JOURNAL OF BACTERIOLOGY, Oct. 2005, p. 6832–6840 Vol. 187, No. 19
0021-9193/05/$08.00+0 doi:10.1128/JB.187.19.6832–6840.2005

Control of the Expression and Compartmentalization of σ^G Activity during Sporulation of *Bacillus subtilis* by Regulators of σ^F and σ^E

Vasant K. Chary, Mauro Meloni,† David W. Hilbert,‡ and Patrick J. Piggot*

Department of Microbiology and Immunology, Temple University School of Medicine, Philadelphia, Pennsylvania

Received 10 June 2005/Accepted 22 July 2005

During formation of spores by *Bacillus subtilis* the RNA polymerase factor σ^G ordinarily becomes active during spore formation exclusively in the prespore upon completion of engulfment of the prespore by the mother cell. Formation and activation of σ^G ordinarily requires prior activity of σ^F in the prespore and σ^E in the mother cell. Here we report that in *spoIIA* mutants lacking both σ^F and the anti-sigma factor SpoIIAB and in which σ^E is not active, σ^G nevertheless becomes active. Further, its activity is largely confined to the mother cell. Thus, there is a switch in the location of σ^G activity from prespore to mother cell. Factors contributing to the mother cell location are inferred to be read-through of *spoIIIG*, the structural gene for σ^G, from the upstream *spoIIG* locus and the absence of SpoIIAB, which can act in the mother cell as an anti-sigma factor to σ^G. When the *spoIIIG* locus was moved away from *spoIIG* to the distal *amyE* locus, σ^G became active earlier in sporulation in *spoIIA* deletion mutants, and the sporulation septum was not formed, suggesting that premature σ^G activation can block septum formation. We report a previously unrecognized control in which SpoIIGA can prevent the appearance of σ^G activity, and pro-σ^E (but not σ^E) can counteract this effect of SpoIIGA. We find that in strains lacking σ^F and SpoIIAB and engineered to produce active σ^E in the mother cell without the need for SpoIIGA, σ^G also becomes active in the mother cell.

Central to cell differentiation is the establishment of distinct programs of gene expression in the different cell types involved. These programs determine the subsequent path of differentiation. Among prokaryotes, formation of spores by *Bacillus subtilis* has become a paradigm for the analysis of cell differentiation. Soon after the start of spore formation, bacteria divide asymmetrically to give the smaller prespore (also called the forespore) and the larger mother cell. The prespore is then engulfed by the mother cell. The prespore develops into the mature spore, whereas the mother cell ultimately lyses. The process of spore formation is characterized by the cell-specific activation of four RNA polymerase σ factors. Immediately after the completion of the spore division septum, σ^F is activated in the prespore. Its activation leads rapidly to activation of σ^E in the mother cell. Upon completion of engulfment, σ^G becomes active in the prespore; its activation, in turn, leads to activation of σ^K in the mother cell (Fig. 1) (reviewed in reference 10). The activation of the successive σ factors is tightly coordinated within and between the two cell types, a process that has been termed crisscross regulation (19). We explore here the activation of σ^G in circumstances in which its normal tight coupling to the prior activation of σ^F and σ^E has broken down.

Both σ^F and σ^E are formed soon after the start of spore formation and before the sporulation division. When first formed they are inactive: σ^F because of interaction with the anti-sigma factor SpoIIAB and σ^E because it is formed as an inactive precursor, pro-σ^E. A complex regulatory system centered on SpoIIAB controls the activation of σ^F, which occurs in the prespore shortly after completion of the sporulation division. Activation of σ^E in the mother cell by processing of pro-σ^E depends on SpoIIGA, which is the putative processing enzyme, and on a σ^F-directed signal from the prespore. The appearance of σ^G activity depends on the activities of both σ^F and σ^E and on morphological signals (10).

The *spoIIIG* locus, which encodes σ^G, is first transcribed early in sporulation by read-through from the upstream *spoIIG* locus (Fig. 2). However, there is little, if any, translation of this transcript, probably because the *spoIIIG* ribosome-binding site is sequestered in a stem-loop structure; further, the transcript is not necessary for spore formation (20, 35). Following septation, the *spoIIIG* locus is transcribed productively from its own σ^F-directed promoter (6, 13), which is active exclusively in the prespore (reviewed in reference 25). Transcription from that promoter, which also depends on a σ^E-directed signal from the mother cell (13, 21), leads to the formation of σ^G (35). When first formed, σ^G is inactive; additional signals are required to activate it. Activation of σ^G requires expression of *spoIIIJ* in the prespore and of *spoIIIA* in the mother cell, and these are thought to act via a direct regulator of σ^G that has yet to be identified (30). Activation also requires completion of engulfment of the prespore by the mother cell (33). SpoIIAB can act as an anti-σ for σ^G as well as for σ^F (5, 15, 30) but is now thought not to be a regulator of σ^G activity in the prespore (32).

Here we explore determinants of σ^G regulation. We find that in the absence of both σ^F and the anti-sigma factor SpoIIAB, σ^G becomes active in the mother cell instead of the prespore. Further, activation follows completion of septation rather than completion of engulfment. We describe a previ-

* Corresponding author. Mailing address: Department of Microbiology and Immunology, 3400 North Broad Street, Philadelphia, PA 19140. Phone: (215) 707-7927. Fax: (215) 707-7788. E-mail: piggotp@temple.edu.

† Present address: Unité des Toxins et Pathogénie Bactérienne, Institut Pasteur, 75724 Paris Cedex 15, France.

‡ Present address: Department of Anatomy and Cell Biology, Columbia University, New York, NY 10032.

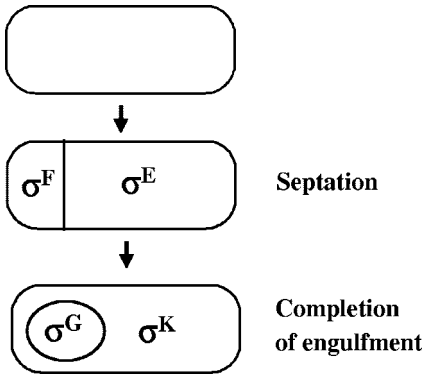

FIG. 1. Schematic representation of stages of spore formation showing the normal location of activity of sporulation-specific sigma factors.

ously unrecognized control, in which SpoIIGA can prevent the appearance of σ^G activity, and pro-σ^E can counteract this effect of SpoIIGA. We also find that premature activation of σ^G can prevent septum formation.

MATERIALS AND METHODS

Media. *B. subtilis* was grown in modified Schaeffer's sporulation medium (MSSM) or on Schaeffer's sporulation agar (23, 28). When required, the medium contained 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 LB (Luria-Bertani lysogeny broth) agar containing ampicillin at 100 μg/ml when required.

Strains. *B. subtilis* 168 strain BR151 (*trpC2 metB10 lys-3*) was used as the parent strain. *B. subtilis* strains used are listed in Table 1. The *spoIIAΔ4* mutation was described previously (24); the deletion encompassed the entire *spoIIA* operon, but the ends of the deletion have not been defined. In the *spoIIA*Δ::*neo* and *spoIIA*Δ::*spc* mutations, the entire *spoIIA* operon, from 48 bp upstream of the first open reading frame (ORF) to 7 bp downstream of the last ORF, was replaced with the antibiotic resistance cassette. In the mutation designated *spoIIAB-AC*Δ::*neo*, the entirety of *spoIIAC* and all but the 5′ 163 bp of *spoIIAB* were replaced with a *neo* cassette. In the *spoIIAC*Δ::*neo* mutation, 543 bp from the 3′ end of the *spoIIAC* ORF were replaced with *neo*. The *spoIIGB*Δ::*spc* mutation was derived from EU8701 of Kenny and Moran (16). The *spoIIG*Δ::*cat* mutation has 388 bp from the 3′ end of *spoIIGA* and 338 bp from the 5′ end of *spoIIGB* replaced with the *cat* cassette. The *spoIIG*(P)Δ::*cat* mutation has the region from 142 bp upstream of *spoIIGA* (including its promoter) to 388 bp into *spoIIGB* replaced with the *cat* cassette. The *spoIIGA*::*cat* mutation has the *cat* cassette inserted at the StuI site located 389 bp from the 3′ end of the ORF. The gene for the pro-less form of σ^E, *sigE*, was inserted at *thrC* under the control of the *spoIIG* promoter; in the encoded protein, N-terminal MH residues are joined to residue 28 (Y) (pro-σ^E numbering). Strain AH2487, containing a translational σ^G-green fluorescent protein (GFP) fusion, was kindly provided by Adriano Henriques. DNA from that strain was used to introduce the fusion into BR151 (*spo*$^+$) to yield SL12673 and into a *spoIIA*Δ::*neo* derivative of BR151 to yield SL12674. The *spoIIIG*::*neo* mutation has the resistance cassette inserted in the Pst1 site within *spoIIIG*. DNA containing the *amyE*::*spoIIIG* construct (35) was kindly provided by Peter Setlow and DNA with the *lonA* disruption by Adriano Henriques. The P_{spoIIE}-*spoIIR* construct was described by Zhang et al. (38). The $P_{spac(hy)}$ vector of Quisel et al. (27) was used to place the entire *spoIIGB* or *spoIIGA* ORF, with its ribosome-binding site, at *thrC* under IPTG (isopropyl-β-D-thiogalactopyranoside)-inducible control. *E. coli* DH5α (Gibco-BRL) was used to maintain plasmids. Details of strain construction are available on request.

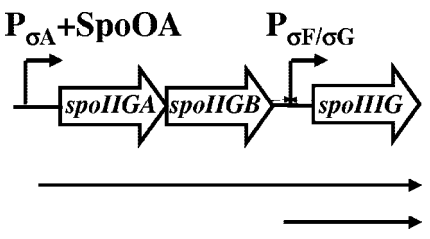

FIG. 2. Schematic representation of the *spoIIG-spoIIIG* region of the chromosome. The promoter for *spoIIG* requires σ^A and activated Spo0A for expression. The promoter specific to *spoIIIG* requires σ^F or σ^G for expression. The different transcripts of *spoIIIG* are indicated. The inverted repeat between *spoIIGB* and *spoIIIG* is indicated by opposed arrows.

Fluorescence microscopy. Cultures were grown in MSSM at 37°C. A 200-μl volume of culture was mixed with 2 μl of FM4-64 (Molecular Probes) that had been previously diluted to 1 mg/ml in phosphate-buffered saline (Gibco-BRL). Samples were incubated at 37°C for 5 min, and 1 μl of unfixed sample was transferred to a slide and visualized essentially as described by Pogliano et al. (26). Images were captured using a Leica DM IRE2 microscope with a TCS SL confocal system, using a 100× oil immersion objective and Leica imaging software. GFP emission was captured between 500 and 550 nm and FM4-64 emission between 600 and 730 nm; excitation for both fluorophors was at 488 nm. Fluorographs shown are projection images generated from a single stack in the Z plane, with four-point line averaging.

Western blot analysis. Procedures for Western blotting were performed essentially as described by Serrano et al. (30). The anti-σ^G polyclonal antiserum was incubated with membranes at a dilution of 1:1,000 in TBS-T (20 mM Tris-HCl [pH 7.6], 136 mM NaCl, 0.1% [vol/vol] Tween 20), containing 5.0% nonfat dry milk. Incubation with an anti-rabbit secondary antibody conjugated to horseradish peroxidase was for 30 min at a 1:5,000 dilution, and detection was with an ECL Plus kit (Amersham). Protein samples of 300 μg were used in each lane.

Other methods. β-Galactosidase was assayed essentially as described previously (23). Specific activity is expressed as nanomoles of ONPG (*o*-nitrophenyl-β-D-galactopyranoside) hydrolyzed per minute per milligram of bacterial dry weight; results of typical experiments are shown in the figures. *B. subtilis* transformation, sporulation by exhaustion in MSSM, and other methods were essentially as described previously (2, 38).

RESULTS

Deletion of the genes encoding σ^F and the anti-sigma factor SpoIIAB causes a breakdown of the tight progression of the activation of sporulation-specific σ factors. When *spoIIAC*, the structural gene for σ^F, is inactivated by point mutation, no activity is detected for the later-expressed σ factors σ^E, σ^G, and σ^K (4, 13, 21). In contrast to that result, we have found that deletion of the entire *spoIIA* operon (designated *spoIIA*Δ) permits activation of σ^G (strain SL12436; Fig. 3), although not σ^E or σ^K (not shown). Thus, σ^G becomes active in the absence of the activities of σ^F and σ^E, effectively disrupting the normal ordered activation of the sporulation-specific sigma factors. Activity was first apparent about 3 h after the onset of spore formation; no activity was detected in a strain with *spoIIIG*, the structural gene for σ^G, disrupted (SL11727; Fig. 3). Similar results were obtained with a different σ^G-directed promoter (not shown). In the *spoIIA* deletion strain, activity was detected earlier during spore formation than for the corresponding *spo*$^+$ strain (SL10369). However, although the normal tight regulation of σ^G had been disrupted in the *spoIIA*Δ mutant, no activity was detected during vegetative growth.

The *spoIIA* operon encodes SpoIIAA and SpoIIAB, as well as σ^F; SpoIIAB is an anti-sigma factor for σ^F, and SpoIIAA is the anti-anti-sigma factor that interacts with SpoIIAB (reviewed in references 10 and 37). We tested to see whether deletion of *spoIIAB* and/or *spoIIAA* was necessary to obtain σ^G activity in the absence of σ^F. The σ^F-independent activation of σ^G was found to require deletion of *spoIIAB* (compare SL12434 with SL12432; Fig. 4). SpoIIAB can act as an anti-

TABLE 1. *B. subtilis* strains used

Strain[a]	Relevant phenotype	Source
SL10969	*sspA-gfp@sspA*[b]	Lab stock
SL10034	*spoIIAΔ4 sspA-gfp@sspA*	Lab stock
SL10153	*spoIIAΔ::spc sspA-gfp@sspA*	This study
SL10162	*spoIIAΔ::spc amyE::spoIIIG-gfp*	This study
SL10215	*spoIIAΔ::spc amyE*::P_{spoIIE}-*spoIIR sspA-gfp@sspA*	This study
SL10369	*sspA-lacZ@sspA*	Simon Cutting
SL11671	*spoIIAΔ::neo spoIIGBΔ::spc sspA-lacZ@sspA*	This study
SL11727	*spoIIAΔ::spc spoIIIG::neo sspA-lacZ@sspA*	This study
SL11758	*spoIIAΔ::neo spoIIGBΔ::spc thrC*::$P_{spac(hy)}$-*spoIIGB sspA-lacZ@sspA*	This study
SL11763	*spoIIAΔ::spc spoIIIG::neo amyE::spoIIIG sspA-lacZ@sspA*	This study
SL11767	*spoIIAΔ::neo spoIIGBΔ::spc sspA-lacZ-cat@sspA lonA::erm*	This study
SL11809	*spoIIAΔ::spc sspA-lacZ-cat@sspA*	This study
SL11813	*spoIIAΔ::neo spoIIGBΔ::spc thrC*::$P_{spac(hy)}$-*spoIIGB sspA-gfp@sspA*	This study
SL11815	*spoIIAΔ::spc spoIIIG::neo amyE::spoIIIG sspA-gfp@sspA*	This study
SL11958	*spoIIAΔ::spc sspA-lacZ@sspA thrC*::P_{spoIIG}-*sigE*	This study
SL12042	*spoIIAΔ::neo spoIIGBΔ::spc thrC*::P_{spoIIG}-*sigE sspA-lacZ@sspA*	This study
SL12137	*spoIIAΔ::neo spoIIGΔ::cat sspA-lacZ-tet@sspA*	This study
SL12306	*spoIIAΔ::neo spoIIGΔ::cat sspA-gfp-spc@sspA*	This study
SL12348	*spoIIAΔ::neo spoIIGAΔ::cat sspA-lacZ-tet@sspA*	This study
SL12359	*spoIIAΔ::neo spoIIGΔ::cat sspA-lacZ-tet@sspA thrC*::$P_{spac(hy)}$-*spoIIGA*	This study
SL12426	*spoIIAΔ::spc spoIIG(P)Δ::cat*[c] *sspA-lacZ-tet@sspA thrC::sigE*	This study
SL12432	*spoIIAB, ACΔ::neo sspA-lacZ-cat@sspA*	This study
SL12434	*spoIIACΔ::neo sspA-lacZ-cat@sspA*	This study
SL12436	*spoIIAΔ::neo sspA-lacZ-cat@sspA*	This study
SL12518	*spoIIAΔ::spc thrC::spoIID-gfp amyE*::P_{spoIIE}-*spoIIR*	This study
SL12538	*spoIIAΔ::spc spoIIG(P)Δ::cat*[c] *sspA-gfp-neo@sspA*	This study
SL12673	*spo*$^+$ *spoIIIG'-'gfp* translational fusion at *spoIIIG*	This study
SL12674	*spoIIAΔ::neo spoIIIG'-'gfp* translational fusion at *spoIIIG*	This study

[a] All strains are in the genetic background of *B. subtilis* 168 strain BR151 (*trpC2 lys-3 metB10*). They have all its auxotrophic markers, except for SL10034, which is *lys*$^+$.
[b] @ indicates that the fusion has been introduced by single-crossover (Campbell-like) recombination.
[c] The promoter and structural genes of *spoIIG* are deleted.

sigma factor for σ^G, as well as for σ^F (5, 15, 32), so that its loss presumably permitted the establishment of a positive-feedback loop of σ^G-directed transcription of *spoIIIG*. Consistent with this interpretation, mutations in either *spoIIIA* or *spoIIIJ*, which ordinarily block σ^G activation in the mother cell through interaction with SpoIIAB (32), did not block σ^G activation in the *spoIIA* deletion background (data not shown).

The presence of *spoIIAA* reduced σ^G activity in the strain with *spoIIAB* and *spoIIAC* deleted (compare SL12432 with SL12436; Fig. 4) but did not abolish it. SpoIIAA is known to inhibit activation of Spo0A (1), which is a central regulator of early sporulation gene expression (reviewed in reference 25). We think it plausible that inhibition of Spo0A activity by SpoIIAA accounts for the effects of SpoIIAA illustrated in Fig. 4, for example, by reducing expression of the *spoIIG* operon (see below). However, we did not explore the role of SpoIIAA further.

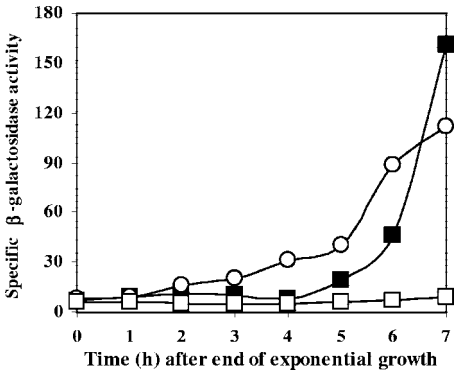

FIG. 3. Activity of σ^G in a strain with the *spoIIA* locus deleted. The activity of σ^G is assessed as β-galactosidase expressed from an *sspA-lacZ* transcriptional fusion in the following strains: filled squares, SL10369, *spo*$^+$; open circles, SL12436, *spoIIA*Δ; open squares, SL11727, *spoIIA*Δ *spoIIIG*::*neo*.

The location of σ^G activation is switched from the prespore to the mother cell in *spoIIA* deletion mutants. During normal spore formation, σ^G activity is confined to the prespore. The prespore specificity is established by the σ^F-directed transcription of *spoIIIG*, which is itself confined to the prespore. Once σ^G becomes active, a positive-feedback loop is then established in which σ^G directs *spoIIIG* transcription from the same promoter, which is recognized by both σ^F and σ^G (35, 36). The question arises, what happens in the absence of σ^F? To answer this, we monitored the expression of σ^G-directed *sspA-gfp* and *spoIIIG-gfp* transcriptional fusions. Consistent with extensive published results (reviewed in reference 10), their expression was largely confined to the prespore in a *spo*$^+$ background (SL10969; Fig. 5 and Table 2). However, we have found that σ^G activity in *spoIIA*Δ strains was, within the limits of detection, confined to the mother cell in the majority of GFP-expressing organisms (Fig. 5, strains SL10034 and SL10153; Table 2, SL10034, SL10153, and SL10162); back-crosses of the *sspA-gfp* fusion into a *spo*$^+$ strain gave recombinants displaying prespore-specific expression, confirming that the fusion was

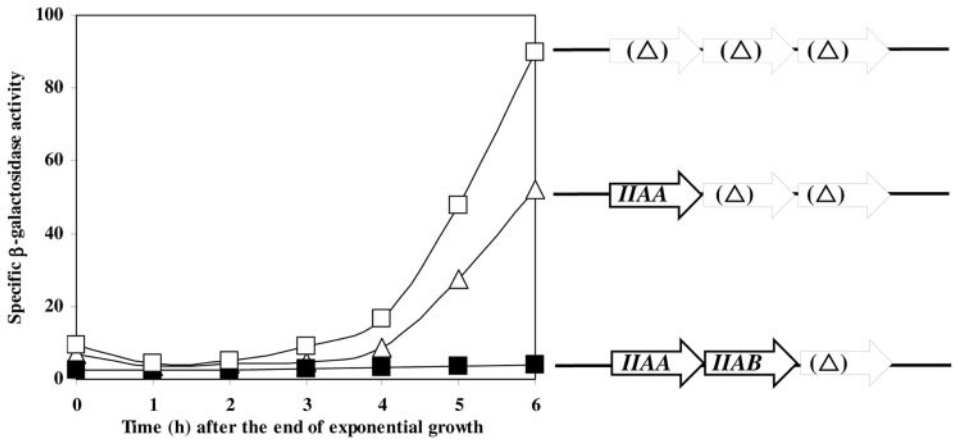

FIG. 4. The presence of SpoIIAB blocks σ^G activity in a strain that lacks σ^F. The activity of σ^G is assessed as β-galactosidase expressed from an *sspA-lacZ* transcriptional fusion in the following strains: filled squares, SL12434, *spoIIAC* (encoding σ^F) deleted; open triangles, SL12432, *spoIIAB* and *spoIIAC* deleted; open squares, SL12436, *spoIIAA*, *spoIIAB*, and *spoIIAC* deleted (*spoIIA*Δ::*neo*). For each strain, the extent of the deletion in the *spoIIA* operon is indicated on the right side, with Δ indicating a deleted gene.

unaltered. The mother cell location of σ^G activity in *spoIIA*Δ strains was surprising. However, it is consistent with recent results of Serrano et al. (32), who have found that SpoIIAB primarily regulates σ^G by preventing its activation in the mother cell, while having at most a redundant role in blocking σ^G activity in the prespore. That σ^G activity is detected after septation rather than completion of engulfment is probably also the result of loss of SpoIIAB control in the mother cell. The few cells that displayed whole-cell fluorescence (Table 2) did not contain a sporulation division septum.

Read-through from the *spoIIG* locus is important for the mother cell location of σ^G expression. A factor contributing to the mother cell location of σ^G activity in *spoIIA* deletion strains might be read through from the *spoIIG* locus, which is upstream of *spoIIIG* (Fig. 2). In *spo*$^+$ strains *spoIIIG* is transcribed by read-through from the *spoIIG* locus, but there is no detectable translation of *spoIIIG* from this read-through transcript (20, 35). Indeed, relocating *spoIIIG* to the distal *amyE* locus does not impair σ^G activity, and gives efficient spore formation, so that read-through from *spoIIG* is not ordinarily required for spore formation (35). However, it may be that read-through is important for σ^G activation in the *spoIIA* deletion strains. Transcription from *spoIIG* through *spoIIIG* has been inferred primarily from results with transcriptional *lacZ* fusions (20, 35) and has proved difficult to detect reproducibly by Northern analysis or S1 mapping (references 16 and 20 and our unpublished observations). We have confirmed by reverse transcription-PCR that under sporulation conditions there was read-through of *spoIIIG* from *spoIIGA* in *spoIIA*Δ as well as in *spo*$^+$ strains (data not shown).

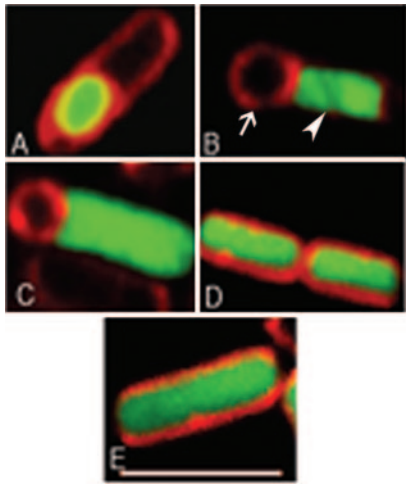

FIG. 5. Examples of GFP-expressing cells illustrating the patterns of localization of green fluorescence obtained with different strains containing a σ^G-directed *sspA-gfp* fusion. Bacteria were stained with FM4-64 to visualize membranes (red). A, SL10969 (*spo*$^+$ *sspA-gfp*); B, SL10034 (*spoIIA*Δ*4 sspA-gfp*); C, SL10153 (*spoIIA*Δ::*spc sspA-gfp*); D, SL11813 (*spoIIA*Δ::*neo spoIIGB*Δ::*spc thrC*::$P_{spac(hy)}$-*spoIIGB sspA-gfp*); E, SL11815 (*spoIIA*Δ::*spc spoIIIG*::*neo amyE*::*spoIIIG sspA-gfp*). An arrow is used to indicate a prespore and an arrowhead a mother cell. A 3-μm scale bar is shown in panel E; all images are on the same scale.

To explore the role of this read-through, we tested the effect of relocating *spoIIIG* to *amyE* and found that *spoIIIG* was actively expressed in a *spoIIA*Δ strain (Fig. 6; SL11763). Thus, read-through from *spoIIG* was not necessary for expression of σ^G activity. However, the relocation changed the pattern of σ^G activity, as σ^G became active earlier in spore formation and became more active than when *spoIIIG* was at its natural locus (Fig. 6; SL12436). The reason for the earlier initiation of transcription of *spoIIIG* at the ectopic locus is not known. Importantly, in the great majority of organisms expressing the σ^G-directed *sspA-gfp* fusion, with *spoIIIG* located at *amyE*, the fluorescence was uncompartmentalized and the sporulation septum was not formed (Fig. 5 and Table 2; SL11815). Thus, read-through of *spoIIIG* from *spoIIG* may be important for obtaining the mother cell specificity of σ^G activity observed in *spoIIA* deletion strains, even though it was not required to obtain the activity. It should be noted that Fujita and Losick (7) have reported greatly increased activity of the *spoIIG* promoter in the mother cell following septation in *spo*$^+$ strains.

A second set of experiments reinforced the idea that read-through from *spoIIG* was indeed important for the mother cell

TABLE 2. Location of GFP expression from different *gfp* fusions[a]

Strain	Relevant genotype	Fusion	No. of cells displaying GFP fluorescence[b]		
			PS	MC	WC
SL10969	*spo*$^+$	*sspA-gfp*	52	0	0
SL10034	*spoIIA*Δ4	*sspA-gfp*	1	36	4
SL10153	*spoIIA*Δ::*spc*	*sspA-gfp*	0	21	1
SL10162	*spoIIA*Δ::*spc*	*amyE*::*spoIIIG-gfp*	0	19	4
SL11813	*spoIIA*Δ::*neo spoIIGB*Δ::*spc thrC*::$P_{spac(hy)}$-*spoIIGB*	*sspA-gfp*	0	4	46
SL11815	*spoIIA*Δ::*spc spoIIIG*::*neo amyE*::*spoIIIG*	*sspA-gfp*	0	3	45
SL10215	*spoIIA*Δ::*spc* P_{spoIIE}-*spoIIR*	*sspA-gfp*	1	20	38
SL12518	*spoIIA*Δ::*spc* P_{spoIIE}-*spoIIR*	*thrC*::*spoIID-gfp*	0	15	18
SL12306	*spoIIA*Δ::*neo spoIIG*::*cat*	*sspA-gfp*	0	58	32
SL12538	*spoIIA*Δ::*spc spoIIG*(P)::*cat*	*sspA-gfp*	0	38	0[c]
SL12673	*spo*$^+$	*spoIIIG'-'gfp*[d]	45	1	0
SL12674	*spoIIA*Δ::*neo*	*spoIIIG'-'gfp*[d]	0	42	2

[a] The pattern of fluorescence was determined for cells expressing GFP 6 h after the end of exponential growth in MSSM. At this time at least 40% of cells displayed an easily scored GFP signal, with the exception of SL12358, where only about 20% were readily scored. Transcription of *sspA-gfp* is directed by σ^G and of *spoIID-gfp* by σ^E; *spoIIIG* is the structural gene for σ^G. Membranes were visualized by staining with FM4-64; with the exception of SL12306, those cells displaying whole-cell GFP fluorescence exhibited no septa. Fusions to *gfp* were located at their own locus unless otherwise indicated.

[b] PS, prespore specific; MC, mother cell specific; WC, whole-cell fluorescence; almost all cells scored as WC had no visible sporulation septum.

[c] A number of other SL12538 cells displayed very weak GFP expression that could not be scored with confidence; they are not included in the table.

[d] Translational fusion; other fusions were transcriptional fusions.

specificity of σ^G expression and that the *spoIIG* promoter contributed to the strength of σ^G expression in the mother cell of *spoIIA*Δ strains. In these experiments, two *spoIIA* deletion strains were compared in which the *spoIIG* locus was also deleted but not *spoIIIG*. In one strain, the *spoIIG* promoter was retained so that it could potentially drive *spoIIIG* transcription, whereas in the other strain the promoter was not retained. The *spoIIG* region was replaced with the same *cat* cassette in the same orientation (away from *spoIIIG*) in both strains so that the insert should not cause a difference between the strains. There was substantial σ^G activity in the strain that retained the promoter (SL12137; Fig. 7) and much-reduced activity in the strain that did not (SL12426; Fig. 7); both strains displayed similar, abortively disporic phenotypes. In a strain that retained the *spoIIG* promoter, but not the *spoIIG* structural genes, σ^G activity was primarily confined to the mother cell (SL12306; Table 2). Assessing the location of σ^G activity in a strain that lacked the promoter was problematic, as the activity was weak; in those cells that expressed sufficient GFP for an unambiguous determination, the activity was confined to the mother cell (SL12538; Table 2). However, in other cells very weak GFP fluorescence was detectable at a level too low to permit determination of its location. Together, the results indicate that the *spoIIG* promoter contributed to strong mother-cell-specific σ^G activity in *spoIIA* deletion strains but that some mother-cell-specific activity could be obtained without that promoter.

Pro-σ^E and SpoIIGA control σ^G activity. We detected no σ^E activity in the *spoIIA*Δ strains, consistent with previous results (14, 38) and indicating that σ^E was not needed for σ^G activity. However, inactivation of *spoIIGB*, which is the structural gene for pro-σ^E (12), blocked the appearance of σ^G activity in *spoIIA*Δ strains (Fig. 8; SL11758 without IPTG).

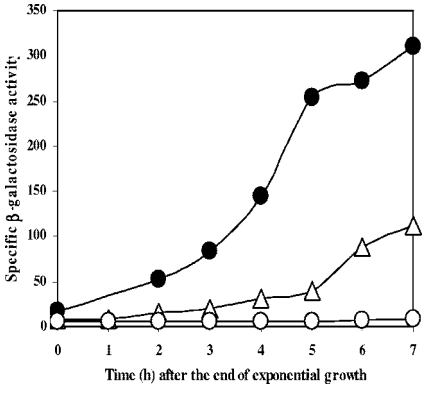

FIG. 6. Effect on σ^G activity of relocating *spoIIIG* to an ectopic locus in a strain with the *spoIIA* locus deleted. The activity of σ^G is assessed as β-galactosidase activity expressed from an *sspA-lacZ* transcriptional fusion in the following strains with the *spoIIA* locus deleted: open triangles, SL12436; open circles, SL11727, *spoIIIG*::*neo*; filled circles, SL11763, *spoIIIG*::*neo amyE*::*spoIIIG*.

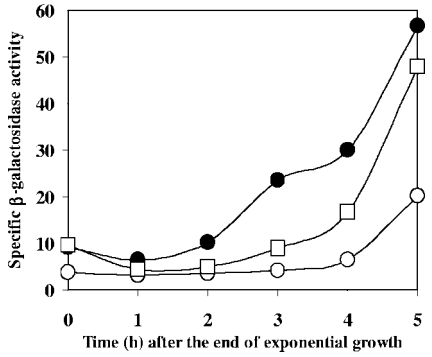

FIG. 7. Effect of deletions of the *spoIIG* operon on σ^G activity in strains with the *spoIIA* locus deleted. The activity of σ^G is assessed as β-galactosidase expressed from an *sspA-lacZ* transcriptional fusion in the following strains with the *spoIIA* locus deleted: open squares, SL12436; closed circles, SL12137 (the *spoIIGA* and *spoIIGB* structural genes are deleted, but the *spoIIG* promoter is retained); open circles, SL12426 (the *spoIIGA* and *spoIIGB* structural genes and the *spoIIG* promoter are deleted).

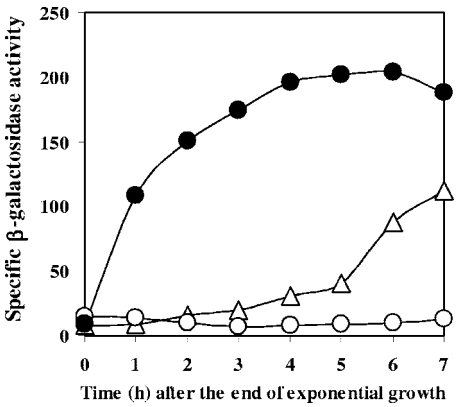

FIG. 8. Effect of ectopic expression of *spoIIGB* on σ^G activity in a strain with the *spoIIA* locus deleted. The activity of σ^G is assessed as β-galactosidase expressed from an *sspA-lacZ* transcriptional fusion in the following strains with the *spoIIA* locus deleted: open triangles, SL12436; open circles, SL11758 (*spoIIGB*Δ::*spc thrC*::$P_{spac(hy)}$-*spoIIGB*) in the absence of IPTG; filled circles, SL11758 in the presence of IPTG.

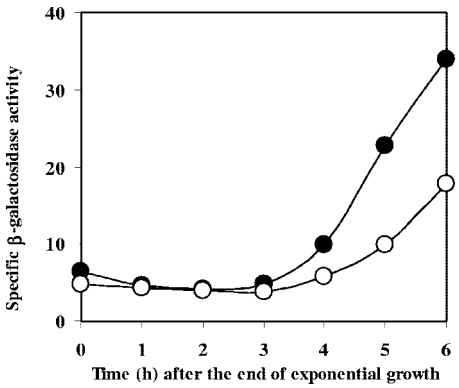

FIG. 9. Effect of ectopic expression of *spoIIGA* on σ^G activity in strains with the *spoIIG* and *spoIIA* loci deleted. The activity of σ^G is assessed as β-galactosidase expressed from an *sspA-lacZ* transcriptional fusion in the following strains with the *spoIIA* locus deleted: filled circles, SL12359 (*spoIIG*Δ::*cat thrC*::$P_{spac(hy)}$-*spoIIGA*) in the absence of IPTG; open circles, SL12359 in the presence of 1 mM IPTG.

Further, activity of σ^G was restored by expression of *spoIIGB* in *trans* from the IPTG-inducible $P_{spac(hy)}$ promoter (Fig. 8; SL11758 with IPTG) so that the effect of *spoIIGB* inactivation on the appearance of σ^G activity cannot be explained by polarity on *spoIIIG*. Rather, the results suggest that either σ^E or pro-σ^E has a role in σ^G activation. Because no σ^E transcriptional activity was detected in *spoIIA*Δ strains, it seemed likely that pro-σ^E is required and not σ^E. Indeed, expression of a pro-less form of σ^E in a *spoIIA*Δ *spoIIGB*Δ mutant did not restore σ^G activity, although the strain did display σ^E activity (data not shown). These results suggest a previously unsuspected role for pro-σ^E that cannot be played by σ^E.

Expression of *spoIIGB* in *trans* resulted in much stronger and earlier σ^G activity in a *spoIIA*Δ strain (Fig. 8; SL11758 with IPTG) than when it was expressed in its natural position as part of the *spoIIG* locus (Fig. 8; SL12436). When *spoIIGB* was expressed in *trans*, the σ^G activity was uncompartmentalized, and no sporulation septa were formed (SL11813; Table 2 and Fig. 5). The lack of septa was consistent with the conclusion presented in the previous section that early activation of σ^G prevented spore septum formation. It remains to be established why σ^G became active earlier in SL11758. The *spoIIGB* gene was expressed earlier than when it was at its natural locus, as the inducer was present throughout growth and sporulation with strains SL11758 and SL12436; presumably, the early appearance of pro-σ^E somehow resulted in the early σ^G activity. Speculatively, pro-σ^E might interact with LonA or some other protease and so protect σ^G from proteolysis.

Pro-σ^E appears to be required only when SpoIIGA is produced. This conclusion is suggested by two sets of experiments. First, when both *spoIIGA* and *spoIIGB* were deleted, there was σ^G activity in a *spoIIA*Δ strain (SL12137; Fig. 7), whereas when *spoIIGB* and not *spoIIGA* was deleted, no activity was detected (SL11758; Fig. 8). Second, when both *spoIIGA* and *spoIIGB* were deleted, induction of *spoIIGA* in *trans* substantially reduced σ^G activity (compare SL12359 in the presence and absence of IPTG; Fig. 9). The expression of σ^G activity in strain SL12359 even in the absence of IPTG was lower than in the corresponding strain, SL12137 (Fig. 7), that did not contain the $P_{spac(hy)}$-*spoIIGA* construct; we think that the reduced expression is a consequence of the leakiness of the inducible promoter. A clue to the possible role of pro-σ^E is provided by the observation that inactivation of *lonA*, which encodes an ATP-dependent protease (29), partly restored σ^G activity in a *spoIIGB* mutant strain (data not shown). LonA can degrade σ^G (29), and it may be that SpoIIGA sensitizes σ^G to proteolysis by LonA (or some other protease) and that somehow pro-σ^E but not σ^E can protect σ^G from the proteolysis. Our result is consistent with a role for SpoIIGA in facilitating LonA-directed proteolysis of σ^G, but it does not prove such a role.

The loss of σ^G-directed transcriptional activity correlates with loss of the σ^G protein in an *spoIIA* deletion strain in which *spoIIGB* is also disrupted. The loss of σ^G activity in *spoIIA*Δ strains with *spoIIGB* inactivated could result from absence of the σ^G protein or from the σ^G protein being held inactive. To distinguish between these possibilities, we used two approaches: first, immunoblotting with antibody directed against σ^G; second, fluorescence from a transcriptionally active σ^G-GFP fusion protein. The σ^G protein was first detected by immunoblotting 4 h after the end of exponential growth in *spo*$^+$ and *spoIIA*Δ strains, and substantially more was detected by 6 h (strains SL10369 and SL12436, respectively; Fig. 10). The presence of the σ^G protein correlated with σ^G activity as detected with an *sspA-lacZ* fusion (not shown). No σ^G protein was detected in an *spoIIIG* knockout mutant (strain SL11727). In contrast to the strong band observed for the *spoIIA*Δ mutant SL12436, the protein was barely detectable in a *spoIIA*Δ mutant with *spoIIGB* also inactivated (strain SL11671; Fig. 10). This result indicated that inactivation of *spoIIGB* resulted in the almost total absence of the σ^G protein, not simply its inhibition, in the *spoIIA*Δ background. The presence of σ^G was not restored by expression in *trans* of a constitutively active form of σ^E in a strain with *spoIIG* deleted (strain SL12042; Fig. 10).

We also utilized strains in which *spoIIIG* was replaced by a translational *spoIIIG-gfp* fusion via single-crossover (Camp-

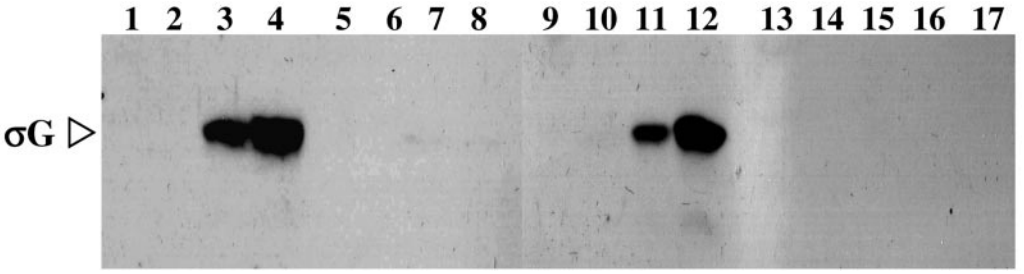

FIG. 10. Effect of deletion of *spoIIA* and *spoIIGB* on the accumulation of σ^G during sporulation. Protein samples (300 μg) were obtained at the indicated time (h) after the start of spore formation in MSSM and fractionated by electrophoresis. They were analyzed for σ^G by Western blotting using a polyclonal antiserum to σ^G. The strains used were SL10369 (*spo*$^+$), lanes 1 to 4; SL12436 (*spoIIA*Δ), lanes 9 to 12; SL11671 (*spoIIA*Δ *spoIIGB*Δ), lanes 5 to 8; SL12042 (*spoIIA*Δ *spoIIGB*Δ *thrC*::*sigE*), lanes 13 to 16; SL11727 (*spoIIA*Δ *spoIIIG*::*neo*), lane 17. Samples were taken at the end of exponential growth (lanes 1, 5, 9, and 13) and 2 h (lanes 2, 6, 10, and 14), 4 h (lanes 3, 7, 11, and 15), and 6 h after the end of exponential growth (lanes 4, 8, 12, 16, and 17). Lanes 1 to 8 and 9 to 17 are from two separate gels.

bell-like) plasmid integration. The fusion protein retained σ^G activity and did not block spore formation in a strain in which it was the sole copy of σ^G; the location of GFP is inferred to be a good indicator of the location of σ^G protein. In a *spo*$^+$ strain, GFP fluorescence was located in the prespore (SL12673; Table 2). When introduced into a *spoIIA*Δ strain, however, GFP fluorescence was confined to the mother cell (SL12674; Table 2), correlating with the location of σ^G activity in *spoIIA*Δ strains. The result is consistent with mother-cell-specific *spoIIIG* transcription. No GFP was detected in a *spoIIA*Δ strain in which *spoIIGB* was also inactivated (data not shown), so there was no indication of σ^G being present in an inactive form in that strain.

σ^E activity in the mother cell does not block σ^G activity. In strains deleted for *spoIIA* and with *spoIIG* intact, pro-σ^E is ordinarily not processed, and so σ^E is not active (38). We tested in two ways the effect on σ^G activity of having active σ^E in *spoIIA* deletion strains. (i) We introduced *spoIIR* under the control of the *spoIIE* promoter. *spoIIR* is the only σ^F-directed gene required for processing of pro-σ^E to its active form, and this construct results in σ^E activity in the absence of σ^F (38). Transcriptional activity of both σ^E and σ^G was detected with the construct (strains SL10215 and SL12518; Table 2). As reported previously for σ^E (38), about half the GFP-expressing bacteria showed mother cell specificity; the rest showed whole-cell activity and had no sporulation septum, probably because the slightly earlier σ^E activation in that part of the population had prevented septum formation. Similar localization was observed for σ^G activity (SL10215; Table 2). (ii) We inserted at *thrC* the gene for a constitutively active, pro-less form of σ^E. This construct resulted in lower σ^E activity than the P_{spoIIE}-*spoIIR* construct but a similar distribution of both σ^E activity and σ^G activity (not shown). Thus, as tested in two ways, σ^E did not have an antagonistic role towards σ^G. That many bacteria displayed mother-cell-specific σ^E activity reinforces the previous view that σ^F has at best a redundant role in directing σ^E activity to be confined to the mother cell (7, 38). The result with the pro-less form of σ^E suggests that processing of the pro sequence is not essential for compartmentalization of σ^E activity.

Both σ^E and σ^G were active before the completion of engulfment in strains SL10215 and SL12518. The σ^E activity in these strains enabled bacteria to complete engulfment (not shown); the corresponding strains, differing only by the lack of active σ^E, did not develop beyond septum formation. We infer that early activation of σ^G in the mother cell does not prevent engulfment. Both σ^E and σ^G activities were detected in the mother cell, suggesting no incompatibility between the two sigma factors, although we did not directly test whether they were active in the same mother cell. Presumably, both activities survive any competition with each other and with σ^A (18) for core RNA polymerase.

DISCUSSION

We report here that in the absence of both σ^F and the anti-sigma factor SpoIIAB, σ^G becomes active in the mother cell and not in the prespore during sporulation of *B. subtilis*. This is the first report, to our knowledge, of an efficient switch between prespore and mother cell of the location of activity of a sporulation-specific σ factor. The switch to mother cell location of the σ^G activity says that, at least in strains with *spoIIAB* and *spoIIAC* deleted, there is no "prespore-only" tag on σ^G and, likewise, no signal in the mother cell saying "no σ^G activity allowed." SpoIIAB acts as an anti-sigma factor for σ^G as well as for σ^F (15, 17) and is thought to act against σ^G in vivo primarily to prevent inappropriate activation in the mother cell (32). Our results are consistent with this interpretation.

The other factor thought to contribute to the mother cell location of σ^G activity in strains with the *spoIIA* locus deleted is transcription of *spoIIIG*, the structural gene for σ^G, from upstream promoters, most notably the *spoIIG* promoter (Fig. 2). In support of this statement, relocating *spoIIIG* away from its normal location, which is downstream of the *spoIIG* locus, abolished the mother cell specificity. Also, mother-cell-specific σ^G activity was detected in a *spoIIA* deletion strain in which the *spoIIG* structural genes were deleted while leaving in place the *spoIIG* promoter upstream of *spoIIIG* (SL12306; Table 2). Extending the deletion to include the *spoIIG* promoter substantially reduced σ^G activity (Fig. 7), indicating the importance of that promoter. However, residual mother-cell-specific σ^G activity remained even in the absence of the *spoIIG* promoter (SL12538; Table 2), suggesting that some other promoter also played a role.

In Spo$^+$ strains *spoIIIG* is transcribed productively (i.e., resulting in σ^G, which becomes active) from its own promoter.

This transcription is primed by σ^F and so occurs only in the prespore. A positive-feedback loop is then established in which transcription is directed from the same promoter by σ^G (reviewed in reference 10). However, the *spoIIIG* locus is also transcribed by read-through from the *spoIIG* locus. The read-through transcript is normally translated poorly, if at all, probably because it forms a hairpin structure that sequesters the presumed ribosome binding site for *spoIIIG* (20, 35). Moving *spoIIIG* to an ectopic locus away from *spoIIG* does not impair spore formation in an otherwise *spo*$^+$ strain, so any read-through transcript is clearly unnecessary for spore formation under the conditions used (35). Nevertheless, *spoIIIG* is located immediately downstream of *spoIIG* in all of the sequenced spore-forming bacteria (34). Such a juxtaposition suggests that in some circumstances the read-through may be important. Presumably in those circumstances the inhibitory effects of mRNA secondary structure can be overcome, as happens for the expression of *rpoH* and *rpoS* in *E. coli* (8), so as to produce some σ^G.

In strains with *spoIIAB* and *spoIIAC* deleted there is no σ^F priming and no SpoIIAB to block the activity of any σ^G formed in the mother cell as a result of read-through from *spoIIG*. In these circumstances, a small amount of active σ^G formed after the burst of *spoIIG* transcription that follows septation (7) may be sufficient to prime a positive-feedback loop of σ^G-directed transcription of *spoIIIG*. But now, the feedback loop is established in the mother cell, so that σ^G activity is confined to the mother cell. With respect to the prespore and the predivisional cell, expression from the *spoIIG* promoter is much reduced compared to that in the mother cell (7). Further, SpoIIAB has at most a redundant role in regulating σ^G in the prespore and also before septum formation, when other unidentified controls are thought to prevent activation (32). The net result is σ^G activity confined to the mother cell in strains with *spoIIAB* and *spoIIAC* deleted strains. Consistent with this interpretation, an *spoIIIG'-'gfp* translational fusion inserted at the *spoIIIG* locus is expressed only in the mother cell in a *spoIIA*Δ strain and only in the prespore in an *spo*$^+$ strain (Table 2).

When *spoIIIG* was moved to an ectopic locus, *amyE*, away from the *spoIIG* promoter in *spoIIA*Δ strains, σ^G became active earlier during spore formation and was more active than when at its natural locus. It is not known why there was this earlier and stronger activity. Whatever the explanation, σ^G activity was uncompartmentalized and no sporulation septum was formed. As neither σ^F nor σ^E was active, the result suggests that σ^G activation can, like that of σ^E (11) and σ^F (3, 9), prevent subsequent septum formation. The function of such an inhibitory role for σ^G in a wild-type genetic background is not clear, but it may relate to the phenomenon of commitment, namely, the ability of an organism to continue to form a spore despite the addition of nutrients that might otherwise trigger an inappropriate restoration of growth and division (22). Thus, σ^G would prevent division of the prespore at later stages of spore formation when σ^F activity is thought to be curtailed (18).

We report a previously unrecognized control of σ^G activity involving pro-σ^E and SpoIIGA, which became apparent in *spoIIA*Δ strains. We found that in the presence of SpoIIGA, σ^G activity is only detected when pro-σ^E is also present. Two lines of evidence suggest that it is pro-σ^E and not σ^E that is required for this effect. First, no σ^E activity was detected in the *spoIIA* deletion strains that displayed σ^G activity; second, σ^G activity was not detected when a pro-less form of σ^E, and not pro-σ^E, was expressed from an ectopic locus, although σ^E activity was now detected. We think that pro-σ^E is needed for σ^G activation only when SpoIIGA is present, because σ^G activity was detected in strains with both *spoIIGA* and *spoIIGB* deleted. Presumably, pro-σ^E works to protect σ^G from protease action or from some other inhibitory mechanism that is stimulated by SpoIIGA. The amount of σ^G protein was dramatically reduced in the strain with *spoIIGB* deleted (Fig. 10), so we think it likely that the effect of pro-σ^E is to stabilize σ^G rather than to activate a preexisting inactive form.

The protease LonA has previously been shown to degrade σ^G (29, 31), and inactivation of *lonA* partly restored σ^G activity to a *spoIIGB*Δ *spoIIA*Δ mutant strain. It may be that LonA and SpoIIGA/pro-σ^E represent separate regulators of σ^G activity and that loss of LonA leads to a large σ^G increase that disrupts the other system. Alternatively, or additionally, pro-σ^E may protect σ^G from SpoIIGA acting to stimulate proteolysis of σ^G by LonA. The mechanism of SpoIIGA/pro-σ^E regulation remains unknown. Nevertheless, our results suggest that several partly overlapping mechanisms ordinarily act to prevent σ^G activation in the mother cell. They indicate that regulators of σ^E and σ^F can also regulate σ^G.

ACKNOWLEDGMENTS

This work was supported by Public Health Service grant GM43577 (to P.J.P.) from the National Institutes of Health.

We thank Bettina Buttaro, Adriano Henriques, and Monica Serrano for helpful discussions. We thank Adriano Henriques and Monica Serrano for the antiserum to σ^G.

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