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Role of Glutathione in the Morphogenesis of the Bacterial Spore Coat

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There is a marked increase in the half-cystine content of bacterial spores, especially the coat layers at the time of formation of the outer coat. When a cysteine auxotroph of *Bacillus cereus* T is grown on limiting cysteine, the spores contain the normal content of half-cystine, suggesting an alternate source. Glutathione appears to be such a supply of cysteine since it is hydrolyzed during sporulation and there are increased activities of the hydrolyzing enzymes at the same time. In addition, a cysteine auxotroph with a second alteration, a temperature-sensitive glutathione disulfide reductase, produces lysozyme-sensitive spores at 40 C. These spores appear to be defective in the formation of outer spore coat. During sporulation at 40 C, the double mutant accumulates oxidized glutathione which is a poor substrate for the hydrolytic enzymes. As a result, sporulating cells are deficient in half-cystines which are essential for outer spore coat morphogenesis. This alteration can be overcome by a shift to 30 C or by addition of cystinyl-pencillamine or cysteinyl-glycine to cultures sporulating at 40 C.

The spore coat of *Bacillus cereus* T, exclusive of the exosporium, consists of two readily distinguishable morphological layers (2, 10, 28). The outer layer which is visible in thin sections contains a relatively high content of cystine and in fact is first apparent in sections at the time of increased cystine content of the spore (2, 32). This layer can be solubilized by treatment of spores or isolated coats with a combination of a reagent which disrupts hydrophobic bonds such as sodium dodecyl sulfate (SDS), guanidine, or urea, plus a reagent which reduces disulfide bonds such as mercaptoethanol or dithioerythritol (DTE) (4). The inner or soft under coat can be seen in thin sections (10, 28) but is most readily observed in freeze-etched preparations (11) and can be selectively solubilized by treatment of spores or coats with DTE or mercaptoethanol at alkaline *p*H (5).

In *B. cereus* T, the total coat appears to be made up of only these two fractions, whereas in some species there may be other components (15, 30). Both of the *B. cereus* T fractions contain almost exclusively protein, and there is evidence that the major protein species in both layers are not only identical but quite possibly there is only a single species of polypeptide as the major structural component of spore coats (5). The morphological differences would then be accounted for by the higher cystine content of the outer layer, implying the unique presence of a cystine-rich polypeptide in this layer. Alternatively, there may be an increased disulfide interchange with preexisting coat polypeptides, resulting in an altered conformation of the polypeptides present in the outer coat. We have presented some evidence in support of the latter mechanism (2, 3, 12) and will further supplement it in the present paper.

Aside from the function of the cystine, the increased incorporation at a specific stage in sporulation implies a mechanism for generating these half-cystine residues. In preliminary experiments (4), we found that sporulating cells could hydrolyze reduced glutathione (GSH) and the half-cystine released was incorporated into the spores. In the present paper, we provide further evidence for the hydrolysis of GSH by measuring changes in the concentration of GSH and GSH-hydrolyzing activities during the course of growth and sporulation. In addition, we have isolated and characterized a temperature-sensitive mutant altered in its capacity to utilize GSH as a source of half-cystine residues. One of the

phenotypic properties of this mutant is the production of spores with defective outer coats. Under certain conditions, GSH hydrolysis appears to be a critical step in spore coat morphogenesis.

MATERIALS AND METHODS

Cell growth. *B. cereus* T wild and various mutants were grown in G medium buffered with 0.03 M tris(hydroxymethyl)aminoethane (Tris), *p*H 7.4, (1) or a synthetic medium (22) in Erlenmeyer flasks (10% vol/vol) at 30 or 40 C in a New Brunswick reciprocal incubator shaker for volumes greater than 100 ml or in a water bath shaker for smaller volumes. Growth was followed in a Coleman 8 colorimeter employing a 655-nm filter. Various stages of sporulation were determined qualitatively by phase-microscopy examination. The most readily distinguishable stages are appearance of a phase-dark region at one end of the cell and the subsequent conversion to a phase-white structure about 1 h later. Biochemical markers such as appearance of extracellular protease shortly after the end of exponential growth (16), the increased rate of incorporation of L-[^{14}C]cystine at the time of phase whitening (2), and the synthesis of dipicolinic acid very late were also used.

Viable counts were done in triplicate on G-Tris agar plates or on a synthetic medium (22).

Isolation of mutants and revertants. A cysteine auxotroph was isolated after treatment with *N*-nitro-*N'*-nitroso-guanidine as previously described (16) and replica plating on synthetic medium supplemented with serine (5 μg/ml) and Na_2S (5 μg/ml) with or without cysteine (3 μg/ml). The serine and Na_2S were added to ensure isolation of a mutant blocked late in the biosynthetic pathway. This auxotroph could utilize cysteinyl-glycine but not GSH as a source of cysteine. As a result, it was possible to isolate by replica plating a spontaneous mutant (designated 10^{cs}) capable of utilizing cysteinyl-glycine at 30 C but not at 40 C. The mutant could grow with cysteine at either temperature, suggesting the alteration specifically affected the utilization of cysteinyl-glycine.

Two kinds of revertants of this double mutant were selected by plating at 40 C on the synthetic medium supplemented with cysteinyl-glycine. Colonies which appeared within 24 h were cysteine prototrophs. Colonies appearing after 2 to 3 days were still cysteine auxotrophs but had regained the ability to utilize cysteinyl-glycine at 40 C.

Enzyme assays. γ-Glutamyl transpeptidase (GGTP) was assayed by the procedure of Orlowski and Meister (24, 25) employing 1-γ-glutamyl-*p*-nitroanilide. The product, *p*-nitroaniline was measured by the procedure of Goldberg and Rutenberg (9). Crude extracts were prepared by sonic treatment of washed cells (Branson no. 4 for 90 s) suspended in 0.05 M Tris-hydrochloride 0.02 M GSH. *p*H 8.0, and centrifuged at 12,000 × *g* for 10 min in an SS34 rotor in a Sorvall RC2B centrifuge. Only freshly prepared extracts were assayed since the activity was very unstable. One unit is defined as the production of 1 μmol of *p*-nitroaniline per min.

Cysteinyl-glycine dipeptidase (CGase, EC 3.4.3.5) was assayed by the procedure of McCorquodale (18) measuring residual cysteinyl-glycine as described by Binkley and Nakamura (6). One unit is defined as the hydrolysis of 0.65 μmol of cysteinyl-glycine per min. For this assay, cells suspended in 0.01 M Tris-hydrochloride, *p*H 7.4, were sonically treated as described above. Ten milligrams of streptomycin was added to each milliliter of crude extract, and the suspension was incubated at 0 C for 40 min before centrifugation at 12,000 × *g* for 20 min. The supernatant liquid was dialyzed against at least 500 vol of 0.01 M Tris-hydrochloride, *p*H 7.4, for 16 h at 4 C.

The assay for glutathione disulfide reductase (GSSG disulfide reductase, EC 1.6.4.2) followed that used for the yeast enzyme (18). Crude extracts were treated with streptomycin (10 mg/ml) and the supernatant fluid was fractionated with $(NH_4)_2SO_4$. The 50 to 80% (wt/vol) precipitate was dialyzed against 0.01 M Tris-hydrochloride, *p*H 7.4, for 8 h at 0 C and assayed. Because of a high nonspecific reduced nicotinamide adenine dinucleotide phosphate (NADPH) oxidase activity in crude extracts of *B. cereus* T (31), the assay was run in Thunberg cuvettes which had been flushed with N_2 and evacuated two to three times. This treatment reduced the initial rate of nonspecific oxidation to less than 30% of the activities measured.

The sensitivity of spores to lysozyme (EC 3.2.1.17) was determined as previously described (3).

Chemical procedures. Protein was determined by the procedure of Lowry et al. (17) employing bovine serum albumin as a standard. Dipicolinic acid was measured by the procedure of Jansen, Lund, and Anderson (14).

One antigenic form of the spore coat, the outer coat, was assayed by employing ^{125}I-labeled antibody which had been purified by adsorption to and elution from intact spores. The details of this quantitative assay and the specificity for outer coat are described elsewhere (12; D. Horn, Ph.D. thesis, Purdue University, 1971).

The GSH or cysteine-cystine content of cells was determined by washing 20-ml samples twice in 8 to 10 ml of 0.85% (wt/vol) NaCl, suspending the cells in 2 ml of 0.85% NaCl and placing the tubes in a bath of boiling water for 15 min. The supernatant fraction (10,000 × *g* for 15 min) was used for a total GSH assay employing yeast GSSG disulfide reductase (33) and for the determination of total ninhydrin-reactive material (20). [^{14}C]GSH was quantitated by chromatographing 5% (wt/vol) trichloroacetic acid extracts ascending in sec-butyl alcohol:HCOOH:H_2O (175:50:25). Cystine-cysteine was measured by the procedure of Schneider, Bradley, and Seegmiller (29).

The relative content of oxidized glutathione (GSSG) was determined by suspending cells from 400 ml of G-Tris medium in 2 ml of cold 5% (wt/vol) trichloroacetic acid, incubating at 0 C for 30 min, and centrifuging at 15,000 × *g* for 20 min. Extraction with trichloroacetic acid may result in some oxida-

tion of the GSH but not greater than 15% of the initial amount. To 1.4 ml of the supernatant fluid was added 0.1 ml of a solution of 0.1 M potassium phosphate (*p*H 7.95)-0.002 M ethylenediaminetetraacetic acid (EDTA)-0.0025 M *N*-ethyl maleimide (NEM), and the mixture was incubated at 27 C for 15 min. Excess trichloroacetic acid and NEM were removed by shaking with ether (2-3 extractions of 4 ml each). To 1 ml of the aqueous phase was added 0.55 ml of 0.1 M sodium phosphate-0.002 M EDTA, *p*H 7.95, Ten milliliters (0.36 units) of yeast GSSG disulfide reductase and 0.1 ml of 0.002 M NADPH were added, and the change in A_{340} was followed for 5 min in a Zeiss spectrophotometer. The initial rates were compared to those obtained with known quantities of GSSG.

Two fractions of spore coat protein or the total coat protein were solubilized as previously described (3). After precipitation and removal of SDS by addition of saturated KCl, the protein was precipitated with 15% (wt/vol) trichloroacetic acid and collected by centrifugation at 12,000 × *g* for 10 min after incubation at 0 C for 30 min. The trichloroacetic acid-soluble fraction was extracted several times with ether and concentrated under vacuum, and a portion was oxidized with excess performic acid (13). A sample of each was chromatographed on the long column of a Beckmann amino acid analyzer to determine cysteic acid content.

For amino acid analyses, the protein precipitate was washed with ether, oxidized in performic acid, dried in vacuo, dissolved in constantly boiling HCl, and hydrolyzed at 110 C for 20 hr. After removal of HCl, the total ninhydrin-reactive material was determined (21) with L-leucine as a standard.

Chemicals. Cysteinyl-glycine was prepared by hydrolysis of GSH as described by Olsen and Binkley (23). Cysteinyl-penicillamine was prepared by the procedure of Crawhill, Scowers, and Watts (7). L-γ-Glutamyl-*p*-nitroanilide was a gift of M. Orlowski. NADPH and 1-arterenol bitartrate were purchased from Sigma Chemical Co.; GSH, GSSG, and yeast GSSH disulfide reductase from California Biochemical Corp.; and L-[^{14}C]cystine (uniformly labeled) (19 μCi/μmol) from Schwarz-Mann.

RESULTS

Half-cystine residues released upon solubilization of the spore coat. The relatively high content of half-cystine residues in spores is primarily due to an enrichment in the spore coat fraction (32) and specifically in the outer coat (2). This enrichment is partly due to the relatively high content of half-cystine (3 mol%) in the spore coat protein (2) and to some half-cystine residues which are found in the trichloroacetic acid-soluble fraction upon solubilization of the coat (Table 1) probably due to disulfide interchanges between DTE and cystine residues. Most of the half-cystines in the trichloroacetic aicd-soluble fraction are found when outer coat is solubilized by treatment with DTE plus SDS in confirmation of previous data showing selective incorporation of ^{14}C-cystine into outer coat fractions late in sporulation (2). There is about one such half-cystine per three half-cystine residues in the outer coat monomer. Very little half-cystine is released when inner coat is solubilized (DTE extract) or when intact spores are treated with performic acid.

Source of cystine for spore coat formation. Both the synthesis of a large amount of spore coat structural protein relatively rich in half-cystines as well as the cystine or cysteine presumably required for the disulfide interchange reaction would necessitate a marked increase in the availability of this amino acid during sporulation. This increase could occur through derepression or activation of the cysteine biosynthetic pathway. In a cysteine auxotroph grown on limiting cysteine, however, spores were formed which contained normal amounts of half-cystine residues in the spore coat (Table 2). A change in the properties of the biosynthetic pathway is thus not essential.

An alternate source of half-cystine was suggested by some earlier work of Roberts et al. (27) where it was found that GSH was degraded during sulfur starvation of *Escherichia coli*. We had previously shown that whereas [^{35}S]GSH is taken into *B. cereus* cells at all stages, only during sporulation is the ^{35}S converted to trichloroacetic acid-insoluble material (4). The GSH content relative to the total extractable ninhydrin-reactive material decreases at the end of growth to a barely measureable amount at the time of appearance of phase-white endospores (Fig. 1). This relative decrease in GSH is qualitatively paralleled by an increase in the half-cystine or cysteinyl-glycine, or both (Fig. 1). The absolute amount of GSH per cell is difficult to determine because of uncertainties in the extraction procedure and losses during handling. For a given number of cells, however, the total extractable ninhydrin-reactive material did not vary by more than a factor of two, suggesting that the post exponential decrease of GSH reflects the absolute change per cell. In addition, the activities of two enzymes implicated in GSH hydrolysis, GGTP and CGAse, increase at 8 h (end of growth) when there is an apparent rapid decline in the GSH concentration (Fig. 2). The GGTP activity then falls to a minimum at 12 h, whereas the CGAse activity appears to increase four- to sixfold at this time. The latter increase may be an artifact because of an entrapment of protein in the forespore which is not released during cell lysis. If this protein is relatively

TABLE 1. *Half-cystine residues released upon solubilization of various B. cereus T spore coat fractions*

Fraction[a]	Total protein[b] (μmol)	Cysteic acid (μmol)	Half-cystine released per half-cystine in coat protein monomer[c]
DTE-SDS extract (total coat)	0.45	0.11	0.081
DTE extract (inner coat)	0.65	0.08	0.041
DTE-SDS extract after DTE extraction (outer coat)	0.15	0.13	0.29
Direct treatment of spores with performic acid		0.03	0.009[d]

[a] Fractions prepared and cysteic acid measured as described in Materials and Methods. Results are the averages of two separate experiments.

[b] Based on a molecular weight of 12,000 (5).

[c] Assuming three half-cystine residues per monomer (2).

[d] Spores (8×10^{10}) were treated with performic acid. Ratio based on assumption of 1.60×10^{-13} g of coat protein per spore (1).

TABLE 2. *Characterization of spores produced by a cysteine auxotroph of B. cereus T*

Cystine added[a] (μg/ml)	Spores × 10^8/ml		g of DPA ($\times 10^{-15}$) per spore[b]	Cysteic acid in spore coat[c] (mol %)
	Direct count[d]	Heat resistant colony formers[e]		
1	1.5	1.6	145	3.8
2	1.9	1.85	150	3.2
5	2.4	1.5	140	3.3
Wild type	4.0	3.5	148	3.4

[a] To synthetic medium (22). Cultures incubated at 30 C.

[b] Direct count in a Petroff-Hauser chamber. Dipicolinic acid (DPA) analysis as described in Materials and Methods.

[c] Spores were first collected by centrifugation at 12,000 × *g* for 10 min in a Sorvall RC II centrifuge, washed twice with distilled water, and then centrifuged through 38% Renografin (Squibb) at 18,000 × *g* for 20 min. The spore pellet was washed three times with distilled water and then suspended for extraction of the coat, hydrolysis and quantitation of the cysteic acid and total ninhydrin-reactive material (see Methods).

[d] Average of triplicate samples counted in a Petroff-Hauser chamber. Error ±15%.

[e] Average of triplicate samples plated on G-Tris agar after heating a suspension at 80 C for 20 min. Error ±12%.

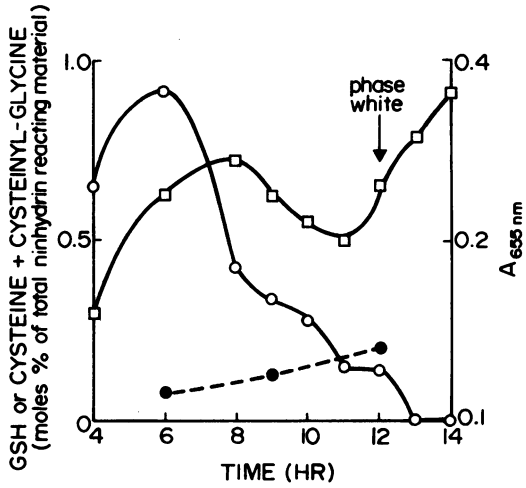

FIG. 1. *Relative changes in the concentration of "pool" GSH or cysteine + cysteinyl-glycine during growth and sporulation of B. cereus T in G-Tris medium. The cells were extracted, and GSH or cystine + cysteinyl-glycine was quantitated relative to the total ninhydrin reactive material as described in Materials and Methods. Growth terminated at 8 h and phase-white endospores (indicative of outer spore coat formation were first seen at 12 h. Symbols:* O, *GSH;* □, A_{655} *(growth curve);* ●, *cysteine + cysteinyl-glycine.*

deficient in CGase, then there would be an apparent increase in the specific activity of the enzyme.

The CGase activities appearing at 8 and 14 h are similar in terms of activation by manganese, heat inactivation, elution profile from Sephadex G200, and specificity for cysteinyl-glycine. While there are other peptidases present in crude extracts, this activity is unique in its specificity for cysteinyl-glycine (H. M. Cheng, Ph.D. thesis, Purdue University, 1972).

The undetectable level of GSH during phase whitening (Fig. 1) does not mean that GSH is not an important source of half-cystine residues at this time (Table 3). About 40% of the [^{14}C]cystine added to the medium is incorporated into GSH at this stage. Since the fraction of radioactivity found in GSH does not increase over a 30-min interval, a continuous synthesis and degradation of GSH is implied.

Isolation of a mutant producing defective spore coats. The above correlations among the time of decrease of GSH, increase of GSH hydrolytic activities, and the formation of spore coat protein are suggestive of a functional relationship but are not conclusive. To confirm the correlation, a mutant with a presumptive alteration in its capacity to hydrolyze GSH was

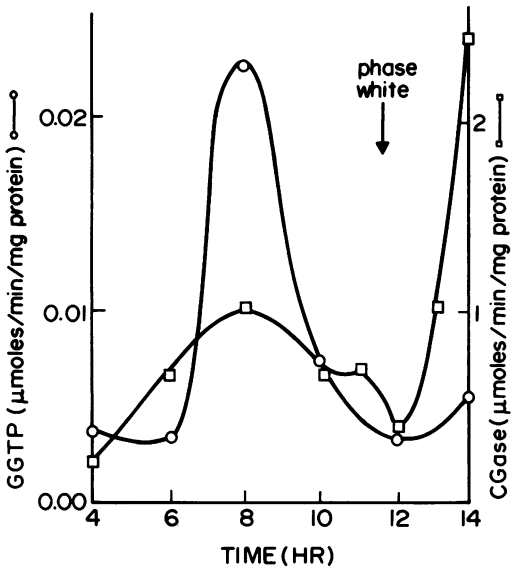

FIG. 2. *Changes in the activities of GSH hydrolytic enzymes during growth and sporulation of B. cereus T grown in G-Tris medium. A 400-ml culture was incubated at 30 C in a 2-liter flask, and 30-ml samples were removed at the indicated times for each assay. The cells were washed with the appropriate buffer, broken by sonic treatment, and in the case of CGase assay, further processed and assayed as described in Materials and Methods. Growth ended at 8 h; phase-white endospores were first seen at 12 h.*

TABLE 3. *Relative distribution of* L-[^{14}C]*cystine in "pool" components of sporulating B. cereus T*[a]

Time of incubation (min)	Total recovered counts/min[b] (%)		
	Cystine	GSH	Other
10	25	40	30
20	27	43	25
30	25	38	32

[a] L-[^{14}C]cystine (uniformly labeled) (0.01 μCi/ml) plus 3 μg of cystine per ml added to 100 ml of a 14-h culture (20% of the cells contained phase-white endospores). Samples (20 ml) were removed at the indicated times, centrifuged at 12,000 × *g* for 10 min, and washed three times at 27 C with G-Tris medium containing 10 μg of cystine per ml. The cells were extracted by suspension in 2 ml of cold 7% (wt/vol) trichloroacetic acid, incubated for 20 min at 0 C, and centrifuged as described above. The trichloroacetic acid extracts were then extracted several times with ether and finally concentrated in vacuo. Cystine and GSH were added as internal markers, and the components were separated by chromatography as described in Materials and Methods. After development with ninhydrin, the total paper was cut into 2-cm squares, placed directly into scintillation vials, and counted.

[b] In all cases 92 to 97% of the added radioactivity was recovered.

isolated as described in Materials and Methods. The mutant grew at 40 C in G-Tris medium at the same rate as the wild type but with a twofold lower cell yield. Of particular interest are the properties of the spores formed by the mutant at 40 C. These spores are sensitive to lysozyme (Fig. 3), a property which can be correlated with the absence (3) or possibly in this case a defect in the spore coat since lysozyme must penetrate the spore coat layers to digest the mucopeptide in the cortex. It is possible, however, that other alterations of the spores could result in lysozyme sensitivity.

Additional evidence that the primary defect is in the outer coat comes from temperature shift experiments (Fig. 4). Cells grown at 30 C in G-Tris medium were shifted to 40 C at the times indicated and allowed to complete sporulation at 40 C. The resulting spores were then assayed for lysozyme sensitivity as a measure of the expression of the mutant phenotype. As can be seen in Fig. 4, lysozyme-resistant spores are produced only when the culture is shifted after 14 h, i.e., the time of initiation of the formation of phase-white endospores and the incorporation of half-cystine into the outer coat. If the culture is shifted to 40 C prior to 14 h and supplemented with an alternate source of half-cystine such as cystinyl-penicillamine (a fairly soluble mixed disulfide) or cysteinyl-glycine, some lysozyme resistant spores are formed.

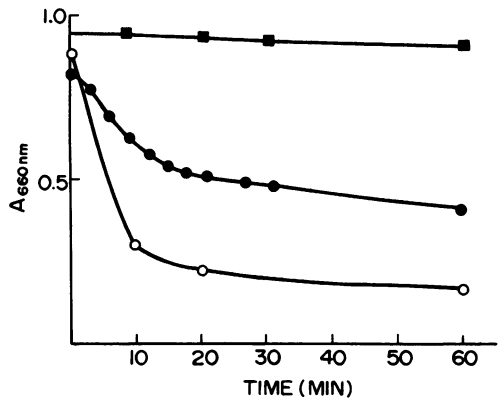

FIG. 3. *Lysozyme sensitivity of mutant 10^{ts} spores produced at 30 C or 40 C in G-Tris medium. Spores were washed several times with distilled water, and suspended in 0.05 M Tris-hydrochloride, pH 7.8, for the assay (see Materials and Methods). Some spores were also incubated in 0.05 M DTE, pH 10.3, at 37 C for 3 h to solubilize inner coat. These spores were then washed, suspended in Tris-hydrochloride, and tested. Symbols:* ■, *spores produced at 30 C with or without DTE extraction;* ●, *spores produced at 40 C;* ○, *spores produced at 40 C, extracted with DTE.*

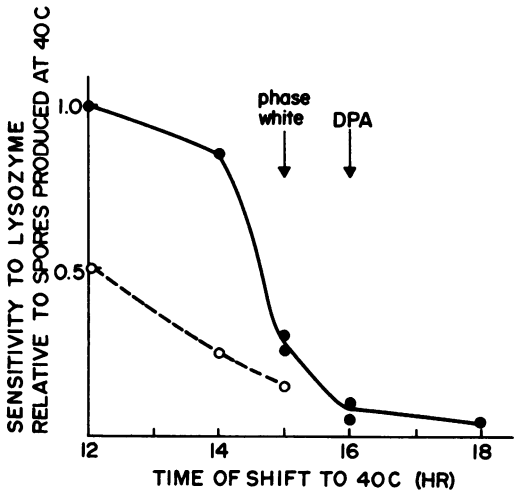

FIG. 4. *Lysozyme sensitivity of spores formed by mutant 10^{ts} after a temperature shift. A 200-ml culture in G-Tris medium was grown at 30 C. At the times indicated (●), 10-ml samples were removed to sterile 125-ml Erlenmeyer flasks and incubated at 40 C until free spores were formed (25–36 h). The spores were washed, suspended in buffer, and analyzed for sensitivity to lysozyme as described in the legend to Fig. 3 and in Materials and Methods. The initial rates of change of A_{665} are plotted relative to the rate for 10^{ts} spores formed at 40 C. Another 10-ml portion of the culture was placed in flasks containing either cystinyl-penicillamine or cysteinyl-glycine (30 μg/ml) at the times indicated and incubated at 40 C until free spores were formed. These spores were also tested for their lysozyme sensitivity relative to 10^{ts} spores formed at 40 C. Arrows refer to times when 50% of the cells contain phase-white endospores and to the initiation of DPA synthesis. Symbols: ●, sporulating cells shifted to 40 C; ○, sporulating cells supplemented with either cystinyl-penicillamine or cysteinyl-glycine.*

A defect in outer spore coat formation can also be shown by employing an immunological assay using ^{125}I-labeled anti-spore coat antibody (12). By adsorption to and elution from intact spores, a species of antibody was isolated which is much more reactive to outer than inner coat. As summarized in Table 4, very little antigenic material is found in extracts of mutant 10^{ts} grown at 40 C.

Characterization of the mutant. Initially, it was suspected that the mutant contained a temperature-sensitive CGase, but the specific activity of this enzyme was the same in mutant or parental strains grown at 30 or 40 C. When cystinyl-diglycine was used as a substrate, however, a twofold lower activity was found for extracts prepared from the mutant grown at 40 C. This is a minimal estimate of the difference, however, since there was 8% cysteinyl-glycine present in the substrate. Since the purified dipeptidase hydrolyzes the reduced form about 5 to 10 times more rapidly than the oxidized form (H. M. Cheng, Ph.D. thesis, Purdue University, 1972) it was suspected that the mutant was defective in an activity essential for reducing cystinyl-diglycine. Since there is no known cystinyl-diglycine disulfide reductase and GSSG disulfide reductase can reduce cystinyl-diglycine at about 3% the rate of reduction of GSSG, we suspected that the latter enzyme may be involved. We were able to detect GSSG disulfide reductase activity in extracts of the mutants (Cys^- or 10^{ts}) if anaerobic cuvettes were employed and found that 10^{ts} contained a heat-labile GSSG disulfide reductase (Fig. 5). The inability of this mutant to utilize cysteinyl-glycine as a source of cysteine at 40 C is probably due to the rapid oxidation of the dipeptide to cystinyl-diglycine (catalyzed by various ions in the medium). As already mentioned, the latter compound is a poor substrate for CGase.

Further evidence that the alteration is at the level of the state of oxidation/reduction of GSH

TABLE 4. *Quantitation of outer spore coat antigen in B. cereus 10^{ts} and parental strains*[a]

Strain	Temp (C)	μg of coat protein/100 μg of total extract protein
Cys^-	30	6.3
	40	5.9
10^{ts}	30	5.5
	40	0.9

[a] Cells grown in G-Tris were centrifuged at 12,000 × *g* for 10 min, washed twice with 0.05 M sodium phosphate, pH 7.0, and broken by sonic treatment as described in Materials and Methods. After centrifugation at 10,000 × *g* for 15 min to remove intact cells and large fragments, the protein content of the extract was determined (17). Coat antigen was quantitated by preparing duplicate sets of dilutions of the extract in 0.05 M sodium phosphate, pH 7.0, plus ^{125}I-antibody. After incubation and processing as described elsewhere (12), the ^{125}I in the precipitates and supernatant fractions were measured in a Baird spectrophotometer. Various amounts of total coat protein (prepared by solubilization from intact spores, 25% [wt/vol] $[NH_4]_2SO_4$ precipitation and dialysis against 0.05 M sodium phosphate, pH 7.0) were also assayed at the same time to provide a standard curve. The values reported are for extracts of endospores at the phase-whitening stage (at least 50% of the cells) and are averages of two separate experiments. The antigen was also quantitated from extracts prepared from cells both earlier and later in sporulation with comparable results.

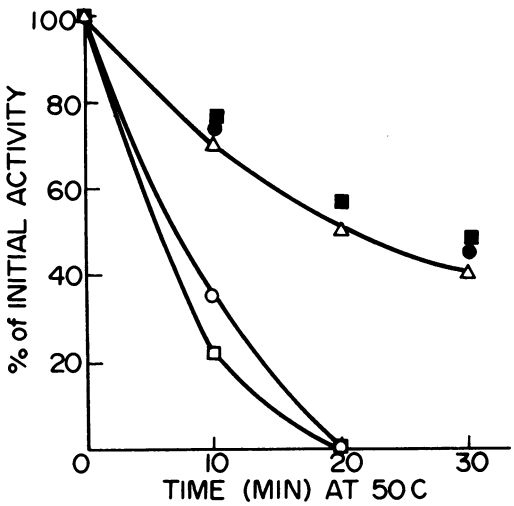

FIG. 5. *Heat sensitivity of GSSG disulfide reductase from various mutants and revertants of B. cereus T. Cells grown at 30 C in G-Tris medium were harvested at the end of exponential growth (9 h) and processed as described in Materials and Methods. Preparations from various mutants or revertants were then incubated at 50 C for the times indicated and assayed. Symbols: ●, B. cereus T wild; △, cysteine auxotroph (Cys⁻); ■, 10ᵗˢ revertant (Cys⁻); ○, 10ᵗˢ; □, 10ᵗˢ revertant (Cys⁺).*

is shown by a direct analysis of the relative amounts of GSSG and GSH in mutant cells grown at 40 C (Table 5). The fraction of GSSG increases markedly in post exponential cells of 10^{ts} which may account for the lower cell yield. Because of the relatively low amount of GSSG present in exponentially growing cells, there must be an alternate mechanism for sustaining GSH during this period.

Two phenotypic revertants of 10^{ts} were found when the mutant was spread on agar plates of synthetic medium containing cysteinyl-glycine and grown at 40 C. Rapidly appearing revertants were cysteine prototrophs which still contained a temperature-sensitive GSSG disulfide reductase (Fig. 5). Surprisingly, these revertants grow like the wild type at 40 C (i.e., same cell yield and produce lysozyme-resistant spores). Revertants appearing more slowly were still cysteine auxotrophs but contained GSSG disulfide reductase activity as heat resistant as the wild type. These revertants also grew like the wild type at 40 C and produced lysozyme-resistant spores. The properties of the revertants and the reversion frequencies are summarized in Table 6. The reversion frequencies suggest that each of the phenotypic properties resulted from initial point mutations.

DISCUSSION

There is evidence that sequential extraction of spores of *B. cereus* T with DTE and then with DTE plus SDS solubilizes first the inner and then the outer spore coat layers (5). Very little, if any, spore coat is visible in electron micrograph sections or freeze-etched preparations after these treatments, suggesting complete solubilization of the coat although other quantitative criteria must be developed. On the basis of gel electrophoresis, isoelectric focusing in gels, NH_2-terminal peptide analysis, and fractionation on agarose columns of these solubilized fractions, it appears that there is only a single major species of coat polypeptide of molecular weight about 12,000 (5; Aronson and FitzJames, *in press*). The possibility that 10 to 20% of the coat protein consists of many different polypeptides with unique components in each morphological layer has not been excluded.

Because of the extensive similarity in the composition of the two coat layers, however, we have assumed that the major morphogenetic change involves disulfide interchange reactions with a subsequent conformational modification of the polypeptides which then polymerize (primarily by hydrophobic interactions) to form the outer coat. It is not known whether the interchange involves cystine and protein-sulfhydryls or protein disulfides with cysteine or some combination.

The proposal of a disulfide interchange reaction is based on the selective incorporation of cystine into outer spore coat late in sporulation (2), the ability of sulfite to substitute for cystine in spore coat formation (2), and the detection of free half-cystine residues after coat solubilization (Table 1). In addition, there are some *in vitro* experiments showing a direct role

TABLE 5. *Relative GSSG content of B. cereus T strains grown at 40 C*[a]

Strain	Culture age (h)	Total glutathione as GSSG (%)
Cys⁻ (parent)	5	0.95
	12	0.00
10^{ts}	5	1.31
	12	28.60[b]

[a] Cells grown in G-Tris were centrifuged, washed, extracted, and assayed as described in Materials and Methods. Five hours is exponential growth; 12 h is 4 h after end of growth (just prior to formation of phase-white endospores).

[b] Extraction with trichloroacetic acid can cause oxidation of as much as 15% of the GSH (Cheng, *unpublished results*).

TABLE 6. *Summary of properties of mutant strains*[a]

Strain	Growth at 40 C on synthetic medium plus Cys-Gly	Spore yield at 40 C in G-Tris (per ml)[b]	Lysozyme-resistant spores at 40 C	GSSG disulfide reductase	Reversion frequency[c]
Wild	NA	7.2×10^8	+	+	
Cys^-	+	6.8×10^8	+	+	1.8×10^{-7}
10^{ts} (Cys^-; GSSG disulfide reductase^{ts})	−	3.2×10^8	−	TS	
Revertants of 10^{ts}					
Cys^+	NA	6.9×10^8	+	TS	2.3×10^{-7}
Cys^-	+	6.5×10^8	+	+	2.0×10^{-7}

[a] Abbreviations: NA, not applicable; TS, temperature sensitive (see Fig. 5).

[b] Direct spore counts in a Petroff-Hauser chamber. The rates of growth are the same in all cases (1.5 generation/h) but the final A_{655} values differ in the same relative way as the spore counts.

[c] Spores formed in G-Tris at 30 C were washed three times with synthetic medium (22) and 10^8 were spread on agar containing synthetic medium plus cysteinyl-glycine (10 μg/ml added as a sterile solution just before spreading spores). The plates were incubated at 40 C and examined periodically. Presumptive revertants were picked and restreaked on the same medium, and finally well-isolated colonies were selected for further studies.

of cystine in converting soluble coat polypeptides to a form which is either morphologically (3) or antigenically (12) like outer coat.

Regardless of the function of the half-cystine, it is evident that an increased supply is needed both for the synthesis of coat polypeptides and for the formation of the outer coat. A derepression of the cysteine biosynthetic pathway does not appear to be essential since a cysteine auxotroph forms spores with a normal content of half cystine in the coat (Table 2). In analogy with sulfur-starved *E. coli* (27), sporulating cells degrade GSH to satisfy the requirement for half-cystine. A second mutant, derived from the Cys^- auxotroph selected for its inability to utilize cysteinyl-glycine as a source of cysteine at 40 C, has a defective GSSG disulfide reductase. The reversion frequency of this second mutation and the simultaneous restoration of the heat stability of the GSSG disulfide reductase to that of the parental strain (as well as restoration of other phenotypic properties of the parent) are consistent with a mutation in the gene coding for this enzyme as being the only alteration. Thus, all of the phenotypic properties found at 40 C (inability to utilize cysteinyl-glycine as a source of cysteine, lower growth yield, and defective spores) are due to this one alteration.

The most direct explanation for the inability of the mutant to utilize cysteinyl-glycine at 40 C is an altered CGase, but the amount and heat stability of this enzyme are the same in the mutant and parental strains. Another quite plausible explanation is based on the oxidation of cysteinyl-glycine. There are a variety of trace metals in the synthetic medium which could catalyze this oxidation, and, in fact, the addition of cysteinyl-glycine to the liquid synthetic medium results in a very rapid and virtually complete conversion to cystinyl-diglycine (Cheng, *unpublished results*). At 40 C, the mutant (10^{ts}) would be unable to reduce cystinyl-diglycine, and thus an effective substrate for CGase would not be present.

The principal effect of this conditional mutant is the production of lysozyme-sensitive spores at 40 C. An immunological assay (Table 4) and the temperature shift experiments (Fig. 4) point to a defect in outer spore coat formation. In addition, gaps in the outer coat layers of mutant spores formed at 40 C can be seen in electron micrographs of thin sections (Holt and Aronson, *unpublished data*).

Because of the accumulation at 40 C of GSSG in sporulating cells of the mutant, there is not an adequate supply of half-cystine residues to complete the morphogenesis of the coat layers. The deficiency of half-cystine residues in the mutant could result in both a decrease in the amount of coat polypeptide synthesized and the inability to carry out disulfide interchange reactions. The synthesis of protein as measured by [^{14}C]leucine incorporation into trichloroacetic acid-precipitable material by sporulating cells is the same in the mutant and parental strains (H. M. Cheng, Ph.D. thesis, Purdue University, 1972), however, and the coat protein extracted from the spores is also identical to the Cys^- parental strain on the basis of gel electrophoresis at *p*H 9.5 and gel isoelectric focusing. It is more likely, therefore, that the most critical effect of the mutation is the inability to carry out disulfide inter-

changes, a conclusion supported by the capacity to overcome the mutation by adding alternate sources of cystine (Fig. 4). Since it is unlikely that the mutant can hydrolyze cystinyl-diglycine, the dipeptide is probably incorporated by disulfide interchanges (2).

If either potential source of cysteine is available, as in the revertants (Table 6), then lysozyme-resistant spores are formed. Either a functional cysteine biosynthetic pathway compensates for the lack of GSH hydrolysis or the cysteine provides an alternate mechanism for sustaining GSH. If the latter is true, then the GSSG disulfide reductase must be a dispensable activity, a result already suggested by the normal growth rate and low GSSG content of exponentially growing 10^{ts} at 40 C. Apparently in this medium, the cell has alternate mechanisms for maintaining GSH at least until one doubling less than the parental strain.

One of the key questions remaining is the mechanism(s) responsible for outer coat formation, i.e., those presumably promoting the disulfide interchange. GSH hydrolysis begins before outer coat is visible in thin sections and probably serves as a source of half-cystine both for coat protein synthesis and the interchange. Apparently the former requirement is satisfied in the mutant because of an adequate supply of GSH at the end of growth, and it is only the latter morphogenetic requirement which cannot be met.

The disulfide interchange could be triggered by the appearance of an enzyme which catalyzes disulfide interchange reactions (8) although no such activity was found in a preliminary survey. The amount of coat polypeptide present could also be important. If, as appears likely, inner coat forms first, then most of the protein would presumably be cross-linked by intermolecular disulfide bonds since DTE solubilizes this layer. The amount of this layer and thus the conformation of the polypeptides may then limit the extent of disulfide bond formation and provide an environment where the disulfide interchange with cystine or cysteine is favored. The contribution of all of these factors to outer spore coat formation must be examined further.

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