Role of the Anti-Sigma Factor SpoIIAB in Regulation of $\sigma^G$ during Bacillus subtilis Sporulation

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RNA polymerase sigma factor $\sigma^F$ initiates the prespore-specific program of gene expression during Bacillus subtilis sporulation. $\sigma^F$ governs transcription of spoIII,G, encoding the late prespore-specific regulator $\sigma^G$. However, transcription of spoIII,G is delayed relative to other genes under the control of $\sigma^F$, and after synthesis, $\sigma^G$ is initially kept in an inactive form. Activation of $\sigma^G$ requires the complete engulfment of the prespore by the mother cell and expression of the spoIII,A and spoIHI loci. We searched for random mutations in spoIII,G that bypassed the requirement for spoIII,A for the activation of $\sigma^G$. We found a mutation (spoIII,G,E156K) that resulted in an amino acid substitution at position 156, which is adjacent to the position of a mutation (E155K) previously shown to prevent interaction of SpoIIAB with $\sigma^G$. Comparative modeling techniques and in vivo studies suggested that the spoIII,G,E156K mutation interferes with the interaction of SpoIIAB with $\sigma^F$. The $\sigma^G$E156K isoform restored $\sigma^G$-directed gene expression to spoIIIA mutant cells. However, expression of spoIE-lacZ in the spoIII,A spoIII,G,E156K double mutant was delayed relative to completion of the engulfment process and was not confined to the prespore. Rather, $\beta$-galactosidase accumulated throughout the entire cell at late times in development. This suggests that the activity of $\sigma^G$E156K is still regulated in the prespore of a spoIIIA mutant, but not by SpoIIAB. In agreement with this suggestion, we also found that expression of spoIII,G,E156K from the promoter for the early prespore-specific gene spoIIQ still resulted in spoIE-lacZ induction at the normal time during sporulation, coincidentally with completion of the engulfment process. In contrast, transcription of spoIII,G,E156K, but not of the wild-type spoIII,G gene, from the mother cell-specific spoIID promoter permitted the rapid induction of spoIE-lacZ expression. Together, the results suggest that SpoIIAB is either redundant or has no role in the regulation of $\sigma^G$ in the prespore.

Gene expression in the prespore and mother cell chambers of sporulating Bacillus subtilis is controlled by RNA polymerase sigma subunits whose activity is restricted to a specific cell type (22, 31, 37, 46). The activation of the sporulation-specific sigma factors is tightly coupled to the completion of key morphological intermediates in the process and also relies on signaling pathways that operate between the two cell types and that keep the prespore and mother cell lines of gene expression in close register (22, 31, 37, 46). Soon after the asymmetric division of the sporangial cell, an event that creates the prespore and the much larger mother cell, the first compartment-specific sigma factor $\sigma^F$ becomes active in the prespore (22, 31, 37, 46). $\sigma^F$ triggers the activation of $\sigma^G$ in the mother cell, which together with $\sigma^F$ drive the migration of the septal membranes around the prespore. This process is termed engulfment and results in the formation of a prospore that is enclosed from the external medium, fully encircled by the mother cell cytoplasm (22, 31, 37, 46). After engulfment, $\sigma^F$ is replaced by $\sigma^G$, which controls late stages of development in this compartment and which also triggers the activation of the late mother cell-specific regulator $\sigma^K$ (22, 31, 37, 46). The activities of both $\sigma^F$ and $\sigma^K$ are required for the assembly of the protective layers that encase the mature spore (22, 31, 37, 46).

Synthesis of $\sigma^F$ occurs in the predivisional cell, but its activation is restricted to the prespore by the action of three regulatory proteins, SpoIIAA, SpoIIAB, and SpoIIE. SpoIIAB is an anti-sigma factor that binds to $\sigma^F$ as a dimer, preventing its association with RNA polymerase, whereas SpoIIAA is an anti-anti-sigma factor that in an unphosphorylated state interacts with SpoIIAB and releases $\sigma^F$ from the SpoIAB-$\sigma^G$ complex (1, 2; reviewed in references 31 and 37). SpoIIE is a septum-bound phosphatase that is also produced in the predivisional cell that promotes the preferential dephosphorylation of SpoIIAA-P in the prespore (reviewed in references 31 and 37).

The transcriptional activity of $\sigma^F$ can be divided into an early phase and a late phase. Transcription of the spoIII,G gene (encoding $\sigma^G$) is induced as part of the late phase, towards the end of the engulfment process (29). After synthesis, $\sigma^G$ does not become active until the engulfment process is complete (29). Once activated and since $\sigma^G$ efficiently recognizes its own promoter, its cellular levels increase rapidly, allowing for the deployment of the $\sigma^G$ regulon (17, 47). Because of this auto-regulation, both the late transcription of spoIII,G and the negative regulation of $\sigma^G$ appear to ensure that its transcriptional activity is effectively coupled to completion of the engulfment process and does not occur prematurely or ectopically (31, 37, 45). The tight coupling of $\sigma^G$ activation to the conclusion of the engulfment sequence may serve to ensure that biogenesis of

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the spore integuments is not initiated during movement of the engulfment membranes (31, 37, 45, 46).

Conclusion of the engulfment process is not sufficient for the activation of $\sigma^G$, which further requires expression of several genes, including the eight cistrons of the spoIIG operon and the spoIIGI gene (6, 19, 34). $\sigma^G$ accumulates in spoIIG or spoIIGI mutant cells but is unable to activate transcription from its target promoters (19, 41). The spoIIGI operon encodes several putative membrane proteins and is expressed in the mother cell under the direction of $\sigma^F$ (15). The spoIIGI gene is expressed during vegetative growth and encodes a membrane protein translocase of the YidC/Oxa1 family (6, 27, 48). Despite the fact that its product may accumulate in both the prespore and the mother cell (6, 27), expression of spoIIGI in the prespore is sufficient for the activation of $\sigma^G$ and sporulation (41).

Two negative regulators of $\sigma^G$ are known, the anti-sigma factor SpoIIB and the LonA protease (3, 8, 19, 21, 35, 40). Expression of spoIIG prior to the asymmetric division of the sporangial cell blocks sporulation, a phenotype that can be suppressed by a multicopy allele of spoIIG (21), and certain point mutations in spoIIB result in expression of $\sigma^G$-dependent genes under conditions that do not support efficient sporulation (8, 35). Moreover, SpoIIB binds to $\sigma^G$ in vitro under conditions that also promote binding of SpoIIB to $\sigma^F$ (19), and the structure of a dimer of Bacillus stearothermophilus SpoIIB in complex with $\sigma^F$ shows that most of the residues involved in the interaction are conserved in $\sigma^G$, but not in other sigma factors (2).

While it seems clear that SpoIIB can regulate $\sigma^G$ under nonsporulation conditions or in the predivisional cell at the onset of sporulation, the evidence for a role in the control of $\sigma^G$ in the prespore is less clear (3, 8, 19, 21, 35). On the one hand, SpoIIB seems to disappear from the prespore coincidently with the first manifestations of $\sigma^G$ activity, but it persists in the prespore of a spoIIGI mutant (21). In addition, production of a form of $\sigma^G$ ($\sigma^G_{GE156K}$) that is not efficiently bound by SpoIIB in vitro allows expression of the $\sigma^G$-controlled sspE gene in spoIIG or spoIIGI mutants, suggesting that the expression of both loci is required to antagonize the inhibitory action of SpoIIB upon $\sigma^G$ (19, 41). However, expression of sspE in spoIIGGE156K cells bearing mutations in either spoIIGI or spoIIGI does not occur prematurly, suggesting that the activity of $\sigma^G_{GE156K}$ is still regulated in the double mutants (19, 41). Also, there seems to be very little, if any, free SpoIIB in the prespore (28), and the anti-sigma factor would have to be able to negatively regulate $\sigma^G$ at a time when SpoIIB itself is antagonized by the anti-sigma-sigma SpoIIBA in order to release active $\sigma^G$ (reviewed in references 31 and 37). Since the interaction of SpoIIB with $\sigma^G$ appears to be very similar to the interaction of SpoIIB with $\sigma^F$ (7), it seems unlikely that at least prior to completion of the engulfment process, SpoIIB decisively contributes to the regulation of $\sigma^G$. Mutations in lonA, coding for the ATP-dependent LonA protease, also result in $\sigma^G$ activity under nonsporulation conditions, and result in some expression of sspE-lacZ in cells of a spoIIGI mutant during sporulation (40).

Here we have analyzed the role SpoIIB plays in the regulation of $\sigma^G$ in sporulating cells. We screened for mutations in spoIIG that allowed expression of the $\sigma^G$-controlled sspE-lacZ fusion in a spoIIGA background and found a single mutation that converted a glutamate at position 156 of $\sigma^G$ to a lysine. However, we found that expression of sspE-lacZ in a spoIIGGE156K spoIIGI double mutant was delayed relative to the completion of the engulfment process and was not confined to the prespore. Rather, $\beta$-galactosidase accumulated throughout the whole cell at late times of sporulation. We also forced the early expression of spoIIGGE156K in the prespore from the spoIIGP promoter and found no premature induction of sspE-lacZ expression. In contrast, expression of spoIIGGE156K in the mother cell readily results in sspE-lacZ expression. The results suggest that the activity of $\sigma^G$ is regulated in the prespore compartment by a SpoIIB-independent mechanism and that SpoIIB is either redundant or plays only a minor role.

MATERIALS AND METHODS

Bacterial strains and general methods. The B. subtilis strains used in this work (listed in Table 1) are congenic derivatives of the Spo+ strain MB24 (trpC2 metC3) (14). Luria-Bertani (LB) medium was used for the maintenance of Escherichia coli DH5a (Bethesda Research Laboratories) and B. subtilis. Sporulation was induced in different sporulation media (DSM) and assayed as described previously (13). All other general methods were performed as described previously (13).

Structure of a complex between B. subtilis SpoIIB and $\sigma^G$ by comparative modelling techniques. The structure of the SpoIIB-$\sigma^G$ complex from B. stearothermophilus (Protein Data Bank code 1LO0) (2) was used here to derive, by comparative modelling techniques, the SpoIIB-$\sigma^G$ and SpoIIB-$\sigma^F$ complexes from B. subtilis (25, 39). The structure of the SpoIIB-$\sigma^G$ complex from B. stearothermophilus (Protein Data Bank code 1LOO) (2) was used here to derive the SpoIIB-$\sigma^G$ and SpoIIB-$\sigma^F$ complexes from B. subtilis. The structure of SpoIIB from B. subtilis can be modelled on the basis of SpoIIB from B. stearothermophilus, because the two sequences present 75% identity and 90% similarity and only five residues at the C terminus cannot be aligned (25, 39). The structure of the SpoIIB-$\sigma^G$ complex from B. stearothermophilus contains information for only partial of $\sigma^G$ (from residues 104 to 160), and modelling of the sigma factors was restricted to the residues that are homologous to this segment. For B. subtilis $\sigma^G$, this segment presents 84% identity and 88% similarity to $\sigma^G$ from B. stearothermophilus, which also suggests that a very good model will be obtained. In contrast, B. subtilis $\sigma^F$ shows 30% identity and 65% similarity with $\sigma^F$ from B. stearothermophilus. The SpoIIB-$\sigma^G$ from B. stearothermophilus has a bound ADP molecule that was not modelled, because no contacts are made between this region and the sigma factor. Modeller (38) version 6.1 was used for all comparative modelling tasks. Sequences for both proteins in the complex were simultaneously aligned against the X-ray structure of SpoIIB-$\sigma^G$ from B. stearothermophilus, and 20 models were generated using these alignments. The model showing the lowest value of the objective function was chosen and analysed using PROCHECK (23). In the case of SpoIIB-$\sigma^G$ from B. subtilis, the Ramachandran plot showed 87.4% residues in most favored regions, 9.5% residues in additional allowed regions, 2.5% in generously allowed regions, and 0.6% residues in disallowed regions. The residues in disallowed regions, Leu 103 and Arg 105, are homologous to residues in SpoIIB-$\sigma^G$ from B. stearothermophilus that are also in disallowed regions. This region corresponds to the ADP binding site, and the conformation of these two residues is the most probable one. In the case of SpoIIB-$\sigma^G$ from B. subtilis, we obtained 88.1% residues in most favored regions, 8.5% residues in additional allowed regions, 3.1% in generously allowed regions, and 0.3% is disallowed regions. In this case, only Arg 105 is in a disallowed zone of the Ramachandran plot, and for the reasons stated above, its conformation was considered the most probable.

Construction of an sspE-lacZ fusion. First, a 500-bp HindIII-to-HincII fragment released from pUC12aspE (10) was inserted between the Smal and HindIII sites of pBluescript SKII(+) (Strategene, La Jolla, Calif.) to generate pAH225. Next, NheI- and EcoRI-digested pAH235 was mixed with the lacZ gene released from pPP207 (49) by digestion with SpeI and MscI, and a neomycin resistance (NmR) determinant was released from pBEST52 (16) with EcoRI and Smal. Linearization of the resulting plasmid, pMS33, with Scal permitted integration of the spoIIB-lacZ fusion into the spoIIB region and the sigma factor. Modeller (38) version 6.1 was used for all comparative modelling tasks. Sequences for both proteins in the complex were simultaneously aligned against the X-ray structure of SpoIIB-$\sigma^G$ from B. stearothermophilus, and 20 models were generated using these alignments. The model showing the lowest value of the objective function was chosen and analysed using PROCHECK (23). In the case of SpoIIB-$\sigma^G$ from B. subtilis, the Ramachandran plot showed 87.4% residues in most favored regions, 9.5% residues in additional allowed regions, 2.5% in generously allowed regions, and 0.6% residues in disallowed regions. The residues in disallowed regions, Leu 103 and Arg 105, are homologous to residues in SpoIIB-$\sigma^G$ from B. stearothermophilus that are also in disallowed regions. This region corresponds to the ADP binding site, and the conformation of these two residues is the most probable one. In the case of SpoIIB-$\sigma^G$ from B. subtilis, we obtained 88.1% residues in most favored regions, 8.5% residues in additional allowed regions, 3.1% in generously allowed regions, and 0.3% is disallowed regions. In this case, only Arg 105 is in a disallowed zone of the Ramachandran plot, and for the reasons stated above, its conformation was considered the most probable.

Conclusion of construction of spoIIG mutations. To create an in-frame deletion of the spoIIG locus, a 1.1-kb DNA fragment containing spoIIG (19) by digestion with BglII and SalI and inserted between the BglII and XhoI sites of pBluescript SKII(+) was used to replace the spoIIGI and spoIIGII operons. The spoIIGI operon encodes several putative membrane proteins and is expressed in the mother cell under the direction of $\sigma^F$ (15). The spoIIGI gene is expressed during vegetative growth and encodes a membrane protein translocase of the YidC/Oxa1 family (6, 27, 48). Despite the fact that its product may accumulate in both the prespore and the mother cell (6, 27), expression of spoIIGI in the prespore is sufficient for the activation of $\sigma^G$ and sporulation (41).
TABLE 1. B. subtilis strains used in this study

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<tr>
<th>Strain</th>
<th>Relevant genotype*</th>
<th>Origin</th>
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<td>AH62</td>
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<tr>
<td>ZB307</td>
<td>Prototrophic</td>
<td>Laboratory stock (52)</td>
</tr>
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* For simplicity, the spoIIGA::Tn917Hu24, ΔspoIIIG::sssp-lacZ, ΔamyE::P_pspo::spoIIG, ΔamyE::P_pspo::spoIIGE156K, ΔamyE::P_pspo::spoIIG::E156K alleles are abbreviated spoIIGA::Tn917, spoIIIG::sssp-lacZ, P_pspo::spoIIG, P_pspo::spoIIGE156K, P_pspo::spoIIG, and P_pspo::spoIIGE156K, respectively.

TABLE 2. Primers used in this study

<table>
<thead>
<tr>
<th>Primer</th>
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<td>spoIIG-24TR</td>
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<tr>
<td>spoIIG-599R</td>
<td>GGACCGGACTGAGACGACATCGC</td>
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<tr>
<td>spoIIG-392D</td>
<td>GGGAAAAAGATCCGAGAAATAAGTGC</td>
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<tr>
<td>spec-R</td>
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<td>spoIIIG-spoIIG</td>
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<td>spoIIG-761R</td>
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<tr>
<td>spoIAB-166D</td>
<td>CACAGGAGGATTTGAGCAGT</td>
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<tr>
<td>spoIAB-665R</td>
<td>CCTCGGATGCTATAAAATCTC</td>
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</table>

served as a PCR template using primers spoIIG::24TR and spoIIG-599R (Table 2). The PCR template was first treated with DpnI and then with PstI and last, it was autoligated, yielding pMS124. Sequencing confirmed the in-frame deletion of codons 13 to 130 of spoIIG. Competent cells of strain MB24 were cotransformed with pMS124 and chromosomal DNA from strain ZB307 (52), with selection for methionine prototrophy. spoIIG::sssp-lacZ recombinants appeared at a frequency of about 3%.

One, shown by PCR to carry a deletion of the spoIIG gene (referred to as ΔspoIIG) was named AH3795 (Table 1). Strain AH3795 was transformed with Scal-linearized pMS33 (sssp-lacZ) to produce AH2452. Strain AH2452 was then transformed with chromosomal DNA from strain AH62 (spoIIB::Tn917Hu24, hereafter abbreviated to spoIIB::Tn917) (Table 1) to create strain AH2456.

To create an insertion-deletion spoIIG mutant, a 922-bp DNA fragment containing the spoIIG gene was first released from pSP72119 (19) by partial digestion with EcoRI and HindIII and inserted between the EcoRI and HindIII sites of pLitmus 29 (New England Biolabs) to create pMS33. Next, a chloramphenicol resistance (Cmr) cassette was released from pMS38 (51) by digestion with NsiI and PstI and cloned into PstI-digested pMS33 to yield pMS40. Strain AH1870 (Cmr) in which disruption of the spoIIG gene by a double-crossover event was verified by PCR resulted from the transformation of strain MB24 with pMS40 (Table 1).

Insertion of an intact copy of the spoIIG gene at amyE. A copy of the spoIIG gene was inserted at amyE in two steps. We first isolated a 427-bp HindIII-to-BamHI fragment from pTK4 encompassing the spoIIG::24TR intergenic region (20), which was introduced between the HindIII and BamHI sites of the amyE integrative vector pMLK83 (18), to create pAH235. Strain AH1043 (amyE::P_amyE) (Table 1) resulted from the transformation of strain MB24 with XbaI-linearized pAH235. Then, a spectinomycin resistance (Sp) cassette was released from pAH256 (13) by digestion with Spel and NcoI and cloned between the same sites of pMS33 (see above), yielding pMS37. Last, a fragment carrying the Sp allele and the spoIIG gene was released from pMS37 by digestion with Smal and EcoRI and cloned between the EcoRI and NruI sites of pDG634 (21). Insertion of the resulting plasmid, pMS45, created AH1842 (Sp::AmyE and spoIIG) (Table 1), in which the presence of an intact spoIIG gene at amyE was verified by PCR. Transformation of strain MB24 with chromosomal DNA from AH1870 (ΔspoIIG::cat) produced AH1843 (ΔspoIIG::cat ΔamyE::spoIIG::E156K) (Table 1).

Random mutagenesis of spoIIG. Strain AH1843 (Table 1) was mutagenized with N-methyl-N'-nitro-N-nitrosoguanidine essentially as described previously (4). The mutagenesis was effective, as 1% of all colonies obtained from the transformation of strain AH1870 (ΔspoIIG::cat) (Table 1) with chromosomal DNA from mutagenized AH1843 selecting for Sp (which selects for the spoIIG copy at amyE) failed to complement the null mutation in the spoIIG gene. In order to select forΔamyE mutants that would bypass the need of the spoIIG locus for ΔamyE activity, chromosomal DNA from mutagenized AH1843 was used to transform AH2456 (ΔspoIIG spoIIB::Tn917 ssp-lacZ [see above]) to Sp.
Transformants that showed β-galactosidase activity on DSM plates supplemented with 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) (dark blue colonies) were selected and purified. The linkage between the Lac+ phenotype and the Sp+ marker was verified by retransforming the screening strain AH2456 with the wild-type allele of spoIIIG at amyE, which was constructed by transformation of AH2456 (ΔspoIIIG spoIIA::Tn917 sspE-lacZ [see above]) with chromosomal DNA from AH1842. Strains AH3786 and AH3787 were constructed by transformation of AH2452 (ΔspoIIIG spoIIA::Tn917 sspE-lacZ [see above]) with chromosomal DNA from AH1842 and AH3791, respectively.

Fusion of the spoIIIG promoter to the spoHII gene. Initially, the 5′ end of the spoHII gene and the spoIIIG promoter region (from positions −256 to −1 relative to the transcriptional start site) were amplified separately, from chromosomal DNA of B. subtilis MB24. Primers PspoIIIG-152D and PspoIIIG-2385R were used for the spoIIIG gene, and primers PspoIIQ-D and PspoIIQ-R were used for the spoHII gene (Table 2). The 370-bp spoIIIG fragment was mixed with the 256-bp spoIIQ fragment, and the resulting fragment of 619 bp was amplified using primers PspoIIQ-D and PspoIIIG-500R. The PspoIIQ-1D fragment was digested with EcoRI and BamHI and ligated to similarly cut pDG364 (4), to yield pMS237. Strains AH3786 and AH3787 (Table 1) were transformed with BamHI-linearized pMS237, selecting for Cm/Sp+ cells, to yield strains AH2490 and AH2491, which carry a fusion of the xylE-inducible PspoIIQ promoter to the spoIIIG and spoHII genes at amyE, respectively (abbreviated to P**spoIIQ** and P**spac**spoIIIGE156K, respectively) (Table 1).

Fusion of the spoIIIGE156K allele to different sporulation promoters. Fusions of spoIIIG to the spoIIQ and to the spoIID promoters were constructed as follows. Initially, the 5′ end of the spoIIIG gene and its promoter region were amplified separately from chromosomal DNA of a wild-type strain that had been mutagenized with N-methyl-N-nitro-N-nitrosoguanidine (Table 1). Strain AH3791 harbored a single-nucleotide change (GAA to AAA) at codon 156 of the spoIIIG gene at amyE causing the replacement of a glutamic acid for a lysine. This allele of spoIIIG was designated spoIIIGE156K. Strains bearing a wild-type spoIIIG gene (AH3786) or the spoIIIGE156K allele (AH3787) at amyE in a spoII mutant background (ΔspoIIIG spoIIA::Tn917 sspE-lacZ) were DNA-positive for the desired allele (Table 1).

Isolation and characterization of the spoIIIGE156K allele. To analyze the mechanism by which αG is kept inactive in cells of a spoIIA mutant, we sought random mutations in spoIIIG at amyE that bypassed the requirement for spoIIA for expression of the αG-controlled spoE operon (26) (see Materials and Methods). We isolated one Lac− mutant called AH3791 (Table 1) upon transformation of B. subtilis strain AH2456 (ΔspoIIIG spoIIA::Tn917 sspE-lacZ) with DNA from strain AH1843 mutated by N-methyl-N-nitro-N-nitrosoguanidine (Table 1). Strain AH3791 harbored a single-nucleotide change (GAA to AAA) at codon 156 of the spoIIIG gene at amyE causing the replacement of a glutamic acid for a lysine.

This allele of spoIIIG was designated spoIIIGE156K. Strains bearing a wild-type spoIIIG gene (AH3786) or the spoIIIGE156K allele (AH3787) at amyE in a spoII mutant background (ΔspoIIIG spoIIA::Tn917 sspE-lacZ) were DNA-positive for the desired allele (Table 1). Nevertheless, while in the spoIIA+ strain AH3787, expression of spoE-lacZ was induced around 4 h after the onset of sporulation (Fig. 1), as in a Spo− strain expressing the wild-type spoIIIG allele at its normal position (26; also data not shown), and reached maximum levels around 6 h after sporulation had started. As expected, expression of spoE-lacZ in strain AH3790 (ΔspoIIIG spoIIA::Tn917 sspE-lacZ ΔamyE::spoIIIG) was severely impaired (19). However, in the congenic strain AH3791, which bears the spoIIIGE156K allele at amyE, expression of spoE-lacZ was restored (Fig. 1). Nevertheless, while in the spoIIA+ strain AH3787, expression of spoE-lacZ was strongly induced around 4 h after the onset of sporulation, in the spoIII4 mutant strain AH3791 (spoIIIGE156K), β-galactosidase accumulated at a reduced rate between 4 and 6 h after the onset of sporulation and reached maximum levels only around 10 h after sporulation had started (Fig. 1). Also, even though the spoIIIGE156K allele restores αG activity to spoIII4 cells, strain AH3791 was still unable to sporulate (Table 3). We also found that the spoIIIGE156K allele restored spoE-lacZ expression (but not sporulation) to a ΔspoIII3::km mutant (data not shown).

To investigate whether the increased activity of αGGE156K in Spo− cells or in the spoIIA mutant relative to wild-type αG could be attributed to its increased accumulation, we compared the levels of αG and αGGE156K throughout sporulation by immunoblot analysis using a previously described anti-αG an-
We found that in agreement with the timing of sspE-lacZ expression, $\sigma^G$ or $\sigma^G_{E156K}$ reached peak levels around 4 h after the onset of sporulation in Spo$^+$ cells (Fig. 2A and B). In a spoIIIA background, the accumulation of the wild-type form of $\sigma^G$ was delayed, reaching maximum levels around 6 h after the start of sporulation (Fig. 2C). In spoIIIJ::Tn197 spoIIIGE156K cells (AH3791), $\sigma^G_{E156K}$ is detected only from 5 h on, and its accumulation reaches a maximum around 8 h after the onset of sporulation (Fig. 2D). The late accumulation of $\sigma^G_{E156K}$ in the spoIIIA mutant suggests that some $\sigma^G_{E156K}$ (but not wild-type $\sigma^G$) escapes inhibition late in sporulation and then amplifies its own synthesis. This late accumulation of $\sigma^G_{E156K}$ in the spoIIIA::Tn197 spoIIIJGE156K double mutant correlates with the late expression of sspE-lacZ (Fig. 1). Note that under our electrophoretic conditions, the $\sigma^G_{E156K}$ form migrates slightly faster than wild-type $\sigma^G$ (compare the mobility of $\sigma^G$ and $\sigma^G_{E156K}$ relative to a background band labeled with an asterisk seen in a sample from a spoIIIG deletion mutant [Fig. 2A and B, for example]). The levels of $\sigma^G_{E156K}$ do not appear to be higher than those of wild-type $\sigma^G$ in either spoIIIA$^+$ or spoIIIA mutant cells (Fig. 2, compare panels A and B and panels C and D). Thus, the difference in expression of sspE-lacZ in spoIIIJGE156K strains AH3787 (spoIIIA$^+$) and AH3791 (spoIIIA mutant) relative to congenic strains expressing a wild-type spoIIIG gene at the amyE locus cannot be explained by an increase in the synthesis or stability of $\sigma^G$. Rather, it may reflect increased activity of the sigma factor. In the case of AH3791 (spoIIIA::Tn197 spoIIIJGE156K), the increased activity of $\sigma^G_{E156K}$ is manifested only at a late time in development.

The E156K mutation is likely to interfere with the interaction between SpoIIAB and $\sigma^G$. Like spoIIIGE156K, a previously described allele of spoIIIG bearing a glutamate-to-lysine substitution at position 155 (spoIIIGE155K) allows expression of sspE-lacZ in cells with the mutant spoIIIA or spoIIIJ gene (19, 41). The E155K substitution was introduced in $\sigma^G$, because a glutamic acid-to-lysine substitution at an equivalent position of $\sigma^F$ (E149K) was found in a genetic screen for $\sigma^F$ mutants with reduced affinity for SpoIAB (5). Moreover, under conditions in vitro that promote binding of SpoIIAB to $\sigma^F$, the anti-sigma factor also binds to $\sigma^G$, but not to $\sigma^G_{E155K}$ (19).

The binding of SpoIAB to $\sigma^F$ or $\sigma^G$ can now be described in molecular terms, using the crystal structure (2) of the SpoIAB-$\sigma^F$ complex from B. stearothermophilus and the comparative models for the SpoIAB-$\sigma^F$ and SpoIIAB-$\sigma^G$ complexes from B. subtilis derived here. These structures show that in B. stearothermophilus $\sigma^F$, the residue (E147) equivalent to E149 in the $\sigma^G$ protein of B. subtilis, as well as three other residues found in genetic screens for mutants resistant to inhibition by SpoIAB are located within a region that contains 17 amino acids (in B. stearothermophilus) found to interact with SpoIAB (2, 5) (Fig. 3A to C). Of the amino acids, 15 are either identical or homologous in $\sigma^G$ (compare Fig. 3, panels C and F) and 3 are uniquely conserved between $\sigma^G$ and $\sigma^F$ (2; also data not shown).

The nature of the E155K and E156K mutations in $\sigma^G$ from B. subtilis can be qualitatively understood by examination of the model for the SpoIIAB-$\sigma^G$ complex (Fig. 3D to F). The model shows that residue E155 of $\sigma^G$ interacts with S17 in one of the SpoIAB molecules present in the dimer (Fig. 3D to F and insert), a contact that is also conserved in the SpoIIAB-$\sigma^F$ complex.

### Table 3: Sporulation of six B. subtilis strains

<table>
<thead>
<tr>
<th>Strain</th>
<th>Relevant genotype</th>
<th>Viable cells</th>
<th>Heat-resistant cells</th>
<th>% Sporulation</th>
</tr>
</thead>
<tbody>
<tr>
<td>AH3786</td>
<td>spoIIIG P$_{spoIIIC}$::spoIIIG</td>
<td>$6.9 \times 10^6$</td>
<td>$5.0 \times 10^6$</td>
<td>72.5</td>
</tr>
<tr>
<td>AH3787</td>
<td>spoIIIG P$_{spoIIIC}$::spoIIIGE156K</td>
<td>$5.0 \times 10^6$</td>
<td>$3.8 \times 10^6$</td>
<td>76.0</td>
</tr>
<tr>
<td>AH3788</td>
<td>spoIIIG P$_{spoIIIC}$::spoIIIG</td>
<td>$5.0 \times 10^6$</td>
<td>$4.8 \times 10^6$</td>
<td>96.0</td>
</tr>
<tr>
<td>AH3789</td>
<td>spoIIIG P$_{spoIIIJ}$::spoIIIGE156K</td>
<td>$1.5 \times 10^6$</td>
<td>$6.0 \times 10^6$</td>
<td>40.0</td>
</tr>
<tr>
<td>AH3790</td>
<td>spoIIIG spoIIIJ P$_{spoIIIC}$::spoIIIG</td>
<td>$1.4 \times 10^6$</td>
<td>$3.0 \times 10^6$</td>
<td>0.02</td>
</tr>
<tr>
<td>AH3791</td>
<td>spoIIIG spoIIIJ P$_{spoIIIC}$::spoIIIGE156K</td>
<td>$1.1 \times 10^6$</td>
<td>$1.0 \times 10^6$</td>
<td>0.01</td>
</tr>
</tbody>
</table>

* The extent of sporulation was measured 24 h after the onset of the process in liquid sporulation medium as described in Materials and Methods.
complex (E149) (Fig. 3A to C). The models also predict that residue E156 of σG contacts residue K41 in the same SpoIIAB molecule contacted by residue E155 of σG (Fig. 3, insert), and again, this contact is conserved in the SpoIIAB-σF complex (D150) (Fig. 3A to C). Therefore, both the E155K and E156K substitutions introduce unfavorable interactions expected to destabilize the interaction of SpoIIAB with σG. We infer that the E156K mutation reduces the binding of SpoIIAB to σG in a manner similar to that observed for σG(K19). However, the interaction of E155 with a serine residue (S17) from SpoIAB is likely to be less strong than the interaction of E156 that forms a salt bridge with K41 from SpoIIAB (Fig. 3, insert). Therefore, the effect of the E156K mutation, placing two positively charged residues in close proximity would create packing problems, and appears more disadvantageous for complex formation than the E155K mutation. Since the models for the two complexes show that most of the contacts between SpoIAB and σF or σG are conserved, our analysis supports the conclusion of Evans et al. that the interaction of SpoIIAB with either σF or σG is very similar (7).

**σG** is less sensitive to SpoIIAB in vivo. To determine whether the σG(K19) form was less sensitive to the inhibitory action of SpoIIAB in vivo, we constructed strains engineered to coexpress spoIAB and either spoIIIG or spoIIIGE156K during vegetative growth in a medium (LB) that does not support efficient sporulation. Strains AH2492 and AH2493 carry a fusion of the xylose-inducible _P_xylA promoter to the _spoIIIG_ and _spoIIIGE156K_ genes, respectively, inserted at the _AmyE_ locus, as well as an IPTG-inducible _P_spac-spoIAB_ fusion inserted at the _thrC_ locus; in addition, the two strains carry an _spcE-lacZ_ fusion (Table 1). Preliminary experiments revealed that expression of _P_spac-spoIIIG_ and _P_spac-spoIIIGE156K_ in the absence of xylose resulted in significant expression of _spcE-lacZ_ (data not shown). Therefore, AH2492 and AH2493 were grown in the absence of xylose and in the absence or presence of IPTG (1 mM) to induce SpoIIAB production.

Expression of _spoIIIG_ or _spoIIIGE156K_ in the presence or absence of SpoIIAB did not result in any detectable growth differences between the strains (Fig. 4A). The _spcE-lacZ_-driven production of β-galactosidase was monitored in the various cultures during the log phase of growth, 1.5, 2, and 2.5 h after inoculation. We found that in the absence of IPTG, the activities of both σG and σG(K19) increased during the experiment, even though the activity of σG(K19) was always lower than that of the wild-type form (Fig. 4B). In the presence of IPTG to induce _spoIAB_ expression, the activity of wild-type σG was immediately reduced and remained at low levels (Fig. 4B). In contrast, the activity of σG(K19) was reduced slowly (Fig. 4B). Moreover, at all the times tested, the fraction of σG(K19) activity remaining after IPTG-induced SpoIIAB synthesis was higher than the fraction of σG activity remaining after SpoIIAB induction (Fig. 4C). These results are consistent with the suggestion that the E156K substitution makes σG less sensitive to the inhibitory action of SpoIIAB.

Activity of **σG** in a _spoIIA_ background is delayed relative to completion of the engulfment process. On the basis of an analogy to the E155K mutation and the results discussed above, we expected that the E156K substitution would also relieve the inhibitory action of SpoIIAB on σG during sporulation. If the interaction of SpoIIAB with σG were reduced, as
suggested by our screen, and because $\sigma^G$ is autoregulatory (17, 47), we would expect premature expression of $\text{sspE-lacZ}$ if SpoIAB were the primary inhibitor of $\sigma^G$ activity in the prespore. In contrast to this expectation, activity of $\sigma^G_{\text{GE156K}}$ was delayed in spoIIIA cells (Fig. 1), which remained unable to sporulate (AH3791) (Table 3).

To determine whether expression of $\text{sspE-lacZ}$ was still coupled to the completion of the engulfment process in strain AH3791 ($\text{spoIII A spoIIIIGE156K}$), we used the membrane stains FM4-64 and MTG to monitor completion of the engulfment process (44). We stained samples of the same strains depicted in Fig. 1, and in parallel, we monitored accumulation of $\beta$-galactosidase to control for the onset of $\sigma^G$ activity. We could not use a fusion of the $\text{sspE}$ promoter to the $\text{gfp}$ gene for this purpose, because even in the absence of $\sigma^G$, most of the cells showed some prespore decoration, presumably due to the activity of $\sigma^F$ (data not shown).

As in the experiment documented in Fig. 1, expression of $\text{sspE-lacZ}$ in the $\text{Spo}^+$ strains AH3786 ($\text{spoIIIIG at amyE}$) and AH3787 ($\text{spoIIIIGE156K at amyE}$) commenced around 4 h after the onset of sporulation, when 42 and 31% of the cells, respectively, had completed the engulfment process (Table 4). Activity of $\beta$-galactosidase peaked 6 h after the onset of sporulation for strain AH3786, when 48% of the cells showed complete engulfment of the prespore, and 6 to 8 h after the start of sporulation for AH3787, when 51 to 74% of the cells had completed the engulfment process (Table 4). These observations are in agreement with the results of a previous study,
FIG. 4. $\sigma^{\text{GE156K}}$ is less susceptible to SpoIIAB in vivo than wild-type $\sigma^G$. (A) B. subtilis strains AH2492 ($\text{P}_{\text{spoIIHIG}} \text{P}_{\text{spoIIAB sspE-lacZ}}$) (circles) and AH2493 ($\text{P}_{\text{spoIIHIGE156K}} \text{P}_{\text{spoIIAB sspE-lacZ}}$) (squares) were grown in LB medium with 1 mM IPTG (closed symbols) to induce spoIIAB expression or in the absence of inducer (open symbols). OD (600 nm), optical density at 600 nm. (B) Samples were taken at the indicated times and assayed for $\beta$-galactosidase production. From left to right, the four bars for each time point show the results for strain AH2492 grown in the absence of IPTG (white bars), AH2492 grown in the presence of IPTG (black bars), AH2493 grown in the absence of IPTG (light grey bars), and AH2493 grown in the presence of IPTG (dark grey bars). (C) Ratio between the activity of $\sigma^G$ (black bars) or $\sigma^{\text{GE156K}}$ (white bars) in the absence and presence of IPTG (expressed as a percentage).

TABLE 4. Time of engulfment completion in four B. subtilis strains

<table>
<thead>
<tr>
<th>Strain</th>
<th>Genotype</th>
<th>Time (h)</th>
<th>No. counted</th>
<th>Sporulating cells (%)</th>
<th>Stage III cells (%)</th>
</tr>
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<tbody>
<tr>
<td>AH3786</td>
<td>spoIIHIG wt</td>
<td>2</td>
<td>279</td>
<td>91/33</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td></td>
<td>4</td>
<td>143</td>
<td>101/71</td>
<td>60/42</td>
</tr>
<tr>
<td></td>
<td></td>
<td>6</td>
<td>132</td>
<td>90/68</td>
<td>64/48</td>
</tr>
<tr>
<td></td>
<td></td>
<td>8</td>
<td>131</td>
<td>100/76</td>
<td>86/66</td>
</tr>
<tr>
<td></td>
<td></td>
<td>10</td>
<td>115</td>
<td>93/81</td>
<td>83/72</td>
</tr>
<tr>
<td>AH3787</td>
<td>spoIIHIGE156K</td>
<td>2</td>
<td>487</td>
<td>153/31</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td></td>
<td>4</td>
<td>178</td>
<td>121/68</td>
<td>55/31</td>
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<td></td>
<td>6</td>
<td>357</td>
<td>221/62</td>
<td>53/51</td>
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<td></td>
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<td>8</td>
<td>158</td>
<td>133/84</td>
<td>117/74</td>
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<tr>
<td></td>
<td></td>
<td>10</td>
<td>172</td>
<td>129/75</td>
<td>114/66</td>
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<td>AH3790</td>
<td>spoIIA spoIIHIG</td>
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<td>157</td>
<td>70/45</td>
<td>0</td>
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<td>121/70</td>
<td>54/31</td>
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<td></td>
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<td>6</td>
<td>167</td>
<td>119/71</td>
<td>98/59</td>
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<td></td>
<td></td>
<td>8</td>
<td>102</td>
<td>70/69</td>
<td>53/52</td>
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<tr>
<td></td>
<td></td>
<td>10</td>
<td>152</td>
<td>67/44</td>
<td>48/32</td>
</tr>
<tr>
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<td>219</td>
<td>77/35</td>
<td>0</td>
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<td>60/54</td>
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<td></td>
<td></td>
<td>10</td>
<td>153</td>
<td>70/46</td>
<td>54/35</td>
</tr>
</tbody>
</table>

a Hours after the onset of sporulation.
b The total number of cells counted is indicated.
c The number of sporulating cells, estimated on the basis of the pattern of staining by FM4-64 and MTG (see text), is shown before the slash, and the percentage of sporulating cells is shown after the slash.
d The number of cells in stage III or above is shown before the slash, and the percentage of cells in stage III or above is shown after the slash.
e spoIIHIG wt, wild-type spoIIHIG.

suggesting that the activity of $\sigma^G$ coincides with the completion of engulfment when sporulation is induced by growth and resuspension in a minimal medium (29). About 20% of AH3791 cells (spoIIA::Tn917 spoIIHIGE156K) showed complete engulfment by 4 h after the onset of sporulation, a fraction that increased to 39% by 6 h (Table 4), and by 8 h after sporulation had started, the number of cells showing clear signs of having completed the engulfment sequence reached a maximum of 54% (Table 4). However, this did not correspond to peak levels of sspE-lacZ expression (Fig. 1). Rather, enzyme production reached a maximum 10 h after the onset of sporulation, when the fraction of cells with clear signs of complete engulfment actually decreased to about 35% (Table 4). The reasons for this decrease may reflect instability of the prespore in cells bearing a spoIIA mutation (see below). Consistent with this interpretation, 10 h after the onset of sporulation in strains bearing a mutation in spoIIA, the pattern of fluorescence decoration resulting from MTG staining tended to change from the ellipsoidal contour of the prespore to a more or less indistinct mass of fluorescence, suggesting coalescence of the prespore membranes (data not shown). We note that a spoIIA mutation per se does not significantly interfere with the timing of engulfment (AH3790) (Table 4). We interpret these observations as indicating that $\sigma^{\text{GE156K}}$ becomes active only about 2 h after the completion of engulfment in a spoIIA mutant.

Activity of $\sigma^{\text{GE156K}}$ in a spoIIA background is not confined to the prespore. The observation that the activity of $\sigma^{\text{GE156K}}$ was delayed relative to the completion of the engulfment process in a spoIIA mutant led us to examine the location of $\beta$-galactosidase produced from the sspE-lacZ fusion in this strain. We used immunofluorescence microscopy to examine samples of the same cultures used in the experiment depicted in Fig. 1 6 and 10 h after the onset of sporulation, as this corresponds to peak levels of sspE-lacZ expression in spoIIA+ or spoIIA mutant cells, respectively (Fig. 1). We were unable to collect reasonable phase-contrast images or images of cells in which the membrane had been stained with FM4-64 or MTG after fixation and permeabilization of the cells with lysozyme (also see reference 33). For that reason, the number of sporulating cells was scored on the basis of the analysis of the pattern of nucleoid staining by DAPI (see Materials and Methods), and for each of these cells, the pattern of $\beta$-galactosidase localization was recorded (see Materials and Methods) (Table 5).
production of β-galactosidase was detected only in cells in which the prespore had been completely engulfed by the mother cell (as defined by DAPI staining, which reveals two equal-size mother cell and prespore chromosomes). Production of the enzyme was always confined to the prespore compartment of strain AH3786 (Fig. 5a to c and Table 5), whereas for strain AH3787, a small percentage of cells (around 1 or 2%) 6 or 10 h after the onset of sporulation showed fluorescence throughout the entire cell (Fig. 5f and Table 5). Interestingly, these specimens did not present any distinctive signs of sporulation as judged from the pattern of DAPI staining (Fig. 5d to f). This effect does not seem to be caused by deficient staining of one of the chromosomes, since the fluorescence signal is distributed by what would be the length of the entire sporulating cell (mother cell plus prespore). These specimens may represent vegetative cells or cells in which the normal compartmentalization of σA activity was lost (see below).

In agreement with the results of sspE-lacZ expression shown in Fig. 1, essentially no β-galactosidase could be detected in cells of AH3790 (∆spoIIIG spoIIA::Tn917 sspE-lacZ ΔamyE::spoIIIG) (Fig. 5i) shows the only specimen found with signs of florescence in the prespore) (Table 5). About 3% of the cells of AH3791 (∆spoIIIG spoIIA::Tn917 sspE-lacZ ΔamyE::spoIIIGE156K) at 6 h after the onset of sporulation showed accumulation of β-galactosidase, a percentage that increased to 23% at 10 h (Table 5). Surprisingly, in most of these cells, β-galactosidase was found to localize throughout the entire cell (Fig. 5j to l and Table 5). The percentage of sporulating cells of AH3791 (two visible nucleoids of about the same size at this stage) decreased from 75% at 6 h after the onset of sporulation to about 32% at 10 h (Fig. 5j to l [6 h] and m to o [10 h] and Table 5). Note that the specimens showing whole-cell fluorescence do not show the DAPI staining pattern of a mid to late stage of sporulation and that specimens in which the prespore can be clearly distinguished do not show sspE-lacZ expression (Fig. 5j to o). The percentage of AH3790 cells (wild-type spoIIIG in a spoIIA background) with two distinct nucleoids also decreased from 6 h (78%) to 10 h after the onset of sporulation (60%) (Table 5) except that in the latter case, the whole-cell pattern of decoration was never found. No decrease in the percentage of sporulating cells was noticed for the spoIIA+ strains.

The results show that σG156K does not become active exclusively in the prespore of a spoIIA mutant. The results suggest that spoIIA may function to antagonize an as yet unknown negative regulator of σG following completion of the engulfment process and that spoIIA may serve an additional function in sporulation related to the maintenance of compartmentalized gene expression in postengulfment cells. Essentially the same observations, i.e., absence of prespore-specific expression of sspE-lacZ, were made for spoIIA cells harboring the E155K mutation (19; E. M. Kelner and C. P. Moran, Jr., unpublished results), reinforcing the view that the E155K and E156K substitutions affect the activity of σG similarly.

Early expression of spoIIIGE156K in the prespore does not result in premature activity of σG. Transcription of the spoIIIG gene by σG is delayed by an unknown mechanism towards the end of the engulfment process relative to the transcription of the first class of σG-dependent genes, which includes the spoIIQ gene (24). We fused the coding region of spoIIIGE156K to the early σG-dependent spoIIQ promoter, reasoning that the fusion allele would bypass both the mechanism that delays transcription of spoIIIG and a possible negative effect of SpoIAB prior to engulfment. The promoter fusion was introduced at the amyE locus, producing strain AH3789 (∆spoIIIG spoIIQ spoIIGE156K). The spoIIQ promoter was also fused to the coding region of the wild-type spoIIIG gene, and the fusion was inserted at amyE to produce AH3788 (∆spoIIIG spoIIQ spoIIGE156K). Both AH3789 and AH3788 sporulate efficiently (Table 3).

In agreement with unpublished work cited by Stragier and Losick (46), the expression of spoE-lacZ in AH3788 began to increase around 4 h after the onset of sporulation, as in strains bearing the wild-type or spoIIIGE156K allele under the control of its native promoter (AH3786 and AH3787) (Fig. 6). Moreover, induction of spoE-lacZ also occurred around 4 h of sporulation in strain AH3789 (Fig. 6); the spoIIQ promoter drives expression of spoIIIGE156K in AH3789.

When produced from their own promoter, σG and σG156K were first detected 3 h after the onset of sporulation, and the

TABLE 5. Patterns of β-galactosidase localization

<table>
<thead>
<tr>
<th>Strain</th>
<th>Genotype</th>
<th>Time (h)a</th>
<th>No. countedb</th>
<th>Sporulating cells%c</th>
<th>Localization of β-galactosidased</th>
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</thead>
<tbody>
<tr>
<td>AH3786</td>
<td>spoIIIG wt</td>
<td>6</td>
<td>411</td>
<td>250/61</td>
<td>Prespore 0/0/0</td>
</tr>
<tr>
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<td></td>
<td>10</td>
<td>360</td>
<td>358/99</td>
<td>Mother cell 9/0/0</td>
</tr>
<tr>
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<td>spoIIIGE156K</td>
<td>6</td>
<td>446</td>
<td>305/68</td>
<td>Prespore 222/0/9</td>
</tr>
<tr>
<td></td>
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<td>397/96</td>
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</tr>
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<td>151/78</td>
<td>Prespore 0/0/0</td>
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<td>178</td>
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<td></td>
<td>10</td>
<td>587</td>
<td>187/32</td>
<td>Mother cell 0/1/135</td>
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</tbody>
</table>

a Hours after the onset of sporulation.
b The total number of cells counted.
c The number of sporulating cells, estimated on the basis of the pattern of DAPI staining (see text), is shown before the slash, and the percentage of sporulation is shown after the slash.
d The number of cells or free spores in prespores, mother cells (MC), and whole cells (cells with no signs of prespore).
level of the \(\sigma^{G}\) factor increased until 4 h (Fig. 2A and B), whereas utilization of the spoIIQ promoter permitted the accumulation of \(\sigma^{G}\) or \(\sigma^{GE156K}\) from 2 h on, with maximum levels between 3 and 4 h after the onset of sporulation (Fig. 2E and F). In all the strains included in the experiment of Fig. 6, induction of spoE-lacZ expression 4 h after the onset of sporulation coincided with completion of the engulfment process (as assayed by FM4-64 or MTG staining) (between 31 and 56% [data not shown]). Both strains bearing the wild-type or spoIIIGE156K allele under the control of the spoIIQ promoter presented a high background of \(\sigma^{G}\) activity starting at the onset of sporulation. The reason for this behavior is not known, but it could be the result of high levels of expression of spoIIIG or spoIIIGE156K from the strong spoIIQ promoter (24). In any event, we note that expression of spoIIIGE156K does not result in a higher background relative to expression of the wild-type spoIIIG gene (Fig. 6). Moreover, we note that in both AH3788 and AH3789, expression of spoE-lacZ remains constant (Fig. 6), while the cellular levels of \(\sigma^{G}\) increase (Fig. 2E and F). It appears that even though \(\sigma^{GE156K}\) accumulates starting 2 h after the onset of sporulation, it directs induction of spoE-lacZ expression only around 4 h, when the process of engulfment of the prespore by the mother cell is complete. Together, the results suggest that SpoIIAB may not contribute decisively to

FIG. 5. Immunolocalization patterns of \(\beta\)-galactosidase produced from the spoE-lacZ fusion. The strains of \(B. subtilis\) were grown in DSM, and samples were taken at the indicated times after the onset of sporulation, stained with DAPI, and processed for immunofluorescence microscopy as described in Materials and Methods. Typical localization patterns of \(\beta\)-galactosidase (\(\beta\)-gal.) produced from the spoE-lacZ fusion for strains AH3786 (\(\Delta\)spoIIIG spoE-lacZ \(\Delta\)amyE::spoIIIG) (wild-type spoIIIG in a wild-type background) (a to c), AH3787 (\(\Delta\)spoIIIG spoE-lacZ \(\Delta\)amyE::spoIIIGE156K) (d to f), AH3790 (\(\Delta\)spoIIIG spoIIIA::Tn917 spoE-lacZ \(\Delta\)amyE::spoIIIG) (wt) (g to i), and AH3791 (\(\Delta\)spoIIIG spoIIIA::Tn917 spoE-lacZ \(\Delta\)amyE::spoIIIGE156K) (j to o) at the indicated times after the onset of sporulation are shown. The complete relevant genotypes of the strains are given in Table 1. The samples were taken 6 or 10 h after the onset of sporulation (T6 and T10, respectively). Arrowheads point to specimens showing spoE-lacZ expression. The specimen labeled with an asterisk in panel f, as well as the specimens in panels l and o, show whole-cell fluorescence (see text). Bars, 2 \(\mu\)m.
the inhibition of σ^G activity in the prespore. The fact that very little free SpoIAB seems to accumulate in the prespore and the similarity of the interaction of SpoIAB with both σ^F and σ^G (7, 28; this work) are consistent with this interpretation.

Activity of σ^G in the mother cell is antagonized by SpoIAB and LonA. Since σ^G is autoregulatory and SpoIAB is present in both the prespore and mother cell compartments, we wanted to determine whether SpoIAB could have a role in the negative regulation of σ^G in the mother cell, as previously suggested (3, 8, 19, 21, 35). To investigate this possibility, we fused the coding regions of the wild-type and spoIIIGE156K alleles to the mother cell-specific, σ^G-dependent spoIID promoter (36). The fusions were transferred to the amyE locus of a strain bearing an in-frame deletion of the spoIIIG gene and an sspE-lacZ fusion (AH2452), yielding strains AH2460 (ΔspoIIIG sspE-lacZ P_{spoIID} spoIIIG) and AH2461 (ΔspoIIIG sspE-lacZ P_{spoIID} spoIIIGE156K) (Table 1). No σ^G activity was detected by monitoring sspE-lacZ-driven β-galactosidase production, when the wild-type spoIIIG allele was expressed in the mother cell (Fig. 7). In contrast, expression of the spoIIIGE156K allele promptly resulted in sspE-lacZ expression, which occurred prior to normal expression of sspE in the prespore, in agreement with the timing of utilization of the spoIID and sspE promoters during sporulation (26, 36) (Fig. 7). This observation supports a role for SpoIAB in the regulation of σ^G activity in the mother cell.

To determine whether the elevated levels of σ^G activity observed in strain AH2461 (P_{spoIID} spoIIIGE156K) relative to strain AH2460 (P_{spoIID} spoIIIG) correlated with increased accumulation of σ^G, we conducted immunoblot experiments. We found that the σ^G_{E156K} protein accumulated starting 2 h after the onset of sporulation, reaching maximum levels around 3 h after sporulation had begun (Fig. 8B), which is in accordance with the temporal pattern of expression of a spoIID-lacZ fusion (36). In contrast, the wild-type form of σ^G was detected only in trace amounts (Fig. 8A), suggesting that σ^G is subjected to proteolysis in the mother cell.

Since the ATP-dependent LonA protease has been implicated in the negative regulation of σ^G (40, 42), we examined whether a mutation in the lonA gene would also result in increased σ^G activity in the mother cell. A ΔlonA::cat allele was introduced into strain AH2460, yielding strain AH2463 (ΔspoIIIG sspE-lacZ P_{spoIID} spoIIIG lonA::cat) (Table 1). Like the spoIIIGE156K allele, the lonA mutation also permitted expression of sspE-lacZ (Fig. 7), and the strain accumulated wild-type σ^G starting 2 h after the onset of sporulation, with peak levels around 3 h after sporulation had begun (Fig. 8C). Even though the levels of wild-type σ^G in AH2463 (lonA) appeared higher than the levels of σ^G_{E156K} in AH2461 (Fig. 8, compare panels B and C), the latter strain showed the highest levels of sspE-lacZ expression (Fig. 7), suggesting that σ^G_{E156K} is still regulated by LonA.

To test this possibility, we introduced the ΔlonA::cat allele into strain AH2461, yielding strain AH2465 (ΔspoIIIG sspE-lacZ P_{spoIID} spoIIIGE156K lonA::cat) (Table 1). Strain AH2465 showed higher levels of sspE-lacZ expression than AH2461 (spoIIIGE156K) (Fig. 7). Moreover, σ^G_{E156K} accumulated at higher levels than in the lonA^- strain AH2461 (Fig. 8B and D). These results show that the σ^G_{E156K} form is only partially resistant to LonA. Since the levels of σ^G_{E156K} in a
inactive in E156K substitution may protect factor SpoIIAB is also partially resistant to LonA (Fig. 8B), the formulation relative to wild-type

the /H9268/H9268 accumulate to significant levels in the mother cell, because of

fig

each sample were subjected to immunoblot analysis using an anti-

after, as indicated by the numbers above the lanes. Proteins (30

1 h after the onset of sporulation in DSM and at hourly intervals there-

(19, 41),

strains

in a

cat

spoIIIJ

spoIID

spoIID

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pattern corresponds to cells that have entered sporulation but that late in development they fail to maintain compartmentalized gene expression because the spoIIA mutation results in instability of the prespore envelope. We favor this idea for two reasons. First, the instability of the prespore membranes has been reported for certain mutants that do not proceed past the stage of the completion of engulfment (30, 32). Second, it agrees with the observation that in cells bearing a spoIIA mutation (expressing either the wild-type allele or spoIIIIGE156K), the percentage of sporulation seems to decrease from 6 to 10 h after the onset of sporulation, as scored by the staining patterns of the prespore membranes by MTG (Table 4) and of the nucleoid by DAPI (Table 5). Thus, expression of sspE-lacZ in the spoIIA spoIIIIGE156K mutant (AH3791) would occur only upon partial lysis of the prespore at a late time in development, permitting access of some SpoIIAB to the mother cell, whereas wild-type SpoIIAB is rapidly proteolyzed, there seems to be compartmentalization of SpoIIAB whereas wild-type SpoIIAB is negatively regulated. It could be that the putative inhibitor of SpoIIAB (together with LonA protease) also impair its activity.

In conclusion, our results suggest that mutations that make SpoIIAB resistant to SpoIIAB do not permit expression of the spoIIA allele in a spoIIA mutant background. While our results support earlier findings indicating that SpoIIAB (together with LonA) are important in the mother cell, they suggest that SpoIIAB is not a decisive regulator of σG in the prespore.

ACKNOWLEDGMENTS

We thank Gonçalo Real and Patrick Pigott for helpful discussions and comments on the manuscript and Ellen Keller and Patrick Pigott for sharing information prior to publication and for helpful discussions. We also thank Filippe Vieira for help with some of the plasmid constructions.

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ADDITION IN PROOF

A recent report shows that compartmentalized gene expression is compromised in spoIIA or spoIII B. subtilis mutants because of prespore instability (Z. Li, F. Di Donato, and P. J. Piggot, J. Bacteriol. 186:2221–2223, 2004). These results support our interpretation that the activity of GGE156K seen in spoIIA cells is due to instability of the prespore and loss of compartmentalized gene expression in this mutant background.

REFERENCES


