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Isolation of a *Bacillus subtilis spoIIGA* Allele That Suppresses Processing-Negative Mutations in the Pro-σ^E Gene (*sigE*)

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σ^E, a sporulation-essential sigma factor of *Bacillus subtilis*, is formed by a developmentally regulated proteolysis which removes 27 to 29 amino acids from the amino terminus of an inactive precursor protein (Pro-σ^E). A mutation which facilitates the conversion of inefficiently processed Pro-σ^E variants into mature σ^E was identified and mapped to *spoIIGA*. The isolation of such a mutation argues that SpoIIGA is directly involved in the Pro-σ^E processing reaction.

The temporal and compartment-specific pattern of gene expression during *Bacillus subtilis* sporulation is largely regulated by the programmed appearance of several distinct RNA polymerase σ factors, including σ^E (reviewed in references 2 and 9). σ^E is activated early in sporulation following partitioning of the cell into mother cell and forespore compartments (stage II), after which it is largely, if not exclusively, responsible for the expression of early mother cell-specific genes (2, 9). Regulation of σ^E is complex. Transcription of its structural gene (*sigE/spoIIGB*) begins at the onset of sporulation under the control of the spore gene activator protein, Spo0A (6, 16, 20). In addition, the product of the σ^E-encoding gene is an inactive proprotein (Pro-σ^E) (7, 19–21). Pro-σ^E is converted into mature σ^E by a developmentally regulated proteolysis that removes 27 to 29 amino acids from the Pro-σ^E amino terminus (Fig. 1) (7, 12). This processing reaction requires the products of several sporulation genes (*spoIIGA*, *spoIIE*, and *spoIIAC*) as well as essential cell division genes (*ftsZ* and *divIC*) (1, 3, 8, 18). The explicit roles of each of these gene products are largely unknown; however, a favored model views SpoIIGA as the likely Pro-σ^E processing enzyme, with the other gene products involved in generating a processing activation signal that is coupled to the stage II septation event (18).

We had previously isolated an allele of *sigE* (*sigE25EK*) whose product is processed inefficiently because of a Glu-to-Lys change at residue 25 of Pro-σ^E (Fig. 1) (13). Cells which express *sigE25EK* as their sole source of σ^E accumulate the proprotein but form little σ^E (Fig. 2) and are Spo$^-$ (Table 1). We sought to isolate mutant *B. subtilis* strains which are able to process the SigE25EK protein more efficiently and, as a consequence, become Spo$^+$. To this end, *B. subtilis* HP91 containing *sigE25EK*, plus a Km^r marker (*apr3′5″*) inserted at the *Eco*RI site (10, 19) approximately 2 kbp upstream of *sigE*, was grown to the mid-exponential stage in LB medium (11) and then treated with nitrosoguanidine (100 μg/ml) for 45 min at 25°C. Fifty percent of the cells survived this treatment and were plated on DS medium (17). After 24 h, brown Spo$^+$ colonies were detected above a lawn of lysing Spo$^-$ cells. Eight independent Spo$^+$ clones were identified. Chromosomal DNA was prepared from these clones and used to transform a Km^s *sigE25EK* strain of *B. subtilis* to Km^r (24). The Km^r transformants were screened for their Spo phenotypes as a measure of the linkage of Spo$^+$ to *sigE*. All eight transforming DNAs demonstrated greater than 90% linkage between these traits. Thus, all of the compensating Spo$^+$ mutations are in or near *sigE*. In order to distinguish *sigE* mutations from extragenic suppressing mutations, we transformed each of the putative suppressor strains with a promoterless copy of *sigE25EK* (1.1 kbp) cloned into a plasmid (pJM102) which cannot replicate in *B. subtilis* but confers Cm^r if integrated into the *B. subtilis* chromosome (13). Cm^r clones, in which the plasmid integrated by single-site (Campbell) recombination at random sites within *sigE*, were isolated. Spo$^-$ clones were found among the transformants of seven of the eight suppressor strains, suggesting that their Spo$^+$ mutations were in *sigE*. By using PCR-based DNA sequencing (Circumvent Thermal Cycle Dideoxy DNA Sequencing Kit; New England Biolabs, Beverly, Mass.), carried out according to the manufacturer's instructions, all seven of the clones were found to have reverted to the original *sigE* sequence (data not shown). Similar sequencing studies verified the presence of *sigE25EK* in the mutant clone (HP93N5) that remained Spo$^+$ following transformation with *sigE25EK* DNA. This clone therefore appeared to contain a suppressor mutation at a site near, but not in, *sigE*. An obvious candidate for the site of such a mutation would be the processing-essential *spoIIGA* gene, which is immediately upstream of and cotranscribed with *sigE* (5, 18). To test this possibility, oligonucleotides corresponding to regions 137 bp upstream and 205 bp downstream of *spoIIGA* were used to amplify the *spoIIGA* gene and its promoter from chromosomal DNA of the mutant clone. Transformation of HP91 (*sigE25EK*) with pJM102 containing this 1.2-kbp *spoIIGA* region yielded 100% Spo$^+$ colonies among the Cm^r transformants. Thus, the suppressor mutation lies within *spoIIGA* and is dominant over the wild-type *spoIIGA* allele. The exact position of the mutation was determined by subcloning fragments of the *spoIIGA* suppressor allele into M13 (23) and then performing DNA sequencing (15). The mutant *spoIIGA* was found to contain a single (C→T) change that results in a Pro-to-Leu substitution at residue 259 of SpoIIGA (10).

Western blot (immunoblot) analysis revealed (Fig. 2A) that the *spoIIGA259PL* allele enhances the level and accelerates the timing of SigE25EK processing to those observed for wild-type Pro-σ^E in the parental strain. This is also reflected in the σ^E activity profile. A σ^E-dependent reporter gene (SPβ *spoIID::lacZ*) (14), introduced into wild-type and *spoIIGA 259PL sigE25EK B. subtilis* strains (JH642 and HP93N5,

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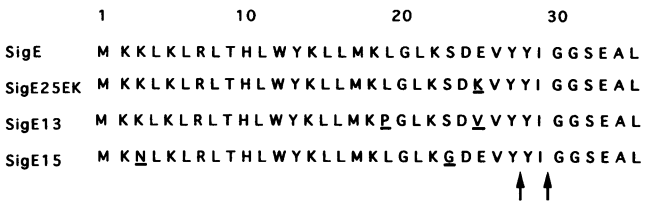

FIG. 1. Structures of mutant *sigE* products. The predicted amino acid sequences of the amino-terminal portions of Pro-σ^E and products encoded by three mutant alleles are shown. The amino acids changed by mutations in each *sigE* allele are underlined. The arrows indicate the positions of the putative processing sites following residues 27 (12) and 29 (7).

respectively), displays virtually identical patterns of induction as the cultures proceed through sporulation in DS medium (Fig. 3A). This contrasts with the induction of *spoIID* that is observed in a strain with only the *sigE25EK* allele. In this case, inefficient Pro-σ^{E25EK} processing leads to delayed activation of the *spoIID* promoter (Fig. 3A). We interpret the patterns of Pro-σ^E processing and σ-factor activity as evidence that SpoIIGA259PL-dependent processing responds to the same developmental cues as does the wild-type reaction.

We next asked whether the altered specificity of SpoIIGA 259PL affects processing of wild-type Pro-σ^E. To this end, we transformed a SpoIIGA$^-$ strain of *B. subtilis* (PEW *spoIIGA*

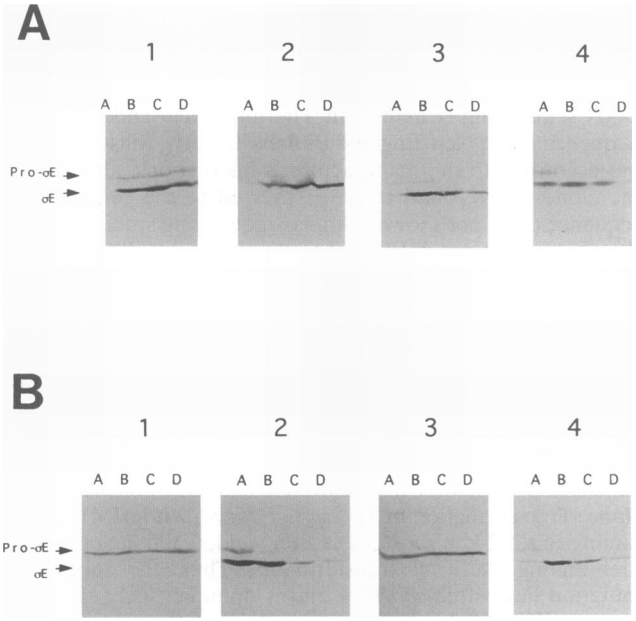

FIG. 2. Western blot analysis of Pro-σ^E processing in strains carrying *spoIIGA*$^+$ and *spoIIGA259PL* alleles. Cultures of JH642 and variants of this strain were grown in DS medium, and samples were harvested at 1.5 (lanes A), 3.0 (lanes B), 4.5 (lanes C), and 6.0 (lanes D) h after the end of exponential growth. Extracts were prepared as previously described and analyzed by Western blot (22). Pro-σ^E and SigE were detected by using a secondary antibody conjugated to alkaline phosphatase. (A) Strains carrying *sigE*$^+$ and *sigE25EK* alleles. Panels: 1, JH642 (wild type); 2, HP91 (*sigE25EK spoIIGA*$^+$); 3, HP 93N5 (*sigE25EK spoIIGA259PL*); 4, HP94N5A (*sigE*$^+$ *spoIIGA259PL*). (B) Strains carrying *sigE13* and *sigE15* alleles. Panels: 1, HP9313 (*sigE 13 spoIIGA*$^+$); 2, HP94N13 (*sigE13 spoIIGA259PL*); 3, HP9415 (*sigE 15 spoIIGA*$^+$); 4, HP94N15 (*sigE15 spoIIGA259PL*).

TABLE 1. Sporulation frequencies of strains containing *spoIIG* alleles[a]

Strain	Relevant genotype	No. of heat-resistant cells/ml
JH642	*sigE*$^+$	1.8×10^7
HP91	*sigE25EK*	$<10^3$
HP93N5	*spoIIGA259PL sigE25EK*	1.9×10^7
HP94N5A	*spoIIGA259PL*	1.9×10^8
HP9313	*sigE13*	$<10^3$
HP94N13	*spoIIGA259PL sigE13*	4×10^8
HP9315	*sigE15*	$<10^3$
HP94N15	*spoIIGA259PL sigE15*	4×10^8
HP94NSA	*spoIIGA259PL/spoIIGA* (merodiploid)	4.9×10^7
PEW	*spoIIGAΔ5*	$<10^3$

[a] Strains were grown in DS medium at 37°C with aeration for 24 h. The resulting cultures were diluted 1/10 in minimal medium salts containing 10% chloroform; briefly vortexed; and incubated at 80°C for 30 min. Samples of the heated cultures as well as untreated controls were plated on DS medium to determine the number of heat-resistant cells (spores) per milliliter of culture.

Δ5) (5) with an integrating plasmid vector (pMJ102) containing *spoIIGA259PL* plus its promoter. The recipient strain is Spo$^-$ because of its failure to process Pro-σ^E. If *spoIIGA 259PL* can function as a wild-type *spoIIGA* allele and permit the processing of the normal Pro-σ^E, cells which acquire it should become Spo$^+$. Transformants that expressed the vector-based antibiotic resistance marker formed brown (Spo$^+$) colonies after 24 h on DS medium plates. A representative clone (HP94N5A) was grown in DS liquid medium and found to sporulate as well or better than the wild-type strain (JH642) from which PEW was derived (Table 1). A wild-type pattern of Pro-σ^E processing and σ^E-dependent promoter induction was also observed (Fig. 2A and 3A, respectively) in this strain. We conclude that the *spoIIGA259PL* mutation did not change the specificity of the processing reaction from the wild-type Pro-σ^E target to the sequence carried by Pro-σ^{E25EK}; rather, it appears to have broadened the specificity of the reaction to include both proproteins as substrates.

As part of an ongoing examination of Pro-σ^E processing, we had used PCR-based mutagenesis to generate additional processing-deficient *sigE* alleles. Two of these alleles (*sigE13* and *sigE15*), with the changes indicated in Fig. 1, encode products that are even poorer substrates for processing than is SigE25 EK; i.e., we are unable to detect the conversion of either SigE13 or SigE15 into mature σ^E by Western blot (Fig. 2B) or *spoIID* promoter activity analyses (Fig. 3B). The changes that occurred in these two mutants (Fig. 1) include alterations at (*sigE13*) or near (*sigE15*) the site of the *sigE25EK* mutation. It therefore seemed possible that the *spoIIGA259PL* allele might also influence the processing of one or both of these variant *sigE* products. We constructed the strains needed to test this possibility by first moving the *spoIIGA259PL* allele into SE84, a *B. subtilis* strain with a *sigE* allele (*sigEΔ84*) that fails to make a detectable product (4, 13). A *spoIIGA259PL sigEΔ84* clone was then transformed with integrating plasmids carrying either *sigE13* or *sigE15* on 1.1-kbp DNA fragments. The deletion in *sigEΔ84* includes the sites of the mutations in *sigE13* and *sigE15* (13). Thus, recombination between the *sigE* alleles to generate a wild-type *sigE* is impossible. Approximately 50% of the transformants formed Spo$^+$ colonies on DS medium. The finding of Spo$^+$ clones among the transformants at a high frequency argues that *spoIIGA259PL* enables *B. subtilis* to process the mutant Pro-σ^Es. Western blot analyses of representative Spo$^+$ clones (Fig. 2B) revealed Pro-σ^E protein is synthesized and converted into mature σ^E in these *spoIIGA*

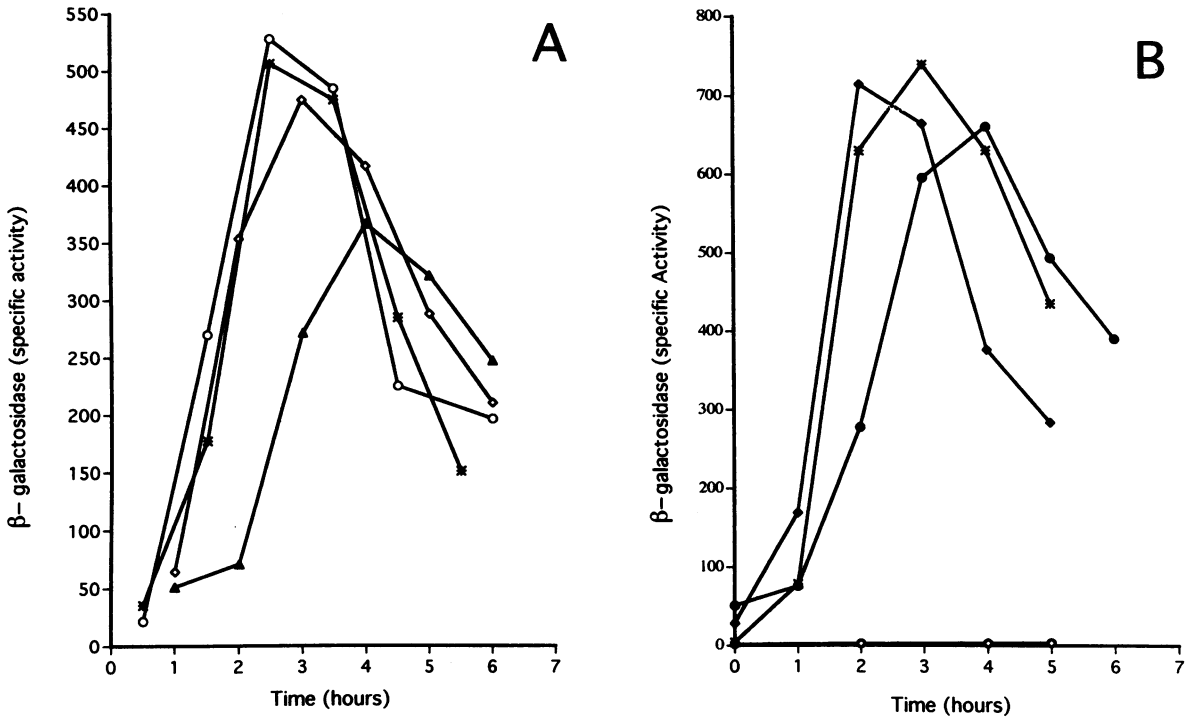

FIG. 3. Expression of *spoIID::lacZ* in *B. subtilis* strains with mutations in *spoIIGA* and *sigE*. *B. subtilis* strains containing SPβ *spoIID::lacZ* were grown in DS medium at 37°C. Samples were taken at the indicated times after the onset of sporulation (time zero) disrupted by passage through a French pressure cell, and centrifuged to remove cellular debris. Cell extracts were assayed for β-galactosidase activity by the method of Miller (11). Protein concentrations were determined by using Bio-Rad protein assay reagent (Bio-Rad Laboratories, Hercules, Calif.) as recommended by the manufacturer. β-Galactosidase specific activity is given as nanomoles of *O*-nitrophenol per minute per milligram of protein. (A) *, *B. subtilis* JH642 (wild type); ◇, HP93N5 (*sigE25EK spoIIGA259PL*); ○, HP94N5A (*sigE*$^+$ *spoIIGA259PL*); ▲, HP91 (*sigE25EK spoIIGA*$^+$). (B) *, *B. subtilis* JH642 (wild type); ◇, HP9313 (*sigE13 spoIIGA*$^+$); ○, HP9315 (*sigE15 spoIIGA*$^+$); ◆, HP94N13 (*sigE13 spoIIGA259PL*); ●, HP94N15 (*sigE15 spoIIGA259PL*).

259PL strains regardless of whether the Pro-σ^E is encoded by *sigE13* or *sigE15*. The processability of the mutant Pro-σ^Es is also reflected in the activity of a σ^E-dependent promoter in these strains. The presence of the *spoIIGA259PL* allele enables both transformant strains to synthesize wild-type levels of *spoIID*-dependent β-galactosidase (Fig. 3B). The expression of *spoIID* in the *spoIIGA259PL sigE15* strain is reproducibly delayed relative to the induction seen in wild-type and *spoIIGA259 sigE13* strains. This may indicate that Pro-σ^{E15} is processed with reduced efficiency, even though we failed to detect this in our Western blot analysis.

SpoIIGA has been hypothesized, on the basis of in vivo activity measurements and limited sequence homologies to proteolytic enzymes (10, 18), to be the Pro-σ^E processing enzyme. The results presented in this paper demonstrate that a mutation in *spoIIGA* can alter the specificity of the Pro-σ^E processing apparatus so that previously unprocessable forms of Pro-σ^E can now be converted into mature σ^E. The alteration in *spoIIGA259PL* is within the putative region of protease homology (10). A simple interpretation of our result is that SpoIIGA is the processing enzyme and that the Pro-to-Leu alteration reduces the stringency of its substrate recognition, thereby allowing processing of a more diverse collection of Pro-σ^Es. This interpretation must, however, be viewed with caution. *spoIIGA259PL* is not an allele-specific suppressor. It is possible that the change in SpoIIGA that reduces the specificity of the processing reaction does so by modifying an interaction between SpoIIGA and a yet-to-be-identified protein that is the actual Pro-σ^E protease. Regardless of whether SpoIIGA proves to interact directly with Pro-σ^E or with another component of the processing apparatus, the finding that a change in SpoIIGA can alter the specificity of the Pro-σ^E processing reaction argues strongly that it is directly involved in this reaction.

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