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A Spore Coat Protein, CotS, of *Bacillus subtilis* Is Synthesized under the Regulation of σ^K and GerE during Development and Is Located in the Inner Coat Layer of Spores

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The spore coat of *Bacillus subtilis* has a unique morphology and consists of polypeptides of different sizes, whose synthesis and assembly are precisely regulated by a cascade of transcription factors and regulatory proteins. We examined the factors that regulate *cotS* gene expression and CotS assembly into the coat layer of *B. subtilis* by Northern blot and Western blot analysis. Transcription of *cotS* mRNA was not detected in sporulating cells of σ^K and *gerE* mutants by Northern blot analysis. By Western blot analysis using anti-CotS antibody, CotS was first detected in protein samples solubilized from wild-type cells at 5 h after the start of sporulation. CotS was not detected in the vegetative cells and spores of a *gerE* mutant or in the spores of mutants deficient in σ^E, σ^F, σ^G, or σ^K. CotS was detected in the sporangium but not in the spores of a *cotE* mutant. The sequence of the promoter region of *cotS* was similar to the consensus sequences for binding of σ^K and GerE. These results demonstrate that σ^K and GerE are required for *cotS* expression and that CotE is essential for the assembly of CotS in the coat. Immunoelectron microscopic observation using anti-CotS antibody revealed that CotS is located within the spore coat, in particular in the inner coats of dormant spores.

Endospore formation by *Bacillus subtilis* is a good model system with which to study fundamental issues of cell biology concerning how the genes involved in cell differentiation are temporally regulated and how structural protein components are assembled at particular sites within a cell. After a final round of chromosomal replication in *B. subtilis*, cells asymmetrically divide into two compartments, the mother cell and the forespore. The forespore is engulfed by the mother cell double-membrane septum as a discrete cell within the mother cell, and the forespore is then surrounded by a cortex layer and coat layers to make a mature spore. The programs of gene expression are distinct in each compartment. Sporulation genes (*spo*) and other related genes that are active in the forespore compartment are governed by RNA polymerase sigma factors σ^F and σ^G. Gene expression in the mother cell is governed by σ^E and σ^K and by the regulatory proteins SpoIIID and GerE (39).

Bacterial spores are morphologically complex structures in which the spore core (cytoplasm), a primordial germ cell wall, a cortical layer (cortex), and a proteinous spore coat are observed. The spore coat, surrounding the cortex, consists of an electron-dense thick outer layer and a thinner, lamella-like inner layer (4), with dozens of proteins ranging in size from 8 to 65 kDa (32). The cortex and coat layers are essential for the remarkable resistance properties of spores. The spore coat, particularly, provides a protective barrier against lysozyme, solvents, and other harsh chemicals and is in part responsible for the prompt response of spores to components capable of triggering germination (4–7, 12, 18, 22, 36, 38).

A recent systematic search of the *B. subtilis* genome listed at least 22 genes that are necessary for the formation of the spore coat (21). Correct formation of the coat is under dual control. A cascade of transcription factors regulates the temporal appearance of the coat components (39), and the action of morphogenetic proteins controls proper assembly of those components to organize the two layers of the coat (38). Temporal control of spore coat genes (*cot*) involves a cascade of four regulatory factors in the sequence, σ^E-SpoIIID-σ^K-GerE (38). σ^E and σ^K are RNA polymerase sigma factors, whereas SpoIIID and GerE are DNA-binding proteins. The *cot* genes and their transcription regulators can be divided into four classes based on their appearance during sporulation (24). The class 1 genes, *spoIIID* and *cotE*, are expressed after the onset of sporulation under the control of σ^E. In class 2, *sigK* and *cotJABC* are expressed by the action of σ^E and SpoIIID. The class 3 genes, *gerE*, *cotA*, *cotD*, *cotF*, and *cotH*, are controlled by σ^K. The last class of genes, *cotB*, *cotC*, *cotG*, *cotYZ*, and *cotVXW*, are expressed under the control of σ^K and GerE. CotE is a morphogenic protein required for the assembly of proteins of the electron-dense outer layer of the spore coat and serves as a basement protein on which the proteins of the outer coat assemble (38). GerE is required for assembly of most of the lamella-like inner coat layer (22).

The *cotS* operon of *B. subtilis* consists of *cotS*, which encodes a spore coat protein (CotS) of 41 kDa, and open reading frame *orfX* (named *ytxN* in the *B. subtilis* genome project [21]) (2). The *cotS* operon is transcribed at about the fifth hour of sporulation (T_5) and has a putative promoter sequence similar to the consensus sequence for σ^K-dependent promoters. Disruption of the *cotS* gene results in no alteration of growth, sporulation, spore germination, or spore resistance to organic solvents (2). A similar observation has been made for other *cot* genes (32). In this study, we examined what regulatory factors direct CotS protein synthesis and which factors direct its assembly into the spore coat. We first purified recombinant CotS having a His_6 tag from *Escherichia coli* and prepared antibody against the

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TABLE 1. Bacterial strains and plasmids used

Strain or plasmid	Genotype or description	Source (reference)
B. subtilis		
168*trpC2*	*trpC2*	BGSC[a]
1S101	*cotA*::*cat trpC2*	BGSC
1S102	*cotB*::*cat trpC2*	BGSC
1S103	*cotC*::*cat trpC2*	BGSC
1S104	*cotD*::*cat trpC2*	BGSC
1S105	*cotE*Δ::*cat trpC2*	BGSC
1S106	*cotF*::*cat trpC2*	BGSC
1S108	*cotT*::*p*Δ *E194 pheA1 trpC2*	BGSC
CB701	*cotS*::*cat trpC2*	This work
1G12	*gerE36 leu-2 trpC2*	BGSC
1S38	*spoIIIC94 trpC2* (σ^K mutant)	BGSC
1S60	*leuB8 spoIIG41 tal-1* (σ^E mutant)	BGSC
SC1159	*spoIIAC1* (σ^F mutant)	S. Cutting (10)
spoIIIGΔ1	*spoIIIG*Δ*1 trpC2* (σ^G mutant)	J. Sekiguchi (31)
E. coli JM109	*relA supE44 endA1 hsdR17 gyrA96 mcrA mcrB$^+$ thi* Δ (*lac-proAB*)/ F′ (*traD36 proAB$^+$ lacI^q lacZ*Δ*M15*)	J. Sambrook (28)
Plasmids		
pCX18S	Cm^r *cotS5′ lacZ′*	A. Abe (2)
pTUE1122	Amp^r *lacI tac* promoter His$_6$	A. Nakane (23)
pBCS1	Amp^r *lacI tac* promoter *cotS* His$_6$	This work

[a] BGSC, *Bacillus* Genetic Stock Center.

protein. Using this antibody, we demonstrated that expression of *cotS* depended on σ^K and *gerE* and that assembly of CotS into the spore coat depended on CotE. Furthermore, immunoelectron microscopy revealed that CotS localized to the inner coat and/or on the outside of the cortex of the mature spore.

MATERIALS AND METHODS

Bacterial strains, plasmids, media, and general techniques. The *B. subtilis* strains used in this study are listed in Table 1 and were all grown in DS medium (30). *E. coli* was grown in LB medium. The conditions for sporulation of *B. subtilis* and method for purification of mature spores have been described previously (2). Recombinant DNA methods were as described by Sambrook et al. (28). Methods for preparing competent cells, for transformation, and for the preparation of chromosomal DNA from *B. subtilis* were as described by Cutting and Vander Horn (11).

Preparation of the *cotS* mutant. Plasmid pCX18S, which had been prepared by ligation of a central portion of the *cotS* gene (302 bp) between the *Pst*I and *Hin*dIII sites of plasmid pCX18 (2.4 kb) (2), was transformed into *B. subtilis* 168*trpC2* to obtain the *cotS* gene disruption mutant CB701. The correct integration of pCX18 in CB701 was verified by restriction analysis of DNA amplified from *cotS* by PCR.

RNA preparation and Northern analysis. *B. subtilis* cells were grown in DS medium, and 5-ml samples were harvested every hour throughout sporulation. The RNA was then prepared as described by Igo and Losick (17). Each 10 μg of the RNA preparation was analyzed by size fractionation through a 1% (wt/vol) agarose gel containing 2.2 M formaldehyde and transferred to a positively charged Hybond-N$^+$ membrane (Amersham). The membrane was stained with 0.04% methylene blue solution containing 0.5 M sodium acetate (pH 5.2) to measure the concentrations of 16S and 23S RNAs in the preparations as described by Herrin and Schmidt (16). The RNA on the membrane was hybridized to a DNA probe corresponding to nucleotides −64 to +467 of the translation initiation codon of *cotS*. This DNA fragment was amplified by PCR using two primers, 5′-GCTTCTAGAGGGTGGCTGAAAA-3′ and 5′-TAATACGACTCACTATAGGGCGATCCTGCAGCTTCCAACGG-3′. Hybridization was performed and hybrids were detected according to the procedure provided by Boehringer Mannheim.

Preparation of whole proteins from sporulating cells. Cultures (5 ml) were harvested every hour throughout sporulation and washed with 10 mM sodium phosphate buffer (pH 7.2). The pellets were suspended in 100 μl of lysozyme buffer (25 mM Tris-HCl [pH 8.0], 10 mM EDTA, 50 mM glucose, 1% lysozyme), kept on ice for 5 min, and then boiled for 5 min in 2% (vol/vol) sodium dodecyl sulfate (SDS)–5% (vol/vol) 2-mercaptoethanol–10% (vol/vol) glycerol–62.5 mM Tris-HCl (pH 6.8)–0.05% (wt/vol) bromophenol blue.

Preparation of spores and solubilization of coat proteins. Cultures (5 ml) were harvested at T_{18} and washed with 10 mM sodium phosphate buffer (pH 7.2). The pellets were suspended in 100 μl of lysozyme solution (10 mM sodium acetate [pH 7.2], 1% lysozyme) and incubated for 15 min at 37°C. After addition of 1.0 ml of 10 mM sodium phosphate buffer (pH 7.2), the suspensions were centrifuged to remove soluble proteins from mother cells. The spores in the pellet fraction were boiled in 2% (vol/vol) SDS–5% (vol/vol) 2-mercaptoethanol–10% (vol/vol) glycerol–62.5 mM Tris-HCl (pH 6.8)–0.05% (wt/vol) bromophenol blue for 5 min.

SDS-PAGE and immunoblotting. Protein samples were analyzed by SDS-polyacrylamide gel electrophoresis (PAGE) (12.0% acrylamide) as described previously (1). For immunoblotting, proteins were transferred onto a polyvinyl difluoridine membrane (Immobilon; 0.45-μm pore size; Millipore), and detected by using rabbit immunoglobulin G (IgG) against CotS as the first antibody and donkey anti-rabbit IgG–horseradish peroxidase conjugate as the second antibody (Amersham). Anti-σ^A antibody and anti-σ^K antibody were provided by M. Fujita and Y. Sadaie (National Institute of Genetics, Mishima, Japan).

Purification of recombinant CotS protein. The DNA sequence from 67 bp upstream of the initiation codon of *cotS* to the stop codon was amplified by PCR using two primers, 5′-GCTTCTAGAGGGTGGCTGAAAA-3′ and 5′-TCAGATCTATTCGCCTCCCGAT-3′. An *Xba*I-to-*Bgl*II fragment carrying the amplified *cotS* gene was then inserted between the *Xba*I and *Bgl*II sites of plasmid pTUE1122, yielding the recombinant plasmid pBCS1. pTUE1122 is a multicopy *E. coli* plasmid containing the *tac* promoter, the *lacI* gene, and a multicloning site followed by a His$_6$ tag coding sequence (23). Consequently, the *cotS* gene in pBCS1 encodes a product with an additional amino acid sequence, RSHHHHHH, at its C-terminal end. Transformants carrying pBCS1 were grown in 200 ml of L broth supplemented with 50 μg of ampicillin per ml at 37°C for 3 h, the culture was made 1 mM in isopropyl-β-D-thiogalactopyranoside, and the cells were incubated for a further 3 h at 37°C. The His-tagged recombinant CotS protein was purified by affinity chromatography on Ni-nitrilotriacetic acid agarose beads (Qiagen Inc., Chatsworth, Calif.) as described previously (35) and was further purified by electroelution from an SDS-gel after SDS-PAGE as described previously (1).

Preparation of antibody against CotS. One milliliter of purified CotS (0.2 μg/μl) and 16 mg of killed *Mycobacterium tuberculosis* cells (Difco) were well mixed with 2 ml of complete Freund's adjuvant (Difco), and 3 ml of the emulsion was injected into a healthy rabbit. After 2 weeks, CotS solutions which were prepared with incomplete Freund's adjuvant (Difco) were injected; 2 weeks after the second immunization, antiserum for CotS was obtained.

Electron and immunoelectron microscopy. Electron and immunoelectron microscopic observations were carried out essentially as described by Sakae et al. (27), with minor modifications as follows. The spores prepared as described previously (2) were fixed with 4% freshly depolymerized paraformaldehyde–0.5% glutaraldehyde buffered at pH 7.0 with 50 mM cacodylate buffer at 4°C for 48 h. Fixed spores were then suspended in 2% agar, and small cubes were cut and dehydrated with a graded series of 30, 50, 70, 80, 90, and 100% ethanol for 1 h at each ethanol concentration at −20°C. The samples were suspended in Lowicryl K4M (Chemische Werke Lowi GmbH)–100% ethanol at 2:1, 1:1, and 1:2 ratios for 2 h at each ratio at 4°C and finally in Lowicryl K4M overnight at 4°C. Samples were then placed in gelatin capsules filled with Lowicryl K4M and polymerized by UV irradiation (model TUV-100 polymerizer; Dosake EM) for 24 h at 4°C. Ultrathin sections were cut with a glass knife on a Reichert Ultracut-E ultramicrotome and mounted on Formvar-coated nickel grids (Veco Ni 200). The grids were placed on droplets of 0.05% Tween 20–0.05 M Tris-HCl

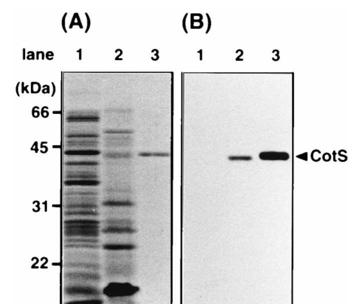


FIG. 1. Detection of CotS protein by immunoblotting using anti-CotS antibody. The protein samples were solubilized from vegetative cells of *B. subtilis* 168*trpC2* (lane 1), the SDS-mercaptoethanol-soluble fraction prepared from dormant spores of *B. subtilis* 168*trpC2* (lane 2), and purified CotS protein with a His$_6$ tag (lane 3). The samples were analyzed by SDS-PAGE (12% gel). (A) Coomassie brilliant blue stain; (B) immunoblotting using anti-CotS antibody. The arrowhead shows the migration position of CotS.

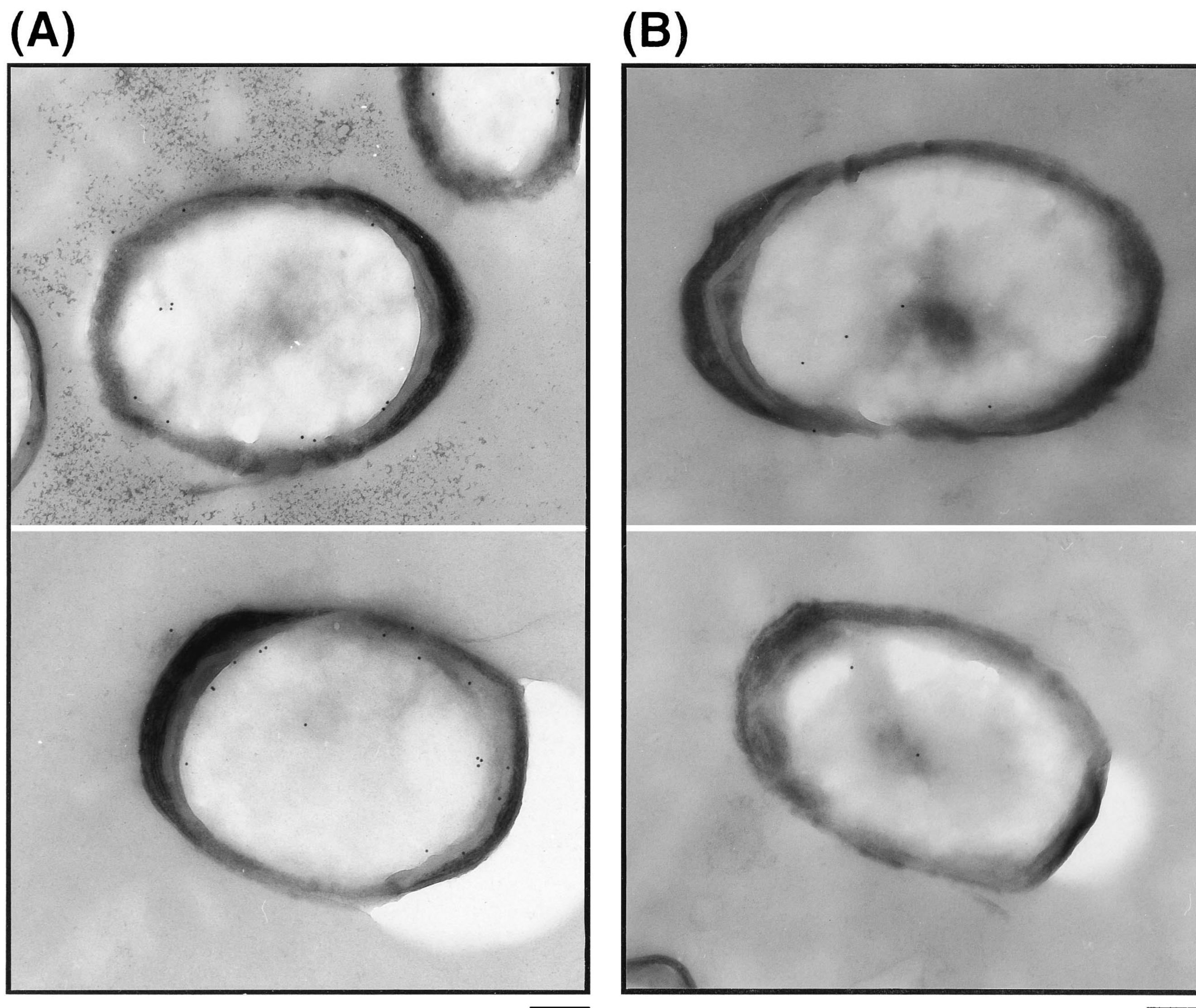


FIG. 2. Immunoelectron microscopic localization of CotS protein in wild-type spores (A) and *cotS* mutant spores. Thin sections of purified wild-type spores (A) and *cotS* mutant spores (CB701) (B) were stained with anti-CotS antibody and a colloidal gold (10 nm)-IgG complex. Bars = 200 nm.

buffered saline (pH 7.2) for 5 min and on Block Ace (Dainippon Pharmaceutical Co., Ltd.) for 1 h at room temperature. The grids were washed, treated with the anti-CotS antibody at a dilution of 1:120 in 1/10 Block Ace for 2 h at room temperature, and then treated with 10-nm gold-conjugated goat anti-rabbit IgG antibody (Zymed Laboratories) at a dilution of 1:40 in 1/10 Block Ace for 1 h at room temperature. The sections were stained with 4% uranyl acetate for 15 min at room temperature. Control sections were treated in a similar way by using preimmune serum from the same rabbit instead of anti-CotS antibody. The sections were observed with a JEM-1200EX electron microscope operating at 80 kV.

RESULTS

Expression of CotS during development. Since *cotS* was identified as the gene encoding the spore coat protein CotS (2), we tried to determine immunologically where during the developmental cycle of *B. subtilis* that CotS is present. The anti-CotS antibody reacted with purified CotS (Fig. 1B, lane 3) and also with CotS protein solubilized from mature spores by treatment with 2% SDS and 5% 2-mercaptoethanol (Fig. 1B, lane 2). However, CotS was not detected in protein samples solubilized from vegetative cells (Fig. 1B, lane 1). These results indicated that CotS protein is present in spores but not in the vegetative cells.

Immunocytochemical localization of CotS. Most spore coat proteins which have been analyzed are presumably located in the outer coat layer, while CotD, CotH, and CotT are thought to be present in the inner coat layer (5, 6, 38). The morphologies of spores of the wild-type and a *cotS* mutant were examined by electron microscopy and found to exhibit similar structures in outer and inner coat layers, cortex, and core regions (data not shown). Therefore, the location of CotS in spores was determined by immunochemical staining using anti-CotS antibody and colloidal gold-labeled second antibody (Fig. 2). In wild-type spores, many gold particles were observed within the spore integument, especially on the inner coat and on the outside region of the cortex, whereas only a few gold particles were seen in the core region (Fig. 2A). In contrast, negligible numbers of gold particles were observed in *cotS* mutant spores (Fig. 2B). Similarly, only a few gold particles were seen with

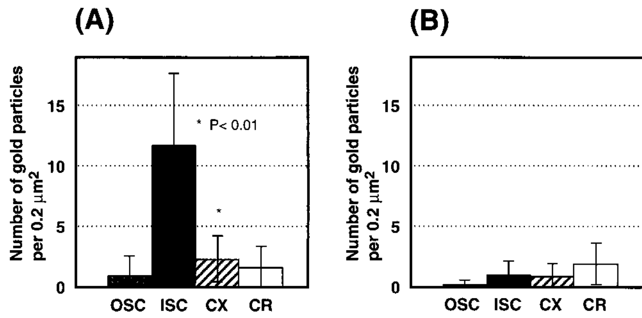


FIG. 3. Number of gold particles in each region of wild-type and *cotS* mutant spores. Each value represents the mean from the analysis of 20 spores (± standard deviation) sectioned and stained as described in the legend to Fig. 2. (A) Wild-type spores; (B) *cotS* mutant (CB701) spores. OSC, outer spore coat; ISC, inner spore coat; CX; cortex; CR, core.

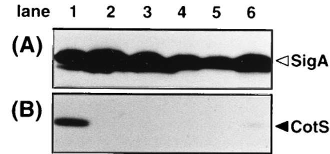


FIG. 5. CotS protein in mutants lacking sporulation-specific transcription factors. The sigma factor-deficient cells and *gerE* mutant cells were harvested from DS medium at T_8. Whole-protein samples were solubilized from the sporulating cells and were analyzed by SDS-PAGE (12% gel) and immunoblotting using anti-σ^A (A) and anti-CotS (B) antibodies. *B. subtilis* 168*trpC2* (lane 1), *spoIIG41* (σ^E mutant) (lane 2), *spoIIAC1* (σ^F mutant) (lane 3), *spoIIIGΔ1* (σ^G mutant) (lane 4), *spoIIIC94* (σ^K mutant) (lane 5), and *gerE36* (*gerE* mutant) (lane 6) were analyzed. The arrowheads indicate the bands for σ^A and CotS.

wild-type and *cotS* mutant spores in assays using preimmune serum (data not shown). To confirm the localization of CotS in wild-type spores, a statistical analysis of 20 photographs of wild-type and *cotS* mutant spores was carried out as described previously (15). The numbers of gold particles per 0.2 μm^2 were clearly highest in the inner spore coat of the wild-type strain and were much higher than in the inner coats of *cotS* mutant spores (Fig. 3). The analysis suggests that CotS is localized in the inner coat and/or subcoat regions of the spores.

Northern blot analysis of *cotS* mRNA. Previous work demonstrated that a *cotS* transcript appears at about T_5 and that the *cotS* operon has a σ^K-controlled promoter (2). To address questions concerning the dependence of *cotS* expression on σ^K and other factors, if any, we first examined expression of a *cotS-lacZ* fusion. Expression of the fusion in wild-type cells was detected first in cells at T_5 and increased during subsequent development; however, no *cotS-lacZ* expression was detected in a σ^K mutant (data not shown). To confirm directly that *cotS* gene expression is regulated by σ^K, we then analyzed *cotS* mRNA in the cells of the wild type, a σ^K mutant, and a *gerE* mutant. Cultures of the wild-type cells were harvested every hour throughout sporulation, and the total RNA was extracted for Northern blot analysis (Fig. 4). All samples contained essentially the same amount of 16S RNA and 23S RNA (Fig. 4A and C). The *cotS* mRNA first appeared as a 2.7-kb band at T_5 (Fig. 4B), and the amount of *cotS* mRNA increased until at least T_8. In contrast to the results with wild-type cells, the *cotS* transcript was not detected in the extracts of σ^K and *gerE* mutant cells prepared at T_8 (Fig. 4D, lanes 4 and 6). These results indicate that both σ^K and GerE are essential for transcription of *cotS*.

Factors affecting synthesis and assembly of CotS. The dependence of *cotS* expression on σ^K and GerE was confirmed by Western blot analysis. Mutants deficient in sporulation-specific sigma factors and a *gerE* mutant were grown in DS medium and harvested at T_8. The SDS-soluble protein samples prepared from those cells were analyzed by Western blotting using anti-CotS antibody. σ^A, a major sigma factor expressed during vegetative growth and sporulation (34), was analyzed as a control by using anti-σ^A antibody and was detectable in all samples (Fig. 5A). However, CotS protein was not detectable in the samples from any of the mutants (Fig. 5B). Since σ^K is the last in a cascade of sigma factors (34), these results strongly suggested that the synthesis of CotS depended on both σ^K and GerE.

We also examined the effects of mutations in other *cot* genes on accumulation of CotS into the spore coat (Fig. 6). The 40-kDa CotS was detected in spores that had mutations in *cotA*, *-B*, *-C*, *-D*, *-F*, or *-T* but not in *cotS* or *-E* mutants (Fig. 6B). CotE is a morphogenic protein and is involved in the proper assembly of the spore coat (13, 24). It is particularly noteworthy in our results that the *cotE* gene product appeared to be involved in incorporation of CotS protein into the coat layer.

Immunoblot analysis of CotS expression in *gerE* and *cotE* mutants. The failure to detect CotS in *gerE* and *cotE* mutants suggested two possibilities: (i) GerE and CotE positively reg-

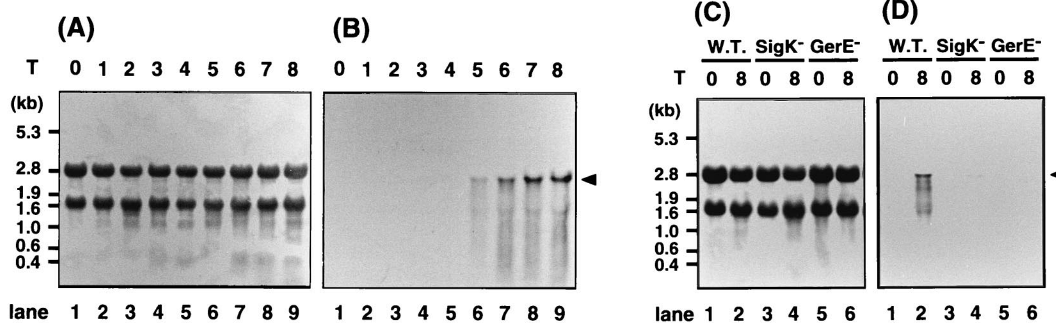


FIG. 4. Northern blot analysis of *cotS* mRNA. *B. subtilis* 168*trpC2* (wild type) (A and B) and *spoIIIC94* (σ^K mutant; SigK$^-$) and *gerE36* (*gerE* mutant; GerE$^-$) (C and D) were grown in DS medium; the cultures were harvested at the indicated times; RNA was prepared, electrophoresed, and transferred to a membrane. (A and C) 16S and 23S RNAs were visualized by staining with methylene blue. (B and D) The gene product of *cotS* was detected by using a digoxigenin-labeled antisense RNA of *cotS* as a probe. T_n shown at the top indicates the harvesting time of cells, where *n* is the number of hours after the end of exponential phase of growth. The arrowhead indicates the band for *cotS* mRNA. W.T., wild-type cell.

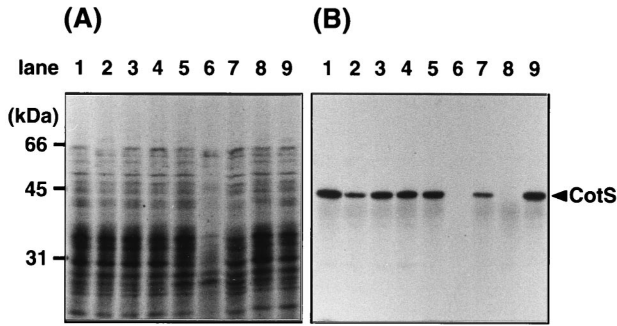


FIG. 6. CotS protein in the spores of *cot* mutants. The *cot* mutant cells were harvested from DS medium at T_{18}. They were then incubated in the presence of lysozyme and washed with buffer to obtain spore preparations. The protein samples solubilized from the spores were analyzed by SDS-PAGE (12% gel). (A) Coomassie brilliant blue stain; (B) immunoblotting using anti-CotS antibody. *B. subtilis* 168*trpC2* (wild type) (lane 1), 1S101 (*cotA* mutant) (lane 2), 1S102 (*cotB* mutant) (lane 3), 1S103 (*cotC* mutant) (lane 4), 1S104 (*cotD* mutant) (lane 5), 1S105 (*cotE* mutant) (lane 6), 1S106 (*cotF* mutant) (lane 7), CB701 (*cotS* mutant) (lane 8), and 1S108(*cotT* mutant) (lane 9) were analyzed. The arrowhead shows the migration position of CotS.

ulate CotS synthesis and (ii) they are responsible for assembly of CotS protein into the coat layer. If the latter is the case, CotS protein synthesized in these mutants may have been degraded by protease(s) in the mother cell compartment. To test these possibilities, the level of CotS protein during sporulation was monitored by Western blotting (Fig. 7). σ^K, a sporulation-specific sigma factor which is expressed at T_2 and later, was also analyzed as a control (19, 33). CotS was detected in the protein samples solubilized from the wild-type cells at T_5 to T_8 (Fig. 7A), consistent with the results of Northern blot analysis as described above. CotS protein was not detected in the vegetative and sporulating cells of the *gerE* mutant (Fig. 7B). In contrast, CotS was detected among the mother cell proteins of the *cotE* mutant from T_5 onward (Fig. 7C), although CotS was

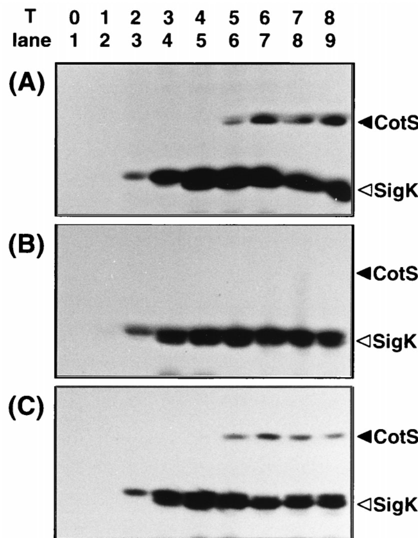
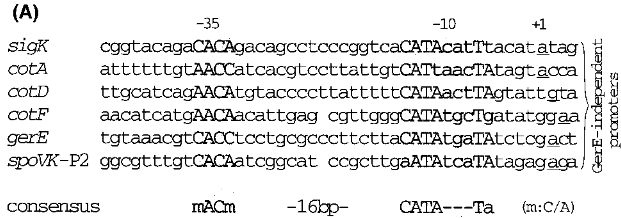
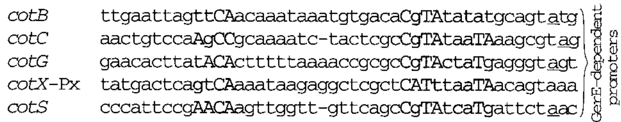
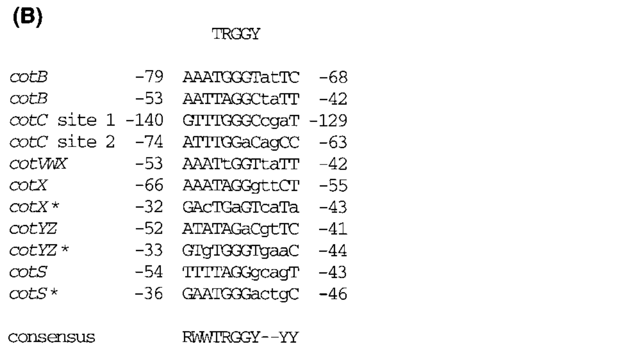


FIG. 7. Immunoblot analysis of CotS expression. *B. subtilis* 168*trpC2* (A), 1G12 (*gerE* mutant) (B), and 1S105 (*cotE* mutant) (C) were grown in DS medium, and the cultures were harvested every hour throughout sporulation (T_0 to T_8). Whole-protein samples were solubilized from the sporulating cells and were analyzed by immunoblotting using anti-CotS and anti-σ^K antibodies. The arrowheads indicate the migration positions of σ^K and CotS.







(C)

GAATTCCCCTCCTCAATGACTTCATGGTTTTCTGCCATTAAAGGACACATCCTTACCTGT 60

TTTACTCCTACCTTATGAATCAAAAGGAAAAAATGATTGAGTTATTTGACTATATTTAAT 120

GerE Binding Site -54 -43

AGATGAATCTGGTTGTTTACCCGTTTTGTTTTTAGGGCAGTCCCATTCCGCACAAGTTGG 180

-46 -36 GerE Binding Site -35

TTGTTCAGCCGTATCATGATTCTAAACTAGTTAAAAATCATGCAGGAATGGAGGCTATGTA 240

-10 +1 SD M cotSA →

TGAAAATAGCACTGATCGCCACAGAGAAGCTTCCTGTCCCATCGGTTCGAGGAGGCGCCA 300

K I A L I A T E K L P V P S V R G G A I

FIG. 8. Alignment of promoter regions of genes transcribed by σ^K RNA polymerase. (A) Sequences near the transcription start sites of genes transcribed by RNA polymerase containing σ^K. Promoters for six genes transcribed in the absence of GerE and five genes whose transcription required GerE in addition to σ^K are shown separately. Nucleotides in each promoter that match the consensus sequence are shown between the groups (m = C or A). The underlined nucleotides correspond to the transcription start point. (B) Alignment of nucleotide sequences of GerE-binding sites. The consensus sequence proposed by Zheng et al. (39) is shown at the top. Numbers refer to positions relative to the transcriptional start site. *, sequence from the opposite DNA strand. The bottom line shows an enlarged consensus sequence for GerE binding based on the sequence shown. R, purine; W, A or T; Y, pyrimidine. (C) Nucleotide sequence of the *cotS* promoter, showing putative −35 and −10 regions, the transcription start point (+1), and a ribosome-binding site (SD) (2). The boxed sequence is a putative $GerE^-$ binding site in the *cotS* operon. References for the sequences of these promoters are as follows: *sigK* (*spoIVCB*), 20; *cotA*, 29; *cotB* and *cotD*, 39; *cotC*, 39 and 40; *cotF*, 9; *gerE*, 8; *spoVK*, 14; *cotVXW*, *cotX*, and *cotYZ*, 36; *cotG*, 26; and *cotS*, 2.

not detected in the spores of *cotE* mutant cells at T_{18} (Fig. 6B, lane 6). These results indicate that while GerE is required for *cotS* expression, CotE is essential for the assembly of CotS in the coat layer.

DISCUSSION

Previously we speculated that expression of the *cotS* operon is dependent on σ^K and the regulatory protein GerE (2). To

test this idea, we examined expression of *cotS* by Northern blot and Western blot analyses. CotS protein was first detectable at T_5 in wild-type cells, whereas almost no CotS protein was synthesized in σ^E, σ^F, σ^G, σ^K, and *gerE* mutants. Northern blot analysis revealed a 2.8-kb mRNA as the product of *cotS*, but the *cotS* transcript was barely detectable in both the σ^K and GerE mutants at T_8 (Fig. 4). CotS was detected in the sporangium but not in the spores of *cotE* mutants by Western blot analysis. These observations suggest that expression of *cotS* was dependent on both σ^K and GerE and that assembly of CotS into the coat layer required CotE.

Alignment of promoter regions of genes transcribed by RNA polymerase containing σ^K is summarized in Fig. 8A. Six of these genes have GerE-independent promoters, whereas the others have GerE-dependent promoters (14, 25, 37, 39, 40). GerE-independent promoters have a good consensus sequence in their −35 and −10 regions (CATA---Ta). In contrast, GerE-dependent promoters do not have this consensus sequence. Comparison of the *cotS* promoter with the consensus sequence indicates that the *cotS* promoter belongs to the GerE-dependent promoter family.

Runoff transcription and DNase I footprinting studies of *cotX*, *cotY*, and *cotZ* of *B. subtilis*, whose transcription is dependent on σ^K and GerE, indicate that the consensus sequence of GerE-binding sites in these genes is RWWTRGGY--YY (R, purine; W, A or T; Y, pyrimidine) (37). Comparison of the *cotS* promoter region with the GerE consensus sequence indicated that the two have similar sequences upstream of the −35 region (Fig. 8B). Two putative consensus regions are possible. One is a 12-bp stretch extending from positions −54 to −43 on the transcribed strand, and the other extends from positions −36 to −46 on the nontranscribed strand. Consequently, we note the presence of a possible GerE-binding site in the *cotS* regulatory region (Fig. 8C).

One of the most striking results in this report concerns the interaction of CotE and CotS. Proper formation of the spore coat depends on the CotE protein (3), and spores from a *cotE* mutant lack the outer coat (37). Immunoelectron microscopic analysis indicated that CotS is localized in the inner coat and/or on the outside region of the cortex of dormant spores. We speculate that CotE protein also functions in assembly of a certain inner coat protein in addition to outer coat morphogenesis. Spore coat components synthesized in the mother cell compartment at an intermediate stage of sporulation could interact with CotE and then be assembled into the inner-laminated layer or outer layer to organize a rigid mature spore coat.

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