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Forespore Expression and Processing of the SigE Transcription Factor in Wild-Type and Mutant *Bacillus subtilis*

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σ^E is a mother cell-specific transcription factor of sporulating *Bacillus subtilis* that is derived from an inactive precursor protein (pro-σ^E). To examine the process that prevents σ^E activity from developing in the forespore, we fused the σ^E structural gene (*sigE*) to forespore-specific promoters (P_{dacF} and $P_{spoIIIG}$), placed these fusions at sites on the *B. subtilis* chromosome which translocate into the forespore either early or late, and used Western blot analysis to monitor SigE accumulation and pro-σ^E processing. *sigE* alleles, placed at sites which entered the forespore early, were found to generate more protein product than the same fusion placed at a late entering site. SigE accumulation and processing in the forespore were enhanced by null mutations in *spoIIIE*, a gene whose product is essential for translocation of the distal portion of the *B. subtilis* chromosome into the forespore. In other experiments, a chimera of pro-σ^E and green fluorescence protein, previously shown to be unprocessed if it is synthesized within the forespore, was found to be processed in this compartment if coexpressed with the gene for the pro-σ^E-processing enzyme, SpoIIGA. The need for *spoIIGA* coexpression is obviated in the absence of SpoIIIE. We interpret these results as evidence that selective degradation of both SigE and SpoIIGA prevent mature σ^E from accumulating in the forespore compartment of wild-type *B. subtilis*. Presumably, a gene(s) located at a site that is distal to the origin of chromosome transfer is responsible for this phenomenon when it is translocated and expressed in the forespore.

Early in the process of endospore formation, *Bacillus subtilis* partitions itself into two unequal compartments with dissimilar developmental fates. The smaller compartment ultimately becomes the spore, while the larger compartment assumes the role of mother cell, engulfing and nurturing the developing forespore and then lysing when the spore matures. Developmental gene expression is unique to each of the two compartments and is dictated by novel sigma (σ) factors which become active only in one or the other compartment (reviewed in reference 39). σ^E is the first of the alternative sigma factors to appear in the mother cell, with σ^F as its counterpart in the forespore (9, 13, 16, 27, 28, 40). Both σ^E and σ^F are synthesized at the onset of sporulation, but neither is active until 1.5 to 2 h later, when the forespore septum establishes the separate mother cell and forespore (9, 13, 21, 22, 27, 28, 41, 44, 45). Each of these sigma factors is kept silent by unique means. σ^F is bound to an anti-σ^F protein (SpoIIAB) which blocks its activity, while σ^E is formed as a pro-protein (pro-σ^E) which becomes active only after 27 amino acids are cleaved from its amino terminus (1, 7, 8, 11, 12, 19, 22, 25, 30, 31, 36–38).

σ^F is freed from SpoIIAB by the action of a second protein (SpoIIAA), which triggers σ^F release by binding to SpoIIAB (1, 8, 11). SpoIIAB is a SpoIIAA-specific kinase, as well as a binding protein (1, 30). Phosphorylated SpoIIAA is ineffective in driving the release of σ^F (10, 11). Before compartmentalization, most of the SpoIIAA is phosphorylated and inactive. SpoIIAA-P is reactivated by a phosphatase (SpoIIE) that becomes bound to the sporulation septum (2–4, 10). It has been speculated that the septal location of the phosphatase might establish a higher phosphatase-to-kinase ratio in the small forespore compartment than in the large mother cell and that this could drive selective σ^F activation in the forespore (10).

Activation of pro-σ^E requires a sporulation-specific protease (SpoIIGA) that is coexpressed with pro-σ^E at the onset of sporulation (18, 33, 38). Although both the protease and substrate are present in the predivisional cell, the processing reaction does not occur until a specific signal protein (SpoIIR) triggers the reaction (20, 26). SpoIIR is produced in the forespore under the control of σ^F (20, 26). It is believed that SpoIIGA is an integral membrane protein that accumulates at the forespore septum membrane. At this site, SpoIIGA is positioned to interact with SpoIIR, which is being secreted by the forespore (15, 19). Thus, the activation of σ^E, as well as σ^F, is tied to the formation of the forespore septum. This dependence on septation explains the timing of σ^F and σ^E activation but leaves the question of compartment-specific σ^E activation unresolved. Both pro-σ^E and SpoIIGA, having been synthesized before the sporulation cell division, should be present in both compartments. An intriguing hypothesis is that there is a directionality to SpoIIGA activation by SpoIIR and that only the mother cell's SpoIIGA is positioned in the septal membrane in an orientation appropriate to receive the SpoIIR signal (15). Although this mechanism is possible, other factors are likely to also be involved. Vegetative *B. subtilis*, expressing *spoIIR* from a gratuitous promoter, can process pro-σ^E if SpoIIGA is present (26). Thus, although transseptal signaling likely occurs, it does not appear to be essential for processing. In addition, a strain of *B. subtilis* in which *spoIIR* was expressed prior to septation still acquired mother cell-specific σ^E activity (48). Apparently, a device other than the forespore-specific expression of *spoIIR* plays a role in establishing the mother cell-specific activity of σ^E. Using a chimera of a portion of pro-σ^E fused to green fluorescent protein (GFP) as a probe of the processing reaction, we had found that pro-σ^E::GFP could be processed following septation if it was synthesized in the predivisional cell but not if it was expressed

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from a forespore-specific promoter (e.g., P_{dacF}) (19). This suggested that the pro-σ^E processing reaction is limited to the mother cell, although the reason for this restriction remained obscure. Recently, Pogliano et al. used fluorescence microscopy to show that pro-σ^E and σ^E are absent from the forespore and that their disappearance requires functional SpoIIIE (35). SpoIIIE is known to be essential for the translocation of the distal 70% of the bacterial chromosome into the forespore (46). In the present study, we examined this phenomenon in greater detail and revisited the question of the mother cell-specific processing of pro-σ^E. We found that the ability of SigE to accumulate, when expressed from a forespore-specific promoter, is dependent on the site of *sigE* expression on the *B. subtilis* chromosome and the state of SpoIIIE. P_{dacF}-*sigE* is more likely to generate a product which can accumulate and be processed into σ^E if it is expressed from a locus on the chromosome that is translocated to the forespore early (e.g., *amyE* or *ctc*) than if it lies at a late entering site (e.g., *dacF*). The absence of SpoIIIE further enhances this accumulation and processing. We also determined that a pro-σ^E::GFP hybrid protein, which had been previously shown to be unprocessed if expressed in the forespore, is processed in that compartment if it is either coexpressed with its processing enzyme (SpoIIGA) or synthesized in a strain which lacks SpoIIIE. Our data are consistent with the notion that a forespore-specific factor, likely a proteolytic activity encoded by a segment of the *Bacillus* chromosome which enters the forespore late, blocks the formation of active σ^E in the forespore by removing both pro-σ^E/σ^E and SpoIIGA from that compartment.

MATERIALS AND METHODS

Bacterial strains and plasmids. The *B. subtilis* strains and plasmids used in this study are listed in Table 1. The transcriptional fusions of the *spoIIIG* and *dacF* promoters to *sigE* or *sigE335* were constructed in two stages. A 0.43-kbp *Hin*dIII/*Pst*I fragment from pGSIIG11 (41), containing the *spoIIIG* promoter, or a 0.42-bp *Hin*dIII fragment from pPP212 (37), containing the *dacF* promoter, were cloned into pUS19 (5) that had been cut with either *Hin*dIII or *Hin*dIII/*Pst*I, as appropriate. The promoters were oriented toward the unique *Pst*I site in the vector. A *Pst*I fragment of approximately 1.1 kbp, containing *sigE* or *sigE335* (32), was cloned into this *Pst*I site, downstream of each of the two promoters. The resulting clones were analyzed by restriction endonuclease digestions to verify proper orientation of *sigE* relative to the promoters. This procedure yields plasmids pFE335, pGE335, pFE-1, and pGE1 (Table 1). A DNA fragment containing *spoIIGA*, with *Sal*I and *Eco*RI sites bracketing it, was generated by PCR techniques and cloned in the *Sal*I/*Eco*RI site downstream site of the *dacF* promoter. This yielded plasmid pFIIGA.

pEL-1 encodes a composite protein with the first 55 amino acids of pro-σ^E fused to LacZ (17). Transcriptional fusions of the *spoIIIG* and *dacF* promoters to *sigE-lacZ* were constructed by joining the *Hin*dIII or *Hin*dIII-*Pst*I promoter pieces that were used in constructing the fusions of these promoters to *sigE* to *Hin*dIII or *Hin*dIII-*Pst*I-cut pEL-1. This procedure yields plasmid pGEL-1 ($P_{spoIIIG}$) and pFEL-1 (P_{dacF}).

PUK19 was constructed by cloning a 1.5-kbp *Cla*I fragment carrying a gene encoding kanamycin resistance (*aph3′5″*) from pJH1 (23) into *Cla*I-cut pUC19. pUK191 is a 0.9-kbp *Eco*RI fragment from pCT050 (42) containing the *ctc* gene cloned into the *Eco*RI site of pUK19. pF1 contains a *sigE55-gfp* in-frame fusion under the control of the *dacF* promoter. An inducible source of σ^F (P_{spac}-*spoIIAC*) was introduced into strains by transformation with chromosomal DNA from SL4342 (37) and selection for the linked Erm marker. Strains containing *dacF* promoter fusions at *amyE* or *ctc* were constructed by transforming the fusion plasmids into strains (SC191 and SL4834) with related plasmids already integrated at these sites. Clones in which the *dacF* fusions had integrated at the *amyE* or *ctc* locus were identified by cotransformation of the antibiotic resistances of the *dacF* and resident plasmids.

Visualization of fluorescence. Fluorescence was visualized as described previously (4, 19, 24, 43). Culture samples of 200 μl were transferred to 1.5-ml microcentrifuge tubes on ice, 50 μl of preservation buffer (40 mM NaN_3, 50% sucrose, 0.5 M Tris [pH 7.45], 0.77 M NaCl) was added, and the suspension was incubated at 4°C for at least 2 h. A 3-μl aliquot of the cell suspension was mixed with 1 μl of 4′,6-diamidino-2-phenylindole (DAPI, 1 μg/ml; Sigma) on a slide precoated with 0.01% polylysine (Sigma) and covered with a polylysine-coated coverslip. Cells were viewed with a Zeiss Axiophot epifluorescence microscope with a 100-W mercury lamp source and a 100× Plan-Neofluar oil immersion objective lens. Images were recorded on Kodak TMZ p3200 professional film. The images were scanned and prepared with Adobe Photoshop version 4.0.

Images of the same field were obtained under conditions for recording fluorescence of GFP, phase-contrast micrographs, and DAPI staining. The camera was set at the automatic mode for all pictures. GFP fluorescence was viewed with a fluorescein isothiocyanate filter set (Chroma Technology; 450-490 exciter filter, FT510 chromatic beam splitter, and LP520 barrier filter). DAPI-stained images were obtained with a fluorescein isothiocyanate filter set (Chroma Technology; BP 365/12 exciter filter, FT395 chromatic beam splitter, and LP397 barrier filter).

Western blot analysis. Crude cell extracts were prepared from *B. subtilis* by disrupting bacteria with a French pressure cell. The protein concentration was determined by a Bio-Rad protein assay in accordance with the manufacturer's instructions. Extracts were fractionated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis with 12% acrylamide. Subsequent steps were as described previously (41), using either locally prepared anti-σ^E monoclonal antibody or commercial anti-GFP monoclonal antibody (Clontech Laboratories, Inc., Palo Alto, Calif.) as the probe. The commercial antibody cross-reacted with

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
RL1059	*spoIIIE::spc*	35
SL4834	*trpC2 metC erm amyE::dacF-lacZ*	37
SEK84	*kan/sigEΔ84*	Laboratory strain
SEK8401	*sigEΔ84 spoIIIE::spc*	RL1059→SEK84
SL4342	*spoIIGΔ1 trpC phe-1 erm* P_{spac}-*spoIIAC*	37
SFE1	*sigE* P_{dacF}-*sigE335*	pFE335→SMY
SFE2	*sigE* P_{dacF}-*sigE335* P_{spac}-*spoIIAC*	SL4342→SFE1
SFE3	*sigE* P_{dacF}-*sigE55-lacZ*	pFEL-1→SMY
SFE4	*sigE* P_{dacF}-*sigE55-lacZ* P_{spac}-*spoIIAC*	SL4342→SFE3
SFE5	*amyE::*P_{dacF}-*sigE335*	pFE335→SL4834
SFE6	*amyE::*P_{dacF}-*sigE335 cat*	pJM102→SFE5
SFE7	*sigE* P_{dacF}-*sigE*	pFE1→SMY
SFE8	*amyE-*P_{dacF}-*sigE*	pSFE1→SL4834
SFE9	P_{dacF}-*sigE cat*	pJM102→SFE7
SFE10	*sigEΔ84 amyE::*P_{dacF}-*sigE335*	SEK84→SFE5
SFE11	*sigEΔ84 amyE::*P_{dacF}-*sigE335 spoIIIE::spc*	SFE5→SEK8401
SFE12	*sigEΔ84* P_{dacF}-*sigE335*	SEK84→SFE1
SFE13	*sigEΔ84 amyE::*P_{dacF}-*sigE335 spoIIIE::spc*	SFE6→SEK8401
SFE14	*sigEΔ84 amyE::*P_{dacF}-*sigE*	SEK84→SFE8
SFE15	*sigEΔ84 amyE::*P_{dacF}-*sigE spoIIIE::spc*	SFE8→SEK8401
SFE16	*sigEΔ84* P_{dacF}-*sigE*	SEK84→SFE7
SFE17	*sigEΔ84* P_{dacF}-*sigE spoIIIE::spc*	SFE9→SEK8401
SFG1	P_{dacF}-*sigE55-gfp*	pF1→SMY
SFG2	P_{dacF}-*sigE55-gfp* P_{dacF}-*spoIIGA*	pFIIGA→SFG1
SFG3	*sigEΔ84* P_{dacF}-*sigE55-gfp spoIIIE::spc*	SFG1→SEK8401
SFG4	*ctc::*P_{dacF}-*sigE55-gfp*	pF1→SC191
SFG5	*ctc::*P_{dacF}-*sigE55-gfp::*P_{dacF}-*spoIIGA*	pFIIGA→SFG4
SFG6	*sigEΔ84 ctc::*P_{dacF}-*sigE55-gfp spoIIIE::spc*	SFG4→SEK8401
SC191	*ctc::pUK191*	pUK191→SMY
SGE1	*sigE* $P_{spoIIIG}$-*sigE355*	pGE335→SMY
SGE2	*sigE* $P_{spoIIIG}$-*sigE355* P_{spac}-*spoIIAC*	SL4342→SGE1
SGE3	*sigE* $P_{spoIIIG}$-*sigE-lacZ*	pGEL-1→SMY
SGE4	*sigE* $P_{spoIIIG}$-*sigE-lacZ* P_{spac}-*spoIIAC*	SL4342→SGE3
Plasmids		
pUS19	*bla spc*	5
pUK19	*bla kan*	This study
pPP212	*bla dacF*	45
pFE335	*bla spc* P_{dacF}-*sigE335*	This study
pGE335	*bla spc* P_{IIG}-*sigE335*	This study
pFE-1	*bla spc* P_{dacF}-*sigE*	This study
pGE-1	*bla spc* P_{IIG}-*sigE*	This study
pF1	*bla cat* P_{dacF}-*sigE55-gfp*	19
pCT050	*cat ctc*	42
pUK191	*bla kan ctc*	This study
pFEL-1	*bla spc* P_{dacF}-*sigE-lacZ*	This study
pGEL-1	*bla spc* P_{IIG}-*sigE-lacZ*	This study
pGSIIG11	*bla cat spoIIGB spoIIIG*	41
pEL-1	*bla cat sigE-lacZ*	17
pFIIGA	*bla spc* P_{dacF}-*spoIIGA*	This study

several proteins in the crude extracts. These were variable with each antibody lot and were identified by comparing the experimental extracts with similar extracts from cells without the fusion. Bound antibody was visualized with an alkaline phosphatase-conjugated goat immunoglobulin against mouse immunoglobulin (American Qualex) by using either an alkaline phosphate substrate kit (Bio-Rad) or CDP-Star (Boehringer Mannheim) as the substrate reagent.

β-Galactosidase assays. Cells were harvested by centrifugation, resuspended in Z buffer (29), and disrupted by passage through a French pressure cell. Cell debris was removed by centrifugation (10,000 × *g*) for 10 min, and the supernatant was analyzed for β-galactosidase by using the reagents described by Miller (29). Protein concentrations were determined by a Bio-Rad assay using the procedures recommended by the manufacturer. β-Galactosidase activity was expressed as $\Delta A_{420} \times 1{,}000 \times \text{min}^{-1} \times \text{mg of protein}^{-1}$.

General methods. DNA manipulation and the transformation of *Escherichia coli* were done in accordance with standard protocols. Transformation of competent *B. subtilis* cells was carried out by the method of Yasbin et al. (47).

RESULTS

Expression of *sigE335* from forespore-specific promoters. The product of the *sigE335* allele lacks 15 amino acids in the pro-σ^E amino terminus (32). It is not recognized for processing into mature σ^E, but it is at least partially active without processing (32). The smaller size of σ^{E335} and its failure to be converted into σ^E allow it to be distinguished from the wild-type *sigE* products in Western blot analyses. To investigate whether there are factors in the forespore that influence σ^E accumulation or activity in this compartment, we joined *sigE335* to promoters (P_{dacF} and $P_{spoIIIG}$) whose transcription depends on the forespore-specific σ factor σ^F (37, 40). When P_{dacF}-*sigE335* or $P_{spoIIIG}$-*sigE335* is transferred on integrative plasmids into *Bacillus*, it can recombine into the chromosomal sites of the promoter sequences (i.e., *dacF* and *spoIIIG*). This results in a duplication of the promoters, with one promoter driving *sigE335* and the second driving its normal operon. Integration is also possible at the *sigE* sequences. At this site, most integration events would exchange *sigE335* for *sigE* as the allele that is expressed from the normal *sigE* promoter (P_{spoIIG}) (41). Cells which carry σ^{E335} as their principal source of σ^E are Spo$^-$ (32). Approximately 20% of the transformants that received either of the *sigE335* plasmids were Spo$^-$. Western blot analysis of the *sigE* proteins in extracts from representative Spo$^-$ clones revealed only σ^{E335}, with its synthesis beginning when P_{spoIIG} becomes active at the onset of sporulation (data not shown). These Spo$^-$ clones likely represent transformants in which the plasmid integrated within *sigE* to place *sigE335* downstream of P_{spoIIG}. When the Spo$^+$ transformants were analyzed for their *sigE* products, pro-σ^E and σ^E were readily observed, but no σ^{E335} could be detected in the strains transformed with either P_{dacF}-*sigE335* (Fig. 1A) or $P_{spoIIIG}$-*sigE335* (Fig. 1C). To verify that the putative P_{dacF}-*sigE335*- and $P_{spoIIIG}$-*sigE335*-containing strains were properly configured to express *sigE335* in response to σ^F activation, we transformed an inducible source of σ^F (i.e., P_{spac}-*spoIIAC*) into these strains and examined the effects of σ^F synthesis on σ^{E335} accumulation during vegetative growth. As shown in Fig. 1B and D, both the P_{dacF}-*sigE335*- and $P_{spoIIIG}$-*sigE335*-containing strains accumulated σ^{E335} in response to σ^F induction.

Our ability to detect σ^{E335} from P_{dacF}-*sigE335* and $P_{spoIIIG}$-*sig335* following σ^F synthesis in vegetatively growing *B. subtilis*, but not during sporulation, suggests either that we are inducing σ^F-dependent transcription more effectively in our artificial system or that there are additional factors in the sporulating cells that inhibit σ^{E335} accumulation. To test the relative activities of the *dacF* and *spoIIIG* promoters under our two conditions, we replaced the *sigE335* alleles in our constructions with a chimeric *sigE55-lacZ* gene. The resulting P_{dacF}-*sigE55-lacZ* and $P_{spoIIIG}$-*sigE55-lacZ* genes have the σ^F-dependent promoters of the previous constructions along with the *sigE* translational regulatory elements and approximately 50 codons from the amino terminus of *sigE* but express a β-galactosidase-like protein as their product. When these constructions were transformed into *B. subtilis*, the resulting clones displayed appreciable sporulation-specific β-galactosidase synthesis (Fig. $1A_2$ and C_2). We then transformed P_{SPAC}-*spoIIAC* into these strains and examined the levels of β-galactosidase following σ^F synthesis during vegetative growth. A comparison of the β-galactosidase levels formed under this circumstance with that observed in sporulating cultures should reflect the relative activities of the *dacF* and *spoIIIG* promoters under these two conditions. We found that the β-galactosidase levels of the vegetatively induced cultures were less than half of those seen when the parent strains were allowed to sporulate (compare Fig. $1B_2$ and D_2 with Fig. $1A_2$ and C_2). Thus, based on the relative levels of β-galactosidase that are seen under the artificial and natural inducing conditions, we would have expected to see twice as much σ^{E335} in the P_{dacF}-*sigE335* and $P_{spoIIIG}$-*sigE335* strains during sporulation as was present following the vegetative inductions. The absence of σ^{E335} in the sporulating cultures argues that there are additional factors that restrict σ^{E335} accumulation within the forespore compartment at the time when the *dacF* promoter becomes active. Given that *sigE-lacZ* and *sigE335* have common transcriptional and translational regulatory elements, selective turnover of σ^{E335} in the forespore becomes a plausible possibility for its failure to accumulate.

Expression of *sigE* alleles from the *amyE* locus in wild-type and *spoIIIE* mutant strains. It has been reported that pro-σ^E/σ^E can persist in the forespore compartment of cells with mutations at *spoIIIE* (35). SpoIIIE is an essential sporulation protein that is needed for the translocation of the forespore's chromosome into that compartment (46). In the absence of SpoIIIE, only the first 20 to 30% of the chromosome enters the forespore (46). For a σ^F-dependent gene to be effectively expressed in a *spoIIIE* mutant, it must be at a site on the chromosome that enters the forespore. We wished to investigate the effects of the loss of SpoIIIE on our ability to synthesize σ^{E335} in the forespore; however, neither the *dacF* nor *spoIIIG* locus enters the forespore in a *spoIIIE* mutant strain. We therefore transferred the P_{dacF}-*sigE335* fusion to a site (*amyE*) that does enter the forespore in a *spoIIIE* mutant background. The strain we used in our analysis had an additional mutation (*sigEΔ84*) which eliminates the synthesis of pro-σ^E/σ^E from its normal locus (*spoIIG*) (17, 32). In this strain, the product of the *amyE*::P_{dacF}-*sigE* fusion is the cell's sole source of SigE and the only protein that will react with our anti-σ^E antibody in Western blots. Prior to investigating the effect of a loss of *spoIIIE* on σ^{E335} accumulation, we examined the expression of *sigE335* at *amyE* in a strain with a wild-type *spoIIIE* allele. Surprisingly, *sigE335* expressed from P_{dacF} at the *amyE* locus, unlike *sigE335* expressed from this same promoter at *dacF*, could be detected in Western blots (Fig. 2A, lane 1). Although σ^{E335} was evident, an abundant higher-mobility band was also detected by the anti-σ^E antibody. We interpret this secondary material to be a σ^{E335} breakdown product. Our ability to now detect σ^{E335} from a forespore-expressed promoter is not due to the absence of wild-type *sigE* in this strain but rather to the expression of *sigE335* from the *amyE* locus. A strain with P_{dacF}-*sigE335* at *dacF* and the *sigEΔ84* allele at *spoIIG* still fails to accumulate σ^{E335} (Fig. 2A, lane 3). Apparently, σ^{E335} can accumulate to detectable levels in the forespore if it is expressed from a site on the *B. subtilis* chromosome that translocates to the forespore early (i.e., *amyE*) but not if it is expressed from a late entering site (i.e., *dacF* or *spoIIIG*). We next examined the accumulation of σ^{E335} in strains with null mutations in *spoIIIE*. *sigE335* expressed from P_{dacF} at *amyE* in

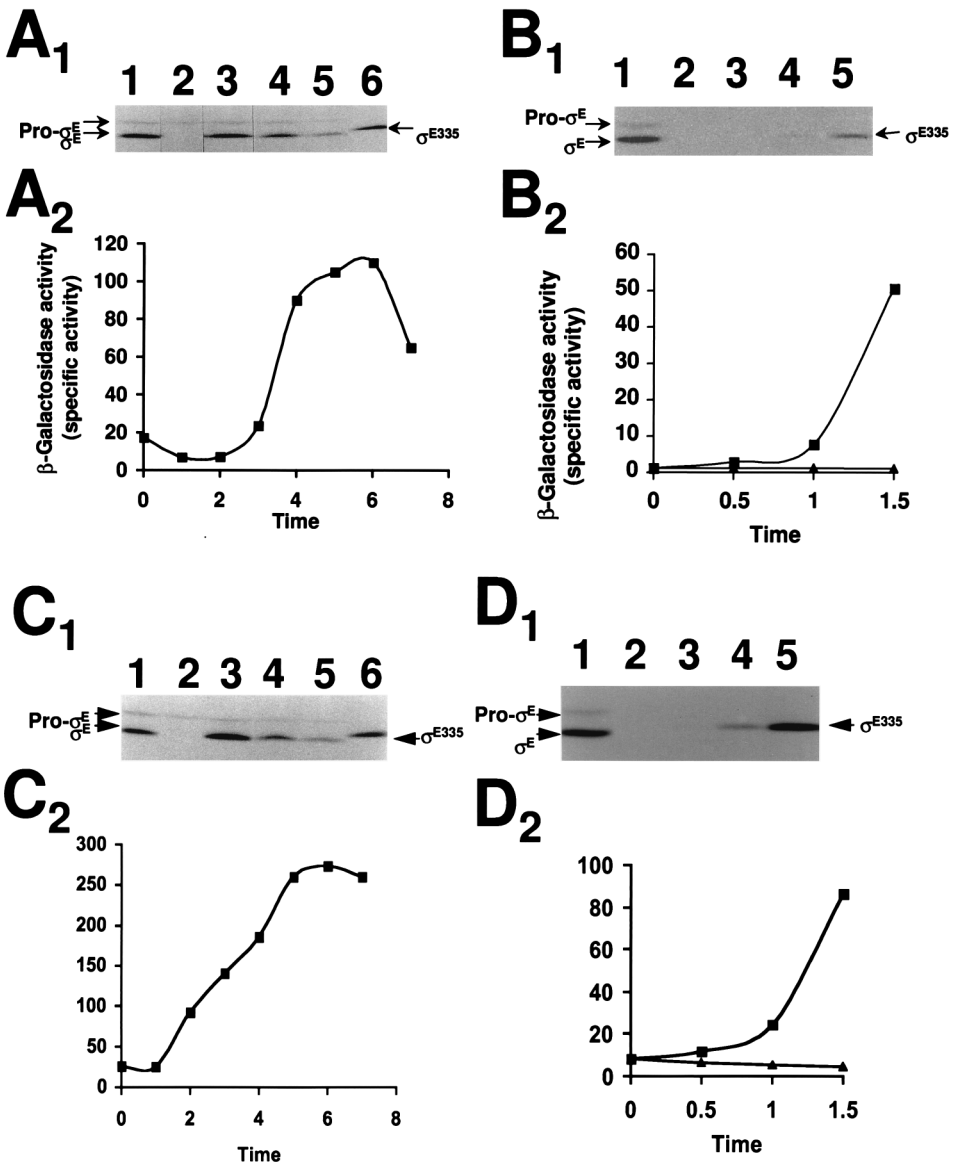

FIG. 1. Accumulation of σ^{E335} and SigE55-LacZ in *B. subtilis* strains. (A_1 and C_1) Western blot analysis of *B. subtilis* SFE1 (P_{dacF}-*sigE335*) and SGE1 ($P_{spoIIIG}$-*sigE335*), respectively. Cells were grown in DS medium with samples taken at 1 (lanes 2), 3 (lanes 3), 5 (lanes 4), and 7 (lanes 5) h after the onset of sporulation. Lanes 1 and 6 contain control extracts from wild-type *B. subtilis* (SMY) and strain SE335 at t_3 of sporulation, respectively. Each lane contained 100 μg of extract which was analyzed for SigE-like proteins by Western blotting as described in Materials and Methods. The positions at pro-σ^E, σ^{E335}, and σ^E are indicated. (A_2 and C_2) β-Galactosidase levels in *B. subtilis* SFE3 (P_{dacF}-*sigE55-lacZ*) and SGE3 ($P_{spoIIIG}$-*sigE55-lacZ*). Cells were grown in DS medium, and samples were taken at the indicated times after the onset of sporulation and analyzed for β-galactosidase activity as described in Materials and Methods. β-Galactosidase units are expressed as $\Delta A_{420} \times 1{,}000 \times \text{min}^{-1} \times \text{mg of protein}^{-1}$. ($B_1$ and D_1) Western blot analysis of strain SFE2 (P_{dacF}-*sigE335* P_{SPAC}-*spoIIAC*) and SGE2 ($P_{spoIIIG}$-*sigE335* P_{SPAC}-*spoIIAC*), respectively. Cells were grown in LB medium and exposed to isopropyl-β-D-thiogalactopyranoside (IPTG; 1 mM) to induce σ^F synthesis. Samples were taken at the time of IPTG addition (lanes 2) and 0.5 (lanes 3), 1 (lanes 4), and 1.5 (lanes 5) h thereafter and analyzed by Western blotting. Lanes 1 contain strain SMY at t_3. The positions of pro-σ^E, σ^{E335}, and σ^E are indicated. (B_2 and D_2) β-Galactosidase levels in strain SFE4 (P_{dacF}-*sigE55-lacZ* P_{SPAC}-*spoIIAC*) and SGE4 ($P_{spoIIIG}$-*sigE55-lacZ* P_{SPAC}-*spoIIAC*), respectively. Cells, grown in LB medium, were exposed to 1 mM IPTG (■) or were allowed to continue to grow in its absence (▲). Samples were taken at the time of IPTG addition and at 0.5-h intervals thereafter and were analyzed for β-galactosidase activity as described above.

a *spoIIIE* mutant strain generated σ^{E335}; however, unlike the SpoIIIE$^+$ strain, the putative breakdown product was minimal (Fig. 2A, lane 2). The presence of likely σ^{E335} breakdown products in the wild-type but not the *spoIIIE* mutant strain suggests that σ^{E335} is more stable in the absence of SpoIIIE. This would be consistent with the finding of Pogliano et al. that SpoIIIE facilitates the disappearance of pro-σ^E/σ^E from the forespore (35). Although the *dacF* locus does not translocate to the forespore in the absence of SpoIIIE, the P_{dacF}-*sigE335* fusion, positioned at *dacF* in the *sigE*Δ*84 spoIIIE* mutant strain, expressed a small amount of σ^{E335} (Fig. 2A, lane 4). Presumably, the small amount of σ^{E335} found in this strain is a consequence of σ^F activation in the mother cell compartment. σ^F has been reported to become partially active in the mother cell compartment in a SpoIIIE mutant background (35, 44) and possibly more active if σ^E is also absent (35).

pro-σ^E is more stable than the products of *sigE* alleles with deletions in their pro sequences (6, 22, 32). Assuming that pro-σ^E might be more readily detectable than σ^{E335} in the forespore environment, where heightened proteolysis is suspected,

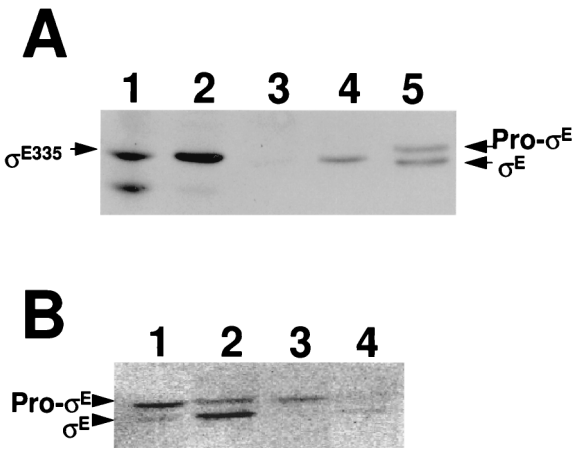

FIG. 2. Western blot analysis of P_{dacF}-*sigE335* (A) and P_{dacF}-*sigE* (B) in sporulating *B. subtilis*. Cells were grown in DS medium and harvested at 4 h after the onset of sporulation. Development of the blots was normalized to a control sample included in the analysis. One hundred micrograms of extract was analyzed for each lane except for lane 5 in panel A, which contains 15 μg of protein from strain SMY at t_3. The positions of pro-σ^E, σ^{E335}, and σ^E are indicated. (A) Lanes: 1, SFE10 (*sigEΔ84 amyE*::P_{dacF}-*sigE335*); 2, SFE11 (*sigEΔ84 spoIIIE*::*spc amyE*::P_{dacF}-*sigE335*); 3, SFE12 (*sigEΔ84* P_{dacF}-*sigE335*); 4, SFE13 (*sigEΔ84 spoIIIE*::*spc* P_{dacF}-*sigE335*); 5, SMY (wild type). (B) Lanes: 1, SFE14 (*sigEΔ84 amyE*::P_{dacF}-*sigE*); 2, SFE15 (*sigEΔ84 spoIIIE*::*spc amy*::P_{dacF}-*sigE*); 3, SFE16 (*sigEΔ84* P_{dacF}-*sigE*); 4, SFE17 (*sigEΔ84 spoIIIE*::*spc* P_{dacF}-*sigE*).

we constructed a P_{dacF}-*sigE* fusion, placed it at *dacF* or *amyE*, and examined the resulting strains for evidence of pro-σ^E accumulation and processing. P_{dacF}-*sigE* expressed in a *sigEΔ84* background from either the *amyE* or *dacF* locus yielded detectable products in Western blot analyses (Fig. 2B). This accumulation was, however, approximately 10% of that which we would have anticipated, based on the strength of the *dacF* promoter (data not shown). The pro-σ^E expressed at *amyE* (Fig. 2B, lane 1) was more abundant than the pro-σ^E expressed at *dacF* (Fig. 2B, lane 3), and a portion of it was processed into mature σ^E. Although there was no apparent processing of the pro-σ^E that was expressed at the *dacF* locus (Fig. 2B, lane 3), the degree of processing observed in the strain that expressed pro-σ^E from *amyE* was minimal (Fig. 2B, lane 1) and the synthesis of pro-σ^E from the *dacF* locus was relatively low (Fig. 2B, lane 3). Thus, it is not clear if the absence of processing in the latter case is due merely to low pro-σ^E abundance and our failure to detect the processed product or to the lack of a processing potential. The difference in the relative abundance of the wild-type *sigE* products synthesized at *amyE* versus those synthesized at *dacF* was less than that which we observed for *sigE335* products. This may be due to the stabilizing effect of the pro sequence on the fusion that was expressed at *dacF*. Disruption of *spoIIIE* resulted in an increase in the ratio of mature σ^E to pro-σ^E in the strain where pro-σ^E was expressed at *amyE* (Fig. 2B, lane 2) and a detectable level of σ^E in the strain where *sigE* was expressed at *dacF* (Fig. 2B, lane 4). As in the experiment described above, we interpret the *sigE* products found in the *spoIIIE sigEΔ84 dacF-*P_{dacF}-*sigE* strain as likely to be expressed by the mother cell due to a partial activation of σ^F in that compartment.

Taken together, the data suggest that forespore-expressed *sigE* products are more likely to accumulate and produce detectable σ^E if they are either encoded at a site that enters the forespore early or synthesized in a strain that lacks a functional *spoIIIE*.

Effect of *spoIIGA* and *sigE* coexpression and *spoIIIE* on pro-σ^E::GFP processing in the forespore. We had recently noted that a pro-σ^E::GFP chimera can be processed into σ^E::GFP if it is expressed in the predivisional cell but not if it is synthesized from P_{dacF} in the forespore (19). Pro-σ^E processing in vegetative *B. subtilis* requires only SpoIIGA and SpoIIR (26). It was therefore surprising that processing did not occur in the forespore, where both of these proteins should also be present. Given that SigE processing in the forespore, like its accumulation, is more likely to occur if the pro-σ^E is synthesized early, it seemed possible that SpoIIGA, like SigE, was disappearing from the forespore. We therefore constructed a *B. subtilis* strain in which both SpoIIGA and pro-σ^E::GFP would be expressed from P_{dacF}. A *B. subtilis* strain containing P_{dacF}-*sigE55-gfp* alone and one that also included P_{dacF}-*spoIIGA* were allowed to sporulate. Samples were taken for Western blot analysis to monitor potential pro-σ^E::GFP processing by using an anti-GFP antibody (Clontech) as a probe. The commercial anti-GFP antibody cross-reacted with several *Bacillus* proteins; however, we were able to identify proteins corresponding to unprocessed and processed pro-σ^E::GFP as bands of the predicted mobilities, whose appearance depended on the presence of the *sigE-gfp* fusion, progression of the culture to the appropriate time in sporulation, and the activity of the gene products needed for pro-σ^E processing (e.g., *spoIIGA*). These bands are indicated in Fig. 3. As we had previously observed, the strain carrying P_{dacF}-*sigE55-gfp* accumulated pro-σ^E::GFP at the time in sporulation when σ^F would be expected to become active in the forespore (t_2) but failed to process it (Fig. 3A, lanes 1 to 4). The strain which expressed both *spoIIGA* and *sigE55-gfp* from P_{dacF} not only formed pro-σ^E::GFP but displayed fusion protein processing (Fig. 3A, lanes 5 to 8). Cells expressing the fusion proteins were viewed by phase-contrast microscopy to visualize their outlines (Fig. $4A_1$ to F_1) and by fluorescence microscopy to localize both the

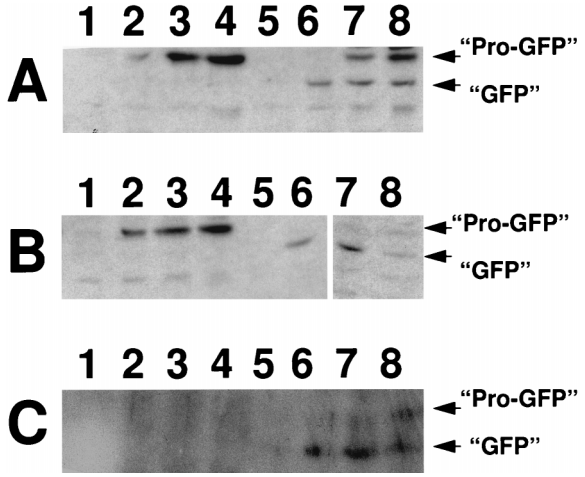

FIG. 3. Western blot analysis of pro-σ^{E55}::GFP accumulation in sporulating *B. subtilis*. Total protein (100 μg) from sporulating cells was fractionated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (12% acrylamide), transferred to nitrocellulose, and probed with an anti-GFP monoclonal antibody (Clontech). Cross-reacting proteins in the extracts were identified by using *B. subtilis* extracts without the fusion protein constructs as controls. Bound antibody was detected, by using a secondary antibody conjugated to alkaline phosphatase (American Qualex) and a chemoluminescent substrate (CDP-Star; Boehringer Mannheim). The position of the unprocessed (Pro-GFP) and processed (GFP) fusion proteins are indicated. (A) Lanes: 1 to 4, SFG1 (P_{dacF}-*sigE55-gfp*); 5 to 8, SFG2 (P_{dacF}-*sigE55-gfp* P_{dacF}-*spoIIGA*). (B) Lanes: 1 to 4, SFG4 (*ctc*::P_{dacF}-*sigE5-gfp*); 5 to 8, SFG5 (*ctc*::P_{dacF}-*sigE55-gfp* P_{dacF}-*spoIIGA*). (C) Lanes: 1 to 4, SFG3 (P_{dacF}-*sigE55-gfp sigEΔ84 spoIIIE*::*spc*); 5 to 8, SFG6 (*ctc*::P_{dacF}-*sigE55-gfp sigEΔ84 spoIIIE*::*spc*). Cells were harvested at t_1 (lanes 1 and 5), t_2 (lanes 2 and 6), t_3 (lanes 3 and 7), and t_4 (lanes 4 and 8).

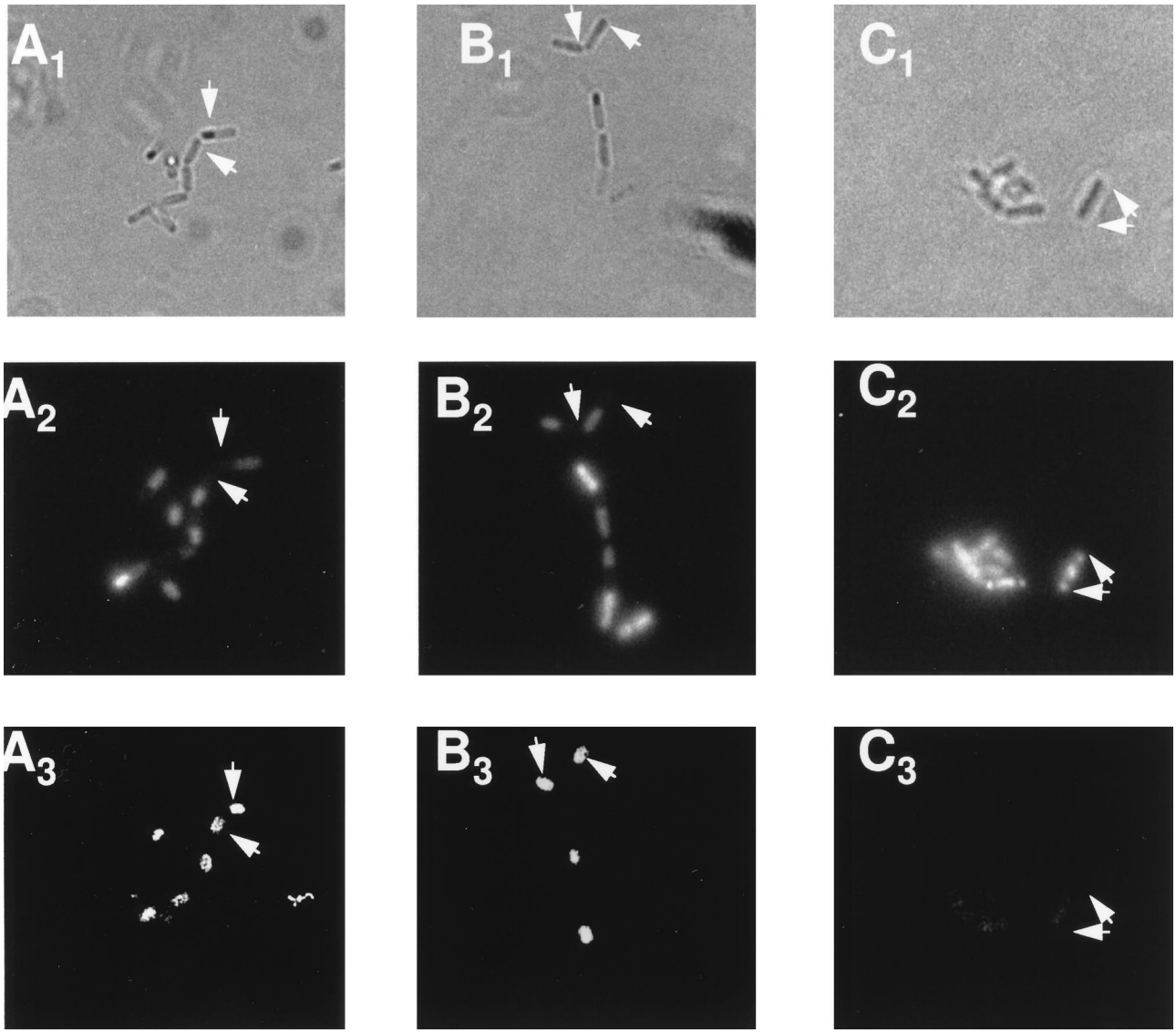

FIG. 4. Localization of pro-σ^{E55}::GFP in sporulating *B. subtilis*. Stationary-phase *B. subtilis* cells were diluted 1/200 in DS medium and incubated for 12 h at 30°C. Sporulation-proficient cells had reached stage III to IV by this time and the stage II mutants (SFG3 and SFG6) displayed a disporic morphology. Samples were treated as described in Materials and Methods to maximize GFP fluorescence and were then stained with DAPI. Phase-contrast microscopy was used to visualize the cells (A_1 to F_1). Fluorescent microscopy was used to detect DAPI-stained chromosomal DNA (A_2 to F_2) and GFP (A_3 to F_3). The arrows indicate the positions of the same forespore compartments in each micrograph of the series. (A) SFG1 (P_{dacF}-*sigE55-gfp*); (B) SFG2 (P_{dacF}-*sigE55-gfp*, P_{dacF}-*spoIIGA*); (C) SFG3 (P_{dacF}-*sigE55-gfp sigE84 spoIIIE*::*spc*); (D) SFG4 (*ctc*::P_{dacF}-*sigE55-gfp*); (E) SFG5 (*ctc*::P_{dacF}-*sigE55-gfp* P_{dacF}-*spoIIGA*); (F) SFG6 (*ctc*::P_{dacF}-*sigE55-gfp sigΔ84 spoIIIE*::*spc*).

GFP fusion proteins (Fig. 4A_3 to F_3) and DAPI-stained chromosomes (Fig. 4A_2 to F_2). As we had observed in the past (19), the DAPI stain preferentially highlighted the mother cell chromosome, presumably due to the difficulty of DAPI entry into the forespore compartments (Fig. 4A_2 and B_2). The chimeric GFP molecules of both P_{dacF}-*sigE55-gfp* strains were restricted to the forespore compartments (Fig. 4A_3 and B_3; Table 2). Thus, the pro-σ^E::GFP processing that we observed in the presence of coexpressed SpoIIGA is occurring in the forespore.

We next examined whether the enhanced conversion of pro-σ^E to σ^E that occurs in the *spoIIIE* mutant could be replicated in the pro-σ^E::GFP system. If it could, the location of the GFP fusion protein, and hence the site of processing, could be verified by fluorescence microscopy. As in the previous experiments, in which we tested the effects of the loss of SpoIIIE on SigE accumulation, we first positioned the fusions at a site on the chromosome (in this case, *ctc*) which would be transferred to the forespore in the absence of SpoIIIE and verified their synthesis in a SpoIIIE$^+$ strain. Pro-σ^E::GFP accumulated normally when expressed at this site (Fig. 3B) and was localized to the forespore compartment (Fig. 4D; Table 2). The pro-σ^E::GFP did not show evidence of obvious processing in the absence of an additional source of SpoIIGA (Fig. 3B, lanes 1 to 4) but was processed if a P_{dacF}-*spoIIGA* fusion was included at *ctc* (Fig. 3B, lanes 5 to 8). Expression of P_{dacF}-*sigE55-gfp* from sites at either *dacF* or *ctc* was then tested in a SpoIIIE$^-$ background. The SpoIIIE$^-$ strain used in this experiment is congenic with the strain used in the *sigE* fusion experiment, i.e., it carries the *sigEΔ84* mutation at *spoIIG*. As a consequence of this, it has a stage II, as well as a SpoIIIE$^-$, terminal phenotype. The stage II phenotype associated with

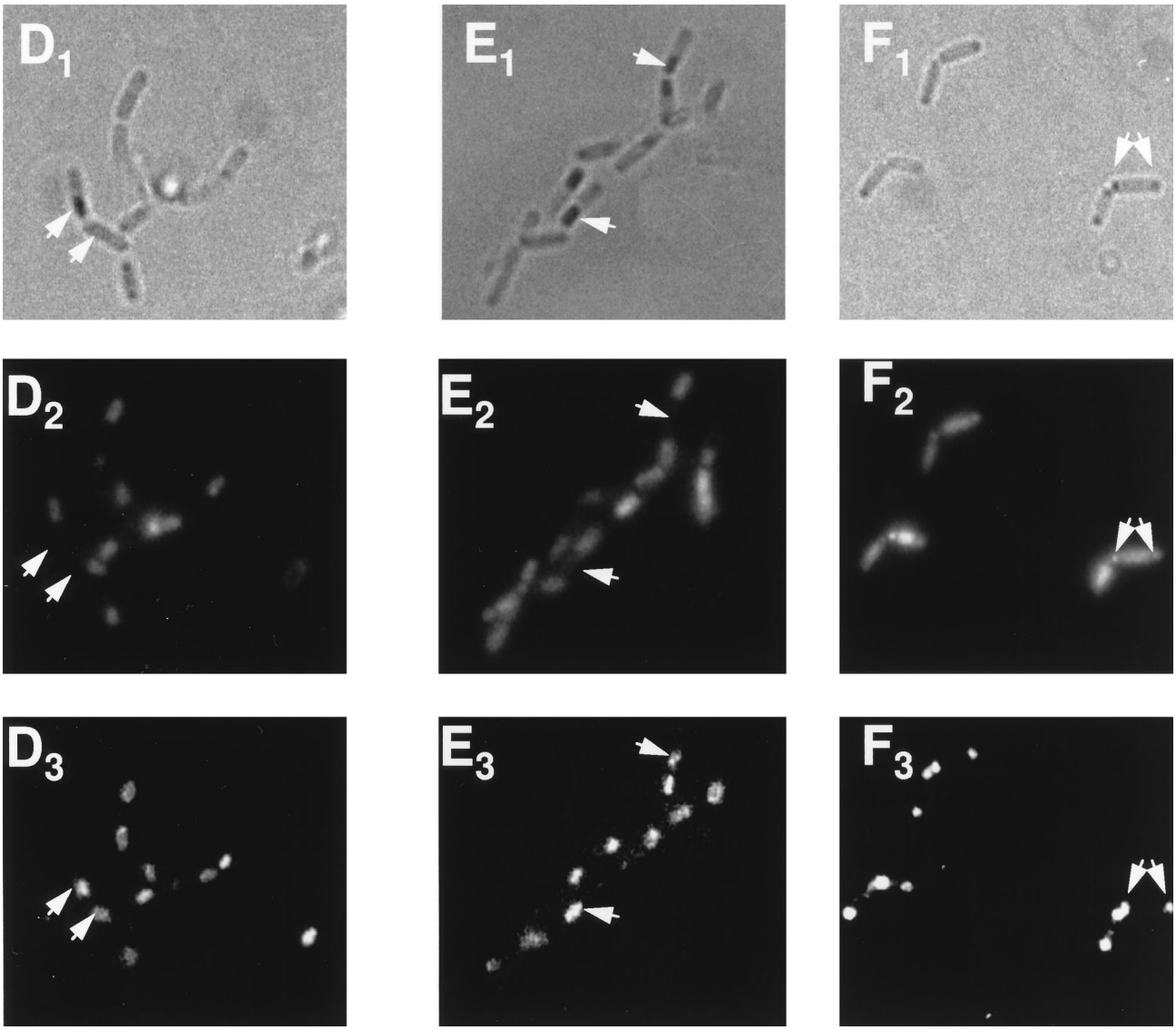

FIG. 4—*Continued.*

the *sigEΔ84* mutation results in the placement of a second septum at the pole of the cell opposite to that at which the first septum is laid down. This is visible in the DAPI-stained micrographs of this strain (Fig. $4C_2$ and F_2), where portions of each of the two chromosomes of the sporulating cell are partitioned into each of these two polar compartments. If P_{dacF}-*sigE-gfp* is positioned at *ctc* in the *spoIIIE*::*spc sigEΔ84* strain, the GFP is localized to the two polar compartments (Fig. $4F_3$; Table 2). Western blot analysis of these cells reveals that virtually all of the pro-σ^{E55}::GFP has been converted to the processed form (Fig. 3C, lanes 5 to 8). We interpret these GFP results as evidence that pro-σ^E processing can occur in the forespore if either additional SpoIIGA is provided or the cell is SpoIIIE$^-$. Very little pro-σ^E::GFP was synthesized in the SpoIIIE$^-$ strain from the P_{dacF} fusion at *dacF*, where expression likely depends on σ^F activation in the mother cell. The Western blot analysis of this strain failed to convincingly detect a pro-σ^E::GFP band (Fig. 3C, lanes 1 to 4), and fluorescence microscopy revealed weak whole-cell GFP fluorescence (Fig. $4C_3$; Table 2).

DISCUSSION

Both pro-σ^E and its processing enzyme (SpoIIGA) are synthesized in the predivisional cell; however, activation of σ^E in wild-type *B. subtilis* is ultimately restricted to the mother cell compartment (27, 39). An immunofluorescence study by Pogliano et al. (35) gave compelling evidence for the absence of pro-σ^E/σ^E from the forespore shortly after septation (35). They also showed that in the absence of SpoIIIE, a protein required for chromosome translocation to the forespore, pro-σ^E/σ^E persisted and σ^E activity could be detected in the forespore.

While attempting to express *sigE* gene products from forespore-specific promoters, we obtained data that support their findings. A variant *sigE* allele (*sigE335*), whose product can be distinguished electrophoretically from pro-σ^E/σ^E, failed to accumulate in wild-type *B. subtilis* (Fig. 1A and C) when it was expressed from the forespore-specific *dacF* promoter. A similar fusion that expressed the wild-type *sigE* allele did generate a product; however, its abundance was approximately 10% of

TABLE 2. GFP fluorescence patterns

Strain (genotype)	No. of cells with a particular pattern[a]		
	F	W	N
SFG1 (P_{dacF}-*sigE55-gfp*)	78	0	5
SFG2 (P_{dacF}-*sigE55-gfp* P_{dacF}-*spoIIGA*)	62	1	4
SFG3 (P_{dacF}-*sigE55 sigEΔ84 spoIIIE*::*spc*)	0	1	43
SFG4 (*ctc*::P_{dacF}-*sigE55-gfp*)	59	0	2
SFG5 (*ctc*::P_{dacF}-*sigE55-gfp* P_{dacF}-*spoIIGA*)	77	4	8
SFG6 (*ctc*::P_{dacF}-*sigE55-gfp sigEΔ84 spoIIIE*::*spc*)	64	1	17

[a] F, fluorescence in forespore compartments; W, whole-cell fluorescence; N, no or weak fluorescence. Cells were grown in DS medium at 30°C and were at approximately stage III to IV of growth when counted.

the level anticipated from the activity of the promoter that drove its expression. Little wild-type SigE and no σ^{E335} were found to accumulate when expressed from the *dacF* locus, even if the strain is unable to express mother cell-specific genes (i.e., it carries *sigEΔ84* at *spoIIG*). Thus, the factors restricting SigE's ability to persist in the forespore do not require ongoing development in the mother cell and instead appear to be independently developed by the forespore itself.

It is believed that the *B. subtilis* chromosome is sequentially translocated into the forespore from a particular origin (14, 46). The position of a gene on the chromosome determines not only its time of transfer but also, as a consequence of transfer time, the likely time at which the gene is expressed in the forespore. Experimental evidence for this notion has recently been obtained in the Piggot laboratory (34). These investigators placed the σ^F-dependent *spoIIR* gene at different sites on the *B. subtilis* chromosome and demonstrated a correlation between its relative time of entry into the forespore and the time at which its activity could be detected. In our experiments, σ^{E335} became visible in Western blots when the P_{dacF}::*sigE335* fusion was moved from late-entry sites (*dacF* and *spoIIIG*) to an early-entry locus (*amyE*) (Fig. 2A, lane 1). We speculate that this heightened SigE accumulation is due to its earlier synthesis. This implies that the factor responsible for SigE's disappearance is itself not present initially but accumulates in the forespore at this time. Presumably, this factor is a forespore-specific protease which needs to be expressed from the translocated chromosome.

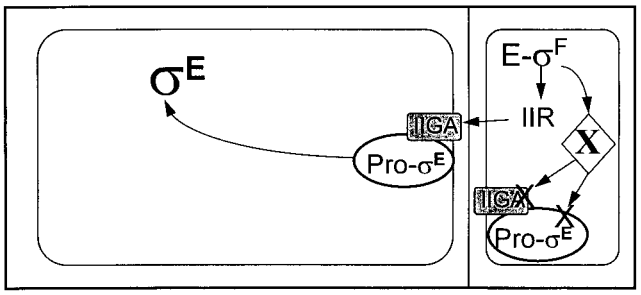

FIG. 5. Model for pro-σ^E activation. SpoIIGA and pro-σ^E are synthesized prior to septation and are likely to be present initially in both mother cell and forespore compartments. As proposed by several investigators (15, 20, 26), σ^F becomes active in the forespore, where it directs the synthesis of the SpoIIGA activator, SpoIIR. SpoIIR then signals SpoIIGA to cleave the pro sequence from pro-σ^E, which along with SpoIIGA appears to be tethered to the septal membrane (15, 19, 20, 26). Very early after septation, this reaction probably occurs in both the mother cell and forespore compartments; however, later, a hypothetical σ^F-dependent gene product (X) initiates the destruction of both SigE and SpoIIGA in the forespore. σ^F-transcribed genes are proposed in this model to be responsible for both the timing of σ^E activation, by directing the synthesis of SpoIIR, and the restriction of σ^E activity to the mother cell, by directing the synthesis of the putative protease that degrades SpoIIGA and SigE.

The result that pro-σ^E, synthesized in the forespore, was more likely to be processed if it was expressed from a site that translocates to the forespore early or if the strain lacked SpoIIIE suggests that the capacity to process pro-σ^E, like the ability to accumulate *sigE* products, is being lost in the forespore due to expression of a gene on a distal part of the translocated chromosome. A similar activity could be responsible for both events, with the SigE-processing enzyme (SpoIIGA), like SigE, becoming unstable in the forespore. In support of this idea, we found that supplemental SpoIIGA allows processing to occur in the forespore (Fig. 3A, lanes 5 to 8). Processing also occurred in the absence of additional SpoIIGA if the cell lacked SpoIIIE (Fig. 3C, lanes 5 to 8). These results are consistent with SpoIIGA, as well as SigE, being degraded by an activity that depends on SpoIIIE, presumably a factor encoded on a distal region of the chromosome.

Our current view of pro-σ^E processing is illustrated in Fig. 5. We had previously shown that the SigE pro sequence tethers proteins which carry it to the forespore septum (19). SpoIIGA, the pro-σ^E-processing enzyme, has the structure of an integral membrane protein (38), and, as proposed by Hofmeister et al. (15), may also reside in the forespore membrane. Hence, pro-σ^E and SpoIIGA could both lie at the forespore septum awaiting the signal to initiate the processing and release of active σ^E into the cell interior. We suspect that σ^F-dependent genes are key for both the timing and the compartmentalization of σ^E activity. Following septation and σ^F activation, *spoIIR*, a gene that is on a region of the chromosome that is translocated to the forespore early, is transcribed and triggers pro-σ^E processing on both sides of the forespore septum. As additional regions of the *B. subtilis* chromosome enter the forespore, a gene(s) encoding a putative protease(s) (X), which is hypothesized to degrade both SigE and SpoIIGA, enters the forespore and is expressed. This results in the elimination of these proteins from the forespore and a block of their further accumulation when their coding sequence (*spoIIG*), on a late-entering segment of the chromosome, is finally transferred to the forespore. This model is highly speculative; however, if our notion of selective proteolysis is true, genetic screens should be able to detect the genes for these hypothetical proteases as the sites of mutations which allow SigE to persist and be active in the forespore.

ACKNOWLEDGMENT

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