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Properties of *Bacillus cereus* Temperature-Sensitive Mutants Altered in Spore Coat Formation

GERALD N. STELMA, JR.,[1] ARTHUR I. ARONSON,[1]* AND PHILLIP FITZ-JAMES[2]

Department of Biological Sciences, Purdue University, West Lafayette, Indiana 47907,[1] and Departments of Bacteriology and Immunology and of Biochemistry, University of Western Ontario Medical School, London, Ontario N6A5C1, Canada[2]

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Three conditional *Bacillus cereus* mutants altered in the assembly or formation of spore coat layers were analyzed. They all grew as well as the wild type in an enriched or minimal medium but produced lysozyme and octanol-sensitive spores at the nonpermissive temperature (35 to 38°C). The spores also germinated slowly when produced at 35°C. Temperature-shift experiments indicated that the defective protein or regulatory signal is expressed at the time of formation of the outer spore coat layers. Revertants regained all wild-type spore properties at frequencies consistent with initial point mutations. Spore coat defects were evident in thin sections and freeze-etch micrographs of mutant spores produced at 35°C. In addition, one mutant contained an extra surface deposit, perhaps unprocessed spore coat precursor protein. A prevalent band of about 65,000 daltons (the same size as the presumptive precursor) was present in spore coat extracts of this mutant and may be incorrectly processed to mature spore coat polypeptides. Another class of mutants was defective in the late uptake of half-cystine residues into spore coats. Such a defect could lead to improper formation of the outer spore coat layers.

The spore coat layers of *Bacillus cereus* consist of an under coat, which is solubilized by the reduction of disulfide bonds, and an outer coat solubilized by treatment with a mercaptan plus sodium dodecyl sulfate (SDS) at alkaline pH (2). Ultrastructural electron microscope studies have shown that the outer coat may be further subdivided into a cross-patched (CP) layer and an underlying pitted layer (5). There is evidence that the major proteins in both outer and under coat layers are identical, predominantly one or a few species with molecular weights of about 13,000 (5, 6). These proteins are keratin-like, relatively rich in half-cystine residues (2, 21), and though generally resistant to proteolysis, they are susceptible to a keratinase isolated from *Streptomyces fradiae* (18, 19). The half-cystine content of the outer coat layers is higher than that of the under coat, apparently due to the selective incorporation late in sporulation of cystine into the outer coat by a disulfide interchange reaction with preexisting coat polypeptides (2, 3, 7, 12).

Spores that have had their coats removed chemically and mutant spores with defective coats, caused either by a cystine deficiency or altered structural proteins, are sensitive to lysozyme (4, 5, 7, 11, 20). These observations have led to the development of a technique for enrichment of mutants with altered spore coats by a series of lysozyme treatments and subsequent separtion of sensitive spores from normal spores by centrifugation in density gradients (4).

To further define the steps required for spore coat morphogenesis, we have isolated mutants producing lysozyme-sensitive spores only at a restrictive temperature. The isolation and partial characterization of some of these conditional mutants are described here.

MATERIALS AND METHODS

Isolation of mutants. Washed spores of wild-type *B. cereus* T were treated with ethyl methane sulfonate (13), washed with 0.05 M tris(hydroxymethyl)-aminomethane (Tris)-hydrochloride, pH 7.4, inoculated into a synthetic glucose-salts medium (CDGS; 17), and grown at 38°C. The resulting spores were harvested, washed as above, and incubated for 2 h at 27°C, pH 7.8, with 70 μg of lysozyme per ml. The spores were then layered on a linear 30 to 60% Renografin gradient (prepared from a 76% stock solution consisting of 66% meglominediatrizoate and 10% sodium diatrizoate) and centrifuged in an SW 50.1 rotor at 25,000 rpm for 40 min. The lysozyme-sensitive spores formed a band at a lower buoyant density than the normal spores; these spores were collected with a syringe, inoculated into fresh CDGS medium, and incubated at 30°C until sporulation was complete. The spores were harvested, washed, and fractionated on a Renografin gradient as described above. This time, the

lysozyme-resistant spore band was collected, inoculated into CDGS medium, and incubated at 38°C. The two cycles of lysozyme treatment, fractionation on Renografin gradients and growth, were repeated and finally lysozyme-resistant spores formed at 30°C were spread on CDGS plates. The plates were incubated at 38°C until spores were formed, and individual colonies were tested for lysozyme sensitivity. All lysozyme-sensitive (Lys^s) mutants were restreaked, grown at 30 and 38°C, and again screened for sensitivity of spores to lysozyme only when formed at 38°C. The mutants studied were isolated in two independent searches: Lys^s(Ts) C in the first screen, and Lys^s(Ts) 9, Lys^s(Ts) 66 subsequently.

Cycloserine-resistant derivatives of these mutants were isolated by enriching in G-Tris plus 50 μg of D cycloserine. The cycloserine resistance property did not alter the phenotypic properties of these mutants (summarized in Table 1).

Growth and preparation of spores. Wild-type *B. cereus T* and the mutants were grown in G medium buffered with 0.03 M Tris-hydrochloride, pH 7.4 (1), or in a chemically defined synthetic medium (CDGS) (17) as previously described (1) at 30 to 38°C. At 38°C, sporulation was often poor, especially for the mutants, so 35°C was used as the nonpermissive temperature in most experiments. Growth was followed on a Coleman 8 colorimeter, using a 655-nm filter. The procedure used for harvesting and washing the spores was as described (6). Either linear 30 to 60% Renografin gradients, as described above, or Renografin step gradients were used in experiments in which lysozyme-sensitive spores were separated from lysozyme-resistant spores. Step gradients were prepared by suspending spores in 2.0 ml of 20% Renografin and layering them over 5.0 ml of a 40% Renografin cushion. After centrifugation at 10,000 rpm for 20 min a Sorvall RC2B refrigerated centrifuge, the lysozyme-sensitive spores remained at the interface, whereas the lysozyme-resistant spores were pelleted.

Gel electrophoresis. Slab gel electrophoresis was carried out by the procedure of Laemmli (15) with the following modifications: the concentrations of acrylamide were 12.5% in the separating gel and 5% in the stacking gel, and 6 M urea was present in both stacking and separating gels. Spore coat proteins and standard proteins were dissolved in 0.005 M cyclohexylaminoethane sulfonic acid, pH 9.5, containing 8 M urea–50 mM dithioerithritol–1.0% SDS and were heated at 100°C for 1 min before electrophoresis. The gels were stained and destained by the method of Fairbanks et al. (9).

For fractionation of labeled proteins, samples were electrophoresed in cylindrical gels, prepared as described above. The gels were frozen immediately after the completion of the run, and 1-mm slices were prepared on a Yeda Macrotome. The slices in 6% NCS tissue solubilizer plus 0.4% Omniflour dissolved in toluene were counted in a Nuclear Chicago Isocap scintillation counter.

Isolation of revertants. Attempts to isolate revertants after treatment of mutant spores with lysozyme and subsequent killing of the sensitive spores by heat treatment did not provide adequate enrichment. Therefore, a method that took advantage of the long delay in the formation of mature spores by the mutants was devised. When cultures of the mutants (cycloserine resistant to ensure that wild-type cells would not be selected) growing at 35°C reached the stage in sporulation at which 60 to 70% of the cells had formed phase-white forespores, samples were removed and treated with 100 μg of lysozyme per ml for 1 h at 27°C. The sensitive spores and remaining vegetative cells were killed by heating at 80°C for 20 min. Surviving spores were spread on G-Tris agar plus 50 μg of cycloserine per ml and incubated at 35°C until the resultant colonies formed spores. The plates were flooded with 5 ml of lysozyme (1 mg/ml) and kept at 27°C for 3 to 4 h (8). Those colonies that were still white after the lysozyme treatment were restreaked and tested for lysozyme sensitivity at both 35 and 28°C.

Analysis of phenotypic properties. Lysozyme sensitivity, germination rates, and heat resistance of spores were determined as previously described (4). Dipicolinic acid was determined colormetrically by the method of Janssen et al. (14). Coat protein per spore was determined after two extractions of spores with 50 mM dithioerithritol plus 1% SDS at 37°C for 90 min. The extracts were pooled and precipitated with cold trichloroacetic acid after removal of SDS by addition of saturated KCl. The precipitates were dissolved in 0.2 N NaOH, and protein was determined by the Folin procedure (16). Spores were counted in a Petroff-Hauser chamber in triplicate. Thin-section electron microscopy and freeze etching were as previously described (4, 10).

Incorporation of L-[^{14}C]cystine and L-[^{3}H]leucine by sporulating cells was measured by growing cells in 300-ml sidearm flasks containing 50 ml of G-Tris medium. At appropriate times, 5-ml samples were removed and incubated in 125-ml flasks with 0.1 μCi of L-[^{14}C]cystine plus 0.2 μCi of L-[^{3}H]leucine per ml. To insure that the isotopes would not be exhausted, 6 ng of unlabeled L-cystine and 4 ng of unlabeled L-leucine were added per ml. At 2-min intervals, 0.2-ml samples were pipetted directly into 2.0 ml of cold 10% trichloroacetic acid containing 100 ng of carrier amino acids. The precipitates were collected on glass-fiber filters, washed with 95% ethanol, dried at 70°C for 1 h, and counted in an Omniflour-toluene cocktail.

Chemicals and isotopes. Dithioerythritol was purchased from Pierce Chemical Co., and SDS came from British Drug House Ltd. Twice-recrystallized lysozyme was from Worthington Biochemicals Corp.; Renografin as a 76% sterile solution was purchased from E. R. Squibb and Sons, Inc.; D-cycloserine was from Sigma Chemical Co. L-[^{14}C]cystine (15 mCi/mmol) and L-[4,5 ^{3}H]leucine (59 Ci/mmol) were obtained from Schwarz/Mann; NCS tissue solubilizer was from Amersham Corp.; Omnifluor was from New England Corp.; glass-fiber filters (no. 25) came from Schleicher and Schuell.

RESULTS

Isolation of mutants. The enrichment procedure, which consisted of three rounds of growth at 30 or 38°C, lysozyme treatment, and separation on Renografin gradients, was highly effective in selecting for lysozyme-sensitive mu-

tants. Approximately 90% of the colonies finally screened on plates contained lysozyme-sensitive spores. Out of several hundred mutants obtained in this manner, three, Lys^s(Ts) C, Lys^s(Ts) 9, and Lys^s(Ts) 66, were temperature sensitive; i.e., spores produced by these mutants at 38°C were highly sensitive to lysozyme, whereas spores produced at 30°C were only slightly sensitive (Fig. 1, Table 1).

Growth and sporulation. Since part of the enrichment procedure involved growth in a minimal, defined medium (17), auxotrophs were excluded. The mutants grew as well as the wild type in CDGS and in G-Tris. When the mutants were grown in G-Tris at 38°C, the formation of phase-white forespores occurred approximately 3 h later than for the wild type, forespore formation was accompanied by extensive cell lysis (up to 50%), and the release of mature spores by the mother cells was delayed by at least 12 h. Lowering the temperature to 35°C was effective in reducing the extent of lysis but had little effect on the lysozyme sensitivity of the spores or on the late release of the spores by the mother cells. To insure higher spore yields, 35°C was used as the restrictive temperature in most experiments.

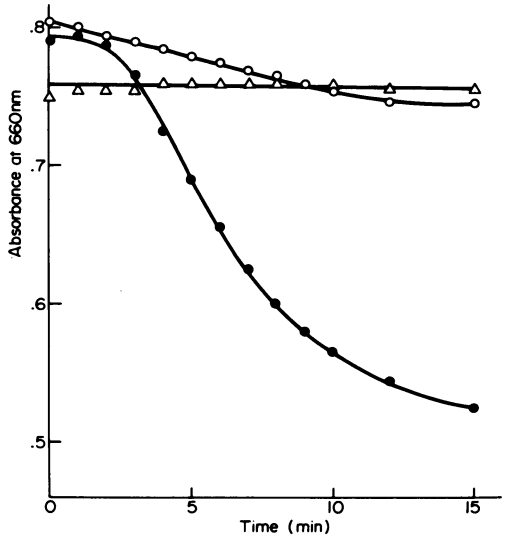


FIG. 1. *Lysozyme sensitivity of mutant and wild-type spores. Spores were washed and suspended in 0.05 M Tris, pH 7.8, plus 100 μg of lysozyme per ml. The loss in absorbance at 660 nm was followed in a Zeiss spectrophotometer.* △, *Wild-type spores;* ●, *Lys^s(Ts) 9 spores produced at 35°C;* ○, *Lys^s(Ts) 9 spores produced at 28°C.*

Properties of the spores. The relative lysozyme sensitivities of spores produced by the mutants at the nonpermissive and permissive temperatures were determined by measuring the loss in absorbance at 660 nm after addition of lysozyme (Fig. 1). The results were nearly identical for the three mutants. Spores produced at 35°C underwent a rapid loss in absorbance that was complete in about 15 min, whereas spores produced at 28°C underwent only a slight loss in absorbance. Wild-type spore suspensions were completely resistant to lysozyme. After 1 h at 27°C, 13 to 25% of the spores formed at 35°C and 70 to 78% of the spores formed at 28°C remained phase bright for all three mutants. The spores that were resistant to lysozyme remained so even after treatments of 12 to 16 h and increases in lysozyme concentrations up to 1 mg/ml.

Several properties of the mutant and wild-type spores are summarized in Table 1. The

TABLE 1. *Summary of properties of mutant and wild-type spores*

Property	Wild type	Mutants produced	
		At 35°C[a]	At 28°C[a]
Resistance to 80°C for 20 min	93–100%	93–99%	93–99%
Lysozyme sensitivity[b]	0	75–87%	22–30%
Resistance to lysozyme plus 80°C for 20 min	93–100%	12–25%	75–80%
Dipicolinic acid (% dry wt)	10–12%	10–12%	10–12%
% Survival after $CHCl_3$ treatment[c]	100%	100%	100%
% Survival after treatment with octanol[d]	67–90%	5–35%	30–60%
Storage in distilled water or 0.01 M sodium phosphate buffer, pH 7.0, at 4°C	100% phase bright after 2 weeks	40–45% phase dark after 48 h.	10–20% phase dark after 48 h.
Reversion frequency		$1/10^7$–$1/10^8$	
Coat protein per spore (grams × 10^{-13}/spore)	1.4–1.6	0.96–1.2	1.5–1.7

[a] The three mutants behaved identically for the properties listed.

[b] Percentage of spores that became phase dark after incubation with lysozyme (100 μg/ml) in 0.03 M Tris-hydrochloride, pH 7.4, at 27°C for 30 min. Counts made in a Petroff-Hauser chamber.

[c] Exposure of spores on G-Tris agar to $CHCl_3$ vapors for 1 min.

[d] As per reference 4.

mutant spores were heat and chloroform resistant and contained the same concentration of dipicolinic acid as the wild type. Mutant spores were more sensitive to octanol, but the difference was difficult to measure accurately due to clumping of the spores in the presence of octanol. When formed at 35°C, spores contained less coat protein than wild type and were unstable when stored at 4°C in distilled water or in phosphate buffer but were stable when stored frozen in buffer. Resistance of the mutant spores to heat and to chloroform was lost after lysozyme treatment (except in that fraction of the spores that remained lysozyme resistant). When the lysozyme-resistant fraction was again grown in G-Tris at 35°C, the same proportion as originally found (about 90%) was sensitive to lysozyme; i.e., resistance is due to phenotypic heterogeneity. Unlike the mutant described by Cheng et al. (7), these mutants could not be cured of their lysozyme-sensitive phenotype by addition of L-cysteine to the medium.

Germination. In general, lysozyme-sensitive spores respond slowly to the germinants L-alanine plus adenosine (or inosine), suggesting that some coat-related function is needed for a rapid response to these germinants (4, 8). For all three mutants, spores produced at 35°C responded slowly to these germinants, whereas spores produced at 28°C responded almost as rapidly as wild type (Fig. 2), implying that both the slow germination and lysozyme-sensitive phenotypes are temperature sensitive.

To further confirm the relationship between the structural integrity of the spore coat and the capacity to germinate rapidly, the lysozyme-resistant fraction of Lys^s(Ts) 9 spores formed at 35°C was purified on a Renografin step gradient. The germination rate of these resistant spores was compared to the rate of the comparable, untreated spore population (Fig. 3). The fraction of spores that was lysozyme resistant germinated at a faster rate than the total population (80% lysozyme sensitive). The germination rate of the resistant fraction was slower than the wild-type spores and about equal to the rate obtained with mutant spores produced at 28°C.

Temperature-sensitive period. To determine the time during which this altered protein (or regulatory signal) must function, a series of temperature-shift experiments was performed. In shift-up experiments, samples were removed from cultures growing in G-Tris medium at 28°C, inoculated into sterile flasks, and incubated at 35°C until mature spores were released from the mother cells. The spores from each subculture were treated with lysozyme, as described in the legend to Fig. 1, and the initial rates of loss of absorbance at 660 nm were de-

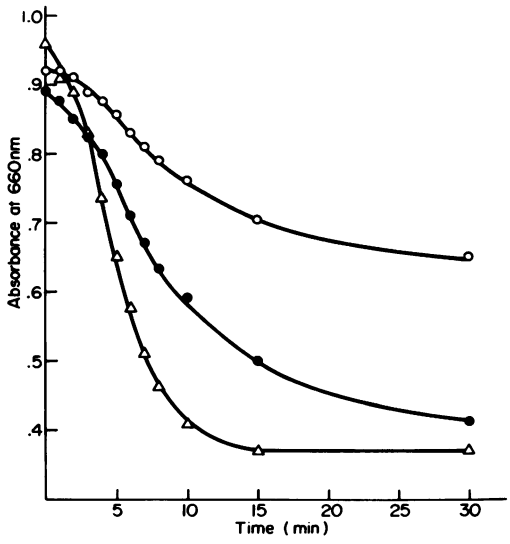


FIG. 2. *Germination of mutant and wild-type spores. Spores were heat activated and germinated in the presence of* L-*alanine plus adenosine as described in Materials and Methods. Loss in absorbance at 660 nm was recorded in a Zeiss spectrophotometer. Symbols:* △, *wild-type spores;* ○, *Lys^s(Ts) 9 spores produced at 35°C;* ●, *Lys^s(Ts) 9 spores produced at 28°C.*

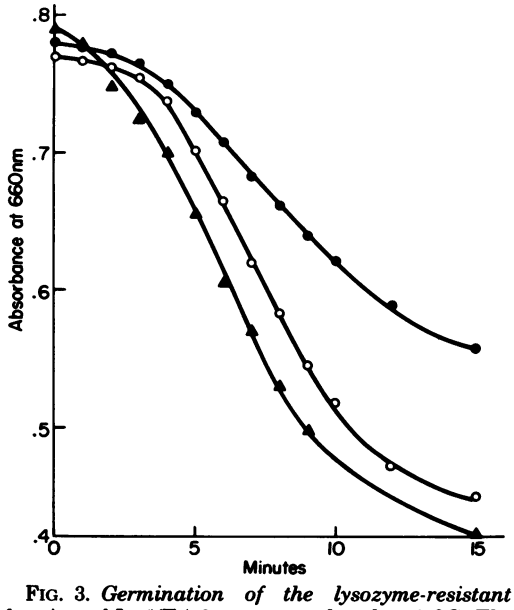


FIG. 3. *Germination of the lysozyme-resistant fraction of Lys^s(Ts) 9 spores produced at 35°C. The lysozyme-resistant spores were separated from the lysozyme-sensitive spores on a Renografin step gradient and germinated as described in Materials and Methods. Symbols:* ●, *mutant spores produced at 35°C;* ○, *lysozyme-resistant fraction of mutant spores produced at 35°C;* ▲, *mutant spores produced at 28°C.*

termined. These rates are plotted relative to the initial rate obtained with control spores from a culture maintained at 35°C throughout sporulation (Fig. 4). The results show that the temperature-sensitive period corresponds closely to the period in which phase-white forespores were formed. The period of change extends over 6 to 8 h and correlates with the time for formation of phase-white forespores but is prior to the time of maximum dipicolinic acid accumulation. This phase whitening has previously been shown to occur at the time of outer spore coat deposition (22). Spores from cultures shifted up before the onset of phase whitening or at the onset of phase whitening were nearly as sensitive to lysozyme as the spores from the 35°C control culture, whereas those from cultures shifted later became progressively less sensitive. Spores from cultures shifted up 16 or more h after the end of growth had the same sensitivity as those from the control culture that was maintained at 28°C throughout sporulation, indicating that the temperature-sensitive period had passed. Similar curves were obtained for the three mutants.

Shift-down experiments were performed in a similar manner (Fig. 5). The results of these experiments correlated with the shift-up experiments, again showing that the critical period for the three mutants corresponded to the phase-whitening stage. Spores from subcultures shifted

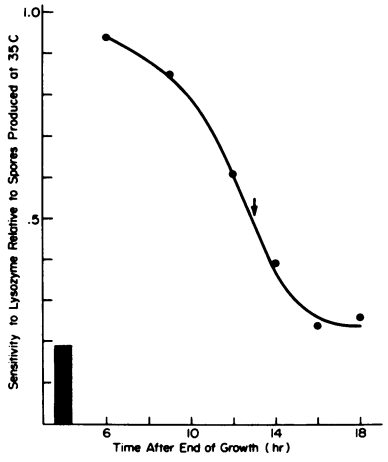


FIG. 4. *Lysozyme sensitivity of Lys⁻(Ts) C spores from cultures shifted up from 28 to 35°C at various times during sporulation. At the times indicated (●), 5-ml samples were removed from a 28°C culture to sterile 125-ml flasks and incubated at 35°C until mature spores were formed. Relative lysozyme sensitivities of the spores were determined as described in the text and legend to Fig. 1. The bar in the lower left-hand corner represents the lysozyme sensitivity of control spores produced at 28°C. The arrow refers to the time when 50% of the cells contained phase-white forespores.*

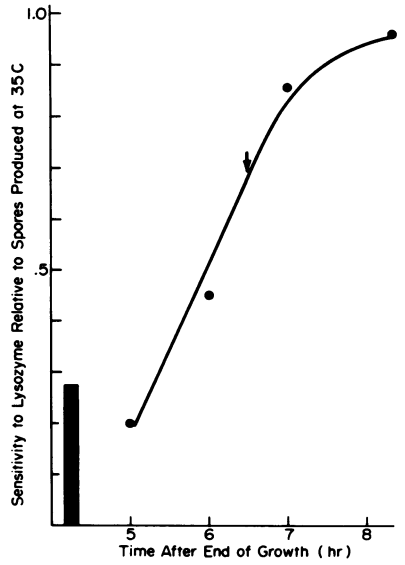


FIG. 5. *Lysozyme sensitivity of Lys⁻(Ts) C spores from cultures shifted down from 35 to 28°C at various times during sporulation. A 50-ml culture in G-Tris medium was grown at 35°C. At the times indicated (●), 5-ml samples were removed to sterile 125-ml Erlenmeyer flasks and incubated at 28°C until free spores were formed. The spores were washed, suspended in buffer, and analyzed for lysozyme sensitivity as described in the legend to Fig. 1. The initial rates of change of absorbance at 660 nm are plotted relative to the rate for spores formed at 35°C. The bar in the lower left-hand corner represents the lysozyme sensitivity of spores produced at 28°C. The arrow refers to the time when 50% of the cells contained phase-white forespores.*

down 5 h after the end of growth, the time of the first appearance of phase-white forespores, were slightly less sensitive to lysozyme than spores from the 28°C control culture. Spores from cultures shifted approximately 30 min after the end of the phase-whitening period (8 h after the end of growth) were as sensitive to lysozyme as spores produced at 35°C.

In some of the temperature-shift experiments the germination rates of the spores were also determined (Fig. 6). In this experiment, the phase-whitening period was 7 to 14 h after the end of growth. Spores from the subculture shifted to 35°C at the onset of phase whitening had a germination rate that was approximately the same as that of spores from a control culture maintained at 35°C throughout sporulation. Spores from subcultures shifted at later times in the phase-whitening period had progressively faster germination rates. Spores from the culture shifted 1 h after the end of the phase-whitening period had germination rates that were approximately 80% of the rates found for spores from

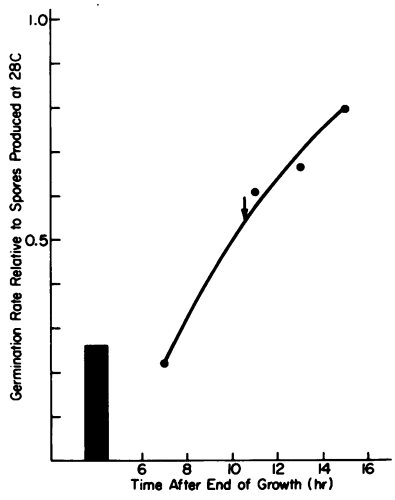


FIG. 6. *Germination rates of Lys^s(Ts) C spores from cultures shifted from 28 to 35°C at various times during sporulation. At the times indicated (●) samples were removed from the 28°C culture and incubated at 35°C. When sporulation was complete, the spores were harvested, washed, and germinated as described in Materials and Methods and the legend to Fig. 2. The initial rates of decrease of absorbance at 660 nm are plotted relative to the rate for spores produced at 28°C. The bar in the lower left-hand corner represents the relative germination rate of spores produced by continuous cultivation at 35°C. The arrow refers to the time when 50% of the cells contained phase-white forespores.*

the 28°C control culture. The temperature-sensitive period for the slow-germination phenotype corresponds closely to that for the lysozyme-sensitive phenotype.

Analysis of spore coat proteins. Since some lysozyme-sensitive spore mutants contain altered spore coat polypeptides (4), total coat proteins extracted from wild-type and mutant spores were compared by gel electrophoresis (Fig. 7). The wild-type coat protein (Fig. 7A and B) contained two major bands, one of 13,000 daltons and a second less prominent band of about 26,000 daltons. The coat protein extracted from spores of Lys^s(Ts) C (Fig. 7A) also contained two major bands, but the 13,000-dalton band was less prominent (especially at 35°C) and the 26,000-dalton band ran slightly ahead of the one from the wild-type spores. In addition there was a major band of about 65,000 daltons in extracts of spores produced at either 35 or 28°C. This protein has the same molecular weight as a presumptive coat precursor (8). Other bands in the 30,000- to 60,000-dalton range, especially prominent in 35°C spore extracts, were not always present and may be due to spore lysis. The gel profile of the Lys^s(Ts) 66 coat extracts was identical to that of Lys^s(Ts) C. The profile of Lys^s(Ts) 9 coat extracts (Fig. 7B) contained the 13,000-dalton and 26,000-dalton bands as in the wild type and some 65,000-dalton protein but less than Lys^s(Ts) C (Fig. 7A).

Disulfide interchange. The selective incorporation of half-cystine into the *B. cereus* spore coat late in sporulation appears to be essential for the maturation of the outer coat layers and the formation of lysozyme-resistant spores (5, 7). This reaction was measured in the mutants by adding [^{14}C]cystine and [^{3}H]leucine during the phase-whitening period (Fig. 8). The latter amino acid provided a measure of the overall rate of protein synthesis. For the wild-type, there was a twofold increase in the initial rate of incorporation of L-cystine during the early stages of the phase-whitening period, whereas the initial rate of [^{3}H]leucine incorporation remained constant (Fig. 8a). Under the same experimental conditions, a similar twofold increase in the initial rate of [^{14}C]cystine incorporation was observed in cultures of Lys^s(Ts) C early in the phase-whitening period (Fig. 8b), suggesting that the disulfide interchange reaction was occurring normally in that mutant. The lower initial rates of incorporation observed in cultures of Lys^s(Ts) C were probably due to the extensive lysis that always occurred in cultures of the mutant growing at the nonpermissive temperature.

The initial rate of [^{14}C]cystine incorporation in Lys^s(Ts) 9 sporulating cells remained constant, whereas the initial rate of protein synthesis decreased slightly (Fig. 8c), implying that the disulfide interchange reaction was impaired. This particular defect was also found in Lys^s(Ts) 9 cultures sporulating at 28°C, although perhaps not as markedly as for 35°C cultures.

Thin-section microscopy. The spores of these three mutants Lys^s(Ts) C, -66, and -9 when formed at 28°C on agar or in fluid culture appeared generally well covered with typical *B. cereus* spore coat profiles and exosporia (Fig. 9). Lysozyme-sensitive spores of mutant Lys^s(Ts) 9 formed at 37°C suffered the loss of outer coat material in segments of the coat. Unlike the spores of the other two mutants, however, most of those of Lys^s(Ts) 9 contained a more complete coat and certainly a better deposit of undercoat material when formed at 37°C (Fig. 10). Occasionally, a spore of mutants C and 66 showed small segments of coat devoid of the outer CP layer or of regions of redundant coat (Fig. 11 and 15). When formed at 37°C, although the exosporium was complete in all mutants, the defect of the spore coats of mutants Lys^s(Ts) C and -66 varied from a small segment missing all coat layers to a similar defect exposing one-third to one-half of the spore cortex (Fig. 11b). Moreover,

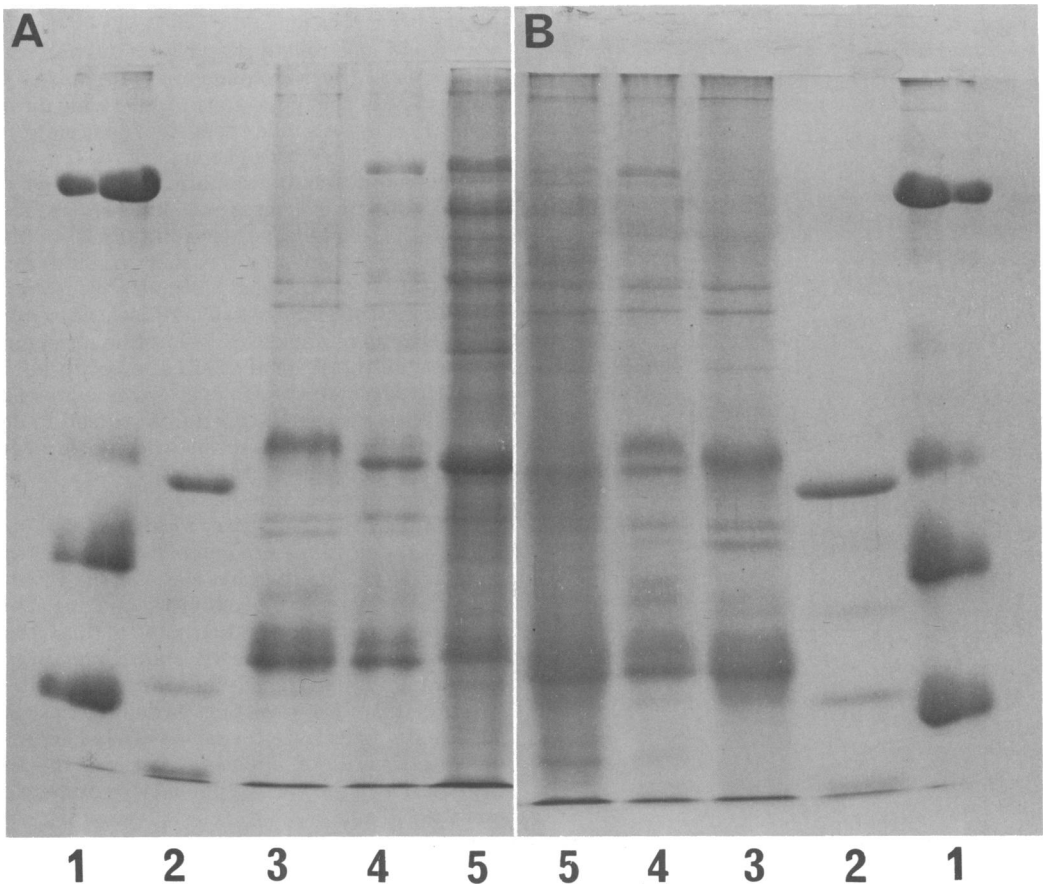


FIG. 7. *SDS-polyacrylamide-stained gel profiles of spore coat protein. Spores were extracted and electrophoresed as described in Materials and Methods. (A) row 1—standards: from top to bottom, bovine serum albumen (65,000 daltons), ribonuclease (13,700 daltons), cytochrome c (12,700 daltons); row 2, chymotrypsinogen (25,000 daltons); row 3, wild-type spore extract (spores produced at 30°C, but an identical profile was obtained for 35°C spore extracts); row 4, extract of Lys^s(Ts) C spores produced at 28°C; row 5, extract of Lys^s(Ts) C spores produced at 35°C. (B) rows 1 and 2, standards as in A rows 1 and 2; row 3, wild-type extract of spores formed at 30°C; row 4, extract of Lys^s(Ts) 9 spores produced at 28°C; row 5, extract of Lys^s(Ts) 9 spores produced at 35°C.*

in both these mutants, coat that was formed appeared much thinner (Fig. 12) than that formed at 28°C. An abnormally heavy and localized deposit of coat that mutant Lys^s(Ts) 66 displayed on some of its spores formed at 28 to 30°C (Fig. 11a and 13) could also be found on a few spores formed at 37°C (Fig. 11b), suggesting a defect independent of the temperature of sporulation.

Freeze-cleave-etch micrographs. In order to compare these mutants with each other and at the two temperatures of sporulation (28 and 37°C), approximately 100 spore replicas of each sample were examined, and some 8 to 10 representative cleaved spores of each were photographed. Although most of the spores of these mutants formed at 28°C showed the CP pattern on the coat surface under the cleaved exosporium, the density of packing of the patches of rods on the surface (Fig. 13, 14, and 16) was less than that normally seen on wild-type *B. cereus* spores (5). The rod lengths also seemed longer than normal on these mutants. Larger areas of pitted layer devoid of CP occasionally encountered proved to be pieces of redundant coat superimposed on the regular coat (Fig. 15). Such extra coat layers are quite common in *B. cereus* T.

When produced at 37°C, the spores showed a marked inability to form the CP layer (Fig. 17, 18, and 19). This defect was common to all three mutants at 37°C. Although occasionally the CP

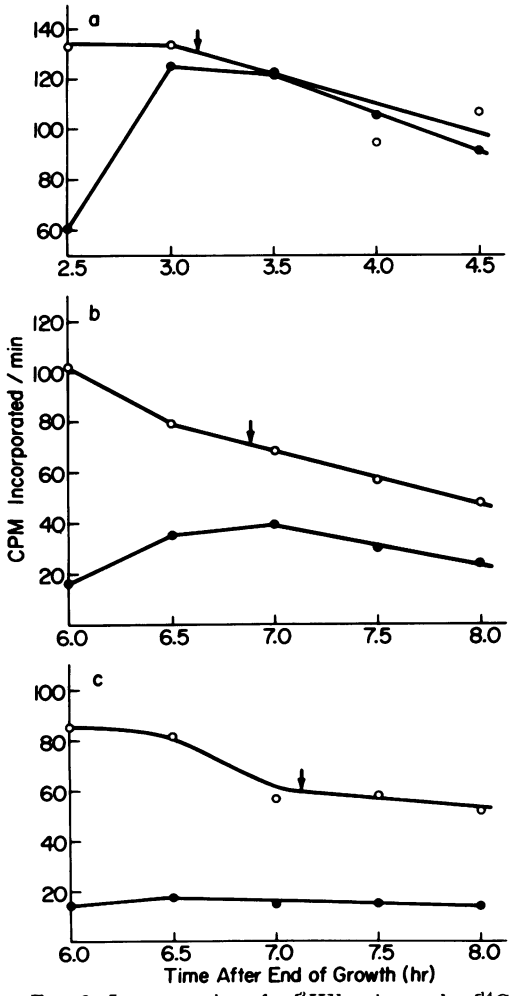


FIG. 8. *Incorporation of L-[^{3}H]leucine and L-[^{14}C] cystine into sporulating cells of wild-type B. cereus (a), Lys^s(Ts) C (b), and Lys^s(Ts) 9 (c) during the phase-whitening stage of forespore formation at 35°C. At the times indicated, 5 ml of cells was placed in 125-ml Erlenmeyer flasks, and carrier plus labeled amino acids were added as detailed in Materials and Methods. Two-tenth-milliliter samples were pipetted into cold 10% trichloroacetic acid at 2-min intervals to establish the initial rates of incorporation (abscissa). The arrows indicate the time at which 50% of the cells contained phase-white forespores. Symbols: ○, ^{3}H counts per minute; ●, ^{14}C counts per minute.*

fiber pattern was encountered, it was even then scarce and incomplete (Fig. 17 and 19) and usually replaced by a granular or unorganized fibrous deposit that covered the underlying pitted layer (Fig. 18). A similar deposit of coat material also covered the cortex of some spores (Fig. 19).

Reversion frequency and properties of revertants. Revertants of Lys^s(Ts) 9 and Lys^s(Ts) C isolated by their ability to form mature spores several hours earlier than the mutants (see Materials and Methods) occurred at a frequency of $1:10^7$ to $1:10^8$. All isolates scored as revertants retained the cycloserine resistance property of the original strain and produced 80 to 99% lysozyme-resistant spores when grown at 35°C. Whereas the 12 revertants studied in detail qualitatively regained the wild-type properties listed in Table 1, there were quantitative variations. For example, increase in germination rates of the revertant spores formed at 35°C did not always correlate completely with lysozyme resistance, i.e., spores from some of the revertants that were 98 to 99% lysozyme resistant germinated more slowly than some of those that were only 80% lysozyme resistant, but always more rapidly than the mutants. We suspect that the procedure for revertant enriching was not sufficiently selective to exclude partial or second-site revertants, but in no case was there a dissociation of the various phenotypic properties analyzed (Table 1).

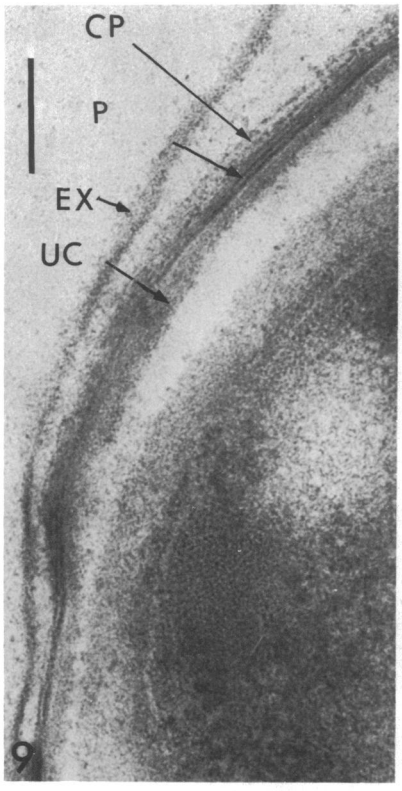
DISCUSSION

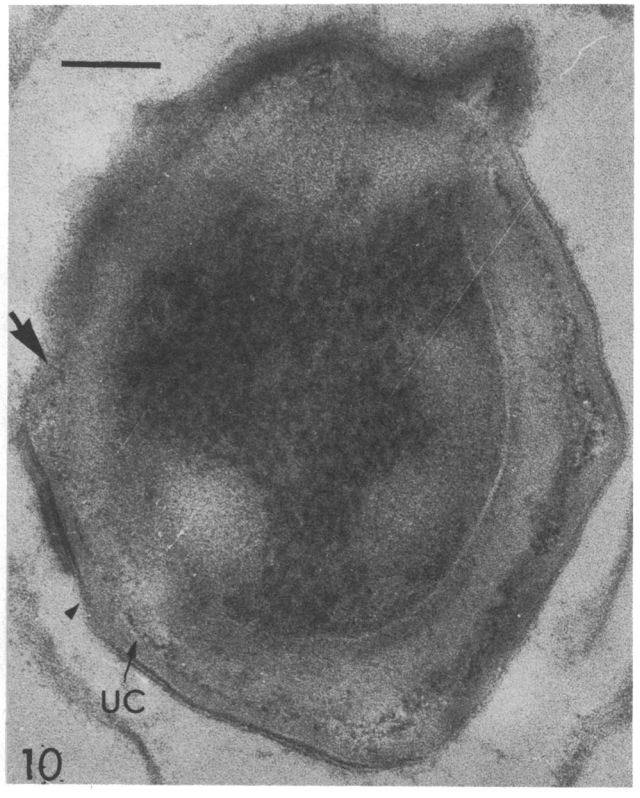
The three conditional mutants are specifically altered in some stage of spore coat synthesis or assembly. They all grow as well as the wild type at 28 to 35°C in an enriched or minimal medium, and the defect is not apparent until the time of morphogenesis of the outer spore coat layers (Fig. 4 to 6). They are all altered not only in coat assembly (lysozyme sensitivity) but also in sensitivity of the spores to octanol and response to the germinants L-alanine plus adenosine. Coat defects in *B. cereus* very often lead to altered germination properties of the spores (4, 5). In all cases, however, the conditional defect was not all or none. Spores produced at 30°C were not identical to the wild type either in structure (Fig. 11 and 13 to 15) or in resistance to lysozyme

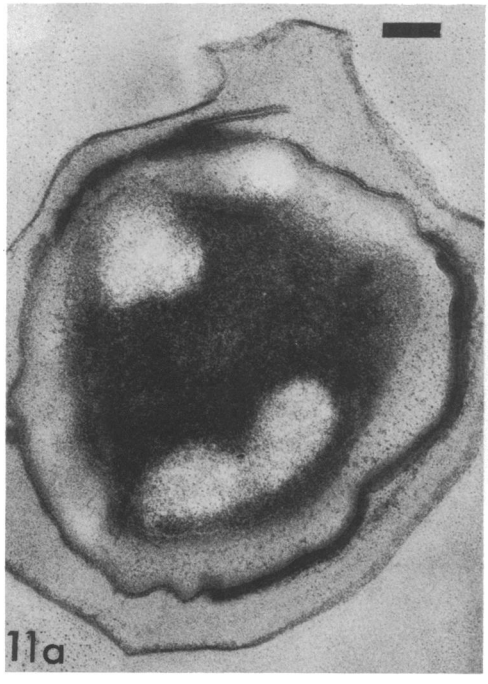
FIG. 9. *Thin section of a spore of B. cereus Lys^s(Ts) 9 showing normal coat found on the majority of spores at 28°C. The outer loose layer, the exosporium (EX), covers the coat which has dense outer profiles: pitted (P) and outermost (CP) layers and a softer undercoat (UC) (see reference 5). ×157,500. Marker is 100 nm in this and subsequent micrographs.*

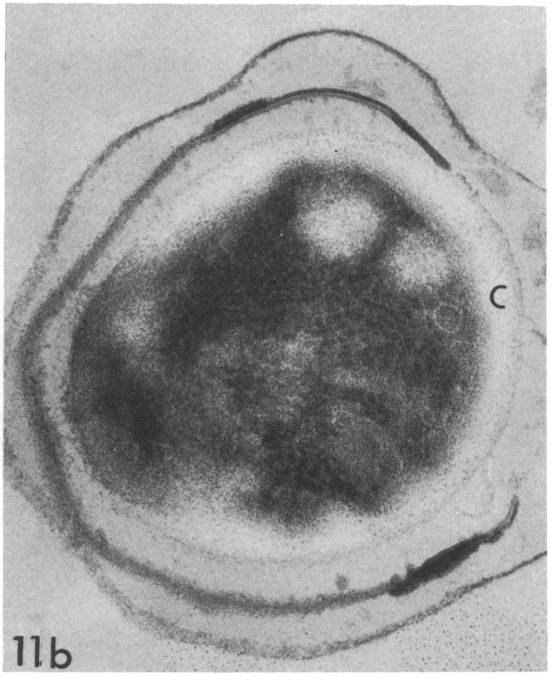
FIG. 10. *Sporulation of mutant Lys^s(Ts) 9 at 37°C produced thinner coats completely defective in some areas. On both sides of a small area of the thicker coat (arrows) the outer coat profiles are absent but undercoat (UC) seems more intact. ×134,000.*

FIG. 11. *Thin section of free spores of mutant Lys^s(Ts) 66. a, The more complete coats formed at 28°C. b, Thinner coats on the lysozyme-sensitive spores formed at 37°C leave a large area of the cortex (C) bare. The denser regions of coat deposit were present on these spores regardless of temperature of sporulation. ×78,500.*


CP
P
EX
UC
9


UC
10


11a


C
11b

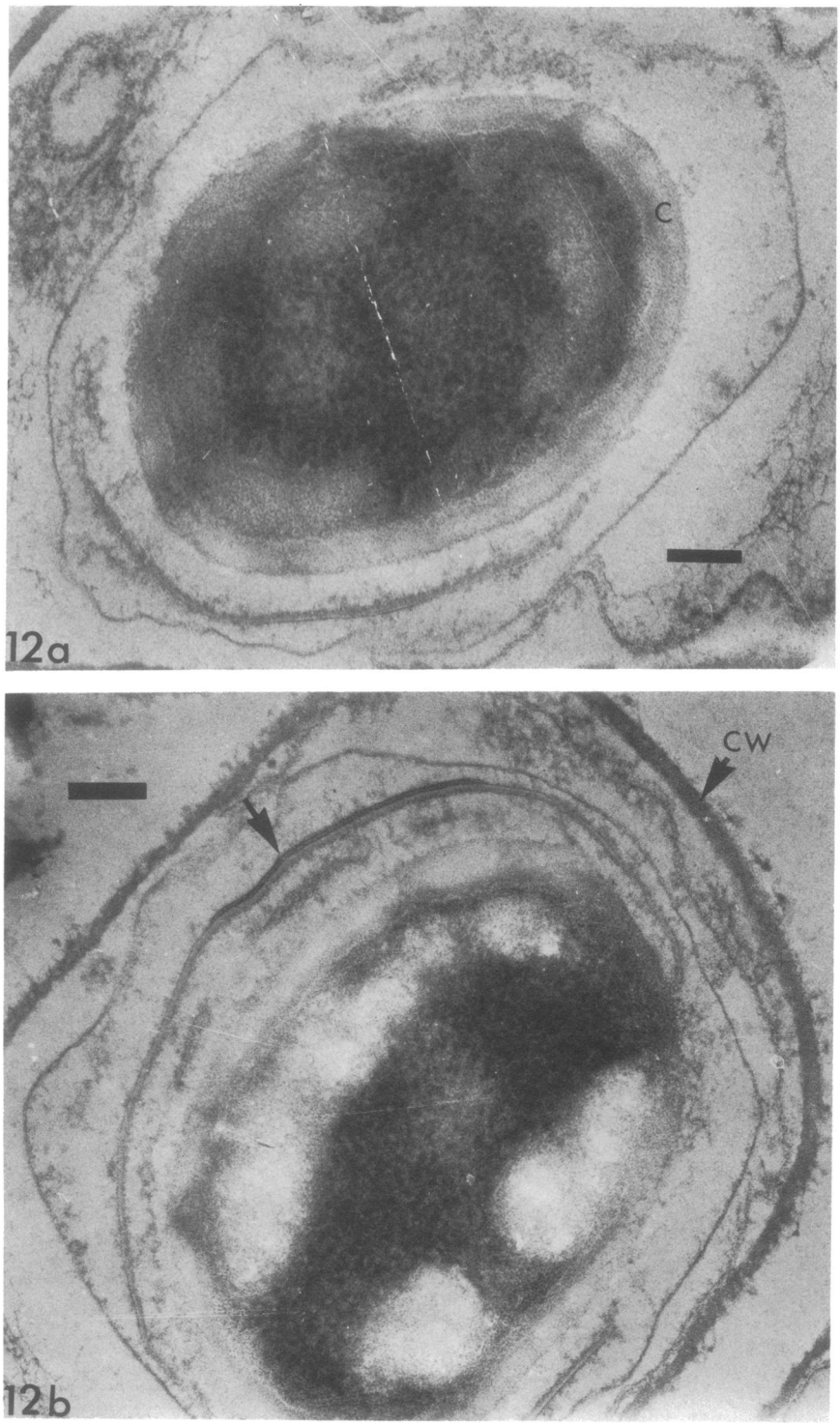
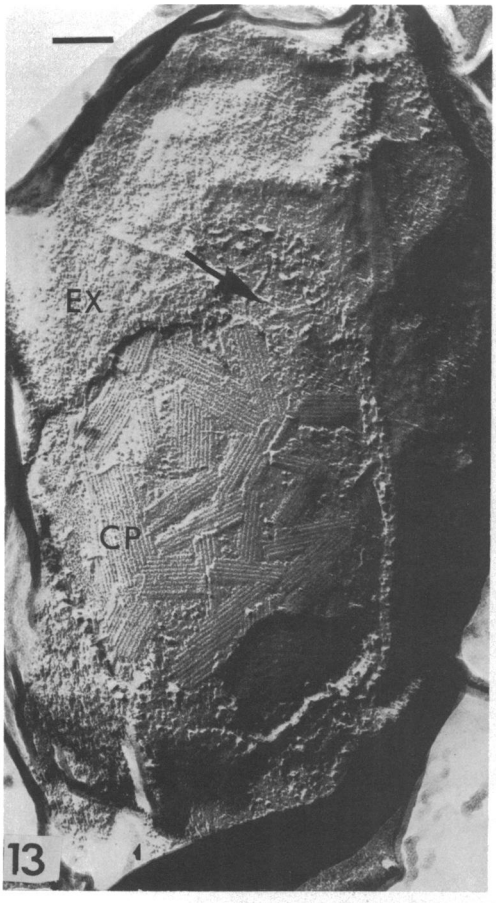
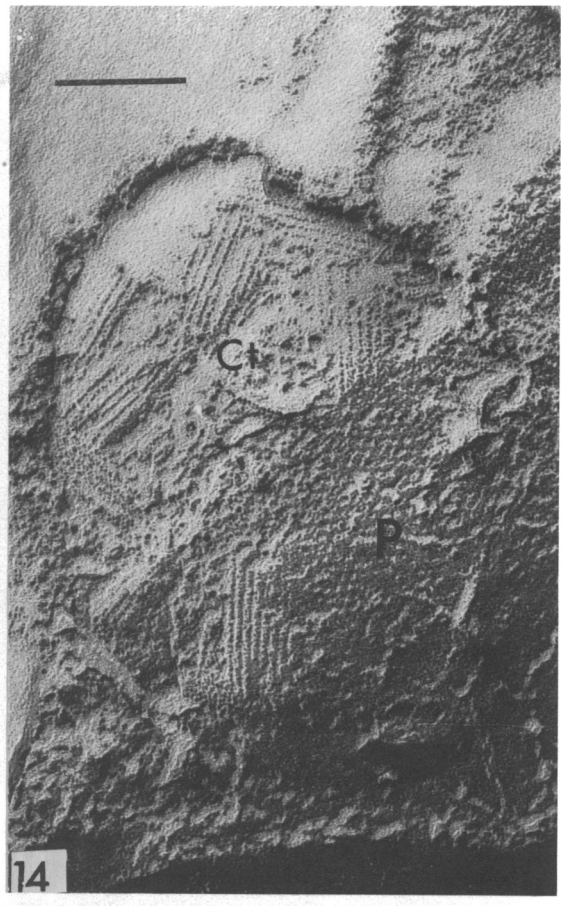


FIG. 12. *Spores of B. cereus Lys⁻(Ts) C formed at 37°C. a, Typical spore showing a very thin coat covering only part of the cortex (C). ×100,000. b, Occasionally a spore is able to form segments of normal coat (arrow). Cell wall (CW) remnant still encloses the spore. ×104,000. (At 28 to 30°C the coats were complete as in Fig. 9).*





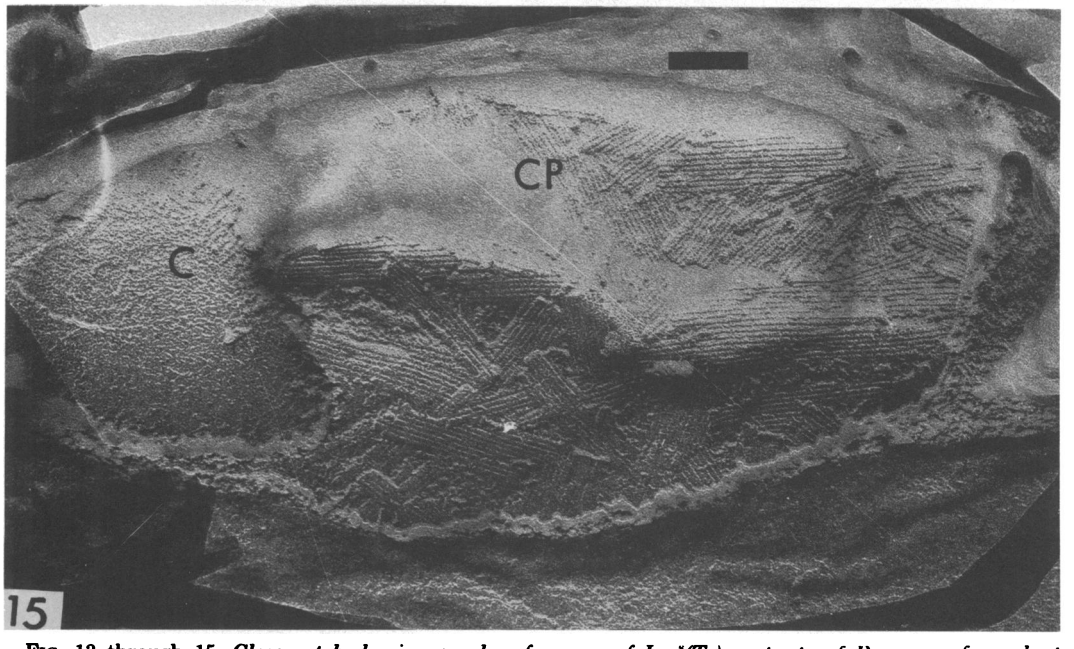


FIG. 13 through 15. *Cleave-etched micrographs of spores of Lys⁻(Ts) mutants of B. cereus formed at permissive temperature (28°C). CP formation is fairly complete in all three mutants. Fig. 13. Lys⁻(Ts) 66 has, as also shown in thin sections (Fig. 11a), some areas of coat (arrow) devoid of organized CP. The exosporium (EX) here and in subsequent micrographs has been cleaved to reveal the coat structure. ×78,500. Fig. 14. Mutant Lys⁻(Ts) C shows a cleavage partly exposing a redundant coat segment that contains predominantly pitted (P) layer. The coat below (ct) is well covered in CP structures. ×157,500. Fig. 15. Spore of Lys⁻(Ts) 9 cleaved in its long axis displaying a well-developed CP coat and clean cortex (C). ×104,000.*

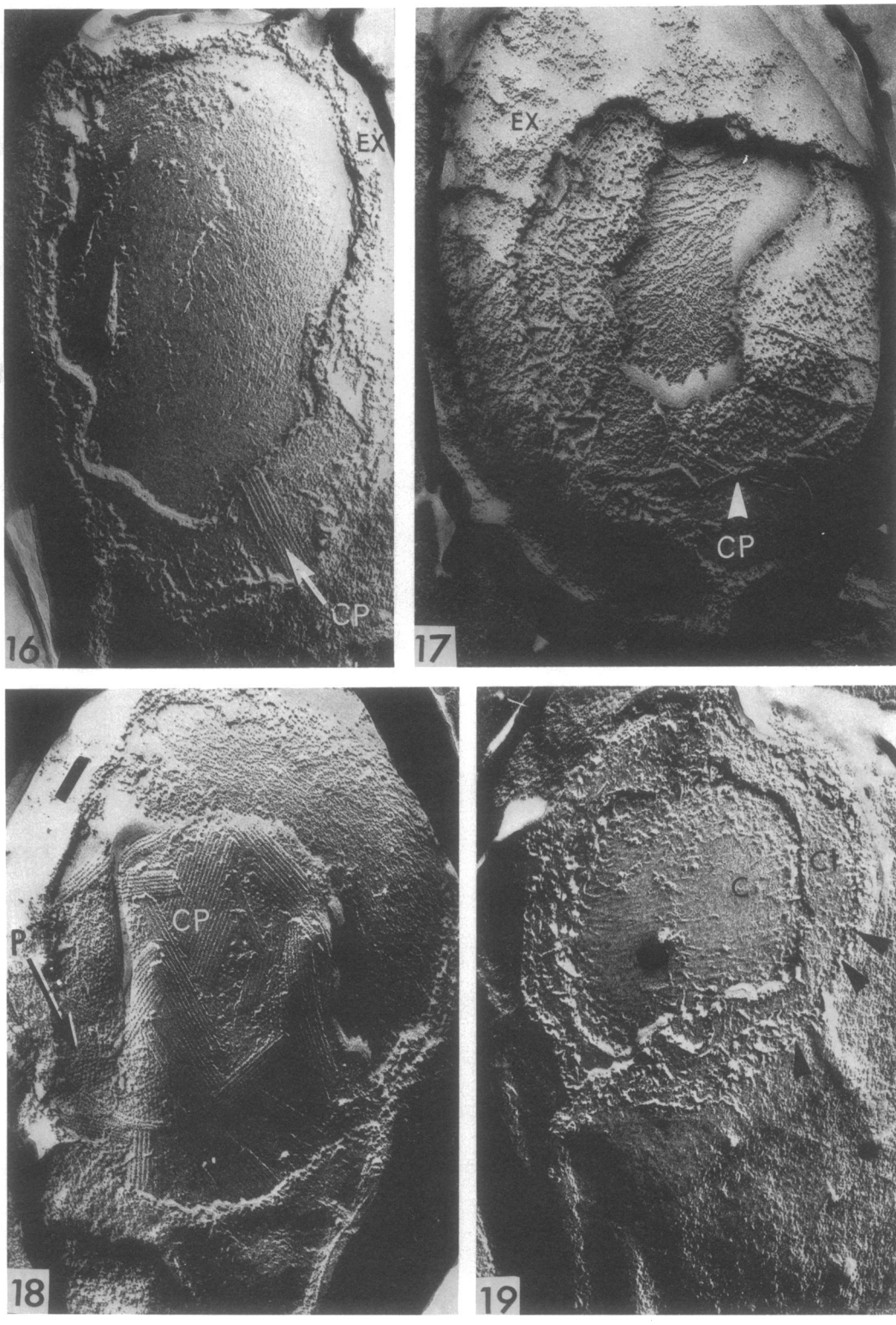


FIG. 16 through 19. *Cleave-etch replicas of mutant spores B. cereus Lys⁻(Ts) C formed at 37°C showing the type of coat development encountered in these three mutants. Fig. 16 and 17. A few strands of CP structure under the cleaved exosporium (EX) were seen on some spores. Fig. 18. A rare spore showed segments of coat with a moderate deposition of CP rods on the pitter layer (P). Fig. 19. most spore replicas were devoid of CP resulting in a thin coat (ct) having a fibrous and granular deposit both on the coat layer and the cortical surface (C). The edge of the sheared exosporium is marked with arrows. ×78,500, As per 100-nm marker in Fig. 18.*

(Fig. 1). There were even some lysozyme-resistant spores produced at 35 to 38°C, but these were phenotypic alterations rather than revertants since recloning resulted in the same predominantly sensitive population as originally isolated.

The most successful procedure for selecting for revertants, enriching for those sporulating rapidly at 35°C, resulted largely in partial revertants. In all cases, all of the phenotypic properties of the wild type as summarized in Table 1 were qualitatively restored. The frequency of these revertants ($1/10^7$ to $1/10^8$) and the fact that all phenotypic properties reverted together suggest that there were original point mutations.

Although the mutants were phenotypically identical, there were some differences in structure and reactions related to spore coat formation. Mutants Lys^s(Ts) C and -66 accumulated on spores a 65,000-dalton polypeptide that may be unprocessed spore coat precursor. This protein is probably the fibrous deposit seen on cleave-etch replicas (Fig. 19). This polypeptide is precipitable by anti-spore coat antibody and does turn over slowly in pulse-chase experiments (G. Stelma and A. Aronson, unpublished data). A major intracellular serine protease that is essential for spore coat precursor processing (8) is present in normal amounts (and heat stability) in the mutant. Processing may be altered either by a defect in the precursor per se or by a labile enzyme essential for modification of the precursor before cleavage. The altered gel profile of the smaller coat polypeptides extracted from Lys^s(Ts) spores, especially the 26,000-dalton species (Fig. 7A), is consistent with such a defect. It should be noted, however, that it is not certain that the 26,000-dalton protein is indeed processed from a larger precursor.

Mutant Lys^s(Ts) 9 accumulates much less 65,000-dalton protein on spores, and the gel profile of coat proteins is very similar to that of the wild type (Fig. 7B). This mutant clearly has a defect at least in the outer CP layer (Fig. 10, 16, and 17) but forms a more complete undercoat than the other two mutants (Fig. 10 to 12). This mutant is defective in the selective incorporation of cystine late in sporulation (Fig. 8c). This defect may reflect the biochemical basis for the alteration, although there was no marked temperature dependence. Disulfide interchange between cysteine and coat polypeptides crosslinked via disulfide bonds seems to be essential for the maturation of the outer coat layers (5). An alteration either in cysteine production or the disulfide interchange could account for the defect. Maintenance of cysteine is dependent on a functional reduced nicotinamide adenine dinucleotide phosphate-dependent disulfide reductase (A. Aronson, unpublished data). A mutant, Lys^s(Ts) 10, previously thought to have a defective glutathione reductase (7) is in fact altered in this more general disulfide reductase. The K_m with oxidized glutathione is much higher than for cystine, which is not surprising in view of the virtual absence of glutathione in bacilli (8a). Mutant Lys^s(Ts) 9 has as much heat-stable disulfide reductase activity as the wild type so, while phenotypically similar to Lys^s(Ts) 10, the actual alteration is different. There may be a disulfide interchange activity selective for incorporation of cysteine into protein that is altered in Lys^s(Ts) 9.

In the absence of altered structural protein or enzyme isolation, it is difficult to establish the basis for the conditional defects. The complementary patterns of the temperature shift-up and -down experiments (Fig. 4 to 6) imply that an altered structural protein or enzyme (or even a defect in the synthesis or regulation of these factors) must either be made (or function) selectively at the time of coat maturation or continuously be produced. Synthesis only at earlier times (prior to stage IV) would result in inability to produce lysozyme-resistant spores in a shift-down experiment at stages prior to phase whitening and the converse for shift-up experiments.

The third mutant, Lys^s(Ts) 66 has not been analyzed as extensively as the others but seems to be very similar to Lys^s(Ts) C biochemically and morphologically. The existence of at least two classes of conditional mutants selectively altered in coat morphogenesis extends the number of reactions required for deposition of spore coat layers. A more complete analysis of these and other specific coat morphogenetic mutants should provide the details of the reactions required for coat assembly.

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