Gene encoding a morphogenic protein required in the assembly of the outer coat of the *Bacillus subtilis* endospore

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Endospores of *Bacillus subtilis* are encased in a two-layer protein shell known as the coat, which consists of a lamellar-like inner layer and an electron-dense outer layer. We report the cloning of the structural gene (designated *cotE*) for an alkali-soluble coat protein of 24 kD and show that the *cotE* gene product is a morphogenic protein required in the assembly of the outer coat. The nucleotide sequence of *cotE* reveals an open reading frame capable of encoding a 181-residue-long polypeptide of 21 kD. A *cotE* mutant was created by replacing the chromosomal gene, which was located at 145° on the chromosome, with an in vitro constructed, deletion-mutated gene. The resulting *cotE* mutant formed normal-looking (optically refractile) spores that were heat resistant but were sensitive to lysozyme and somewhat impaired in germination. Ultrastructural analysis indicated that the mutant spores lacked the electron-dense outer layer of the coat but retained a normal-looking inner coat. The mutant spores were pleiotropically deficient in several coat proteins, including the product of *cotE* and the products of previously cloned *cot* genes A–C. Based on experiments in which expression of the *cotA* and *cotC* genes was found to be unimpaired in *cotE* mutant cells, we infer that the *cotE* gene product is involved in the assembly of the products of *cotA–cotC* and certain other proteins into the electron-dense outer layer of the coat.

[Key Words: Spore coat assembly, *Bacillus subtilis*; *cot* genes]

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Endospores of the gram-positive bacterium *Bacillus subtilis* are encased in a tough protein shell known as the coat, which provides a protective barrier against the environment (Aronson and Fitz-James 1976; Jenkinson et al. 1981). The coat consists of about a dozen proteins ranging in size from 8 to 65 kD which, together, make up 50–60% of the spore protein and are organized into an electron-dense outer coat and a lamellar-like inner coat. In earlier work, we cloned and characterized genes known as *cotA*, *cotB*, *cotC*, and *cotD*, encoding coat proteins of 65, 59, 12, and 11 kD, respectively (Donovan et al. 1987). None of these genes is needed for the formation of normal-looking spores (optically refractile) with normal resistance properties, but a mutant of *cotA* fails to develop the brown color characteristic of colonies of wild-type sporulating cells, and spores of a *cotD* mutant are slightly defective in germination. Here, we report the identification of an additional coat gene (*cotE*) that encodes a coat protein of 21 kD. We show that spores of a *cotE* mutant are deficient in the electron-dense outer coat, highly sensitive to lysozyme, and moderately impaired in their responsiveness to germinants. In contrast to *cotA*, *cotB*, *cotC*, and *cotD* mutant spores, each of which lacks only the corresponding product of the mutant *cot* gene, spores of the *cotE* mutant were deficient in several coat proteins, including the products of *cotA*, *cotB*, and *cotC*, as well as the *cotE* gene product. Evidence is presented indicating that CotE is a morphogenic protein required for the assembly of proteins of the electron-dense outer layer of the coat.

Results

**Cloning a B. subtilis gene encoding a spore coat protein of 24 kD**

Figure 1 is an SDS–polyacrylamide gel electropherogram of coat polypeptides obtained by extraction of purified spores with NaOH at 0°C [Fig. 1A] or with SDS and dithiothreitol at 65°C [Fig. 1B]. Four coat proteins of 65, 59, 12, and 11 kD had been identified in earlier work (Donovan et al. 1987). As part of an ongoing effort to dissect the spore coat genetically, we attempted to clone the structural genes for an additional pair of prominent,
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Figure 1. SDS–polyacrylamide gel electrophoresis of spore coat polypeptides from mutant and wild-type spores. Spores from the cotE mutant (tracks 2 and 4) and from its wild-type parent (tracks 1 and 3) were purified by sedimentation in Reno-grafin. Coat proteins were extracted in 0.1 M NaOH at 0°C for 15 min [A] or in 1% (wt/vol) SDS and 50 mM dithiothreitol at 65°C for 30 min. [B], as described previously [Donovan et al. 1987], and subjected to electrophoresis in a gel containing 15% polyacrylamide. The sizes of selected coat proteins are indicated in daltons × 10⁻³.

alkali-soluble coat proteins of ~24 kD (Fig. 1A). The 24-kD proteins were purified by electrophoresis in a preparative polyacrylamide slab gel, but the two 24-kD species could not be separated from each other entirely. Nonetheless, a mixture of the polypeptides yielded a single amino-terminal sequence of 20 amino acids when subjected to sequential Edman degradation (underlined residues in Fig. 2). This suggests that the 24-kD polypeptides are related proteins with identical amino-terminal amino acid sequences or that one member of the pair was refractory to the Edman degradation reactions.

The partial amino-terminal amino acid sequence was used to design a 56-base-long oligonucleotide, which was used as a hybridization probe to identify and clone a B. subtilis HindIII fragment of 2 kb in Escherichia coli that hybridized strongly with the synthetic DNA [see Materials and methods]. The presence of the 24-kD protein-coding sequence in the 2-kb HindIII fragment was verified by nucleotide sequencing, which revealed an open reading frame as described. The deduced amino acid sequence of the open reading frame was determined for the 24-kD protein (Fig. 2). The entire open reading frame encodes a polypeptide of 181 residues, whose predicted molecular weight (21,000) was similar to that estimated for the 24,000-m.w. protein on the basis of its electrophoretic mobility. We designate the structural gene for the 24,000-m.w. protein cotE and its protein product CotE. A noteworthy feature of the predicted CotE sequence is the presence at the carboxyl terminus of a region 32 amino acids long (positions 150–181), which is rich in Glu and Asp.

Construction of a cotE insertion and deletion mutant

To study cotE genetically, we constructed a deletion and insertion mutation in the cloned gene in vitro, which we then substituted for the wild-type gene in the chromosome. As described in Materials and methods, a 1.3-kb fragment of DNA bearing a chloramphenicol-resistance gene (cat) was inserted between the Sphi site located just before the cotE open reading frame and a RsaI site at codon 65 in the coding sequence [Fig. 3]. This resulted in an insertion and deletion mutation in which the cat cassette replaced a 200-bp-long Sphi–RsaI segment of DNA from the S' end of cotE. Next, we substituted the cotE gene in the chromosome with the insertion and deletion mutation by means of marker replacement [double] recombination. Linear DNA bearing the cotE : : cat insertion mutation was used to transform competent cells of B. subtilis strain PY17 [trpC2] to chloramphenicol resistance. That the mutated cotE gene had replaced the wild-type gene in the chromosome in one such drug-resistant transformant (BZ213) was verified by Southern hybridization analysis (data not shown).

Mapping the cotE gene

The chromosomal location of cotE was determined by phage PBS1-mediated transduction using the cat insertion as selectable genetic marker. cotE : : cat was found to be cotransduced with the auxotrophic mutations thyA1 and pyrD1 [Dedonder et al. 1977] at frequencies of 30% and 23%, respectively, and with the sporulation insertion mutation spoVM : : Tn917HUO2324 [Sandman et al. 1987] at a frequency of 38%. These results and the results of three-factor crosses (data not shown) are consistent with the gene order purD1-spoVM-cotE-thyA1 and indicate a position of about 145° on the genetic map of Piggot and Hoch [1985].

Effect of the cotE : : cat mutation on the pattern of spore coat proteins

Like mutations in other cot genes studied previously, the cotE : : cat mutation did not prevent the formation of normal-looking [optically refractile] spores. Therefore, it was possible to obtain purified spores from cells bearing the cotE mutation and to investigate the effect of the absence of the cotE gene product on the overall pattern of coat polypeptides. Figure 1 shows that spores of the cotE mutant were deficient in many alkali-soluble coat proteins and in several proteins solubilized by alkali treatment, the cotB and cotA proteins, and in several proteins solubilized by treatment of spores with SDS and dithiothreitol. The effect of the cotE : : cat mutation varied somewhat in different mutant spore preparations and was not always quite as pronounced as that observed in the experiment of Figure 1. Nonetheless, several proteins were found to be reproducibly deficient in spores of the cotE : : cat mutant. These included the products of the cotC and cotE genes [Fig. 1A], among coat proteins preferentially solubilized by alkali treatment, the cotA and cotB gene products and highly prominent polypeptides of 34 and 38 kD (whose structural genes have not been identified).
among proteins preferentially solubilized by treatment with SDS and dithiothreitol [Fig. 1B]. A '36-kD' coat protein, presumably corresponding to the 34- or 38-kD species, was implicated previously in spore lysozyme resistance [Jenkinson 1981], and the absence of this protein in cotE mutant spores is in keeping with the observed sensitivity of the mutant spores to the bacteriocidal enzyme (see below).

**Figure 2.** Nucleotide sequence of the nontranscribed strand of cotE and flanking DNA. The nucleotide sequence of both strands of the DNA was determined by the chain-termination method of Sanger et al. [1977], following the strategy described immediately beneath the endonuclease restriction map of Figure 3. Endonuclease restriction sites corresponding to those shown in Figure 3 are identified by thin underlining. The predicted amino acid sequence of CotE is shown below the DNA sequence. The putative ribosome-binding site preceding the cotE protein-coding sequence is indicated with asterisks (*). The 20 amino acids of the amino-terminal sequence determined experimentally by Edman degradation are indicated by bold underlining.

**Effect of the cotE mutation on the ultrastructure of the spore coat**

The pleiotropic effect of the cotE mutation on the protein composition of the coat prompted us to examine the ultrastructure of the coat of cotE mutant spores. The coat of wild-type spores consists of an electron-dense outer layer and a lamellar-like inner layer. In contrast, spores of the cotE mutant contain a normal-looking inner coat but lack the electron-dense outer layer. An example of a comparison between mutant and wild-type spores is shown in the electron micrograph of Figure 4. Electron-micrographs (not shown) of mutant sporangia [i.e., sporulating cells containing a developing spore] frequently revealed small blebs of electron-dense material around the inner coat and deposits of electron-dense material in the mother cell. This material may be occlusions of outer coat proteins that have failed to assemble properly around the spore.

**Effects of the cotE mutation on sporulation pigmentation and spore properties**

Three additional effects of the cotE mutation were: (1) the absence of sporulation-associated brown pigment, (2) spore sensitivity to lysozyme, and (3) altered spore germination. Cells of wild-type *B. subtilis* produce colonies on solid sporulation medium that turn dark brown at late stages of sporulation. The brown color is due to the appearance of CotA, a 65-kD coat protein, which is encoded by the pigment-determining gene known as pig [or cotA; Rogolsky 1968, Donovan et al. 1987]. Cells of the cotE mutant were found to form white colonies that eventually turned light brown when grown on solid sporulation medium. These observations are in keeping with the finding [above] that spores of the cotE mutant were deficient in CotA [Pig] protein.

The absence of the outer coat prompted us to investigate the resistance properties of cotE mutant spores. Spores of the mutant were found to be resistant to chloroform and heat treatment [data not shown] but exhibited high sensitivity to the enzyme lysozyme. The experiment depicted in Figure 5 shows that suspensions of...
Figure 3. Endonuclease restriction map of cotE and flanking DNA. The thick arrow above the map shows the location and orientation of the cotE-coding sequence. A complete map of HindIII, EcoRI, PstI, and SphI sites is shown, but only selected HpaII, Rsal, and SspI sites (used in the nucleotide sequencing) are indicated. The HpaII–Rsal DNA segment at the 5' end of cotE that was substituted with the cat-bearing cassette in the construction of the cotE::cat insertion/deletion mutation is indicated above the thick arrow. The thin arrows immediately below the map show the segments of DNA that were cloned into M13 phages mp18 and mp19 and subjected to nucleotide sequencing analysis in determining the composite sequence shown in Figure 2. The horizontal lines [bottom] identify the DNA inserts of the integration plasmids described in the text. The integration vectors were constructed by insertion of the indicated DNA insert into a derivative of plasmid pUC18, bearing a chloramphenicol-resistance gene. The phenotypes of transformants in which the plasmids had been integrated into the chromosome of B. subtilis are summarized (right) [+ in the Pig (pigmentation) column] colonies of the plasmid-bearing cells exhibited a wild-type level of pigmentation; [R in the Tzm (tetrazolium) column] spores of the corresponding plasmid-bearing cells rapidly reduced the indicator dye to a red color; [W] the spores were substantially slower or unable to reduce the tetrazolium dye.

spores of the cotE mutant and — by comparison — spores of the germination mutant getE, which is known to be lysozyme sensitive (Moir 1981; Jenkinson and Lord 1983), underwent a rapid drop in optical density following exposure to the cell-wall-degrading enzyme. This corresponded to a decrease in viability of >95% (data not shown). Microscopic examination revealed that enzyme treatment had caused the cotE mutant spores to lyse or lose refractility.

Finally, experiments in this laboratory and experiments of R. Sammons of the University of Birmingham [pers. comm.] indicate that cotE mutant spores display somewhat altered germination properties. For example, cotE mutant spores exhibit a short lag (~5 min) in re-

Figure 4. Electron micrograph of the coat of a wild-type and a cotE mutant spore. (A) The coat profile of a wild-type [PY17, the cotE mutant parent] spore showing the thin outer exosporium [Ex], the thick electron-dense outer coat [OC], the low-density, lamellar-like inner coat [IC] of five to six layers, and an amorphous undercoat [UC] of variable thickness filling the space between the spore cortex [Ctx] and the inner coat. (B) In the cotE mutant [BZ213], the inner coat [IC] is the prominent layer deposited on the spore. Bits of undercoat material adhere to the inner surface of the inner coat. Magnification of A and B is indicated by the 100-nm marker in A. Samples for electron microscopy were harvested from the surface of DS medium after 4 days [ripe spores] at 37°C and prepared for electron microscopy, as detailed previously (Rosenbluh et al. 1981).
nm was measured at the indicated times of incubation at room temperature and is expressed as a percent of the optical density (OD600) or OD540 of spores to lysozyme. The decrease in the optical density at 600 nm was measured at the indicated times of incubation at room temperature and is expressed as a percent of the optical density at time 0.

Figure 5. Sensitivity of wild-type (○), cotE (△), and getE (■) spores to lysozyme. The decrease in the optical density at 600 nm was measured at the indicated times of incubation at room temperature and is expressed as a percent of the optical density at time 0.

The effects of the cotE :: cat mutation could be due to the absence of the cotE gene product itself or the absence of a downstream gene in the cotE transcription unit, upon whose expression the insertion mutation exerts a polar effect. To distinguish between these possibilities, we constructed a series of integration plasmids, pLZ173, pLZ195, and pLZ139, each bearing one of three contiguous segments [HindIII–SphI, HpaI–Rsal, and Rsal–HpaII, respectively] of B. subtilis DNA from within or adjacent to cotE (Fig. 3). The integration plasmids lacked a B. subtilis chloramphenicol-resistance gene (cat). Competent cells of wild-type B. subtilis were transformed separately with each of the integration plasmids, and transformants were recovered by selection for resistance to chloramphenicol. Verification that the drug-resistant transformants had arisen by integration of the plasmid vectors into the chromosome by single, reciprocal [Campbell] recombination between the B. subtilis DNA insert in the integration vector and the corresponding region of homology in the chromosome was obtained by Southern hybridization analysis [not shown].

Cells bearing an integrated copy of pLZ173 (bearing an insert just upstream of cotE) produced normally pigmented colonies [Pig+] on sporulation medium and spores that germinated normally as judged by their ability to cause tetrazolium to turn dark red (Tzm+) in the presence of germinants. We interpret this finding to indicate that integration of pLZ173 did not disrupt the cotE transcription unit and, hence, the promoter for cotE is located within the 630-bp HindIII–SphI segment of DNA immediately upstream of the CotE-coding sequence. In contrast, cells bearing an integrated copy of pLZ195 (bearing an insert within the 5′ end of cotE) produced colonies of white or light brown color (Pig−) and spores that were slow to cause tetrazolium to turn red (Tzm−). This result was anticipated because integration of pLZ195 disrupted the 5′ half of the cotE gene.

Finally, we consider the case of pLZ139, the integration of which did not cause either a Pig− or Tzm− phenotype. The insert in pLZ139 was entirely internal to the CotE-coding sequence, extending from a Rsal site in the 5′ half of the gene to a HpaII site located nine codons from the carboxyl terminus. Thus, Campbell integration of pLZ139 was expected to create a slightly truncated cotE gene and to disrupt the cotE transcription unit at the downstream end of the CotE-coding sequence. We draw two conclusions from the absence of a noticeable phenotype caused with the integration of pLZ139: (1) The effect of mutations in cotE are not due to a polar effect on the expression of a downstream gene(s), and (2) the last nine amino acids of CotE are evidently not required for CotE function.

The cotE :: cat mutation does not block expression of cotA

Two explanations for the requirement of CotE protein in the formation of the outer coat are [1] the CotE is a morphogen protein required for assembly of the protein components of the outer coat, and [2] in addition to being a component of the coat, CotE is a regulatory protein required for expression of cotA, cotB, cotC, and other genes encoding outer coat proteins. The availability [Sandman et al. 1988] of transcriptional and translational fusions of cotA to the lacZ gene of E. coli provided an opportunity to test conveniently the possible effect of the cotE mutation on the expression of cotA, a coat protein gene whose protein product, a 65-kD protein, was among the coat proteins found to be deficient in cotE mutant spores. The time course experiment shown in Figure 6 shows that the patterns of induction and level of expression of a cotA–lacZ transcriptional fusion in cotE mutant cells and in cotE+
Like was not shown). In other work (L. Zheng and R. Losick, unpubl.), we have constructed an in-frame fusion of cotC publ.), we have constructed an in-frame fusion of cotC.


duplication, and (4) a marked reduction in the amount of CotA, CotB, CotC and the prominent 34- and 38-kD proteins in the spore coat, in addition to the absence of the cotE gene product. In contrast, mutants of cot genes A–D produce spores whose coats lack only the product of the corresponding mutated coat gene and differ only slightly in structure and function from wild-type spores [Donovan et al. 1987]. Experiments in which we disrupted the cotE gene near the terminus of its coding sequence demonstrated that these pleiotropic effects were the consequence of the absence of CotE per se, rather than the lack of expression of one or more downstream genes in the cotE transcriptional unit.

We propose that the CotA, CotB, CotC, CotE and the prominent 34- and 38-kD proteins found to be deficient in cotE mutant spores constitute the electron-dense outer layer of the coat, which we infer functions as a protective shield against certain bacteriocidal agents, such as lysozyme. The somewhat impaired responsiveness of cotE spores to 1-alanine suggests that the outer coat may influence the accessibility of the spore to germinants. Jenkinson et al. [1981] have proposed a model for the structure and assembly of the coat based on surface labeling of coat proteins with 125I at various times during spore morphogenesis. It is not possible to interpret their model in terms of spore ultrastructure, as visualized by electron microscopy, and only some of the radioactively labeled proteins in their study can be assigned unambiguously [by virtue of distinctive size or high relative abundance] to known cot gene products. In any event, their suggestion that the 65-kD (CotA) and 59-kD (CotB) kD proteins are located at a layer in the coat lower than the major 11-kD (cotD) protein is inconsistent with our present results, which suggest that CotD is in the inner coat and that CotA and CotB are located in the electron-dense outer coat layer.

Our results suggest that CotE is a morphogenic protein required for the incorporation of CotA–CotC and certain other proteins into the outer coat. The basis for this inference is that [1] CotE is a coat protein itself, and [2] expression of the cotA and cotC genes is not blocked in cotE mutant cells, as judged by the use of transcriptional and translational fusions of lacZ to the cotA gene and a translation fusion of lacZ to cotC. CotE could influence the expression of cotA and cotC in a way [e.g., stabilization of cotA and cotC mRNAs] not revealed by our lacZ fusions, but we consider this to be unlikely. Rather, we suppose that CotE is deposited on the outside surface of the inner coat during spore morphogenesis and serves as a kind of basement protein upon which CotA, CotB, CotC and other outer coat proteins assemble. It would be instructive to know the precise location of CotE in the coat, through the use of antibodies against CotE in immunogold labeling experiments.

Finally, we compare and contrast the role of cotE in spore coat assembly to that of the germination gene gerE, a mutant of which is known to be altered profoundly in the structure of the spore coat [Moir 1981; Jenkinson and Lord 1983]. Like cotE mutant spores, gerE mutant spores are sensitive to lysozyme, but unlike cotE spores, gerE spores are highly defective in germination, ultrastructure studies [Moir 1981; P.C. Fitz-James and L. Zheng, unpubl.] show that gerE mutant spores substantially lack the lamellar-like inner coat (seemingly unaltered in cotE spores), as well as being partially deficient in the electron-dense outer coat. In other work [L. Zheng, unpubl.], we find that in contrast to cotE spores,
gerE spores lack the 11-kD cotD protein and contain enhanced levels of the pigment-determining cotA (pig) protein. It is not known whether the product of gerE is a coat protein itself, but at least some of the effects of a mutation of gerE are exerted at the level of gene expression; a mutation of gerE enhances the level of transcription of cotA severalfold [Sandman et al. 1988] and causes a complete block in the transcription of cotC [L. Zheng, unpubl.]. Thus, it is becoming increasingly clear that the cot gene expression and of proteins that act at the level of cot gene expression and of proteins that act at the level of the assembly of the products of cot genes into a multilayered superstructure.

Materials and methods

Purification of the 24-kD spore coat protein and determination of its amino-terminal sequence

Spores were obtained by growth of B. subtilis strain PY17 [CU1056 (trpC2, SPp) pCU1056 (Piggot and Hoch 1985)] cells in Difco sporulation (DS) medium. The spores were purified by washing and lysozyme treatment, as described by Jenkinson et al. [1981]. Coat proteins were extracted from the purified spores in 0.1 M NaOH at 0°C for 15 min and were subjected to electrophoresis in a preparative polyacrylamide slab gel, as described previously [Donovan et al. 1987]. The doublet of 24-kD coat proteins was obtained by electrophoresion from a slice cut from the gel [Hunkapiller et al. 1983]. The partial amino-terminal sequence of the gel-purified coat protein was determined by automated sequential Edman degradation, carried out using an Applied Biosystems gas-phase sequenator in the Biological Laboratories Microchemistry Facility.

Cloning the structural gene for the 24-kD polypeptide

The following 56-base long oligonucleotide was synthesized as a hybridization probe to clone the 24-kD structural gene: 5’-GAATATCGT GAAATTATTA CAAAAGCAGT TGTTGCAA-GGCCGTAAATTTACAC A-3’.

The oligonucleotide was designed to encode the second through nineteenth residues of the partial amino-terminal sequence. We relied on a compilation of codon usage in B. subtilis [Piggot and Hoch 1985] to choose most likely base assignments at positions that were uncertain due to the degeneracy of the code. The oligonucleotide was radioactively labeled at its 5’ terminus with [32P]phosphate, using phage T4 kinase. Southern [1977] hybridization analysis revealed a B. subtilis HindIII fragment of ~2 kb, to which the radioactive probe annealed strongly. HindIII fragments of ~2 kb were partially purified by electrophoresis in a preparative agarose gel and were cloned in E. coli strain HB101, using the plasmid vector pBR322. Colony hybridization was used to identify E. coli clones containing B. subtilis DNA that hybridized with the radioactive probe. Hybrid plasmid DNA from one such colony contained a 2-kb HindIII fragment insert, whose size was indistinguishable from that of the corresponding HindIII fragment identified by Southern hybridization of total genomic DNA.

Construction of an insertion/deletion mutant

To construct a null mutation in cotE, we created a hybrid plasmid in which the 1.3-kb cot-bearing cassette from pBD201 [Donovan et al. 1987] was flanked with the 0.7-kb HindIII–SpeI fragment that spans the region immediately 5’ of cotE and the 0.3-kb Rsal–HpaII segment that contains the 3’ half of cotE. This created a mutant gene in which the 5’ half of cotE [i.e., the region between SpeI and Rsal] had been replaced with the cot gene cassette. To replace the wild-type gene in the chromosome with the in vitro-created mutant gene, the hybrid plasmid was linearized with HindIII and used to transform competent cells of B. subtilis strain PY17 to chloramphenicol resistance.

Phenotypic studies on cotE mutant spores

Because spores of the cotE mutant are sensitive to lysozyme, spore purification in all experiments involving the characterization of the mutant and comparison with wild-type and the gerE mutant was carried out by sedimentation of the spores through 50% Renografin-76 [Aronson and Horn 1972] in place of the lysozyme treatment procedure used in the purification of 24-kD protein [above]. Sensitivity to lysozyme was measured by suspending mutant and wild-type spores in 10 mM Tris [pH 7.0] buffer containing lysozyme [50 μg/ml] at room temperature. The decrease in optical density was monitored at 595 nm. Spore viability was measured after 30 min as colony-forming units on LB agar plates. Germination was assessed qualitatively by the tetrazolium assay of Irie et al. [1982], as modified by Cutting and Mandelstam [1986]. Patches of cells were sporulated on agar plates containing DS medium for 2 days, blotted onto nitrocellulose, heated at 65°C for 1 hr to kill vegetative cells, and exposed to agar plates containing tetrazolium. Colony pigmentation was determined by observing the color of colonies formed on DS medium after several days of incubation.

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