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Sigma Factor Displacement from RNA Polymerase during *Bacillus subtilis* Sporulation

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As *Bacillus subtilis* proceeds through sporulation, the principal vegetative cell σ subunit (σ^A) persists in the cell but is replaced in the extractable RNA polymerase (RNAP) by sporulation-specific σ factors. To explore how this holoenzyme changeover might occur, velocity centrifugation techniques were used in conjunction with Western blot analyses to monitor the associations of RNAP with σ^A and two mother cell σ factors, σ^E and σ^K, which successively replace σ^A on RNAP. Although the relative abundance of σ^A with respect to RNAP remained virtually unchanged during sporulation, the percentage of the detectable σ^A which cosedimented with RNAP fell from approximately 50% at the onset of sporulation (T_0) to 2 to 8% by 3 h into the process (T_3). In a strain that failed to synthesize σ^E, the first of the mother cell-specific σ factors, approximately 40% of the σ^A remained associated with RNAP at T_3. The level of σ^A-RNAP cosedimentation dropped to less than 10% in a strain which synthesized a σ^E variant (σ^{ECR119}) that could bind to RNAP but was unable to direct σ^E-dependent transcription. The E-σ^E-to-E-σ^K changeover was characterized by both the displacement of σ^E from RNAP and the disappearance of σ^E from the cell. Analyses of extracts from wild-type and mutant *B. subtilis* showed that the σ^K protein is required for the displacement of σ^E from RNAP and also confirmed that σ^K is needed for the loss of the σ^E protein. The results indicate that the successive appearance of mother cell σ factors, but not necessarily their activities, is an important element in the displacement of preexisting σ factors from RNAP. It suggests that competition for RNAP by consecutive sporulation σ factors may be an important feature of the holoenzyme changeovers that occur during sporulation.

The pivotal event establishing sporulation-specific gene expression in *Bacillus subtilis* is the reprogramming of the bacterium's RNA polymerase (RNAP) (52). This occurs when the principal promoter recognition subunit (σ^A) of the vegetative cell RNAP is replaced by analogous sporulation-specific subunits (σ^E, σ^F, σ^G, and σ^K) (18, 52). These alternative σ factors control both the timing and localization of spore gene expression.

Early in sporulation, *B. subtilis* partitions itself into two unequal compartments with unique developmental fates. The smaller, forespore compartment is eventually engulfed by the larger, mother cell compartment, which nurtures the forespore as it develops into a mature endospore. Each of the sporulation-specific σ factors is active in only one of these two compartments. Mother cell gene expression is controlled by the sequential appearance of σ^E followed by σ^K, while forespore-specific genes are activated first by σ^F and then by σ^G (7, 8, 10, 18, 20, 30, 37, 41, 42, 44, 52, 60). The genes encoding each of the early sporulation σ factors (σ^E and σ^F) are expressed at the onset of sporulation (31, 36, 56); however, neither of these factors is active until later in development, when the two separate compartments are formed. σ^E and σ^F are each kept silent by a unique means. σ^F is bound to an inactivating anti-σ^F protein (SpoIIAB), while σ^E is formed as an inactive proprotein, pro-σ^E (9, 13, 36, 43, 51). Pro-σ^E becomes active only after 27 amino acids are cleaved from its amino terminus (36). The septation event initiates a process that leads to the release of σ^F from its antagonist in the forespore (1, 3, 12). σ^F then directs the transcription of a gene, *spoIIR*, whose product in turn triggers pro-σ^E processing in the mother cell (23, 29, 39). Once active in their particular compartments, σ^E and σ^F induce expression of the genes for the sigma factors which will ultimately replace them (σ^K and σ^G, respectively) (14, 34, 35, 44, 53). σ^K and σ^G, like their predecessors, are initially inactive. σ^K is formed as a proprotein which is processed and activated in response to a signal from the developing forespore (7, 8, 41). σ^G's activity is restricted by SpoIIAB, the same anti-σ protein that inhibited σ^F (16, 30). Turnover of SpoIIAB in the forespore is thought to be an important factor in activating σ^G (30, 33).

Although much is known about the mechanisms by which the sporulation-specific σ factors are expressed and activated, the process by which they replace σ^A on RNAP is still unclear. σ^A persists in sporulating *B. subtilis* (54), and yet by 2 to 3 h after the onset of sporulation, the RNAP extracted from these bacteria is virtually devoid of σ^A (19, 38). Early studies of σ^A's displacement from RNAP revealed that treatment of a sporulating *B. subtilis* culture with the protein synthesis inhibitor chloramphenicol allowed σ^A to again become extractable as an RNAP subunit (47). This result was interpreted as evidence for the existence of a short-lived protein inhibitor of σ^A that appears in sporulating *B. subtilis* to block σ^A's binding to RNAP.

Anti-σ factor proteins are now known to restrict the ability of several *B. subtilis* factors to form RNAP holoenzymes. In addition to σ^F and σ^G (9, 13, 16, 30, 33), the general stress response (σ^B) and motility (σ^D) σ factors of *B. subtilis* are controlled by inhibitory binding proteins (4, 6, 11). It is plausible that a similar protein could be involved in restricting σ^A's access to RNAP in sporulating *B. subtilis*. Alternatively, the presence of the sporulation-specific σ factors themselves could

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TABLE 1. *B. subtilis* strains and plasmids used in this study

Strain or plasmid	Relevant genotype or features	Source, construction, or reference
Strains		
SMY	*trpC*	Laboratory strain
BK410	*spoIIIC94*	35
EUR9030	*spoIIAC*::*erm*	30
M01027	*spoIVCB*::*erm*	P. Stragier
AG514	Δ*spo0A*::*cat*	A. Grossman
SF9030	*spoIIAC*::*erm*	EUR9030→SMY
SK1027	*spoIVCB*::*erm*	701027→SMY
SOA514	Δ*spo0A*::*cat*	AG514→SMY
BK410-1	*sigKB109CR*	pUS109CR→BK140
S410	*sigK109CR*	BK410-1→SMY
SE117CR	*sigE117CR*	pUS117CR→SMY
SE500	*spoIIGA*::pGEM3C500	pGEM3C500→SMY
SEF500	*spoIIAC*::*erm* *spoIIGA*::pGEM3C500	EUR9030→SE500
Plasmids		
pUS19	Ap^r Spc^r	4
pGEM3Zf(+)*cat*	Ap^r Cm^r	58
pGEM3C500	Ap^r Cm^r 500-bp *spoIIGA* DNA fragment in pGEM3Zf(+)*cat*	This study
pBZ1	Ap^r Km^r P_{spac}::*sigK109CR*	L. Kroos
pUS109CR	Ap^r Spc^r P_{spac}::*sigK109CR*	This study
pJ89CR117	Km^r *sigE117CR*	27
pUS117CR	Ap^r Spc^r *sigE117CR*	This study

be the basis for σ^A's exclusion from RNAP. If the sporulation σ factors are effective competitors for a limited pool of core RNAP, their mere presence might be sufficient to deny σ^A access.

Using velocity sedimentation techniques to separate RNAP from unassociated σ factors and Western blot analyses to locate RNAP, σ^A, σ^E, and σ^K in these gradients, we revisited the problem of σ^A release during sporulation. The pattern of σ factor association with RNAP in wild-type and mutant *B. subtilis* was found to be consistent with a model in which competition for RNAP by sporulation σ factors is necessary for E-σ^A decrease and the changeover from E-σ^E to E-σ^K that occurs later in development. If unknown sporulation factors facilitate this process, they do not appear to be adequate for separating the preexisting σ factors from RNAP in the absence of the σ proteins that will replace them.

MATERIALS AND METHODS

Plasmids and bacterial strain constructions. The *B. subtilis* strains and plasmids used in this study are listed in Table 1. pGEM3C500 is the *Escherichia coli* vector pGEM-3Zf(+)*cat* (58) containing a 500-bp *Pst*I DNA fragment from the interior of the *spoIIGA* coding sequence (25). Transformation of this plasmid into *B. subtilis*, followed by selection for chloramphenicol acetyltransferase, generates transformants (SE500) in which the plasmid has inserted into *spoIIGA* and separated *sigE* (*spoIIGB*) from its promoter element. pBZ1 was obtained from Lee Kroos (Michigan State University). It carries the *sigKCR109* allele cloned downstream of P_{spac}. An *Eco*RI/*Hin*dIII fragment from pBZ1, carrying P_{spac}::*sigKCR109*, was cloned into *Eco*RI- and *Hin*dIII-cut pUS19 (5) to create pUS109CR. *B. subtilis* BK410 (35) has a deletion of the 3′ end of *sigK* (*spoIIIC94*). Transformation of pUS109CR into BK410 and selection for the vector-encoded Spc^r results in single-site recombinants in which the vector has integrated within the 5′ end of *sigK*. This creates a single intact *sigK* gene, controlled by its normal promoter, and an inactive, truncated *sigK* gene downstream of the vector sequence. P_{spac}. Spo^- clones would have the *sigK109CR* mutation included in the intact gene. BK410-1 was one such clone. S410 is SMY transformed with chromosomal DNA from BK410-1 and with selection for the *sigK109CR*-linked Spc^r. pUS117CR is a 1.1-kbp DNA fragment carrying the *sigE117CR* allele cut from pJ89CR117 (27) with *Pst*I and cloned into *Pst*I-cut pUS19. SE117CR is SMY transformed with pUS117CR, with selection for the vector-encoded Spc^r, followed by screening for the Spo^- phenotype of the *sigE117CR* allele. SF9030 is SMY transformed with chromosomal DNA from EUR9030 (30) and selection for the *spoIIAC*-disrupting *erm* cassette. The $SigE^-$ $SigF^-$ strain (SEF500) was generated by transforming SE500 ($SigE^-$) with this same EUR9030 DNA.

Induction of sporulation. *B. subtilis*, grown overnight in Luria broth, was diluted 1/20 into Difco Sporulation medium (DSM) and incubated at 37°C. The onset of sporulation (T_0) was taken as the time that the culture stopped exponential growth.

Velocity gradient analysis. Cells were harvested into equal volumes of crushed ice at various times after the onset of sporulation, washed in 1 M NaCl, concentrated 20-fold, and disrupted in a resuspension buffer (10 mM Tris [pH 8.0], 1 mM EDTA, 50 mM NaCl, 10 mM $MgCl_2$, 0.3 g of phenylmethylsulfonyl fluoride per liter, 3 mM dithiothreitol) by passage (three times) through a French press at 12,000 lb/in^2. Cell debris was removed by a low-speed centrifugation (10,000 rpm, 45 min, SS-34 rotor [Sorvall]). Supernatant samples (0.5 ml) were applied onto 11-ml linear glycerol gradients (15 to 30% glycerol in resuspension buffer) and centrifuged for 22 to 24 h at 37,000 rpm and 4°C in a Sorvall TH641 rotor. After centrifugation, 0.5-ml samples were collected from the bottom of the tube. Two volumes of ethanol were added to this sample. This precipitated 80 to 90% of the protein, without apparent preference for any of the proteins under investigation. The precipitated material was resuspended in sample buffer and analyzed by Western blotting. When Triton was used in the analysis, it was added to both the sample buffer (1.0%) and the glycerol gradient (0.1%).

Western blot analysis. Fractions from the velocity gradient were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis on gels containing 10% acrylamide. Subsequent steps were as done previously (11). The anti-β′ and anti-σ^A mouse monoclonal antibodies were obtained as previously described, using *B. subtilis* core RNAP and σ^A-His_6 as the inoculated antigens (11). The anti-σ^E monoclonal antibody has been described previously (55). A sample of rabbit polyclonal antibody against σ^K (60) was provided by L. Kroos. Bound antibody was detected with alkaline phosphatase-conjugated goat anti-mouse or goat anti-rabbit antibodies, as appropriate. Western blot data were quantitated with an AlphaImager 2000 (Alpha Innoteck Corp., San Leandro, Calif.) and its associated software.

General methods. DNA manipulations were performed by standard protocols. Transformation of naturally competent *B. subtilis* cells was carried out as described by Yasbin et al. (57).

TABLE 2. Relative σ^A abundance

Time	Avg pixel content[a]		σ^A/β'
	β′	σ^A	
T_0	81,830	53,452	0.65
$T_{1.5}$	76,464	48,952	0.64
$T_{3.0}$	77,395	51,982	0.67
$T_{4.5}$	67,968	36,579	0.54

[a] Average pixel contents of the β′ and σ^A portions of Western blots of crude *B. subtilis* extracts from cells harvested at the time indicated. Each number is the average of eight determinations. The average deviation was approximately 10% for samples analyzed on the same membrane.

RESULTS

Patterns of association of σ^A with RNAP. RNAP purified from sporulating *B. subtilis* contains little σ^A; nevertheless, σ^A can be immunoprecipitated from crude extracts of such cells (54). In an attempt to better define the status of σ^A in sporulating *B. subtilis*, we prepared an anti-σ^A monoclonal antibody and used it as a probe to monitor σ^A's abundance and RNAP association in *B. subtilis* extracts.

In an initial experiment, we examined the σ^A levels in sporulating *B. subtilis* and compared this value to the σ^A level which existed previously. Extracts were prepared from *B. subtilis* that had been harvested at the onset of sporulation and at 1.5-h intervals thereafter and analyzed by Western blotting as described in Materials and Methods. As noted by others (40, 54), the ratio of σ^A to the core RNAP β′ subunit was found to be essentially unchanged as the cells proceeded through 4.5 h of sporulation ($T_{4.5}$) (Table 2). Thus, a significant drop in the σ^A/core RNAP ratio is not responsible for σ^A's disappearance from the sporulating cell's extractable RNAP.

Velocity centrifugation techniques can readily separate RNAP holoenzymes (approximately 5×10^5 Da) from free σ subunits (typically 2.5×10^4 to 5×10^4 Da) and had been used

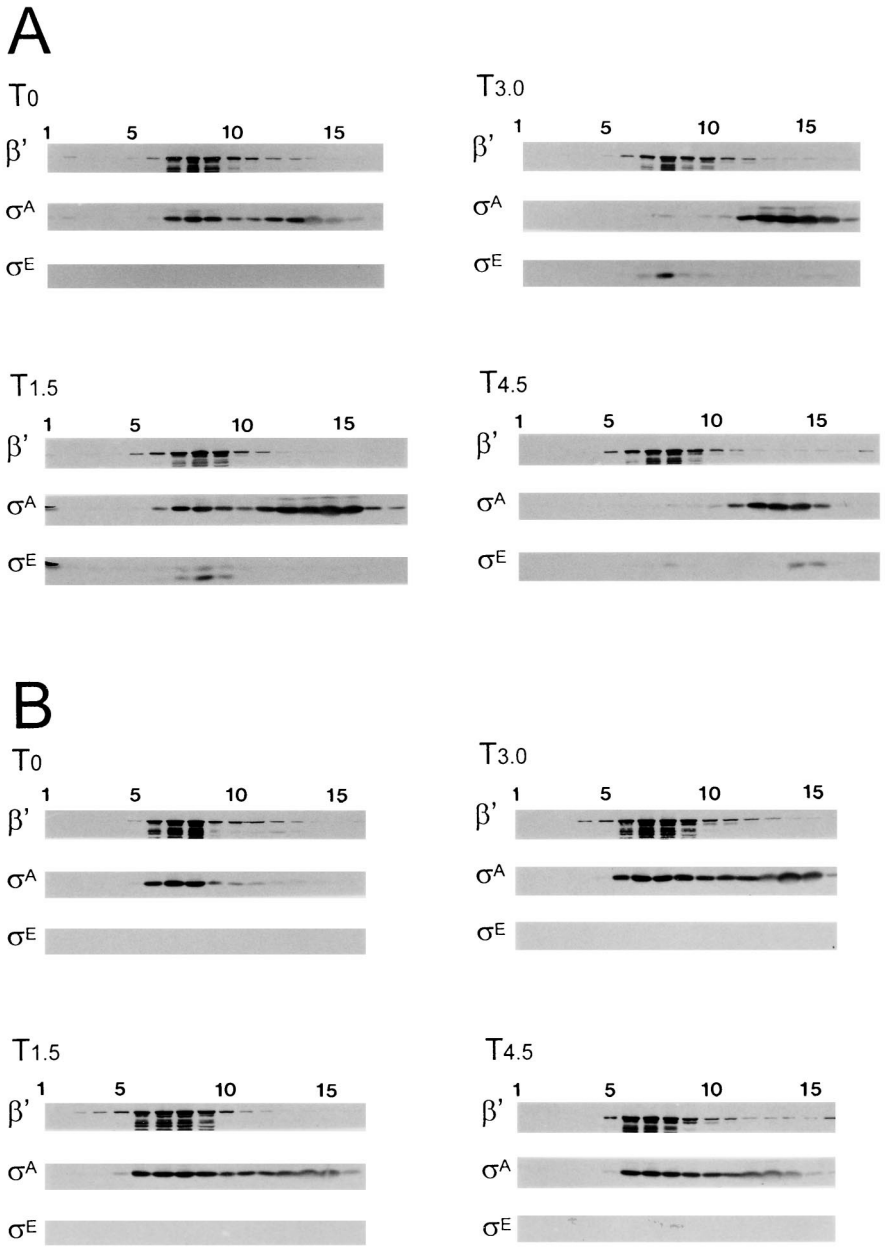

FIG. 1. Fractionation of extracts from sporulating and Spo0A$^-$ *B. subtilis*. Wild-type *B. subtilis* SMY (A) and its congenic Spo0A$^-$ variant S0A514 (B) were grown in DSM. Samples were harvested at the end of exponential growth (T_0) or at 1.5-h intervals thereafter ($T_{1.5}$, T_3, and $T_{4.5}$), the cells were disrupted, and the resulting crude extracts were fractionated by centrifugation through a linear gradient of 15 to 30% glycerol, as described in Materials and Methods. Fractions were collected and analyzed by Western blotting with anti-β′, anti-σ^A, and anti-σ^E antibodies as probes. Fraction 1 represents the bottom of the centrifuge tube. The two bands detected by the anti-σ^E antibody in panel A at $T_{1.5}$ represent σ^E and its slightly larger precursor (pro-σ^E).

by others to monitor the association of mutant σ factors with RNAP (49). We applied this technique, in conjunction with Western blot analyses, to address the question of σ factor-RNAP associations during sporulation. We first asked whether the RNAP-σ factor fractionation profile seen following velocity centrifugation would resemble the sporulation-specific changes in RNAP holoenzyme composition that had been documented previously.

The most obvious change in the RNAP profile of sporulating *B. subtilis* occurs at approximately T_2 to T_3. At this time, E-σ^A becomes rare and E-σ^E, a holoenzyme carrying the first of the mother cell-specific σ factors, becomes evident. The loss of σ^A from RNAP and the synthesis of σ^E do not occur if sporulation is blocked by mutation in the *spo0A* gene (19). In such cells, E-σ^A remains extractable under culture conditions that induce σ^A displacement in wild-type *B. subtilis*. Crude extracts were prepared from wild-type and *spo0A* mutant cells and subjected to centrifugation through 15 to 30% glycerol gradients. The gradients were then fractionated and analyzed by Western blotting with monoclonal antibodies against σ^A, σ^E, and the core RNAP β′ subunit as probes. At the onset of sporulation, approximately half of the σ^A present in the wild-type *B. subtilis* extract cosedimented with the RNAP marker (β′) (Fig. 1A, T_0). By 3 h into the process (Fig. 1A, T_3), most of the σ^A no

TABLE 3. Partitioning of σ^A and σ^E at T_3

Strain	Protein	% in fractions[a]:		
		1–3	4–10	11–16
Wild type	β	0	87.8	12.2
	σ^A	0	2.0	98
	σ^E	0	69.4	30.6
	Pro-σ^E	0	100	0
Spo0A$^-$	β	0	82.7	17.3
	σ^A	0	52.5	47.5
SigE$^-$	β	1.3	84.5	14.2
	σ^A	0	30.5	69.5
SigF$^-$	β	14.3	65.2	20.4
	σ^A	0	32.8	67.1
	Pro-σ^E	50.8	42.9	6.1
SigE$^-$ SigF$^-$	β	0	82.7	17.3
	σ^A	0	29.1	70.9
SigECR117	β	6.0	79.3	14.7
	σ^A	0	7.5	92.5
	σ^E	5.6	81.2	0
	Pro-σ^E	18.8	75.1	19.3

[a] Percentage of the bound antibody that was detected in a Western blot of the indicated fractions obtained after a nonequilibrium centrifugation experiment (see Materials and Methods). Fraction 1 represents the bottom of the gradient; fraction 16 represents the top.

longer moved with the RNAP marker and instead was found higher in the gradient. A similar analysis of the *spo0A* extract (Fig. 1B) revealed a persistent association of σ^A with RNAP throughout this same period.

The Western blot data for the T_3 extracts were quantitated by densitometry to estimate the relative abundance of each protein in the separate fractions (Table 3). In both the wild-type and Spo0A mutant extract gradients, fractions 4 to 10 contained approximately 85% of the RNAP β′ marker. These same fractions contained approximately 50% of the Spo0A extract's σ^A but only 2% of the σ^A that was present in the wild-type extract. In order to verify that the estimates of σ^A in the various fractions were based on measurements that were in a linear response range, fractions that corresponded to the RNAP-containing fractions and those that did not contain RNAP were pooled, serially diluted, and analyzed by Western blotting for RNAP and σ factor contents. The amount of the σ^A component cosedimenting with the RNAP marker was consistently less than 10% of the σ^A that was detectable in cells that had progressed beyond the initial stages of sporulation, while it remained at 50% or greater in Spo0A$^-$ extracts (data not shown). The experiment also revealed that the amount of extract that we analyzed, although optimal for the σ factor signal, had begun to saturate the β′ signal in the peak β′ fractions. Thus, the fractions indicated as the principal RNAP-containing fractions contain even more of the RNAP than depicted in the figures or tables.

The Western blot analyses of the wild-type *B. subtilis* strain also revealed some interesting aspects of the distributions of pro-σ^E and σ^E in these extracts (Fig. 1A). Pro-σ^E and σ^E were detectable in the $T_{1.5}$ extract. Virtually all of the σ^E, and a portion of the pro-σ^E, cosedimented with the RNAP marker, while most of the pro-σ^E moved to a position below RNAP in the gradient. The "pro" sequence of σ^E is believed to tether pro-σ^E to the *B. subtilis* cytoplasmic membrane (22, 23, 28). It is likely that the fast-sedimenting forms of pro-σ^E represent molecules of pro-σ^E that are associated with membrane components. In Triton-treated extracts, pro-σ^E no longer sediments to the bottom of the gradient but is found either in the RNAP fractions or higher in the gradient, while the sedimentation pattern of σ^E is unaltered (data not shown). At T_3, only mature σ^E was evident in the gradient fractions. In this and the earlier ($T_{1.5}$) sample, almost all of the detectable σ^E was restricted to the RNAP-containing fractions, while by $T_{4.5}$ most of the σ^E was no longer associated with RNAP but sedimented as if it was free within the extract (Fig. 1A). Apparently, σ^E readily associates with RNAP early in sporulation, but between T_3 and $T_{4.5}$, its ability to remain bound to RNAP declines.

We conclude from these experiments that the velocity gradient technique allows us to monitor σ-RNAP associations during sporulation, that σ^A displacement from RNAP is largely complete by T_3, and that σ^E, like σ^A, becomes less likely to be RNAP bound as sporulation progresses.

Effect of sigma factor mutations on E-σ^A persistence. σ^A dissociates from RNAP in sporulating *B. subtilis* but continues to form an RNAP holoenzyme in Spo0A-deficient cells. Among the genes whose expression depends on Spo0A are those which encode the first of the mother cell- and forespore-specific sigma factors, σ^E and σ^F, respectively (2, 45, 46, 56). To determine whether either of these early-sporulation sigma factors is required, directly or indirectly, for the displacement of σ^A from RNAP, we repeated the fractionation analysis with extracts that had been prepared from *B. subtilis* strains lacking one or both of these transcription factors. Representative Western blot analyses of extracts fractionated by velocity gradient centrifugation are illustrated in Fig. 2. The extracts were prepared from sporulating cultures of the mutant strains that had been harvested at T_3. The distribution of the σ factors in these gradients is quantitated in Table 3. The loss of σ^E, σ^F, or both proteins allowed a substantial retention of the σ^A in the RNAP fractions (Fig. 2). In each of the three mutant strains, approximately 30% of the extract's σ^A sedimented in the peak RNAP fractions (Table 3).

σ^A is believed to be dispersed at equivalent concentrations in the mother cell and forespore (40). It is therefore reasonable to assume that any factor that causes a significant displacement of σ^A from RNAP would have to be active in the large mother cell compartment, where the bulk of the σ^A should lie. The loss of either the forespore-specific σ^F or the mother cell-specific σ^E caused a similar retention of σ^A in the RNAP fraction. This suggests that the mother cell factor responsible for displacing σ^A requires σ^F for its appearance. σ^F-dependent transcription in the forespore is needed for pro-σ^E processing (29, 39). Thus, mature σ^E could be the missing factor necessary for the bulk of the σ^A displacement. A need for mature σ^E would indicate either that processed σ^E, but not its proprotein, can compete with σ^A for RNAP or that a σ^E-dependent gene product is the actual effector of σ^A displacement.

To distinguish between these possibilities, we next analyzed the σ^A-RNAP association profile in a *B. subtilis* strain whose only source of σ^E is an allele (*sigECR117*) which encodes a product that can bind to RNAP but fails to recognize σ^E-dependent promoters (27). If competition between σ^E and σ^A for RNAP is needed for σ^A's dissociation from RNAP, then σ^A should still be displaced in the *sigECR117* mutant strain. Alternatively, if a σ^E-dependent gene product is responsible, the *sigECR117* allele should be equivalent to a *sigE* null mutation and allow E-σ^A to persist. When the σ^A pattern in cells harvested at T_3 of sporulation was analyzed, only 7.5% of the σ^A remained associated with the RNAP during the centrifugation analysis (Table 3). It therefore appears that the presence of the mature σ^E protein itself is more important than σ^E's transcriptional activity for σ^A displacement from RNAP.

Effect of chloramphenicol on σ^E persistence and σ^A RNAP displacement. We next revisited the observation that E-σ^A could be more readily isolated from sporulating *B. subtilis* if the

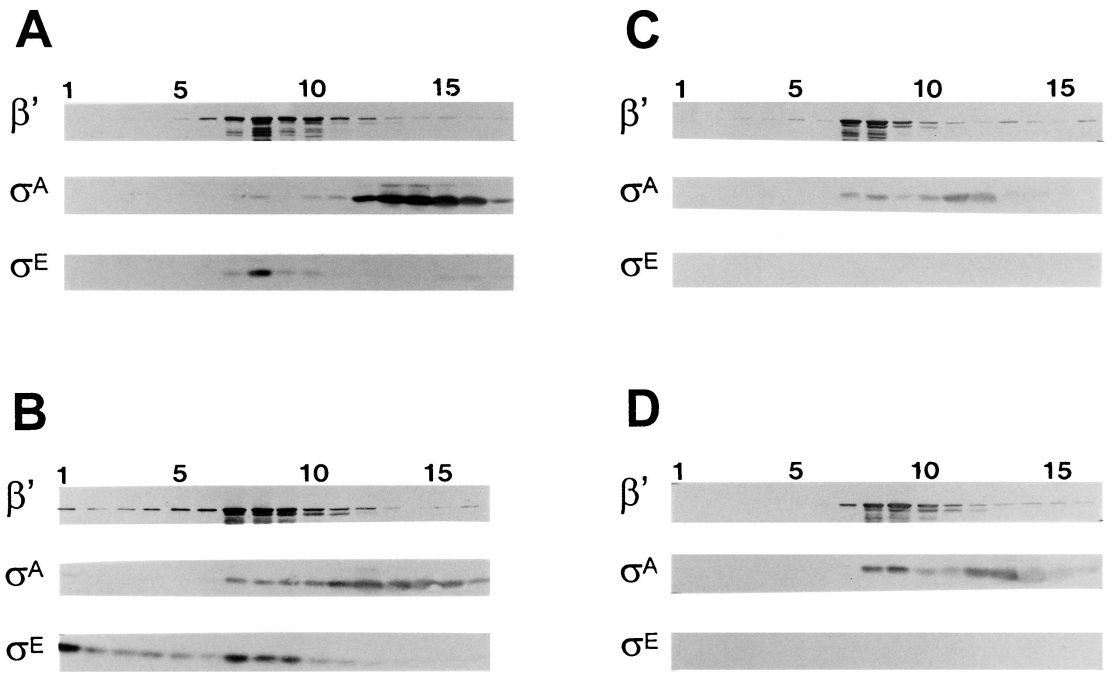

FIG. 2. Fractionation of SigE$^-$ and SigF$^-$ *B. subtilis.* Wild-type *B. subtilis* (SMY) (A) and its congenic SigF$^-$ (SF9030) (B), and SigE$^-$ (SEF500) (C), and SigE$^-$ SigF$^-$ (SEF500) (D) variants were grown to T_3 in DSM and analyzed as described for Fig. 1. The protein band reacting with the anti-σ^E antibody is σ^E in wild-type *B. subtilis* (A) and pro-σ^E in the SigF$^-$ strain (B).

culture was pretreated with chloramphenicol (47). In a previous study of the effects of antibiotics on σ^E processing and turnover, we found that σ^E, once formed, persists in chloramphenicol-treated cells (26). We therefore looked at the effect of chloramphenicol treatment on the σ^A association with RNAP at different times in sporulation. Chloramphenicol treatment could influence the cosedimentation of σ^A with RNAP, but the time at which treatment was applied had a significant effect on the outcome. When chloramphenicol was added to the culture before significant σ^E accumulation and σ^A displacement, at $T_{1.5}$, σ^E synthesis was compromised, and cells harvested 0.5 h later, at T_2, did not show as great a degree of σ^A displacement as an untreated culture at T_2 (Fig. 3). However, when a culture was treated with chloramphenicol after σ^E

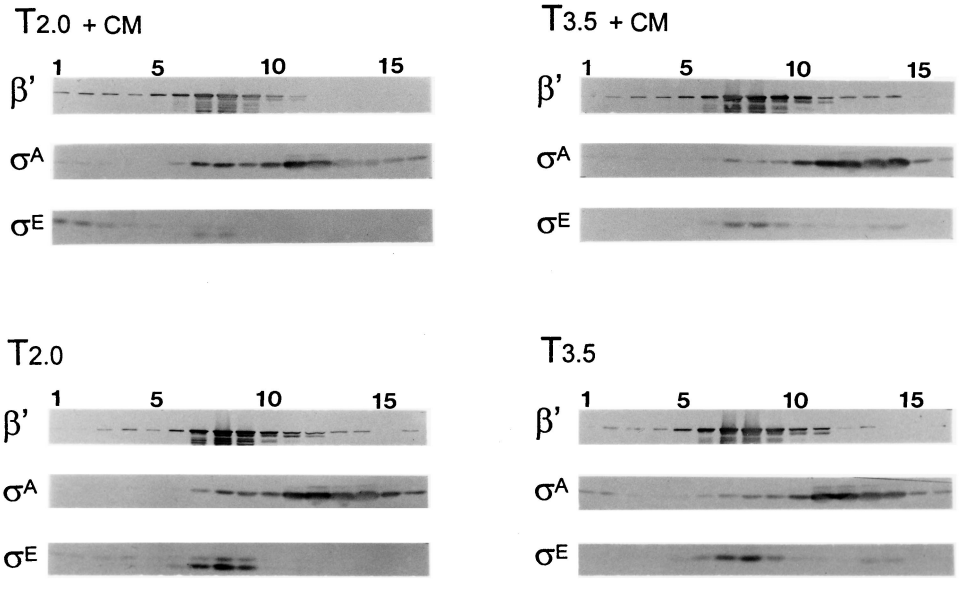

FIG. 3. Effect of chloramphenicol treatment on E-σ^A persistence. Wild-type *B. subtilis* (SMY) was grown in DSM. At 1.5 and 3 h after the end of exponential growth, chloramphenicol was added to portions of the culture, which were harvested 0.5 h later. $T_{2.0}$ + CM and $T_{2.0}$ represent portions of the culture harvested at 2 h with and without chloramphenicol treatment, respectively. $T_{3.5}$ + CM and $T_{3.5}$ are similar cultures harvested at $T_{3.5}$. The culture samples were analyzed as described for Fig. 1. The anti-σ^E antibody detected pro-σ^E and σ^E at T_2 and only σ^E at $T_{3.5}$.

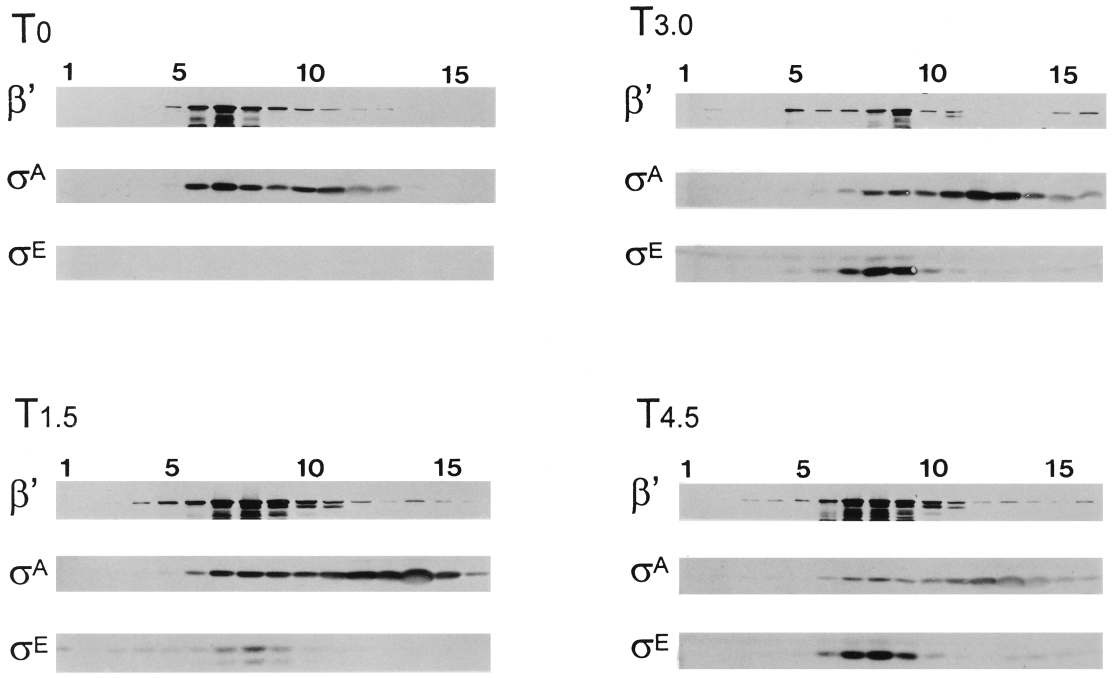

FIG. 4. Fractionation of crude extracts from SigK$^-$ *B. subtilis*. SigK$^-$ *B. subtilis* (SK1027) was grown in DSM, and samples were harvested at the end of growth (T_0) and at 1.5-h intervals thereafter ($T_{1.5}$, $T_{3.0}$, and $T_{4.5}$). The samples were analyzed as described for Fig. 1. The anti-σ^E antibody detects pro-σ^E and σ^E at $T_{1.5}$ and T_3 and primarily σ^E at $T_{4.5}$.

had accumulated and σ^A was largely displaced, at T_3, σ^E persisted, and there was no obvious difference at $T_{3.5}$ in the amount of σ^A that cosedimented with the RNAP in either the treated or untreated cultures (Fig. 3). Thus, in our hands, chloramphenicol treatment did not return displaced σ^A to RNAP but could block its initial displacement. The data sustain a correlation between the presence of σ^E and the decrease in E-σ^A.

Replacement of E-σ^E by E-σ^K. Sigma factor substitution is an ongoing process during sporulation (52). In the mother cell compartment, σ^E, which becomes the principal σ factor on RNAP by T_3, is itself supplanted on RNAP after T_3 (Fig. 1A). There is an aspect to the E-σ^E loss that is distinct from E-σ^A replacement. σ^A persists in the cell even though E-σ^A declines. In contrast, both E-σ^E and σ^E protein levels fall after σ^E's period of activity (55, 60, 61). The previously documented decline of σ^E, as well as its dissociation from RNAP, can be noted in Fig. 1A ($T_{4.5}$). By $T_{4.5}$ the bulk of the remaining σ^E has become displaced from the RNAP-containing fractions and sediments higher in the gradient. Presumably, this slower-migrating species represents free σ^E. Zhang and Kroos have found that σ^E is negatively regulated by σ^K (60, 61). They showed that σ^K reduced σ^E-dependent transcription and, more recently, the synthesis of σ^E itself (60, 61). We therefore examined the effects of a σ^K loss on the σ^E profile in our systems. Our data (Fig. 4) confirm the finding of Zhang and Kroos that σ^E persists in the absence of σ^K and, in addition, show that σ^E continues to cofractionate with RNAP in the σ^K-deficient strain (e.g., compare Fig. 1A, $T_{4.5}$, with Fig. 4, $T_{4.5}$). σ^K itself, or a σ^K-dependent process, thus affects both σ^E levels and σ^E's ability to associate with RNAP.

In an attempt to distinguish between σ^K, as competitor of σ^E, or an unknown gene product under σ^K's control as an effector of σ^E displacement and loss, we examined the σ^E profile in a *B. subtilis* mutant whose σ^K (σ^{KCR109}) could bind RNAP but could not recognize σ^K-dependent promoters (61). There is a limitation to this experiment which was not encountered in the similar experiment with the σ^{ECR119} mutant. *sigE* is expressed from a σ^A-dependent promoter (32), and therefore, σ^E synthesis is unaffected by the activity of the σ factor product that is made. *sigK*, in contrast, is transcribed first by E-σ^E and then by E-σ^K (34, 35). Given that the σ^{KCR109} protein is ineffective as a σ factor, the σ^K protein levels in this strain remain dependent on E-σ^E. Western blot analyses of extracts prepared from sporulating wild-type and *sigKCR109* mutant strains revealed that the mutant strain formed approximately 25% of the σ^K protein that was found in wild-type *B. subtilis* (data not shown). Perhaps due to this lower σ^K level and a continued dependence on E-σ^E for σ^K synthesis, σ^E persisted in the σ^{KCR109} strain but nevertheless became increasingly displaced from RNAP as the mutant σ^K protein accumulated (Fig. 5). Quantitation of the antibody reactions in Fig. 5 revealed that the σ^E abundance was essentially unchanged between $T_{4.5}$ and T_6 (i.e., the σ^E antibody signal at $T_{4.5}$ was 94.3% of the signal at $T_{6.0}$ [62.5×10^3 and 66.3×10^3 pixels, respectively]); however, the percentage of unbound σ^E rose from 14.5 to 30%. During this same period, the abundance of σ^{KCR109} increased 2.4-fold, with virtually all of the σ^K being in the RNAP-containing fractions. The disproportionate association of σ^K with RNAP, relative to that of σ^E, and the coincidence of an approximately twofold increase in σ^K abundance with a twofold increase in free σ^E suggest that σ^K can compete with σ^E and displace it from RNAP in these extracts.

The anti-σ^K antibody detects both σ^K and its larger precursor form (pro-σ^K). Pro-σ^K, like pro-σ^E, is believed to be associated with cell membrane components (59). This likely accounts for pro-σ^K's distribution at the bottom, and throughout, the gradient (Fig. 5). The distribution of pro-σ^K in these gradients differs somewhat from the profile which we had observed for pro-σ^E in similar gradients. Pro-σ^E was more highly

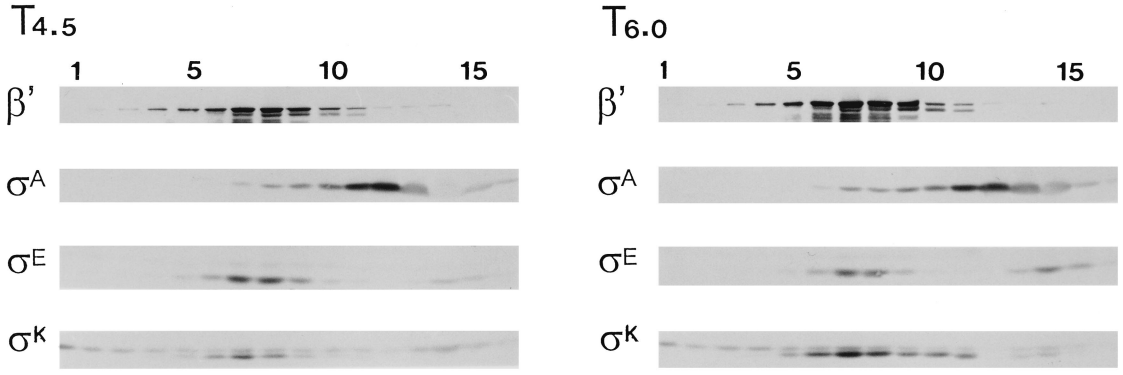

FIG. 5. Fractionation of crude extracts from *sigK109CR B. subtilis*. *B. subtilis* S410 (*sigK109CR*) was grown in DSM. Samples harvested at 4.5 h ($T_{4.5}$) and 6 h (T_6) after the end of growth were analyzed as described for Fig. 1 with antibodies against β′, σ^A, σ^E, and σ^K as probes. The anti-σ^E antibody detected σ^E. The anti-σ^K antibody detected σ^K and its slower-migrating precursor, pro-σ^K.

enriched in the RNAP-containing fractions and appeared to either cosediment with RNAP or migrate as faster-sedimenting, and presumably membrane-bound, forms (Fig. 1A [$T_{1.5}$], 2B, 3, and 4 [$T_{1.5}$]). The pro-σ^K, in contrast, was spread more diffusely throughout the gradient, with less of it in the RNAP fractions and a portion of it near the top of the gradient, where it is free from high-molecular-weight associations (Fig. 5). Pro-σ^E thus appears more likely than pro-σ^K to be associated with RNAP. Whether this is due to inherent differences between the two proproteins in their capacity for RNAP binding or to an effect of other components in the extract at the time of their synthesis (e.g., σ^E present to compete with pro-σ^K) is not clear.

DISCUSSION

As *B. subtilis* proceeds into sporulation, its extractable RNAP contains decreasing amounts of the principal vegetative cell σ factor, σ^A, and increasing amounts of sporulation-specific σ factors (18, 52). Early in sporulation (T_2 to T_3), the principal RNAP holoenzyme species that can be isolated contains σ^E (19), the first sporulation-specific σ factor that is present in the large mother cell compartment (10). After T_4, the level of σ^E falls and the second mother cell holoenzyme, E-σ^K, appears and continues the next stage of transcription in that compartment (55, 60). In the present study we have examined some of the properties of the E-σ^A→E-σ^E→E-σ^K progression. We found, as have others (40), that σ^A and RNAP levels remain relatively constant until at least $T_{4.5}$ of sporulation. Nevertheless, by 3 h into this process, the percentage of σ^A that cosediments with RNAP falls from 50% to less than 10% (Fig. 1A). In contrast, σ^A association with RNAP remains at 50% in a *B. subtilis* strain which is unable to sporulate due to a mutation in the sporulation-essential *spo0A* gene (Fig. 1B). Thus, as initially reported more than 20 years ago, σ^A persists in sporulating *B. subtilis* but becomes displaced from RNAP by sporulation-specific factors (47, 54).

Although our gradient analyses showed a progressive loss of E-σ^A as *B. subtilis* proceeds into sporulation, a recent study, using a histidine tag to withdraw RNAP from *B. subtilis* extracts, found E-σ^A to persist throughout sporulation (17). We are unable to explain the differences in our findings. The absence of σ^A has long been a hallmark of RNAP from sporulating *B. subtilis* (19, 38, 47, 54). Given that σ^A persists during sporulation, it is possible that some feature of the Ni^{2+} resin extraction technique allowed it to reassociate with RNAP during the purification.

Based on immunofluorescence experiments, σ^A is believed to be in roughly equal concentrations in the mother cell and forespore (40). The mother cell compartment of a typical *B. subtilis* cell is at least five times the size of the forespore. Therefore, whatever is responsible for σ^A displacement during sporulation needs to be active in the large mother cell, where the bulk of the RNAP and σ^A is likely to be found. Our data implicate σ^E, the first of the mother cell-specific σ factors, as having a direct role in σ^A displacement. Mutations which block σ^E synthesis alter the σ^A retention on RNAP from the few percent found in wild-type *B. subtilis* to a level almost as great as that seen in the *spo0A* mutant. σ^E's role in σ^A dissociation from RNAP appears to be related to the presence of the σ^E protein rather than its transcriptional activity. A mutant σ^E (σ^{ECR119}), which can bind to RNAP but cannot direct RNAP to σ^E promoters, reduced the amount of σ^A that cosedimented with RNAP from the 30% observed in the absence of σ^E to below 10%. The notion that σ^E is preferentially bound to RNAP in early sporulating cells is supported by the distribution of σ^E in the gradient analyses. Until σ^K appears, virtually all of the detectable σ^E in the crude extracts cosediments with RNAP (Fig. 1A, 2, 3, and 4), while a significant portion of the σ^A in the extracts is unassociated. Either a sporulation-specific factor, which does not depend on σ^E for its expression, weakens σ^A's ability to compete with σ^E or σ^E is an inherently more potent RNAP binding protein than is σ^A. The latter possibility is attractive. σ^A, but not σ^E, is released from RNAP when these holoenzymes are bound to phosphocellulose (19, 48). This implies that σ^E binds more tightly to RNAP than does σ^A. In addition, the synthesis of sporulation σ factors in vegetatively growing *B. subtilis* induces the expression of genes whose transcription depends on them (see, e.g., references 24, 44, 46, 50, and 51). Thus, sporulation σ factors can gain access to RNAP to at least some degree in the presence of σ^A without the aid of additional sporulation factors.

In wild-type strains, active σ^E initiates the synthesis of σ^K, the second of the two mother cell σ factors. In such strains, there is a displacement of σ^E from RNAP and a drop in σ^E protein levels. Zhang and Kroos have documented that the decrease in σ^E levels is dependent on active σ^K (60, 61). Our current data confirm the need for σ^K in σ^E disappearance and also show that the presence of σ^K protein alters the profile of σ^E's association with RNAP. Before the appearance of σ^K, or

in mutant *B. subtilis* that cannot synthesize σ^K, most of the σ^E present in crude extracts cosediments with RNAP (Fig. 1A [T_3] and 4); however, after σ^K's appearance, a significant portion of the σ^E becomes displaced from RNAP (Fig. 1A [$T_{4.5}$] and 5). Coincident with the σ^E displacement, virtually all of the mature σ^K is found in the RNAP-containing fractions (Fig. 5). Thus, σ^K effectively competes with σ^E for RNAP, with the result that E-σ^K becomes a preferred holoenzyme species.

Competition among σ factors has been implicated as an element of postexponential gene expression in both *E. coli* and *B. subtilis*. The stationary-phase σ factor of *E. coli*, σ^S, appears to compete with this organism's housekeeping σ^{70} during the transition to stationary phase (15). Overproducing either σ^{70} or σ^S was found to shift the pattern of transcription in stationary-phase *E. coli* in favor of those promoters recognized by the σ factor that was overexpressed (15). More germane to our present study, Hicks and Grossman have found that altering σ^A levels in *B. subtilis* affects gene expression by the sporulation-essential, transition state σ factor σ^H (21). Enhanced or restricted σ^A production during growth was found to decrease or increase, respectively, σ^H-dependent gene expression. Overproduction of σ^A also delayed the production of heat-resistant spores, an outcome that might be expected if, as Hicks and Grossman suggested, sporulation-specific σ factors had to compete with this σ^A pool for a limited population of RNAP core (21).

Recently, Lord et al. analyzed the replacement of σ^A by the forespore-specific σ factor σ^F (40). They noted, as did we, that the intracellular concentrations of core RNAP and σ^A were virtually unchanged during the first 3 h of sporulation. In addition, they determined that by the time the first sporulation σ factors are activated (i.e., after septation), the concentration of σ^A and σ^F exceeds the concentration of RNAP and that competition for core RNAP must be occurring (40). Although the simplest model for σ factor substitution would have σ^F as a more effective competitor for RNAP than σ^A, Lord et al. found that σ^A's affinity for RNAP was 25-fold greater than that of σ^F (40). Based on this disparity, they proposed that an anti-σ^A factor is synthesized or activated during sporulation to allow σ^F to successfully compete for RNAP. If this or a similar hypothetical factor also participates in the exchange of σ^E for σ^A, our data argue that its appearance depends on Spo0A, but not active σ^E or σ^F, and that it cannot in itself remove σ^A from RNAP but would instead enhance the competitiveness of the sporulation σ factors to displace σ^A. In vitro studies of the affinities of purified σ^A, pro-σ^E, and σ^E for RNAP, as well as transcription competition analyses with purified proteins, will be useful in determining whether σ^E has the affinity for RNAP that would allow it to directly supplant σ^A on RNAP.

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