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σ^K Can Negatively Regulate *sigE* Expression by Two Different Mechanisms during Sporulation of *Bacillus subtilis*

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Temporal and spatial gene regulation during *Bacillus subtilis* sporulation involves the activation and inactivation of multiple sigma subunits of RNA polymerase in a cascade. In the mother cell compartment of sporulating cells, expression of the *sigE* gene, encoding the earlier-acting sigma factor, σ^E, is negatively regulated by the later-acting sigma factor, σ^K. Here, it is shown that the negative feedback loop does not require SinR, an inhibitor of *sigE* transcription. Production of σ^K about 1 h earlier than normal does affect Spo0A, which when phosphorylated is an activator of *sigE* transcription. A mutation in the *spo0A* gene, which bypasses the phosphorelay leading to the phosphorylation of Spo0A, diminished the negative effect of early σ^K production on *sigE* expression early in sporulation. Also, early production of σ^K reduced expression of other Spo0A-dependent genes but not expression of the Spo0A-independent *ald* gene. In contrast, both *sigE* and *ald* were overexpressed late in development of cells that fail to make σ^K. The *ald* promoter, like the *sigE* promoter, is believed to be recognized by σ^A RNA polymerase, suggesting that σ^K may inhibit σ^A activity late in sporulation. To exert this negative effect, σ^K must be transcriptionally active. A mutant form of σ^K that associates with core RNA polymerase, but does not direct transcription of a σ^K-dependent gene, failed to negatively regulate expression of *sigE* or *ald* late in development. On the other hand, the negative effect of early σ^K production on *sigE* expression early in sporulation did not require transcriptional activity of σ^K RNA polymerase. These results demonstrate that σ^K can negatively regulate *sigE* expression by two different mechanisms, one observed when σ^K is produced earlier than normal, which does not require σ^K to be transcriptionally active and affects Spo0A, and the other observed when σ^K is produced at the normal time, which requires σ^K RNA polymerase transcriptional activity. The latter mechanism facilitates the switch from σ^E to σ^K in the cascade controlling mother cell gene expression.

In response to nutrient depletion, *Bacillus subtilis* undergoes a developmental process that culminates with the formation of a dormant spore (62). Two compartments, the mother cell and the forespore, are formed early during the sporulation process due to the synthesis of an asymmetric septum. The forespore is later engulfed within the mother cell, being completely surrounded by the two membranes of the septum. The mother cell contributes to the synthesis of many components necessary for forespore maturation, including a thick layer of peptidoglycan called cortex and a tough proteinaceous spore coat, and is discarded by lysis at the end of sporulation, releasing the mature spore.

Sporulation involves highly ordered programs of gene expression in the two compartments that are regulated primarily by the ordered appearance of two series of alternate sigma factors (33, 62). Upon starvation, multiple signals impinge on a phosphorelay system composed of protein kinases and phosphatases, a phosphotransferase, and at least one kinase inhibitor (5, 13, 22, 53, 65). The result is an elevated level of phosphorylated Spo0A (Spo0A~P), a transcription factor that activates σ^A RNA polymerase (RNAP) and σ^H RNAP to transcribe the genes encoding σ^E and σ^F, respectively (2, 4, 5, 67). After formation of the asymmetric septum, σ^F becomes active in the forespore and directs transcription of the gene encoding σ^G (18, 41, 48, 52, 64). Similarly, σ^E becomes active in the mother cell and directs transcription of the gene encoding σ^K (10, 18, 36).

Communication between the mother cell and the forespore regulates sigma factor activity (33, 43). All the compartment-specific sigma factors are initially inactive. In the forespore, σ^F and σ^G are held inactive by an anti-sigma factor, SpoIIAB (11, 28, 31, 50). In the mother cell, σ^E and σ^K are first synthesized as inactive precursor proteins, pro-σ^E and pro-σ^K (8, 38, 44). Compartmentalized activation of these sigma factors, except for σ^F, depends on intercompartmental signal transduction (33, 43). In this way, the programs of gene expression in the two compartments are coupled. In addition to controlling the synthesis and activation of subsequent sigma factors in the cascade, each sigma factor directs core RNAP to transcribe different genes whose products drive morphogenesis (62).

Although the synthesis and activation of sigma factors during *B. subtilis* sporulation have been relatively well studied, little is known about how later sigma factors replace the earlier ones. We showed previously that in the mother cell compartment, the appearance of σ^K accelerates the disappearance of σ^E (73). In mutants that fail to produce σ^K, the σ^E level at 5 to 8 h into development was two- to fivefold higher than in wild-type cells. In a mutant that produces σ^K earlier than normal, twofold less σ^E accumulated than in wild-type cells. σ^K seems to affect the synthesis of σ^E, because β-galactosidase activity from a *lacZ* transcriptional fusion to the promoter of the *spoIIG* operon (referred to as *sigE-lacZ* since *spoIIGB*, also called *sigE*, encodes pro-σ^E) mirrored the σ^E level during sporulation of wild-type cells or *sigK* mutant cells that either fail to make σ^K or make σ^K earlier than normal. Also, σ^K did not detectably alter the stability of σ^E. Taken together, these results suggest that σ^K initiates a negative feedback loop that regulates transcription of *sigE* in the mother cell compartment of developing cells.

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TABLE 1. *B. subtilis* strains used

Strain	Relevant characteristics	Reference or source
PY79	Spo$^+$ prototroph	70
BK556	*spoIVCB23*	34
VO48	*spoIVCBΔ19 cat*	8
BZ48	*spoIVCBΔ19 spc*	This study
IS432	*sinR*::*cat*	12
EU8743	SPβ::*spoIIG-lacZ*	29
AG919	*rvtA11 cat*	14
AG1431	*spo0FΔspo0BΔ*	14
KI1202	SPβ::*spoIIA-lacZ*	A. Grossman (66)
KY9	SPβ::*spoIIE-lacZ*	15
KI220	*ald*::Tn*917lac MLS*	59
BK410	*spoIIIC94*	34
BZ410	*spoIIIC94 sigKC109R spc*	This study
SC284	SPβ::*gerE-lacZ*	9
BSL50	*spoIIID83 bofA*::Tn*917Δlac*::pTV21Δ2 *cat*	45
PS1	*bofA*::Tn*917Δlac*::pTV21Δ2 *cat*	This study
PS2	*spoIIIC94 sigKC109R spc* *bofA*::Tn*917Δlac*::pTV21Δ2 *cat*	This study
BSL1	*sspB-lacZ cat*	49
KI1261	*spo0A-lacZ cat*	24
KH566	*spo0H-lacZ cat*	21
KH586	*spo0K-lacZ cat*	21
ZB456	SPβ::*spoVG42-lacZ*	74

We have further investigated how σ^K negatively regulates *sigE* expression during sporulation. Transcription of *sigE* is carried out by σ^A RNAP and is activated by Spo0A~P (2, 4, 30, 57) and repressed by SinR (46, 47). Here, we show that SinR is not required for the negative effect of σ^K on *sigE* expression. The negative effect of early σ^K production appears to involve Spo0A, but the negative effect of σ^K late in sporulation was also observed for the Spo0A-independent *ald* gene. Since *sigE* is known to be transcribed by σ^A RNAP, and *ald* is believed to be, we tested the idea that σ^K inhibits σ^A RNAP activity late in sporulation by competing with σ^A for binding to core RNAP. We found instead that σ^K must be transcriptionally active to exert its negative effect late in development. These results give further insight into why σ^K activity is temporally regulated and how σ^K switches the mother cell pattern of gene expression.

MATERIALS AND METHODS

Bacterial strains. The *B. subtilis* strains used in this study are listed in Table 1. BZ48 was constructed by replacing the chloramphenicol resistance gene (*cat*) of VO48 with a spectinomycin resistance gene (*spc*) as described previously (60). To introduce gene fusions and mutations into the wild-type strain PY79 and its derivatives BK556 and BZ48, chromosomal DNA was prepared from a strain containing the desired fusion or mutation and used to transform competent cells of the recipient strain (19). Specialized transduction was used to move *lacZ* fusions carried on SPβ phages into various strains (19). Transformants or transductants were selected on LB plates containing appropriate antibiotics. Chloramphenicol was used at 5 μg/ml, and spectinomycin was used at 100 μg/ml. Resistance to macrolide-lincosamide-streptogramin B (MLS) was selected by using a combination of erythromycin (1 μg/ml) and lincomycin (25 μg/ml). Colonies of cells containing the *sinR* null mutation displayed a characteristic rough phenotype (12). The *rvtA11* mutation in AG919 is 80 to 90% linked by cotransformation to a downstream chloramphenicol resistance gene marker (14). To verify the presence of the *rvtA11* mutation in a chloramphenicol-resistant transformant, chromosomal DNA was used to transform competent AG1431 cells. DNA from isolates containing the *rvtA11* mutation rescued the Spo$^-$ and Pig$^-$ AG1431 cells to Spo$^+$ and Pig$^+$ at a frequency of 80 to 90%. A derivative of PY79 containing *ald*::Tn*917lac* sporulated poorly in DS medium, consistent with a previous report (59). However, the sporulation efficiency of this strain was comparable to that of the wild-type strain in SM resuspension medium (data not shown). Apparently, the *ald* locus is dispensable for sporulation in SM medium.

Construction of a strain that makes transcriptionally inactive σ^K during sporulation. A 1.4-kb *Pst*I-*Hin*dIII fragment containing the *sigK* gene (44) was cloned into phage M13mp19. A single base pair mutation was made in *sigK* by site-directed mutagenesis (37). The mutation resulted in a cysteine-to-arginine change at position 109 (C109R) in the region of σ^K thought to interact with promoter −10 regions. The sequence of the oligonucleotide used to make the C109R mutation was 5′-CAGCGAGGCGTATTGAA-3′. The entire *sigK* gene was sequenced to confirm the desired mutation and to ensure that no other mutations had been introduced. A 1.2-kb *Sac*I-*Hin*dIII DNA fragment containing the mutation was used to replace the corresponding fragment in pSL1 (44) to generate pBZ1, in which the mutated *sigK* gene was fused to the P_{spac} promoter. A 1.5-kb *Eco*RI-*Hin*dIII fragment from pBZ1 was then cloned into the integrational vector pUS19 (3). The resulting plasmid was transformed into BK410, where it integrated via homologous recombination. Recombination upstream of the mutation in *sigK* resulted in a copy of the mutated gene fused to the *spoIVCB* promoter followed by a wild-type copy of *spoIVCB* fused to P_{spac}. *spoIVCB* encodes the N-terminal part of σ^K and is joined to *spoIIIC*, encoding the C-terminal part of σ^K, by a DNA rearrangement that forms the composite *sigK* gene during *B. subtilis* sporulation (61). Since the *spoIIIC94* mutation in BK410 is a deletion of *spoIIIC* (35), the wild-type copy of *spoIVCB* cannot recombine with *spoIIIC* during sporulation and no wild-type σ^K is made. Therefore, transformants in which recombination occurred upstream of the mutation were expected to produce only mutant σ^K and exhibit a Spo$^-$ phenotype. Spo$^-$ transformants were further screened by Western blot analysis (73), and one isolate (BZ410) that produced mutant σ^K during development at a level similar to that observed in wild-type cells was used further. Mutations and *lacZ* fusions were introduced into BZ410 as described above.

Cell growth and sporulation. LB medium (19) was used for growth of *Escherichia coli* and *B. subtilis*. Sporulation was induced by growing cells in the absence of antibiotic and resuspending cells in SM medium as described previously (19). The onset of sporulation (T_0) is defined as the time of resuspension. The sporulation efficiency was measured as described previously (19).

Analysis of *lacZ* fusion expression. Strains containing a *lacZ* fusion were constructed by transformation or transduction as described above. In each case, at least 10 isolates were screened by placing each isolate on DSM agar (19) containing 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (20 μg/ml). This qualitative assay was used to eliminate occasional isolates with abnormally high or low β-galactosidase activity. Two or more isolates that displayed average blue colony color were induced to sporulate by the resuspension method, and samples collected at hourly intervals were subjected to quantitative β-galactosidase assays, using toluene to permeabilize cells and *o*-nitrophenol-β-D-galactopyranoside as the substrate (19). One unit of enzyme hydrolyzes 1 μmol of substrate per min per A_{600} of initial cell density. We found that the maximum β-galactosidase specific activity of a given isolate often varied between experiments, but that the relative maximum β-galactosidase activity of different strains in the same experiment was reproducible. Therefore, strains to be compared were induced to sporulate in parallel, and β-galactosidase specific activities were normalized within each experiment. Minimally, two isolates of each strain were induced to sporulate in each of two separate experiments. The normalized data from separate experiments was averaged to obtain the points shown in the figures. Statistical analysis of the data was performed using the program GraphPad InStat (GraphPad Software).

RESULTS

SinR is not required for the negative effect of σ^K on *sigE* expression. Using a transcriptional fusion between the *spoIIG* promoter and *lacZ* (29), which we referred to as *sigE-lacZ* since it provided an indirect measure of *sigE* transcription, we showed previously that in *spoIVCB23* (*spoIVCB* encodes the N-terminal part of σ^K) mutant cells that fail to produce σ^K, *sigE-lacZ* was overexpressed late in development (73). In *spoIVCBΔ19* cells that make active σ^K 1 h earlier than normal due to a deletion in the prosequence of pro-σ^K, *sigE-lacZ* expression was reduced (73). To further explore the mechanism by which σ^K inhibits expression of *sigE*, we introduced a *sinR* null mutation into wild-type cells and mutants with altered σ^K production. SinR is a transcription factor that inhibits the transcription of some early sporulation genes, including *sigE* (46, 47). In cells containing only the *sinR* mutation, *sigE-lacZ* expression increased and decreased with similar timing as in wild-type cells but reached a twofold-higher maximum level (130 U versus 70 U) (73), consistent with the finding reported previously that SinR inhibits *sigE* expression (46, 47). In *sinR spoIVCB23* mutant cells that fail to make σ^K, *sigE-lacZ* expression late in development was higher than in cells containing only the *sinR* mutation (Fig. 1). Statistical analysis of the data (i.e., a nonparametric test of the two-sided *P* value) indicated that at T_4 to T_7, *sigE-lacZ* expression was significantly higher in the strain that fails to make σ^K. In *sinR spoIVCBΔ19* cells that make σ^K earlier than normal, the level of *sigE-lacZ* expression

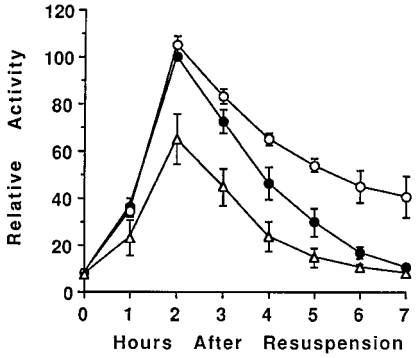

FIG. 1. Effect of a *sinR* mutation on *sigE-lacZ* expression. The *sinR* null mutation in IS*432* was transformed into wild-type cells (PY79 [●]), *sigK* (*spoIVCB23*) mutant cells (BK556 [○]), and *spoIVCBΔ19* cells that produce σ^K earlier than normal (BZ48 [△]). The resulting strains were lysogenized with phage SPβ::*sigE-lacZ*, and expression of *lacZ* during development was analyzed as described in Materials and Methods. In each of two separate experiments, the β-galactosidase specific activities were normalized to the average maximum specific activity in two isolates containing the *sinR* mutation in the wild-type background (typically 130 U). Points on the graph are averages of the normalized values (four determinations), and error bars show 1 standard deviation of the data.

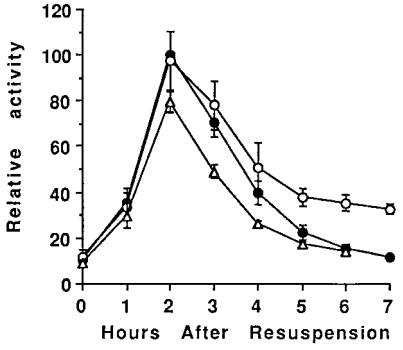

FIG. 2. Effect of bypassing the phosphorelay on *sigE-lacZ* expression. The *rvtA11* mutation was introduced into wild-type cells (PY79 [●]), *sigK* (*spoIVCB23*) mutant cells (BK556 [○]), and *spoIVCBΔ19* cells that produce σ^K earlier than normal (BZ48 [△]). The resulting strains were lysogenized with phage SPβ::*sigE-lacZ*, and expression of *lacZ* during development was analyzed as described in Materials and Methods. In each of two separate experiments, the β-galactosidase specific activities were normalized to the average maximum specific activity in two or three isolates containing the *rvtA11* mutation in the wild-type background (typically 70 U). Points on the graph are averages of the normalized values (five determinations), and error bars show 1 standard deviation of the data.

at T_2 to T_6 was significantly lower than that in *sinR* mutant cells (Fig. 1). Since the *sinR* null mutation did not change the effect of the *spoIVCB23* or *spoIVCBΔ19* mutation on *sigE-lacZ* expression, we conclude that σ^K does not affect *sigE* expression by increasing the level or activity of SinR.

The negative effect of early σ^K production depends on Spo0A, but the negative effect of σ^K late in sporulation involves another mechanism. A second mutation that we tested for an effect on the σ^K-dependent inhibition of *sigE* expression was a missense mutation in *spo0A* called *rvtA11* (58). At the onset of sporulation, multiple signals activate a multicomponent phosphorelay system to phosphorylate Spo0A (5, 13, 22, 53). Spo0A~P then activates transcription of *sigE* and other early sporulation genes (2, 4). The *rvtA11* mutation bypasses the need for the phosphorelay and renders Spo0A able to be phosphorylated by an alternate kinase (39). If σ^K inhibits *sigE* transcription by affecting a component of the phosphorelay so as to lower the level of Spo0A~P, then the *rvtA11* mutation might bypass this effect and relieve the inhibition of *sigE* transcription by σ^K.

We introduced the *rvtA11* mutation into wild-type cells and into *spoIVCB23* and *spoIVCBΔ19* mutant cells. The *sigE-lacZ* transcriptional fusion, carried on phage SPβ, was then integrated into the chromosomes of these strains, and developmental β-galactosidase activity was measured. Figure 2 shows that in general, the pattern of *sigE-lacZ* expression was the same in these strains as in the parental strains without the *rvtA11* mutation (73). In cells containing only the *rvtA11* mutation, *sigE-lacZ* expression increased and decreased with similar timing, and reached a similar maximum level, as in wild-type cells (73). In *rvtA11 spoIVCB23* mutant cells that fail to make σ^K, *sigE-lacZ* expression late in development was higher than in cells containing only the *rvtA11* mutation (Fig. 2). The only difference was that the *rvtA11* mutation diminished the negative effect of early σ^K production in *spoIVCBΔ19* cells. As shown in Fig. 2, *sigE-lacZ* expression in *rvtA11 spoIVCBΔ19* cells reached, on average (five determinations), 80% of the maximum level observed (at T_2) in cells containing only the *rvtA11* mutation. Statistical analysis of the data collected for these two strains at T_2 of sporulation yielded a two-sided *P* value of 0.095 in a nonparametric test, which is considered not quite a significant difference. In contrast, *sigE-lacZ* expression in *spoIVCBΔ19* cells without the *rvtA11* mutation reached only 55% of the maximum level observed in wild-type cells (73), a difference that is significant ($P = 0.016$) when the same statistical test is applied. Likewise, *sigE-lacZ* expression in *sinR spoIVCBΔ19* cells reached only 65% of the maximum observed in cells containing only the *sinR* mutation (Fig. 1), which is a significant difference ($P = 0.029$). While the *rvtA11* mutation clearly diminished the negative effect of early σ^K production on *sigE-lacZ* expression, *rvtA11 spoIVCBΔ19* cells did exhibit significantly ($P \leq 0.05$) less *sigE*-directed β-galactosidase activity at T_3 and T_4 than cells containing only the *rvtA11* mutation (Fig. 2). Taken together, these results suggest that early σ^K production inhibits *sigE-lacZ* expression, in part, by reducing Spo0A~P formation by the phosphorelay, because bypassing the phosphorelay with the *rvtA11* mutation partially restored *sigE-lacZ* expression in *spoIVCBΔ19* cells; however, early σ^K production also inhibits *sigE* expression by another mechanism that is not bypassed by the *rvtA11* mutation, because *sigE-lacZ* expression in *rvtA11 spoIVCBΔ19* cells was not completely restored to the level observed in cells containing only the *rvtA11* mutation.

If early σ^K production reduces Spo0A~P formation, then expression of other genes that depend on Spo0A~P for activation may be reduced in *spoIVCBΔ19* cells. Therefore, we examined expression of transcriptional *lacZ* fusions to the *spoIIE* and *spoIIA* promoters. The *spoIIE* promoter, like the *spoIIG* promoter (which drives *sigE* expression), is recognized by σ^A RNAP (1, 69), and the *spoIIA* promoter is recognized by σ^H RNAP (1, 66). All three promoters are activated by Spo0A~P (2, 4, 5, 67, 69). Expression of the *spoIIE-lacZ* and *spoIIA-lacZ* fusions in *spoIVCBΔ19* cells reached, on average (two or three determinations), 79 and 65%, respectively, of the maximum level observed in wild-type cells (data not shown), consistent with the idea that early σ^K production reduces the Spo0A~P level early in sporulation.

The incomplete restoration of *sigE-lacZ* expression in *rvtA11 spoIVCBΔ19* cells (Fig. 2) indicated that early σ^K production also inhibits *sigE* expression by another mechanism that is not bypassed by the *rvtA11* mutation. To determine whether this is due to a general effect on transcription of genes induced early

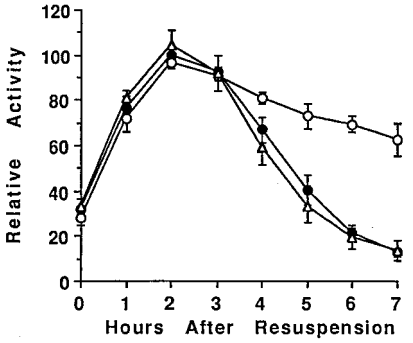

FIG. 3. Effect of altered σ^K production on *ald-lacZ* expression. Wild-type cells (PY79 [●]), *sigK* (*spoIVCB23*) mutant cells (BK556 [○]), and *spoIVCBΔ19* cells that produce σ^K earlier than normal (VO48 [△]) were transformed with DNA from KI220 to introduce *ald*::Tn*917lac*. Expression of *ald-lacZ* was analyzed as described in Materials and Methods. In each of two separate experiments, the β-galactosidase specific activities were normalized to the average maximum specific activity in two isolates containing *ald-lacZ* in the wild-type background (typically 300 U). Points on the graph are averages of the normalized values (four determinations), and error bars show 1 standard deviation of the data.

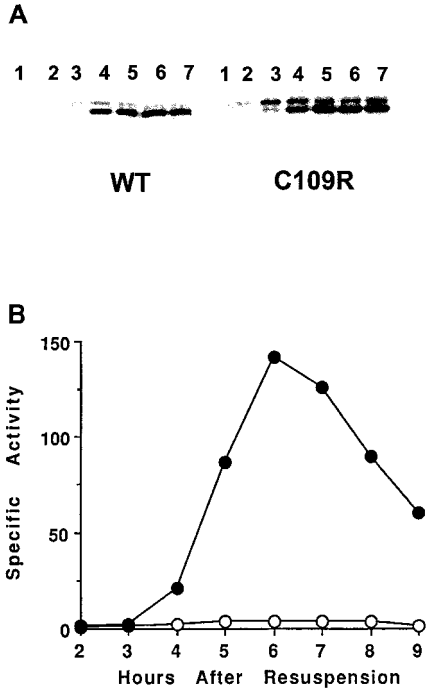

FIG. 4. Production of transcriptionally inactive σ^K during sporulation. Wild-type PY79 cells (WT) and BZ410 cells engineered to produce transcriptionally inactive σ^K (C109R) were lysogenized with phage SPβ::*sigE-lacZ* and induced to sporulate in SM medium. Samples were collected at the indicated hours after the onset of sporulation. (A) Whole-cell extracts (5 μg) were subjected to Western blot analysis using anti-pro-σ^K antibodies as described previously (73). (B) β-Galactosidase specific activity from *gerE-lacZ* in wild-type (●) and *sigKC109R* mutant (○) cells.

in sporulation, we examined expression of a Spo0A-independent gene in mutants with altered σ^K production. The *ald* gene (encoding alanine dehydrogenase) is induced at the onset of sporulation by an unknown mechanism that does not depend on Spo0A (59). Figure 3 shows that expression of an *ald-lacZ* transcriptional fusion was unaffected by the *spoIVCBΔ19* mutation. Thus, early σ^K production does not inhibit expression of all genes induced early in sporulation. On the other hand, *ald-lacZ*, like *sigE-lacZ* (73) (Fig. 1 and 2), was overexpressed late in development of *spoIVCB23* cells that fail to make σ^K (Fig. 3). Taken together, these results suggest that the negative effect of early σ^K production is exerted, at least in part, through Spo0A, but the negative effect of σ^K produced at the normal time in sporulation involves another mechanism.

Transcriptionally active σ^K RNAP is required for the negative effect of σ^K late in sporulation, but not for the negative effect of early σ^K production. One means by which σ^K might inhibit expression of early genes is by competing with other sigma factors for binding to core RNAP. Alternatively, inhibition might require that σ^K not only bind to core RNAP but also direct transcription. To distinguish between these possibilities, we mutated the *sigK* gene to produce a single amino acid substitution in σ^K that was predicted to abolish transcriptional activity but not core RNAP binding ability. The mutation in σ^K was C109R in subregion 2.4, which is thought to be involved in interaction with the −10 region of cognate promoters (20, 42). A similar mutation in σ^E (C117R) did not prevent binding of the sigma to core RNAP or binding of the holoenzyme to a cognate promoter but did prevent initiation of transcription (25).

We mutated the *sigK* gene and integrated it into the chromosome of a *sigK* mutant, creating BZ410, in which only the *sigKC109R* allele is expressed (see Materials and Methods). Figure 4A shows that in this mutant pro-σ^{KC109R} was processed to σ^{KC109R}, which accumulated abundantly at T_4 and persisted at least until T_7, as did σ^K in wild-type cells. The σ^{KC109R} was transcriptionally inactive because it failed to direct expression of a *gerE-lacZ* fusion (Fig. 4B), and the cells failed to form heat-resistant spores (data not shown). When an extract of cells producing σ^{KC109R} was fractionated by gel filtration chromatography as described previously (72), σ^{KC109R} coeluted with the subunits of core RNAP (data not shown), demonstrating that σ^{KC109R} binds to core RNAP.

Expression of *sigE-lacZ* (Fig. 5A) and *ald-lacZ* (Fig. 5B) was higher late in development of *sigKC109R* cells than wild-type cells. The levels of expression in *sigKC109R* cells making σ^{KC109R} were not significantly different from the levels observed in *spoIVCB23* cells which fail to make σ^K (Fig. 5). These results suggest that σ^K must be transcriptionally active to exert its negative effect on *sigE-lacZ* and *ald-lacZ* expression late in sporulation.

To determine whether σ^K RNAP transcriptional activity is also necessary for the negative effect of early σ^K production on *sigE* expression, we introduced a *bofA* mutation into *sigKC109R* cells. The *bofA* mutation uncouples processing of pro-σ^K from its normal dependence on a signal from the forespore, causing σ^K to be produced about 1 h earlier than normal (8, 23, 56). Figure 6 shows that in cells containing only the *bofA* mutation, the level of *sigE-lacZ* expression at T_2 and T_3 was lower than that in wild-type cells. This effect is similar to that observed previously for *bofB8* or *spoIVCBΔ19* mutations (73), which also cause early σ^K production (8, 16). In *bofA sigKC109R* cells, *sigE-lacZ* expression early in sporulation was indistinguishable from that in cells containing only the *bofA* mutation (Fig. 6). Thus, early production of transcriptionally inactive σ^{KC109R} inhibited *sigE* expression early in sporulation as effectively as early production of wild-type σ^K. On the other hand, the level of *sigE-lacZ* expression in *bofA sigKC109R* cells was significantly higher than that in *bofA* or wild-type cells at T_6 to T_8 (Fig. 6), consistent with the idea that transcriptionally active σ^K RNAP is required for the negative effect on *sigE* expression late in development.

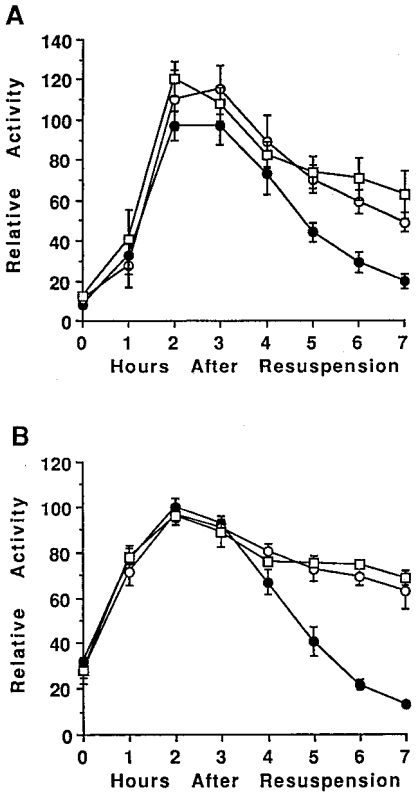

FIG. 5. Effect of making transcriptionally inactive σ^K during sporulation on *sigE-lacZ* and *ald-lacZ* expression. Wild-type cells (PY79 [●]), *sigK* (*spoIVCB23*) mutant cells (BK556 [○]), and *sigKC109R* cells that produce transcriptionally inactive σ^K (BZ410 [□]) were lysogenized with phage SPβ::*sigE-lacZ* (A) or transformed with DNA from KI220 to introduce *ald*::Tn*917lac* (B). Expression of *lacZ* during development was analyzed as described in Materials and Methods. In each of two separate experiments, the β-galactosidase specific activities were normalized to the average maximum specific activity in two isolates containing the *lacZ* fusion in the wild-type background (typically 70 U for *sigE-lacZ* and 300 U for *ald-lacZ*). Points on each graph are averages of the normalized values (four determinations), and error bars show 1 standard deviation of the data.

DISCUSSION

We have found that the appearance of σ^K during *B. subtilis* sporulation can negatively regulate expression of the *sigE* gene by two different mechanisms, depending on whether σ^K is produced at the normal time or 1 h earlier (Fig. 7). The mechanism operative when σ^K is produced earlier than normal does not require σ^K to be transcriptionally active (Fig. 6) and appears to affect Spo0A~P, as evidenced by the partial restoration of *sigE-lacZ* expression in *rvtA11 spoIVCBΔ19* cells (Fig. 2) and the reduced expression in *spoIVCBΔ19* cells of both *sigE* (73) and other Spo0A-dependent genes (*spoIIA* and *spoIIE* [data not shown]) but not a Spo0A-independent gene (*ald* [Fig. 3]). As depicted in Fig. 7, a second mechanism, observed when σ^K is produced at the normal time, inhibits *sigE* expression late in development. This mechanism requires σ^K RNAP transcriptional activity (Fig. 5 and 6) and inhibits expression of the Spo0A-independent *ald* gene (Fig. 3 and 5B). Since *ald* is believed to be transcribed by σ^A RNAP, we speculate that transcription of one or more genes by σ^K RNAP creates a feedback loop that normally lowers transcription of early genes by σ^A RNAP (Fig. 7). The resulting inhibition of *sigE* expression, together with turnover of σ^E, would help switch the mother cell from σ^E-directed transcription to the σ^K-directed pattern.

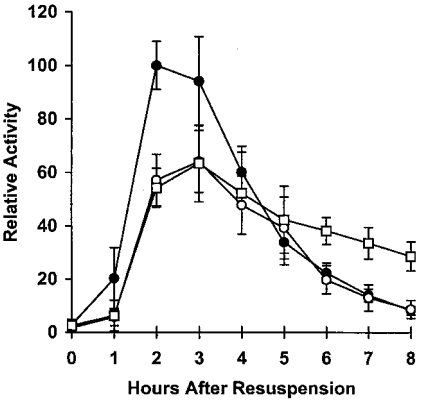

FIG. 6. Effect on *sigE-lacZ* expression of making transcriptionally inactive σ^K earlier than normal during sporulation. The *bofA*::*cat* mutation in BSL50 was transformed into wild-type PY79 cells and BZ410 cells engineered to produce transcriptionally inactive σ^K, resulting in PS1 (○) and PS2 (□), respectively. These strains, and wild-type PY79 (●), were lysogenized with phage SPβ::*sigE-lacZ*, and expression of *lacZ* during development was analyzed as described in Materials and Methods. In each of two separate experiments, the β-galactosidase specific activities were normalized to the average maximum specific activity in two isolates containing *sigE-lacZ* in the wild-type background (typically 70 U). Points on the graph are averages of the normalized values (four determinations), and error bars show 1 standard deviation of the data.

Previous work has shown that it is important not to make σ^K too early during sporulation (8). Proteolytic processing of pro-σ^K to σ^K in the mother cell is governed by a signal transduction pathway that emanates from the forespore (7, 8, 33, 44). Bypassing this step by deleting the prosequence (i.e., the *spoIVCBΔ19* mutation) or mutating components of the pathway (i.e., the *bof* mutations) causes a 10-fold decrease in sporulation efficiency, and the spores that are produced germinate poorly (8). Our results provide a plausible explanation for these defects. σ^K produced earlier than normal inhibits expression of Spo0A-dependent sporulation genes, including *sigE*. This lowers the level of σ^E (73), and SpoIIID (16) produced. SpoIIID is a transcription factor that activates or represses many genes in the σ^E and σ^K regulons (17, 71). The cumulative effects of aberrant early gene regulation presumably cause the observed sporulation and germination defects.

How might early production of σ^K inhibit the expression of Spo0A-dependent genes? The finding that early production of transcriptionally inactive σ^{KC109R} has the same effect (Fig. 6) suggests that competition of σ factors for binding to a limiting amount of core RNAP may be responsible. Evidence for such competition between σ^A and σ^H at the onset of sporulation has been presented previously (22). Recently, Ju et al. (27) have

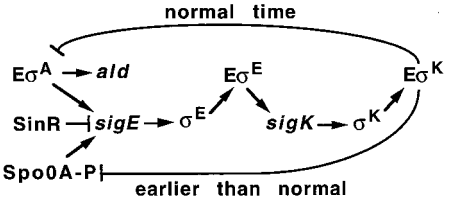

FIG. 7. Model showing the mother cell sigma factor cascade and two different mechanisms by which σ^K can negatively regulate *sigE* expression. σ^K produced earlier than normal may compete with other sigma factors for binding to core RNAP (E) and inhibit formation of Spo0A~P, an activator of *sigE* transcription. Transcriptional activity of $E\sigma^K$ produced at the normal time during sporulation may inhibit $E\sigma^A$ activity, reducing transcription of *sigE*, *ald*, and other early genes late in development.

used velocity centrifugation and Western blot analysis to monitor the association of σ^A, σ^E, and σ^K with core RNAP during sporulation. Their results suggest that σ^E partially displaces σ^A from core and that σ^K further displaces σ^A and also displaces σ^E. Transcriptionally inactive σ^{EC117R} and σ^{KC109R} were as effective as their wild-type counterparts at displacing other σ factors from core RNAP. These results suggest that later-acting σ factors in the mother cell cascade have successively higher affinity for core RNAP. If this is the case, then early σ^K production would prematurely displace σ^A, σ^H, and σ^E from core RNAP. The resulting changes in the pattern of mother cell gene expression could reduce Spo0A-dependent gene expression in more than one way. For example, the complex phosphorelay system that governs the level of Spo0A~P provides many potential regulatory targets (5, 13, 22, 53, 65). Reduced expression of a phosphorelay component that leads to the formation of Spo0A~P may explain the portion of reduced *sigE-lacZ* expression in *spoIVCBΔ19* cells (73) that was restored by the *rvtA11* mutation which bypasses the phosphorelay (Fig. 2). Reduced expression of the *spo0A* gene, which is transcribed by σ^A RNAP (6) and σ^H RNAP (55, 63) from different promoters, might account for the reduced *sigE-lacZ* expression that could not be restored by the *rvtA11* mutation (Fig. 2). Alternatively, early σ^K production may act more directly to inhibit *sigE* expression, displacing enough σ^A from core RNAP to reduce transcription of *sigE* by σ^A RNAP.

Early σ^K production did not lower *ald* expression (Fig. 3). If *ald* is transcribed by σ^A RNAP, as has been proposed (59), then apparently the σ^K produced earlier than normal in *spoIVCBΔ19* cells does not displace enough σ^A from core RNAP to inhibit *ald* transcription. It has been postulated that an unidentified regulatory factor is involved in *ald* induction early in sporulation (59). Perhaps this putative factor can stimulate *ald* transcription even when the σ^A RNAP level is low.

Pro-σ^K does not inhibit expression of Spo0A-dependent genes, even if it is produced earlier than normal (45), because it does not associate with core RNAP (72). The prosequence targets pro-σ^K to membranes (72) and prevents premature σ competition in the mother cell.

Sigma competition does not seem to account for the negative effect of σ^K produced at the normal time during sporulation, because σ^{KC109R} was completely ineffective at inhibiting *sigE-lacZ* (Fig. 5A and 6) or *ald-lacZ* (Fig. 5B) expression late in development. How, then, does σ^K RNAP transcriptional activity inhibit expression of early genes late in development? We considered the possibility that expression of genes in the σ^K regulon causes morphological changes that make some of the products of early gene expression inaccessible, due to sequestering in the forespore. In our previous study (73) and in the experiments shown here, we used toluene to permeabilize developing cells to a substrate for β-galactosidase. Lysozyme treatment is much more effective than toluene for the detection of β-galactosidase activity in the forespore (7), a finding that we verified in assays using cells containing *sspB-lacZ* (data not shown), which is expressed specifically in the forespore (49). However, there was no significant difference between the two methods for detection of *sigE-lacZ* expression during sporulation (data not shown), indicating that β-galactosidase produced from this fusion is predominantly in the larger mother cell compartment. Perhaps β-galactosidase partitioned to the forespore is degraded, as appears to be the case for σ^E produced in the forespore (26, 40, 54). Thus, it is very unlikely that σ^K-dependent sequestering of early gene products in the forespore accounts for the lower level of *sigE*-directed β-galactosidase activity (73) (Fig. 1 and 2), *ald*-directed β-galactosidase activity (Fig. 3), or σ^E protein (73) observed late in development of wild-type cells than *spoIVCB23* mutant cells that fail to make σ^K, nor do we think that σ^K RNAP activity leads to increased turnover of σ^E and β-galactosidase in the mother cell. The *spoIVCB23* mutation did not affect turnover of pro-σ^E or σ^E at T_3 to T_5 in a pulse-chase experiment (73). Also, this mutation did not alter the decay of β-galactosidase activity after T_1 in cells containing *lacZ* fused to the *spo0A*, *spo0H*, or *spo0K* promoter (data not shown). Unlike these promoters, which probably are no longer transcribed after T_1, the *spoIIA*, *spoIIE*, and *spoVG42* promoters continue to be transcribed, judging from the continued increase of β-galactosidase activity after T_1 in cells containing *lacZ* fusions to these promoters. Interestingly, β-galactosidase activity from these fusions is reproducibly higher at T_5 to T_7 in *spoIVCB23* cells than in wild-type cells (data not shown), although the difference is smaller than observed for *sigE-lacZ* (73) or *ald-lacZ* (Fig. 3). It is possible that transcription by σ^K RNAP drains mother cell nucleotide pools late in development, inhibiting expression of all early genes. Alternatively, the product(s) of a specific gene(s) under σ^K control may be involved in the feedback loop. In either case, it appears that the feedback loop inhibits expression of many early genes, including ones transcribed by σ^A RNAP (*sigE*, *ald*, and *spoIIE*) and ones transcribed by σ^H RNAP (*spoIIA* and *spoVG42*).

We do not know how important the feedback loop created by σ^K RNAP transcriptional activity is for sporulation. The lowering of the σ^E level (73) is expected to reduce transcription of all genes for which σ^E RNAP is the limiting factor. In addition, σ^K RNAP activity may inhibit σ^E RNAP activity by draining nucleotide pools or by a more specific mechanism, as proposed above. At least one key gene in the σ^E regulon, *spoIIID*, is overexpressed in mutants that fail to make σ^K (34, 73). We have proposed previously that the feedback loop ensures the timely disappearance of SpoIIID from the mother cell, relieving SpoIIID repression of several *cot* genes that encode spore coat proteins (16, 17, 73). Both σ^E RNAP and σ^K RNAP transcribe genes whose products are involved in formation of the spore cortex and coat (62). In order for these structures to form properly, it may be critical to regulate the transition from σ^E- to σ^K-directed transcription.

Negative feedback loops control the transcription of genes involved in flagellar biosynthesis in *E. coli* (32) and *Caulobacter crescentus* (51, 68). In some cases, negative control turns off expression of genes encoding structural proteins once those proteins assemble properly. Determination of whether the feedback loop initiated by σ^K RNAP activity monitors morphogenesis or whether it simply acts as a timer governing the switch from early to late gene expression must await further elucidation of the molecular mechanism.

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