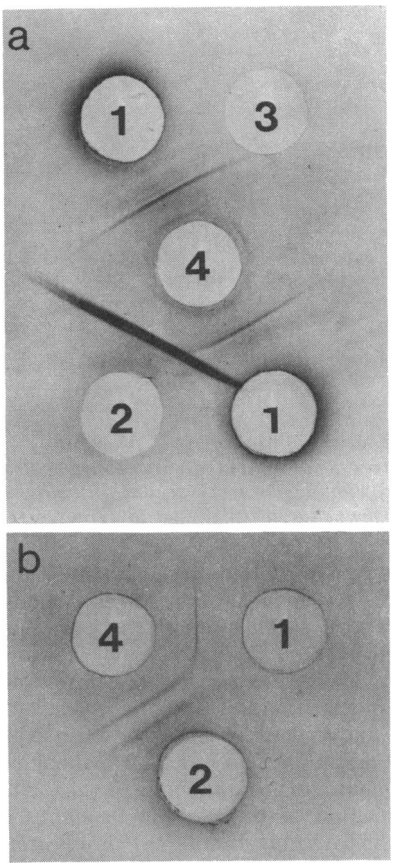

FIG. 1. *(a) Precipitin pattern formed by urea-mercaptoethanol extracts of (1) B. megaterium spores (200 μg of protein), (2) B. thuringiensis crystal (100 μg of protein), and (3) phosphate extract of ruptured B. thuringiensis spores (400 μg of protein). Incubation was for 48 hr at 30 C. (b) As above, but with 200 μg of a urea-mercaptoethanol extract of B. cereus T in well 1.*

C. roseum, contain significant amounts of protein, or type of protein, characterized by the following common properties: extractable, in part, from intact spores by urea-mercaptoethanol; indistinguishable from the crystal protein of *B. thuringiensis* by acrylamide-gel electrophoresis under several conditions; and similar in amino acid composition. In addition, the protein from each of the *Bacillus* spores was immunologically reactive with antisera prepared against the solubilized protein crystal of *B. thuringiensis* and partially homologous with the crystal protein in the Ouchterlony double diffusion test. The demonstration of protein similar to the crystal protein of *B. thuringiensis* in spores of four additional species chosen at random suggests

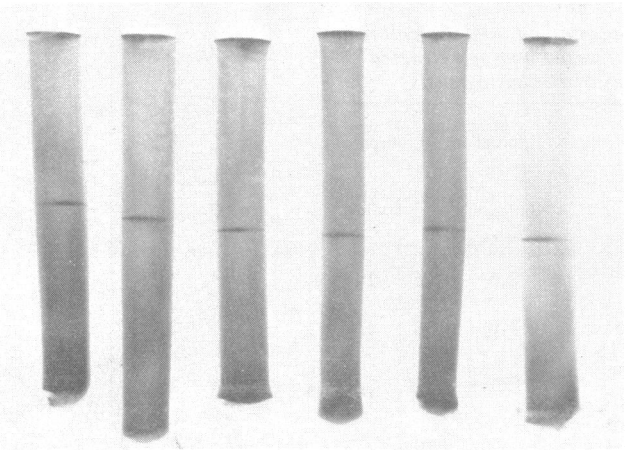

FIG. 2. *Disc electrophoresis at pH 9.5 in 5% acrylamide gel. About 5 to 10 μg of protein in each sample of dissolved crystal or of urea-mercaptoethanol extract of complete spores of the named strains. Split gels (54 by 5 mm diameter) with (left to right): crystal, B. thuringiensis var. alesti cr⁻; crystal, B. subtilis; crystal, B. megaterium; B. thuringiensis var. alesti, crystal; B. cereus, crystal; crystal, C. roseum.*

that this type of protein may be an important and widespread component of the bacterial endospore.

The above listed properties of the protein extracted by urea-mercaptoethanol from broken spores of *B. thuringiensis* plus the close similarity of the peptide maps of tryptic digests of this protein and crystal protein led us to conclude that the crystal protein was a normal component of the spore of *B. thuringiensis* (4, 11). The demonstration of this protein in spores of an acrystalliferous mutant of *B. thuringiensis* adds support to the previous conclusion.

The data, although indicating similarity, do not permit the conclusion that the protein extracted by urea-mercaptoethanol from the different species are identical to each other or to the crystal protein. The critical test of comparing the peptide maps of their tryptic digests remains to be done. Additionally, although their amino acid analyses are remarkably similar considering their diversity of origin and the crudeness of the fractionation procedure, they are not identical. In part, these differences in analyses may be due to differences in the degree of contamination of these preparations with other types of spore protein, and, in part, disparities in the contents of certain amino acids may result from their instability under the conditions used in preparing the samples for analysis. In any event, in the comparison between different organisms, the complete identity in composition of proteins is of secondary significance as compared to identity of function, since it has been well established that proteins identical in function but from different organisms may differ in amino acid composition, presumably reflecting to some extent their evolutionary diversity (8).

With respect to the function(s) of this protein, only the spores of *B. thuringiensis* have been investigated. A comparison of the properties of intact and extracted spores indicates that the protein is not required for the viability of spores, nor is it involved with the resistance of spores to heat or to ultraviolet irradiation. These findings are in agreement with those of Gould et al. (5) who found no alteration in resistance to heat and ultraviolet irradiation by spores of *B. cereus* treated with reducing agents.

Extraction of the protein did increase the sensitivity of the spores to the action of lysozyme and also increased their rate of initiation of germination while lowering the nutrient concentrations required for the process. The former effect was previously reported (5) for spores of *B. cereus* treated with urea-mercaptoethanol or heated at *p*H 3. Since both treatments remove part of the crystal-like protein from intact spores, the changes may perhaps be attributable to this loss and not merely to rupture of disulfide bonds as suggested by Gould and Hitchins (5). It is not excluded that the changes in these two spore properties result from parallel effects of the extracting reagents that are unrelated to either protein removal or action on disulfide bonds, and further investigation of the problem is required.

We previously suggested (11) that the crystal-like protein makes up one or more of the spore coats of *B. thuringiensis* because of the similarities of its properties to those reported for partially purified spore coat protein (9). Recently Aronson and Fitz-James (1) published an amino acid analysis of spore coat protein of *B. cereus* which is similar to that of the crystal protein. These

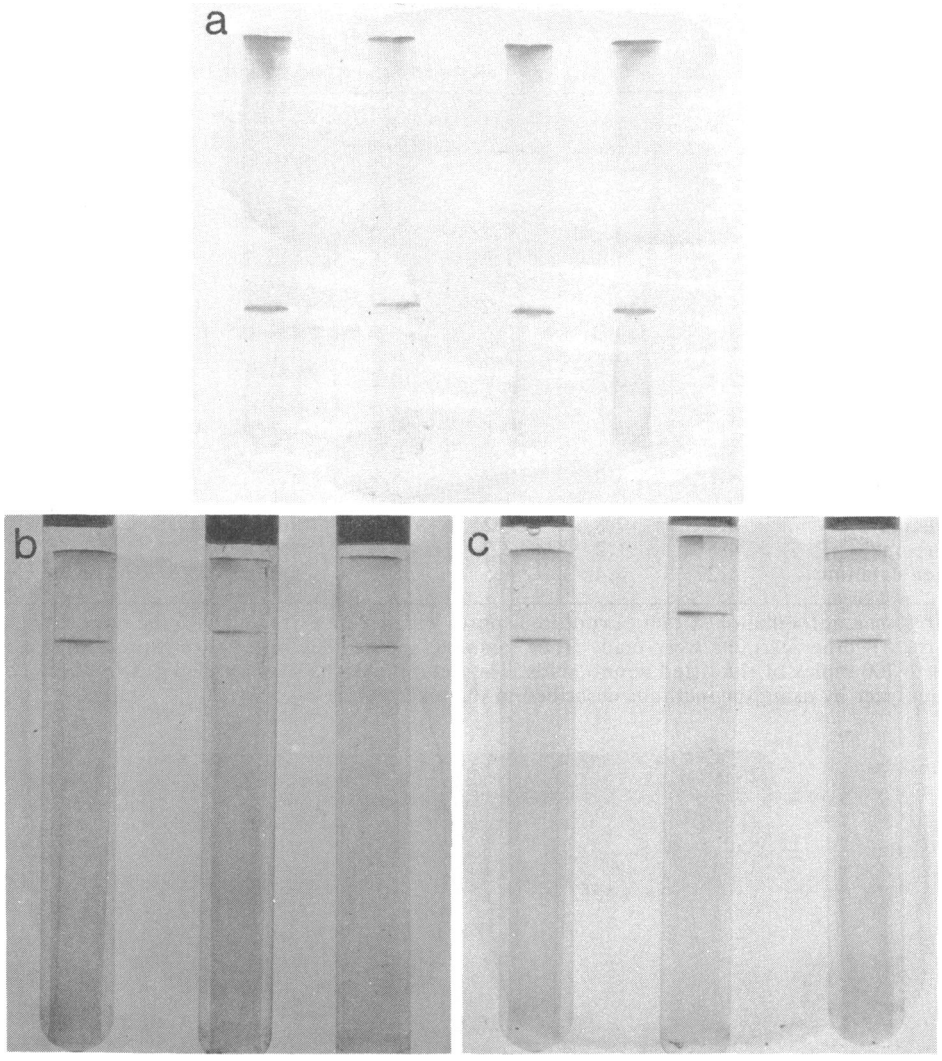

FIG. 3. *Disc electrophoresis at pH 12 in 7.5% acrylamide gel; about 5 to 10 μg of protein in each sample of dissolved crystal or of urea-mercaptoethanol extract of complete spores of the named strains. (a) Split gels (54 by 5 mm diameter) with the first named sample on the left in each case. Left to right; crystal, B. thuringiensis var. alesti cr⁻; B. cereus, crystal; crystal, B. megaterium; crystal, B. thuringiensis var. alesti. (b) Gels (65 by 7 mm diameter) left to right: crystal; split gel crystal, B. subtilis; B. subtilis. (c) Gels as in b left to right: crystal; split gel crystal, C. roseum; C. roseum.*

investigators showed that part of the coat could only be solubilized in the presence of sodium lauryl sulfate. Likewise, our data show that extraction with the sodium lauryl sulfate reagent solubilizes protein not removed by the unsupplemented urea-mercaptoethanol solution. As already noted, the two fractions were indistinguishable on the basis of the tests applied.

These results suggest that the spore coat of *B. thuringiensis* and possibly that of other bacterial spores may consist of two similar protein eomponents. As only about half of each component is extracted from complete spores (as compared to broken spores), one may speculate that a layer of each kind is located on either side of yet another layer impermeable to the solvents. The existence of such a layer would explain the apparent failure of either urea-mercaptoethanol, or urea-mercaptoethanol-sodium lauryl sulfate to extract the phosphate (or urea)-soluble fraction

TABLE 3. *Amino acid analyses of urea-mercaptoethanol extracts of spores*[a]

Amino acid	*B. thuringiensis* var. *alesti* crystal protein	*B. thuringiensis* var. *alesti*	*B. thuringiensis* var. *alesti* [a]	*B. thuringiensis* var. *alesti* cr⁻	*B. cereus*	*B. megaterium*	*B. subtilis*	*C. roseum*
Lysine	3.2	4.1	6.2	4.0	3.5	3.9	3.6	5.2
Histidine	2.2	2.1	1.9	1.6	1.6	1.9	2.1	1.6
Arginine	6.4	3.8	3.4	2.9	2.9	3.5	1.9	1.4
Aspartic acid	12.4	10.9	11.2	11.2	10.6	10.5	10.3	10.9
Threonine	6.3	10.8	6.6	8.6	8.9	10.5	6.1	9.4
Serine	7.3	5.6	5.8	6.5	7.1	7.1	8.7	5.4
Glutamic acid	12.0	6.6	11.6	10.2	9.2	7.3	10.9	8.6
Proline	4.8	7.6	2.3	2.4	4.8	6.9	5.5	7.3
Glycine	7.7	11.0	10.8	10.9	11.3	10.6	12.0	13.3
Alanine	5.4	8.7	9.7	9.4	9.2	9.5	12.0	7.0
Valine	8.1	8.0	8.7	8.9	8.0	7.4	7.4	7.7
Isoleucine	6.0	6.1	7.2	6.9	6.6	5.8	6.1	8.2
Leucine	8.3	7.8	7.2	8.9	8.8	8.1	7.6	6.8
Tyrosine	4.8	1.2	2.7	2.5	2.1	1.6	2.3	3.2
Phenylalanine	5.2	5.4	4.5	5.2	5.5	5.5	3.6	3.7

[a] Urea-mercaptoethanol extract of ruptured spores which were extracted with the same reagent before rupture. All other extracts were made from complete spores. The results are expressed as a ratio of moles to 100 moles of the listed amino acids. The determinations were carried out in a Spinco amino acid analyzer by using the methods described in the text.

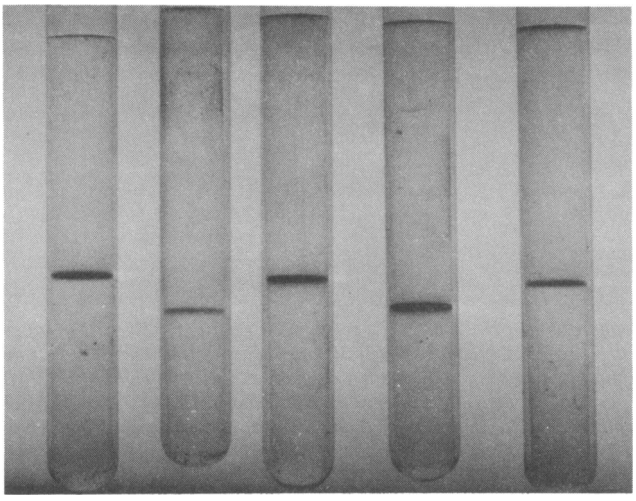

FIG. 4. *Disc electrophoresis at pH 9.5 in 5% acrylamide gel. Gels as in Fig. 3b. About 10 μg of protein in each sample of the following extracts of crystal or spores of B. thuringiensis var. alesti, left to right: 0.1 N NaOH extract of spores; extract of spores at 70 C and pH 3; UMS extract of UM-extracted spores; UMS extract of spores plus UM crystal extract; UM crystal extract. Abbreviations as in Table 6.*

TABLE 4. *Heat resistance of complete, naked, and extracted naked spores of B. thuringiensis var. alesti*

Min heated at 85 C	Percentage of spores surviving		
	Complete spores	Naked spores	Extracted, naked spores
0	100	100	100
10	59	52	39
20	51	40	39
30	31	16	18

TABLE 5. *Resistance to ultraviolet irradiation of complete, naked, and extracted naked spores of B. thuringiensis var. alesti*

Min exposure to ultraviolet irradiation	Percentage of spores surviving		
	Complete spores	Naked spores	Extracted, naked spores
0	100	100	100
2	44	45	45
5	4	4	4
10	0.4	1	0.3

TABLE 6. *Sensitivity of extracted spores of B. thuringiensis to lysozyme measured by phase-darkening*[a]

Spore type and treatment	Per cent of spores dark in phase-contrast microscope after lysozyme treatment for indicated time in hr								
	0	0.25	0.5	1.0	1.25	2	4.5	8.5	20.5
Complete spores									
Untreated	5				5			5	5
UM[b]	5		20	30		60	65		
UM and UMS[b]	5	100							
*p*H 3.0 at 23 C	5		20	30		65	75		
*p*H 3.0 at 70 C	5	30	70	76		88			
Naked spores									
Untreated	11				9			13	11
UM	14				20			69	90

[a] No increase in the number of phase-dark spores was observed in parallel control experiments without lysozyme.

[b] Abbreviations: UM, 8 M urea-1% mercaptoethanol; UMS, UM + 1.5% sodium lauryl sulfate.

TABLE 7. *Effect of extraction on germination requirements of spores of B. thuringiensis*

Hr of incubation	Percentage of spores germinated									
	Complete spores						Naked spores			
	Untreated in mixture		UM[a] extracted in mixture		UM and UMS[a] extracted in mixture		Untreated in mixture		UM and UMS[a] extracted in mixture	
	a[b]	b	a	b	a	b	a	b	a	b
0	8	8	6	6	5	5	11	11	7	7
1			62	80	56	89				
4	11	62	83	91	86	94	22	65	88	92

[a] Abbreviations as in Table 6.

[b] Mixture a: 0.02 M phosphate (*p*H 7.2), 1 mM glucose, 6 mM L-alanine, 0.01 mM adenosine. Mixture b: 0.02 M phosphate (*p*H 7.1), 5 mM glucose, 30 mM L-alanine, 0.05 mM adenosine.

from unbroken spores. The hypothesis is also compatible with electron microscope observations which show several layers outside the spore cortex (9), and should be directly verifiable by an electron microscope study comparing thin sections of extracted and unextracted spores.

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ADDENDUM IN PROOF

We are indebted to W. M. Waites who has pointed out that the strain of *Clostridium roseum* used in this

work has recently been identified as *Clostridium bifermentans* (H. O. Halvorson and A. Swanson, Spores IV, p. 121–132, 1969).

LITERATURE CITED

1. Aronson, A. I, and P. C. Fitz-James. 1968. Biosynthesis of bacterial spore coats. J. Mol. Biol. **33**:199–212.
2. Berger, J. A., and A. G. Marr. 1960. Sonic disruption of spores of *Bacillus cereus*. J. Gen. Microbiol. **22**:147–157.
3. Crowle, A. J. 1960. Immunodiffusion. Academic Press Inc. New York.
4. Delafield, F. P., H. J. Somerville, and S. C. Rittenberg. 1968. Immunological homology between crystal and spore protein of *Bacillus thuringiensis*. J. Bacteriol. **96**:713–720.
5. Gould, G. W., and A. Hitchins. 1963. Sensitization of bacterial spores to lysozyme and to hydrogen peroxide with agents which rupture disulphide bonds. J. Gen. Microbiol. **33**:413–424.
6. Janssen, F. W., A. J. Lund, and L. E. Anderson. 1958. Colorimetric assay for dipicolinic acid in bacterial spores. Science **127**:26–27.
7. Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall. 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. **193**:265–275.
8. Margoliash, E., and E. L. Smith. 1965. Structural and functional aspects of cytochrome in relation to evolution, p. 221–242. *In* V. Bryson and H. J. Vogel (ed.), Evolving genes and proteins. Academic Press Inc., New York.
9. Murrell, W. G. 1967. The biochemistry of the bacterial endospore. p. 133–252. *In* A. H. Rose and J. F. Wilkinson (ed.), Advances in microbial physiology, I. Academic Press Inc., New York.
10. Sacks, L. E., P. B. Percell, R. S. Thomas, and Glenn F. Bailey. 1964. Kinetics of dry rupture of bacterial spores in the presence of salt. J. Bacteriol. **87**:952–960.
11. Somerville, H. J., F. P. Delafield, and S. C. Rittenberg. 1968. Biochemical homology between crystal and spore protein of *Bacillus thuringiensis*. J. Bacteriol. **96**:721–726.