

Exchange of Precursor-Specific Elements between Pro- σ^E and Pro- σ^K of *Bacillus subtilis*

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σ^E and σ^K are sporulation-specific σ factors of *Bacillus subtilis* that are synthesized as inactive proproteins. Pro- σ^E and pro- σ^K are activated by the removal of 27 and 20 amino acids, respectively, from their amino termini. To explore the properties of the precursor-specific sequences, we exchanged the coding elements for these domains in the σ^E and σ^K structural genes and determined the properties of the resulting chimeric proteins in *B. subtilis*. The pro- σ^E - σ^K chimera accumulated and was cleaved into active σ^K , while the pro- σ^K - σ^E fusion protein failed to accumulate and is likely unstable in *B. subtilis*. A fusion of the *sigE* “pro” sequence to an unrelated protein (bovine rhodanese) also formed a protein that was cleaved by the pro- σ^E processing apparatus. The data suggest that the σ^E pro sequence contains sufficient information for pro- σ^E processing as well as a unique quality needed for σ^E accumulation.

During endospore formation, *Bacillus subtilis* partitions itself into two compartments (mother cell and forespore) that follow distinct developmental paths. To achieve this specialization, the bacterium must activate mother cell- and forespore-specific genes in the appropriate compartment at the proper time. Mother cell gene expression is principally regulated by the sequential appearance of two RNA polymerase σ factors, σ^E and σ^K (3, 10, 12). These proteins are unique among known σ factors in that they are synthesized as proproteins which are subsequently processed into their active forms (8, 11). Processing of the σ factor precursors is developmentally regulated and involves the removal of a portion of each proprotein's amino terminus (8, 11). These processing reactions appear to tie activation of each σ factor to particular developmental events (2, 4, 9, 10, 17, 21).

Pro- σ^E is synthesized at the onset of sporulation and activated approximately 1 h later in the newly formed mother cell compartment (5, 8, 24). Transcription of the pro- σ^K structural gene (*sigK*) is σ^E dependent (7, 23). Pro- σ^K appears within the mother cell 3 h after the onset of sporulation, where it is converted into mature σ^K within the following hour (11). Pro- σ^E activation involves the removal of 27 amino acids from its amino terminus, while the pro- σ^K processing reaction cleaves 20 amino acids from the pro- σ^K amino terminus (16, 17, 23). The “pro” sequences of σ^E and σ^K silence their respective σ factor's activity. In addition, the σ^E pro sequence, but not the corresponding σ^K pro sequence, is also needed for its σ factor to accumulate within *B. subtilis* (8, 11, 17, 18). An altered *sigE* gene, lacking its pro sequence, encodes a product that is virtually undetectable in *B. subtilis* (18), while the product of a similarly altered *sigK* gene is readily found (17). The processing of either pro- σ^E or pro- σ^K is unique. Each reaction employs separate collections of gene products which appear to be activated by signals that are generated by the developing forespore (2, 4, 9–12, 17, 21, 24, 25).

To investigate whether the σ^E pro sequence is sufficient to target its removal and to determine if it has qualities for sta-

bilizing σ^E that are absent in the σ^K pro sequence, we employed standard techniques of oligonucleotide-directed mutagenesis (19) to place convenient restriction endonuclease recognition sites before and after the *sigE* and *sigK* pro sequences and used these sites to exchange pro sequence domains between the *sigE* and *sigK* genes. Figure 1 depicts portions of the *sigE* (panel A) and *sigK* (panel B) structural genes and the sequence changes that were created by introducing the restriction sites. The *sigK* gene with the σ^E pro sequence was formed in several steps. A 1.5-kbp *PstI*-*HindIII* DNA fragment containing the *sigK* coding sequence (6) and a 1.1-kbp *PstI* DNA fragment carrying *sigE* (22) were mutagenized to introduce unique *Bam*HI restriction sites at codon 23 of *sigK* (Fig. 1B, *sigK23*) and codon 30 of *sigE* (Fig. 1A, *sigE30*).

The *PstI*-*Bam*HI fragment of *sigE30* containing the *sigE* pro sequence and upstream DNA was joined to the *sigK* sequence at the *Bam*HI site of *sigK23*. This resulted in an in-frame fusion of *sigE* at codon 29, plus the two amino acids specified by the *sigE30* *Bam*HI mutation, with codon 25 of *sigK*. The composite *sigE*-*sigK* gene was cloned as a 1.5-kbp *PstI*-*HindIII* fragment into a plasmid (pJM102) (18) which confers *Cm*^r when integrated into the *B. subtilis* chromosome. *sigK* composes approximately 80% of the *B. subtilis* DNA that is carried on the plasmid. Recombination between the plasmid and the *B. subtilis* chromosome would therefore be expected to occur predominantly within *sigK*. In growing cells, *sigK* coding sequences are present at two loci, *spoIVCB* and *spoIIIC*. A DNA rearrangement generates the composite *sigK* gene during sporulation (23). Recombination between the *sigE*-*sigK* plasmids and *spoIVCB* yields *sigK* fused to its normal promoter with the *sigE*-*spoIVCB* gene lying downstream of the vector sequences and unexpressed, whereas recombination at *spoIIIC* leaves *spoIVCB* and *spoIIIC* unchanged, followed by vector sequences and an unexpressed *sigE*-*sigK* gene. In either case, clones such as these should remain *Spo*⁺. If, however, recombination occurs within the *sigE* sequence, the *sigE*-*sigK* chimera would become the expressed allele at *sigE*. The wild-type *sigE* would then be separated from its promoter and remain silent. This deprives the cells of a source of σ^E and converts them to *Spo*⁻. When the *sigE*-*sigK*-containing plasmid was transformed into *B. subtilis* (JH642), approximately 20% of the *Cm*^r colonies were *Spo*⁻ (i.e., their colonies failed to turn brown when plated

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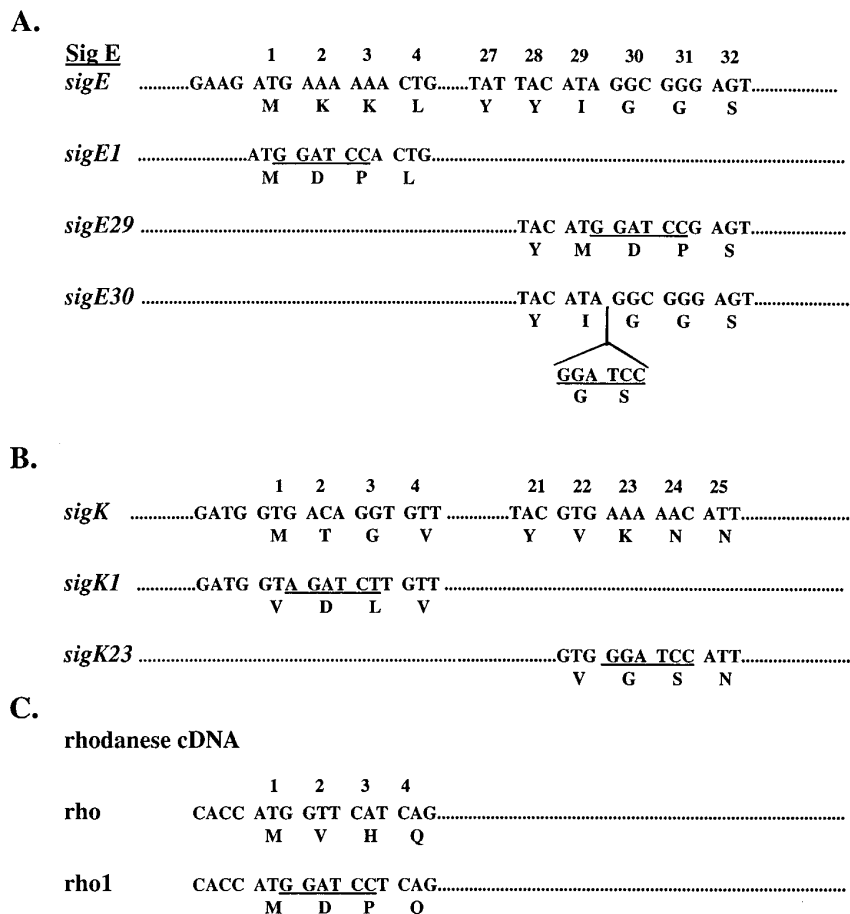


FIG. 1. Sequence of altered *sigE*, *sigK*, and *rho* alleles. Portions of the wild-type *sigE* (22) (A), *sigK* (23) (B), and rhodanese (14) (C) coding sequences are illustrated. Changes engineered into the indicated alleles are depicted below the wild-type sequences. The mutations were constructed as described previously (19) with 30-mer oligonucleotides centered at the site of the introduced mutations. The codons for each gene are numbered in sequence beginning with the initiating codon as 1. Breaks in the illustrated sequence are depicted as dotted lines. The single-letter amino acid code for each position is placed under the respective codon. Restriction endonuclease recognition sites (*Bam*HI or *Bgl*II) introduced by the mutations are underlined.

on Difco sporulation (DS) medium [20]). This is the expected frequency for recombination within the *sigE* region. A representative Spo⁻ colony was picked and examined by Western blot (immuno-blot) for the appearance of the *sigE-sigK* gene product by using anti- σ^K antibody (11) as a probe (Fig. 2A). Consistent with expression of *sigE-sigK* from the *sigE* locus, the anti- σ^K antibody detected a protein at 1 h after the end of log-phase growth (T_1) which continued to accumulate and be converted into a lower-molecular-weight form by T_2 . This is the pattern that is normally observed for the appearance and processing of pro- σ^E (24). Pro- σ^K , expressed from *sigK*, does not begin to accumulate within the sporulating cell until T_3 (11).

In order to correlate the appearance of the anti- σ^K -reacting proteins with σ^K activity, the *sigE-sigK*-expressing strain was transduced with a bacteriophage containing a fusion of a σ^K -dependent promoter (*cotD*) to the *Escherichia coli lacZ* gene (SP β ::*cotD-lacZ*) (6). *cotD* expression normally occurs in wild-type *B. subtilis* following the processing of pro- σ^K at T_4 (11). *cotD*-dependent β -galactosidase synthesis in the *sigE-sigK* mutant strain occurred at T_2 , coincident with the appearance of the processed form of pro- $\sigma^{E/K}$ (Fig. 2B). Expression levels of *cotD-lacZ* were similar to that of wild-type cells (6), suggesting that the processed " σ^K " is quite active, despite the fact that its N-terminal amino acid sequence cannot be the same as that of

σ^K . We interpret the Western blot and *cotD* promoter activity data as evidence that pro- $\sigma^{E/K}$ can be recognized and cleaved by the pro- σ^E processing apparatus and that the removal of the *sigE* pro sequence is necessary for σ^K activity.

The apparent conversion of pro- $\sigma^{E/K}$ into a σ^K -like protein suggests that recognition for pro- σ^E processing lies entirely within the SigE pro sequence. However, σ^E and σ^K are both σ factors and as such have many common features. It is possible that the pro- σ^E processing apparatus recognizes elements common to this class of protein as well as the SigE pro sequence. To explore this possibility, we transferred the *sigE* pro sequence to an unrelated protein, the bovine mitochondrial protein rhodanese (thiosulfate:cyanide sulfurtransferase). We chose this protein as our model for several reasons. Rhodanese has been extensively characterized *in vivo* and *in vitro*, it is stable when expressed in bacteria (e.g., *E. coli*), and it is small enough (i.e., 33 kDa) so that the loss of the 27-amino-acid pro sequence would be detectable by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (13, 14). In addition, we had access to both the cDNA for the rhodanese sequence (14) and a collection of monoclonal antibodies (13) that we could use to detect the protein in crude extracts.

We inserted a unique *Bam*HI site into the rhodanese cDNA at its first codon (Fig. 1C, rho1) and used this site to join the rhodanese coding sequence to either the upstream 270-bp *Pst*I-

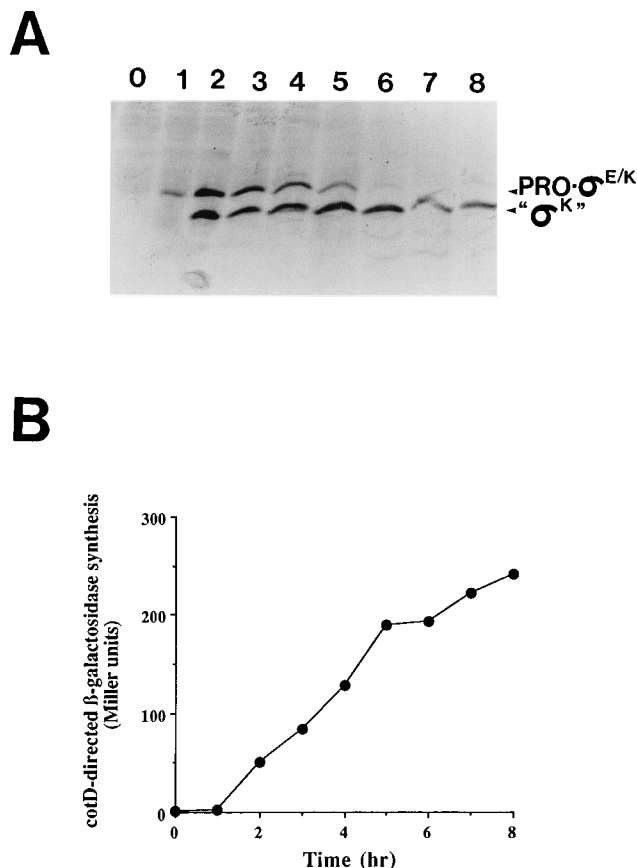


FIG. 2. Expression and activity of a pro- σ^E - σ^K fusion protein. A 1.5-kbp DNA fragment carrying the pro- σ^E coding sequence through codon 29 fused to *sigK* at codon 25 (i.e., *sigE30::sigK23* fusion [Fig. 1]) was transformed into *B. subtilis* (JH642) as the expressed *sigE* allele. (A) *B. subtilis* (JH642) expressing *sigE-sigK* was grown in DS medium (20) and analyzed by Western blot using anti- σ^K antibody as a probe (11) at the onset of sporulation (lane 0) and hourly intervals (lanes 1 and 2, etc.) thereafter. The positions of the unprocessed (pro- $\sigma^{E/K}$) and processed (σ^K) proteins are indicated. (B) The *B. subtilis* strain examined in panel A was transduced with a bacteriophage carrying a σ^K -dependent promoter fused to the *E. coli lacZ* gene (SP β *codD::lacZ*) (6). The strain was sporulated in DS medium with samples taken for β -galactosidase analysis (15) at the same time points as those taken in panel A.

*Bam*HI fragment of *sigE29* (Fig. 1A) or a 340-bp *Pst*I-*Sau*3A fragment that can be cleaved from the 5' end of the wild-type *sigE* sequence (22). Both *sigE* fragments, when joined to *rho1*, form in-frame fusions with the entire rhodanese gene. The larger *Pst*I-*Sau*3A piece includes 25 codons from the amino terminus of mature σ^E in addition to the *sigE* pro sequence (22). When either the *sigE30-rho1* or the *sigE52-rho1* fusion, cloned into pJM102, was transformed into *B. subtilis*, all of the transformants displayed the Spo⁻ phenotype anticipated for the disruption of *sigE* by the *sigE-rho* chimera. Western blot analysis of representative clones revealed substantially higher levels of rhodanese in *sigE52-rho1* than in *sigE30-rho1* transformant strains (1). This is presumably due to differences in fusion protein stability. The Western blot data from a typical *sigE52-rho1*-expressing strain are depicted in Fig. 3. Early in sporulation, at $T_{1.5}$ (Fig. 3, lane A), two proteins are detected by the antirhodanese antibody, with the lower-molecular-weight form predominating by T_3 (Fig. 3, lane B). A protein with the mobility of the larger protein is the only rhodanese-like protein seen in a *B. subtilis* strain containing *sigE52-rho1* plus a mutation (*spoIIE::Tn917*) which blocks pro- σ^E process-

ing (Fig. 3, lanes D and E). This protein, as well as a likely degradation product, are observed when *sigE52-rho1* is expressed from *P_{lac}* in *E. coli* (Fig. 3, lane C). The results are consistent with the SigE52-Rho1 protein being synthesized and processed in *B. subtilis*. Thus, the *sigE* pro sequence appears to contain sufficient information for its recognition and cleavage by the pro- σ^E processing apparatus.

σ^E fails to accumulate in *B. subtilis* if it is synthesized without at least a portion of its pro sequence (19). We next asked whether the σ^K pro sequence could substitute for the σ^E pro sequence and allow σ^E to accumulate. Using the mutated *sigE* and *sigK* alleles employed in the pro- σ^E - σ^K construction (i.e., *sigE30* and *sigK23*) (Fig. 1A and B), we joined the *Pst*I-*Bam*HI fragment containing the 5' end of *sigK* through codon 22, plus the two amino acids specified by the *sigK23 Bam*HI mutation, to a *Bam*HI-*Pst*I fragment containing the 3' end of *sigE* beginning at codon 30. This formed a composite *sigK-sigE* gene on a *Pst*I fragment. The DNA fragment was then mutagenized to place a unique *Bgl*III site at codon 1 of the *sigK* sequence (Fig. 1B, *sigK1*). The resulting allele was cut at this *Bgl*III site, and the downstream *Bgl*III-*Pst*I fragment, containing the *sigK-sigE* chimera, was joined at a *Bam*HI site (Fig. 1A, *sigE1*) to *sigE* regulatory elements. This formed a 1.1-kbp DNA fragment encoding the initiating codon of *sigE* with approximately 200 bp of upstream DNA joined, in frame, to the *sigK* pro region and the coding sequence for mature σ^E . The structure of the *sigK-sigE* gene was verified by DNA sequencing. This fragment was cloned into an integrating vector (pJM103) (18) and transformed into *B. subtilis*. Recombination events within the *sigE* sequence downstream of the σ^K pro sequence would form wild-type *sigE* genes with silent *sigK-sigE* alleles separated from promoter elements. Transformants of this type should remain Spo⁺. Representative Spo⁺ clones, analyzed by Western blot using anti- σ^E antibody as a probe, displayed the normal pro- σ^E - σ^E profile (data not shown). Approximately 20% of the vector-encoded Cm^r transformants formed Spo⁻ colonies on DS plates. On the basis of the size of the cloned DNAs, most of these Spo⁻ clones would be expected to arise from plasmid recombinations into the chromosomal *sigE* regulatory elements rather than within the *sigK* pro element. Recombination at *sigE* was verified for the Spo⁻ clone illustrated in Fig. 4 by PCR amplification and restriction endonuclease analysis (data not shown). The Spo⁻ clones, which we expected to express *sigK-sigE*, failed to form proteins of the size anticipated for pro- $\sigma^{K/E}$. Instead, a smaller, low-abundance protein was seen reacting with the anti- σ^E antibody in extracts from these strains

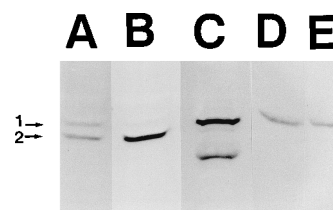


FIG. 3. Expression of a pro- σ^E -rhodanese fusion protein. A DNA fragment containing the full-length rhodanese coding sequence (*rho1* [Fig. 1]) joined to 52 codons of the *sigE* amino terminus was transformed into *B. subtilis* (JH642) as the expressed *sigE* allele. The *sigE52-rho1*-expressing strain was grown in DS medium with samples taken at 1.5 h (lane A) and 3 h (lane B) after the onset of sporulation. The samples were processed and analyzed as in Fig. 2 with antirhodanese monoclonal antibody (13) as the probe. Lane C, *sigE52-rho1* allele expressed in *E. coli* from *P_{lac}* and analyzed by Western blot; lane D and E, *sigE52-rho1* expressed in a *B. subtilis* mutant (*spoIIE::Tn917*) with samples taken at $T_{1.5}$ and T_3 , respectively. Arrows 1 and 2 indicate the positions of pro- σ^E -rhodanese and the putative processed form, respectively.

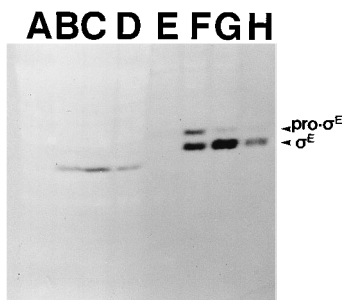


FIG. 4. Expression of a pro- $\sigma^{\text{K}}\text{-}\sigma^{\text{E}}$ fusion protein. A DNA fragment encoding the initiating codon of *sigE*, joined in frame to the *sigK* pro region and the coding sequence of mature σ^{E} , was transformed into *B. subtilis* (JH642) on an integrating vector as the expressed *sigE* allele. Strains of *B. subtilis* expressing chimeric (lanes A to D) or wild-type (lanes E to H) *sigE* alleles were sporulated in DS medium and analyzed for their *sigE* proteins (24) at T_0 (lanes A and E), $T_{1.5}$ (lanes B and F), T_3 (lanes C and G), and $T_{4.5}$ (lanes D and H). The positions of pro- σ^{E} and σ^{E} are indicated.

(Fig. 4, lanes A to D). This likely represents a SigK-SigE breakdown product. It is similar to the proteins that we observe from *sigE* alleles with pro sequence alterations which compromise product stability (19). Apparently the *sigK* pro sequence cannot substitute for the *sigE* pro region in allowing σ^{E} to accumulate in *B. subtilis*. Given that wild-type SigK readily accumulates (11), it is unlikely that the 20 codons from the *sigK* amino terminus, which we placed at the 5' end of *sigE*, represent a block on translation. It is more probable that the SigE pro sequence has an element that is not shared by the *sigK* sequence which facilitates proper SigE folding or contributes to other aspects of pro- σ^{E} stability. The data suggest that the σ^{E} pro sequence contains sufficient information for pro- σ^{E} processing as well as a unique quality needed for σ^{E} accumulation.

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