Involvement of the Spore Coat in Germination of
Bacillus cereus T Spores†

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Bacillus cereus T spores were prepared on fortified nutrient agar, and the spore coat and outer membrane were extracted by 0.5% sodium dodecyl sulfate–100 mM dithiothreitol in 0.1 M sodium chloride (SDS-DTT) at pH 10.5 (coat-defective spores). Coat-defective spores in L-alanine plus adenosine germinated slowly and to a lesser extent than spores not treated with SDS-DTT, as determined by decrease in absorbance and release of dipicolinic acid and Ca2+. Spores germinated in calcium dipicolinate only after treatment with SDS-DTT. Biphasic and triphasic germination kinetics were observed with normal and coat-defective spores, respectively, in an environment with temperature increasing from 20 to 65°C at a rate of 1°C/min. Therefore, the physical and biochemical processes involved in germination are modified by coat removal. The data suggest that a portion of the germination apparatus located interior to the coat may be protected by the coat and outer membrane or that the coat and outer membrane otherwise enhance germination. Therefore, coat and outer membrane are essential for fast germination.

Germination is the process by which a dormant spore is converted into a vegetative cell. Once triggered to germinate, spores sequentially lose heat resistance, release dipicolinic acid (DPA) and Ca2+, and lose refractivity when viewed by phase contrast microscopy, and their ability to scatter light decreases. The mechanism(s) by which bacterial spore germination is initiated and progresses is not known. To understand germination, knowledge of the role(s) of bacterial spore components in germination is essential.

The bacterial spore structure consists of an inner core surrounded by the inner membrane, a cortex surrounded by the outer membrane, and an exterior coat. The coat frequently is surrounded by a loosely attached exosporium (8,13). The spore coat consists largely of structural proteins (2,29) with small amounts of lipid and carbohydrate (6). The spore coat appears to have a role in germination; mutants lacking spore coats or with defective coats and spores from which the coats and outer membrane have been removed chemically have altered, typically more fastidious, germination requirements. Such spores can be germinated artificially by addition of cortex-lytic enzymes, such as lysozyme, or spore-specific enzymes (1,4,27). Coatless spores have been observed to germinate at a decreased rate (13,17,18,28), and in some cases mutants lacking spore coats germinate at a rate comparable to normal spores (26). The exact involvement of the coat and outer membrane in germination is unclear. The coat or outer membrane may serve as one location where the germinant interacts with the spore (trigger site), protect the germination trigger sites, be affiliated with cortex-lytic enzymes, or otherwise be involved in germination. This paper reports studies characterizing the germination of normal and coat-defective Bacillus cereus T spores to identify the significance of the coat and outer membrane to spore germination.

MATERIALS AND METHODS

Bacterial strain and preparation of spore suspension. B. cereus T was from the North Carolina State University culture collection and originally was obtained from K. Johnson and F. Busta at the University of Minnesota. The culture was sporulated on fortified nutrient agar by the procedures of Johnson et al. (11), except that growth and sporulation were at 35°C. The spores were harvested, washed approximately eight times, and suspended in distilled water, with clumps dispersed as detailed by Foegeding et al. (9).

Extraction of the coat plus outer membrane. The spore coats and outer membranes were removed by the methods of Fitz-James (8) and Vary (28). A spore suspension containing approximately 108 spores per ml was sedimented, and the pellets were suspended in 100 mM dithiothreitol (DTT; Eastman Kodak Co., Rochester, N.Y.) plus 0.5% sodium dodecyl sulfate (SDS; Fisher Scientific Co., Raleigh, N.C.) in 0.1 M NaCl. The suspension was adjusted to pH 10.5 and incubated for 18 h at 37°C. Following incubation, the spores were washed approximately eight times in 0.14 M NaCl–0.3 M sucrose (pH 9.0), and the supernatant liquids were pooled for later use. Spores were suspended in sterile distilled water, shaken (100 rpm) overnight at 4°C with sterile glass beads to prevent clumping, and stored at 4 ± 2°C. The SDS-DTT-treated spores are referred to as coat-defective spores, and nontreated spores are referred to as normal spores. The extracted supernatant liquids were dialyzed (membrane cutoff molecular weight, 12,000; Spectrum Medical Industries, Inc., Los Angeles, Calif.) overnight against 20% polyethylene glycol at 4°C. The extract was suspended in 1/50 of the original volume of 0.1 M sodium phosphate buffer, pH 7.0, and stored at 4 ± 2°C.

Testing for spore coat removal. The efficiency of spore coat removal was tested on the basis of the ability to adhere to n-hexadecane and observed by transmission electron microscopy.

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(i) Hydrophobicity. Cell surface hydrophobicity was determined by the difference in partitioning of normal and coat-defective spores in n-hexadecane (7, 21, 22). Spores were diluted in 0.1 M sodium phosphate buffer, pH 7.0, to an optical density at 450 nm between 0.5 and 1.0. Duplicate test tubes containing 3.0-ml portions of the spore suspension and volumes of n-hexadecane ranging from 0 to 3.0 ml were preincubated for 10 min at 35°C before being vortexed vigorously for 2 min. After the phases were allowed to separate for 15 min, the absorbance of the lower aqueous phase was measured at 450 nm. The results of this procedure were confirmed by phase contrast microscopic examination of the upper and lower phases.

(ii) Electron microscopy. Spores in Nobel agar were fixed in 3% glutaraldehyde and 1% OsO₄, embedded in Epon, and stained with uranyl acetate and lead citrate as detailed by Beaman et al. (4). All reagents were from Ladd Research Industries, Inc., Burlington, Vt. Observations were made with a Jeol 100S transmission electron microscope.

Spore germination experiments. Spores were heated activated for 15 min at 80°C after being diluted in filter-sterilized (0.45-μm filter) 0.1 M sodium phosphate buffer (pH 7.0) and suspended at a density of about 10⁶ spores per ml in germination medium tempered as desired. Spores were germinated within 2 h after heat activation. Germination was monitored by four procedures, including spectrophotometric absorbance decrease, loss of heat resistance, release of DPA, and release of Ca²⁺, as detailed below. Frequently, germination was confirmed by phase contrast microscopy. Unless stated otherwise, duplicate or triplicate trials of all germination studies were performed.

(i) Spectrophotometric evaluation. Germination at 35°C was monitored for 10 min by measuring the absorbance decrease at 660 nm (model 2600 spectrophotometer, connected to a thermoprocessor; Gilford, Oberlin, Ohio). Spores and germination medium were mixed in microcuvettes, and measurements commenced within 10 s of exposure of the spores to the germinant. Typically the initial absorbance was 0.8. The percent decrease in absorbance was used to indicate the extent of germination.

(ii) Loss of heat resistance. Portions (2 ml) of normal and coat-defective spores germinating at 35°C were removed at 5-min intervals, heated at 80°C for 20 min (or not heated), serially diluted in 0.1% peptone–water, and enumerated on tryptic soy agar incubated for 18 h at 35°C. The total population was approximately 10⁸ spores per ml. The percentage of heat-resistant (not germinated) spores at a given sampling time was calculated.

(iii) Determination of DPA and Ca²⁺. DPA and Ca²⁺ released during germination of normal and coat-defective spores were measured on three occasions. Duplicate DPA and Ca²⁺ measurements were obtained at each sampling time, and averages of the six values at each time are reported. At appropriate intervals 2-ml samples of germinating spores were removed, added to centrifuge tubes containing 1 ml of cold 40 mM d-aldarone to block further germination, mixed, and kept on ice. The suspensions were centrifuged at 4,000 × g for 15 min at 4°C, and the supernatant liquids were filtered through 0.45-μm membrane filters. DPA in the filtered medium was determined by a modification of the method of Warth (31). Samples (20 μl) were injected into an organic packed C₁₈ reverse-phase column (PRP-1 reverse-phase column; Hamilton, Las Vegas, Nev.). Elution was conducted with 8% tert-amylalcohol in 0.2 N sulfuric acid, pH 1.4, at a flow rate of 0.6 ml/min, and peak heights at 271 nm were measured. Chromatography of each sample was complete within 5 min, and samples were injected at 6-min intervals. All samples were preceded within 30 min by freshly dissolved DPA (Aldrich Chemical Co., Milwaukee, Wis.) external standard solutions.

The filtered supernatant medium not used for DPA assays was frozen (~20°C) for calcium determination by atomic absorption spectrophotometry (model 5000 atomic absorption spectrophotometer; Perkin-Elmer, Norwalk, Conn.) at 422.7 nm. Samples were thawed, and lanthanum oxide (0.1%) was added to each sample to overcome suppression of calcium absorption by phosphate ions. Results are reported as the percent DPA and Ca²⁺ released at each time when the total DPA or Ca²⁺ contents of each spore preparation were determined for spores burst by autoclaving. The DPA content of autoclaved normal and coat-defective spores was 171.5 and 165.2 nmol/10⁸ spores, respectively; Ca²⁺ content was 90.1 and 79.6 nmol/10⁸ spores, respectively.

(iv) Germination media and temperatures. To select a system permitting rapid germination, germination of normal, nonextracted B. cereus T spores was monitored by adding spores to L-alanine solutions ranging from 0.2 to 20 mM, each with 10 mM adenine, and in 10 mM glucose, 10 mM t-alanine, 20 mM t-alanine plus 10 mM glucose, and 20 mM t-alanine plus 10 mM adenosine. The solutions were in distilled water in each case. In most subsequent experiments, 20 mM t-alanine plus 10 mM adenosine at 35°C was used. Germination also was evaluated at 35°C by adding spores to 20 mM calcium dipicolinate (CaDPA), 20 mM DPA (each in distilled water), and tryptic soy broth (TSB) with nalidixic acid (0.232 mg/ml) added to prevent outgrowth. The germination media represented defined and complex media including both nutrient and non-nutrient germinants. Spores in phosphate buffer were added to the germination media so that the actual medium concentrations during germination were 50% of those indicated and the final buffer concentration was <2.5 mM. The spore-medium ratio was approximately 1:2 for all germination procedures. Normal and coat-defective spores also were studied in an environment where the temperature increased from 20 to 65°C at a rate of 1°C/min. The temperature increase was controlled by a thermoprogrammer connected to the spectrophotometer as detailed previously.

Coat-defective spore germination with spore extract. A suspension of coat-defective spores prepared as detailed above was heat activated with dialyzed spore extract (1:2 [vol/vol] ratio) and germinated by addition to 20 mM t-L-alanine plus 10 mM adenosine at 35°C. Additionally, spores and extract were heated separately, mixed, and added to the germination medium, and spores without added extract were similarly germinated. Germination was monitored spectrophotometrically for 15 min as detailed previously.

RESULTS AND DISCUSSION

The rate of B. cereus T spore germination (determined by absorbance) in L-alanine plus adenosine increased as the L-alanine concentration increased from 0.2 to 10 mM, with no further change in rate in 20 mM L-alanine; adenosine or glucose alone had little effect on germination (data not shown). However, adenosine enhanced germination in L-alanine. Therefore, 20 mM L-alanine plus 10 mM adenosine was used as the germination medium for most subsequent studies.

The spore coat and outer membrane were partially removed or disrupted by alkaline SDS-DTT treatment (3, 7, 13, 18, 28); the resultant spores are referred to as coat-
The release of DPA and Ca\(^{2+}\) by coat-defective spores during germination in L-alanine plus adenosine at 35°C was slower and less overall than by normal spores (Fig. 4). For example, about 59% of the DPA in coat-defective spores was released in the first 5 min of germination at 35°C, compared with 88% released from normal spores for the same period. Detection of both components 30 s after addition of the germinant was expected, since release of DPA and Ca\(^{2+}\) is associated with an early germination event (23).

The patterns of DPA and Ca\(^{2+}\) release from normal and coat-defective spores were similar to the observed absorbance data. Therefore, heat resistance data appear to overestimate germination of coat-defective spores. Loss of heat resistance is an early germination event. Given the complex nature of spore germination, loss of heat resistance may proceed rapidly in coat-defective spores, while the later germination changes (Ca\(^{2+}\) and DPA release and absorbance decrease) may be rate limiting and result in an overall slower response of coat-defective spores. Alternatively, coat-

![FIG. 1. Partitioning in n-hexadecane of normal B. cereus T spores with intact coats (●, ■) and coat-defective (○, □) B. cereus T spores. Duplicate spore suspensions in 0.1 M sodium phosphate buffer (pH 7.0) were mixed with n-hexadecane and the amount of adherence was determined by absorbance (ABS) at 450 nm of the aqueous phase after phase separation.](image)

defective spores. To confirm the removal of these outer layers, normal and coat-defective spores were examined for their ability to partition in n-hexadecane and by electron microscopy. Normal spores adhered to n-hexadecane more than the coat-defective spores (Fig. 1), indicating at least partial removal of the outer spore layers and exposure of the cortex. The hydrophobic nature of the spore coat, and not the coatless spore, indicates that the coat constituents are responsible for the hydrophobicity (S. E. Craven and L. C. Blankenship, Abstr. Annu. Meet. Am. Soc. Microbiol. 1986, 1103, p. 182). Murrell (16) reported that bacterial spores do not absorb water as well as protoplasts do, suggesting that spores are hydrophobic. Warth (30) attributed the ability to bind hydrophobic solvents to the presence of sites for adherence of hydrocarbons provided by the proteinaceous coats. Neither vegetative cells nor coatless spores contain significant amounts of surface proteins (15) and thus lack hydrophobic binding sites provided by the proteinaceous spore coat. Transmission electron micrographs confirmed that treatment of spores with SDS-DTT removed about half of the spore coat plus the outer membrane (Fig. 2). The coat-defective spores appeared refractile by phase-contrast microscopy and retained their refractility for at least 4 months when stored in cold, sterile, deionized water. However, the percentage of refractile spores decreased with the length of storage. Others have indicated that coat-extracted spores cannot be stored because of rapid, spontaneous germination (8, 13).

The rates of germination of normal and coat-defective spores in L-alanine plus adenosine at 35°C were compared (Fig. 3). Coat-defective spores germinated more slowly and to a lesser extent in 10 min than normal spores, as determined by absorbance (Fig. 3). The maximum rates of absorbance decrease were 19 and 6% per min for normal and coat-defective spores, respectively. More than 90% of the normal and only ca. 70% of the coat-defective spores lost refractility in about 5 min, as determined by phase-contrast microscopy. The germination differences of normal and coat-defective spores in L-alanine plus adenosine at 35°C observed spectrophotometrically were not substantiated by heat resistance data; coat-defective spores showed a slightly higher rate and extent of loss of heat resistance than did normal spores in the first 10 min (Fig. 4). Greater than 99% of both normal and coat-defective spores lost heat resistance by 30 min.

![FIG. 2. Transmission electron micrographs of normal (A) and coat-defective (B) B. cereus T spores. The coat (c) removal or disruption in coat-defective spores is evident between the arrows and in other regions. Bar, 0.2 μm.](image)
defective spores may be less heat resistant, making this a poor germination indicator. When the heat resistance of normal and coat-defective spores was measured in phosphate buffer at 90.3°C, the D values were 11 and 8 min, respectively. Comparing changes in absorbance, Ca2+ and DPA release, and phase appearance, it appears that the absorbance changes in coat-defective and normal spores correspond to comparable changes in the other germination indicators (Table 1). Thus, a given percent absorbance change represents comparable changes in phase appearance and fractions of DPA and Ca2+ released for both coat-defective and normal spores. The slow germination rate at 35°C for coat-defective spores, as indicated by the decrease in absorbance and release of DPA and Ca2+, suggests that the overall ability of spores to germinate in nutrient medium is impaired by disruption of the spore coat or the outer membrane or both.

The germination response of spores added to 20 mM L-alanine plus 10 mM adenosine was studied in environments in which the temperature was constantly rising (1°C/min from 20 to 65°C) to evaluate further the effect of defective coat and outer membrane on germination. The normal spores exhibited biphasic germination kinetics, while coat-defective spores showed triphasic germination kinetics (Fig. 5). The first phase of germination in normal spores was the most rapid (6.7% absorbance decrease per min) and extensive, occurring before the temperature reached 30°C. The second phase exhibited a slower rate (1.8% per min) of absorbance decrease and occurred between 56 and 62°C. The first and second phases of germination of coat-defective spores exhibited greater absorbance decreases and rates (3.5% and 4.7% per min, respectively) than the third phase (1.3% per min). The total percent absorbance decrease for normal spores was 10 to 17% more than for coat-defective spores. These data suggest that the extracted components

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**FIG. 3.** Germination at 35°C when added to 20 mM L-alanine plus 10 mM adenosine of coat-defective and normal B. cereus T spores, measured by spectrophotometric absorbance (ABS) decrease as detailed in the legend to Fig. 1. These data are representative of four replicate trials.

**FIG. 4.** Germination at 35°C of normal (A) and coat-defective (B) B. cereus T spores as measured by loss of heat resistance (■), release of DPA (▲), and release of calcium (▲). Spores were heat activated (80°C, 15 min) and incubated in prewarmed 20 mM L-alanine plus 10 mM adenosine germinating at 35°C. At timed intervals, samples were removed for determining the heat resistance and DPA and Ca2+ released as detailed in the text. The data are averages of duplicate assays at each time point and three replicate trials.

**FIG. 5.** Germination of coat-defective (———, three trials presented) and normal (———, two trials presented) B. cereus T spores in an environment with temperature (—-—-) constantly rising from 20 to 65°C at 1°C/min. Spores were prepared and germinated in L-alanine plus adenosine, and germination was monitored by decrease in absorbance (ABS) as detailed in the legend to Fig. 1.

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**TABLE 1.** Comparison of DPA released, Ca2+ released, and phase-dark spots appearing which correspond to a 1% absorbance decrease during germination* of normal and coat-defective spores

<table>
<thead>
<tr>
<th>Spores</th>
<th>Change represented by 1% decrease in absorbance*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>DPA (% release)</td>
</tr>
<tr>
<td>Normal</td>
<td>1.38</td>
</tr>
<tr>
<td>Coat-defective</td>
<td>1.04</td>
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</tbody>
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* Germination of spores added to 20 mM L-alanine plus 10 mM adenosine at 35°C. 
* Estimated within first 5 min.
Germination of coat-defective B. cereus T spores added to 20 mM l-alanine plus 10 mM adenosine at 35°C. Germination of spores plus extract heat activated separately, heat-activated spores with no extract, and spores plus extract heat activated together was measured by absorbance (ABS) decrease. The data are representative of quadruplicate trials.

FIG. 6. Germination at 35°C of normal (A) and coat-defective (B) B. cereus T spores in DPA, CaDPA, TSB, and l-alanine plus adenosine as measured by absorbance (ABS) decrease.

FIG. 7. Effect of spore extract on the germination of coat-defective B. cereus T spores added to 20 mM l-alanine plus 10 mM adenosine at 35°C. Germination of spores plus extract heat activated separately, heat-activated spores with no extract, and spores plus extract heat activated together was measured by absorbance (ABS) decrease. The data are representative of quadruplicate trials.

The inability of CaDPA and CaCl₂ to induce measurable germination in normal spores within 15 min could reflect lack of permeability due to the coat. This could prevent Ca²⁺ or CaDPA from penetrating to the active sites. The removal of the coat may expose the germination sites to Ca²⁺ or reduce hydrophobic repulsion of the charged ion (10) so that it can penetrate to the sites and result in the eventual germination of coat-defective spores.

This study characterizes the unique role of the spore coat and outer membrane in germination of B. cereus T spores. Slow rates of decrease in absorbance and of DPA and Ca²⁺ release by coat-defective spores germinating in nutrient media suggest an impairment to germination that is attributable to spore coat and membrane removal. Similar results have been reported by other researchers (17, 18, 28). These data agree with those of Nakatani et al. (17), who reported that the coats of B. megaterium may not be essential in germination but that the coats are necessary for fast germination of spores. The ability of coat-defective spores to germinate indicates that a required spore germination apparatus is not located in the spore coat or outer membrane, that an autonomous germination system(s) is interior to these SDS-DTT-extractable components, and that some component(s) to initiate germination or promote rapid germination is located in the coat or outer membrane. The coat or outer membrane may protect underlying germination sites, and SDS-DTT disruption of the exterior structure may permit or cause damage to the site(s). Rapid germination of coat-defective spores in CaDPA probably is a consequence of permeability changes. Skomurski et al. (24, 25) suggest that the spore germination trigger apparatus is located in the inner membrane. They observed changes in the anisotropy of the inner membrane in the presence of germinants, indicating the existence of a substance in the inner membrane which binds the germinant, resulting in a conformational change.
the germinants (Fig. 7). Heat-activating spore extract plus normal spores together did not enhance germination of normal spores (data not shown). Although the rate of germination was not equivalent to that of normal spores, the spore extract did enhance germination of coat-defective spores when the spores and extract were heated together prior to germination. This illustrates the importance of the spore coat or outer membrane during spore germination and implies that a component(s) in the coat or outer membrane with a molecular weight of ≥12,000 is responsible for germination enhancement. Aronson and Fitz-James (3) reported that if alkaline SDS-DTT-treated spores were mixed with solubilized extract or partially purified coat, the resulting spores had a density close to that of normal spores, showing that the extract and treated spores could reassociate. Despite apparent reassociation, germination comparable to normal spores did not occur and indeed may be impossible due to irreversible damage of the reconstitution apparatus during extraction of the coat and prior to readdition of the extract. Coat-defective spores and spore extract had to be heat activated together to enhance germination, suggesting that heat activation causes a biophysical or chemical change within the germination mechanism.

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LITERATURE CITED