

## The Timing of *cotE* Expression Affects *Bacillus subtilis* Spore Coat Morphology but Not Lysozyme Resistance<sup>∇</sup>

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**The synthesis of structural components and morphogenetic factors required for the assembly of the *Bacillus subtilis* spore coat is governed by a mother cell-specific transcriptional cascade. The first two temporal classes of gene expression, which involve RNA polymerase sigma  $\sigma^E$  factor and the ancillary regulators GerR and SpoIID, are deployed prior to engulfment of the prespore by the mother cell. The two last classes rely on  $\sigma^K$ , whose activation follows engulfment completion, and GerE. The *cotE* gene codes for a morphogenetic protein essential for the assembly of the outer coat layer and spore resistance to lysozyme. *cotE* is expressed first from a  $\sigma^E$ -dependent promoter and, in a second stage, from a promoter that additionally requires SpoIID and that remains active under  $\sigma^K$  control. CotE localizes prior to engulfment completion close to the surface of the developing spore, but formation of the outer coat is a late,  $\sigma^K$ -controlled event. We have transplanted *cotE* to progressively later classes of mother cell gene expression. This created an early class of mutants in which *cotE* is expressed prior to engulfment completion and a late class in which expression of *cotE* follows the complete engulfment of the prespore. Mutants of the early class assemble a nearly normal outer coat structure, whereas mutants of the late class do not. Hence, the early expression of CotE is essential for outer coat assembly. Surprisingly, however, all mutants were fully resistant to lysozyme. The results suggest that CotE has genetically separable functions in spore resistance to lysozyme and spore outer coat assembly.**

The timing and fidelity of several morphogenetic processes in bacterial cells, such as the assembly of the flagellum and other surface appendages, or spore development, often rely on transcriptional cascades that link gene expression to morphogenesis (15, 46). Spores of *Bacillus subtilis* are encased in a complex proteinaceous structure called the coat, whose formation extends for several hours during the deployment of a mother cell-specific transcriptional cascade (13, 14, 51, 56) (Fig. 1A) and involves the coordinated production and deposition of over 50 protein components (11, 21, 22). The coat, which consists of two main layers, a laminated lightly staining inner layer, and a thick electron-dense outer coat (11, 21, 22), confers protection against lytic enzymes, small toxic molecules, and predation and allows prompt and efficient spore germination (3, 11, 22, 29, 36, 37, 55). Assembly of the coat begins soon after the polar division of the sporulating cell, concurrently with the activation of RNA polymerase sigma factor E ( $\sigma^E$ ) in the larger mother cell compartment (13, 14, 17, 18, 24, 30) (Fig. 1A). Coat assembly proceeds following engulfment of the smaller compartment (or prespore) by the mother cell, with the activation of a second mother cell-specific sigma factor,  $\sigma^K$ , which governs late stages of development (7, 13, 23, 54, 56) (Fig. 1A). Some coat proteins are produced under the control

of  $\sigma^E$  whereas the synthesis of other relies on  $\sigma^K$ , but it is only when  $\sigma^K$  becomes active that the coat layers start gaining their characteristic appearance as viewed by electron microscopy (11, 21, 22).

Biogenesis of the spore coat further relies on a class of morphogenetic proteins that act by directing the assembly of the structural components (11, 21, 22, 35). Soon after polar division of the sporangial cell, the morphogenetic protein SpoIVA localizes at or very close to the outer prespore membrane, which faces the mother cell cytoplasm (12, 42, 43) (Fig. 1A). The localization of SpoIVA is required for the assembly of another morphogenetic protein, CotE (10, 56), at a distance of about 75 nm from SpoIVA (12) (Fig. 1A). The gap defined by the positions of SpoIVA and CotE, or matrix, is of unknown composition. When  $\sigma^K$  becomes active, the matrix is thought to be converted into the inner coat (12). At this postengulfment stage in coat formation, CotE appears to act from the edge of the inner coat region to nucleate assembly of the outer coat, presumably in part by direct interaction with other coat proteins (27, 34). Because spores of a *cotE* deletion mutant lack the outer coat layer and are highly susceptible to lysozyme, the outer coat was proposed to serve as a barrier against lytic enzymes which would otherwise gain access to the underlying cortex peptidoglycan layer of the spore (55). Additionally, *cotE* spores are impaired in germination and susceptible to predation (2, 29, 55).

Here, we are concerned with the function of *cotE* in relation to its time of expression during coat assembly. Consistent with its early assembly, production of CotE relies on tandem promoters, designated P1 and P2, under  $\sigma^E$  control (55, 56) (Fig.

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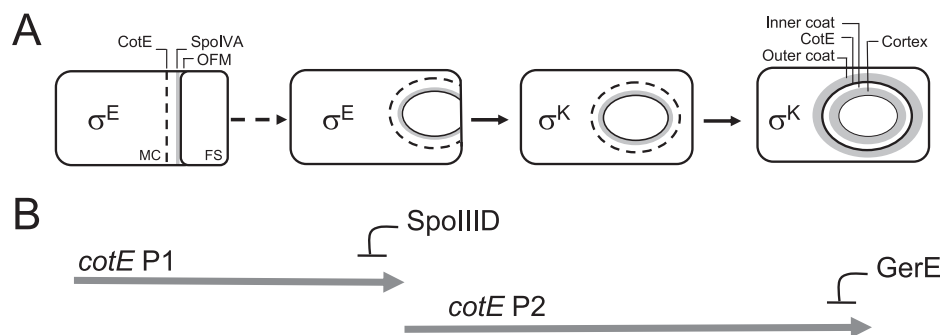


FIG. 1. Stages in coat formation and CotE expression in *B. subtilis* in relation to the mother cell line of gene expression. Panel A illustrates the main stages in coat morphogenesis and the periods of activity of the two main mother cell-specific transcriptional regulators  $\sigma^E$  and  $\sigma^K$ .  $\sigma^E$  is activated in the mother cell (MC) soon after polar division of the sporulating cell and drives synthesis of coat morphogenetic proteins SpoIVA and CotE. SpoIVA localizes near the outer forespore membrane (OFM), which faces the mother cell cytoplasm, and CotE localizes at a distance of about 75 nm, in an SpoIVA-dependent manner. The region between CotE and SpoIVA (or matrix) is of unknown composition.  $\sigma^K$  is activated in the mother cell following engulfment completion and replaces  $\sigma^E$ . The acquisition of the structural features of the inner and outer coat layers, as viewed by electron microscopy, is a postengulfment,  $\sigma^K$ -dependent event. The position of CotE is thought to define the site of assembly of the outer coat, whereas the matrix region is converted into the inner layer of the coat. Panel B shows the periods of expression of *cotE*. CotE is the only coat morphogenetic protein to be produced from tandem promoters, P1 and P2. P1 is under the control of  $\sigma^E$  and is shut off by SpoIIID prior to the activation of P2, which requires SpoIIID. Expression of *cotE* is maintained following engulfment completion, under  $\sigma^K$  control, from P2, and is shut off by GerE during the latest stages of morphogenesis. FS, forespore.

1B). Transcription from P1 is initiated soon after asymmetric division and is shut off by the repressive action of SpoIIID, a transcription factor that works together with  $\sigma^E$  prior to activation of transcription from the P2 promoter (13, 33) (Fig. 1B). Transcription from P2 appears to be under the joint control of  $\sigma^E$  and SpoIIID but remains on after the activation of  $\sigma^K$ , to be repressed in the final stages of sporulation by the GerE regulatory protein, itself the product of a  $\sigma^K$ -controlled gene (13, 56) (Fig. 1B). Thus, the *cotE* gene is expressed both before and after the engulfment process is concluded (Fig. 1B). It has been hypothesized that the early assembly of CotE is a prerequisite for its role in guiding the assembly of the outer coat components once  $\sigma^K$  becomes active (12), but it is not known whether expression of *cotE* following engulfment completion is sufficient for outer coat assembly.

To test the possibility that the assembly of the outer coat requires the early expression of *cotE* and to examine the contribution of the *cotE* promoters for the assembly and functionality of the outer coat structure, we have fused *cotE* to either its

P1 or P2 promoter, to the  $\sigma^K$ -dependent *gerE* promoter, and to the GerE-dependent *cotG* promoter. These fusions created two main classes of mutants, an early class in which *cotE* is expressed prior to engulfment completion and a late class in which expression takes place following engulfment completion. The early, but not the late, mutants are able to assemble a nearly normal outer coat structure. Hence, the early expression of *cotE* is essential for the late stages in outer coat formation. However, spores of all mutants were resistant to lysozyme. The results indicate that CotE has a function in lysozyme resistance that is genetically separable from its role in promoting assembly of the outer coat structure.

#### MATERIALS AND METHODS

**Bacterial strains, media, and general techniques.** The *B. subtilis* strains used in this study derive from the Spo<sup>+</sup> strain MB24 (Table 1). Plasmids were constructed using *Escherichia coli* strain DH5 $\alpha$  (Bethesda Research Laboratories). Luria-Bertani (LB) medium was used for routine growth of *E. coli* and *B. subtilis* strains, with appropriate antibiotic selection when needed. Sporulation was in-

TABLE 1. Strains used in this study

Strain	Relevant genotype	Source or reference
AH1835	BL21(DE3) (pLysS) (pMS17)	This work
MB24	<i>trpC2 metC3</i> ; Spo <sup>+</sup>	Laboratory stock
AOB68	<i>trpC2 metC3 ΔsafA::sp</i> ; Sp <sup>r</sup>	Laboratory stock
IS105	$\Delta cotE::cat$ ; Cm <sup>r</sup>	56 and BGSC <sup>a</sup>
AH2835	<i>trpC2 metC3 ΔcotE::cat</i> ; Cm <sup>r</sup>	This work
AH2914	<i>trpC2 metC3 ΔcotE::cat ΔamyE::P<sub>gerE</sub>-cotE</i> ; Cm <sup>r</sup> Nm <sup>r</sup>	This work
AH2915	<i>trpC2 metC3 ΔcotE::cat ΔamyE::P<sub>cotG</sub>-cotE</i> ; Cm <sup>r</sup> Nm <sup>r</sup>	This work
AH2920	<i>trpC2 metC3 ΔcotE::cat ΔamyE::P<sub>cotEP2</sub>-cotE</i> ; Cm <sup>r</sup> Nm <sup>r</sup>	This work
AH2921	<i>trpC2 metC3 ΔcotE::cat ΔamyE::P<sub>cotEP1</sub>-cotE</i> ; Cm <sup>r</sup> Nm <sup>r</sup>	This work
AH2942	<i>trpC2 metC3 ΔcotE::cat ΔamyE::P<sub>cotEP1P2</sub>-cotE</i> ; Cm <sup>r</sup> Nm <sup>r</sup>	This work
AH2654	<i>trpC2 metC3 SPβ::cotA-lacZ</i> ; Cm <sup>r</sup> Erm <sup>r</sup>	This work
AH4113	<i>trpC2 metC3 ΔcotE::cat ΔamyE::P<sub>gerE</sub>-cotE SPβ::cotA-lacZ</i> ; Cm <sup>r</sup> Erm <sup>r</sup> Nm <sup>r</sup>	This work
AH4114	<i>trpC2 metC3 ΔcotE::cat ΔamyE::P<sub>cotG</sub>-cotE SPβ::cotA-lacZ</i> ; Cm <sup>r</sup> Erm <sup>r</sup> Nm <sup>r</sup>	This work
AH4116	<i>trpC2 metC3 ΔcotE::cat SPβ::cotA-lacZ</i> ; Cm <sup>r</sup> Erm <sup>r</sup>	This work

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duced by growth and nutrient exhaustion in Difco sporulation medium (DSM), and the titers of heat- or lysozyme-resistant spores were measured 24 h after sporulation onset, as described previously (39). Genetic manipulations of *B. subtilis* were as previously described (9).

**Insertion of *cotE* at *amyE*.** A PCR product obtained with primers *ymcA664D* and *cotE761R* (the sequences of all primers are available on request), including the *cotE* gene and promoter region, was cut with HindIII-EcoRI and inserted between the same sites of pMLK83 (26), producing pTC84. In this and all constructions described below, the absence of mutations in the cloned inserts was verified by sequencing. Transformation of a *cotE* null mutant, AH2835 ( $\Delta$ *cotE::cat*), generated by transforming MB24 with DNA from strain 1S105 (55) (Table 1), with PstI-digested pTC84, produced the Nm<sup>r</sup> AmyE<sup>-</sup> strain AH2942 ( $\Delta$ *cotE::cat*  $\Delta$ *amyE::P<sub>cotEPI</sub>P2-cotE*) (Table 1).

**Expression of *cotE* from its P1 or P2 promoters.** First, primers *ymcA664D* and *cotE499R* were used for PCR amplification of a fragment encompassing the *cotE* promoter region and part of its coding region, which was doubly digested with HindIII and AatII, and the product was cloned between the same sites of pAH256 (19) to yield pTC18. Second, we used pTC18 and primers *cotE178D* and *cotE222R* to create a 6-bp deletion of the -10 region of the *cotEP2* promoter using the QuickChange system (Stratagene). Third, a 611-bp DNA fragment encompassing the entire *ymcA* coding region was PCR amplified using primers *ymcA74D* and *ymcA681R*, digested with BglIII and NsiI, and cloned between the NsiI and BglIII sites of pAH256 (19) to yield pTC20. Lastly, a 633-bp XhoI-BglIII *ymcA* fragment obtained from pTC20 was cloned between XhoI and BglIII sites of pTC19 to yield pTC21 which, after ScaI-linearization, was used to transform MB24 to spectinomycin resistance (Sp<sup>r</sup>). A double crossover at the *cotE* locus (verified by PCR) generated AH1936, in which deletion of the -10 region of *cotEP2* (herein called the P<sub>*cotEPI*</sub>-*cotE* allele) was confirmed by sequencing. To insert P<sub>*cotEPI*</sub>-*cotE* at *amyE* in a *cotE* null mutant, we used DNA from AH1936 to PCR amplify the mutant promoter with primers *ymcA664D* and *cotE761R*. The resulting product was cloned between the HindIII and EcoRI sites of pMLK83 (26) to create pTC63. AH2835 was transformed with PstI-linearized pTC63, resulting in the neomycin-resistant (Nm<sup>r</sup>) Cm<sup>r</sup> AmyE<sup>-</sup> strain AH2921 ( $\Delta$ *cotE::cat*  $\Delta$ *amyE::P<sub>cotEPI</sub>-cotE*) (Table 1).

A *cotE* promoter fragment excluding its P1 promoter was amplified by PCR with primers *cotE123D* and *cotE761R* and inserted between the HindIII and EcoRI sites of pMLK83 (26) to create pTC56. AH2835 (see above) was transformed to Nm<sup>r</sup> with PstI-linearized pTC56 to yield the Cm<sup>r</sup> AmyE<sup>-</sup> strain AH2920 ( $\Delta$ *cotE::cat*  $\Delta$ *amyE::P<sub>cotEP2</sub>-cotE*) (Table 1).

**Expression of *cotE* from the *gerE* or *cotG* promoters.** A 314-bp PCR product obtained with primers *gerE1D* and *gerE314R*, encompassing the *gerE* promoter and part of its coding sequence (7), was cut with HindIII and SpeI and inserted between the same sites of pMLK83 (26) to form pTC35. A 587-bp fragment encompassing the *cotE* ribosome binding site, and the coding region was obtained by PCR with primers *cotE174D* and *cotE761R*, doubly digested with SpeI and EcoRI and inserted between the same sites of pTC35 to yield pTC36. PstI-linearized pTC36 was used to transform a *B. subtilis* strain to Nm<sup>r</sup>, from which chromosomal DNA was prepared and then used to transform AH2835 to Nm<sup>r</sup>, yielding the Cm<sup>r</sup> AmyE<sup>-</sup> strain AH2914 ( $\Delta$ *cotE::cat*  $\Delta$ *amyE::P<sub>gerE</sub>-cotE*) (Table 1).

For the expression of *cotE* from the *cotG* promoter (47), a fragment carrying the *cotG* promoter region, extending 173 bp and 88 bp upstream and downstream, respectively, from its transcriptional start site was PCR amplified with primers *cotG28D* and *cotG288R* and digested with HindIII and SpeI. Next, the *cotG* fragment and the 587-bp SpeI- and EcoRI-digested *cotE* fragment generated with primers *cotE174D* and *cotE761R* (see above) were ligated with HindIII- and EcoRI-cleaved pMLK83 (26), yielding pTC69. PstI-linearized pTC69 transformed AH2835 to Nm<sup>r</sup>, yielding the Cm<sup>r</sup> AmyE<sup>-</sup> strain AH2915 ( $\Delta$ *cotE::cat*  $\Delta$ *amyE::P<sub>cotG</sub>-cotE*) (Table 1).

**Overproduction and purification of a six-His-S-tagged CotE for antiserum production.** The *cotE* coding sequence was amplified by PCR with primers *cotE224D* and *cotE808R*, and the resulting product was cut with EcoRI and XhoI and inserted between the same sites of pET-30b(+) (Novagen). The resulting pMS17 was transformed into BL21(DE3) (pLysS) (Novagen) to create AH1835, in which a six-His-S-tagged CotE fusion protein could be produced from the T7lac promoter (Table 1). AH1835 was grown in LB to an optical density at 600 nm (OD<sub>600</sub>) of 0.3 and induced for 3 h with 1 mM isopropyl- $\beta$ -D-thiogalactopyranoside. The fusion protein, mostly present in inclusion bodies, was solubilized in 20 mM sodium phosphate, pH 7.4, 0.5 M NaCl, 10 mM imidazole, and 8 M urea and purified over a HisTrap column as described by the manufacturer (Amersham Biosciences). Gel-purified CotE was sent to Eurogentec (Belgium) for antibody production in rabbits.

**Whole-cell lysates and immunoblot analysis.** Whole-cell lysates were prepared from samples of DSM cultures using a French press, and proteins were separated and immunoblotted as described previously (6, 49, 58). The anti-CotE antibody was used overnight at 4°C at a dilution of 1:1,000 in phosphate-buffered saline-Tween 20 containing 0.5% low-fat milk.

**Preparation of spore fractions.** Spore fractions of sporulating cells were isolated as described before (25) except that whole-cell lysates were prepared as described above. The whole-cell lysates were then fractionated by centrifugation (20 min at 13,000 rpm in a microcentrifuge), and samples (5  $\mu$ g) of the cell sediment (spore fraction) were subjected to immunoblot analysis with the anti-CotE antibody (see above).

**Purification of spores and extraction of coat proteins.** Spores were collected from DSM cultures 24 h after the onset of sporulation and purified on Gastrografin gradients, and the coat proteins were extracted and separated as described previously (6, 19, 20, 49, 58). Twenty OD<sub>580</sub> units of the purified spore suspension was subjected to an alkaline spore de-coating procedure prior to lysozyme susceptibility testing, as described before (41, 44). One OD<sub>580</sub> unit of de-coated spores was tested for heat or lysozyme resistance (see above).

**Enzyme assays.** Chromosomal DNA of AH2654 (*cotA-lacZ*) (Table 1) was used to transform strains AH2914 (P<sub>*gerE*</sub>), AH2915 (P<sub>*cotG*</sub>), and AH2835 (*cotE*), yielding strains AH4113, AH4114, and AH4116, respectively (Table 1). The activity of  $\beta$ -galactosidase was determined with the substrate o-nitrophenyl- $\beta$ -D-galactopyranoside (6, 40).

**Microscopy.** Samples (0.6 ml) from DSM cultures of MB24 and of its congeneric derivatives AH2835, AH2921, AH2920, AH2914, AH2915, and AH2942 (Table 1) were collected 2, 3, 4, 6, and 8 h after the initiation of sporulation, resuspended in 0.2 ml of phosphate-buffered saline supplemented with 200 nM Mito-Tracker Green (MTG; Molecular Probes, Eugene, OR) for visualization of membranes. Aliquots were applied to agarose-coated microscope slides and visualized with a standard filter for fluorescence, as described before (6, 45). For electron microscopy, spores were collected from 24 h DSM cultures of the same strains, purified on Gastrografin gradients (see above), and processed as described before (22). Electron microscopy analysis and photography were conducted on a Phillips EM301, operated at 80 keV.

**Protein identification by peptide mass fingerprinting.** Proteins were excised from Coomassie brilliant blue R250-stained gels and subjected to matrix-assisted laser desorption-ionization time-of-flight analysis as previously described (6).

## RESULTS

### Morphogenesis in a series of *cotE* heterochronic mutants.

To begin addressing the function of CotE in relation to its time of expression during sporulation, we engineered strains in which *cotE* was placed under the control of either its P1 (strain AH2921; P<sub>*cotEPI*</sub>) or P2 (strain AH2920; P<sub>*cotEP2*</sub>) promoters (Table 1). Note that AH2921 bears a 6-bp deletion of the -10 region of the downstream *cotE* P2 promoter and that cloning of a *cotE* fragment extending just 82 bp upstream of the P2 transcriptional start site allowed elimination of the upstream *cotE* P1 promoter in strain AH2920 (see Material and Methods) (56). In addition, we have produced strains expressing *cotE* from the  $\sigma^K$ -dependent *gerE* promoter (strain AH2914; P<sub>*gerE*</sub>) (7) or from the  $\sigma^K$ - and GerE-dependent *cotG* promoter (AH2915; P<sub>*cotG*</sub>) (47). All the promoter fusions were inserted at the nonessential *amyE* locus of a strain bearing a deletion of *cotE* (AH2835) at its normal locus. To control for any effects of expressing *cotE* at *amyE*, we constructed AH2942, a derivative of AH2835 that bears a wild-type copy of *cotE* (under the control of both P1 and P2 [P<sub>*cotEPI*</sub>P2]) at *amyE* (Table 1). Results described below indicate that expression of *cotE* from both P1 and P2 at *amyE* (strain AH2942) largely mimics the expression pattern and functionality of *cotE* at its normal locus. We first wanted to test whether under our conditions any of the *cotE* alleles interfered with the normal course of morphogenesis. To do this, we used the hydrophilic membrane dye MTG and fluorescence microscopy to quantify the kinetics of appear-

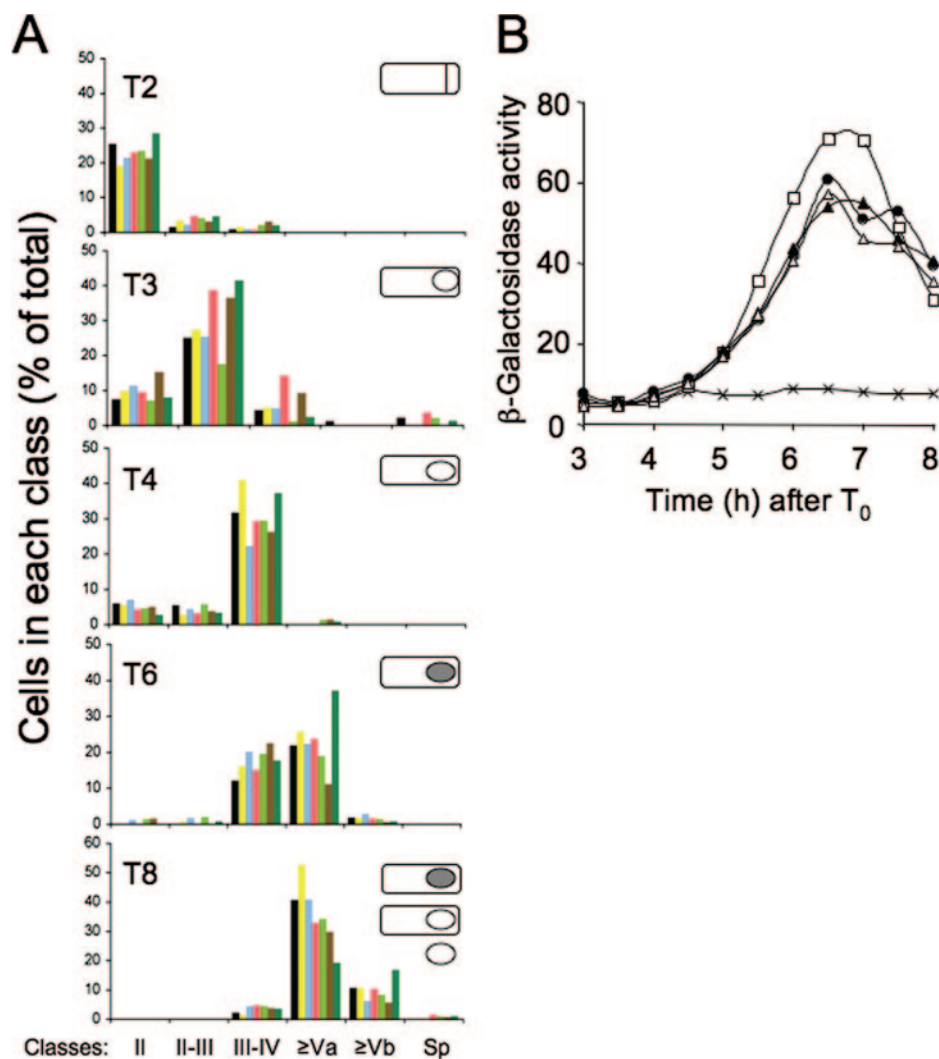


FIG. 2. The course of morphogenesis in the various *cotE* mutants. Panel A represents the evolution of various morphological classes during sporulation of the different mutants. Samples from sporulating cultures were collected at various times (T) in hours (2, 3, 4, 6, and 8) after the onset of sporulation, as indicated. Strains are represented as follows: MB24 (wild type), black; AH2942 ( $P_{cotEP1P2}$ ), yellow; AH2921 ( $P_{cotEP1}$ ), blue; AH2920 ( $P_{cotEP2}$ ), magenta; AH2914 ( $P_{gerE}$ ), light green; AH2915 ( $P_{cotG}$ ), brown; and AH2835 (*cotE*), dark green. Classes were defined as follows: II, straight septa; II-III, curved septa; III-IV, cells in which engulfment is completed; Va, phase-gray spores; Vb, phase-bright spores; Sp, free mature spores. Scoring was based on fluorescence microscopy following staining with the vital membrane stain MTG to visualize septa and on phase-contrast microscopy for the visualization of phase-gray, phase-bright, and released spores (Material and Methods). The inserts in each panel represent the main morphological class found at each time: straight septa (hour 2), curved septa (hour 3), engulfed prespores (hour 4), and phase-gray spores (hour 6 and 8). The hour 8 panel also includes phase-bright spores, as well as free spores. Phase-bright spores were the predominant class at hour 10 of sporulation (not represented). Panel B shows the levels of  $\beta$ -galactosidase activity (expressed in Miller units) resulting from expression of a *cotA-lacZ* transcriptional fusion at the indicated times after the onset of sporulation (T<sub>0</sub>) in the following strains: MB24 (wild type), crosses; AH2654 (*cotA-lacZ*), open squares; AH4113 ( $P_{gerE}$ -*cotE cotA-lacZ*) closed circles; AH4114 ( $P_{cotG}$ -*cotE cotA-lacZ*), closed triangles; and AH4116 ( $\Delta$ *cotE cotA-lacZ*), open triangles.

ance of asymmetric septa, curved septa, and fully engulfed prespores, and we used phase contrast microscopy to quantify the accumulation of phase-gray and phase-bright spores and released mature spores in the various strains. The results in Fig. 2A indicate that the various *cotE* promoter mutants did not cause any discernible effect on the timing of the main events that take place during spore morphogenesis. In particular, in all the strains examined, the engulfment process is concluded in the majority of the population 4 h after the onset of the sporulation (Fig. 2A). Engulfment completion represents the signal for  $\sigma^K$  activation and triggers expression of a

large subset of coat genes, among which is *cotA*, coding for an abundant outer coat component (48). The results in Fig. 2B show that expression of *cotA-lacZ* is induced in the  $P_{gerE}$  and  $P_{cotG}$  strains, as in the wild type or in the *cotE* null mutant, at hour 4 of sporulation. Expression of *cotA-lacZ* was also unaltered in the  $P_{cotEP1P2}$ ,  $P_{cotEP1}$ , and  $P_{cotEP2}$  strains (not shown in Fig. 2B for clarity).

**Synthesis and assembly of CotE.** Next, we wanted to investigate the time of synthesis and assembly of CotE in the series of promoter mutants. Cells of the wild-type strain MB24 and of the various *cotE* mutants were harvested at various times dur-



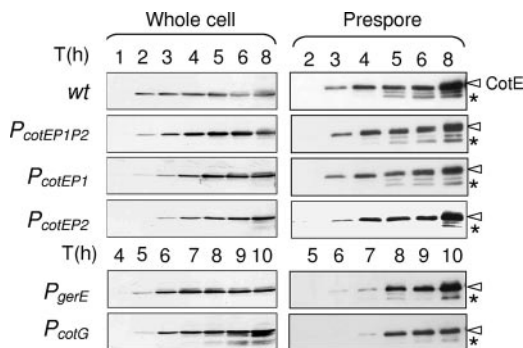


FIG. 3. Assembly of CotE. Panels A and B represent the accumulation of CotE in the whole-cell and prespore extracts of sporulating cells in strains MB24 (wild type), AH2942 (*P<sub>cotEP1P2</sub>*), AH2921 (*P<sub>cotEP1</sub>*), AH2920 (*P<sub>cotEP2</sub>*), AH2914 (*P<sub>gerE</sub>*), and AH2915 (*P<sub>cotG</sub>*), as indicated. Samples were collected at the indicated times (in hours) after the onset of sporulation. Proteins (30  $\mu$ g for the whole-cell lysates or 5  $\mu$ g for the prespore fraction) were resolved on 12.5% SDS-PAGE gels and transferred to nitrocellulose membranes, which were then probed with an anti-CotE polyclonal antibody. Please note that in case of the *P<sub>cotEP2</sub>* prespore fraction, 15  $\mu$ g of protein was used, as the mutant accumulates reduced levels of CotE. The arrowheads indicate the positions of the main CotE antigen, and the asterisks indicate the positions of a presumptive processed product of about 21 kDa.

ing sporulation and lysed, and the presence of CotE in whole-cell extracts or in spore fractions was examined by immunoblot analysis using a CotE-specific antibody (25) (see also Material and Methods). The presence of CotE in whole-cell extracts reflects its time of synthesis, whereas its detection in the spore fractions indicates the time of its recruitment to the surface of the forming spore (25). CotE was first detected in whole-cell extracts from hour 2 of sporulation onward in MB24, AH2942 (*P<sub>cotEP1P2</sub>*), and AH2921 (*P<sub>cotEP1</sub>*) and in the prespore fraction of the same strains from hour 3 onward (Fig. 3). Note, however, that the absence of CotE in the prespore fraction prior to hour 3 may result in part from the inefficiency with which this

fraction is prepared by centrifugation at this time in development (25, 39, 58). On the whole, our observations are consistent with previous studies in which CotE was found to be targeted to the surface of the developing spore at an early stage in development (12, 42, 53). In strain AH2920 (*P<sub>cotEP2</sub>*), CotE was only detected from hour 3 onward in both whole-cell and prespore fractions (Fig. 3). However, this strain differed from the wild type or the two strains in which expression of *cotE* at *amyE* involved the P1 promoter, as the level of CotE assembled appeared lower (Fig. 3). Indeed, three times more protein had to be analyzed for this strain in order to detect CotE in the prespore fractions (Fig. 3, legend).

In strain AH2914 (*P<sub>gerE</sub>*), CotE was detected in whole-cell lysates at hour 5 and in the prespore fraction at hour 6 (Fig. 3). In AH2915 (*P<sub>cotG</sub>*), CotE was found in the cell extracts at hour 5, albeit weakly, and more noticeably at hour 6, but its association with the spore fraction was only clearly seen at hour 7 of sporulation (Fig. 3). Together, these results show that when produced from P1 or P2, CotE is assembled essentially at the same time of sporulation as in the wild type and with little if any delay relative to its time of synthesis. In contrast, the postengulfment synthesis of CotE (from either the *gerE* or *cotG* promoters) appears to result in a delay of 1 to 2 h in the assembly of CotE, relative to its time of synthesis.

**Levels of CotE in purified spores.** The results described in the two preceding sections indicated that transplanting *cotE* to the  $\sigma^K$  regulon interfered with the assembly of CotE (Fig. 3). However, these experiments do not allow a direct comparison of the levels of CotE among the various mutants. This is because the samples were analyzed by loading an equal amount of total protein and because prespores of the various mutants (which predominate in the hour 8 sample) may have different protein contents (19, 20). We therefore compared the levels of CotE in equal numbers of purified spores of the various mutants. The results shown in Fig. 4A indicate that CotE is extracted from wild-type spores not only as a main band of about

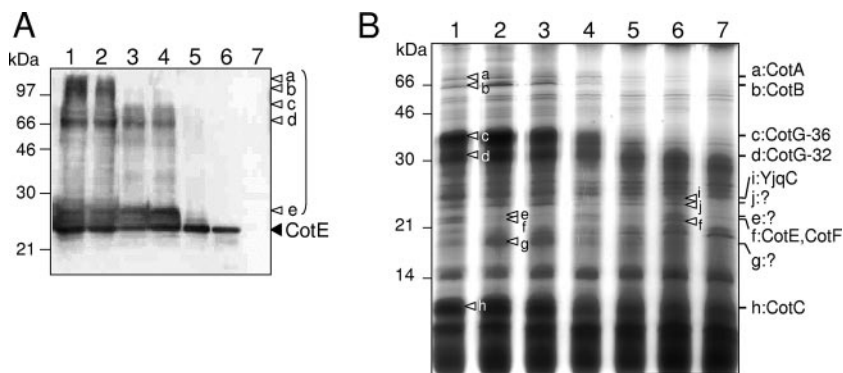


FIG. 4. Coat polypeptide composition in spores of the various *cotE* mutants. Panel A shows the accumulation of CotE in purified mature spores of strains MB24 (wild type; lane 1), AH2942 (*P<sub>cotEP1P2</sub>*; lane 2), AH2921 (*P<sub>cotEP1</sub>*; lane 3), AH2920 (*P<sub>cotEP2</sub>*; lane 4), AH2914 (*P<sub>gerE</sub>*; lane 5), AH2915 (*P<sub>cotG</sub>*; lane 6), and AH2835 (*cotE*; lane 7). Coat-extractable proteins were resolved by SDS-PAGE, transferred to nitrocellulose membranes, and probed with an anti-CotE antibody. The position of the CotE monomer is indicated by the black arrowhead, and the parenthesis indicates multimeric forms of CotE (bands a to e, white arrowheads). The positions of molecular mass markers (in kDa) are also shown. Panel B shows a Coomassie-stained gel of electrophoretically resolved coat proteins extracted from purified spores of the same strains indicated in panel A. White arrowheads in lane 1 (wild type) point to bands reduced or missing in the late or the null *cotE* mutants (lanes 5 to 7); they are identified on the right side of the panel. Bands designated as e, f, g, i, and j were excised and subjected to mass spectrometry analysis (see Material and Methods); the identification of bands i and f is indicated on the right side of the panel. No identification was possible for bands e, j, and g. The positions of molecular mass markers (in kDa) are shown on the left side of the panel.

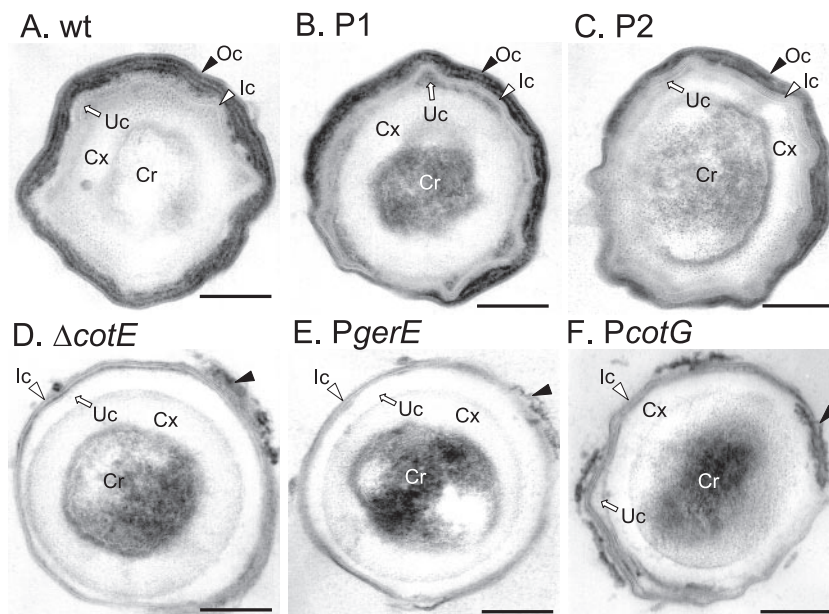


FIG. 5. Electron microscopy of wild-type and various *cotE* mutant spores. Spores were collected 24 h after the initiation of sporulation from DSM cultures of MB24 wild-type (wt) strain (A), AH2921 ( $P_{cotEP1}$ ) (B, P1), AH2920 ( $P_{cotEP2}$ ) (C, P2), AH2835 (*cotE*) (D,  $\Delta cotE$ ), AH2914 ( $P_{gerE}$ ) (E), and AH2915 ( $P_{cotG}$ ) (F). The spores were purified and processed for electron microscopy analysis as described in Material and Methods. The white and black arrowheads point to the inner and outer coat structures, respectively, and the arrow points to the undercoat (the slightly more electro-dense region between the inner coat and the cortex) or undercoat region. Ic, inner coat; Oc, outer coat; Uc, under coat; Cx, cortex; Cr, spore core. Bar, 0.2  $\mu$ m.

24 kDa (black arrowhead) which is the size of monomeric CotE but also as several multimeric forms, in agreement with previous immunoblot analyses (2) and with previous results in which mass spectrometry techniques have been used to analyze the coat composition (31, 32). The multimeric forms of CotE comprise five main species, labeled a through e in Fig. 4A (white arrowheads and lane 1). Essentially the same levels of monomeric CotE and of species a through e are seen in the  $P_{cotEP1P2}$  control strain (Fig. 4A, lanes 1 and 2). In the P1 and P2 strains, however, bands a and b are considerably reduced (Fig. 4A, lanes 3 and 4), whereas in  $P_{gerE}$  and  $P_{cotG}$  (lanes 5 and 6) bands a to e are almost undetectable. In all strains, the levels of monomeric CotE remains very similar (Fig. 4A). Together, the results suggest that the late expression of *cotE* drastically reduces the multimerization of CotE at the spore surface but has no proportional effect on the level of monomeric CotE.

**The overall composition of the coat.** To examine the overall composition of the coat in the various mutants, coat protein extracts were prepared from equivalent numbers of purified spores of the various strains and resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and the gels were stained with Coomassie (see Material and Methods). Overall, the coat polypeptide composition of AH2942 ( $P_{cotEP1P2}$ ) and AH2921 ( $P_{cotEP1}$ ) spores was very similar to that of wild-type spores (Fig. 4B, lanes 1 to 3), except for two species above the 21-kDa marker band that appeared reduced (Fig. 4B, lane 2, bands e and f) and another below the 21-kDa marker (band g) that was more extractable. Band f was identified by mass spectrometry as containing CotE and CotF species (see below). Attempts to identify species present on bands e and g were inconclusive (not shown). In contrast to the

$P_{cotEP1}$  strain, the representation of several coat components was altered in spores produced by AH2920 ( $P_{cotEP2}$ ) relative to AH2942 ( $P_{cotEP1P2}$ ) spores (Fig. 4B, lanes 2 and 4). The use of mutants in conjunction with immunoblot analysis indicated that CotA (band a), CotB (band b), two forms of CotG of 32 (band c) and 36 kDa (band d), and CotC (band h) were reduced in  $P_{cotEP2}$  spores (not shown) (10, 33, 58). All these proteins have been assumed to reside mainly in the spore outer coat because they are greatly reduced or missing from the coats of a *cotE* null mutant (55) (Fig. 4B, lane 7). It seems that proper coat assembly relies to a greater extent upon the expression of *cotE* from P1 than from P2 and that expression solely from P2 results in spores with reduced levels of some outer coat proteins. Nevertheless, the coat protein profile of AH2920 ( $P_{cotEP2}$ ) spores resembled more that of wild-type spores (Fig. 4B, lane 1) than that of *cotE* spores (Fig. 4B, lane 7). Strikingly, the coat protein profiles of AH2914 ( $P_{gerE}$ ) and AH2915 ( $P_{cotG}$ ) spores appeared very similar to the profile of *cotE* spores (Fig. 4B, lanes 5 to 7). In particular, outer coat proteins CotA, CotB, CotC, and CotG (10, 47, 56, 58) were greatly reduced or undetected. The results suggest that spores of the  $P_{gerE}$  and  $P_{cotG}$  mutants have a compromised outer coat structure. The results further advocate that the early expression of *cotE* is important for proper outer coat assembly.

**Early expression of *cotE* is essential for assembly of the outer coat structure.** To assess the status of the coat layers, purified spores were examined by thin-section electron microscopy. The coats of MB24 (wild type) spores showed the typical organization of the coat layers, with a lamellar inner coat and a thick, electron-dense striated outer coat closely apposed to the inner coat (Fig. 5A). Spores of AH2942 ( $P_{cotEP1P2}$ ) (not

TABLE 2. Spore resistance properties of various *cotE* mutants

Strain	Relevant genotype	Cell count (CFU/ml) <sup>a</sup>			Cell count after decoating (CFU/ml) <sup>a</sup>		
		Viable	Heat <sup>R</sup>	Lys <sup>R</sup>	Viable	Heat <sup>R</sup>	Lys <sup>R</sup>
MB24	Wild type	7.9 × 10 <sup>8</sup>	6.4 × 10 <sup>8</sup>	5.8 × 10 <sup>8</sup>	3.4 × 10 <sup>7</sup>	1.9 × 10 <sup>7</sup>	5.0 × 10 <sup>3</sup>
AH2835	Δ <i>cotE</i>	3.3 × 10 <sup>8</sup>	3.7 × 10 <sup>8</sup>	1.5 × 10 <sup>6</sup>	2.6 × 10 <sup>8</sup>	1.3 × 10 <sup>8</sup>	≤10 <sup>3</sup>
AH2914	Δ <i>cotE</i> Δ <i>amyE</i> ::P <sub><i>gerE</i></sub> - <i>cotE</i>	6.1 × 10 <sup>8</sup>	6.4 × 10 <sup>8</sup>	3.5 × 10 <sup>8</sup>	9.8 × 10 <sup>7</sup>	5.1 × 10 <sup>7</sup>	1.0 × 10 <sup>4</sup>
AH2915	Δ <i>cotE</i> Δ <i>amyE</i> ::P <sub><i>cotG</i></sub> - <i>cotE</i>	4.2 × 10 <sup>8</sup>	6.2 × 10 <sup>8</sup>	9.9 × 10 <sup>7</sup>	7.9 × 10 <sup>7</sup>	7.7 × 10 <sup>7</sup>	≤10 <sup>3</sup>
AH2920	Δ <i>cotE</i> Δ <i>amyE</i> ::P <sub><i>cotEP2</i></sub> - <i>cotE</i>	6.9 × 10 <sup>8</sup>	6.0 × 10 <sup>8</sup>	1.7 × 10 <sup>8</sup>	6.0 × 10 <sup>7</sup>	6.3 × 10 <sup>7</sup>	5.0 × 10 <sup>3</sup>
AH2921	Δ <i>cotE</i> Δ <i>amyE</i> ::P <sub><i>cotEP1</i></sub> - <i>cotE</i>	3.0 × 10 <sup>8</sup>	2.2 × 10 <sup>8</sup>	1.0 × 10 <sup>8</sup>	9.5 × 10 <sup>7</sup>	6.5 × 10 <sup>7</sup>	1.1 × 10 <sup>4</sup>
AH2942	Δ <i>cotE</i> Δ <i>amyE</i> ::P <sub><i>cotEP1P2</i></sub> - <i>cotE</i>	1.9 × 10 <sup>8</sup>	2.0 × 10 <sup>8</sup>	1.2 × 10 <sup>8</sup>	6.6 × 10 <sup>7</sup>	2.0 × 10 <sup>7</sup>	2.9 × 10 <sup>4</sup>

<sup>a</sup> The titer of viable cells and heat- and lysozyme-resistant (Heat<sup>R</sup> and Lys<sup>R</sup>) spores was measured 24 h after the onset of sporulation in DSM (see Material and Methods).

shown) and those of AH2921 (P<sub>*cotEP1*</sub>) (Fig. 5B) were indistinguishable from wild-type spores. Spores of AH2920 (P<sub>*cotEP2*</sub>) also showed no major alterations in the structure of the coat layers (Fig. 5, compare panels A and C). Thus, expression of *cotE* from either P1 or P2 still permitted the formation of spores essentially with a normal ultrastructural organization.

In contrast, electron micrographs of AH2914 (P<sub>*gerE*</sub>) (Fig. 5E) and AH2915 (P<sub>*cotG*</sub>) (Fig. 5F) spores revealed a seemingly normal inner coat but the almost complete absence of the outer coat layer (Fig. 5E and F). An expanded undercoat region is also particularly evident for AH2914 (P<sub>*gerE*</sub>) spores (Fig. 5E). These morphological features are characteristic of spores of a *cotE* null mutant (Fig. 5D) (2, 49, 55). Occasionally, remnants of electro-dense material were observed in association with the defective spores of AH2914 or AH2915 (Fig. 5E and F). However, this was also observed for *cotE* spores (Fig. 5D). Thus, spores of AH2914 or AH2915 did not differ structurally in any discernible way from spores produced by a *cotE* null mutant. Hence, expression of *cotE* under the control of the *gerE* or *cotG* promoters, while causing no discernible structural alterations at the level of inner coat, does not permit assembly of a visible outer coat structure.

**Spore resistance to lysozyme can be uncoupled from outer coat formation.** Spores of a *cotE* null mutant are unable to assemble the outer coat layer and are highly susceptible to lysozyme treatment (55). Because shifting *cotE* to late classes of mother cell-specific gene expression blocked outer coat assembly, we wanted to determine whether spores of the various *cotE* promoter mutants retained resistance to lysozyme. Spores produced by the various promoter mutants were purified 24 h after the onset of sporulation and assayed for their resistance to lysozyme and heat (see Material and Methods). The *cotE* null mutant AH2835 formed only 10<sup>6</sup> lysozyme-resistant spores per ml of culture (Table 2). To our surprise, all the *cotE* promoter mutants presented close to parental levels of lysozyme resistance, about 10<sup>8</sup> CFU/ml (Table 2). We noted that the resistance levels observed for the AH2915 (P<sub>*cotG*</sub>) spores were reproducibly lower than for the other mutants but still close to 10<sup>8</sup> CFU/ml (Table 2). To exclude the possibility that lysozyme was inactivated by a coat-associated protease exposed due to abnormal coat assembly in the mutants, we treated spores of the *cotE* null mutant with the lysozyme solutions recovered after treatment of AH2914 and AH2915 spores and found the same spore titer as with a freshly prepared solution (10<sup>6</sup> CFU/ml) (data not shown). In all the

promoter mutants, formation of lysozyme-resistant spores still required expression of the morphogenetic loci *spoIVA*, *spoVID*, and *safA* (11, 21, 22) (not shown). None of the *cotE* promoter mutations affected the resistance of spores to heat treatment (Table 2).

Remarkably, while spore resistance to lysozyme demands expression of the *cotE* gene, it is largely independent of the time of expression of *cotE*. Because the “late” mutants fail to assemble the outer coat but form lysozyme-resistant spores, it follows that outer coat assembly is not a strict requirement for spore resistance to lysozyme.

**CotE and spore resistance to lysozyme.** We presume that the lysozyme resistance of spores produced by the various *cotE* promoter mutants, including those unable to assemble the outer coat, is due to the assembly of coat components around the developing spore and not to any other alteration of the spore not apparent by transmission electron microscopy. To investigate this, we subjected purified wild-type and *cotE* spores, as well as spores of the various *cotE* promoter mutants, to a standard alkaline decoating regime (4, 41) and assayed for lysozyme resistance. For all strains the decoating regime reduced the lysozyme-resistant cell count to about 0.01% of the level obtained prior to decoating (Table 2). In contrast, spores of all strains remained heat resistant following decoating (Table 2), indicating that the spore cortex was not affected by the treatment. Moreover, the SDS-PAGE profile of the proteins extracted by the alkaline treatment (not shown) resembled that obtained by the SDS-dithiothreitol extraction of the coat proteins from spores of the same strains (Fig. 4B). This suggests that the alkali treatment removed most of the coat proteins present or remaining in spores of the various *cotE* promoter mutants.

Since species of about 26 kDa (Fig. 4B, band i in lane 6), 24 kDa (band j), and 22 kDa (band f) that were still extracted from AH2914 or AH2915 spores (lysozyme resistant) were missing from coat extracts of the *cotE* null mutant (lysozyme susceptible), we attempted to identify them by matrix-assisted laser desorption–ionization time-of-flight mass spectrometry. Note that band e is also seen in AH2914 or AH2915 spores but absent from the null mutant, but this species is also reduced in the lysozyme-resistant spores of AH2921 (P<sub>*cotEP1*</sub>) (see above). In any case, no clear identification by peptide mass fingerprinting was possible for bands e (above) or j. Coat proteins CotE and CotF were identified in band f (see above). The identification of CotE (21 kDa) is in agreement with the immunodetection of CotE in spores of AH2915, but the 19-kDa product

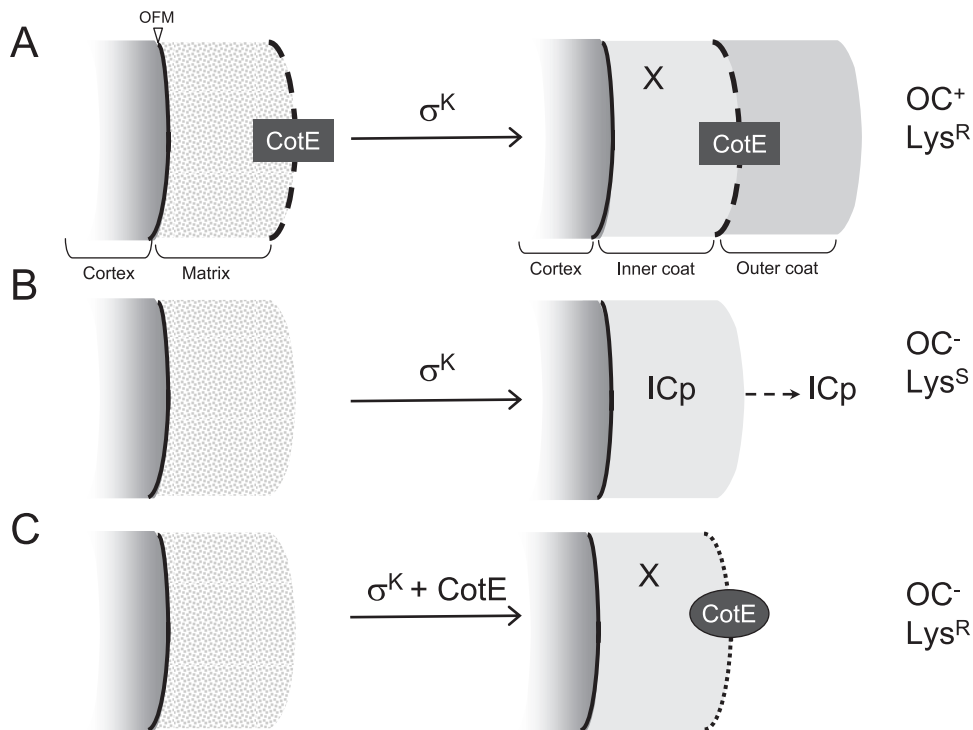


FIG. 6. Dual function of CotE in spore resistance to lysozyme and assembly of the outer coat. Morphogenetic protein SpoIVA localizes at or close to the outer forespore membrane (OFM). The localization of SpoIVA is required for the assembly of CotE (black box and dashed line) at the edge of a matrix of unknown composition, which is adjacent to the cortex. Following engulfment completion and the activation of  $\sigma^K$ , the matrix is transformed into the inner coat. In wild-type cells (A), CotE remains at the edge of the changing matrix from where it nucleates outer coat assembly ( $\text{OC}^+$ ), and the resulting spores are resistant to lysozyme ( $\text{Lys}^R$ ). In the absence of CotE (B), the matrix still develops into an inner coat, but the resulting spores lack the outer coat ( $\text{OC}^-$ ) and are susceptible to lysozyme ( $\text{Lys}^S$ ). Some inner coat proteins (ICp) are lost from these spores. The expression of *cotE* under  $\sigma^K$  control (C) causes abnormal assembly of CotE (black oval and dotted line; CotE is represented at the edge of the inner coat, but it may also be deposited within the inner coat). The resulting spores lack an outer coat ( $\text{OC}^-$ ) yet are fully resistant to lysozyme ( $\text{Lys}^R$ ). Inner coat proteins that may contribute to spore lysozyme resistance are represented by X.

of the *cotF* gene is normally proteolytically converted into forms of about 5 and 8 kDa by the YabG protease (8). The 19-kDa CotF band may reflect abnormal assembly of YabG or, alternatively, a cross-linked product of mature CotF (31). In any event, CotF is not required for spore resistance to lysozyme (8). Lastly, and in good agreement with its predicted size, we found band i to contain the YjqC protein (31.2 kDa). YjqC was recently identified as a spore-associated protein in the proteomics study of Kuwana et al. (31), and *yjqC* was identified as a  $\sigma^K$ -dependent gene (13, 51). YjqC appears to be an Mn-dependent catalase related to inner coat protein CotJC (20, 50). In contrast to CotJC, inactivation of which has no major impact on spore properties (20, 50), we found that a *yjqC* insertional mutant produced heat- and lysozyme-sensitive spores (about  $10^3$  CFU/ml) (data not shown). Heat sensitivity implies the absence or deficiency of the cortex peptidoglycan layer (16). Undoubtedly, YjqC plays an important role in sporulation but does not appear to have a specific role in spore protection against lysozyme.

## DISCUSSION

The early localization of CotE close to the forespore outer membrane in an *spoIVA*-dependent manner has been hypothesized to help establish a topological plan important for guid-

ing the assembly of the outer coat when, following engulfment completion,  $\sigma^K$  becomes active in the mother cell (12) (Fig. 1A). The position of CotE, which forms a ring around the forespore after the completion of engulfment, has been viewed as the site of outer coat assembly, whereas the region interior to the CotE ring is presumably converted into the inner coat (11, 12, 21, 22).

However, *cotE* is expressed both before and after engulfment completion, and since the inner coat forms essentially in a CotE-independent manner (12, 55), it would seem plausible that CotE produced in the postengulfment sporangium could still localize to the edge of the matrix region and still nucleate deposition of the outer coat. An important finding of our study is that a class of "early" mutants, in which *cotE* is expressed from either P1 or P2, is able to form the outer coat, whereas a class of "late" mutants, expressing *cotE* under the control of  $\sigma^K$ -dependent promoters, fails to assemble a visible outer coat structure (Fig. 4 and 5). It thus appears that the early expression of *cotE* (as part of the  $\sigma^E$  regulon) is essential for outer coat assembly. The incapacity of the late mutants to support assembly of the outer coat structure is not likely to be due to changes in expression levels inherent to the various promoters used because the greatly reduced levels of CotE that accumulate from the weak P2 promoter, turned on just before engulfment completion (13, 14, 51, 56), supported outer coat assem-



bly while the much higher levels that accumulate from the *gerE* promoter, activated just after engulfment completion (7, 13, 14, 51), did not (Fig. 3). P2 has an atypical  $-35$  promoter element, which could explain the need for SpoIIID for its utilization in vivo (56), and was found to be active during late stages of development, under  $\sigma^K$  control (13). The capacity of P2 to support outer coat assembly (in contrast to the stronger *gerE* promoter) underscores the importance of *cotE* expression prior to engulfment completion for outer coat assembly.

Why the late *cotE* mutants fail to nucleate normal coat assembly is unclear. When made under  $\sigma^K$  control, CotE assembly appears reduced (Fig. 4), and it seems plausible that CotE is only productively assembled at the edge of the matrix region prior to the stage (following engulfment completion) when this region begins to be converted into a visible inner coat. In this view, assembly of CotE produced under  $\sigma^K$  control would be abnormal. We note that in the early class of mutants as in the wild type, CotE appears to undergo extensive multimerization at the spore surface (2, 31, 32) (Fig. 4). In contrast, in the late mutants, multimerization of CotE, which may represent formation of SDS-dithiothreitol-resistant cross-linked species (2, 31, 32), is greatly reduced, in spite of the fact that the level of the monomeric form of CotE does not vary much among the early or late mutants (Fig. 4). Reduced multimerization could be due to decreased assembly of proteins with which CotE interacts, but it is also tempting to speculate that multimerization of CotE is a factor in its ability to nucleate assembly of the outer coat.

Because spores of a *cotE* deletion mutant lack the outer coat and are sensitive to lysozyme treatment, the outer coat has been viewed as a barrier to the access of lytic enzymes to the spore cortex peptidoglycan (55). However, the observation that the late *cotE* mutants, which lack the outer coat, are still resistant to lysozyme suggests that CotE has genetically separable functions in lysozyme resistance and outer coat assembly. The apparent lack of correlation between outer coat assembly and spore resistance to lysozyme has been documented before. For example, a *cotE* mutant with a deletion of residues 58 to 75 failed to assemble the outer coat but showed a small decrease in lysozyme resistance (22% of the wild type, compared to 6% for a *cotE* null mutant) (5). A modest decrease in lysozyme resistance along with the absence of an outer coat was also seen for other deletions in the N or C termini of CotE (5). Also, in *Bacillus anthracis* in which a highly similar CotE ortholog is present, disruption of the gene for the Cot $\alpha$  protein strongly interferes with assembly of the darkly staining outer coat but not with lysozyme resistance (28).

The basis for the lysozyme resistance of the P<sub>*gerE*</sub> and P<sub>*cotG*</sub> mutants is not presently known. One possibility is that CotE per se is sufficient to confer lysozyme resistance. It could be that sufficient CotE is assembled so as to form a shell around the spore surface capable of blocking access of lysozyme to the spore cortex (Fig. 6). However, we cannot exclude the possibility that in the P<sub>*gerE*</sub> and P<sub>*cotG*</sub> mutants residual assembly of one or several CotE-controlled proteins mainly associated with the outer coat layer. However, CotE may also control the assembly of inner coat proteins important for lysozyme resistance (Fig. 6, X). Deletion of *cotE* also affects the inner coat (although not sufficiently to cause any alteration visible by electron microscopy), from which some proteins are missing or

tend to be lost when the spore is released into the environment (1, 6, 44, 52) (Fig. 6). The involvement of the inner coat layers in lysozyme resistance has also been suggested before (38, 57).

Our analysis of the species present in the coats of spores of the P<sub>*cotG*</sub> strain (lysozyme resistant) but absent from *cotE* spores (lysozyme sensitive) did not lead to the identification of proteins (other than CotE itself) with roles in lysozyme resistance. Also, we note that no CotE-controlled protein is presently known to have a key role in lysozyme resistance (11, 21, 22). However, recent studies suggest the existence of a number of as-yet-uncharacterized proteins that may associate with the coat layers, and some of these could be important for spore resistance to lysozyme (13, 14, 31, 32). Together, our results indicate that assembly of the outer coat structure is not a strict requirement for spore resistance to lysozyme and raise the possibility that CotE itself or as-yet-uncharacterized CotE-controlled proteins have a key role in resistance to this agent.

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