JOURNAL OF BACTERIOLOGY, July 1992, p. 4629–4637
0021-9193/92/144629-09$02.00/0

Vol. 174, No. 14

Mutational Analysis of the Precursor-Specific Region of *Bacillus subtilis* σ^E

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Received 1 April 1992/Accepted 15 May 1992

σ^E is a sporulation-specific sigma factor of *Bacillus subtilis* that is formed from an inactive precursor protein (pro-σ^E) by the removal of 27 to 29 amino acids from the pro-σ^E amino terminus. By using oligonucleotide-directed mutagenesis, sequential deletions were constructed in the precursor-specific region of *sigE* and analyzed for their effect on the gene product's activity, ability to accumulate, and susceptibility to conversion into mature σ^E. The results demonstrated that the first 17 residues of the pro sequence contribute to silencing the σ-like activity of pro-σ^E and that the amino acids between positions 12 and 17 are also important for its conversion into σ^E. Deletions that remove 21 or more codons from *sigE* reduce σ^E activity in cells which carry it, presumably by affecting pro-σ^E stability. A 26-codon deletion results in a gene whose product is not detectable in *B. subtilis* by either reporter gene activity or Western blot (immunoblot) assay. The primary structure as well as the size of the pro region of σ^E contributes to the protein's stability. The placement of additional amino acids into the pro region reduces the cell's ability to accumulate pro-σ^E. Additional *sigE* mutations revealed that the amino acids normally found at the putative processing site(s) of pro-σ^E are not essential to the processing reaction; however, a Glu residue upstream of these sites (position 25) was found to be important for processing. These last results suggest that the pro-σ^E processing apparatus does not recognize the actual site within pro-σ^E at which cleavage occurs but rather sequence elements that are upstream of this site.

Bacillus subtilis synthesizes at least four sporulation-specific sigma factors (σ^E, σ^F, σ^G, and σ^K) that are critical for orchestrating the temporal and compartmental patterns of gene expression that occur throughout endospore formation (12). σ^E is the most abundant of these regulatory proteins during the early stages of spore formation (3). If a cell fails to synthesize σ^E, its development proceeds to the point where the cell partitions itself into forespore and mother cell compartments (stage II) but fails to progress beyond that stage (11). Transcription of the operon that encodes σ^E (*spoIIG* [22]) begins at the onset of sporulation and requires the sporulation gene activator, Spo0A, for its expression (7, 8, 17). In addition to transcriptional regulation, synthesis of σ^E is also controlled posttranslationally. The initial protein product of the σ^E structural gene (*sigE*/*spoIIGB*) is an inactive precursor form of σ^E (pro-σ^E) (11, 23). Pro-σ^E is converted into σ^E by proteolytic activity that removes 27 to 29 amino acids from the pro-σ^E amino terminus (11, 12). Mutants that fail to convert pro-σ^E into σ^E have the same Spo$^-$ phenotype as those with null mutations in σ^E itself (4). The activity that cleaves the pro sequence from σ^E is itself developmentally regulated (24). It appears approximately 1 h after the onset of pro-σ^E synthesis if the products of at least six *spo* genes (4) and the cell division gene *ftsZ* are present (1). In an initial study of the pro sequence of σ^E, we isolated a fortuitous *sigE* mutation (*sigE*Δ*48*) in which a portion of the coding region of the σ^E pro sequence was lost and another sequence was added (5). The product of this unusual *sigE* allele is not converted into mature σ^E, yet it is active. An additional deletion mutation in *sigE* (*sigE*Δ*84*) which removed the entire pro-sequence coding region yielded an apparently unstable product that accumulated to only approximately 1% of the level of wild-type gene product (5). These results suggested that the precursor-specific region of pro-σ^E was multifunctional and contained distinct domains involved in silencing σ^E activity and stabilizing the σ^E protein. In order to further characterize the pro region of σ^E, we have performed a sequential deletion analysis of its coding region and monitored the effects of these deletions, as well as other *sigE* mutations, on pro-σ^E activity, processability to σ^E, and ability to accumulate. In addition, the need for specific amino acids at the putative processing sites was tested by directly changing the codons specifying these residues.

The analyses demonstrated that pro-σ^E processability, inactivity, and stability decrease as deletions extend into the pro sequence. Inactivation of pro-σ^E and its ability to be processed were lost in parallel prior to a loss in the ability of σ^E-like products to accumulate. An amino acid change near the putative processing sites was found to markedly inhibit processing, while changes in the specific amino acids occupying these sites did not block processing. This result suggests that the processing apparatus recognizes particular features of the pro sequence and not a specific sequence at the site of processing.

MATERIALS AND METHODS

Bacterial strains and plasmids. *B. subtilis* JH642 (*pheA trpC2*) and 1A287 (*rpsL*) were obtained from J. Hoch and the *Bacillus* Genetic Stock Center (Ohio State University), respectively. Strain SE84 (*pheA trpC2 rpsL sigE*Δ*84*) is JH642 into which *sig*Δ*84* was substituted. SE84 was constructed by transforming JH642 with chromosomal DNA from 1A287 (Str^r) mixed with an excess of linearized pJMΔ84 (pJM102 carrying *sigE*Δ*84* [5]) selecting for Str^r and screening the streptomycin-resistant clones for a Spo$^-$ Cm^s phenotype. The *Escherichia coli* mutator DM2516 (*mutD5*) was obtained from J. Walker (University of Texas at Austin).

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The M13 host strains CJ236 (*dut ung*) and JM103 (*dut*$^+$ *ung*$^+$) were obtained from V. Deretic (University of Texas Health Science Center at San Antonio). Plasmids pSR5 (*spoIID/lacZ*) (14) and pGSIIG11 (*sigE*) (20) have been described. pJM102, obtained from J. Hoch, is pUC18 (26) with a chloramphenicol acetyltransferase gene (950 bp) cloned into its unique *Nde*I site.

Oligonucleotide-directed mutagenesis. The 1.1-kbp *Pst*I fragment of pGSIIS11 was cloned into M13 mp18 (26), and its orientation within the vector was determined by restriction endonuclease analysis of replicative-form DNA. M13 mp18 containing the 1.1-kbp *sigE* fragment was grown on *E. coli* CJ236 to incorporate uracil into the phage DNA. To construct the various *sigE* alleles, oligonucleotides (30-mers) that carried that sequence 15 bases upstream and downstream of the region to be deleted or altered were hybridized to single-stranded M13 DNA containing its complementary sequence within the cloned 1.1-kbp *Pst*I fragment. The oligonucleotides used in these experiments were as follows: *sigEΔ388*, 5′-GAGGTGCGTCAACCGCATCTTCCTCTCC CT-3′, *sigEΔ388.5*, 5′-CATCAGCTTATACCACATCTTCC TCTCCCT-3′; *sigEΔ335*, 5′-TTTCAGCCCAAGTTTCATC TTCCTCTCCCT-3′; *sigE335.5*, 5′-ATAGACTTCATCACT CATCTTCCTCTCCCT-3′; *sigEΔ78*, 5′-ACTCCCGCCTAT GTACATCTTCCTCTCCCT-3′; *sigEBAM1*, 5′-AGGGCTT CACTCGGATCCATGTAATAGACT-3′, *sigET1*, 5′-CAGC TTATACCATCTTAAGTGCGTCAACCG-3′; *sigET2*, 5′-C TTATACCATCTGGCCAGTAAGTGCGTCAA-3′; *sigEY2 F2*, 5′-ACTCCCGCCTATGAAGAAGACTTCATCACT-3′. Following hybridization, Sequenase (version 2.0; U.S. Biochemical Corp., Cleveland, Ohio) was used to synthesize the complementary strand. Selection for the stand polymerized in vitro was accomplished by plating the polymerization mixture on the *dut*$^+$ *ung*$^+$ *E. coli* host strain (JM103) (10). The resulting phage clones were screened for changes in the size of the cloned *B. subtilis* DNA fragment or the introduction of new restriction endonuclease sites indicative of some of the engineered mutations. The creation of the mutation was verified by DNA sequencing.

Strain construction. Strains of JH642 in which one of the engineered *sigE* mutations replaced the resident gene as the expressed copy were constructed by transforming either JH642 (Spo$^+$) or SE84 (Spo$^-$) with pJM102 containing the mutant *sigE* allele on a 1.1-kbp DNA fragment. The 1.1-kbp DNA fragment encodes the entire *sigE* gene but not its promoter (6, 8). Integration of the pJM102 (*sigE*) plasmid with its homologous sequences on the *B. subtilis* chromosome would result in only one of the *sigE* alleles joined to the *sigE* promoter. Clones in which the plasmid had integrated were selected on the basis of a plasmid-borne Cm^r gene (5 μg/ml) and screened for an altered sporulation phenotype on the basis of either turning brown (Spo$^+$) or remaining white (Spo$^-$) following incubation on nutrient broth (Difco sporulation [DS]) plates (18) for 24 h. Those in which the sporulation phenotype had changed as a consequence of plasmid integration were screened by Western blot (immunoblot) analysis using an anti-σ^E monoclonal antibody to detect the altered *sigE* gene product (24). Strains which incorporated and expressed a *sigE* allele were named after the allele that they carry (e.g., strain SEP388 is strain JH642 with *sigEΔ388*, etc.).

Analysis of extracts for *sigE* products. *B. subtilis* cultures were grown in DS medium, cells were harvested, and protein extracts were prepared as previously described (24), except that the ammonium sulfate step was omitted. Protein samples (100 μg) (determined by the Coomassie method [Bio-Rad Laboratories]) were precipitated with 2 volumes of cold ethanol, suspended in sample buffer, and fractionated on sodium dodecyl sulfate-polyacrylamide gels (12% acrylamide). Following electrophoretic transfer to nitrocellulose and blocking of the nitrocellulose with BLOTTO, the protein bands were probed with an anti-σ^E monoclonal antibody (24). Bound antibody was visualized using a alkaline phosphatase-conjugated goat immunoglobulin against mouse immunoglobulin (Hyclone Laboratories, Inc.).

Isolation of *sigE* mutants deficient in processing. The 1.1-kbp *Pst*I fragment encoding *sigE* but not its promoter was cloned into pJM102 and transformed into the *E. coli* mutator strain DM2516. pJM102 can replicate in *E. coli* but not in *B. subtilis*. Isolated clones of DM2516 carrying this plasmid were grown overnight in Luria broth. Plasmid DNA was prepared from these cells and used to transform *B. subtilis* JH642 to Cm^r. Cm^r clones arise by the integration of the plasmid into the *B. subtilis* chromosome by a Campbell-like recombination event resulting in a duplication of the *sigE* locus with only one of the alleles being joined to its promoter. Clones in which mutant *sigE* alleles are expressed were detected by screening for a Spo$^-$ phenotype on DS plates. Spo$^-$ clones were further screened by Western blot analysis for those clones which synthesize a protein with the mobility of pro-σ^E which is inefficiently converted into a protein with the mobility of σ^E. Three such clones were detected following transformation by one of the plasmid preparations. The mutant sequence was recovered by cutting chromosomal DNA from the *B. subtilis* clones with a restriction endonuclease that maintained the connection between the vector and *sigE* sequence followed by joining the ends with DNA ligase and transformation into *E. coli*. The site of the mutation responsible for the phenotype was determined by DNA sequencing. All three mutant clones contained the same G→A transition mutation at codon 25 of pro-σ^E (GAA [Glu] →AAA [Lys]).

β-Galactosidase assays. *B. subtilis* strains carrying pSR5 were grown in DS medium (18) and harvested at various times during growth and sporulation. Cells were disrupted by passage through a French pressure cell (two passages at 20,000 lb/in^2) and analyzed for β-galactosidase as described by Miller (13).

DNA sequencing. DNA sequencing was performed by the method of Sanger et al. (16), using the Sequenase reagents (U.S. Biochemical Corp.) and protocol provided by the manufacturer.

***B. subtilis* transformation.** Plasmid DNA was transformed into *B. subtilis* following the generation of competence for DNA uptake by the method of Yasbin et al. (27).

Determination of sporulation frequencies. *B. subtilis* strains to be tested were inoculated into DS medium (18) and incubated with shaking for 24 h at 37°C. Cultures were diluted 1/10 into 2 ml of minimal medium salt base (19). Chloroform (100 μl) was added, and after being vortexed, the cultures were incubated for 30 min at 80°C. Samples of the heated cultures and the untreated parental culture were diluted and plated on DS medium for counting of viable cells.

RESULTS

Construction of pro-σ^E deletion strains. A series of mutagenic oligonucleotides (30-mers) complementary to both the region immediately upstream of the second codon of pro-σ^E and the region immediately downstream of the sequences to be deleted were synthesized and used, as described in Materials and Methods, to generate a family of *sigE* genes

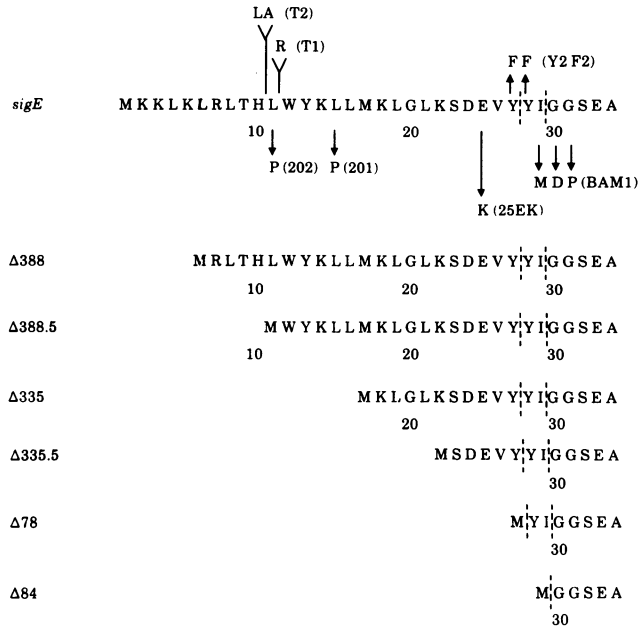

FIG. 1. Structures of mutant *sigE* products. The predicted amino acid sequences of the amino termini of pro-σ^E and several mutant *sigE* products are given. All of the mutant *sigE* genes have the same sequence upstream of the initiating Met codon and downstream of residue 30. The codon for the initiating Met of pro-σ^E begins at bp 179 of a 1.1-kbp *Pst*I fragment used to both generate the mutations and transfer them to *B. subtilis*. The broken lines between residues 27 and 28 and residues 29 and 30 represent putative processing sites. The arrows indicate the residues altered by mutations and illustrate the amino acid changes that accompany the mutations. The *sigE201* and *sigE202* alleles (isolated by S. Rong and A. L. Sonenshein) were previously described (5, 15). Each of the insertion mutations (T1 in *sigE12* and T1 and T2 in *sigE13*) was generated by placing codons for the indicated amino acids within *sigE* at the sites depicted.

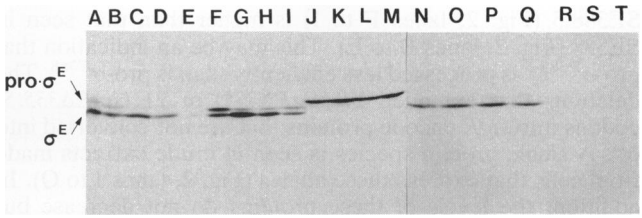

FIG. 2. Western blot analysis of *sigE* deletion mutants. Cultures of *B. subtilis* JH642 and isolates of this strain into which mutant *sigE* alleles had been transformed for expression were grown in DS medium and harvested at one or more time points after the end of exponential growth (T_0). Samples were processed and assayed by Western blot analysis as described in Materials and Methods. Anti-σ^E antibody was detected by an alkaline phosphatase-conjugated goat anti-mouse immunoglobulin antibody. The two bands in lane A depict pro-σ^E and σ^E. Lane A, JH642 (*sigE*$^+$) harvested at T_2 (i.e., 2 h after T_0); lanes B to E, SEP388 (*sigEΔ388*); lanes F to I, SEP388.5 (*sigEΔ388.5*); lanes J to M, SEP335.5 (*sigEΔ335.5*); lanes N to Q, SEP335 (*sigEΔ335*) harvested at $T_{1.5}$, T_3, $T_{4.5}$, and T_6, respectively; lanes R to T, SEP78 (*sigEΔ78*) harvested at T_2, T_4, and T_6, respectively.

with progressively longer deletions into the precursor-specific portion of *sigE*. The amino termini of the σ^E-like proteins specified by these mutant alleles are illustrated in Fig. 1. The 1.1-kbp DNA fragments carrying the entire *sigE* coding region of each of the *sigE* deletion mutations but not their promoters were separately cloned into the integrating vector pJM102 and introduced into the wild-type *B. subtilis* strain (JH642) by transformation (27). Cells that had integrated one of these nonreplicating plasmids into their chromosomes by a Campbell-like recombination at *sigE* were isolated by selecting for plasmid-encoded chloramphenicol resistance. Homologous recombination between the plasmids and the *B. subtilis* chromosome could occur at their *sigE* regions either upstream or downstream of the engineered deletions. Recombination upstream of a deletion would place the mutant allele under the control of the *spoIIG* promoter, allowing it to be expressed. In such a recombination, the wild-type allele would be separated from the promoter by the vector sequence and become silent. A recombination event downstream of the deletion in the mutant allele would leave the wild-type allele as the expressed copy. The deletions in *sigE* occur at approximately 20% of the distance from the upstream end of the 1.1-kbp fragment that was cloned into pJM102. Assuming that recombination can occur at random along this sequence, we anticipated that approximately 20% of the chloramphenicol-resistant transformants would have the mutant allele as the expressed copy. This is the result that we had obtained in a previous study in which the *sigEΔ84* allele was transformed into *B. subtilis* using this same vector system (5). Deletions that affect the activity or stability of σ^E are likely to affect the sporulation ability of a cell that carries that *sigE* allele as its sole source of σ^E. We therefore tested transformants for their sporulation phenotype on DS medium (18). Spo$^+$ colonies turn brown after 24 h at 37°C on DS medium, while Spo$^-$ colonies remain white and begin to lyse. Approximately 20% of the chloramphenicol-resistant colonies that had been transformed with plasmid containing the *sigEΔ335*, *sigEΔ335.5*, or *sigEΔ78* allele were Spo$^-$ by this criterion. All of the cells that received either *sigEΔ388* or *sigEΔ388.5* DNA turned brown (i.e., were Spo$^+$). We inferred from this result that the two smallest deletions (*Δ388* and *Δ388.5*) did not sufficiently compromise pro-σ^E to prevent it from performing its sporulation-essential functions, while the more extensive deletions altered a critical aspect of pro-σ^E. In order to verify that the *Δ388* and *Δ388.5* deletions do not compromise a cell's sporulation ability and to easily identify possible *B. subtilis* clones that express these alleles, we transformed a *B. subtilis* strain (SE84) whose wild-type *sigE* gene had been previously replaced with *sigEΔ84* in a congression experiment. Following transformation with the *sigEΔ388*- or *sigEΔ388.5*-containing plasmids, approximately 20% of the SE84 cells that had become Cm^r were also Spo$^+$. This result substantiated the Spo$^+$ phenotype of the *sigEΔ388* or *sigEΔ388.5* allele and identified clones that were expressing these alleles.

To verify that each strain was expressing the anticipated altered *sigE* product, representative clones (Spo$^-$ clones of JH642 transformed with *sigEΔ335*, *sigEΔ335.3*, or *sigEΔ78* or Spo$^+$ clones of SE84 transformed with *sigEΔ338* or *sigEΔ338.5*) were sporulated in DS medium and screened by Western blot analysis with an anti-σ^E monoclonal antibody for pro-σ^E-like proteins with altered mobilities (24). Figure 2 illustrates the results of this analysis. Crude cell extracts of SE388 and SE388.5 contain pro-σ^E-like proteins that appear smaller than authentic pro-σ^E but are still converted into proteins of the size of authentic σ^E (Fig. 2, lanes B to E and F to I, respectively). Although both mutant pro proteins are converted into mature σ^E, the ratio of pro-σ^E to σ^E in strain

SE388.5 (Fig. 2, lanes F to I) is higher than that seen in SE388 (Fig. 2, lanes B to E). This may be an indication that pro-$\sigma^{E388.5}$ is processed less efficiently than is pro-σ^{E388}. The deletions that extended 17 (*sigEΔ335*) or 21 (*sigEΔ335.5*) codons into *sigE* encode proteins that are not converted into σ^E. A single protein species is seen in crude extracts made from cells that express these alleles (Fig. 2, lanes J to Q). In addition, the levels of these proteins do not decrease but remain relatively constant during the course of the experiment. This is likely to be a reflection of the Spo$^-$ status of cells that express these alleles: having a dysfunctional σ^E, they are unable to proceed into the later stages of sporulation, and so σ^E synthesis persists. The pro-σ^E-like proteins encoded by *sigEΔ388*, *sigEΔ388.5*, and *sigEΔ335* have the greater mobilities predicted of proteins that are smaller than their wild-type counterpart (Fig. 2, lanes B to I and N to Q); however, pro-$\sigma^{E335.5}$ has the mobility of as a protein that is as large or larger than wild-type pro-σ^E (Fig. 2, lanes J to M). We have sequenced, cloned, and expressed several different isolates of this allele. They all have the proper sequence but the unusual mobility. We do not know the basis of the aberrant mobility of pro-$\sigma^{E335.5}$. It may be due to an unusual conformation or altered sodium dodecyl sulfate (SDS) binding caused by the amino acids at its amino terminus, or perhaps it reflects a modification of the protein by the bacterium. If its mobility is due to a modification, it is unlikely that it is related to a specific role for the protein in *B. subtilis*. Pro-$\sigma^{E335.5}$ has reduced mobility on SDS-polyacrylamide gels regardless of whether it is synthesized in *B. subtilis* or *E. coli* (data not shown). An aberrant mobility is also seen in the product of another mutant *sigE* allele (*sigE30*) discussed below.

The deletion that extends to codon 27 (*sigEΔ78*) specifies a product that is undetectable in *B. subtilis* (Fig. 2, lanes R to T). This is similar to the result that we had previously obtained with the deletion mutation (*sigEΔ84*) that extended to codon 29, the putative σ^E amino terminus (5). The difference in abundance between the *sigEΔ335.5* and *sigEΔ78* products suggests that the sequence between codons 22 and 28 has a significant effect on the ability of the *sigE* product to accumulate in *B. subtilis*.

***spoIID* promoter activity in *sigE* deletion mutants.** Deletions that extend up to 10 codons into the pro-σ^E coding region do not significantly affect the ability of strains with these deletions to sporulate under normal laboratory conditions. In contrast, deletions which extend 15 or more codons into *sigE* confer a Spo$^-$ phenotype. In order to test whether this phenotype is due to a loss in σ^E activity, strains with these deletions were transformed with a replicating plasmid (pSR5) that contains a transcriptional fusion of the σ^E-dependent *spoIID* promoter to the *E. coli lacZ* gene (14). Plasmid-bearing cells were selected on the basis of kanamycin resistance and grown and sporulated in DS medium, with samples taken and analyzed for β-galactosidase levels. Induction of the *spoIID* promoter in the wild-type *B. subtilis* strain (JH642) normally occurs 2 h after the onset of sporulation (T_2) (Fig. 3). This is the time in development when pro-σ^E is converted into an active sigma factor (24). Results similar to these were obtained by using the two deletion strains (SEP388 and SEP388.5) that synthesize a processable pro-σ^E (data not shown). There was substantial *spoIID* expression in the two deletion strains (SEP335 and SEP335.5) that accumulate an unprocessed pro-σ^E-like protein; however, the onset of β-galactosidase synthesis in these strains is delayed by 1 or 2 h relative to the time at which its induction occurs in the wild-type strain (Fig. 3).

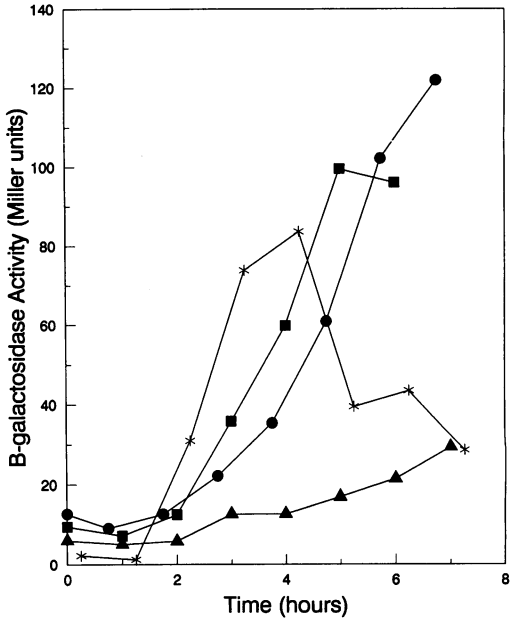

FIG. 3. Expression of *spoIID::lacZ* in *B. subtilis* strains with *sigE* deletion mutations. *B. subtilis* JH642(pSR5) with the wild-type *sigE* allele (*) or the *sigEΔ335* (■), *sigEΔ335.5* (●), or *sigEΔ78* (▲) allele was grown in DS medium at 37°C. When the cultures left exponential growth (0 h), samples were taken at the indicated times, disrupted by passage through a French pressure cell, and assayed for β-galactosidase activity as described by Miller (13).

Besides a delay in induction, these strains do not display the drop in β-galactosidase activity that is observed in the wild-type strain. This persistence parallels the unchanging levels of the pro-σ^E-like proteins in these strains (Fig. 2). Thus, unlike the wild-type and mutant *sigE* strains (SEPΔ388 and SEPΔ388.5) in which the levels of σ^E fall after T_4 and *spoIID* expression decrease as a likely consequence, the levels of pro-σ^{E335} and pro-$\sigma^{E335.5}$ remain high and *spoIID*-associated β-galactosidase levels also remain elevated. As expected, *spoIID* promoter activity in the deletion strain which failed to accumulate σ^E (SEP78) was very low (Fig. 3).

The failure of the Western blot analyses to detect a protein with σ^E-like mobility in extracts of SEP335 and SEP335.5 even though these strains contained significant *spoIID* promoter activity suggests that the products of the *sigE* alleles in these strains are active without being processed. It is also possible that one or both of the short pro-σ^Es that are processed is active without processing but that this activity is masked in a processing-competent background. In order to verify the first suggestion and test the second possibility, the *sigE* alleles were moved by transformation into a *B. subtilis* strain that carries both the *spoIID::lacZ* gene fusion (pSR5) and a mutation in a gene (*spoIIE*) whose product is needed for processing (4). In this background, *spoIID*-dependent β-galactosidase levels would be due to the activity of the unprocessed *sigE* product. Figure 4 illustrates the induction of β-galactosidase in several of these strains. The wild-type *sigE* allele gives no detectable *spoIID* induction in this genetic background: however, the *sigEΔ388*, *sigEΔ388.5*, and *sigEΔ335* deletion mutants show progressively higher levels of induction that parallel the extent of their deletions. The *sigEΔ335.5* mutant displays a higher level of induction than does the *sigEΔ388.5*-containing strain

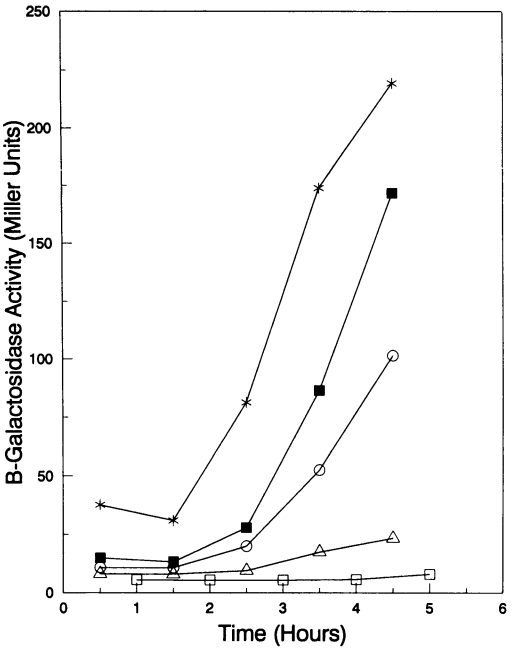

FIG. 4. Expression of *spoIID::lacZ* in *B. subtilis* strains with mutations in *spoIIE* and *sigE*. *B. subtilis* IS35 (*spoIIE64*) carrying pSR5 with the wild-type *sigE* allele (□) or the *sigEΔ388* (△), *sigEΔ388.5* (○), *sigEΔ335* (*), or *sigEΔ335.5* (■) allele was grown in DS medium and assayed as described in the legend to Fig. 3.

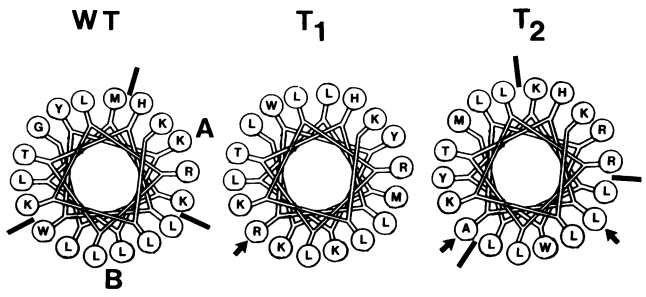

FIG. 5. Helical models of pro-σ^E amino termini. Amino acids 3 through 21 of the pro-σ^E amino terminus are represented on a helix wheel. In the wild-type sequence (WT), the residues form a surface of basic amino acids (His, Lys, Arg) (A) and an adjoining surface (B) of hydrophobic amino acids (Leu, Trp). In *sigE12* (T_1), an extra amino acid (arrow) is inserted into the helix at position 12 (Fig. 1, T1) which rotates portions of the faces out of alignment. In *sigE13*, two additional amino acids (arrows) are inserted into *sigE12* (Fig. 1, T1 and T2) as residues 11 and 12. This rotates the basic and hydrophobic components into an alignment more similar to that seen in the wild-type protein.

but less than that seen in the *sigEΔ335* mutant (Fig. 4). The lower level of activity displayed by the product of the *sigEΔ335.5* allele relative to *sigEΔ335* was also seen in the wild-type background (Fig. 3) and may be due to a slightly decreased stability of $\sigma^{E335.5}$ relative to that of σ^{E335}. Although the lower activity of $\sigma^{E335.5}$ complicates the role of amino acids 17 to 21, it seems clear that at least through the first 17 residues of pro-σ^E, removal of amino acids can be directly correlated with σ^E activation.

Addition of amino acids to the pro-σ^E sequence. The region of the pro sequence that is required to silence the activity of pro-σ^E as well as for processing of the precursor into σ^E is composed of amino acid residues that are compatible with the formation of an alpha-helix (25). The notion that an alpha-helix may actually be formed in this region was also suggested by the observation that the *sigE* mutations *sigE201* and *sigE202* (Fig. 1) which substitute the helix-disrupting amino acid Pro for Leu at two different sites in this region substantially destabilize the *sigE* product (5). If an alpha-helix is drawn, the residues align to form a structure with hydrophobic and positively charged faces (Fig. 5, WT). It appeared possible that these different faces of the helix might be selectively interacting with other proteins or other regions of σ^E itself to give this region its properties. To test this, we used oligonucleotide-directed mutagenesis to insert an additional amino acid residue into the helix at a site approximately midway between the ends of the amphipathic region so as to rotate the faces out of alignment (Fig. 5, T_1). The region of the putative helix in which the changes were made was also chosen because it was an area of the pro sequence in which one of the Leu→Pro mutations (Fig. 1, 202) which substantially destabilized pro-σ^E occurred (5, 15). Having constructed the first insertion mutation, we then incorporated additional residues into the mutant sequence that should reestablish the amphipathic faces of the helix (Fig. 5, T_2). As described in Materials and Methods, the first mutation involved inserting an Arg codon between codons 11 and 12 (Fig. 1, T1) forming *sigE12*. The second mutation (*sigE13*) was then introduced into *sigE12* by inserting codons for Leu and Ala between codons 10 and 11 (Fig. 1, T2).

The sequences of the first (*sigE12*) and second (*sigE13*) mutations were verified by direct DNA sequencing and then cloned, as with the previous mutations, on a 1.1-kbp fragment into pJM102. When pJM102 carrying either *sigE12* or *sigE13* was moved into the wild-type *B. subtilis* strain, *sigE13* but not *sigE12* yielded Spo$^-$ transformant clones. The Spo$^+$ quality of the *sigE12* allele was verified by its transformation into SE84 and the appearance of the predicted percentage of Spo$^+$ colonies among the chloramphenicol-resistant transformants (data not shown). Western blot analysis of strains that should be expressing the *sigE12* and *sigE13* alleles revealed (Fig. 6) that both mutations had a deleterious effect on pro-σ^E accumulation. On the basis of the degree of antibody binding, the incorporation of the single Arg residue reduced the level of σ^E-like proteins (Fig. 6, lanes D to F) in the strain which carried it to approximately 10% of that found in the wild-type strain (Fig. 6, lanes

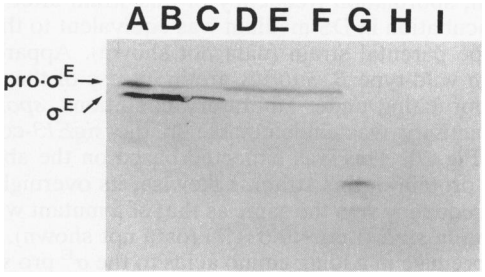

FIG. 6. Western blot analysis of *sigE12* (T_1) and *sigE13* (T_1 and T_2) mutations. Cultures of *B. subtilis* JH642 (lanes A to C) or isolates of this strain in which *sigE12* (lanes D to F) or *sigE13* (lanes G to I) are the expressed *sigE* alleles were grown in DS medium and harvested at 2 (lanes A, D, and G), 4 (lanes B, E, and H), or 6 (lanes C, F, and I) h after the end of exponential growth. Samples were processed and analyzed as described in the legend to Fig. 2.

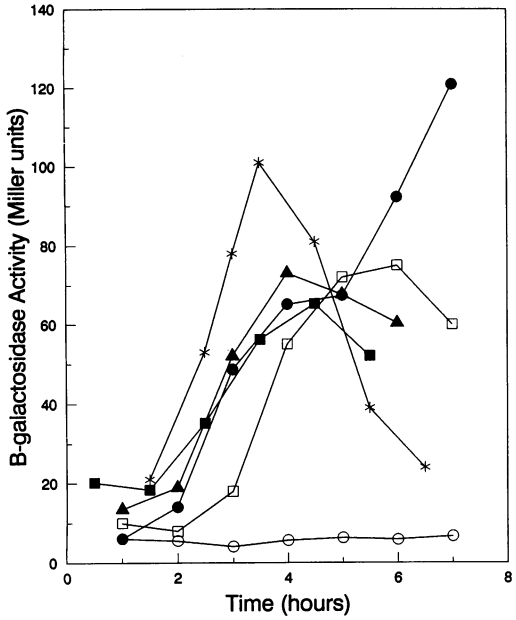

FIG. 7. Expression of *spoIID*::*lacZ* in *B. subtilis* strains with missense and insertion mutations in *sigE*. *B. subtilis* JH642(pSR5) with either the wild-type *sigE* allele (*), or the *sigE30* (Fig. 1, BAM1) (●), *sigE27* (Fig. 1, Y2 F2) (■), *sigE12* (Fig. 1, T1) (▲), *sigE13* (Fig. 1, T1 T2) (○), or *sigE25* (Fig. 1, 25EK) (□) allele was grown in DS medium and analyzed as described in the legend to Fig. 3.

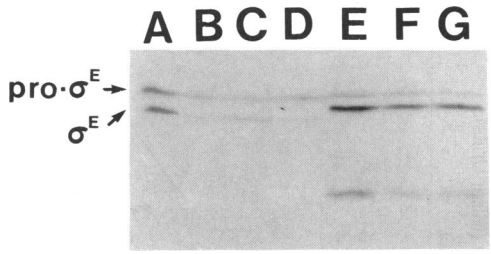

FIG. 8. Western blot analysis of pro-σ^E proteins with amino acid substitutions at the putative processing sites. *B. subtilis* JH642 (lane A) or isolates of this strain in which *sigE27* (lanes B to D) or *sigE30* (lanes E to G) are the expressed alleles were grown in DS medium and harvested at 2 (lanes A, B, and E), 4 (lanes C and F), or 6 (lanes D and G) h after the end of exponential growth. Samples were processed and analyzed as described in the legend to Fig. 2.

A to C). Crude cell extracts from the strain which carried the double mutation (*sigE13*) contained no proteins of the size of pro-σ^E or σ^E but did contain lower-molecular-weight proteins, presumably breakdown products, that reacted with the anti-σ^E monoclonal antibody (Fig. 6, lanes G to I). The pro-σ^E-like protein encoded by *sigE12* appeared to be processed into σ^E; however, on the basis of the relative abundance of antibody-reacting protein present at the pro-σ^E versus σ^E positions, its processing did not appear to be as efficient as that of the wild-type pro-σ^E (Fig. 6, lanes A to C versus lanes D to F). Although the strain which carries the *sigE12* allele produces only a fraction of the mature σ^E formed in a strain carrying the wild-type allele, this level appears to be sufficient to meet the sporulating cell's need for σ^E. *spoIID* promoter activity in the *sigE12* strain was nearly equal to the levels seen in wild-type strains (Fig. 7). In addition, sporulation frequency of this strain after an overnight incubation in DS medium was equivalent to that of the wild-type parental strain (data not shown). Apparently σ^E levels in wild-type *B. subtilis* are in excess of the needs of cells sporulating under laboratory conditions. *spoIID* promoter activity was undetectable in the *sigE13*-containing strain (Fig. 7). This was expected based on the absence of σ^E-like protein in this strain. Likewise, its overnight sporulation frequency was the same as that of a mutant with a null mutation in *sigE* (i.e., <0.001%) (data not shown).

Our premise in adding amino acids to the σ^E pro sequence was that we would distort the polarity of the putative helix that could be formed in this region which would alter the activity of the pro sequence. This outcome was not realized. Although the potential helix distortion did affect processing and stability, it was not reversed by a restoration of the hypothetical amphipathic structure. Instead, it appears that either the sequence in this region or the distance between the residues of pro-σ^E upstream and downstream of the sites of the insertion mutations is important in specifying pro-σ^E stability and processability. Insertion of additional amino acids had a significant effect on both properties, which did not correlate with their potential effect on the polarity of the pro-sequence helix.

Alteration of amino acids at the putative σ^E processing sites. Microsequencing of the σ^E amino terminus suggested that the processing of pro-σ^E occurs either between residues 27 and 28 (Tyr-Tyr) (12) or 29 and 30 (Ile-Gly) (11). The two Tyr residues present at positions 27 and 28 are similar to the two Tyr residues near the putative processing site of another *B. subtilis* σ-factor precursor, pro-σ^K (9, 21). Having observed that even conservative changes to the pro sequence can have dramatic effects on apparent protein stability, we engineered a conservative change into the pro sequence at the upstream processing position. Using a mutagenic oligonucleotide, we changed the codons for residues 27 and 28 from those specifying Tyr to those designating Phe (Fig. 1, Y2 F2). Inserting this mutant allele (*sigE27*) into the *B. subtilis* chromosome on pJM102 yielded clones that had a Spo$^+$ phenotype on DS plates but proved to be oligosporogenous when tested for the appearance of heat-resistant cells after overnight incubation in DS medium (approximately 10% of the parental frequency) (data not shown). A *sigE27*-containing strain induced the *spoIID* promoter at near wild-type levels (Fig. 7) but accumulated reduced levels of pro-σ^E (Fig. 8, lanes B to D). This pro-σ^{E27} was converted into a protein with the apparent mobility of authentic σ^E, although less σ^E is formed than in a wild-type cell (Fig. 8, lane A). We cannot say whether this lower level of σ^E is solely due to a lower level of precursor or if the efficiency of processing the precursor could also be reduced. We conclude that the presence of Tyr residues at positions 27 and 28 is not essential for processing but contributes to the stability of pro-σ^E and possibly the efficiency with which it is processed.

Changes were also made at the second putative processing site (residues 29 and 30). The changes at this site were quite substantial, primarily to introduce a convenient restriction endonuclease site (*Bam*HI) for use in other studies, but also to assess the effects of such a substitution. An oligonucleotide was used to convert the pro-σ^E Ile-Gly-Gly sequence at positions 29 to 31 into Met-Asp-Pro (Fig. 1, BAM1). *B. subtilis* that expressed this allele (*sigE30*) are Spo$^-$ ([<0.001%] heat-resistant cells after an overnight incubation in DS medium), but they have a high level of *spoIID* promoter activity (Fig. 7). Western blot analysis of crude cell

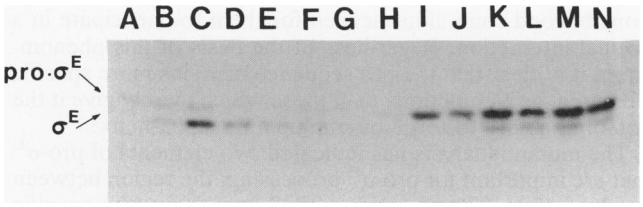

FIG. 9. Western blot analysis of a processing-defective allele of *sigE*. *B. subtilis* JH642 (lanes A to G) or a JH642 isolate (lanes H to N) expressing *sigE25* was grown in DS medium and harvested at hourly intervals from 0 to 7 h after the end of exponential growth. Samples were processed and analyzed as described in the legend to Fig. 2.

extracts prepared from *B. subtilis* expressing the *sigE30* allele revealed the presence of pro-σ^E and σ^E-like proteins with unexpectedly slow mobilities in the SDS-polyacrylamide gel system. Both the pro-σ^E and σ^E of *sigE30* migrated more slowly than their wild-type counterparts (Fig. 8). We do not know whether the mobility of the σ^E-like protein formed from pro-σ^{E30} is aberrant because of cleavage of pro-σ^E at a different site or an inherent property of σ^{E30} due to the changes at its amino terminus. The conversion of pro-σ^{E30} to σ^{E30} is, however, dependent on the cell's normal processing apparatus in that neither σ^{E30} formation nor *spoIID* induction occurs in a *B. subtilis* strain with a mutation in a gene (*spoIIE*) essential for processing (data not shown). The Spo$^-$ phenotype of the *sigE30*-containing strain is puzzling, given the activation of the *spoIID* promoter. Apparently the mutant protein lacks some other sporulation-essential feature of pro-σ^E or σ^E.

We infer from the phenotypes conferred by the *sigE27* and *sigE30* alleles that the amino acids at the putative pro-σ^E processing sites are not essential for the processing reaction to occur and that at least some changes at these sites are tolerated.

Isolation of a *sigE* mutant defective in pro-σ^E processing. Having failed to significantly and specifically block processing by altering the amino acid residues that bracketed the putative pro-σ^E processing sites, we adopted a more general strategy to identify residues that are critical for processing. The 1.1-kbp *Pst*I fragment that encodes the entire pro-σ^E gene cloned was passaged on pJM102 through a strain of *E. coli* (DM2516) which replicates DNA with poor fidelity. The resulting biologically mutagenized plasmid DNA was introduced by transformation into *B. subtilis*. Transformants in which the plasmid had integrated were selected and screened for their Spo phenotype. Spo$^-$ transformants were further screened by Western blot analysis for those that formed pro-σ^E but failed to efficiently convert it into σ^E. Three mutants of this class were identified, sequenced and found to have the identical change: GAA to AAA at codon 25 of the pro-σ^E sequence (Fig. 1, 25EK). A Western blot analysis of one of these mutants (SEP25) is illustrated in Fig. 9. During sporulation, substantial amounts of pro-σ^E are synthesized, but little of it is converted into σ^E (Fig. 9, lanes H to N). Unlike the parental strain in which σ^E is abundant only after 3 h (Fig. 9, lanes A to G), mutant pro-σ^{E25} persists throughout the experiment. Cells which express *sigE25* as their source of σ^E are as deficient in sporulation (<0.001% heat-resistant cells after 24 h in DS medium) as cells with null mutations in *sigE* or its processing machinery. The terminal phenotype of SEP25 cells, on the basis of electron microscopic examination (13a) is that of a disporic stage II mutant. This is also the terminal phenotype of a *sigE* null mutant. Unlike the case for a *sigE* null mutant, however, there is a substantial but delayed induction of the *spoIID* promoter in the *sigE25* strain (Fig. 7). This induction requires the cell's normal processing apparatus, as it does not occur in processing-deficient (*spoIIE* or *spoIIGA*) strains (data not shown). Apparently the processing of pro-σ^{E25EK} occurs at a level adequate to induce eventual *spoIID* expression but not efficiently enough to permit sporulation to occur. The σ^E that does eventually form may appear too late relative to other sporulation events to permit proper spore development. The identification of a residue at a position close to the putative processing sites that is important for the processing reaction indicates that at least some residues near the cleavage site(s) are needed for processing to occur.

DISCUSSION

Pro-σ^E is recognized by a developmentally regulated processing function that converts it into active σ^E (11, 24). In order to identify regions of pro-σ^E that are important for it to be processed, as well as to localize elements of the pro sequence that give the precursor form of σ^E its unique properties, we used oligonucleotide-generated mutagenesis to specifically alter or delete regions of the pro sequence. Sequential deletions from the amino terminus of pro-σ^E resulted in a parallel loss of both the ability of the pro-σ^E to be converted into σ^E and the gain of its activity as a sigma factor. Western blot analyses of the products of a series of *sigE* deletion mutations revealed that deletion of residues 2 to 6 does not significantly affect pro-σ^E processing and that the loss of residues 2 to 11 has a minor effect on this reaction. Deletion of residues 2 to 17, however, totally blocks the ability of pro-σ^E to be converted into σ^E. Analysis of the activity of the σ^E-dependent *spoIID* promoter in strains which express these mutant alleles as their sole source of σ^E and lack the capacity to process pro-σ^E demonstrated that the deletions in the pro-σ^E region caused σ^E activation in reverse order to their effect on processability (i.e., the smallest deletion resulted in the least active but most efficiently processed pro-σ^E). The similar sensitivity of pro-σ^E inactivation and susceptibility for processing to the same deletions suggests that the same region of the pro sequence could be involved in both functions. In a simple model, the inactivation of pro-σ^E in *B. subtilis* could be due to its sequestation by the processing apparatus. In such a model, the loss of a pro-sequence element required for the association would permit pro-σ^E to remain free and potentially active. Although this notion is appealing, it is not likely to be true in its simplest form. When the *sigE* deletion alleles are present and expressed from pJM102 in *E. coli*, the effects of the deletions are evident on the colony morphology of the *E. coli* clones which carry them. *E. coli* harboring mutant *sigE* genes with substantial deletions (e.g., *sigEΔ335*) form small, partially lysed colonies, while those carrying plasmids encoding pro-σ^E proteins with small pro-sequence deletions (e.g., *sigEΔ388*) form normal-sized colonies. If we assume that the compromised growth characteristics of the *E. coli* strains expressing σ^{E335} is a reflection of the deleterious effects of an active σ^E on these cells, then the more normal growth rate of the σ^{E388}-containing cells indicates that the larger pro sequence is silencing some aspect of the σ^E activity in the absence of a processing apparatus. The apparent ability of the pro sequence to restrict σ^E's activity in *E. coli* suggests that it is interacting with some site on σ^E itself rather than with another protein. This interaction may

mask a region of σ^E which is necessary for a productive interaction with RNA polymerase, or it may cause the pro-σ^E to assume a structure incompatible with σ-factor activity. The coincidence of the same region being required for both processability and inactivity may be an indication that this structure is also the target of the processing apparatus. We have recently placed the pro sequence of σ^E onto another *B. subtilis* σ factor (σ^K). In collaboration with members of the Kroos laboratory, it was observed that the σ^E pro sequence was cleaved from σ^K at the normal time in development for pro-σ^E processing (2). This result suggests that the σ^E pro sequence contains most, if not all, of the elements required for its recognition by the processing apparatus.

Deletions that remove 21 or more residues from the pro-σ^E amino terminus reduce σ^E activity in cells with the mutations. This reduction appears to be caused by a lower abundance of σ^E within the cell. Genes with deletions that extend to either of the likely σ^E amino termini encode proteins that accumulate at barely detectable levels in *B. subtilis* (i.e., approximately 1% of the wild-type pro-σ^E level) and reduced levels (10% of pro-σ^E) in *E. coli* (data not shown). The basis for the failure of these proteins to accumulate has not been rigorously tested. It is possible that their synthesis is compromised in some way; however, on the basis of the dramatic changes in product levels that are seen in mutant *sigE* alleles which have minor (single-base) changes along their sequence, it is thought that failure to accumulate is primarily due to a susceptibility of the *sigE* gene products to proteolysis (5). If we assume that the differences in pro-σ^E abundance are due to differences in their susceptibility to degradation, then a difference of only five amino acids at the pro-σ^E amino terminus is sufficient to distinguish a protein ($\sigma^{E335.5}$) that can accumulate to appreciable levels from one (σ^{E78}) that is neither detectable in conventional Western blots nor displays significant activity in reporter gene assays. The fact that such a short sequence (five amino acids) is able to protect pro-σ^E from degradation implies that this stability is not due to a general conformation conferred on σ^E by the pro sequence but rather a masking of a degradation signal present in the mature σ^E sequence. In support of the idea that the amino terminus of mature σ^E contains a signal for degradation, we have observed that a deletion of 10 amino acids from the N terminus of σ^{E84} increases its accumulation in *B. subtilis* 10-fold (2a). This hypothetical masking may involve the binding of some unidentified protein to the pro sequence or merely the absence of the amino-terminal sequence of mature σ^E at the end of the protein. We have speculated that the unusual instability of the σ^E protein may have biological relevance as a means to ensure σ^E removal from *B. subtilis* during ongoing differentiation and σ-factor exchange (5).

The structure of the σ^E pro sequence as it relates to both protein stability and processability is highly ordered. The insertion of a single amino acid into the structure (*sigE12*) is sufficient to reduce both precursor stability and processing efficiency. Although the addition of this single amino acid residue should have disturbed the charge distribution on the pro sequence's putative alpha-helix and this might explain its properties, the insertion of additional amino acids which would restore the pro sequence's configuration do not correct the effect of the first change. Instead, they enhance the degradation of pro-σ^E. The simplest interpretation of this result is that either the insertions were fortuitously placed in a critical region of the pro sequence or their placement separated regions upstream and downstream of the insertions beyond some limit needed for them to participate in a critical interaction. Regardless of the basis of this phenomenon, it is clear that the pro sequence contains more specific elements for its functions than those which merely give it the potential ability to form an amphipathic alpha-helix.

The mutant analysis has indicated two elements of pro-σ^E that are important for pro-σ^E processing: the region between residues 12 to 17 and residue 25. Surprisingly, the specific amino acids present at the processing sites themselves are less critical than these two regions. This suggests that the processing apparatus recognizes not the cleavage site itself but rather sequence elements upstream of it. This may be the reason for the ambiguity in determining the actual amino terminus of σ^E. If recognition for processing does not depend on the identity of the residue where cleavage occurs, the cutting of the pro sequence could be imprecise, with a resulting mixed population of cleavage products. The reconstruction of the processing reaction in vitro and the identification of the factors involved will be needed to clarify much of the confusion and speculation surrounding this reaction.

ACKNOWLEDGMENTS

This work was supported by NSF grant DMB-8613978 and Texas Advanced Technology program grant 003659-003 to W.G.H.

We thank D. Kolodrubetz for helpful discussions and a critical reading of the manuscript.

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