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Loss of Compartmentalization of σ^E Activity Need Not Prevent Formation of Spores by *Bacillus subtilis*▽†

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Compartmentalization of the activities of RNA polymerase sigma factors is a hallmark of formation of spores by *Bacillus subtilis*. It is initiated soon after the asymmetrically located sporulation division takes place with the activation of σ^F in the smaller cell, the prespore. σ^F then directs a signal via the membrane protease SpoIIGA to activate σ^E in the larger mother cell by processing of pro-σ^E. Here, we show that σ^E can be activated in the prespore with little effect on sporulation efficiency, implying that complete compartmentalization of σ^E activity is not essential for spore formation. σ^E activity in the prespore can be obtained by inducing transcription in the prespore of *spoIIGA* or of *sigE, which encodes a constitutively active form of σ^E, but not of *spoIIGB*, which encodes pro-σ^E. We infer that σ^E compartmentalization is partially attributed to a competition between the compartments for the activation signaling protein SpoIIR. Normally, SpoIIGA is predominantly located in the mother cell and as a consequence confines σ^E activation to it. In addition, we find that CsfB, previously shown to inhibit σ^G, is independently inhibiting σ^E activity in the prespore. CsfB thus appears to serve a gatekeeper function in blocking the action of two sigma factors in the prespore: it prevents σ^G from becoming active before completion of engulfment and helps prevent σ^E from becoming active at all.**

Formation of spores by *Bacillus subtilis* results from a primitive cell differentiation involving two distinct cell types, the smaller prespore (also called the forespore) and the larger mother cell. These cells are formed following an asymmetrically located division that takes place about 1 h after the start of spore formation. The prespore is subsequently engulfed by the mother cell and develops into a mature spore. The mother cell is necessary for spore formation but ultimately lyses. Formation of a heat-resistant, dormant spore takes about 7 h at 37°C. A fundamental trait of cell differentiation is the existence of distinct programs of gene expression in the different cell types. This is exemplified in the sporulation of *B. subtilis* by the action of distinct RNA polymerase sigma factors (reviewed in reference 18). The expression of different genes in the two cells is governed to a considerable extent by the activation of four RNA polymerase σ factors. The first sigma factor to become active in the prespore is σ^F, and it does so soon after the formation of the division septum (17). σ^F directs transcription of about 50 genes (42, 46), including one critical for intercellular signaling, *spoIIR*. Expression of *spoIIR* leads rapidly to the activation of σ^E in the mother cell (27, 33); σ^E directs transcription of about 250 genes (10, 42). Upon completion of engulfment, σ^F and σ^E are replaced by σ^G and σ^K, respectively. σ^G directs transcription of about 110 genes in the prespore (42, 46), and σ^K directs transcription of about 140 genes in the mother cell (10, 42). In this paper, we focus on the compartmentalized activation of σ^E.

σ^E is synthesized as pro-σ^E, an inactive precursor (45). It is activated by cleavage of the 27-residue pro sequence (31, 43). Pro-σ^E is encoded by *spoIIGB*, which is cotranscribed with *spoIIGA* (34, 43). Transcription of the *spoIIG* operon is directed by the housekeeping σ factor, σ^A (29). Transcription is activated by the master regulator Spo0A (41) and commences soon after the start of spore formation, before the spore septum is formed (16, 28). Ordinarily, pro-σ^E is processed to active σ^E only after septation because it also requires the action of SpoIIR, which is synthesized after septum formation (27). SpoIIR is synthesized in the prespore but is exported to the intermembrane septal space, where it is thought to activate SpoIIGA. σ^E becomes active exclusively in the mother cell (8). It is activated within 4 min of *spoIIR* transcription in the prespore (11). The activity of σ^E is strictly confined to the mother cell, and it is thought that specific regulatory controls exist to prevent it from becoming active in the prespore (14, 25, 37). In addition, after septum formation there is a large increase in Spo0A-directed transcription of the *spoIIG* operon in the mother cell but not in the prespore (14, 15).

There have been several reports of strains engineered to activate σ^E before formation of the spore septum. Generally in these cases, the σ^E activity prevented septum formation so that the prespore was not formed (14, 37, 48). Fujita and Losick (14) did report rare organisms displaying σ^E activity in both the prespore and the mother cell of a strain in which the *spoIIG* operon was expressed constitutively; however, they did not comment further about those organisms. Attempts to obtain substantial σ^E activity in the prespore of *B. subtilis* by placing its structural gene under the control of a σ^F-directed promoter have been largely unsuccessful, even when *sigE**, which en-

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TABLE 1. *B. subtilis* strains used

Strain[a]	Relevant genotype
SL14574	*spoIIG*::P_{spoIIQ}-*spoIIG thrC*::P_{spoIID}-*gfp*
SL14578	*spoIIG*::P_{spoIIQ}-*spoIIG thrC*::P_{spoIID}-*gfp* Δ*csfB*::*cat*
SL14613	*spoIIG*::P_{spoIIQ}-*spoIIG thrC*::P_{spoIID}-*gfp* Δ*csfB*::*cat amyE*::*spoIIG*
SL14631	*spoIIG*::P_{spoIIQ}-*spoIIG thrC*::P_{spoIID}-*gfp amyE*::*spoIIG*
SL14656	*amyE*::P_{spoIIQ}-*sigE* thrC*::P_{spoIID}-*gfp* Δ*csfB*::*cat* Δ*spoIIGB*::*spc*
SL14657	*amyE*::P_{spoIIQ}-*sigE* thrC*::P_{spoIID}-*gfp* Δ*spoIIGB*::*spc*
SL14679	*amyE*::P_{spoIIQ}-*spoIIGB thrC*::P_{spoIID}-*gfp* Δ*csfB*::*cat*
SL14711	*amyE*::P_{spoIIQ}-*spoIIG* P_{sspA}-*gfp@sspA*[b]
SL14712	*amyE*::P_{spoIIQ}-*spoIIG thrC*::P_{spoIID}-*gfp*
SL14715	*amyE*::P_{spoIIQ}-*spoIIG thrC*::P_{spoIID}-*gfp* Δ*csfB*::*spc*
SL14816	*amyE*::P_{spoIIQ}-*spoIIG thrC*::P_{spoIIQ}-*gfp*
SL14868	*amyE*::P_{spoIIQ}-*spoIIG thrC*::P_{cotEp1}-*gfp*
SL15041	*amyE*::P_{spoIIQ}-*spoIIG* Δ*csfB*::*spc* Δ*spoIIIG*::*cat*
SL15062	*amyE*::P_{spoIIQ}-*spoIIGA thrC*::P_{spoIID}-*gfp* Δ*csfB*::*spc*
SL15139	*spoIIR*::P_{spoIIQ}-*spoIIR thrC*::P_{spoIID}-*gfp* Δ*csfB*::*cat*
SL15143	*amyE*::P_{spoIIQ}-*spoIIG thrC*::$P_{spoIIID}$-*gfp*
SL15145	*amyE*::P_{spoIIQ}-*spoIIG* Δ*csfB*::*cat thrC*::P_{spoIID}-*gfp*
SL15175	*amyE*::P_{spoIIQ}-*spoIIG* Δ*csfB*::*cat*
SL15365	*spoIIG*::P_{spoIIQ}-*spoIIG amyE*::P_{spoIIG}-*spoIIG* Δ*csfB*::*cat thrC*::P_{spoIID}-*gfp* Δ*spoIIIG*::*spc*
SL15366	*spoIIG*::P_{spoIIQ}-*spoIIG amyE*::*spoIIG thrC*::P_{spoIID}-*gfp* Δ*spoIIIG*::*spc*

[a] All strains are in the genetic background of *B. subtilis* 168 strain BR151 (*trpC2 lys-3 metB10*). They have all its auxotrophic markers. All strains were developed in the course of the present study.

[b] @, the fusion has been introduced by single-crossover (Campbell-like) recombination.

codes a form of σ^E (σ^{E*}) that is active without the need for processing (25), was used. It is thought that degradation of σ^E (or σ^{E*}) in the prespore is a critical factor in restricting σ^E activity to the mother cell (14, 25, 37).

In this paper, we reexamine the question of activating σ^E in the prespore. We reasoned that the prespore-specific degradation of σ^E might be under developmental control and tested the possibility that such a control might be directed from the mother cell. We find evidence of a σ^E-directed signal from the mother cell that blocks σ^E from becoming active in the prespore. We also find evidence that the product of a σ^F-directed gene, CsfB, inhibits σ^E activity in the prespore. We find that under some conditions, it is possible to obtain σ^E activity exclusively in the prespore or in both the prespore and the mother cell. We show that organisms displaying σ^E activity in both the prespore and the mother cell are capable of forming heat-resistant spores so that complete compartmentalization of σ^E activity is not essential for spore formation.

MATERIALS AND METHODS

Media. *B. subtilis* was grown in modified Schaeffer's sporulation medium (MSSM) or on Schaeffer's sporulation agar as described previously (5, 36). When required, the medium was supplemented with chloramphenicol at 5 μg/ml, erythromycin at 1.5 μg/ml, neomycin at 3.5 μg/ml, spectinomycin at 100 μg/ml, or tetracycline at 10 μg/ml. *Escherichia coli* was grown on Luria-Bertani lysogeny broth agar containing 100 μg of ampicillin/ml when required.

Strains. The *B. subtilis* strains used are listed in Table 1. *B. subtilis* 168 strain BR151 (*trpC2 metB10 lys-3*) was used as the parent strain. Plasmids were constructed in *E. coli* DH5α, and their structures were confirmed by restriction enzyme digestions and also by PCR.

The *sigE** gene, encoding σ^{E*}, which is active without processing, was cloned under the control of the σ^F-directed P_{spoIIQ}. The pro-less form of the open reading frame (ORF), starting with the 28th codon of pro-σ^E and including the rest of the ORF, was amplified by PCR. The PCR product was cloned downstream of P_{spoIIQ} in pEIA99 (4) to give pVK333, which is designed to integrate the P_{spoIIQ}-*sigE** construct at the *amyE* locus of *B. subtilis* by double crossover. As a result of the cloning, the codon for Tyr, which is the 28th codon of pro-σ^E, is preceded by just two codons, for Met and His, and a strong ribosome binding site ([RBS] GGAGG).

The *spoIIG* operon, retaining its RBS but not its promoter, was amplified by PCR and cloned downstream of P_{spoIIQ} in pEIA99 to give pVK339, containing *amyE*::P_{spoIIQ}-*spoIIG*. The promoterless *spoIIGA* gene, retaining its RBS, was amplified by PCR and cloned downstream of P_{spoIIQ} in pEIA99 to give pVK239, containing *amyE*::P_{spoIIQ}-*spoIIGA.* The constructs from pVK333, pVK339, and pVK239 were introduced by double crossover into the *amyE* locus of *B. subtilis* BR151 by transformation, selecting for the appropriate resistance marker. The constructs were subsequently moved into other strains by transformation.

P_{spoIID}-*gfp* fusions in two locations were used as indicators of σ^E activity, located at *ppsD* (169°) and *thrC* (284°); the origin of replication of the circular chromosome is at 0°. Construction of *thrC*::P_{spoIID}-*gfp* was described previously (3). The *ppsD*::P_{spoIID}-*gfp* fusion was constructed in two stages. First, a 450-bp portion of *ppsD* was PCR amplified and inserted upstream of the *spoIID* promoter (−291 to +22) in pMLK5169 (a gift from Margaret Karow) to form pVK177. Next, a promoterless *gfp* was isolated from a derivative of pGreenTIR (35) described previously (2) and inserted downstream of the *spoIID* promoter in pVK177, replacing *lacI*, to form pVK196. *B. subtilis* BR151 was transformed with pVK196, selecting for Cm^r and screening for single-crossover (Campbell-like) integration of the plasmid at *ppsD.* The site of integration of pVK196 was confirmed by linkage analysis. DNA was prepared from one such transformant and used to transform appropriate strains. To construct P_{cotEp1}-*gfp*, the same promoterless *gfp* fragment was inserted downstream of P_{cotEp1} in pDF1264, which was a gift from Patrick Stragier. As a result of this manipulation, the *spoIIQ* of pDF1264 was replaced with *gfp*. The construct was introduced by double crossover into the *thrC* locus of *B. subtilis* BR151. Vectors for the expression of $P_{spoIIID}$-*gfp* and P_{gerE}-*gfp* were also designed for insertion into *thrC* by double crossover. For $P_{spoIIID}$-*gfp*, a 400-bp DNA region that contains the promoter of *spoIIID* was PCR amplified from *B. subtilis* BR151 chromosomal DNA and cloned upstream of *gfp* in pVK370, which is a laboratory construct. For P_{gerE}-*gfp*, the *lacZ* gene of *thrC*::P_{gerE}-*lacZ* in *B. subtilis* (6) was replaced with *gfp* using the *lacZ*-to-*gfp* replacement vector pVK209 (2). Details of plasmid and strain construction are available on request.

Fluorescence microscopy. The conditions for growth and imaging of the samples were essentially as described previously (5). Images were captured using a Leica DM IRE2 microscope with a TCS SL confocal system.

Other methods. The methods for transformation of *B. subtilis* and for sporulation by exhaustion in MSSM and other methods were essentially those described previously (4). The initiation of spore formation in MSSM is taken to occur at the end of exponential growth; time after the initiation of spore formation is indicated as T2 for 2 h, T3 for 3 h, etc.

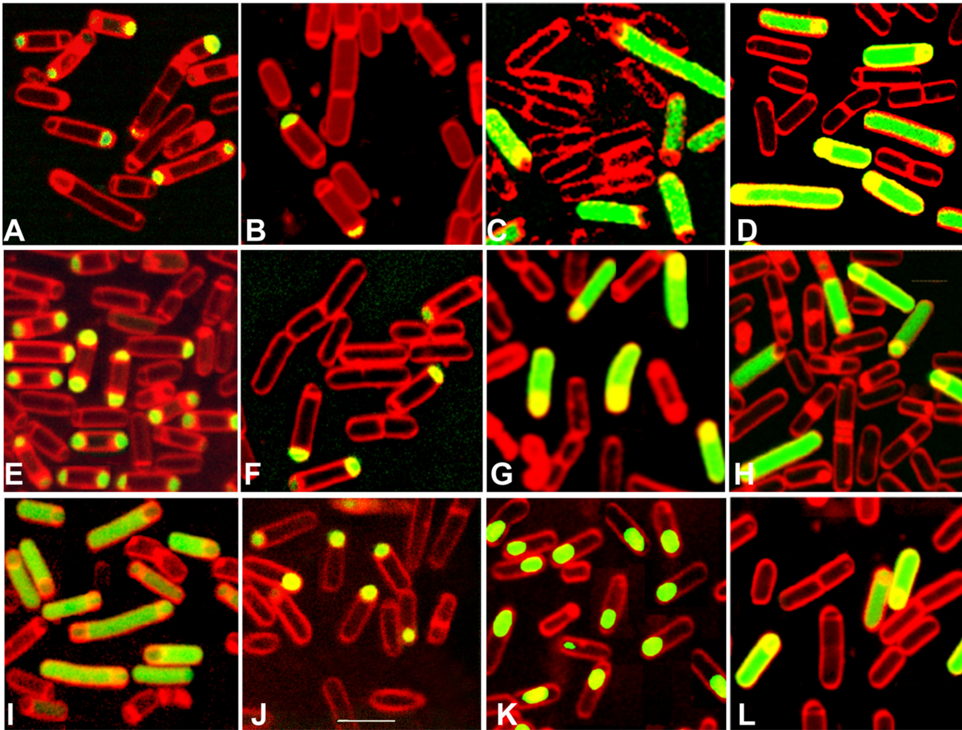

FIG. 1. Localization of activity of σ^E and other sporulation-associated sigma factors in strains with various modifications to expression of the *spoIIG* operon. Examples of bacteria stained with FM4-64 (red) and expressing GFP (green) from *gfp* transcriptional fusions to promoters directed by different sigma factors are shown. Promoters are as follows: σ^E-directed P_{spoIID} (A to H and L), σ^E-directed P_{cotEp1} (I), σ^F-directed P_{spoIIQ} (J), and σ^G-directed P_{sspA} (K). Strains are either *csfB*+ (A to D and I to K) or *csfB* null (E to H and L). (A) Strain SL14657 at T5 (*amyE*::P_{spoIIQ}-*sigE** Δ*spoIIGB*::*spc*). (B) SL14574 at T2.5 (*spoIIG*::P_{spoIIQ}-*spoIIG*). (C) SL14631 at T2.5 (*spoIIG*::P_{spoIIQ}-*spoIIG amyE*::*spoIIG*). (D) SL14712 at T2.5 (*amyE*::P_{spoIIQ}-*spoIIG*). (E) SL14656 at T5 (*amyE*::P_{spoIIQ}-*sigE** Δ*spoIIGB*::*spc* Δ*csfB*::*cat*). (F) SL14578 at T2.5 (*spoIIG*::P_{spoIIQ}-*spoIIG* Δ*csfB*::*cat*). (G) SL14613 at T2.5 (*spoIIG*::P_{spoIIQ}-*spoIIG amyE*::*spoIIG* Δ*csfB*::*cat*). (H) SL14715 at T2.5 (*amyE*::P_{spoIIQ}-*spoIIG* Δ*csfB*::*spc*). (I) SL14868 at T2.5 (*amyE*::P_{spoIIQ}-*spoIIG*). (J) SL14816 at T2.5 (*amyE*::P_{spoIIQ}-*spoIIG*). (K) SL14711 at T5 (*amyE*::P_{spoIIQ}-*spoIIG*). (L) SL15062 at T2.5 (*amyE*::P_{spoIIQ}-*spoIIGA* Δ*csfB*::*spc*). The scale bar shown in panel J is 3 μm and is applicable to all images.

RESULTS AND DISCUSSION

σ^E activity in the prespore. In order to obtain σ^E activity in the prespore, we performed three genetic manipulations. First, we utilized *sigE**, which encodes a form of σ^E (σ^{E*}) that is active without the need for processing (25). Second, we placed *sigE** under the control of the strong σ^F-directed P_{spoIIQ} promoter so that it would be expressed only in the prespore and located the construct at the origin-proximal *amyE* locus to ensure optimal transcription. Third, we inactivated the parental *spoIIGB* locus, which encodes pro-σ^E, so as to block any σ^E-directed signal from the mother cell that might prevent σ^E from becoming active in the prespore. The resulting strain, SL14657, displayed σ^E activity in the prespore (Fig. 1A). By 2.5 h after the end of exponential growth (T2.5), 20% of organisms had undergone the asymmetrically located sporulation division. Of those, 21% displayed σ^E activity, and in all cases it was localized to the prespore (Table 2). At T5, 70% of organisms had formed the sporulation septum, and 37% of those displayed a signal in the prespore; again, none displayed any σ^E activity in the mother cell. The strains were blocked in spore formation and had the abortively disporic phenotype typical of *spoIIGB* mutants. Interestingly, the σ^E activity was often confined to just one of the two prespores in the abortively disporic organisms (Fig. 1A; Table 2). Time-lapse studies (data not shown) indicated that it was the first of the two prespores to be formed that displayed σ^E activity. We obtained a similar result with *spoIIGB* intact but *spoIIR* disrupted so that pro-σ^E was made under its normal control but could not be processed to σ^E (data not shown).

Processing of pro-σ^E in the prespore. We next tested to see if the wild-type form of pro-σ^E could be processed to active σ^E in the prespore. Ordinarily, expression of the *spoIIG* operon, which encodes pro-σ^E and SpoIIGA, is induced soon after the start of spore formation and before the sporulation division (16, 28). However, processing of the inactive pro-σ^E to active σ^E by SpoIIGA is confined to the mother cell following septum formation (8) and requires the action of SpoIIR, which is formed in the prespore from the σ^F-controlled *spoIIR* locus (27, 33). Thus, σ^E activation in the mother cell is dependent on an intercompartmental signal across the septum.

To test for processing of pro-σ^E in the prespore, we replaced the promoter of the *spoIIG* operon with the σ^F-directed *spoIIQ* promoter so as to confine its transcription to the prespore. This substitution indeed resulted in σ^E activity being largely confined to the prespore (Fig. 1B; Table 2, strain SL14574) although at T5, for reasons that are not clear, some organisms showed activity in the mother cell. The activation of σ^E was blocked by inactivation of *spoIIR* (data not shown) so that in

this genetic background SpoIIR acts as an intracompartmental signal to activate processing of pro-σ^E. The strain did not form spores and was blocked at stage II of sporulation. The septum, as indicated by FM4-64 staining, appeared to be disrupted in a number of organisms in the T5 sample, making quantitation for that sample difficult.

Activation of σ^E in the mother cell can prevent it from becoming active in the prespore. The strains described in the experiments in the previous sections had a genetic background in which normal *spoIIG* expression was disrupted. This was done to block a suspected σ^E-directed signal from the mother cell that would prevent σ^E from becoming active in the prespore. To test for such a signal, the strain SL14631, which is isogenic with SL14574 (*spoIIG*::P_{spoIIQ}-*spoIIG*) except for the insertion of an intact copy of the *spoIIG* operon under its own promoter at the ectopic *amyE* locus, was tested. The change restored σ^E activity to the mother cell. All organisms displaying σ^E activity now had activity in the mother cell (Fig. 1C and Table 2). Importantly, few organisms showed any σ^E activity in the prespore, and that activity was weak. The strain formed spores efficiently, and no abortively disporic organisms were detected.

These results are consistent with a σ^E-directed signal from the mother cell largely preventing σ^E from becoming active in the prespore. However, they also fit an alternative explanation, namely, that the critical factor is the activation of σ^E, not its activity. In particular, enhanced expression of the protease SpoIIGA in the mother cell outcompetes any SpoIIGA in the prespore for activation by SpoIIR, thus ensuring that pro-σ^E is processed by activated SpoIIGA only in the mother cell. This possibility is considered in detail below, in the section on compartmentalization of σ^E activity.

σ^E activity in the prespore is regulated by CsfB. The *csfB* locus is transcribed in the prespore from a σ^F-directed promoter (7). The CsfB protein has been shown to inhibit premature activation of σ^G in the prespore (1, 6, 26). CsfB can also impair σ^E activity (6). We wished to test the possibility that CsfB might be a developmentally regulated check on σ^E becoming active in the prespore. Consistent with this possibility, inactivation of *csfB* increased σ^{E*} activity in the prespores of a *spoIIGB* mutant that was expressing *sigE** from a σ^F-directed promoter. Most notably, a higher proportion of abortively disporic organisms displayed σ^{E*} activity in both prespores in the *csfB* mutant SL14656 than in the *csfB*$^+$ strain SL14657 (37% and 12%, respectively, at T5) (Table 2; compare Fig. 1A and E). The effect is unlikely to be an indirect consequence of CsfB acting on σ^G because a strain isogenic with SL14656 but containing a reporter for σ^G displayed no σ^G activity (data not shown). Inactivation of *csfB* also resulted in a substantial increase in the proportion of prespores expressing σ^E activity in a strain expressing the *spoIIG* locus under the σ^F-directed P_{spoIIQ} promoter (Table 2, compare strains SL14578 and SL14574).

The effect of *csfB* inactivation was dramatic in a strain that had a second copy of *spoIIG* expressed from the σ^F-directed P_{spoIIQ} promoter, in addition to *spoIIG* expressed from its natural promoter. Expression was largely confined to the mother cell of the *csfB*$^+$ strain (SL14631) (Fig. 1C and Table 2). However, with *csfB* inactivated, the location of σ^E activity changed: the majority of green fluorescent protein (GFP)-

TABLE 2. Expression of σ^E activity in the prespore

Strain	Relevant genotype	*csfB*[a]	Time[b]	No. of organisms showing the indicated GFP fluorescence pattern[c]										
				(pattern 1)	(pattern 2)	(pattern 3)	(pattern 4)	(pattern 5)	(pattern 6)	(pattern 7)	(pattern 8)	(pattern 9)	(pattern 10)	(pattern 11)
SL14657	*amyE*::P_{spoIIQ}-*sigE* spoIIGB*::*spc*	+	T2.5	187	38	1	0	0	6	3	0	0	0	0
			T5	79	61	20	0	0	53	35	12	0	0	0
SL14656	*amyE*::P_{spoIIQ}-*sigE* spoIIGB*::*spc*	−	T2.5	212	13	6	0	0	7	11	8	0	0	0
			T5	90	47	24	0	0	42	25	40	0	0	0
SL14574	*spoIIG*::P_{spoIIQ}-*spoIIG*	+	T2.5	102	35	4	0	0	7	6	4	0	0	0
			T5	84	43	1	4	0	48	14	5	1	0	22
SL14578	*spoIIG*::P_{spoIIQ}-*spoIIG*	−	T2.5	229	28	4	0	0	6	18	15	0	0	0
			T5	30	26	27	0	0	12	12	50	0	0	18
SL14631	*spoIIG*::P_{spoIIQ}-*spoIIG* *amyE*::*spoIIG*	+	T2.5	432	13	0	18	0	0	0	0	0	0	4
			T5	63	32	0	104	0	0	0	0	0	0	6
SL14613	*spoIIG*::P_{spoIIQ}-*spoIIG* *amyE*::*spoIIG*	−	T2.5	259	31	0	21	22	0	0	0	0	0	0
			T5	79	38	0	23	85	0	0	0	0	0	1
SL15366	*spoIIG*::P_{spoIIQ}-*spoIIG* *amyE*::*spoIIG spoIIIG*::*spc*	+	T2.5	221	25	0	20	0	1	0	0	0	0	0
SL15365	*spoIIG*::P_{spoIIQ}-*spoIIG* *amyE*::*spoIIG spoIIIG*::*spc*	−	T2.5	588	36	0	43	16	1	0	0	0	0	0

[a] Strains were either *csfB*$^+$ or *csfB* null (−).

[b] Samples were analyzed at the indicated times given in h after the start of spore formation.

[c] σ^E activity was visualized using a P_{spoIID}-*gfp* fusion. Cultures were induced to form spores in MSSM. Schematic representations are given of the different types of organisms present in the culture; shaded areas indicate GFP fluorescence. Membranes were visualized by staining with FM4-64. Some organisms of strains SL14575 and SL14578 appeared to have defective septa, indicated by a dotted line; the defective septa would disrupt compartmentalized GFP fluorescence.

TABLE 3. Expression pattern of different sigma factors in strains with enhanced *spoIIG* transcription in the prespore

Strain[a]	σ factor tested	Reporter	*csfB*[b]	Time[c]	No. of organisms showing the indicated GFP fluorescence pattern[d]					
SL14712	σ^E	P_{spoIID}-*gfp*	+	T2.5	264	36	2	1	37	3
				T5	123	56	0	27	74	0
SL14715	σ^E	P_{spoIID}-*gfp*	−	T2	359	34	0	0	36	0
				T3	327	53	5	2	49	2
				T5	216	21	0	8	117	3
SL14816	σ^F	P_{spoIIQ}-*gfp*	+	T2.5	51	12	62	0	0	0
SL14711	σ^G	P_{sspA}-*gfp*	+	T2.5	141	35	10	0	0	0
				T5	137	23	162	0	0	0
SL15143	σ^E	$P_{spoIIID}$-*gfp*	+	T2.5	128	5	1	0	7	0
				T5	161	45	0	97	26	0
SL15145	σ^E	$P_{spoIIID}$-*gfp*	−	T2.5	369	6	1	0	23	0
				T5	128	1	0	26	89	0
SL14868	σ^E	P_{cotEp1}-*gfp*	+	T2.5	203	44	0	3	41	0
				T5	60	3	0	96	24	1

[a] Cultures were induced to form spores in MSSM.
[b] Strains were either *csfB*$^+$ or *csfB* null (−).
[c] Samples were analyzed at the indicated times given in hours after the start of spore formation.
[d] All strains were *spoIIG*$^+$ and also contained *amyE*::P_{spoIIQ}-*spoIIG* to enhance early transcription of *spoIIG* in the prespore. Schematic representations are given of the different types of organism present in the cultures; shaded areas indicate GFP fluorescence. Membranes were visualized by staining with FM4-64.

expressing organisms now displayed activity in both the mother cell and the prespore (Fig. 1G and Table 2, strain SL14613). This distinction was retained when the structural gene for σ^G was inactivated: in the *csfB*$^+$ strain σ^E activity was confined to the mother cell, whereas in the *csfB* mutant, there was substantial σ^E activity in the prespore (Table 2, strains SL15366 and SL15365, respectively).

Together, the results indicate that CsfB helps curtail σ^E activity in the prespore. CsfB does so independently of its role in inhibiting σ^G. CsfB also acts to curtail the activity of σ^{E*}, which is active without processing. We think it plausible that, by analogy with its action on σ^G (1, 6, 26), CsfB directly inhibits σ^E activity. That is to say, CsfB inhibits the activity of two sigma factors. It serves a gatekeeper function in the prespore, helping to prevent σ^E from becoming active at all in the prespore and to prevent σ^G from becoming active prematurely before completion of engulfment. In an otherwise wild-type background, this gatekeeper role of CsfB for σ^E is redundant as inactivation of *csfB* does not result in σ^E activity in the prespore (data not shown), but the role is revealed in the genetic backgrounds used here. The results show that it is possible to have σ^E active in both the prespore and the mother cell. In the following section we explore how σ^E activity in both compartments can also be achieved by enhancing *spoIIG* transcription even if *csfB* is intact.

Enhanced *spoIIG* transcription in the prespore can result in σ^E being active in both the prespore and the mother cell. Results described above suggested that activation of σ^E in, or a σ^E-directed signal from, the mother cell helped prevent σ^E from becoming active in the prespore (Table 2, compare strains SL14574 and SL14631). Implicit in the conclusion is the importance of timing: if σ^E was activated sooner in the prespore, then its activity in the prespore might not be blocked by activation and/or activity in the mother cell. Additionally, earlier expression of σ^E in the prespore might help it escape the inhibitory effects of CsfB. A key factor in the timing of gene expression directed by σ^F in the prespore is the location of the gene on the chromosome (30, 49); origin-distal genes enter the prespore later than origin-proximal genes so that their expression is delayed (47). In strain SL14631 the σ^F-directed *spoIIG* locus is at an origin-distal position, 135°, so that its transcription in the prespore is delayed relative to origin-proximal genes. We tested the effect of earlier transcription of *spoIIG* in the prespore by introducing the P_{spoIIQ}-*spoIIG* construct at the origin-proximal *amyE* locus, at 25°, in a strain that has *spoIIG* expressed from its own promoter at its natural locus. The change in location to 25° indeed altered the location of σ^E activity. In a sample taken 2.5 h after the start of spore formation, over 90% of organisms with active σ^E displayed activity in both the prespore and the mother cell (Fig. 1D and Table 3, strain SL14712,). For SL14715, at T5, the figure was about 78%, with 20% having activity predominantly or exclusively in the mother cell and about 2% displaying activity predominantly in the prespore. In the same genetic background, σ^F and σ^G activities were completely confined to the prespore (strains SL14816 and SL14711, respectively) (Fig. 1J and K and Table 3) so that the presence of σ^E activity in both compartments was not a consequence of loss of septum integrity. A likely cause of the heterogeneity of location for σ^E activity with SL14712 is variability (noise) in the expression of the genes encoding σ^E, or its activators, combined with some type of switch that results in a bimodal distribution in σ^E expression (9, 12).

Expression in both the mother cell and the prespore was also observed with two other σ^E-directed promoters, *cotEp*$_1$ (strain SL14868) (Fig. 1I and Table 3) and *spoIIID* (strain SL15143) (Table 3). These promoters were weaker than the promoter for *spoIID*. In samples taken later in sporulation (T5), but not in earlier samples (T2.5), fluorescence in the prespore was often very faint for *cotEp*$_1$ and *spoIIID*, making scoring difficult (only prespores displaying substantial fluorescence are scored as positive in Table 3). We suspect that this behavior indicates relatively short-lived expression of σ^E in the prespore, combined with its turnover. Inactivation of *csfB* increased the proportion of organisms at T5 clearly displaying σ^E activity in both

TABLE 4. Effect on spore formation of σ^E being active in both the prespore and the mother cell

Relevant genotype (strain)	Location of σ^E activity[a]	No. of heat-resistant spores/ml ($\times 10^8$)[b]
spo$^+$ (SL4)	MC	5.0, 3.2
amyE::P$_{spoIIQ}$-*spoIIG* (SL14712)	PS + MC	5.0, 4.0, 4.0, 4.6
amyE::P$_{spoIIQ}$-*spoIIG csfB*::*cat* (SL14715)	PS + MC	3.0, 2.0
amyE::P$_{spoIIQ}$-*spoIIG csfB*::*cat* (SL15175)	PS + MC	4.0, 3.5

[a] MC, mother cell; PS, prespore.
[b] Sporulation was assessed 18 to 20 h after the end of exponential growth. Heat treatment was for 25 min at 80°C. Each value represents a different culture.

the prespore and the mother cell for the weakly expressed *spoIIID* promoter (Table 3, strain SL15145 compared to SL15143); it had much less effect on the stronger *spoIID* promoter (Table 3, compare strains SL14715 and SL14712).

σ^E activity in the prespore does not block spore formation. Surprisingly, strains displaying σ^E activity in both the prespore and the mother cell did not appear compromised in their abilities to form heat-resistant spores. Results of typical experiments are shown in Table 4. Much of the sporulating population of strain SL14712 showed σ^E activity in both the prespore and the mother cell (Table 3). To analyze this behavior further, individual sporulating organisms of strain SL14712 were tracked by time-lapse microscopy. Activity was detected in both compartments at various stages of prespore engulfment (Fig. 2). The development of phase-bright spores in organisms that had σ^E activity in both the prespore and the mother cell is illustrated in a movie (see Fig. S1 in the supplemental material). Because of GFP stability, it was not possible to say for how long σ^E remained active during the maturation of the prespore. However, the results do indicate that complete compartmentalization of σ^E activity is not essential for spore formation even though σ^E activity is ordinarily confined to the mother cell.

The *skin* element is not excised in the prespores of strains that display σ^E activity in the prespore. The σ^E regulon contains more than 200 genes (10, 42). They direct a variety of functions that are required for spore formation. A striking example of a late-expressed function is the excision of the 48-kb skin element from the genome of the mother cell (44). Transcription of the gene encoding the recombinase for *skin* excision, *spoIVCA*, is directed by σ^E. We tested for *spoIVCA* promoter activity but found the signal too weak to reach any conclusion about the location of its expression. Excision of *skin* is required to join together the 5′ and 3′ ends of *sigK*, the structural gene for σ^K, whose activity in the mother cell is required for spore formation. The mother cell ultimately dies, and *skin* is not excised in the developing spore (the germ line); many strains of *B. subtilis* retain *skin* in their genome. We detected σ^K activity only in the mother cell of strains with the *amyE*::P$_{spoIIQ}$-*spoIIG* construct that gave σ^E activity in both the prespore and the mother cell (data not shown). Consequently, we suspected that the *skin* element had not been excised from the prespore. To test directly for *skin* excision, we utilized a *skin* element containing a *cat* gene (23) which conferred chloramphenicol resistance. This construct was present in a strain expressing σ^E activity in both the prespore and the mother cell. The strain was maintained and induced to form spores in the absence of chloramphenicol. We tested 25 colonies derived from heat-resistant spores formed in each of 12 separate sporulation experiments (i.e., a total of 300 colonies). We detected no loss of the *cat* gene. This result indicated that the *skin* element was not excised in the prespore. Similar results were obtained with *csfB*$^+$ and *csfB* strains. Three different promoters, P$_{spoIID}$, P$_{cotEp1}$ and P$_{spoIIID}$, were used to demonstrate σ^E activity in the prespore. We infer that *B. subtilis* is able to tolerate a considerable amount of σ^E activity in the wrong cell compartment without compromising its ability to form spores. However, *skin* was not excised in the prespore, indicating that the σ^E regulon was not fully functional in the prespore. The intensity of GFP expression from the different promoters suggests that σ^E activity in the prespore is lower than its normal activity in the mother cell although we do not have firm quantitative data. We had previously shown that σ^F and σ^G do not impair each other's activity when they are coexpressed in the prespore, indicating that their activity is not limited by competition for core polymerase (5); thus, we think it unlikely that σ^E activity is limited by competition with σ^F although we cannot exclude that possibility. Structural factors might also limit the functioning of various members of the regulon in the prespore. Suggestive of such an explanation, Ramamurthi and Losick (38) have shown that a product of the σ^E regulon, SpoVM, homes only to convex membranes so that even if it were expressed in the prespore, it would not localize properly.

Compartmentalization of σ^E activity. The ability to change the location of σ^E activity helped us to explore the mechanism of compartmentalization of σ^E activity. Pro-σ^E and SpoIIGA are synthesized before the spore septum is formed. Ordinarily, pro-σ^E is processed to active σ^E only after septation because it requires the action of SpoIIR, which is expressed only after

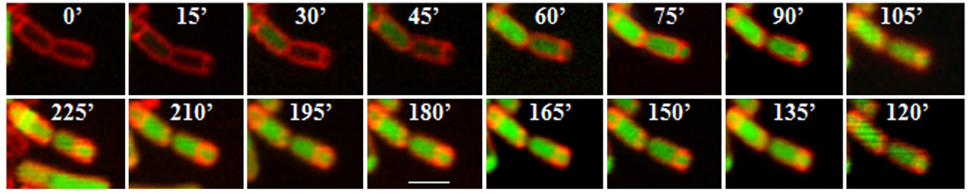

FIG. 2. Engulfment of a prespore expressing σ^E activity. Successive images from time-lapse microscopy of strain SL14712, *amyE*::P$_{spoIIQ}$-*spoIIG thrC*::P$_{spoIID}$-*gfp*. Membranes were stained with FM4-64 (red); GFP (green) indicates the location of σ^E activity. Time in each image is the time in minutes (′) from the first frame. Scale bar, 2 μm. Bacteria were placed on agarose pads containing MSSM and incubated at 33°C.

TABLE 5. Enhanced expression of *spoIIGA* in the prespore results in σ^E activity in the prespore

Strain	Relevant genotype	No. of organisms showing the indicated GFP fluorescence pattern[a]					
SL14679	*amyE*::P$_{spoIIQ}$-*spoIIGB*	178	18	0	60	1	0
SL15062	*amyE*::P$_{spoIIQ}$-*spoIIGA*	274	51	0	7	23	0
SL15139	*spoIIR*::P$_{spoIIQ}$-*spoIIR*	115	25	0	101	9	0
SL14715	*amyE*::P$_{spoIIQ}$-*spoIIG*	359	34	0	0	36	0

[a] σ^E activity was visualized using a P$_{spoIID}$-*gfp* fusion. Strains contained the *csfB*::*cat* mutation. Cultures were induced to form spores in MSSM. Samples were analyzed 2.5h after the start of spore formation. Schematic representations are given of the different types of organism present in the cultures; shaded areas indicate GFP fluorescence. Membranes were visualized by staining with FM4-64. For clarity, data for SL14715 (2h) have been recapitulated from Table 3.

septation (27). σ^E becomes active in the mother cell within 4 min of *spoIIR* transcription in the prespore (11) but does not become active in the prespore (8). The *spoIIR* locus is weakly transcribed (27), but once it is expressed, SpoIIR or pro-σ^E or SpoIIGA could be the limiting factor that helps restrict σ^E activation to the mother cell. These possibilities were tested by increasing expression of each of the corresponding structural genes in the prespore from the strong σ^F-directed *spoIIQ* promoter. The strains contained the intact *spoIIG* operon at its natural location. They also contained a *csfB* mutation in order to prevent inhibition of σ^E activity in the prespore by CsfB.

Enhanced transcription of *spoIIGB* (encoding pro-σ^E) in the prespore did not change the location of σ^E activity, which remained in the mother cell (Table 5, strain SL14679). However, enhanced transcription of *spoIIGA* in the prespore resulted in a substantial number of organisms expressing σ^E activity in the prespore as well as the mother cell (23 organisms out of 30 displaying σ^E activity) (Table 5, strain SL15062; Fig. 1L). When expression of *spoIIR* was increased by replacing its natural promoter with the stronger *spoIIQ* promoter, σ^E activity remained predominantly in the mother cell (Table 5, strain SL15139). Some organisms also displayed σ^E activity in the prespore (9 out of 110), but the effect was less dramatic than with enhanced *spoIIGA* expression. As discussed above, enhanced expression of the *spoIIG* operon also resulted in σ^E activity in both the prespore and the mother cell. Together, the results suggest that limitation of the processing machinery, SpoIIGA and SpoIIR, normally helps prevent σ^E from becoming active in the prespore.

The SpoIIR protein is exported from the prespore into the intercellular space (19) and is thought to interact with membrane-located SpoIIGA, activating it to cause the conversion of pro-σ^E into active σ^E (22). The *spoIIR* locus is poorly transcribed (27), and once it is expressed, we suggest that SpoIIGA proteins in the membranes of the prespore and the mother cell compete for the limited amount of SpoIIR that has been made. Ordinarily, SpoIIGA in the mother cell predominates, and so σ^E activation is confined to the mother cell. However, increased SpoIIGA expression in the prespore can result in σ^E also becoming active in the prespore. This interpretation also fits with the results of Ju et al. (24, 25), who expressed a fusion of the pro sequence to *gfp* in the prespore and detected no processing unless *spoIIGA* was also expressed in the prespore. Less effectively, increased expression of SpoIIR improves the chances of SpoIIGA being activated in the prespore.

The question then becomes, Why is SpoIIGA normally predominantly in the mother cell? We think that a diffusion and capture model (13, 40) may account, at least in part, for the initial compartmentalization of σ^E activation. This model is illustrated in Fig. 3. SpoIIGA is a membrane protein that likely inserts nonspecifically into the cell membrane before septation (13, 32, 43). As a consequence, we suggest that upon septation, the majority of SpoIIGA that has been synthesized before septation will be in the membrane surrounding the mother cell (perhaps a 5-fold excess over the amount in the membrane around the prespore). SpoIIGA is freely diffusible within its membrane and can diffuse into the face of the septal mem-

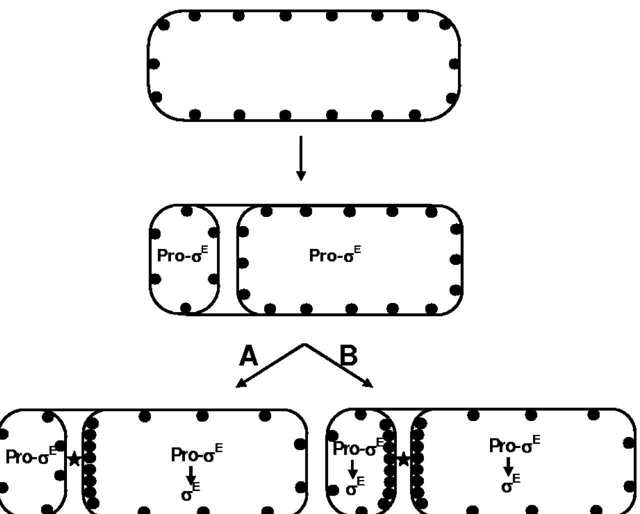

FIG. 3. Schematic representation of a model for the compartmentalization of σ^E activation. The membrane protein SpoIIGA is indicated by filled circles, and SpoIIR is indicated by a star. SpoIIGA is first synthesized before the spore septum is formed and is distributed randomly throughout the cell membrane. As a consequence, upon septation the large majority of SpoIIGA is located in the mother cell. Some component of the recently completed septum captures SpoIIGA that has diffused from the peripheral membrane. When SpoIIR is synthesized, it is secreted into the space between the septal membranes. There, it can interact with SpoIIGA from either the mother cell or the prespore. (SpoIIR may or may not be the component that captures SpoIIGA in the septum.) Because the preponderance of SpoIIGA is in the mother cell, mother cell SpoIIGA wins out in the competition for the very limited amount of SpoIIR. As a consequence, only SpoIIGA in the mother cell is activated, and so conversion of pro-σ^E to active σ^E occurs only in the mother cell (shown in path A). However, if expression of SpoIIGA in the prespore is artificially enhanced, then activation of σ^E can also occur in the prespore (path B).

brane. SpoIIR is exported from the prespore and localizes to the sporulation septum (39), where presumably it interacts with SpoIIGA from either the prespore or the mother cell membrane. Because there is excess SpoIIGA in the mother cell membrane, the mother cell wins out in the competition for the limited SpoIIR available, and σ^E becomes active only in the mother cell. SpoIIGA functions as dimer or higher multimer (22), which may enhance this selectivity. Additionally, some component of the septum (not necessarily SpoIIR) may capture and hence help concentrate SpoIIGA in the septum. Once σ^E is active in the mother cell, it may direct a signal to the prespore to block activation in the prespore. The studies of Fawcett et al. (13) on SpoIIGA-GFP localization in *B. subtilis* and *Bacillus megaterium* are consistent with the model in Fig. 3. Fawcett et al. found that SpoIIGA is initially located in the peripheral membrane and then becomes concentrated at the septum; indeed, they proposed that SpoIIGA is sequestered (captured) by some unknown component of the septum (13).

A second factor in the normal compartmentalization of σ^E activity is enhanced transcription in the mother cell of the *spoIIG* operon (14). It remains unclear if the increase in *spoIIG* transcription occurs before or after σ^E first becomes active in the mother cell. The increase occurs soon after septation (14). However, σ^E becomes active in the mother cell within 4 min of σ^F-directed *spoIIR* transcription in the prespore (11), and σ^F activation is thought to follow very rapidly after septum formation (17, 20, 21). If increased *spoIIG* transcription occurs before σ^E activation, then it could be important for both establishing and maintaining compartmentalization; if *spoIIG* transcription increases after σ^E activation, then its role is in maintaining compartmentalization. This Spo0A-mediated increase in *spoIIG* transcription (14, 15) would "lock in" σ^E activity in the mother cell.

Prespore-specific proteolysis of σ^E is also important for enforcing compartmentalization (14, 25, 37), and we think that this proteolysis may be activated by a σ^E-directed signal from the mother cell. CsfB provides an additional barrier to inappropriate σ^E activation in the prespore. The existence of such multiple controls suggests that compartmentalizing σ^E activity to the mother cell is important for spore formation. It is therefore surprising to find that *B. subtilis* can still form spores efficiently despite a substantial loss of σ^E compartmentalization. However, only a small selective advantage, below differences detectable in our assay, would be sufficient to ensure complete compartmentalization of σ^E activity in the mother cell in a wild-type strain.

It is interesting to compare the proposed model for compartmentalizing σ^E in the mother cell (Fig. 3) with the models for compartmentalization of σ^F in the prespore (20, 21). Models for both sigma factors invoke cell size asymmetry together with sequestration in the septum of a membrane protein critical to activation, SpoIIGA for σ^E and SpoIIE for σ^F. Yet opposite results are achieved with respect to cell compartment. The actual activation mechanisms are very different: release from the anti-sigma factor SpoIIAB for σ^F because of activation of the anti-anti-sigma factor SpoIIAA by SpoIIE-mediated dephosphorylation (20, 21) and cleavage of the inactive precursor pro-σ^E for σ^E (31, 43). All the components for activation of σ^F are already present before septation. Indeed, SpoIIE is involved in formation of the septum and initially is probably present in comparable levels on both faces of the septum (18). It is thought to be the sudden change in ratio of soluble components to septum-bound components, which accompanies formation of the prespore but not the mother cell, that shifts the equilibrium, triggering σ^F activation in the prespore; this switch is then locked in by hysteresis (20, 21).

For σ^E, the critical component SpoIIR is synthesized only after the septum has formed (48). In addition, we suggest that SpoIIGA comes from peripheral membrane to the septum after completion of septation so that it is predominantly on the mother cell face (Fig. 3) (13). Consequently, the excess of SpoIIGA already present in the mother cell face of the septum wins out in the competition to be activated by the small amount of SpoIIR that has been made so that σ^E activation is confined to the mother cell (Fig. 3).

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