

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

Liangbiao Zheng,¹ William P. Donovan,^{1,3} Philip C. Fitz-James,² and Richard Losick^{1,4}

¹Department of Cellular and Developmental Biology, The Biological Laboratories, Harvard University, Cambridge, Massachusetts 02138 USA; ²Department of Microbiology and Immunology, Medical Faculty, University of Western Ontario, London, Ontario, N6S 5C1 Canada

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,

³Current address: Ecogen Incorporated, Langhorne, Pennsylvania 19047 USA.

⁴Corresponding author.

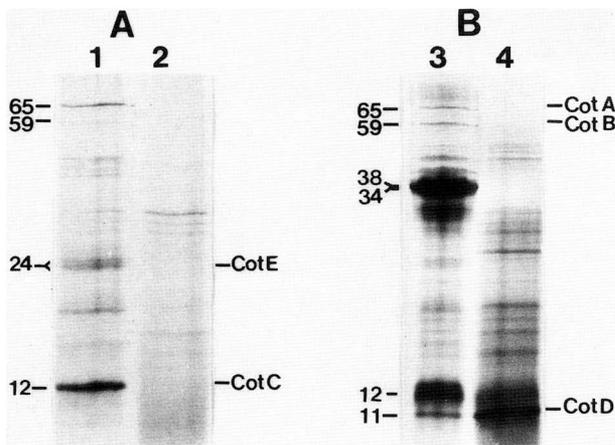


Figure 1. SDS-polyacrylamide gel electrophoresis of spore coat polypeptides from mutant and wild-type spores. Spores from the *cotE* mutant (tracks 2 and 4) and from its wild-type parent (tracks 1 and 3) were purified by sedimentation in Renografin. Coat proteins were extracted in 0.1 M NaOH at 0°C for 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 $\times 10^{-3}$.

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* *Hind*III 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 *Hind*III fragment was verified by nucleotide sequencing, which revealed an open reading frame whose deduced amino acid sequence from codons 2 through 21 conformed exactly to the partial amino-terminal amino acid sequence determined for the 24-kD protein (Fig. 2). The entire open reading frame could encode 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 *Sph*I site located just before the *cotE* open reading frame and a *Rsa*I 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 *Sph*I–*Rsa*I segment of DNA from the 5' 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* : : Tn917HUN324 (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 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)

*****^{SphI HpaII}
 CCAGCATAAGATAACACGAAGAAGAACAAGGAGGCATGCCGGA

ATG TCT GAA TAC AGG GAA ATT ATT ACG AAG GCA GTA GTA GCG AAA GGC CGA AAA
 Met Ser Glu Tyr Arg Glu Ile Ile Thr Lys Ala Val Val Ala Lys Gly Arg Lys

TTC ACC CAA TGC ACC AAC ACC ATC TCG CCT GAG AAA AAA CCG AGC AGC ATT TTG
 Phe Thr Gln Cys Thr Asn Thr Ile Ser Pro Glu Lys Lys Pro Ser Ser Ile Leu

GGT GGT TGG ATT ATT AAC CAC AAG TAT GAC GCT GAA AAA ATT GGA AAA ACG GTA
 Gly Gly Trp Ile Ile Asn His Lys Tyr Asp Ala Glu Lys Ile Gly Lys Thr Val

RsaI

GAA ATT GAA GGG TAT TAT GAT ATA AAC GTA TGG TAC TCT TAC GCG GAC AAC ACA
 Glu Ile Glu Gly Tyr Tyr Asp Ile Asn Val Trp Tyr Ser Tyr Ala Asp Asn Thr

AAG ACA GAG GTT GTC ACA GAA CGG GTA AAA TAT GTA GAT GTC ATT AAA CTC AGA
 Lys Thr Glu Val Val Thr Glu Arg Val Lys Tyr Val Asp Val Ile Lys Leu Arg

TAC AGA GAC AAT AAT TAC TTA GAT GAT GAG CAT GAA GTG ATT GCC AAA GTG CTT
 Tyr Arg Asp Asn Asn Tyr Leu Asp Asp Glu His Glu Val Ile Ala Lys Val Leu

CAG CAG GCA AAC TGC CTT GAA GTG ACC ATT TCG CCG AAT GGA AAT AAA ATC GTT
 Gln Gln Pro Asn Cys Leu Glu Val Thr Ile Ser Pro Asn Gly Asn Lys Ile Val

GTG CAG GCA GAA AGA GAA TTT TTG GCG GAA GTG GTA GGG GAA ACA AAG GTA GTT
 Val Gln Ala Glu Arg Glu Phe Leu Ala Glu Val Val Gly Glu Thr Lys Val Val

GTT GAG GTC AAT CCT GAC TCG GAA CAG GAT GAC GAG GAA GAT TGG GAA GAT GAG
 Val Glu Val Asn Pro Asp Trp Glu Glu Asp Asp Glu Glu Asp Trp Glu Asp Glu

HpaII

CTT GAT GAA GAG CTT GAA GAC ATC AAC CCG GAG TTT TTA GTG GGA GAT CCT GAA
 Leu Asp Glu Glu Leu Glu Asp Ile Asn Pro Glu Phe Leu Val Gly Asp Pro Glu

RsaI

GAA TAA AAAAGGGACTAGGGGAGACAGTAC
 Glu ***

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.

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).] A conspicuous example of a protein that was not eliminated from the coat as a result of the *cotE* mutation was the 11-kD product of *cotD* (Fig. 1B).

Effect of the *cotE* : : cat 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 ma-

terial 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* : : cat 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

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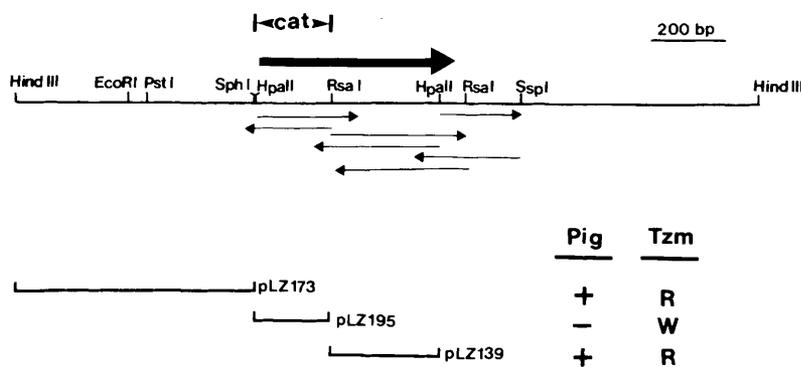


Figure 3. Endonuclease restriction map of *cotE* and flanking DNA. The thick arrow above the map shows the location and orientation of the *cotE*-coding sequence. A complete map of *Hind*III, *Eco*RI, *Pst*I, and *Sph*I sites is shown, but only selected *Hpa*II, *Rsa*I, and *Ssp*I sites (used in the nucleotide sequencing) are indicated. The *Hpa*II-*Rsa*I 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 *gerE*, 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 en-

zyme 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-

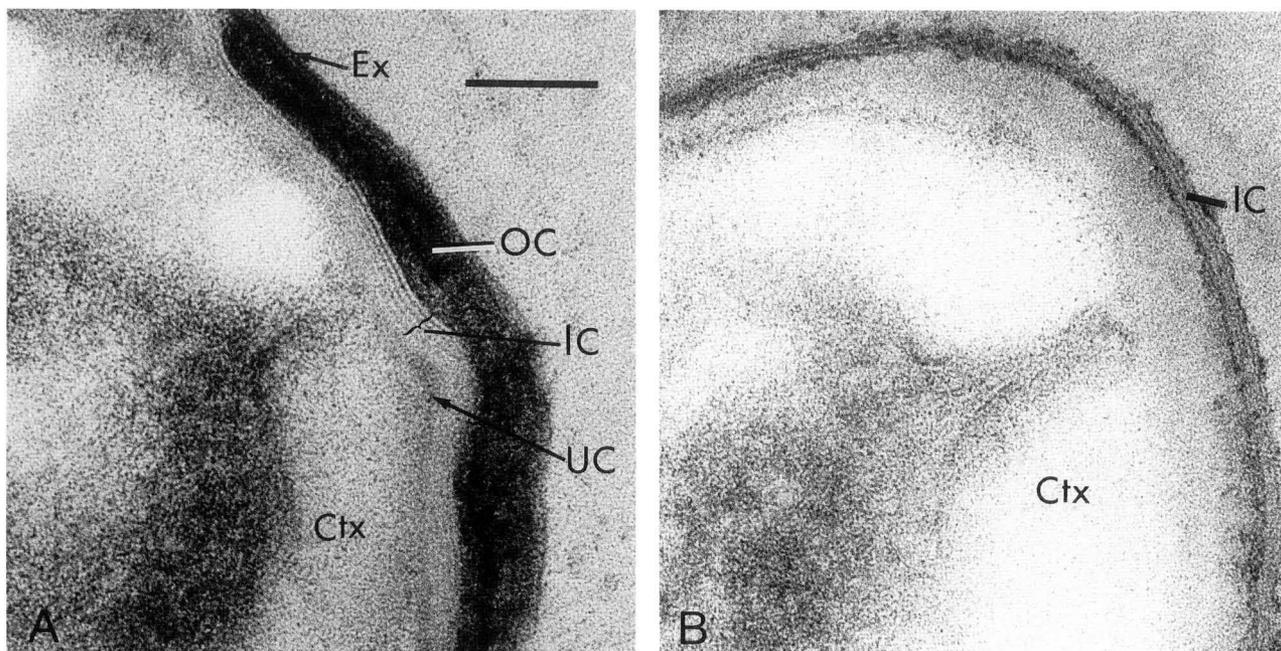


Figure 4. Electron micrograph of the coat of a wild-type and a *cotE* mutant spore. (A) The coat profile of a wild-type (PY17, the *cotE* mutant parent) spore showing the thin outer exosporium (Ex), the thick electron-dense outer coat (OC), the low-density, lamellar-like inner coat (IC) of five to six 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).

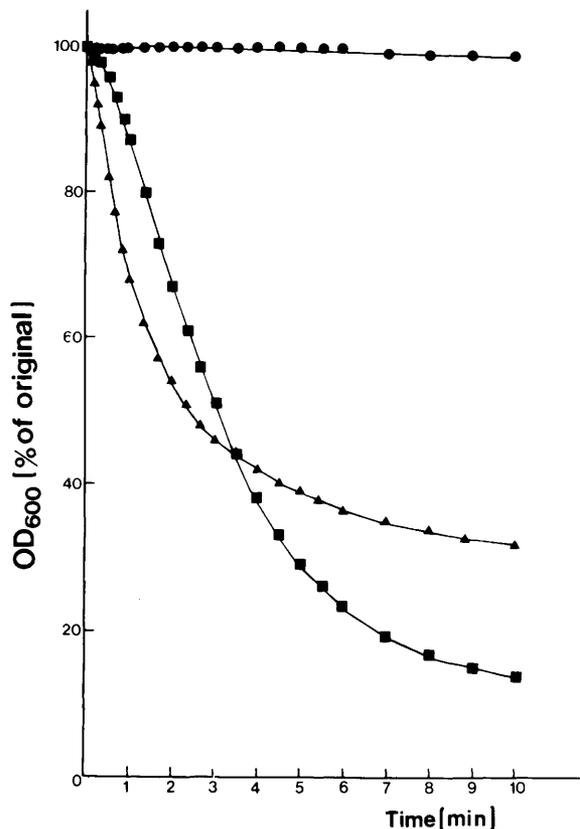


Figure 5. Sensitivity of wild-type (●), *cotE* (▲), and *gerE* (■) spores to lysozyme. The decrease in the optical density at 600 nm was measured at the indicated times of incubation at room temperature and is expressed as a percent of the optical density at time 0.

sponding to the germinant L-alanine (but not to the germination mixture L-asparagine, glucose, fructose, and KCl), as monitored by the loss of optical density of spore suspensions. *cotE* spores also germinate less efficiently than wild-type spores do, as judged by their impaired ability to reduce the dye tetrazolium in the presence of a mixture of germinants.

Defining the functional boundaries of *cotE*

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*, *HpaII*–*RsaI*, and *RsaI*–*HpaII*, respectively) of *B. subtilis* DNA from within or adjacent to *cotE* (Fig. 3). The integration plasmids lacked a *B. subtilis* replicon and, hence, were incapable of autonomous replication in *B. subtilis* but contained a *B. subtilis* chloramphenicol-resistance gene (*cat*). Competent cells of wild-type *B. subtilis* were transformed separately with each of the integration plasmids, and trans-

formants 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^R) 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^W). 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 *RsaI* 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 morphogenic 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*⁺

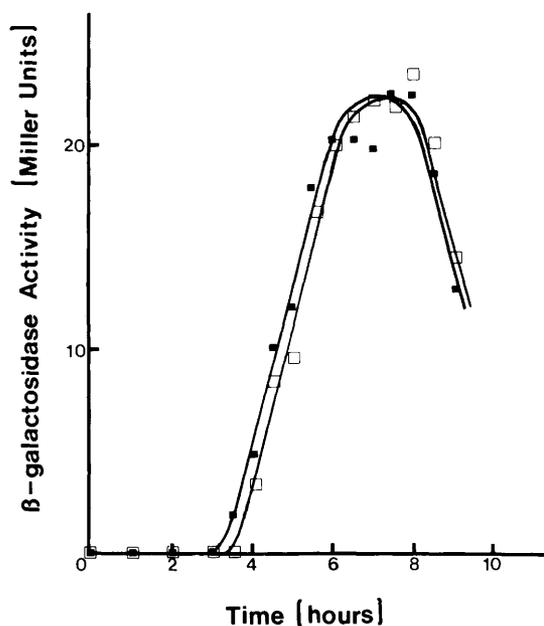


Figure 6. Transcription of the *cotA-lacZ* operon fusion during sporulation in mutant (□) and wild-type (■) cells. Cells of the *cotE* mutant and its wild-type parent were lysogenized with the SPβ::*cotA-lacZ* transducing phage described previously (Sandman et al. 1988). Fusion-bearing cells were grown and sporulated in DS medium. Samples of cells were collected at the times indicated after the end of the exponential phase of growth and assayed for fusion-directed β-galactosidase synthesis, as described previously (Miller 1972; Sandman et al. 1988).

wild-type cells were indistinguishable. Similarly, the patterns of expression of a *cotA-lacZ* in-frame gene fusion were the same in mutant and wild-type cells (data not shown). In other work (L. Zheng and R. Losick, unpubl.), we have constructed an in-frame fusion of *cotC* to *lacZ*. Like *cotA* expression, expression of *cotC* was not measurably impaired by the *cotE* mutation. Evidently, the deficiencies in *cotE* mutant spores of CotA and CotC are not attributable to a block in the expression of these genes.

Discussion

We have identified a new spore coat gene in *B. subtilis*, called *cotE*, encoding a 181-residue-long, alkali-soluble polypeptide of 21 kD. *cotE* maps at 145° on the chromosome and is well separated from *cot* genes studied previously. *cotE* differs from other known *cot* genes in that its inactivation has striking pleiotropic effects on spore morphogenesis. These effects include (1) a substantial deficiency in the electron-dense outer layer of the coat, (2) high spore sensitivity to lysozyme, (3) slightly impaired germination, 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 L-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- (CotA) and 59- (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 spore coat is a highly complex structure whose morphogenesis involves the interplay 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*, $\text{SP}\beta^s$) Zahler et al. 1977] 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 electroelution from a slice cut from the gel (Hunkapillar 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 sequencer 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-AAGGCCGTAAATTTACACA-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* *Hind*III fragment of ~2 kb, to which the radioactive probe annealed strongly. *Hind*III 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 clone contained a 2-kb *Hind*III fragment insert, whose size was indistinguishable from that of the corresponding *Hind*III 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 *cat*-bearing cassette from pBD201 (Donovan et al. 1987) was flanked with the 0.7-kb *Hind*III-*Sph*I

fragment that spans the region immediately 5' of *cotE* and the 0.3-kb *Rsa*I-*Hpa*II 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 *Sph*I and *Rsa*I) had been replaced with the *cat* gene cassette. To replace the wild-type gene in the chromosome with the in vitro-created mutant gene, the hybrid plasmid was linearized with *Hind*III 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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References

- Aronson, A.I. and P. Fitz-James. 1976. Structure and morphogenesis of the bacterial spore coat. *Bacteriol. Rev.* **40**: 360–402.
- Aronson, A.I. and D. Horn. 1972. Characterization of spore coat protein of *Bacillus cereus* T. In *Spore V* (ed. H.O. Halvorson, R. Hanson, and L.L. Campbell), pp. 19–27. American Society for Microbiology, Washington, D.C.
- Cutting, S. and J. Mandelstam. 1986. The nucleotide sequence and the transcription during sporulation of the *gerE* gene of *Bacillus subtilis*. *J. Gen. Microbiol.* **132**: 3013–3024.
- Dedonder, R.A., J. Lepesant, J. Lepesant-Kejzlarova, A. Billault, M. Steinmetz, and F. Kunst. 1977. Construction of a kit of reference strains for rapid genetic mapping in *Bacillus subtilis* 168. *Appl. Environ. Microbiol.* **33**: 989–993.
- Donovan, W.P., L. Zheng, K. Sandman, and R. Losick. 1987. Genes encoding spore coat polypeptides from *Bacillus subtilis*. *J. Mol. Biol.* **196**: 1–10.
- Hunkapillar, M.W., E. Lujan, F. Ostrander, and L.E. Hood. 1983. *Methods Enzymol.* **91**: 227–236.
- Irie, R., T. Okamoto, and Y. Fujita. 1982. A germination mutant of *Bacillus subtilis* deficient in response to glucose. *J. Gen. Appl. Microbiol.* **28**: 345–354.

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- Jenkinson, H.F. 1981. Germination and resistance defects in spores of a *Bacillus subtilis* mutant lacking a coat polypeptide. *J. Gen. Microbiol.* **127**: 81–91.
- Jenkinson, H.F. and H. Lord. 1983. Protease deficiency and its association with defects in spore coat structure, germination and resistance properties in a mutant of *Bacillus subtilis*. *J. Gen. Microbiol.* **129**: 2727–2737.
- Jenkinson, H.F., W.D. Sawyer, and J. Mandelstam. 1981. Synthesis and order of assembly of spore coat proteins in *Bacillus subtilis*. *J. Gen. Microbiol.* **123**: 1–16.
- Miller, J.H. 1972. *Experiments in molecular genetics*. Cold Spring Harbor Laboratory, Cold Spring Harbor, New York.
- Moir, A. 1981. Germination properties of a spore coat-defective mutant of *Bacillus subtilis*. *J. Bacteriol.* **146**: 1106–1116.
- Piggot, P. and J.A. Hoch. 1985. Revised genetic linkage map of *Bacillus subtilis*. *Microbiol. Rev.* **49**: 158–179.
- Rogolsky, M. 1968. Genetic mapping of a locus which regulates the production of pigment associated with spores of *Bacillus subtilis*. *J. Bacteriol.* **95**: 2426–2427.
- Rosenbluh, A., C.D.B. Banner, R. Losick, and P.C. Fitz-James. 1981. Identification of a new developmental locus in *Bacillus subtilis* by construction of a deletion mutation in a cloned gene under sporulation control. *J. Bacteriol.* **148**: 341–351.
- Sanger, F., S. Nicklen, and A.R. Coulson. 1977. DNA sequencing with chain terminating inhibitors. *Proc. Natl. Acad. Sci.* **74**: 5463–5467.
- Sandman, K., R. Losick, and P. Youngman. 1987. Genetic analysis of *Bacillus subtilis* *spo* mutations generated by Tn917 mediated insertional mutagenesis. *Genetics* **117**: 603–617.
- Sandman, K., L. Kroos, S. Cutting, P. Youngman, and R. Losick. 1988. Identification of the promoter for a spore coat protein in *Bacillus subtilis* and studies on the regulation of its induction at a late stage of sporulation. *J. Mol. Biol.* **200**: 461–473.
- Southern, E.M. 1975. Detection of specific sequences among DNA fragments separated by gel electrophoresis. *J. Mol. Biol.* **98**: 503–517.
- Zahler, S., R.Z. Korman, R. Rosenthal, and H.E. Hemphill. 1977. *Bacillus subtilis* bacteriophage SP β : Localization of the prophage attachment site and specialized transduction. *J. Bacteriol.* **129**: 556–558.



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L B Zheng, W P Donovan, P C Fitz-James, et al.

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