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Genetics

Processing of the mother-cell σ factor, σ^K, may depend on events occurring in the forespore during *Bacillus subtilis* development

(gene expression/proteolysis/RNA polymerase/sporulation/transcription)

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ABSTRACT **During sporulation of the Gram-positive bacterium *Bacillus subtilis*, transcription of genes encoding spore coat proteins in the mother-cell compartment of the sporangium is controlled by RNA polymerase containing the σ subunit called σ^K. Based on comparison of the N-terminal amino acid sequence of σ^K with the nucleotide sequence of the gene encoding σ^K (*sigK*), the primary product of *sigK* was inferred to be a pro-protein (pro-σ^K) with 20 extra amino acids at the N terminus. Using antibodies generated against pro-σ^K, we have detected pro-σ^K beginning at the third hour of sporulation and σ^K beginning about 1 hr later. Even when pro-σ^K is expressed artificially during growth and throughout sporulation, σ^K appears at the normal time and expression of a σ^K-controlled gene occurs normally. These results suggest that pro-σ^K is an inactive precursor that is proteolytically processed to active σ^K in a developmentally regulated fashion. Mutations that block forespore gene expression block accumulation of σ^K but not accumulation of pro-σ^K, suggesting that pro-σ^K processing is a regulatory device that couples the programs of gene expression in the two compartments of the sporangium. We propose that this regulatory device ensures completion of forespore morphogenesis prior to the synthesis in the mother-cell of spore coat proteins that will encase the forespore.**

Upon starvation the Gram-positive bacterium *Bacillus subtilis* undergoes a series of morphological changes that result in endospore formation (1). The first easily observed morphological change is asymmetric septum formation, which divides the cell into two compartments, the mother-cell and the forespore, each receiving a copy of the genome. A complex regulatory circuit ensures the correct temporal and spatial pattern of gene expression during sporulation. Critical to this regulatory circuit are the synthesis and activation of σ subunits of RNA polymerase that direct the enzyme to transcribe different gene sets (2). Two σ factors are compartment-specific: σ^G, the product of the *spoIIIG* gene, is produced predominantly, if not exclusively, in the forespore and controls the expression of forespore-specific genes (3–5). The counterpart to σ^G in the mother-cell is σ^K (6), which controls the expression of mother-cell-specific genes such as *cotA* (7), *cotD* (8), and *gerE* (9) (referred to as the *cotA* regulon). The *cotA* and *cotD* genes encode spore coat proteins that assemble on the forespore surface (10), and *gerE* encodes a regulator of spore coat synthesis (11).

σ^K is encoded in a composite gene (*sigK*) generated by a mother-cell-specific chromosomal rearrangement that joins two loci, *spoIVCB* (encoding the N-terminal portion) and *spoIIIC* (encoding the C-terminal portion) (12, 13). Transcription of the *sigK* promoter is also confined to the mother-cell (14) and compartmentalization of both the DNA rearrangement and *sigK* transcription appear to result from mother-cell-specific expression of *spoIIID* (15). A third possible regulatory mechanism for σ^K was inferred from a comparison of the N-terminal amino acid sequence of σ^K and the nucleotide sequence of *sigK* (6, 12) and by analogy to σ^E, a sporulation-specific *B. subtilis* σ factor that is activated by proteolytic processing (16–18). The primary product of *sigK* was predicted to be a pro-protein (pro-σ^K) bearing 20 extra amino acids at the N terminus. Here we present evidence that σ^K is first made as an inactive precursor and is processed to the active σ factor in a developmentally regulated fashion. Furthermore, mutations in forespore regulatory genes (e.g., *spoIIIG*, encoding the forespore σ factor, σ^G) appear to block processing of pro-σ^K to σ^K, suggesting that the previously noted dependence of mother-cell-specific gene expression on forespore events (7–9, 14) is mediated at the level of proteolytic activation of the mother-cell σ factor.

MATERIALS AND METHODS

Bacterial Strains. *Escherichia coli* strain AG115 [*araD139*, Δ(*ara*, *leu*)*7697*, Δ*lacX74*, *galU*$^-$, *galK*$^-$, *hsr*$^-$, *hsm*$^+$, *strA*, (F′, *proAB*, *lacI*^{q}*Z*::Tn*5*)] was obtained from A. Grossman. *B. subtilis* strains were obtained from R. Losick. *B. subtilis* cells were made competent (19) and transformants were selected on Luria–Bertani (LB) agar (20) with kanamycin sulfate at 5 μg/ml. Use of the specialized transducing phage SPβ::*cotD–lacZ* (obtained from L. Zheng and R. Losick) has been described (8).

Construction of Plasmids. All plasmids were derived from pSK5, which contains *sigK* (13), and from pDG148 (18), which permits isopropyl β-D-thiogalactopyranoside (IPTG)-inducible expression of an inserted gene from the P_{spac} promoter (21) in *E. coli* or *B. subtilis*. To fuse *sigK* expression to the P_{spac} promoter, a 1.4-kilobase-pair (kbp) *Ssp* I–*Hin*dIII fragment from pSK5 (including 141 bp upstream and 556 bp downstream of the *sigK* open reading frame) was ligated to *Hin*dIII-digested pDG148, the unligated end of the vector was made blunt using the fill-in reaction of Klenow enzyme, and ligation was continued (20). Ampicillin-resistant *E. coli* transformants were obtained (20) and the structure of pSL1, a plasmid containing the insert in the proper orientation to fuse *sigK* transcription to P_{spac}, was verified by restriction mapping. pSL2 and pSL4 were derived from pSL1 and pDG148, respectively, by deletion of the *Eco*RI fragment containing the origin of replication and the kanamycin-resistance gene that function in *B. subtilis*.

Production of Pro-σ^K and Preparation of Antibodies. To produce pro-σ^K in *E. coli*, strain ESL2 (strain AG115 containing pSL2) was induced with 1 mM IPTG during the late logarithmic phase of growth at 37°C in LB medium (22). One hour after the addition of IPTG, cells were harvested by

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Abbreviations: IPTG, isopropyl β-D-thiogalactopyranoside; T_n, hour n of sporulation.

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centrifugation (6 min, 7500 × *g*). The cell pellet was resuspended in 0.05 volume of sample buffer (0.125 M Tris·HCl, pH 6.8/2% SDS/5% 2-mercaptoethanol/10% glycerol/0.1% bromophenol blue) and the sample was boiled for 5 min to produce a whole-cell extract. From 0.5 ml of extract, ≈100 μg of pro-σ^K was purified by preparative SDS/PAGE (10–15% polyacrylamide gradient) and electroelution. Pro-σ^K (65 μg) was precipitated with acetone, dissolved in phosphate-buffered saline (23), emulsified with Freund's complete adjuvant (BRL), and injected into or near the popliteal gland of a New Zealand White rabbit. Three weeks later a booster injection [30 μg of pro-σ^K emulsified with Freund's incomplete adjuvant (BRL)] was given at the same site. The rabbit was bled 1 week after the boost and serum was prepared (23).

Sporulation and Western Blot Analysis. Sporulation of *B. subtilis* was initiated by nutrient exhaustion in Difco sporulation (DS) medium (24) as described (7). Cells were harvested by centrifugation (5 min, 16,000 × *g*) and whole-cell extracts were prepared (22). Extract protein was quantitated by the Bradford method (25). After addition of 0.5 volume of 3× sample buffer, proteins were separated by SDS/12.5% PAGE and electroblotted to a poly(vinylidene difluoride) membrane (26). The membrane was incubated in TBS (20 mM Tris·HCl, pH 7.5/0.5 M NaCl) with 2% nonfat dry milk for 4 hr at room temperature with shaking to block nonspecific antibody binding and then incubated overnight at room temperature with shaking in polyclonal antiserum diluted 1:2000 into TBS/2% nonfat dry milk/0.05% Tween 20. Immunodetection using a goat anti-rabbit alkaline phosphatase conjugate was performed according to the manufacturer's instructions (Bio-Rad).

RESULTS

Antibodies to Pro-σ^K Detect Pro-σ^K and σ^K in Sporulating *B. subtilis*. To purify pro-σ^K for the generation of a polyclonal antiserum, the protein was expressed in *E. coli*. Transcription of *sigK* (encoding pro-σ^K) was fused to an IPTG-inducible promoter in plasmid pSL2. Whole-cell extracts of IPTG-induced and uninduced *E. coli* containing pSL2 or a control plasmid (pSL4, which does not contain *sigK*) were analyzed by gel electrophoresis (Fig. 1). A protein of the expected mobility for pro-σ^K [≈29 kDa, since pro-σ^K is predicted to contain 20 amino acids at its N terminus that are absent from σ^K, which migrates at 27 kDa (6)] increased upon IPTG induction of cells containing pSL2, but not upon IPTG induction of cells containing the control plasmid. This protein was assumed to be pro-σ^K, the predicted primary translation product of *sigK*, since pSL2 contains no other 29-kDa-protein-encoding open reading frames downstream of the IPTG-inducible promoter.

Antibodies to gel-purified pro-σ^K were generated in a rabbit and used in Western blot analyses. The antibodies detected pro-σ^K and one larger protein in a whole-cell extract of IPTG-induced *E. coli* containing pSL2 (Fig. 2*A*, lane 2), while only the larger protein was detected for cells containing the control plasmid without *sigK* (lane 1). The antibodies, referred to hereafter as "anti-pro-σ^K antibodies," easily detected 15 ng of pro-σ^K gel-purified from *E. coli* (Fig. 2*B*, lane 1). The anti-pro-σ^K antibodies also recognized σ^K (6) gel-purified from *B. subtilis* (Fig. 2*B*, lane 2) and these antibodies detected either pro-σ^K or σ^K with similar sensitivity. The antibodies detected proteins that comigrated with pro-σ^K and σ^K in a whole-cell extract of sporulating *B. subtilis* (Fig. 2*B*, lane 3), while these proteins were not detected in extracts of growing *B. subtilis* (lane 4) or in extracts of *sigK* mutants (i.e., *spoIVCB* or *spoIIIC* mutants; see below). Thus, Western blot analysis using the anti-pro-σ^K antibodies provides a sensitive assay for the level of pro-σ^K and σ^K in *B. subtilis*.

Levels of Pro-σ^K and σ^K Are Developmentally Regulated. To examine the levels of pro-σ^K and σ^K in *B. subtilis* at various times during sporulation, cells were harvested at hourly intervals during growth and sporulation in DS medium. Under these conditions, the end of exponential growth defines the initiation of sporulation (T_0), prespores that appear gray in the phase-contrast microscope begin to appear 4 hr later (T_4), and phase-bright free spores (released by mother-cell lysis at the end of sporulation) begin to appear at T_8. Whole-cell extracts were subjected to Western blot analysis using the anti-pro-σ^K antibodies and the result for the Spo$^+$ strain PY79 (27) is shown in Fig. 3. A similar result was obtained for the Spo$^+$ strain SG38 (28) (data not shown). Pro-σ^K was first observed at 3 hr into the sporulation process (T_3), reached a maximum at T_5, and then decreased to a barely detectable level by T_8. σ^K was first observed at T_4 (1 hr later than pro-σ^K), increased to a maximum at T_6, and decreased thereafter. These results demonstrate that the levels of pro-σ^K and σ^K are regulated during sporulation. Since the appearance of pro-σ^K precedes the appearance of σ^K and since the N terminus of σ^K corresponds to codon 21 of *sigK* (6, 12), σ^K may be derived from pro-σ^K by proteolytic processing.

Mutations in Many Sporulation Genes Block Accumulation of σ^K. Mutations at many different loci in the *B. subtilis*

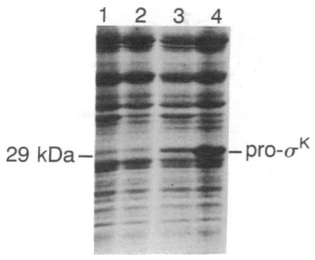

FIG. 1. Production of pro-σ^K in *E. coli*. Proteins in whole-cell extracts (10 μl) of IPTG-induced (lanes 2 and 4) and uninduced (lanes 1 and 3) *E. coli* were separated by SDS/PAGE (10–15% polyacrylamide gradient) and visualized by Coomassie blue staining. Strains ESL4 (lanes 1 and 2) and ESL2 (lanes 3 and 4) were constructed by transformation of strain AG115 with the control plasmid (pSL4) and the P_{spac}–*sigK* fusion plasmid (pSL2), respectively. Only the 35- to 25-kDa region of the gel is shown; the positions of a 29-kDa marker protein (carbonic anhydrase) and the protein assumed to be pro-σ^K are indicated.

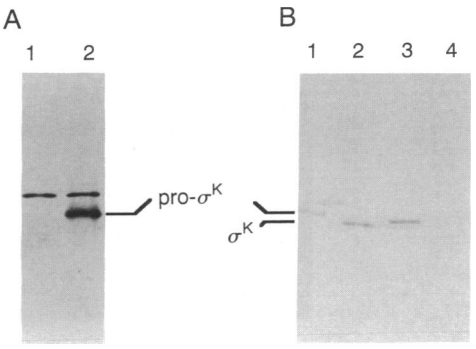

FIG. 2. Characterization of the anti-pro-σ^K antiserum by Western blot analyses. (*A*) Whole-cell extracts (1 μl of a 1:100 dilution) from IPTG-induced *E. coli* strains ESL4 (lane 1) and ESL2 (lane 2), containing the control plasmid (pSL4) and the P_{spac}–*sigK* fusion plasmid (pSL2), respectively, were prepared as described for the production of pro-σ^K (see *Materials and Methods*). (*B*) Pro-σ^K (15 ng) from *E. coli* (lane 1) and σ^K (15 ng) from sporulating *B. subtilis* (lane 2) were gel-purified. Whole-cell extracts (10 μg of protein) were from *B. subtilis* harvested during growth (lane 4) and at 6 hr into sporulation (lane 3) in DS medium.

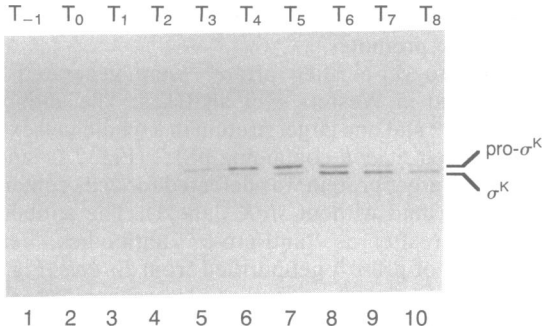

FIG. 3. Pro-σ^K and σ^K in sporulating *B. subtilis*. Wild-type strain PY79 (27) was harvested at hourly intervals during growth and sporulation in DS medium. Whole-cell extracts (10 μg of protein) were subjected to Western blot analysis using the anti-pro-σ^K antibodies. Lanes 1–10, samples harvested at hourly intervals beginning 1 hr before the end of exponential growth (T_{-1}) and ending 8 hr into sporulation (T_8). Pro-σ^K gel-purified from *E. coli* served as a marker on the blot and the inferred position of σ^K is indicated.

genome block or reduce expression of genes in the σ^K-controlled *cotA* regulon (7–9). To further investigate these effects, 16 mutants with altered *cotA* regulon expression were examined for pro-σ^K and σ^K by using the anti-pro-σ^K antibodies in Western blot analyses. Samples collected at hourly intervals from T_0 through T_7 in DS medium were tested for each mutant, but only the result at T_6, the time when both pro-σ^K and σ^K are abundant in the Spo$^+$ strains (see above), is shown in Fig. 4 for each mutant. Five mutants accumulated neither pro-σ^K nor σ^K (lanes 3, 5, 6, 12, and 13). These mutants have either a mutation in the *sigK* gene [*spoIVCB* and *spoIIIC* (12)] or a mutation in a gene whose product is essential for the chromosomal rearrangement that generates *sigK* [*spoIIGB*, *spoIIID*, and *spoIVCA* (12, 13)]. Nine mutants accumulated pro-σ^K but not σ^K (lanes 1, 2, 4, 7, 8, 11, 14, 15, and 16). Interestingly, this group includes 4 strains with mutations in genes required for forespore-specific gene expression [*spoIIIA*, *spoIIIE*, and *spoIIIG* (32, 33)] and/or in genes expressed predominantly, if not exclusively, in the forespore [*spoIIIG* (4, 5) and *spoIVB* (S. Cutting and R. Losick, personal communication)], suggesting that accumulation of σ^K in the mother-cell compartment of the sporangium depends on events occurring in the forespore compartment. In addition, this group includes 2 mutants (*spoIIB* and *spoIID*) blocked early in sporulation at the stage of asymmetric septum formation and 3 strains with mutations in the *spoIVF* locus, which is not required for expression of a forespore-specific gene (32). Finally, 2 strains with mutations in *spoIVA* accumulated a normal amount of pro-σ^K but accumulated much less σ^K (lanes 9 and 10) than the wild-type strain. The *spoIVA* mutants express the *cotA* regulon at a reduced level, whereas all the other mutants examined in this study fail to express the *cotA* regulon (7–9). Thus, for all the mutants examined, the impaired *cotA* regulon expression observed previously (7–9) may be due to impaired accumulation of σ^K. If σ^K is derived from pro-σ^K by proteolytic processing as suggested above, at least eight loci (*spoIIB*, *spoIID*, *spoIIIA*, *spoIIIE*, *spoIIIG*, *spoIVA*, *spoIVB*, and *spoIVF*) may be directly or indirectly involved in processing pro-σ^K and/or stabilizing σ^K.

Processing of Pro-σ^K to σ^K Is Required to Produce an Active σ Factor and Is Developmentally Regulated. *In vitro* and *in vivo* approaches were used to address whether pro-σ^K can direct transcription of σ^K-controlled promoters. For the *in vitro* approach, pro-σ^K gel-purified from *E. coli* was tested for its ability to direct transcription of the *sigK* [previously called *spoIVCB* (6, 14)] and *cotD* (8) promoters upon addition to *B. subtilis* core RNA polymerase (6). As a positive control, σ^K (60 ng) partially purified from sporulating *B. subtilis* was eluted from a gel, renatured (34), and added to core RNA polymerase (60 ng). The reconstituted enzyme produced a run-off transcript from the *sigK* promoter in the presence of the SpoIIID protein (120 ng) and from the *cotD* promoter in the absence of SpoIIID (data not shown), as shown previously (6). Under these conditions, pro-σ^K (300 ng) failed to direct transcription of the *sigK* promoter in the presence of SpoIIID (120 ng) and also failed to direct transcription of the *cotD* promoter in the absence of SpoIIID (data not shown). These results suggest that pro-σ^K is inactive as a σ factor.

To determine whether pro-σ^K could direct transcription of a σ^K-controlled gene *in vivo*, we used a multicopy plasmid bearing *sigK* fused to an IPTG-inducible promoter to express pro-σ^K in *B. subtilis* during growth and sporulation and a *cotD–lacZ* fusion (8) to monitor the transcriptional activity of a σ^K-controlled promoter. A *sigK* mutation prevented production of pro-σ^K or σ^K from the chromosome in this experiment. Production of pro-σ^K from the plasmid was induced with IPTG $\approx$2 hr before the end of exponential growth, and samples collected at hourly intervals were tested for β-galactosidase production from the *cotD–lacZ* fusion and were also subjected to Western blot analysis using the anti-pro-σ^K antibodies (Fig. 5). Even though a large amount of pro-σ^K was present 1 hr prior to the end of exponential growth (T_{-1}) and throughout the early stages of sporulation (Western blot, *Inset*), *cotD*-directed β-galactosidase activity remained low

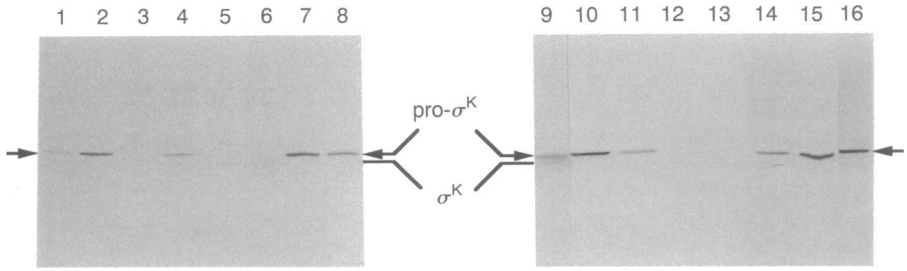

FIG. 4. Pro-σ^K and σ^K in *B. subtilis* sporulation mutants harvested 6 hr after the end of exponential growth in DS medium. Whole-cell extracts (10 μg of protein) were subjected to Western blot analysis using the anti-pro-σ^K antibodies. Arrows indicate the position of pro-σ^K, which served as a marker on the blots; the inferred position of σ^K is also indicated. Lanes: 1, strain 131.5 (*spoIIB131*, *trpC2*); 2, KS298 (*spoIID*::Tn*917*Ω*HU298*); 3, KS440 (*spoIIG41*); 4, KS13 (*spoIIIA*::Tn*917*Ω*HU13*); 5, BK410 (*spoIIIC94*), 6, BK395 (*spoIIID83*); 7, SC622 (*spoIIIE36*); 8, BK338 (*spoIIIG*Δ*1*); 9, KS194 (*spoIVA*::Tn*917*Ω*HU194*); 10, strain 67 (*spoIVA67*, *trpC2*); 11, BK750 (*spoIVB*::*ermG*); 12, BK558 (*spoIVCA133*); 13, BK556 (*spoIVCB23*); 14, SC834 (*spoIVF152*); 15, KS301 (*spoIVF*::Tn*917*Ω*HU301*); 16, KS179 (*spoIVF*::Tn*917*Ω*HU179*). These strains are isogenic to PY79 (27), except 131.5 and 67 are isogenic to SG38 (28). These strains have been described (7, 14, 15, 29), except BK750 was constructed by transformation of DNA prepared from JH12719 (30) into PY79 with selection for the erythromycin-resistance gene (*ermG*) inserted in the *spoIVB* gene (B. Kunkel and R. Losick, personal communication) and KS301 has Tn*917* inserted in the *spoIVF* locus (S. Cutting and R. Losick, personal communication). The Tn*917* insertions *HU194* and *HU179* were thought to define new loci designated *spoVP* and *spoVL*, respectively (31), but are now assigned to the indicated loci (8).

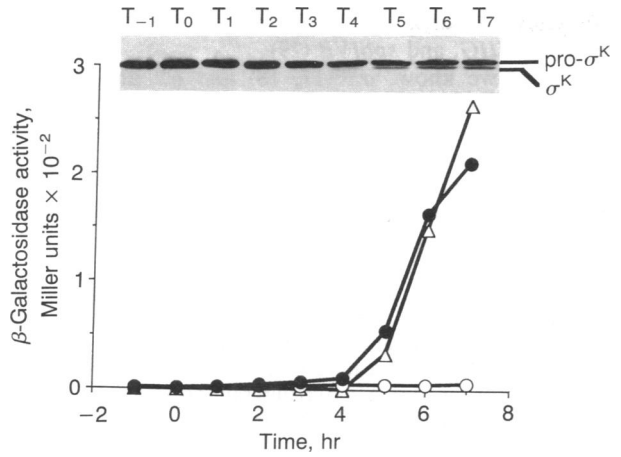

FIG. 5. Effect of producing pro-σ^K from a plasmid during growth and sporulation of *B. subtilis*. Strain BK410 (*spoIIIC94*; ref. 15) was transformed with the P_{spac}–*sigK* fusion plasmid (pSL1) or the control plasmid (pDG148), and both resulting strains were lysogenized with phage SPβ::*cotD–lacZ*, resulting in strains BSL3 and BSL4, respectively. The Spo$^+$ strain PY79 (27) was also lysogenized with SPβ::*cotD–lacZ*, resulting in strain BSL5. Cells were grown and sporulated in DS medium with the addition of 1 mM IPTG ≈2 hr before the end of exponential growth. Samples were harvested at hourly intervals and β-galactosidase activity was determined (35) using the substrate *o*-nitrophenol β-D-galactoside. One unit of enzyme hydrolyzes 1 μmol of substrate per min per OD_{595} unit of initial cell density. Background activity (ranging from 0.5 to 6 units) of PY79 at each time point was subtracted from the values obtained for strains containing the *cotD–lacZ* fusion. *cotD*-directed β-galactosidase activity was determined for strains BSL3 (●), BSL4 (○), and BSL5 (△). (*Inset*) Western blot analysis of whole-cell extracts (10 μg of protein) of strain BSL3, using the anti-pro-σ^K antibodies.

until T_4 (●). Pro-σ^K produced in *B. subtilis* appears to be inactive as a σ factor, unless the presence or absence of another regulatory factor(s) prevents *cotD* transcription during growth and early in sporulation. Beginning at T_4, and more noticeably at T_5, σ^K was observed by Western blot analysis and *cotD*-directed β-galactosidase activity increased significantly compared to the level observed in a control strain harboring a plasmid without *sigK* (Fig. 5, ○). The increase in *cotD*-directed β-galactosidase activity paralleled that observed from the *cotD–lacZ* fusion in wild-type *B. subtilis* (△). Thus, σ^K was first detected at T_4 and increased through T_6 in wild-type cells (Fig. 3) or in cells expressing pro-σ^K from a plasmid (Fig. 5), and in both cases the increase in the σ^K level coincided with the increase in *cotD*-directed β-galactosidase activity (Fig. 5). The finding that σ^K accumulated at the normal time in cells expressing pro-σ^K from a plasmid during growth and early in sporulation demonstrates that production of pro-σ^K is not the limiting factor in the production of σ^K. This suggests that if σ^K is derived from pro-σ^K by proteolytic processing, the processing step itself may be a developmentally regulated event that begins at about T_4.

DISCUSSION

The primary product of *sigK* was inferred to be a pro-protein (pro-σ^K) with 20 extra residues at the N terminus based on a comparison of the N-terminal amino acid sequence of σ^K (6) with the nucleotide sequence of *sigK* (12). Using anti-pro-σ^K antibodies in Western blot analyses of whole-cell extracts of sporulating *B. subtilis*, we detected proteins that we believe are pro-σ^K and σ^K for the following reasons: (*i*) the proteins comigrated with gel-purified pro-σ^K and σ^K (Fig. 2), (*ii*) the proteins were not observed in Western blot analyses of whole-cell extracts prepared from growing wild-type cells (Fig. 2) or from developing cells of five strains that were expected to be unable to produce pro-σ^K and σ^K due to a mutation either in the *sigK* structural gene or in a gene whose product is required to generate the composite *sigK* gene (Fig. 4), and (*iii*) the protein that comigrated with pro-σ^K was first observed in Western blot analysis of wild-type cells at T_3 (Fig. 3), which is consistent with the timing of *sigK* expression (14), while the protein that comigrated with σ^K was first observed at T_4 (Fig. 3), which is consistent with the timing of expression of the σ^K-controlled *cotA* regulon (7–9).

Proteolytic processing has been shown to control the activity of σ^E (16, 17), another sporulation-specific σ factor in *B. subtilis*. By analogy to σ^E and based on the finding that the N terminus of σ^K corresponds to codon 21 of *sigK*, it was proposed that pro-σ^K may be an inactive precursor that is proteolytically processed to active σ^K (6, 12). Several of our results are consistent with this model. First, the appearance of pro-σ^K preceded the appearance of σ^K during sporulation of wild-type *B. subtilis* (Fig. 3), and the timing of appearance of *cotD*-directed β-galactosidase activity (Fig. 5, △) coincided with the appearance of σ^K, not pro-σ^K. Second, mutations in eight sporulation loci (*spoIIB*, *spoIID*, *spoIIIA*, *spoIIIE*, *spoIIIG*, *spoIVA*, *spoIVB*, and *spoIVF*) blocked or reduced accumulation of σ^K, but not accumulation of pro-σ^K (Fig. 4), and the impaired *cotA* regulon expression in strains with these mutations (7–9) correlates with the impaired accumulation of σ^K, not with the level of pro-σ^K, which was normal in all these mutants except the *spoIIB* mutant (see below). Third, when pro-σ^K was gel-purified from *E. coli* and renatured under the same conditions that permit recovery of activity of σ^K gel-purified from *B. subtilis*, it failed to promote transcription of σ^K-controlled promoters *in vitro* (data not shown). Fourth, production of pro-σ^K from a plasmid in a *B. subtilis sigK* mutant resulted in production of σ^K during sporulation (Fig. 5 *Inset*), and, just as in wild-type cells, the timing of appearance of *cotD*-directed β-galactosidase activity (Fig. 5, ●) coincided with the appearance of σ^K, not with the level of pro-σ^K, which was high during growth and throughout sporulation. Our data do not rule out the interpretation that σ^K is produced by translational initiation at an alternative site; however, this possibility is unlikely since no apparent ribosome-binding site or initiation codon exists at the appropriate position in the *sigK* mRNA. Nevertheless, it may be possible to use a pulse–chase experiment to demonstrate directly a precursor–product relationship between pro-σ^K and σ^K, as has been done in the case of σ^E and its precursor (17). Proof that pro-σ^K is an inactive precursor that can be proteolytically processed to active σ^K will require reconstitution of the processing reaction *in vitro*.

The accumulation of σ^K is a developmentally regulated event that begins at about T_4 in wild-type cells (Fig. 3) or in *sigK* mutant cells expressing pro-σ^K from a plasmid (Fig. 5). This event directly or indirectly requires proper functioning of the products of at least eight sporulation loci since, as noted above, mutations in eight loci blocked or reduced accumulation of σ^K but not accumulation of pro-σ^K. If σ^K is derived from an inactive precursor by a developmentally regulated proteolytic processing event, what purpose might this regulatory device serve? In the case of σ^E, processing has been suggested to be a mechanism for coupling formation of the sporulation septum to activation of σ^E and the subsequent pattern of gene expression (17, 18). Our finding that *spoIIIA*, *spoIIIE*, *spoIIIG*, and *spoIVB* mutants accumulate pro-σ^K, but not σ^K, and the results of Cutting *et al.* (29), discussed below, suggest that pro-σ^K processing may couple activation of the mother-cell σ factor to events occurring in the forespore compartment.

A regulatory mechanism connecting mother-cell-specific gene expression to forespore events was inferred (7–9, 14) from the observation that mutations in *spoIIIA*, *spoIIIE*, and

spoIIIG that impair forespore-specific gene expression (32, 33) also impair mother-cell-specific gene expression. Although little is known about the functions of the *spoIIIA* and *spoIIIE* gene products, *spoIIIG* is expressed predominantly, if not exclusively, in the forespore compartment and it encodes a σ factor, σ^G, that directs forespore-specific gene expression (4, 5). Recently, *spoIVB* (30) has been shown to be expressed specifically in the forespore, yet mutations in this gene impair mother-cell-specific gene expression (S. Cutting and R. Losick, personal communication). Cutting *et al.* (29) isolated mutants (called *bof* mutants for *b*ypass *o*f *f*orespore) that bypass the dependence of *cotA* regulon expression on *spoIIIA*, *spoIIIE*, *spoIIIG*, and *spoIVB* mutations. Using the anti-pro-σ^K antibodies described here, it was shown that *bof* mutations restore production of σ^K in *spoIIIA* and *spoIIIG* mutant cells (29). Thus, *bof* mutations appear to uncouple mother-cell-specific gene expression from forespore events by permitting pro-σ^K processing. Furthermore, replacement of *sigK* with a deletion-mutated version lacking codons 2–20 (so that the protein produced, $\sigma^{K\Delta 19}$, would differ from σ^K only by a methionine residue at its N terminus) relieved the dependence of *cotA* regulon expression on the *spoIIIG* gene product (29). In this case the proposed coupling between forespore events and pro-σ^K processing appears to be circumvented by producing the truncated, active $\sigma^{K\Delta 19}$ instead of pro-σ^K. A protein that was presumably $\sigma^{K\Delta 19}$, since it comigrated with σ^K in Western blot analysis using the anti-pro-σ^K antibodies, was detected beginning at T_3 in a *spoIIIG* mutant containing the deletion-mutated *sigK* gene (data not shown). This finding suggests that the failure of the *spoIIIG* mutant to accumulate σ^K when it contains an intact *sigK* gene (Fig. 4, lane 8) results from a failure to process pro-σ^K rather than from instability of σ^K, unless $\sigma^{K\Delta 19}$ is significantly more stable than σ^K in the *spoIIIG* mutant. Cells containing the deletion-mutated *sigK* gene also began expressing a *cotA–lacZ* fusion at T_3, 1 hr earlier than normal (29), as would be expected if $\sigma^{K\Delta 19}$ but not pro-σ^K were able to function as a σ factor. The results presented here and the results of Cutting *et al.* (29) strongly suggest that pro-σ^K processing is a regulatory device that couples mother-cell gene expression to forespore morphogenesis. The *spoIIIA*, *spoIIIE*, *spoIIIG*, and *spoIVB* mutations are inferred to block forespore morphogenesis at a stage that is incompatible with pro-σ^K processing.

Mutations in the *spoIIB*, *spoIID*, and *spoIVF* loci also blocked accumulation of σ^K, but not pro-σ^K (Fig. 4). The *spoIIB* mutant used in this study was shown previously to express only 6% of the β-galactosidase activity normally expressed from a *sigK–lacZ* fusion during sporulation (14). This may explain the reduced amount of pro-σ^K detected by the anti-pro-σ^K antibodies in this mutant (Fig. 4, lane 1). Production of pro-σ^K was unimpaired in the *spoIID* mutant (Fig. 4, lane 2). Since *spoIID* mutations have been shown to impair forespore-specific gene expression (5, 32, 33), perhaps these mutations also block forespore morphogenesis at a stage that is incompatible with pro-σ^K processing. The *spoIVF* locus is the best candidate to encode a protein directly involved in the pro-σ^K processing reaction. Mutations in *spoIVF* block expression of the *cotA* regulon (7–9) and our results show that these mutants accumulate pro-σ^K but not σ^K (Fig. 4, lanes 14–16). Like a *spoIIIG* mutant, a *spoIVF* mutant engineered to produce truncated, active $\sigma^{K\Delta 19}$ expresses the *cotA* gene (29). However, a *spoIVF* mutation does not block the expression of a forespore-specific gene (32). Furthermore, a *bofA* mutation does not bypass the dependence of *cotA* expression on a *spoIVF* mutation, and the *bofB* mutations are alleles of the *spoIVF* locus (29). These results have led to the proposal that the *spoIVF* gene product(s) governs processing of pro-σ^K to σ^K and that *bofB* mutations alter the *spoIVF* gene product(s) so as to relieve its dependence on the products of *spoIIIA*, *spoIIIE*, *spoIIIG*, and *spoIVB* (29).

As far as we know, σ^E (17, 18) and σ^K are the only transcription factors thought to be synthesized as inactive precursor proteins and activated by specific proteolytic cleavages. In both cases, proteolytic processing may couple completion of a morphogenetic step to the subsequent, new pattern of gene expression, but in each case the molecular mechanism of the coupling remains to be elucidated.

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