A *Bacillus cereus* Mutant Defective in Spore Coat Deposition

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Spores of *Bacillus cereus* mutants selected for slow response to germinants and sensitivity to lysozyme were found to be deficient in coat, but were heat-resistant and contained the same quantity of dipicolinic acid as the wild-type. While the average coat protein content of the spores was 25% of the wild-type, many spores were coatless with large whorls of coat deposited in the cytoplasm. These coat deposits were isolated in Renografin gradients and found to cross-react immunologically with wild-type coat. The proteins extractable from these deposits were virtually identical to those extracted from wild-type spores. The morphology of the coat deposits was very similar to coats of wild-type spores, but with a deficiency of the outermost cross-patched layer. The sites of formation and deposition were altered. Since the mutant reverted to a phenotype identical to the parental strain with a frequency consistent with an initial point mutation, apparently a single defect resulted in alteration of the deposition of the spore coats on to the outer forespore membrane. Despite this defect, mutant cells were able to synthesize and process spore coat precursors into an array of morphological layers very similar to the wild-type. There are apparently distinct morphogenetic pathways for the formation of the spore body and coat layers.

INTRODUCTION

The spores of *Bacillus* species contain a complex array of proteinaceous layers on the outer surface. These coats not only have a protective function but apparently some role in germination (Aronson & Fitz-James, 1975, 1976; Stelma et al., 1978). In *Bacillus cereus*, there are three distinct morphological layers comprised of keratin-like polypeptides stabilized by disulphide bonds and hydrophobic interactions (Aronson & Fitz-James, 1976). These low molecular weight polypeptides are synthesized rather early in sporulation, before the coats are visible, as a larger precursor that is then processed by a major intracellular protease (Cheng & Aronson, 1977).

The site(s) of synthesis of the coat precursor is not known, although there is some immunological evidence for coat formation within the forespore (Short et al., 1977). Coat deposits are first noted 3 to 4 h after the commencement of sporulation as small, separate pieces assembled in the cytoplasm close to the outer forespore membrane (Ohye & Murrell, 1973; Aronson & Fitz-James, 1976). Subsequently the coat is extended around the forespore as larger, interrupted fragments and eventually is found in close proximity to the outer forespore membrane with no intervening cytoplasm.

A variety of coat mutants have been isolated in *B. cereus* and more recently in *B. subtilis* (Aronson & Fitz-James, 1975; Stelma et al., 1978; Smith et al., 1978). In most of these, coat is deposited irregularly resulting in gaps exposing the cortex. The spores are thus very sensitive to lysozyme and simultaneously lose their ability to respond rapidly to germinants.
There are also reports of stage IV mutants in *B. subtilis* that form coat-like deposits in the cytoplasm in addition to heat-sensitive coatless spores (Coote, 1972; Piggot, 1973). There is some preliminary evidence that these deposits contain coat antigens (D. Wood, personal communication).

In our efforts to characterize the morphogenetic reactions required for spore coat formation, we have isolated *B. cereus* mutants producing spores that are extremely sensitive to lysozyme. In two of these mutants the typical products of sporulation were a coatless spore plus a deposit adjacent in the cytoplasm. The coat deposits and spore bodies have been isolated and characterized. The deposits appear to be very similar to mature coats in morphology, antigenicity and proteins present. The ability of these mutants to assemble complete spore coats separate from the spore body implies that distinct pathways exist for the formation of the two structures.

**METHODS**

**Cell growth and isolation of mutants.** *Bacillus cereus* T was grown in G-tris medium (Aronson *et al.*, 1971) at 30 or 37 °C in a rotary shaker as previously described (Stelma *et al.*, 1978). Growth was monitored in a Coleman 8 colorimeter employing a 655 nm filter. For microscopy samples and for some batches of antigens, mutants were grown in liquid or on agar GBBM (Young & Fitz-James, 1959) modified by the replacement of the papain digest of beef by 0.32% (w/v) nutrient broth (Difco), the replacement in the salts (Grelet, 1951) of half of the KH₂PO₄ by K₂HPO₄ and the omission of KOH.

For mutant isolation, washed spores (Aronson & Pandey, 1978) were treated with ethyl methanesulphonate (Ito & Spizizen, 1971), collected by centrifugation at 12000 g for 10 min in a Sorvall SS34 rotor and washed twice with 20 ml sterile 0-05 M-Tris/HCl (pH 7-4). The spores were then inoculated into G-tris medium and the resulting cultures were incubated at 37 °C for 24 to 36 h until spores were again formed. The spores were washed once with and suspended in 0-1 M-sodium phosphate (pH 7-0), heat-activated at 80 °C for 20 min and germinated by addition of inosine (0.5 mM) plus L-alanine (5 mM) for 20 min. The spores were again heated at 80 °C for 20 min to kill those that had germinated. The suspension was again inoculated into G-tris medium and the cycle of spore production, germination and killing was repeated twice more. After the third cycle, spores were spread on G-tris agar and the plates were incubated at 37 °C for 24 to 36 h. Spores were picked from individual colonies, suspended in 30 to 50 ml 0-05 M-Tris/HCl (pH 7-8) plus 5 μg lysozyme (EC 3.2.1.17) and incubated at 27 °C for about 1 h. The suspensions were examined under a Zeiss phase contrast microscope for darkening of the spores. Colonies containing lysozyme-sensitive spores were restreaked on G-tris agar and again tested for lysozyme sensitivity.

**Isolation of revertants.** To ensure against scoring wild-type contaminants as revertants, spontaneous streptomycin-resistant derivatives of the mutants were selected by first growing cells in G-tris medium containing 100 μg streptomycin ml⁻¹ for 2 to 3 h. Cells were then spread on G-tris agar containing 100 μg streptomycin ml⁻¹.

Mutants were grown in G-tris medium at 37 °C and the resulting spores were washed and suspended in 0-05 M-Tris/HCl (pH 7-8) at about 5 × 10⁶ spores ml⁻¹. Filter-sterilized lysozyme was added (200 μg ml⁻¹) and the suspension was incubated on a rotary shaker at 37 °C for 90 min. The spores were then placed in a boiling water bath for 5 min to kill those that were lysozyme-sensitive. The suspension was spread on G-tris agar containing 100 μg streptomycin ml⁻¹ and the plates were incubated at 37 °C for 30 to 40 h until free spores were present. The plates were then covered with 1-5 ml lysozyme (1 mg ml⁻¹ in 0-05 M Tris/HCl, pH 7-8) and reincubated for 2 to 3 h at 37 °C. Those colonies that did not turn dark (Cheng & Aronson, 1977) were restreaked on G-tris streptomycin agar and the spores were again tested for lysozyme resistance.

**Characterization of spores.** The quantity of coat protein per spore was determined as previously described (Stelma *et al.*, 1978). Heat resistance (80 °C for 20 min) and germination rates were determined as previously described (Aronson & Fitz-James, 1975). Dipicolinic acid was determined colorimetrically by the method of Janssen *et al.* (1958). Slab gel electrophoresis of solubilized spore proteins was carried out by the procedure of Laemmli (1970) modified as previously described (Stelma *et al.*, 1978). Rates of incorporation of [³⁵S]-cystine were determined by methods previously outlined (Aronson & Fitz-James, 1968) using glass-fibre filters (Whatman, GF/A). Spore yields were measured by direct counting in diluted samples of cultures in a Petroff–Hauser counting chamber.

**Isolation of spore coats from mutants.** Cytoplasmic deposits of spore coats were isolated by growing the mutants on G-tris or GBBM agar at 30 or 37 °C for 30 to 40 h. Spores and other material were removed from the surface by scraping with a glass rod and suspending in sterile saline (0-14 M-NaCl, pH 8-0). After centrifuging at 17 300 g for 15 min in a Sorvall SS34 rotor, the pellet was suspended in a small volume of saline (about 1 ml per content of one agar plate). Renografin [76% (w/v) stock solution consisting of 66% (w/v)
Defective spore coat deposition

Fig. 1. Phase contrast photomicrograph of *B. cereus* G101 after 22 h aeration at 30 °C in liquid GBBM. Most of the spores are fully refractile. Note the oblique, transverse and lateral displacement of the spores. The parasporal coat deposits are not readily seen in the dense cytoplasm. Bar marker represents 5 μm.

diatrizoate meglumine plus 10% (w/v) sodium diatrizoate] was then added to 20% (w/v) final concentration and the suspension was placed on ice for 15 min before layering over 8 ml 30 to 60% linear Renografin gradients. The tubes were centrifuged in a Sorvall HB4 swinging bucket rotor at 17300 g for 30 min. An opaque band at a density somewhat less than that of spores was removed with a syringe and shown to contain spore coat antigen (Ouchterlony diffusion plates), spore coat proteins (Fig. 4) and many structures with the appearance of spore coats in thin sections (P. C. Fitz-James, unpublished observations). Some of the spores banded but most pelleted, indicative of high density spores that were probably coat deficient. The spore coat band was diluted four- to fivefold with deionized water and collected by centrifuging at 17300 g for 15 min in a Sorvall SS34 rotor. The pellet was extracted with reagents used for spore coat solubilization (Stelma et al., 1978) or mixed with Freund's complete adjuvant and injected into rabbits for antibody production (Horn et al., 1973).

**Microscopy.** Dark phase contrast microscopy of cover-slip smears was routinely used to monitor sporulating cultures. Photomicrographs were made on Ektapan cut film (Kodak) at 1995 x magnification and enlarged twice in printing.

Samples of sporulating cells for thin-section electron microscopy were taken at various times from liquid cultures growing at 30 °C. After chilling to 2 °C they were fixed first with cold 2% (v/v) glutaraldehyde then with osmium tetroxide using the methods previously described (Fitz-James, 1971). In some samples, preservation of mature spores was achieved by the triple fixation procedure of Stevenson et al. (1972).

Samples for freeze-fracturing and cleave-etching were taken from both liquid and agar sporulated cultures and processed as previously described (Aronson & Fitz-James, 1975).

**RESULTS**

**Properties of the mutants**

Enrichment for slowly germinating mutants was also effective for obtaining mutants forming lysozyme-sensitive spores. After three cycles, 10 to 20% of the colonies screened contained lysozyme-sensitive spores. Of 133 isolates, two, labelled G101 and G102, exhibited an extreme sensitivity to lysozyme and have been studied in detail.

Both mutants grew at the same rate as the wild-type at either 30 or 37 °C in G-tris or in a minimal medium, CDGS (Nakata, 1964). By phase contrast microscopy, sporulation proceeded normally up to whitening (stage IV) when the absence of a developing coat
Fig. 2. Lysozyme sensitivity of mutant and wild-type spores. Spores were washed and suspended in 0.05 M-Tris/HCl (pH 7.8) and lysozyme was added to the final concentrations indicated below. The decrease in $A_{660}$ was followed in a Zeiss spectrophotometer. △ Wild-type spores plus 100 µg lysozyme ml$^{-1}$; ◆ G101 spores plus 5 µg lysozyme ml$^{-1}$; ○, G102 spores plus 10 µg lysozyme ml$^{-1}$.

Fig. 3. Germination of mutant G101 and wild-type spores. Spores were heat-activated at 80 °C for 20 min and germinated in 0.1 M-sodium phosphate buffer (pH 7.0) plus 5 mM-L-alanine and 0.5 mM-adenosine. The decrease in $A_{660}$ was followed in a Zeiss spectrophotometer. ○, Wild-type spores; ◆, G101 spores.

Table 1. Summary of properties of mutant and wild-type spores

<table>
<thead>
<tr>
<th>Property</th>
<th>Wild-type</th>
<th>Mutant G101</th>
<th>Mutant G102</th>
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</thead>
<tbody>
<tr>
<td>Resistance to 80 °C for 20 min (%)</td>
<td>95-100</td>
<td>93-100</td>
<td>93-100</td>
</tr>
<tr>
<td>Dipicolinic acid (% dry wt)</td>
<td>12 ± 2</td>
<td>15 ± 2</td>
<td>14 ± 2</td>
</tr>
<tr>
<td>Coat protein per spore* (10$^{-13}$ g)</td>
<td>1.6 ± 0.15</td>
<td>0.48 ± 0.08</td>
<td>0.4 ± 0.05</td>
</tr>
<tr>
<td>Reversion frequency</td>
<td>—</td>
<td>10$^{-7}$-10$^{-8}$</td>
<td>10$^{-7}$-10$^{-8}$</td>
</tr>
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* Value for crude spore pellet after washing and extraction as described in Methods.

allowed many spores to become twisted from the normal longitudinal alignment of wild-type B. cereus spores. This oblique or even transverse alignment of the spore was seen in agar and liquid sporulating cultures of both mutants (Fig. 1).

As with previously studied lysozyme-sensitive mutants (Stelma et al., 1978), the formation of a refractile endospore was somewhat slower than in the wild-type and the release of the mature spore from the mother cell was very slow and asynchronous. The free spores of G101 or G102 were unstable. Within 24 h of liberation most were phase dark and about half completely lysed. Spore suspensions could be stored at −20 °C in aqueous buffer in 60% (v/v) glycerol or in 1 M-NaCl. The spore yield of G101 aerated in liquid culture at 30 °C and counted prior to the lysis of any spores was 2.0 x 10$^8$ spores ml$^{-1}$; that of the parent culture B. cereus T under identical conditions was 4.7 x 10$^8$ spores ml$^{-1}$.

Both mutants were very sensitive to lysozyme at 5 to 10 µg ml$^{-1}$ (Fig. 2), in contrast to other lysozyme sensitive mutants previously studied (Aronson & Fitz-James, 1975; Stelma et al., 1978) where treatment with lysozyme at 100 µg ml$^{-1}$ resulted in only a partial decrease in absorbance in 15 min. There was also a very slow response to the germinants L-alanine plus inosine (or adenosine, Fig. 3).
Defective spore coat deposition

Fig. 4. Gel electrophoresis profiles of wild-type, Gl01 and Gl02 coat extracts. Electrophoresis was performed as described in Methods. Gels contained 12.5% (w/v) acrylamide, 0.1% (w/v) sodium dodecyl sulphate and 6 M-urea.

(a) Spore extracts. 1, Standards (from top to bottom) bovine serum albumin (65000 daltons), RNAase (13700 daltons) and cytochrome c (12700 daltons); 2, chymotrypsinogen (25000 daltons); 3, wild-type spores; 4 to 6, different amounts of Gl01 spores; 7 and 8, wild-type spores; 9 to 11, Gl02 spores; 12, wild-type spores.

(b) Extracts of Gl01 fractionated on Renografin gradients. 1, Standards as in (a, 1 and 2); 2, extract of Gl01 spores prior to gradient fractionation; 3 and 4, extract of spore coat band; 5, extract of spore pellet.

Other properties of these mutant spores are summarized in Table 1. Revertants to lysozyme resistance occurred at a frequency of $1 \times 10^{-8}$ and they all responded to germinants as did the wild-type. The mutants were heat-resistant, contained the same quantity of dipicolinic acid as the parental strain but only about 30% of the wild-type coat protein per spore. As mentioned in Methods, there were two spore populations on the basis of density in Renografin. Most of the spores were very dense and pelleted through 60% Renografin; the remainder banded at a somewhat greater density than wild-type spores. The value for coat protein per spore is an average of these two populations (plus any fragments trapped in the spore pellet). As shown by electron microscopy (see below) most (60 to 80%) of the spores are virtually devoid of coat and others may have 70 to 80% of the wild-type amount.

Characterization of spore coat proteins

Protein extracted either directly from the spores or from the isolated spore coats showed after electrophoresis (Fig. 4) an array of proteins virtually identical to that from wild-type spores, with predominant components of 13000 and 26000 daltons. Since one or more of these low molecular weight proteins is processed from a 65000 dalton precursor (Cheng & Aronson, 1977) at least this portion of coat synthesis was completed in the mutants. In addition, antibody to the isolated mutant spore coats completely cross-reacted with wild-type coats (and vice versa) on Ouchterlony plates. Antibody to the mutant spore coats very effectively precipitated spore coat antigen from extracts of wild-type sporulating cells. The amount of coat protein found in the free coat band was 30 to 100% of that present on an
equivalent number of wild-type spores (1.6 \times 10^{-13} \text{ g per spore}; \text{Aronson} \& \text{Fitz-James}, 1976). The variation was probably due to lysis of the spores during washing and incomplete recovery of the coat band from Renografin gradients. In any case, there does not appear to be a major alteration in the rate of coat protein synthesis.

The electron microscopic appearance of the coat deposits, however, indicated a reduction in the formation of the cross-patch layer (Fig. 15). Since the rearrangement of coat polypeptides into cross-patch is apparently dependent on an exchange with half cystine residues (Aronson \& Fitz-James, 1976), we compared the uptake of [^{14}\text{C}]cystine in mutant GlOl with that of the parent culture during late stages of sporulation. The rate of uptake by the mutant was greatly reduced (Fig. 5) and the decrease was much greater than could be accounted for by reduction in the number of cells forming spores in the mutant (twofold) or by comparison to the total rate of protein synthesis as measured by [^{3}\text{H}]isoleucine incorporation (results not shown).

**Electron microscopy**

No abnormality in growth or sporulation in these two mutants was evident from micrographs of thin-sectioned material up to and including stage III of sporulation. In stage IV development of the germ cell wall and cortex appeared normal. However, coat deposition which normally begins in stage IV and is localized about the spore was first seen in these mutants as a whorl nearby in the cytoplasm (Fig. 6). Through stage V the deposit of coat developed and in stage VI changes appeared in the spore body (Fig. 7) which are normally associated with the refractility stage, i.e. dipicolinic acid synthesis, calcium uptake and spore dehydration. The micrographs indicate that much of the deposited coat material consisted of flexible whorls of exosporium and less flexible deposits of pitted layer and accompanying undercoat (Fig. 7). The patterned cross-patch layer was only occasionally seen in sections of the deposit. A rare spore could be found with a coat deposit but even these were incomplete and often redundant (Fig. 8). Likewise some of the coat deposits assumed a near normal arrangement of their layers around a whorl of exosporium (Fig. 9). It is interesting that the clearing of the cytoplasm at the lytic stage appeared to start in the region of the coat deposit, particularly around the whorls of exosporium, suggesting a site of release or activation of lytic enzymes (Fig. 7).
Defective spore coat deposition

Fig. 6. Stage IV of sporulation in *B. cereus* G101 after 14.5 h aeration in liquid GBBM at 30 °C. With the formation of the cortex (CX) the changes associated with early refractility are taking place: a peculiar density of the cytoplasm, crystalline appearance of the nucleoid (N) and the poor preservation of the pericortical or outer membrane zone. Coat (CT) deposition is occurring in the cytoplasm rather than on the spore. Bar marker represents 100 nm.

Freeze etchings confirmed the cytoplasmic deposition of coat and exosporium. Spores cleaved while still in the cell, although fully ripe, showed only outer membrane (Fig. 11) or, in a rare case, a disorganized globular mass over the cortical surface (Fig. 10). Profiles of extensive whorls of exosporium and spore coat were evident in most cells (Figs 10 and 12). The exposure of the outer membrane on ripe spores by freeze etching is unusual. It is not seen in cleavages of ripe wild-type spores (Aronson & Fitz-James, 1976) perhaps due to sporulation lysis or to the incorporation of the membrane into the undercoat layer. In any event, cleavage planes of ripe coated spores passed either through the coat or over the cortex but with no revelation of the outer membrane leaflets as shown here in Figs 11 and 12.

The free spores of these mutants showed little or no outer membrane on the cortical surface (Fig. 13). Cleavage into a spore revealed the physical state peculiar to coated spores. Other than the six or so lamellae of the cortex, the germ cell wall, inner membrane
Fig. 7. After 19 h aeration in liquid GBBM at 30 °C, the fully refractile spores of B. cereus G101 share the cell space with the deposited coat material arranged in the form of a wound layer of exosporium (EX) and stacked massed coat layers (CT). The spore body is severely displaced. Stage VII lysis appears to begin in the region of the deposited coat. Bar marker represents 1 μm.

Fig. 8. An occasional mutant has a coat, but the deposition seldom covers the spore completely and often shows bare cortex and redundancy of layers. The exosporium on such rare spores is apparently complete. Bar marker represents 100 nm.

Fig. 9. Rarely the coat and exosporium are found arranged in near normal spore coat structures apart from the adjacent spore body. Bar marker represents 100 nm.
Defective spore coat deposition

Fig. 10. *Bacillus cereus* G101 after 4 d aeration in liquid GBBM. Many spores are still not liberated. This freeze-etch shows the exosporium and spore coat deposit adjacent to the spore body. The remaining outer membrane (OM) appears to be sheared or undergoing lysis over the surface of the cortex (CX). Bar marker represents 100 nm.
Figs 11 and 12. *Bacillus cereus* G101 after 7 d on GBBM agar. About 70% of the spores are free (see Fig. 13). Bar markers represent 100 nm.

Fig. 11. Outer membrane (OM) is still present on the surface but cleaved revealing the convex outer surface of its inner sheet. The opening in the OM revealing the cortex (CX) could be from cleavage or, more likely, lysis.

Fig. 12. The extensive floret of coat material is close to, but not on, the spore body represented here by a glimpse of the concave inner surface of the outer half of the outer membrane (OM).
and spore coat were undifferentiated (Fig. 13). The rare spore that did attain a coat showed the typical pattern of the cross-patch layer, with occasionally some pitted coat and the underlying cortex. Unlike wild-type *B. cereus* these rare coated forms were incompletely covered (Fig. 14). Cleavage of the liberated coat deposits confirmed the observation from sections that these structures, in keeping with the finding of reduced cystine uptake, were deficient in the cross-patch layer. They showed largely the pitted or under coat patterns (Fig. 15).

**DISCUSSION**

The two mutants, G101 and G102, were very similar in phenotypic properties, with slight differences in sensitivity to lysozyme (Fig. 2) and perhaps in the gel profile of spore coat proteins (Fig. 4a). The differences in the latter were in minor bands that may have been due to contaminants or differential spore lysis during extraction.
Both mutants produced a completely refractile, heat-resistant spore body that was apparently normal in cortex structure and in content of dipicolinic acid. It is interesting that the outer forespore membrane was present on these bodies prior to maturation. Its loss or disintegration seemed to occur even in the absence of coat deposition and may be dependent on the release of a refractile, heat-resistant spore body.

The mutants were capable of producing and processing spore coat protein and of assembling these monomers into structural layers. The amount of coat protein synthesized (30 to 100% of that of the wild-type) was not accurately determined for technical reasons, but was probably close to that produced by the wild-type. The primary defect in the mutants is probably in coat deposition, perhaps an alteration in the outer forespore membrane. The absence of a cross-patched layer in the coat deposits (and the low incorporation of half cystine, Fig. 5) is likely to be due to the extensive folding of these coat deposits (Figs 7, 10, 12 and 15) and thus the inaccessibility of certain coat proteins for the disulphide interchange
necessary for the formation of the cross-patched layer (Aronson & Fitz-James, 1976). In the rare cases when coat deposited on the spore, a cross-patched layer was present (Fig. 14), implying that the mutant did have the capacity to carry out disulphide exchange.

There was no morphological evidence that the coat was deposited on the forespore membrane in the mutants and subsequently removed, but rather that the sites of deposition were abnormal. It appears, therefore, that two series of morphogenetic reactions, coat maturation and formation of a dormant spore body, proceed independently.

Morphologically similar mutants have been isolated in B. subtilis and partially characterized (Coote, 1972; Piggot, 1973). They were classified as being blocked at stage IV because of the lack of coat deposition around the spore. The coat-like deposits from these mutants should be isolated and characterized to see if synthesis of the total array of B. subtilis spore coat proteins, as well as the cross-linking reactions (Aronson & Pandey, 1978; Goldman & Tipper, 1978), have occurred.

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REFERENCES


