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# Crystal Protein Formed by *Bacillus subtilis* Cells

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**Electron microscopic investigation of ultrathin sections of *Bacillus subtilis* Cgr4 cells revealed the presence of crystal-like inclusions which were formed of spheric homogeneous subunits. The frequency of cells with a crystal-like inclusion in the culture approached 1%. The appearance of the crystal protein in cells coincided in time with spore morphogenesis. However, the process of crystal protein formation and sporulation are two alternatives: the cells either form the crystal protein or continue spore morphogenesis. Fractionation of cells in the stationary growth phase on a Percoll density gradient showed that the cells containing the crystal protein accumulated in the fraction corresponding to a 1.14-g/ml Percoll density. The cells were disintegrated by sonication, and alkaline-extracted proteins were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. After sodium dodecyl sulfate-gel electrophoresis, the fraction enriched with crystal-containing cells showed practically a single band with a molecular weight of 47,000 that corresponded to the crystal-forming protein. The antigenic features and amino acid composition indicated certain similarities between the crystal-forming protein in *B. subtilis* Cgr4 cells and the spore coat protein.**

As shown by electron microscopy of numerous ultrathin sections of *Bacillus subtilis* cells, crystal-containing cells appear but rarely (3, 8, 9, 13). The frequency of such cells in cultures of different strains under different growth conditions varies from 0.1 to 3.0%. The crystals are formed by globular protein subunits. The crystal-forming protein was assumed to be a precursor or a mature form of a protein which forms the spore coat structure (9). Such an assumption was based on analogy with the supposed nature of the *Bacillus thuringiensis* crystal.

Crystal protein inclusion was detected in sections of *B. subtilis* Cgr4 mutant cells, which are conditionally resistant to the presence of glucose in the medium and differ from the wild type in the course of the stationary phase of growth (12). In cultures of mutant cells at the time of the appearance of thermoresistant spores, the crystal proteins were produced by 1% of the cells.

In the present paper we describe the conditions under which the frequency of the crystal formation in *B. subtilis* Cgr4 cells was increased, fractionation and electron microscopic study of the cells, analysis of the protein composition of the cells, and the extraction and biochemical and immunological characterization of the crystal protein.

## MATERIALS AND METHODS

**Strain, media, and growth conditions.** We used *B. subtilis* Cgr4, a mutant obtained in our laboratory after chemical mutagen treatment of *B. subtilis* WB-746 cells as previously described (12). *B. subtilis* Cgr4 is a mutant conditionally resistant to high concentrations of glucose in the medium. Despite the presence of almost half of the added glucose in the medium at the end of logarithmic phase, comparatively high levels of both aconitase activity in the cells and serine protease activity in the medium have been observed during the growth of the mutant in enriched medium. Secondary induction of serine protease is another feature of the mutant developed during growth in enriched medium. It coincides with the induction of spore morphogenesis and occurs at about hour 10 of incubation. The media used were previously described (5): medium I, mineral sporulation medium; medium II, mineral sporulation medium enriched by glucose up to 2% and by enzymatic casein hydrolysate (E. Merck, AG, Darmstadt, Federal Republic of Germany) up to 1%; medium III, tryptone agar (Ferack, Berlin, Federal Republic of Germany).

The culture of *B. subtilis* Cgr4 was grown and synchronized by Halvorson's method of active culture (6). The culture, after growth on medium III for 17 h, was washed with 2 ml of medium I and inoculated in 50 ml of medium I until an optical density at 670 nm of 0.25 was attained. The culture was grown at 37°C in a shaker until its optical density reached 1.0; then it was centrifuged, and the cells were suspended in 125 ml of medium II and grown at 30°C for 17 to 19 h.

**Microscopy.** Living bacteria were submitted to phase-contrast microscopy. The fixed preparations of bacterial cells were stained by the method of Smirnoff (14) with amidoschwartz 10 B (Reanal, Budapest, Hungary) and carbol-fuchsine. For electron microscopy, cells were prefixed with glutaraldehyde, followed by osmium fixation by the method of Kellenberger et al. (10). The sections were prepared with a Reich ultramicrotome and then stained with lead citrate and examined on a JEM 100 B electron microscope.

**Fractionation of cells and protein extraction.** Cells were fractionated on a Percoll gradient (Pharmacia, Uppsala, Sweden). After being grown in medium II for 17 to 19 h, *B. subtilis* Cgr4 culture was centrifuged at 5,000 × *g* for 15 min, and the cells were suspended in 0.14 M NaCl solution containing $10^{-2}$ M phenylmethylsulfonyl fluoride (Serva, Heidelberg, Federal Republic of Germany) and $10^{-2}$ M EDTA (Serva). A 1-ml sample of the cell suspension (0.167 g [wet weight] of bacteria) was layered on 29 ml of 98% isotonic Percoll and centrifuged at 30,000 × *g* for 1.5 h. The cells of separate fractions were collected and then washed from the Percoll two or three times by centrifugation with 0.14 M NaCl. Then the cells were suspended in 0.14 M NaCl

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containing $10^{-2}$ M phenylmethylsulfonyl fluoride and $10^{-2}$ M EDTA and sonicated three times for 3 min at 44 kH, using sonicator UZDN-2T (Minsviaz, Moscow, USSR).

After sonication, the suspension was centrifuged. The sediment was extracted with the following. (i) 0.05 M NaCl (pH 12.3) for 30 min at 20°C. (ii) 0.05 M Tris buffer (pH 8.5) containing 1% mercaptoethanol (Loba-Chemie, Vienna, Austria) and 8.0 M urea (Fluka, Buchs, Federal Republic of Germany). The extraction mixture was heated for 5 min in water bath at 100°C. (iii) 8 M urea for 30 min at 20°C. After extraction, the suspension was centrifuged to remove cells and debris from the cell membrane. For further study, a supernatant was used.

The proteins extracted from the sonicated cells in the logarithmic growth phase were used as control samples. Electrophoresis was performed by the Weber and Osborn procedure (16) with 5 and 10% polyacrylamide gels (acrylamide *N*,*N*$^1$-methylene-bisacrylamide, *N*,*N*,*N*′,*N*′-tetramethylenediamine, ammonium persulfate; Bio-Rad Laboratories, Richmond, Calif.) which contained 0.1% sodium dodecyl sulfate (SDS; Bio-Rad) and 8 M urea. The gels were stained with 0.25% Coomassie R-250 (Bio-Rad) in 7% acetic acid and then were washed at 100°C with 7% acetic acid solution.

**Immunological procedures.** For immunological identification of the crystal protein, rabbits were immunized with the proteins extracted from cells in the logarithmic growth phase or with the spore coat proteins. The spores were prepared by the following procedure. After being grown in mineral sporulation medium for 40 h, *B. subtilis* Cgr4 culture was centrifuged at 4,000 × *g* for 1.5 h. The sediment was suspended in 100 ml of 0.03 M Tris buffer (pH 8.0) containing 100 mg of lysozyme per ml and incubated for 30 min at 42°C. Then the suspension was centrifuged, and the sediment was washed in succession with 1 M NaCl, double-distilled water, 1 mM NaOH, double-distilled water, 0.1% SDS, and three times with double-distilled water. The washed spores were disrupted by stirring them with glass beads, 0.2 mm in diameter, for 7 min. The suspension of disrupted spores was decanted, and 0.03 M Tris buffer (pH 8.0) was used to wash the glass beads three times. The suspension and the washing buffer were collected together and centrifuged at 12,000 × *g* for 15 min to concentrate the spore coats. The sediment was washed by the method of Pandey and Aronson (11). Protein was extracted from the spore coats by the method of Horn et al. (7). The immune gamma globulins were purified on DEAE-Sephadex A-50 (Pharmacia) by the method of Exum and Bowser (4).

Double immunodiffusion reactions were done in 1% agarose gel (Chemapol, Prague, Czechoslovakia) containing 0.9% NaCl and 0.01% merthiolate (Loba-Chemie). The gels were incubated at 20°C in a damp chamber. Precipitin bands appeared after 10 to 15 h.

**Amino acid composition analysis.** The proteins extracted from polyacrylamide gel by an electrode buffer containing 1% SDS were dialyzed against distilled water and then lyophilized. The protein sample was hydrolyzed with 5.7 N HCl for 24 h at 105°C, and then the amino acid content was assayed with a Biotronic C 5001 analyzer.

## RESULTS

*B. subtilis* Cgr4 cells were synchronized at 37°C, grown for 17 to 19 h at 30°C, and fractionated by centrifugation on a Percoll gradient. The cells, which had formed the crystal protein, were concentrated in the fraction corresponding to a Percoll density of 1.14 g/ml. Optical microscopy of stained preparations and electron microscopy of ultrathin sections of the cells showed that the majority of the cells contained in this fraction had crystal protein. Inclusion formation was easily noticeable: two-thirds of the cell volume or even more was occupied by a regular crystal-like structure, consisting of globular subunits of 7.0 to 8.0 nm (Fig. 1). The cells containing crystal protein did not form spores: crystal protein formation and sporulation appear to be alternative states of the cell.

The protein composition of *B. subtilis* Cgr4 cells was determined by polyacrylamide gel electrophoresis in 0.1% SDS and 8 M urea. Electrophoretic protein patterns of the cells harvested at the logarithmic and stationary phases were compared. Numerous protein bands were characteristic of these electropherograms (Fig. 2, lanes b and c). On the contrary, electropherograms of the cells containing protein crystal-like inclusions showed practically only one clear-cut protein band (Fig. 2, lane d). The latter was attributed to the crystal protein. The molecular size of the crystal protein was estimated by SDS-polyacrylamide gel electrophoresis to be 47 kilodaltons (kDa). Comparison of electropherograms and densitograms indicated that the crystal protein was absent in cells in the logarithmic phase (Fig. 3), whereas it was present as a weak band on the electropherograms of unfractionated stationary-phase cells.

The tendency of the protein to aggregate prevented its dissolution in water. A complete dissolution could be achieved only at convenient pH values in alkaline medium (pH 12 or even higher). Joint action of substances that reduce disulfide bridges (2-mercaptoethanol, dithioerythritol) and urea led to complete crystal dissolution also, although these reagents when used separately caused only partial dissolution. Solubilization was performed in the presence of $10^{-2}$ M EDTA and $10^{-2}$ M phenylmethylsulfonyl fluoride to avoid proteolysis. NaOH (0.05 M) seemed to be an optimal solvent since a single protein component was found in this solution when it was studied by SDS-urea-polyacrylamide gel electrophoresis.

The amino acid content of the crystal protein was determined (Table 1). The amino acid composition of the *B. subtilis* Cgr4 crystal protein indicated that the major amino acids were glutamic acid (13%), aspartic acid (11.2%), and glycine (10.3%); the amount of tyrosine obtained turned out to be very small (0.22%). Comparison of the amount of hydrophilic and hydrophobic amino acids in the crystal protein indicated that the former were the major amino acids.

The antigenic properties of the crystal protein were investigated by double immunodiffusion. The crystal protein of *B. subtilis* Cgr4 did not form precipitation bands with antisera to either the crystal protein of *B. thuringiensis* or the protein from *B. subtilis* Cgr4 logarithmic-phase cells. On the contrary, the precipitation bands appeared with antiserum to *B. subtilis* Cgr4 spore coat proteins, indicating the antigenic relationship between these two proteins.

## DISCUSSION

Crystal protein inclusions are rather widespread among bacilli. *B. thuringiensis* crystals have been thoroughly studied as they are endowed with insecticidal activity. It was found that crystal protein formation coincided with sporogenesis (2, 15) and that the protein existed in the cell along with a spore. A connection between crystal protein formation and the presence of the plasmids in the cells was

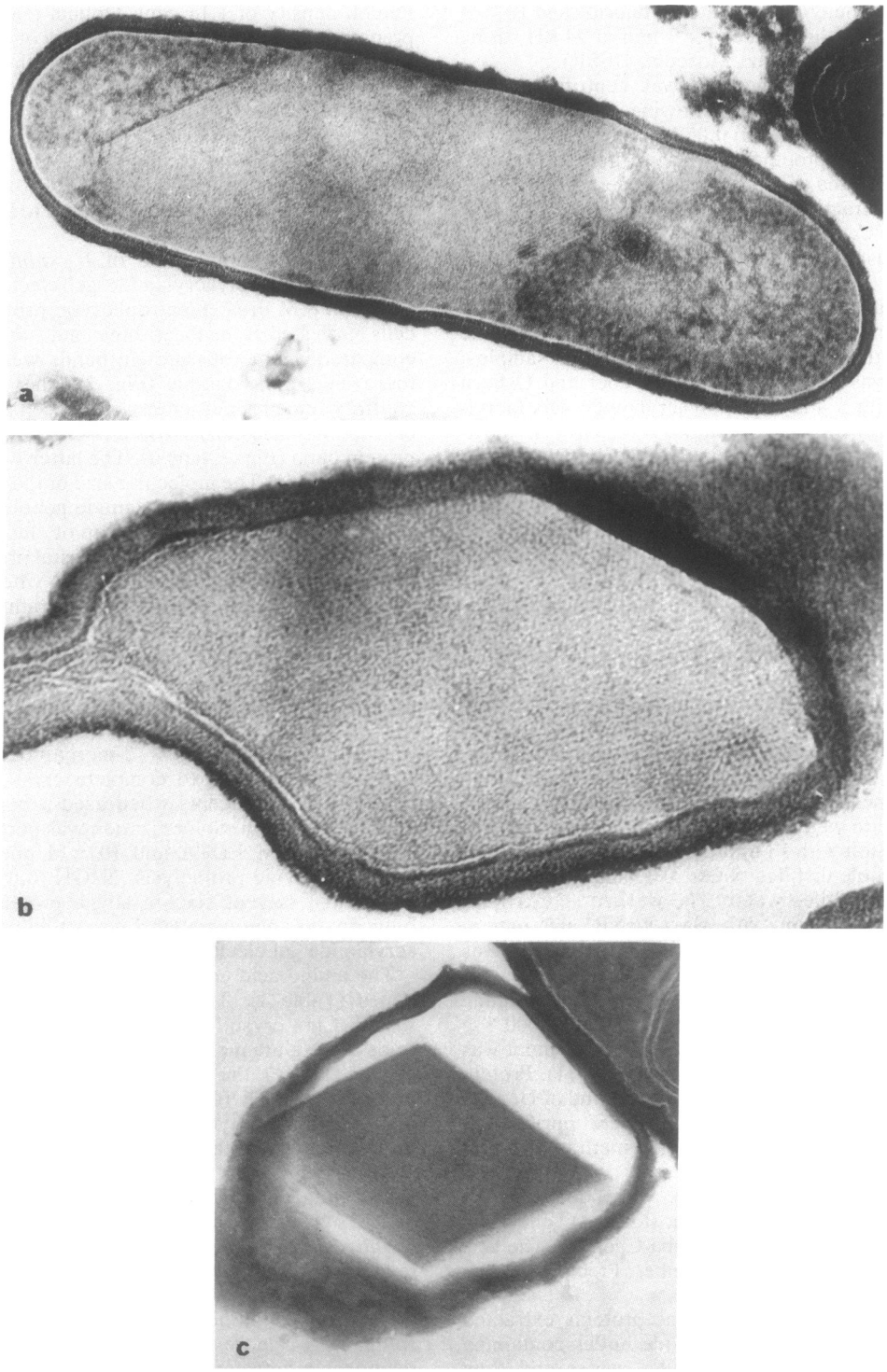


FIG. 1. Electron micrographs of crystal protein in *B. subtilis* Cgr4 cells. The crystal after pushing out the cytoplasm. It occupies the whole inner volume of the bacterium, thus changing the cell shape. (a and c) Magnification, ×135,000. (b) Magnification, ×200,000.

established. Crystal protein synthesis was shown to be under the control of genes situated in plasmids.

It appears that the synthesis of the crystal protein within *B. subtilis* cells proceeds in a different way. Crystal synthesis is related to one of the two alternative states of a cell: either protein crystal is synthesized or the cell develops a spore. Cells forming the crystal appear to be self-killers; they neither produce the spores nor multiply and form colonies. In the samples taken from Percoll gradient fractions, in which crystal-containing cells prevailed, practically no sporeforming cells were found.

Crystal protein appearance and spore formation in *B. subtilis* Cgr4 culture coincide in time. It may be assumed that the crystal protein is a precursor of one of the mature spore

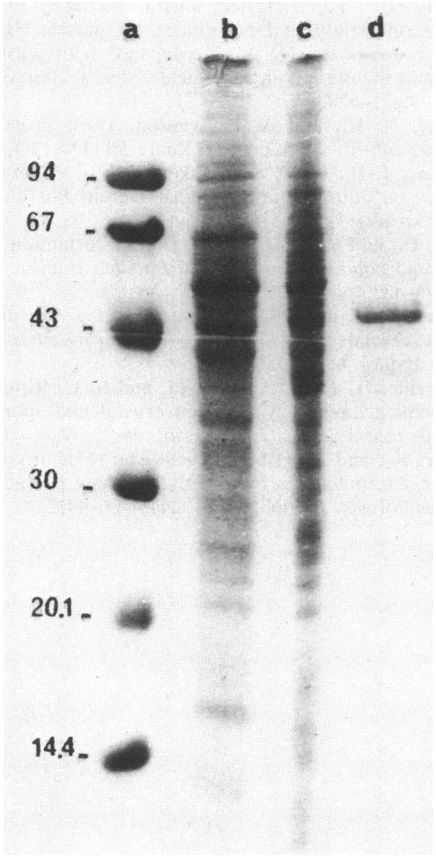


FIG. 2. Protein electropherograms of *B. subtilis* Cgr4 (10% polyacrylamide gel containing 0.1% SDS and 8 M urea). Lanes: a, molecular weight markers ($\times 10^3$); b, proteins of cells in the logarithmic phase of growth; c, proteins of cells in the stationary phase of growth; d, proteins of the fraction of cells containing the crystal.

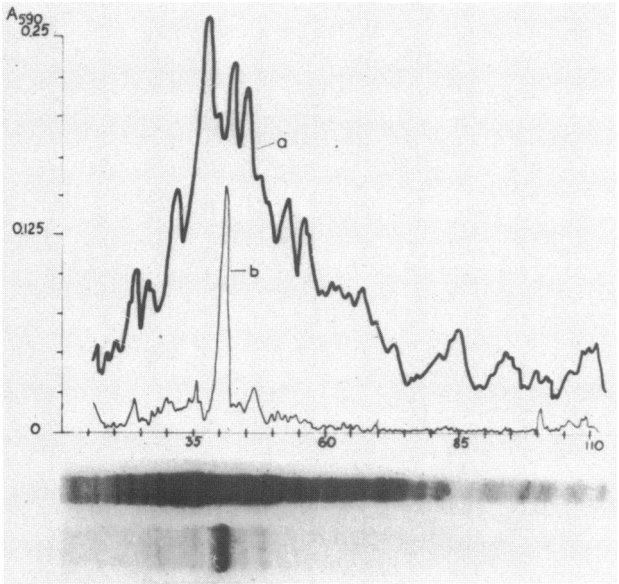


FIG. 3. Density analysis of protein bands in SDS-urea-polyacrylamide gel after electrophoresis of logarithmic-phase cell proteins (upper line, curve a) and of stationary-phase crystal-containing cell proteins (lower line, curve b).

TABLE 1. Amino acid composition of *B. subtilis* Cgr4 crystal protein and of *B. subtilis* spore coat soluble protein

| Amino acid | Crystal protein of *B. subtilis* Cgr4 | | *B. subtilis* spore coat soluble proteins[a] (mol/100 mol) |
|---|---|---|---|
| | Residues[b] | mol/100 mol | |
| Lysine | 29.8 | 6.4 | 9.2 |
| Histidine | 16.3 | 3.65 | 3.4 |
| Arginine | 19.6 | 4.6 | 4.9 |
| Aspartic acid[c] | 50.3 | 11.2 | 12.2 |
| Threonine | 22.8 | 5.1 | 5.0 |
| Serine | 30.3 | 6.8 | 7.3 |
| Glutamic acid[c] | 61.7 | 13.8 | 6.8 |
| Proline | 23.9 | 5.4 | 3.6 |
| Half-cystine | 3.0 | 0.7 | |
| Glycine | 46.2 | 10.3 | 14.3 |
| Alanine | 36.6 | 8.2 | 8.7 |
| Valine | 27.1 | 6.05 | 5.2 |
| Methionine | 7.5 | 1.67 | 1.7 |
| Isoleucine | 20.2 | 4.51 | 4.3 |
| Leucine | 34.2 | 7.64 | 5.6 |
| Tyrosine | 1.0 | 0.22 | 8.4 |
| Phenylalanine | 18.3 | 4.08 | 3.8 |
| Total | 448 | 100 | 100 |

[a] According to Pandey and Aronson (11).
[b] Amino acid residues were calculated from that of tyrosine.
[c] Aspartic and glutamic acid values include asparagine and glutamine, respectively.

coat proteins. This assumption is confirmed by the positive precipitation reaction of the protein with antiserum to spore coat protein and by the similarity of its amino acid composition to that of the spore coat protein (with the obvious exception of tyrosine and glutamic acid content). According to published data (1), there are two fractions of soluble proteins in the *B. subtilis* spore coat: a major fraction (50%) with a molecular size of 8 to 12 kDa and a minor fraction (20 to 30%) with a molecular size of 40 kDa. These proteins are formed from a 60-kDa precursor protein. We found a protein with a molecular size of 47 kDa forming crystal-like inclusions which could be a precursor of spore coat proteins or a partially processed intermediate. In some cells, misregulation of sporogenesis occurred, followed by a highly increased synthesis of spore coat protein precursor. This led to a crystal-like structure formation. Crystal production occurs in sporeforming cultures of *B. subtilis* 168 as well. However, this is a more frequent event in cultures of *B. subtilis* Cgr4, especially when the cells are grown under catabolite repression conditions; the course of the stationary phase is altered as well as the regulation of the cell cycle. The temperature decrease from 37°C at which synchronization was performed to 30°C at which incubation proceeded in the enriched medium produced a shocklike effect on the culture. Presumably, it caused the alteration in the regulation of stationary-phase processes accompanied by slower spore morphogenesis and frequent appearance of the crystal protein.

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