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One Perturbation of the Mother Cell Gene Regulatory Network Suppresses the Effects of Another during Sporulation of *Bacillus subtilis*▽†

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In the mother cell of sporulating *Bacillus subtilis*, a regulatory network functions to control gene expression. Four transcription factors act sequentially in the order σ^E, SpoIIID, σ^K, GerE. σ^E and σ^K direct RNA polymerase to transcribe different regulons. SpoIIID and GerE are DNA-binding proteins that activate or repress transcription of many genes. Several negative regulatory loops add complexity to the network. First, transcriptionally active σ^K RNA polymerase inhibits early sporulation gene expression, resulting in reduced accumulation of σ^E and SpoIIID late during sporulation. Second, GerE represses *sigK* transcription, reducing σ^K accumulation about twofold. Third, SpoIIID represses *cotC*, which encodes a spore coat protein, delaying its transcription by σ^K RNA polymerase. Partially circumventing the first feedback loop, by engineering cells to maintain the SpoIIID level late during sporulation, causes spore defects. Here, the effects of circumventing the second feedback loop, by mutating the GerE binding sites in the *sigK* promoter region, are reported. Accumulation of pro-σ^K and σ^K was increased, but no spore defects were detected. Expression of σ^K-dependent reporter fusions was altered, increasing the expression of *gerE-lacZ* and *cotC-lacZ* and decreasing the expression of *cotD-lacZ*. Because these effects on gene expression were opposite those observed when the SpoIIID level was maintained late during sporulation, cells were engineered to both maintain the SpoIIID level and have elevated *sigK* expression late during sporulation. This restored the expression of σ^K-dependent reporters to wild-type levels, and no spore defects were observed. Hence, circumventing the second feedback loop suppressed the effects of perturbing the first feedback loop. By feeding information back into the network, these two loops appear to optimize target gene expression and increase network robustness. Circumventing the third regulatory loop, by engineering cells to express *cotC* about 2 h earlier than normal, did not cause a detectable spore defect.

When starved, the gram-positive bacterium *Bacillus subtilis* initiates a process called sporulation in order to form a dormant spore (for reviews, see references 8 and 23). Sporulation is a complex developmental process in which morphological changes are coupled to temporal and spatial regulation of gene expression. An early morphological change is the formation of an asymmetrically positioned septum that divides the cell into a larger mother cell (MC) compartment and a smaller forespore (FS) compartment. Completion of DNA replication ensures that a copy of the chromosome is available for each cell type. Distinct temporal programs of gene expression occur in the MC and FS, but signaling pathways between the two cell types coordinate the programs and morphogenesis. The MC engulfs the FS, pinching it off as a protoplast within the MC. Cortex, a modified peptidoglycan, is synthesized between the two membranes that surround the FS after engulfment. Proteins assemble on the surface of the FS, producing the coat. Eventually, the mature spore is released by lysis of the MC. The spore is resistant to harsh conditions, such as high temperature and exposure to UV light, lytic enzymes, and chemicals. The spore germinates in the presence of nutrients and grows vegetatively.

In the MC, a regulatory network controls gene expression during sporulation (for a review, see reference 23). The backbone of the network consists of a cascade of four transcription factors (σ^E, SpoIIID, σ^K, and GerE) (43), but several regulatory loops are also present in the network (Fig. 1). Synthesis of σ^E requires σ^A RNA polymerase (RNAP), the major form of RNAP in growing cells, and phosphorylated Spo0A (Spo0A-P), a response regulator that governs initiation of sporulation (for a review, see reference 15). The initial product of *sigE* is inactive pro-σ^E. It is cleaved to form active σ^E in response to a signal from the FS (18, 22, 27). σ^E RNAP transcribes many genes, including *spoIIID* and *sigK* (Fig. 1), as well as other genes whose products contribute to engulfment and synthesis of the spore cortex and coat (10, 11, 34). SpoIIID is a DNA-binding protein that activates transcription of *sigK* by σ^E RNAP and σ^K RNAP (13, 24) (Fig. 1). SpoIIID also positively regulates at least seven other transcription units and negatively regulates at least 62 transcription units in the σ^E regulon (10). In addition, SpoIIID negatively regulates certain genes in the σ^K regulon, such as *cotC* and *cotD* (14, 20, 24) (Fig. 1), which encode spore coat proteins (7). Like SpoIIID, GerR is a tran-

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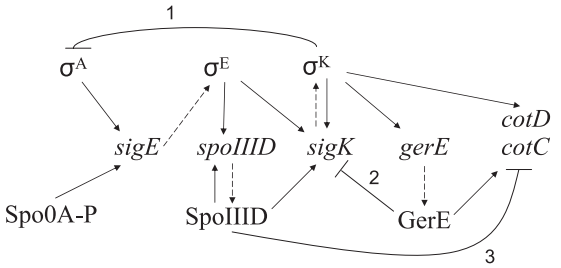

FIG. 1. Gene regulatory network in the MC during sporulation of *B. subtilis*. The dashed arrows indicate gene-protein relationships. The solid arrows and the lines with bars at their ends indicate positive and negative regulation, respectively. The numbers 1, 2, and 3 indicate three negative regulatory loops. See the text for an explanation and references.

scription factor under σ^E control, but it is not shown in Fig. 1 since it is not known to affect expression of other transcription factors in the cascade, although it does negatively regulate 10 transcription units in the σ^E regulon (10). σ^K, like σ^E, is first made as an inactive precursor protein that is cleaved in response to a signal from the FS (4, 28). σ^K RNAP transcribes many genes, including *gerE* (Fig. 1) and other genes whose products contribute to synthesis of the spore cortex and coat (10, 34). GerE is a DNA-binding protein that activates transcription of certain *cot* genes, such as *cotC* and *cotD* (19, 20, 42), and has an effect opposite that of SpoIIID (Fig. 1). In addition, GerE positively regulates at least 25 other transcription units in the σ^K regulon and negatively regulates at least 36 transcription units (10), including *sigK* (19, 42) (Fig. 1).

Three negative regulatory loops in the MC network are numbered in Fig. 1. Loop 1 involves transcriptionally active σ^K RNAP inhibition of early gene expression under σ^A control by an unknown mechanism, resulting in reduced accumulation of σ^E and SpoIIID late during sporulation (12, 39, 40). Loop 2 involves GerE repression of *sigK* transcription, lowering the σ^K level about twofold (19). Loop 3 involves SpoIIID repression of *cotC* transcription, delaying its expression by at least 1 h compared with other genes under positive control of σ^K RNAP and GerE (20).

How important are the three negative regulatory loops for sporulation? To begin to address this question, we previously engineered *B. subtilis* to circumvent the decrease in the level of SpoIIID late during sporulation, which is normally brought about by loop 1 (37). We found that maintaining the SpoIIID level late during sporulation resulted in altered expression of σ^K-dependent genes, lower numbers of resistant spores, and a structural defect in the coat of most spores that were produced. Here, we report the effect of mutating the GerE binding site in the *sigK* promoter, which eliminated loop 2 (Fig. 1). As expected, the levels of pro-σ^K and σ^K were increased during sporulation. This did not cause detectable spore defects. However, expression of σ^K-dependent genes was altered, and the effects were opposite those observed when the SpoIIID level was maintained late during sporulation. Interestingly, elimination of GerE negative feedback for *sigK* transcription suppressed the spore defects caused by maintaining the SpoIIID level late during sporulation. Also, expression of σ^K-dependent genes in a strain with both perturbations was restored to wild-type levels. We also report that expression of *cotC* approximately 2 h earlier than normal (circumventing loop 3) did not cause detectable spore defects. Taken together, these results suggest that loops 2 and 3 are less important for sporulation than loop 1 but that loops 1 and 2 exert opposing effects, fine-tuning the expression of target genes in order to optimize spore formation.

MATERIALS AND METHODS

Site-directed mutagenesis and construction of plasmids. A plasmid bearing the *sigK* promoter (starting at position −108) and the entire coding region was constructed in several steps. First, pRG2 (32) was digested with BamHI and annealed oligonucleotides (5′-GATCCCACCACCACCACCACCACTAA-3′ and 5′-GATCTTAGTGGTGGTGGTGGTGGTGG-3′) were inserted in the orientation that preserved the BamHI site at the 3′ end of *sigK*, generating pHP1. Second, a QuikChange site-directed mutagenesis kit (Stratagene) was used with primers 5′-GGCTTTTGCCTACAAGCTTTTGTGGAGGTGACG-3′ and 5′-CGTCACCTCCACAAAAGCTTGTAGGCAAAAGCC-3′ (mutant nucleotides are underlined) and pHP1 as the template to introduce a HindIII site between the *sigK* promoter and the ribosome binding site, generating pHP12, for which the *sigK* sequence was verified. Third, site-directed mutagenesis was likewise performed with primers 5′-CCCGAAAAGTGCCACCTGGTGTCTAAG AAACC-3′ and 5′-GGTTTCTTAGACACCAGGTGGCACTTTTCGGG-3′ and with pDG364 as the template to eliminate its AatII site, generating pHP13. Finally, the *sigK*-containing EcoRI-BamHI fragment from pHP12 was gel purified and ligated into EcoRI-BamHI-digested pHP13, generating pHP14, which due to the BamHI site and vector added four codons (specifying the amino acids GSPA) to the 3′ end of *sigK*. This *sigK* allele was flanked by the 5′ and 3′ ends of *amyE*, and it complemented a *sigK* mutant when it replaced the normal *amyE* gene in the *B. subtilis* chromosome (see below). Plasmid pHP14 was the template for site-directed mutagenesis designed to eliminate the GerE binding site in the *sigK* promoter region. Four nucleotide changes, GG to CC at positions 4 and 5 and CC to GG at positions 12 and 13, were made using primers 5′-CCGGTCAC ATACATTTACATATACCCTTTTGGGTACATACTTTTGTGGAGG-3′ and 5′-CCTCCACAAAAGTATGTACCCAAAAGGGTATATGTAAATGTATGT GACCGG-3′. The resulting allele in pJP16 was designated *sigKmut* and was sequenced to confirm that only the desired mutations were present.

In order to express *cotC* earlier than normal, we used the *gerE* promoter to drive its expression. A DNA fragment containing *cotC* and spanning the region from slightly upstream of its ribosome binding site to slightly downstream of its stop codon was synthesized by performing PCR with primers 5′-CGAAGCTTT AAAGGAGGAGTATATATGGGTTATTAC-3′ and 5′-GCGGATCCACCCG GCAATAGCCGGG-3′, which contained HindIII and BamHI restriction sites (underlined), respectively, and with chromosomal DNA from *B. subtilis* PY79 as the template. The PCR product was digested with HindIII and BamHI and ligated with HindIII-BamHI-digested pJP1 (37), generating pJP4. The sequence of the entire P_{gerE}-*cotC* fusion was determined to ensure that no errors occurred during the PCR.

Bacterial strains. *Escherichia coli* strain AG115 [*araD139* Δ(*ara*,*leu*)*7697* Δ*lacX74 galU galK hsr hsm*$^+$ *strA* (F′ *proAB lacI*^{q}*Z*::Tn*5*)] was obtained from A. Grossman (Massachusetts Institute of Technology). It was used during construction and maintenance of plasmids. Luria-Bertani (LB) medium (33) was used to grow *E. coli* and *B. subtilis* and was supplemented with appropriate antibiotics. *B. subtilis* strains used in this study are listed in Table 1. Plasmid pJP4 was transformed into PY79 with selection on LB agar containing kanamycin sulfate (5 μg/ml) to generate strain BJP3. Plasmids pHP14 and pJP16 bearing wild-type *sigK* and the *sigKmut* allele, respectively, were transformed into BK556 with selection on LB agar containing chloramphenicol (5 μg/ml). Strains in which *amyE* was replaced by *sigK* (BLW1) or *sigKmut* (BLW2) were identified as described previously (6). pJP1 (a multicopy plasmid with a P_{gerE}-*spoIIID* fusion) (37) was transformed into BLW1 and BLW2 with selection on LB agar containing chloramphenicol (5 μg/ml) and kanamycin sulfate (5 μg/ml) to generate strains BLW3 and BLW4, respectively. The chloramphenicol resistance (Cm^r) gene of BLW1, BLW2, BLW3, and BLW4 was replaced with the spectinomycin resistance (Sp^r) gene of pCm::Sp as described previously (35), generating strains BLW5, BLW6, BLW7, and BLW8, respectively. A lysate containing SPβ::*cotC-lacZ* was obtained by heat induction of strain OR825 as described previously (6). SPβ::*cotD-lacZ* and SPβ::*gerE-lacZ* have been described previously (4, 5). Specialized transduction was used to move *lacZ* fusions into BLW5, BLW6, BLW7, and BLW8 as described previously (16). Transductants were selected on LB agar containing chloramphenicol (5 μg/ml). In each case, at least 10 transductants were transferred onto a DSM agar (16) plate with 5-bromo-4-chloro-3-indolyl-

TABLE 1. *B. subtilis* strains used

Strain	Phenotype, genotype, and/or derivation	Reference or source
PY79	Spo$^+$ prototroph	38
OR825	SPβ::*cotC-lacZ* Cm^r	3
EUDC9901	*trpC2 pheA1 gerE*::*kan* Km^r	2
BK556	*spoIVCB23*	26
BD071	*cotC*::*cat* Cm^r	7
BJP1	PY79 transformed with pJP1(P_{gerE}-*spoIIID*), Km^r	37
BJP2	PY79 transformed with pJP2(P_{gerE}), Km^r	37
BJP3	PY79 transformed with pJP4(P_{gerE}-*cotC*), Km^r	This study
BLW1	BK556 transformed with pHP6(*amyE*::*sigK*), Cm^r	This study
BLW2	BK556 transformed with pJP16(*amyE*::*sigKmut*), Cm^r	This study
BLW3	BLW1 transformed with pJP1, Km^r Cm^r	This study
BLW4	BLW2 transformed with pJP1, Km^r Cm^r	This study
BLW5	BLW1 transformed with pCm::Sp, Sp^r	This study
BLW6	BLW2 transformed with pCm::Sp, Sp^r	This study
BLW7	BLW3 transformed with pCm::Sp, Km^r Sp^r	This study
BLW8	BLW4 transformed with pCm::Sp, Km^r Sp^r	This study

β-D-galactopyranoside (40 μg/ml). Three or more isolates with average blue color were saved for further analysis, excluding occasional isolates with abnormally high or low β-galactosidase activity.

Cell growth and sporulation. Sporulation was induced by resuspension of cells in SM medium as described previously (16). The time of resuspension was defined as the onset of sporulation (zero time).

Western blot analysis. Starting at 3 h into sporulation and at hourly intervals thereafter until 9 h into sporulation, 0.5-ml samples were subjected to centrifugation (14,000 × *g* for 1 min), the supernatants were removed, and the cell pellets were stored at −70°C. Preparation of whole-cell extracts, electrophoresis, and electroblotting were performed as described previously (12, 28). The blots were probed with anti-SpoIIID (12), anti-pro-σ^K (28), or anti-CotC (21) antibody diluted 1:10,000. Immunodetection of primary antibodies was performed as described previously (25).

Analysis of β-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 using toluene as described previously (30). The β-galactosidase specific activity was determined as described previously (30) using *o*-nitrophenol-β-D-galactopyranoside as the substrate. One unit of the enzyme hydrolyzed 1 μmol of substrate per min per unit of initial culture optical density at 595 nm.

Spore purification and germination and resistance assays. Spores were harvested at 24 h into sporulation by centrifugation at 7,000 × *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 consisting of 20 to 50% RenoCal-76 (Bracco Diagnostics Inc.) as described previously (17). 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 (31). Assays to determine resistance to heat, lysozyme, and organic solvents were performed at 24 h into sporulation without spore purification, as described previously (16).

Transmission electron microscopy of spores. For transmission electron microscopy spores were harvested at 24 h into sporulation, washed with water, and immediately fixed as described previously (29).

RESULTS

Engineering *B. subtilis* to circumvent GerE repression of *sigK*. GerE binds to a site overlapping the *sigK* transcriptional start site and represses transcription about twofold, lowering the level of pro-σ^K and σ^K about twofold (19). Within the GerE binding site are two sequences that match the consensus sequence for GerE binding (Fig. 2). To assess the importance of *sigK* repression by GerE, we mutated two nucleotides in each match to the consensus (Fig. 2). A previous study showed that changing GG to TT near the center of the GerE binding site in the *cotH* promoter region impaired GerE repression of

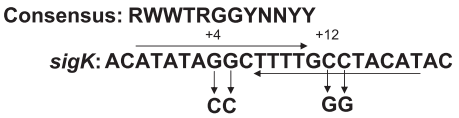

FIG. 2. Mutation in the GerE binding site in the *sigK* promoter region. The top line shows the consensus sequence for GerE binding (41) (R = A or G; W = A or T; Y = C or T; N = A, G, C, or T). The bottom line shows the sequence in the *sigK* promoter region where GerE binds, as defined by DNase I footprinting (19). The numbering is relative to the transcriptional start site. The rightward arrow indicates a perfect match to the consensus. The leftward arrow indicates a match at 9 of 12 positions to the consensus on the strand not shown. The downward arrows show the four changes in the *sigKmut* allele.

cotH transcription (1). We changed GG at positions 4 and 5 to CC and CC at positions 12 and 13 to GG in the *sigK* promoter region (Fig. 2). The resulting mutant allele of *sigK*, including the promoter and the entire coding region, was integrated at the *amyE* locus of *spoIVCB23* mutant *B. subtilis* BK556, creating BLW2 (*spoIVCB23 amyE*::*sigKmut*). The *spoIVCB23* mutation prevents production of pro-σ^K and σ^K (28) from the native *sigK* locus, which is created in the MC during sporulation by site-specific recombination between the *spoIVCB* and *spoIIIC* genes (36). As a control, a wild-type *sigK* allele was integrated at the *amyE* locus of the *spoIVCB23* mutant, creating BLW1 (*spoIVCB23 amyE*::*sigK*). BLW1 and BLW2 were grown and induced to sporulate along with *B. subtilis* wild-type strain PY79 as an additional control. Samples were collected during sporulation, and whole-cell extracts were subjected to Western blot analysis to detect pro-σ^K and σ^K. The levels of pro-σ^K and σ^K were higher in the *spoIVCB23 amyE*::*sigKmut* strain than in either control strain, starting at 5 h into sporulation (Fig. 3). The pro-σ^K and σ^K levels were similar in the two control strains. The effect of the *sigKmut* allele on *sigK* expression was similar to the effect of a *gerE* null mutation (19), suggesting that GerE repression of *sigK* is eliminated in BLW2.

Effects of circumventing GerE repression of *sigK*. Because σ^K acts after SpoIIID in the MC gene regulatory network (Fig.

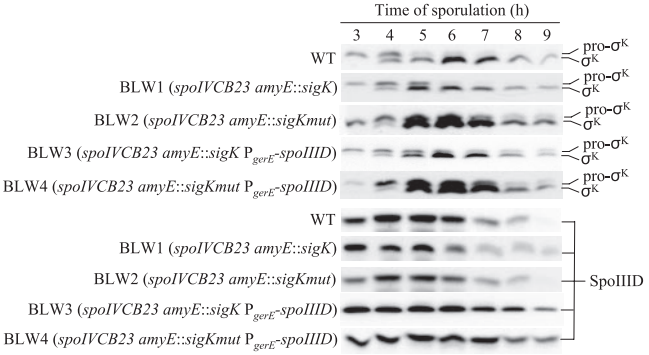

FIG. 3. Levels of pro-σ^K and σ^K and levels of SpoIIID during sporulation. *B. subtilis* wild-type strain PY79 (WT) and the other strains indicated were induced to sporulate by resuspension in SM medium. Samples were collected at hourly intervals beginning at 3 h after the onset of sporulation. Equal volumes (5 μl) of whole-cell extracts were fractionated on sodium dodecyl sulfate-14% polyacrylamide gels and subjected to Western blot analysis with anti-pro-σ^K or anti-SpoIIID serum.

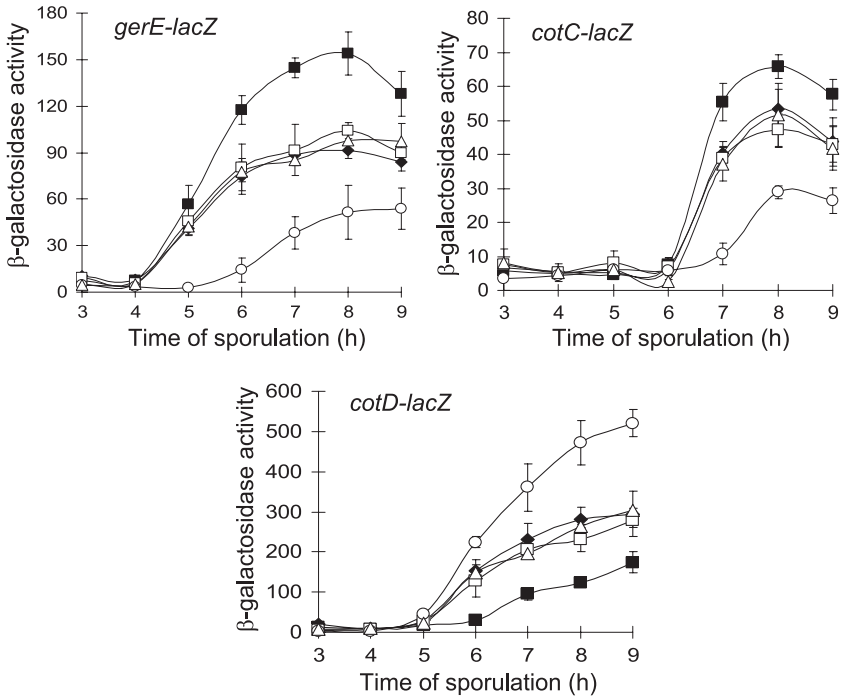

FIG. 4. Effects on gene expression. β-Galactosidase activity during sporulation after resuspension in SM medium was measured for *B. subtilis* containing the indicated *lacZ* fusions in wild-type strain PY79 (△), BLW5 (*spoIVCB23 amyE*::*sigK*) (◆), BLW6 (*spoIVCB23 amyE*::*sigKmut*) (■), BLW7 (*spoIVCB23 amyE*::*sigK* P_{gerE}-*spoIIID*) (○), and BLW8 (*spoIVCB23 amyE*::*sigKmut* P_{gerE}-*spoIIID*) (□). Each symbol indicates the average of three determinations, and the error bars indicate one standard deviation.

1), we did not expect the SpoIIID level during sporulation to be altered in the *spoIVCB23 amyE*::*sigKmut* strain. Indeed, the level was indistinguishable from that in the wild type and the *spoIVCB23 amyE*::*sigK* strain (Fig. 3).

To test whether expression of σ^K-dependent genes was affected by the elevated level of σ^K in BLW2, we measured β-galactosidase activity from *lacZ* fusions to *gerE*, *cotC*, and *cotD* during sporulation. In order to introduce *lacZ* fusions with associated Cm^r markers into strains BLW2 and BLW1, we replaced their Cm^r cassettes with Sp^r cassettes, creating BLW6 and BLW5, respectively. Expression from *gerE-lacZ* and *cotC-lacZ* was elevated in BLW6 relative to the BLW5 and PY79 control strains (Fig. 4). Apparently, elevated σ^K levels in sporulating cells can increase transcription of σ^K-dependent genes. Interestingly, expression from *cotD-lacZ* was reduced in BLW6 relative to the control strains (Fig. 4). This might have been due to an elevated level of GerE since a high level of GerE represses *cotD* transcription by σ^K RNAP in vitro (19).

Circumventing GerE repression of *sigK* compensates for persistent *spoIIID* expression. The effects on expression of the three σ^K-dependent reporters (Fig. 4) were the opposite of the effects observed previously when cells were engineered to maintain the SpoIIID level late during sporulation (37). In the previous study, SpoIIID was expressed ectopically from a multicopy plasmid bearing a P_{gerE}-*spoIIID* fusion (pJP1). Introduction of multicopy P_{gerE}-*spoIIID* into the *spoIVCB23 amyE*::*sigKmut* background (creating BLW4 initially; then the Cm^r cassette was replaced with an Sp^r cassette to create BLW8 for introduction of *lacZ* fusions [Table 1]) restored expression of *gerE-lacZ*, *cotC-lacZ*, and *cotD-lacZ* to the levels observed in the wild-type PY79 and *spoIVCB23 amyE*::*sigK* (BLW5-derived) control strains (Fig. 4). In contrast, introduction of multicopy P_{gerE}-*spoIIID* into the *spoIVCB23 amyE*::*sigK* background (creating BLW3 initially; then the Cm^r cassette was replaced with an Sp^r cassette to create BLW7 for introduction of *lacZ* fusions [Table 1]) decreased expression of *gerE-lacZ* and *cotC-lacZ* and increased expression of *cotD-lacZ* (Fig. 4), similar to the effects of multicopy P_{gerE}-*spoIIID* in an otherwise wild-type background (37). As expected, both BLW3 and BLW4 exhibited a higher level of SpoIIID late during sporulation than strains without P_{gerE}-*spoIIID* (Fig. 3). Also as expected, the levels of pro-σ^K and σ^K were higher starting at 5 h into sporulation in BLW4 than in strains without *amyE*::*sigKmut* (Fig. 3). An elevated level of σ^K in BLW4 appears to compensate for an elevated level of SpoIIID, restoring *gerE*, *cotC*, and *cotD* expression to wild-type levels.

Does circumventing GerE repression of *sigK* with the *sigKmut* allele compensate for the other effects of persistent *spoIIID* expression from P_{gerE}-*spoIIID*, which include defects in spore resistance properties and coat assembly (37)? Figure 5 shows that BLW4 produced heat- and lysozyme-resistant spores as efficiently as the wild type. These spores germinated normally in response to L-alanine (see Fig. S1 in the supplemental material) and were indistinguishable from wild-type strain PY79 spores when they were examined by thin-section transmission electron microscopy (data not shown). On the basis of the criteria that we tested, elevated levels of σ^K in BLW4 appeared to fully suppress the sporulation defects caused by maintaining the SpoIIID level during the late stages.

In contrast to strain BLW4 and as expected from our pre-

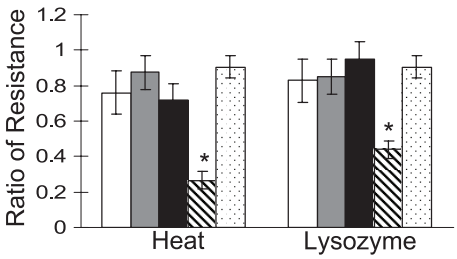

FIG. 5. Resistance properties of spores. The ratio of the number of CFU after the indicated treatments to the number of CFU before the treatments was determined for *B. subtilis* wild-type strain PY79 (open bars), BLW1 (*spoIVCB23 amyE*::*sigK*) (gray bars), BLW2 (*spoIVCB23 amyE*::*sigKmut*) (black bars), BLW3 (*spoIVCB23 amyE*::*sigK* P_{gerE}-*spoIIID*) (striped bars), and BLW4 (*spoIVCB23 amyE*::*sigKmut* P_{gerE}-*spoIIID*) (dotted bars) at 24 h after resuspension in SM medium. The bars indicate the averages of three determinations, and the error bars indicate one standard deviation. An asterisk indicates that the *P* value is <0.05 for a comparison of BLW3 with PY79 or BLW4.

vious study (37), strain BLW3, in which the SpoIIID level was maintained late during sporulation without an elevated level of σ^K, produced significantly fewer heat- and lysozyme-resistant spores (Fig. 5). Also, these spores germinated normally (see Fig. S1 in the supplemental material), but about 90% showed a defect in coat assembly when they were examined by electron microscopy (data not shown). The inner and outer coat layers were thinner and less organized than those of wild-type spores, and the coat was not as closely apposed to the cortex and its ridges were less evident, as seen previously for strain BJP1 (containing multicopy P_{gerE}-*spoIIID* in an otherwise wild-type background) (37).

Despite elevated σ^K levels (Fig. 3) and altered expression of σ^K-dependent genes (Fig. 4) in strain BLW2, this strain produced heat- and lysozyme-resistant spores as efficiently as the control strain BLW1 and wild-type strain PY79 (Fig. 5). The spores of all three strains germinated similarly (see Fig. S1 in the supplemental material) and were indistinguishable when they were examined by electron microscopy (data not shown).

We also measured the ability of strains BLW1, BLW2, BLW3, and BLW4 to produce spores resistant to phenol, ethanol, and chloroform. None of the strains differed significantly (i.e., yielded a *P* value of <0.05 as determined by a Student *t* test) from wild-type strain PY79 (data not shown).

Effects of circumventing the delay in CotC production. Expression of *cotC-lacZ* is delayed by 2 h relative to *gerE-lacZ* expression during sporulation (Fig. 4). To determine whether this delay is important for sporulation, we fused *cotC* to the *gerE* promoter in a plasmid (pJP4) that can be maintained in *B. subtilis* in multiple copies. The plasmid was transformed into *B. subtilis* wild-type strain PY79, creating BJP4 with multicopy P_{gerE}-*cotC*. BJP2 (containing P_{gerE} without *cotC* in the PY79 background) (37) served as a control. Western blot analysis of whole-cell extracts from sporulating cells showed that in BJP4, CotC accumulates to a low level at 2 to 4 h into sporulation, perhaps due to readthrough transcription from a constitutive promoter on the plasmid, and the level of CotC rises beginning at 5 h into sporulation, presumably due to transcription from P_{gerE} (see Fig. S2 in the supplemental material). As expected, the CotC level began to rise about 2 h later, at 7 h into sporulation, in BJP2 and PY79. Taken together, these results demonstrate that multicopy P_{gerE}-*cotC* in BJP4 circumvents the 2-h delay in CotC accumulation.

We measured the ability of strains BJP4 and BJP2 to produce spores resistant to heat, lysozyme, phenol, ethanol, and chloroform. Neither strain differed significantly (i.e., yielded a *P* value of <0.05 as determined by a Student *t* test) from wild-type strain PY79 (data not shown). Also, the germination kinetics in response to L-alanine were similar for spores produced by strains BJP4 and BJP2 compared to spores produced by PY79 (data not shown). We concluded that accumulating CotC 2 h earlier than normal did not detectably alter spore resistance or germination properties.

DISCUSSION

Our study resulted in two novel findings about loop 2 in the MC gene regulatory network (Fig. 1). First, circumvention of GerE repression of *sigK* elevated the σ^K level and probably the GerE level (since expression of a *gerE-lacZ* translational fusion increased), but this did not detectably alter spore resistance, germination, or structure. This revealed robustness in the network. Second, circumvention of loop 2 suppressed the effects on sporulation of persistent *spoIIID* expression. This suggests that negative regulatory loops with opposing effects enhance network robustness and presumably optimize target gene expression.

The results of a previous study aided our effort to circumvent GerE repression of *sigK*. Initially, we deleted nucleotides from position 5 to position 15 relative to the *sigK* transcriptional start site, but this did not result in elevated pro-σ^K and σ^K levels (data not shown), perhaps due to interference with promoter utilization by RNAP. After learning that a mutation in the GG sequence within the GerE binding site in the *cotH* promoter region increased *cotH-lacZ* expression (1), we made the *sigKmut* allele shown in Fig. 2. This allele resulted in levels of pro-σ^K and σ^K accumulation similar to those observed previously in a *gerE* null mutant, which accumulated about twofold more total pro-σ^K/σ^K than the wild type (19).

What limits the total pro-σ^K/σ^K accumulation to about twofold more than that in the wild type? SpoIIID activates *sigK* transcription by σ^E RNAP (13) or σ^K RNAP (24). Late in sporulation, the SpoIIID and σ^E levels decrease due to negative feedback by σ^K RNAP (12, 39, 40). This suggested that SpoIIID might become limiting for *sigK* transcription by σ^K RNAP; however, this is not the case because introduction of P_{gerE}-*spoIIID* into the *spoIVCB23 amyE*::*sigKmut* background did not change the pro-σ^K or σ^K level (Fig. 3). Perhaps processing of pro-σ^K to active σ^K or degradation of σ^K limits accumulation of SigK products under these conditions. Such posttranslational mechanisms would contribute to network robustness by limiting *sigK* autoregulation in the event that negative regulatory loops (i.e., GerE repression and loss of the SpoIIID activator) that operate at the transcriptional level fail.

The elevated σ^K level due to the *sigKmut* allele did not detectably alter the final product of the MC gene regulatory network, the spores. Elevated σ^K levels did not significantly alter the number of heat-, lysozyme-, phenol-, ethanol-, or chloroform-resistant spores produced (Fig. 5 and data not shown), nor did they alter the spore germination kinetics in

response to L-alanine, as measured by changes in optical density (see Fig. S1 in the supplemental material). Moreover, we detected no difference in spore structure compared to that of the wild type upon examination of thin sections by electron microscopy. On the basis of these criteria, the network is robust in terms of its final output (the spore) when the σ^K level is elevated. On the other hand, expression of all three σ^K-dependent genes tested was altered (Fig. 4). Increased expression of *gerE* and *cotC* can be understood in terms of the dependence of these genes on σ^K RNAP and, in the case of *cotC*, activation by GerE (42). Transcription of *cotD* also depends on σ^K RNAP (24), but a high level of GerE represses *cotD* transcription in vitro (19), so elevated GerE levels in the strain with elevated σ^K levels might account for the observed decrease in *cotD* expression (Fig. 4). Based on the findings for these three genes, it seems likely that expression of many of the 108 other genes in the σ^K regulon (10) is altered by elevated σ^K levels. In terms of gene expression, the network seems quite susceptible to perturbation of loop 2 (Fig. 1). This provides one rationale for retention of loop 2 during evolution; mutations in loop 2 components may subtly alter MC gene expression, optimizing it for a particular ecological niche.

A second rationale for evolutionary retention of loop 2 is its ability to suppress sporulation defects caused by persistent *spoIIID* expression. Preliminary results suggest that transposon insertion mutations in several genes elevate expression of an *spoIIID-lacZ* fusion (L. Wang and L. Kroos, unpublished data). Hence, the MC gene regulatory network appears to be quite susceptible to mutational perturbation leading to elevated SpoIIID levels, which can cause spore defects (37). We show here that mutating the GerE binding site in the *sigK* promoter elevates the σ^K level and probably the GerE level (since expression of a *gerE-lacZ* translational fusion increased), compensating for persistent *spoIIID* expression by restoring MC gene expression (including expression of *gerE-lacZ*) and formation of spores with normal resistance properties and coat structure. Undoubtedly, other mutations could elevate the σ^K level as well, but loop 2 increases network robustness by providing additional targets for mutations that can compensate for changes in the SpoIIID level.

Is GerE repression of *sigK* (loop 2) likely present in sporeformers related to *B. subtilis*? A search for orthologs of MC transcription factors revealed that σ^K is present in *Bacillus* and *Clostridium* species, but GerE is absent from *Clostridium* (10). Among *Bacillus* species, we searched for a GerE binding site in the *sigK* promoter region. As shown in Fig. S3A in the supplemental material, *Bacillus licheniformis* differed from *B. subtilis* at only one position, which did not affect either match to the GerE consensus binding sequence (Fig. 2). However, most strains of *Bacillus cereus*, *Bacillus weihenstephanensis*, all strains of *Bacillus anthracis*, and one strain of *Bacillus thuringiensis* have two changes that create mismatches to the TRGGY core of the GerE consensus binding sequence (see Fig. S3B in the supplemental material). One of these changes is a G-to-A transition at a position predicted to interact with Lys41 in *B. subtilis* GerE (9). This Lys residue is perfectly conserved among GerE homologs of the organisms shown in Fig. S3 in the supplemental material. Moreover, the strains in Fig. S3B in the supplemental material do not exhibit a second match to the GerE consensus binding sequence, as *B. subtilis* does (Fig. 2). Therefore, GerE is likely to bind more weakly, if at all, to the corresponding position in the *sigK* promoter region of the strains in Fig. S3B in the supplemental material. For the strains in Fig. S3C in the supplemental material, it seems even less likely that GerE represses *sigK* transcription. On the other hand, the strains in Fig. S3D in the supplemental material retain more characteristics of the GerE binding site in the *B. subtilis sigK* promoter region, suggesting that GerE represses *sigK* transcription in these species. We speculate that about one-half of the distinct species shown in Fig. S3 in the supplemental material have loop 2 in their MC gene regulatory network.

Elevated σ^K levels in the *B. subtilis sigKmut* strain did not detectably hasten the decrease in the level of SpoIIID during sporulation (Fig. 3). We infer from this result that σ^K is not the rate-limiting factor in the regulatory loop (loop 1 in Fig. 1) by which σ^K RNAP leads to a decrease in the SpoIIID level (12, 39, 40). As depicted in Fig. 1, the evidence suggests that one or more genes transcribed by σ^K RNAP inhibit the activity of σ^A RNAP, decreasing transcription of *sigE* and other early genes, including *spoIIID*, but the mechanism of inhibition remains a mystery.

Expression of *cotC* 2 h earlier than normal from the heterologous *gerE* promoter did not detectably alter spore resistance or germination properties. Why, then, is *cotC* expression normally delayed? Perhaps repression of *cotC* by SpoIIID prevents wasteful expression before CotC can assemble into the spore coat. CotH is required for assembly of CotC into the outer coat (21, 31). The *cotH* gene is expressed by 5 h into sporulation (1) and CotH accumulates by 6 h (44), but earlier times have not been examined. Under our conditions, *cotC-lacZ* was expressed by 7 h into sporulation (Fig. 4), and CotC began to accumulate at that time (see Fig. S2 in the supplemental material). Earlier expression of *cotC* from P_{gerE}-*cotC* on a multicopy plasmid may be inconsequential because CotH availability limits CotC assembly. Interestingly, GerE represses *cotH* expression, but circumventing this negative regulatory loop by mutating the GerE binding site in the *cotH* promoter region had no detectable effect on spore structure or function, although it did allow accumulation of CotC in the MC (1). Normally, CotC does not accumulate in the MC, probably because it assembles immediately into the spore coat (21). In cells with GerE-independent expression of *cotH*, an elevated CotH level appears to stabilize CotC in the MC, suggesting that the spore has a limited capacity to incorporate CotC (1).

In summary, circumvention of GerE repression of *sigK* elevated the σ^K level and probably the GerE level, two nodes in the MC gene regulatory network, likely altering expression of many genes in the σ^K regulon but not detectably changing spore resistance, germination, or structure. However, this perturbation compensated for the effects of persistent *spoIIID* expression, which also appears to alter two nodes in the network; the SpoIIID level is maintained late during sporulation (when it normally decreases), and the GerE level is probably reduced (since expression of a *gerE-lacZ* translational fusion is diminished) (37). We infer that the decrease in SpoIIID normally brought about, at least in part, by loop 1 (Fig. 1) promotes *gerE* expression. In contrast, loop 2 normally inhibits further *gerE* expression

because less σ^K RNAP is produced. It appears that two negative feedback loops with opposing effects on *gerE* expression and different effects on other nodes (loop 1 reduces the σ^E and SpoIIID levels [12, 39, 40], whereas loop 2 reduces the σ^K level [19] [Fig. 3]) enhance the robustness of the MC network and optimize the expression of target genes.

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