

# Maintaining the Transcription Factor SpoIIID Level Late during Sporulation Causes Spore Defects in *Bacillus subtilis*<sup>∇</sup>

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**During sporulation of *Bacillus subtilis*, four regulatory proteins act in the order  $\sigma^E$ , SpoIIID,  $\sigma^K$ , and GerE to temporally control gene expression in the mother cell.  $\sigma^E$  and  $\sigma^K$  work sequentially with core RNA polymerase to transcribe different sets of genes. SpoIIID and GerE are small, sequence-specific DNA-binding proteins that activate or repress transcription of many genes. Previous studies showed that transcriptionally active  $\sigma^K$  RNA polymerase inhibits early mother cell gene expression, reducing accumulation of SpoIIID late in sporulation. Here, the effects of perturbing the mother cell gene regulatory network by maintaining the SpoIIID level late during sporulation are reported. Persistent expression was obtained by fusing *spoIIID* to the  $\sigma^K$ -controlled *gerE* promoter on a multicopy plasmid. Fewer heat- and lysozyme-resistant spores were produced by the strain with persistent *spoIIID* expression, but the number of spores resistant to organic solvents was unchanged, as was their germination ability. Transmission electron microscopy showed structural defects in the spore coat. Reporter fusions to  $\sigma^K$ -dependent promoters showed lower expression of *gerE* and *cotC* and higher expression of *cotD*. Altered expression of *cot* genes, which encode spore coat proteins, may account for the spore structural defects. These results suggest that one role of negative feedback by  $\sigma^K$  RNA polymerase on early mother cell gene expression is to lower the level of SpoIIID late during sporulation in order to allow normal expression of genes in the  $\sigma^K$  regulon.**

Upon sensing nutrient limitation, the gram-positive bacterium *Bacillus subtilis* initiates an adaptive process called sporulation that culminates in the formation of a dormant spore (reviewed in references 12 and 28). During sporulation, *B. subtilis* undergoes an asymmetric division, forming a larger mother cell (MC) compartment and a smaller forespore (FS) compartment. Different genes are expressed in each compartment. Signaling between the MC and the FS ensures precise temporal and spatial control of gene expression. The products of genes expressed in the two cell types cause further morphological changes. The FS is engulfed by the MC membrane, surrounding the FS with two membranes and pinching it off as a protoplast within the MC. Then, cell wall-like material called cortex is deposited between the two membranes of the FS. Next, the spore coat is assembled on the surface of the FS and its interior or core is dehydrated. The mature spore, which is resistant to environmental insults such as heat, UV light, lytic enzymes, and chemicals, is released by lysis of the MC. Under favorable environmental conditions, the dormant spore germinates and forms a growing cell.

In the MC, a hierarchical cascade of four regulatory factors,  $\sigma^E$ , SpoIIID,  $\sigma^K$ , and lastly GerE, governs gene expression during sporulation (55) (Fig. 1). The *sigE* gene is transcribed by  $\sigma^A$  RNA polymerase (RNAP), the major form of RNAP in growing cells, prior to asymmetric division, but initiation of transcription at the promoter of the *spoIIG* operon, which

includes *sigE*, requires phosphorylated Spo0A, a key transcription factor that governs entry into the sporulation process (reviewed in reference 20). After formation of the asymmetric septum, phosphorylated Spo0A persists in the MC and the product of *sigE*, pro- $\sigma^E$ , accumulates primarily in the MC (15, 16). Pro- $\sigma^E$  is cleaved in response to a signal from the FS (23, 27, 33), forming active  $\sigma^E$  RNAP in the MC, where it transcribes more than 260 genes, including those that encode SpoIIID and pro- $\sigma^K$  (13, 14, 46) (Fig. 1). SpoIIID is a small, sequence-specific DNA-binding protein (18) that positively regulates at least eight transcription units in the  $\sigma^E$  regulon, including the *sigK* gene (Fig. 1), and negatively regulates at least 62 transcription units (13). SpoIIID also negatively regulates genes in the  $\sigma^K$  regulon, including *cotC* and *cotD* (18, 25, 29) (Fig. 1), which encode spore coat proteins (9). GerR (not shown in Fig. 1) is a second transcription factor under  $\sigma^E$  RNAP control that negatively regulates 10 transcription units but is not known to affect the expression of the four regulators in the cascade (13). The *sigK* gene encodes pro- $\sigma^K$  (49). Like pro- $\sigma^E$ , pro- $\sigma^K$  is activated by proteolytic cleavage in response to an FS signal (6, 34), although the mechanisms of signaling and proteolysis are different (reviewed in reference 28).  $\sigma^K$  RNAP transcribes more than 100 genes, including many *cot* genes that encode spore coat proteins, and the gene encoding GerE (13, 46) (Fig. 1). Like SpoIIID, GerE is a small, sequence-specific DNA-binding protein (54). It positively regulates at least 27 transcription units, including the *cotC* and *cotD* genes, and negatively regulates at least 36 transcription units (13), including the *sigK* gene (24, 54) (Fig. 1).

All of the regulatory interactions depicted in Fig. 1 are direct effects based on *in vitro* studies, with one exception. The negative-feedback loop from  $\sigma^K$  to  $\sigma^A$  is inferred from

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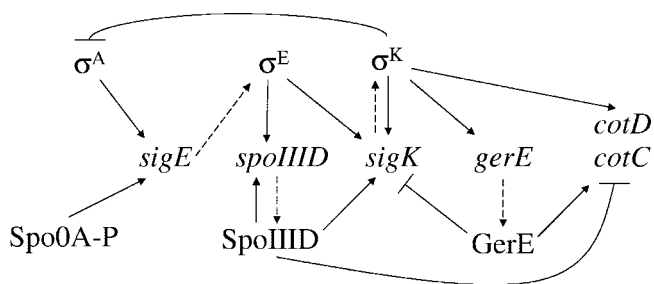


FIG. 1. Gene regulatory network in the MC during sporulation of *B. subtilis*. Dashed arrows indicate gene-to-protein relationships. Solid arrows or lines with barred ends represent positive or negative regulation, respectively. See the text for explanation and references.

in vivo studies (17, 52, 53). Transcriptionally active  $\sigma^K$  RNAP inhibits early MC gene expression, including transcription of *sigE* by  $\sigma^A$  RNAP (53). This results in reduced accumulation of  $\sigma^E$  and SpoIIID late during sporulation (17, 52). Neither the mechanism nor the significance of this negative-feedback loop is known. To access its significance, we engineered ectopic expression of *spoIIID* such that the SpoIIID level was maintained late during sporulation. This resulted in fewer heat- and lysozyme-resistant spores being produced, and most of the spores exhibited structural changes in their coats. Perturbing the MC gene regulatory network in this way also uncovered a novel connection that was unexpected from in vitro studies; persistent *spoIIID* expression lowered expression of a *gerE-lacZ* fusion. These results demonstrate the importance of lowering the SpoIIID level late during sporulation, which is one consequence of the  $\sigma^K$ -dependent negative-feedback loop.

#### MATERIALS AND METHODS

**Construction of plasmids.** A DNA fragment containing the *gerE* promoter region was synthesized by PCR using primers 5'-GGGAATTCCTGGACGGA TGAGGAGAAAG-3' and 5'-GGGAAGCTTGC GAATCAGAAACGAATG G-3', which contain EcoRI and HindIII restriction sites (underlined), respectively. This fragment spans from bp -96 to bp +33 relative to the transcriptional start site of *gerE* and does not include the ribosomal binding site (7). The PCR product was digested with EcoRI and HindIII and ligated into EcoRI-HindIII-digested pDG364 (26), resulting in plasmid pRB1 (R. Burri and L. Kroos, unpublished data). The sequence of this insert was determined by the Research Technology Support Facility at Michigan State University in order to ensure that no errors occurred during the PCR. A DNA fragment containing the *spoIIID* gene, from bp +145 to bp +463 relative to the transcriptional start site of *spoIIID* (50), including the ribosomal binding site and the entire coding region, was amplified by PCR using primers 5'-GGAAGCTTAGGGAGGTCGAGTGGTGTG-3' and 5'-CGGATCCCAAGAAGCAATGCCAGG-3', which contain HindIII and BamHI restriction sites (underlined), respectively. The PCR product was digested with HindIII and BamHI and ligated with HindIII-BamHI-digested pRB1, resulting in pRB2 (R. Burri and L. Kroos, unpublished). The sequence of the entire  $P_{gerE}$ -*spoIIID* fusion in pRB2 was determined as described above. Next, pRB2 was digested with EcoRI and BamHI, the  $P_{gerE}$ -*spoIIID* fragment was purified, and it was ligated with a 6.6-kb fragment of EcoRI (partial digestion)-BamHI-digested pDG148 (48), resulting in pJP1. To construct a control plasmid containing only  $P_{gerE}$ , pJP1 was digested with BamHI and HindIII to remove the *spoIIID* insert. The resulting 6.7-kb fragment was treated with the Klenow fragment of DNA polymerase I, and the blunt ends were ligated by T4 DNA ligase, resulting in pJP2.

**Bacterial strains.** *Escherichia coli* strain AG115 [*araD139*  $\Delta$ (*ara leu*)7697  $\Delta$ *lacX74 galU galK hsr hsm*<sup>+</sup> *strA* (F' *proAB lacI*<sup>qZ</sup>::Tn5)] was obtained from A. Grossman (Massachusetts Institute of Technology). It was used during construction and maintenance of plasmids. Luria-Bertani (LB) medium (44) was used to grow *E. coli* and *B. subtilis* and was supplemented with appropriate antibiotics.

PY79 (Spo<sup>+</sup> prototroph) (51), BK556 (*spoIVCB23*) (31), and OR825 (PY79 SP $\beta$ ::*cotC-lacZ*) (5) were provided by R. Losick (Harvard University). EUDC9901 (*trpC2 pheA1 gerE::kan*) (4) was provided by C. Moran (Emory University). pJP1 was transformed into PY79 and OR825 as described previously (21), with selection by addition of kanamycin sulfate (5  $\mu$ g/ml) to the medium, generating strains BJP1 and BJP3, respectively. Similarly, pJP2 was transformed into PY79 and OR825 to generate strains BJP2 and BJP4, respectively. SP $\beta$ ::*cotD-lacZ* and SP $\beta$ ::*gerE-lacZ* have been described previously (6, 7). Specialized transduction was used to move *lacZ* fusions into BJP1, BJP2, and PY79 as described previously (21). Transductants were selected on LB agar containing chloramphenicol (5  $\mu$ g/ml). In each case, at least 10 candidates were transferred onto a DSM agar (21) plate with 5-bromo-4-cholro-3-indolyl- $\beta$ -D-galactopyranoside (40  $\mu$ g/ml). Three or more isolates with average blue color were saved for further analysis, excluding occasional isolates with abnormally high or low  $\beta$ -galactosidase activity.

**Cell growth and sporulation.** Sporulation was induced by resuspension of cells in SM medium as described previously (21). The time of resuspension is defined as the onset of sporulation ( $T_0$ ).

**Western blot analysis.** Starting at 3 h into sporulation ( $T_3$ ) and at hourly intervals thereafter until  $T_9$ , 0.5-ml samples were subjected to centrifugation (14,000  $\times g$  for 1 min), the supernatants were removed, and cell pellets were stored at -70°C. Preparation of whole-cell extracts, electrophoresis, and electroblotting were as described previously (17, 34). The blots were probed with anti-SpoIIID antiserum diluted 1:10,000 (17) or polyclonal anti-pro- $\sigma^K$  antiserum diluted 1:10,000 (34). Immunodetection of primary antibodies was as described previously (30).

**Analysis of  $\beta$ -galactosidase activity.** Samples were collected during sporulation as described above. Cell pellets were stored at -70°C prior to the assay. Cells were resuspended and then treated with lysozyme and permeabilized by toluene as described previously (38). The  $\beta$ -galactosidase specific activity was determined as described previously (38), using *o*-nitrophenol- $\beta$ -D-galactopyranoside as the substrate. One unit of the enzyme hydrolyzed 1  $\mu$ mol of substrate per minute per unit of initial-culture optical density at 595 nm.

**Spore purification and germination and resistance assays.** Spores were harvested at  $T_{24}$  by centrifugation at 7,000  $\times g$  for 10 min, washed with 4°C water once, and stored at 4°C overnight. The next day, spores were purified on a step gradient of 20% to 50% RenoCal-76 (Bracco Diagnostics Inc.) as described previously (22). The purity of the spores was verified by microscopy. The germination assay was performed with purified spores, using L-alanine (10 mM) as the germinant as described previously (39). Assays for resistance to heat, lysozyme, and organic solvents were performed at  $T_{24}$  without spore purification, as described previously (21).

**Transmission electron microscopy of spores.** Spores were harvested at  $T_{24}$ , washed with water, and immediately fixed as described previously (37).

#### RESULTS

**Engineering *B. subtilis* to maintain the SpoIIID level late during sporulation.** In wild-type PY79, the level of SpoIIID reaches a maximum at 5 h into sporulation and decreases thereafter (17) (Fig. 2). To test whether the SpoIIID decrease is important, we initially constructed a *B. subtilis* strain with a single copy of a  $P_{gerE}$ -*spoIIID* fusion integrated at the *amyE* locus in the chromosome. Unlike the native *spoIIID* promoter, which is under  $\sigma^E$  RNAP control, the *gerE* promoter is under the control of the later-acting  $\sigma^K$  RNAP (Fig. 1). However, at single copy, the  $P_{gerE}$ -*spoIIID* fusion had no apparent effect on the level of SpoIIID during sporulation (R. Burri and L. Kroos, unpublished). Therefore, we constructed pJP1, a multicopy plasmid bearing the  $P_{gerE}$ -*spoIIID* fusion. pJP1 was transformed into PY79, resulting in *B. subtilis* BJP1. As a control, a plasmid with only  $P_{gerE}$ , pJP2, was also transformed into PY79, resulting in strain BJP2. During sporulation, the SpoIIID level in BJP2 decreased after 5 h, as observed for PY79, but in BJP1 the level of SpoIIID remained about the same at least until 9 h into sporulation (Fig. 2). We conclude that BJP1 maintains the level of SpoIIID late during sporulation.

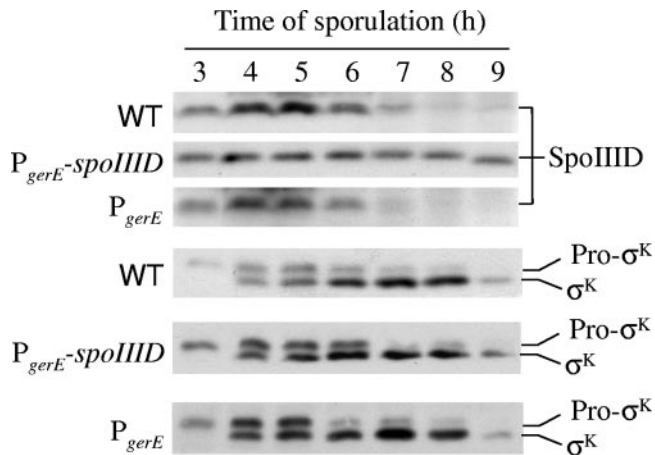


FIG. 2. Levels of SpoIIID, pro- $\sigma^K$ , and  $\sigma^K$  during sporulation. *B. subtilis* wild-type (WT) PY79, BJP1 bearing pJP1 with the  $P_{gerE}$ -*spoIIID* insert, and BJP2 bearing pJP2 with the  $P_{gerE}$  insert were induced to sporulate by resuspension in SM medium, and samples were collected at hourly intervals beginning at 3 h after the onset of sporulation. Equal volumes (5  $\mu$ l) of whole-cell lysates were fractionated on SDS-14% polyacrylamide gels and subjected to Western blot analysis with anti-SpoIIID or anti-pro- $\sigma^K$  serum.

**Pro- $\sigma^K$  and  $\sigma^K$  levels are unchanged in *B. subtilis* strains that maintain the SpoIIID level late during sporulation.** SpoIIID activates or represses many genes in the MC (13, 18, 19). One key gene that it activates is *sigK* (Fig. 1), which encodes pro- $\sigma^K$  (49). SpoIIID activates *sigK* transcription by

RNAP containing either  $\sigma^E$  or  $\sigma^K$  (18). Hence, maintaining the SpoIIID level late during sporulation might elevate the pro- $\sigma^K$  and/or  $\sigma^K$  level at late times if SpoIIID is limiting for transcription of *sigK* and/or for processing of pro- $\sigma^K$  to  $\sigma^K$ .

The levels of pro- $\sigma^K$  and  $\sigma^K$  were indistinguishable in PY79, BJP1, and BJP2 (Fig. 2). This result indicates that SpoIIID is not the limiting factor for pro- $\sigma^K$  or  $\sigma^K$  production late during sporulation. Negative-feedback loops in the MC network (Fig. 1) may limit  $\sigma^E$  and  $\sigma^K$  production late during sporulation, counteracting potential activation by SpoIIID of *sigK* transcription.

**Maintaining the SpoIIID level late during sporulation alters gene expression.** SpoIIID strongly represses transcription of *cotD* (18, 19) and *cotC* (25) by  $\sigma^K$  RNAP in vitro and weakly activates transcription of *gerE* by  $\sigma^K$  RNAP in vitro (19). Using *lacZ* reporter fusions to these three promoters, we examined the effect of persistent *spoIIID* expression late during sporulation. Surprisingly, *gerE-lacZ* expression was reduced in the strain that maintains the SpoIIID level late during sporulation, compared with the *gerE-lacZ* levels in the wild type and the control strain with multicopy  $P_{gerE}$ , which were indistinguishable (Fig. 3A). This result was unanticipated from the in vitro study (19). It suggests that SpoIIID can directly or indirectly inhibit *gerE* expression.

Since SpoIIID represses *cotC* transcription by  $\sigma^K$  RNAP in vitro (25), it was not surprising that *cotC-lacZ* expression was lower in the strain with multicopy  $P_{gerE-spoIIID}$  than in the wild type or the control strain bearing multicopy  $P_{gerE}$  (Fig. 3B). Moreover, GerE activates *cotC* transcription by  $\sigma^K$  RNAP

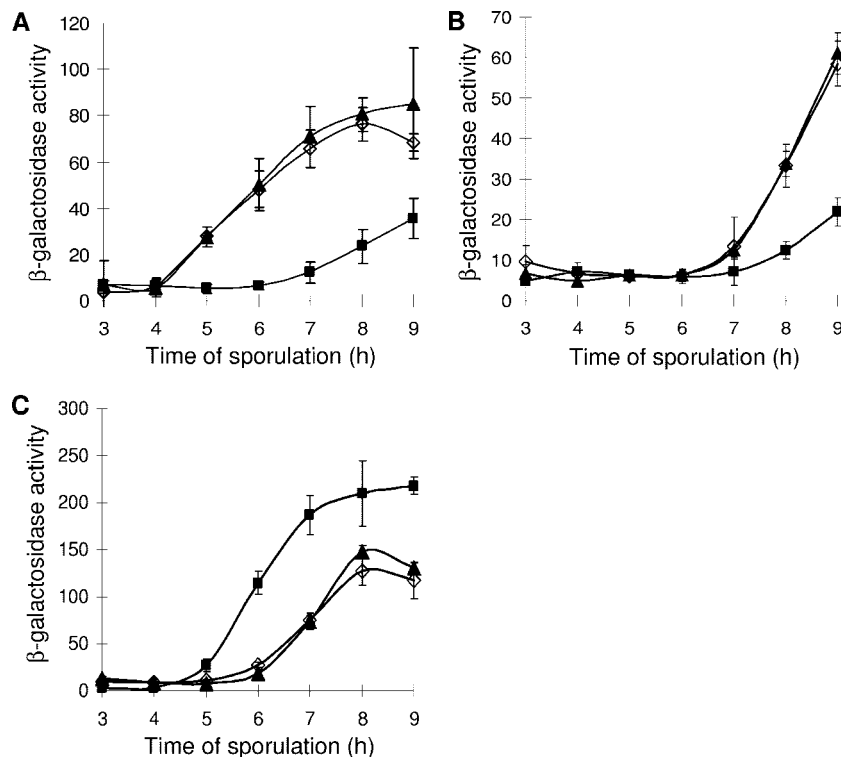


FIG. 3. Effects on gene expression.  $\beta$ -Galactosidase activity during sporulation after resuspension in SM medium was measured for *B. subtilis* containing *lacZ* fused to the *gerE* (A), *cotC* (B), or *cotD* (C) promoter in wild-type PY79 ( $\diamond$ ) or strains bearing multicopy  $P_{gerE-spoIIID}$  ( $\blacksquare$ ) or  $P_{gerE}$  ( $\blacktriangle$ ). Each point is the average for three determinations, and error bars show 1 standard deviation of the data.

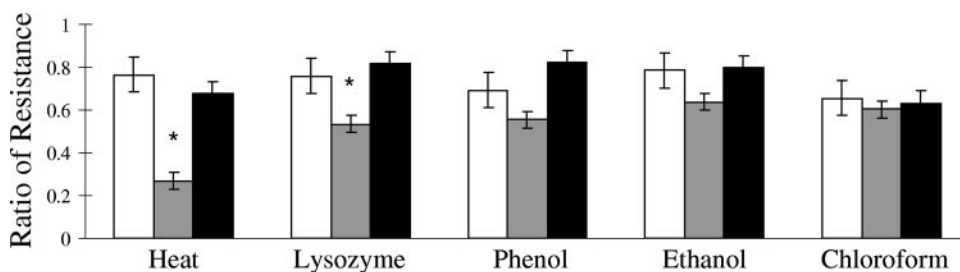


FIG. 4. Resistance properties of spores. Ratios of CFU counts after the indicated treatments to those before the treatments were determined for *B. subtilis* wild-type PY79 (white bars), strain BJP1 bearing multicopy  $P_{gerE}$ -*spoIIID* (gray bars), and strain BJP2 bearing multicopy  $P_{gerE}$  (black bars) at 24 h after resuspension in SM medium. Bars show the averages for three determinations, and error bars show 1 standard deviation of the data. Asterisks indicate  $P$  values of  $<0.05$  in the comparisons between BJP1 and both PY79 and BJP2.

in vitro (54), so diminished *gerE* expression in the  $P_{gerE}$ -*spoIIID* strain (Fig. 3A) may also contribute to its diminished *cotC-lacZ* expression (Fig. 3B).

Expression of *cotD-lacZ* rose earlier and reached a higher level in the  $P_{gerE}$ -*spoIIID* strain than in the wild type or the  $P_{gerE}$  control strain (Fig. 3C). This may not be surprising in light of the diminished *gerE* expression in the  $P_{gerE}$ -*spoIIID* strain (Fig. 3A), because a low level of GerE activates *cotD* transcription by  $\sigma^K$  RNAP in vitro, but at a higher level, GerE represses (24). Although SpoIIID strongly represses transcription of *cotD* by  $\sigma^K$  RNAP in vitro (18, 19), the SpoIIID level in the  $P_{gerE}$ -*spoIIID* strain may not be high enough for repression to be the dominant effect.

We conclude that maintaining the SpoIIID level late during sporulation lowers *gerE-lacZ* expression and alters the expression levels of other genes in the  $\sigma^K$  regulon, increasing or decreasing their expression levels depending on the effects of SpoIIID and GerE at particular promoters.

**Persistent *spoIIID* expression affects certain spore resistance properties.** The effects of maintaining the SpoIIID level late during sporulation on the numbers of heat-, lysozyme-, phenol-, ethanol-, and chloroform-resistant spores were measured. *B. subtilis* strain BK556 (*spoIVCB23*), which fails to make  $\sigma^K$  (34), served as a negative control. In each assay, BK556 produced at least  $10^5$  fewer resistant spores (data not shown) than the wild type (PY79 in Fig. 4).

About 70% of the viable cells for wild-type PY79 and the multicopy  $P_{gerE}$  control strain BJP2 at 24 h after the onset of sporulation were heat resistant (Fig. 4). However, for the multicopy  $P_{gerE}$ -*spoIIID* strain BJP1, only about 30% of the viable cells were heat resistant. Statistical analysis of the data indicated no significant difference between PY79 and BJP2, but the differences between these two strains and BJP1 were significant ( $P$  values of less than 0.05 in Student  $t$  tests).

About 80% of the viable PY79 and BJP2 cells were lysozyme resistant at 24 h after the onset of sporulation, while the number was only about 50% for BJP1 (Fig. 4). Again, statistical analysis indicated a significant difference ( $P$  values of less than 0.05 in Student  $t$  tests) between BJP1 and either PY79 or BJP2 but no significant difference between the two control strains.

Resistance to three types of organic solvents was tested: the water-immiscible solvent chloroform, the water-miscible solvent ethanol, and the organic acid phenol. On average, BJP1 exhibited slightly less resistance than the two control strains for each treatment (Fig. 4), but these differences were not consid-

ered statistically significant, because no pairwise comparison between the data obtained for two strains treated with the same organic solvent yielded a  $P$  value of less than 0.05 in Student  $t$  tests.

**Maintaining the SpoIIID level late during sporulation does not impair spore germination.** Spores were harvested 24 h after the onset of sporulation and purified. Germination was assayed as the change in absorbance at 580 nm over time after exposure to the germinant L-alanine. A germination mutant, *B. subtilis* EUDC9901 (*gerE*), showed very little change in absorbance even after 55 min (Fig. 5). In contrast, the absorbance levels of wild-type PY79 spores decreased rapidly after exposure to L-alanine, and similar results were observed for strains bearing multicopy  $P_{gerE}$ -*spoIIID* or  $P_{gerE}$ . We conclude that spores produced by a strain with persistent *spoIIID* expression germinate normally.

**Persistent *spoIIID* expression causes a defect in spore coat structure.** To assess the consequences of persistent *spoIIID* expression on the assembly of cellular structures, we carried out thin-section transmission electron microscopy of spores.

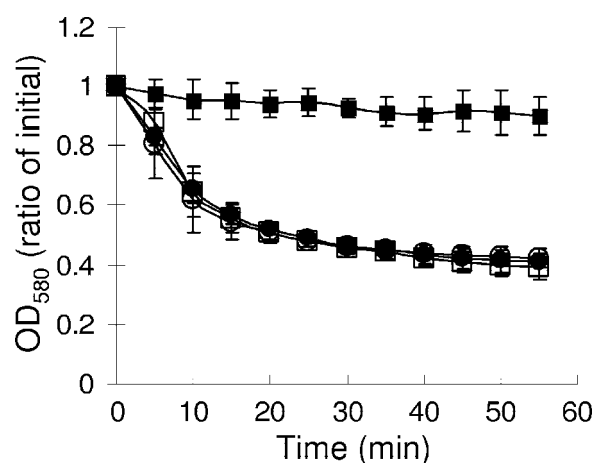


FIG. 5. Spore germination. *B. subtilis* wild-type PY79 (□), strain BJP1 bearing multicopy  $P_{gerE}$ -*spoIIID* (●), strain BJP2 bearing multicopy  $P_{gerE}$  (○), and *gerE* mutant EUDC9901 (■) were induced to sporulate by resuspension in SM medium; samples were collected after 24 h; spores were purified; and quantitative germination assays were performed. Each point is the average for three determinations, and error bars show 1 standard deviation of the data. OD<sub>580</sub>, optical density at 580 nm.



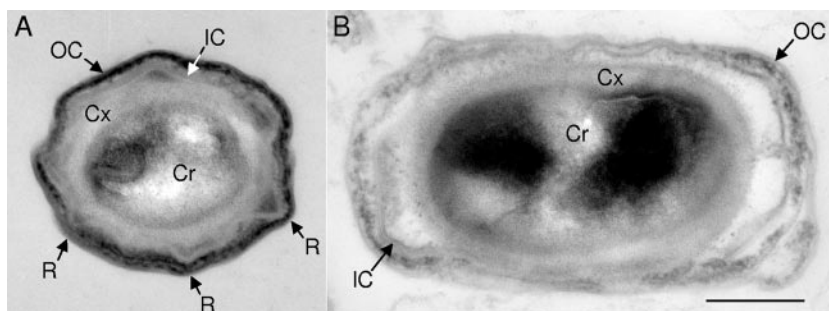


FIG. 6. Thin-section electron microscopic analysis of spores. Spores from *B. subtilis* BJP2 bearing multicopy  $P_{gerE}$  (A) or BJP1 bearing multicopy  $P_{gerE-spoIIID}$  (B) were harvested 24 h after resuspension in SM medium, washed, and immediately fixed before further processing. In panel A, a section through the short axis of the spore is shown. In panel B, the cross section is through the long axis. Therefore, the difference in the shapes of the two spores is insignificant (i.e., both strains, like wild-type PY79, showed mixtures of spherical and ovoid sections). The core (Cr), cortex (Cx), inner coat (IC), outer coat (OC), and ridges (R) are indicated. The bar applies to both panels and indicates 200 nm.

The spores from the wild type (data not shown) and the control strain with multicopy  $P_{gerE}$  (Fig. 6A) were indistinguishable. As is characteristic of mature spores, the core, cortex, and inner and outer coat layers were readily identified, and the coats had ridges. Approximately 10% of the spores from the strain with multicopy  $P_{gerE-spoIIID}$  resembled wild-type spores (data not shown). The remaining 90% showed significant defects in coat assembly. While structures resembling the inner and outer coat layers were present, these were thinner and disorganized (Fig. 6B). Additionally, the coat was no longer in close apposition to the cortex and ridges were less evident.

## DISCUSSION

Our results demonstrate that maintaining the SpoIIID level late during sporulation alters the expression levels of genes in the  $\sigma^K$  regulon, lowers the numbers of heat- and lysozyme-resistant spores produced, and causes a defect in spore coat assembly. Clearly, it is important that the SpoIIID level decreases in order for spore formation to proceed normally during the late stages. One contributor to the SpoIIID decrease is a negative-feedback loop initiated by  $\sigma^K$  RNAP that inhibits early gene expression in the MC (17, 52, 53). Our finding that a single copy of the  $P_{gerE-spoIIID}$  fusion was insufficient to detectably boost the SpoIIID level late during sporulation was surprising, as was the possibly related observation that SpoIIID can negatively regulate *gerE* expression. While multiple copies of  $P_{gerE-spoIIID}$  did allow the SpoIIID level to be maintained late during sporulation, the levels of pro- $\sigma^K$  and  $\sigma^K$  were unchanged, indicating that the MC regulatory network is somewhat resistant to perturbation. Such robustness is a common feature of regulatory networks (1, 47). Our results reveal elements of both robustness and susceptibility to perturbation in the MC regulatory network.

A single copy of  $P_{gerE-spoIIID}$  did not result in perceptibly higher accumulation of SpoIIID late during sporulation. We chose the *gerE* promoter because it is strongly transcribed by  $\sigma^K$  RNAP in vitro (54) with little effect of SpoIIID (19). However, we discovered that in cells bearing multicopy  $P_{gerE-spoIIID}$ , the level of SpoIIID is elevated late during sporulation (Fig. 2) and *gerE-lacZ* expression is reduced (Fig. 3A). Hence, SpoIIID might negatively autoregulate  $P_{gerE-spoIIID}$  expression, necessitating a high copy number to maintain the

SpoIIID level late during sporulation. Alternatively or in addition, one or more posttranscriptional mechanisms might inhibit SpoIIID accumulation late during sporulation. If so, such a mechanism(s) would presumably contribute to the decrease in SpoIIID level observed in wild-type cells late during sporulation (17).

Multicopy  $P_{gerE}$  exhibited none of the effects of multicopy  $P_{gerE-spoIIID}$ . Both plasmids were derived from pUB110, which is maintained at about 50 copies/cell (36). Most of these copies would presumably be in the MC (owing to its larger size) after polar septation. Considering that  $\sigma^K$  RNAP transcribes approximately 71 genes or operons in the MC during sporulation (13, 46), the  $P_{gerE}$ -containing plasmid is expected to significantly increase the number of  $\sigma^K$ -dependent promoters in the MC. Yet, the strain containing this plasmid showed no differences from the wild type in any of our assays. Moreover, a multicopy  $P_{gerE-cotC}$  fusion did not lower the numbers of heat- and lysozyme-resistant spores produced (L. Wang, J. Perpich, and L. Kroos, unpublished data), as did multicopy  $P_{gerE-spoIIID}$  (Fig. 4). We conclude that neither  $P_{gerE}$  nor  $P_{gerE-cotC}$  titrates  $\sigma^K$  RNAP or other cellular resources sufficiently to inhibit sporulation. An earlier study showed that when  $\sigma^K$  is produced at a much lower level than normal, it is sufficient for considerable  $\sigma^K$ -dependent gene expression and sporulation (35). Taken together, our results suggest that  $\sigma^K$  is normally made in excess during sporulation.

Our results also suggest that the regulatory network resists elevating the levels of pro- $\sigma^K$  and  $\sigma^K$  late during sporulation. Transcription of *sigK* depends absolutely on SpoIIID (32). However, maintaining the SpoIIID level late during sporulation did not elevate the pro- $\sigma^K$  or  $\sigma^K$  level (Fig. 2). This suggests that some other factor limits *sigK* expression late during sporulation. By that time, the level of  $\sigma^E$  is decreased due to negative feedback by  $\sigma^K$  RNAP (52), so only  $\sigma^K$  RNAP can transcribe *sigK*. One factor known to limit *sigK* transcription is GerE (24, 54). In a *gerE* mutant, *sigK* expression is elevated about twofold (24). A mutation in the GerE binding site in the *sigK* promoter region elevates the pro- $\sigma^K$  and  $\sigma^K$  levels approximately twofold during sporulation (L. Wang, J. Perpich, and L. Kroos, unpublished). However, no further enhancement of the pro- $\sigma^K$  or  $\sigma^K$  level was observed when multicopy  $P_{gerE-spoIIID}$  was introduced into the binding site mutant (L.

Wang, J. Perpich, and L. Kroos, unpublished). Under these conditions, neither SpoIIID (acting positively) nor GerE (acting negatively) should limit *sigK* expression late during sporulation. Perhaps under these conditions, the ability to process pro- $\sigma^K$  to active  $\sigma^K$  or degradation of  $\sigma^K$  limits *sigK* expression.

Given the unchanged level of  $\sigma^K$  in cells engineered to maintain the SpoIIID level late during sporulation, we would not have predicted the observed decrease in *gerE-lacZ* expression (Fig. 3A), because  $\sigma^K$  RNAP transcribes *gerE* and SpoIIID has little effect, based on in vitro studies (19). The same *gerE* promoter-containing fragment (bp -96 to +170) used in the in vitro study was used to create the *gerE-lacZ* translational fusion used here, so it is unlikely that the *gerE-lacZ* fusion has an additional SpoIIID binding site. Nevertheless, further experiments are warranted to test whether maintaining the SpoIIID level late during sporulation inhibits *gerE* expression (e.g., by measuring *gerE* mRNA and GerE protein levels), since this appears to be a previously unknown connection in the MC regulatory network, coordinating the disappearance of SpoIIID with the appearance of GerE.

Maintaining the SpoIIID level late during sporulation decreased expression of *cotC-lacZ* (Fig. 3B) and increased that of *cotD-lacZ* (Fig. 3C). These effects can be understood from the effects of SpoIIID and GerE on transcription of *cotC* (25, 54) and *cotD* (18, 19, 24) by  $\sigma^K$  RNAP in vitro, as noted in Results. We predict that the expression levels of other genes in the  $\sigma^K$  regulon whose transcription is influenced by SpoIIID and/or GerE are also altered in cells engineered to maintain the SpoIIID level late during sporulation. Only 4 genes in the  $\sigma^K$  regulon have been shown to be influenced by SpoIIID (18, 25), but at least 53 genes or operons have been shown to be positively or negatively regulated by GerE, based on genome-wide DNA microarray expression profiling experiments (13, 46).

Altered expression of genes in the  $\sigma^K$  regulon presumably causes the altered spore resistance properties (Fig. 4) and the spore coat structural defects (Fig. 6B) observed for spores derived from cells with persistent *spoIIID* expression. Interestingly, the coat protein compositions of these spores are not detectably altered, as determined by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis analysis of SDS-extractable coat proteins (data not shown). A possible interpretation of this is that proper coat assembly depends on regulation of coat protein levels in the MC cytoplasm prior to deposition around the FS and not just on the final composition of the coat. In this view, the normal changes in SpoIIID and GerE levels regulate coat protein levels so that interactions interfering with coat assembly are suppressed. Persistent expression of *spoIIID* might lead to the abnormal simultaneous appearance of proteins that can interact nonproductively and, as a result, affect the usual course of coat formation without altering its final composition. Alternatively, it is possible that coat composition is altered in spores produced by the  $P_{gerE}$ -*spoIIID* fusion-bearing strain. This would be the case if the changes were in protein species that were not extracted by SDS or did not enter the SDS-polyacrylamide gel. That such species do exist is highly likely (37, 40). Regardless of whether the coat composition is altered by persistent *spoIIID* expression, our data support the notion that proper regulation of coat protein levels during sporulation is required for proper coat assembly.

Recently, it was shown that the timing of *cotE* expression is important for normal assembly of the spore outer coat (3).

The nature of the spore defect caused by maintaining the SpoIIID level late during sporulation is intriguing. The resistance of the spore to environmental insults is due both to the complex structure of its cortex and coat and to the unique physiological state of the core (reviewed in references 2 and 12). It is believed that the cortex confines the core of the spore by forming a woven fabric-like structure, maintaining a highly dehydrated state that is resistant to heat. On the other hand, lysozyme resistance is largely due to the coat, which shields the cortex from the enzyme. The defect in lysozyme resistance of spores derived from cells with persistent *spoIIID* expression (Fig. 4) might be explained by their defect in spore coat structure (Fig. 6B). Although gross cortex and core defects were not evident by electron microscopic analysis of these spores, subtle defects are implied since more than half lost their ability to resist the heat treatment (which does not depend on the coat) (Fig. 4).

The electron microscopic analysis of spores derived from cells with persistent *spoIIID* expression revealed that typically much of the coat did not contact the cortex and that ridges were less evident than for wild-type spores (Fig. 6). These observations suggest an inability of the coat to maintain the folded, contracted state that appears to be responsible for the ridges (2, 10, 11, 41–43). Previous work implicated CotE (possibly indirectly) in coat folding (2). While that data indicate that the absence of a single protein can prevent ridge formation, the present study suggests that the ability of the wild-type coat to fold requires proper global regulation of coat protein levels. This supports the view that the flexibility of the coat is a function of multiple coat proteins.

The impact of maintaining the SpoIIID level late during sporulation depends to some extent on the conditions used to initiate the sporulation process. In the experiments documented in Results, sporulation was initiated by centrifugal collection of growing cells followed by resuspension in SM medium lacking nutrients. When sporulation was instead initiated by growth in Difco sporulation (DS) medium followed by nutrient exhaustion (21), results similar to those shown in Fig. 2 and 3 were observed, but the effects of persistent *spoIIID* expression on spore resistance properties were diminished such that none of the differences were statistically significant by the criterion (*P* value of less than 0.05 in a Student *t* test) that we used (data not shown). These results indicate that the method of sporulation initiation influences susceptibility to perturbation of the SpoIIID level late during sporulation, at least in terms of spore resistance properties. A connection between the method of sporulation initiation and expression of *cotC* late during sporulation was observed previously (55). Recently, aconitase, a Krebs cycle enzyme, was shown to be required for efficient late-sporulation gene expression, apparently because it binds to the 3' untranslated region of *gerE* mRNA, facilitating its accumulation (45). Aconitase provides a plausible link between early and late events during sporulation since the *citB* gene encoding aconitase is induced during late exponential phase in nutrient-exhausted medium (8). Our preliminary results suggest that aconitase accumulates to a considerably higher level late during sporulation in nutrient-

exhausted DS medium than after resuspension in SM medium (L. Wang and L. Kroos, unpublished data). The higher level of aconitase under nutrient exhaustion conditions might lead to a higher level of GerE, counteracting some of the effects of maintaining the SpoIIID level late during sporulation. Obviously, the MC gene regulatory network is more complex than that depicted in Fig. 1 and much remains to be learned about how entry into sporulation influences network characteristics and output.

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