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A Single Amino Acid Substitution in σ^E Affects Its Ability To Bind Core RNA Polymerase

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We have examined the role of the most highly conserved region of bacterial RNA polymerase sigma factors by analyzing the effect of amino acid substitutions and small deletions in σ^E from *Bacillus subtilis*. σ^E is required for the production of endospores in *B. subtilis* but not for vegetative growth. Strains expressing each of several mutant forms of *sigE* were found to be deficient in their ability to form endospores. Single amino acid substitutions at positions 68 and 94 resulted in sigma factors that bind with less affinity to the core subunits of RNA polymerase. The substitution at position 68 did not affect the stability of the protein in *B. subtilis*; therefore, this substitution probably did not have large effects on the overall structure of the sigma factor. The substitution at position 68 probably defines a position in σ^E that closely contacts a subunit of RNA polymerase, while the substitution at position 94 may define a position that is important for protein stability or for binding to core RNA polymerase.

Sigma factors are a family of proteins that associate with the core subunits of RNA polymerase to form the RNA polymerase holoenzyme, which can initiate promoter-specific transcription. Each sigma factor confers a different specificity for promoter utilization on RNA polymerase. Nevertheless, sigma factors have highly conserved structural features that can be used to group sigma factors into two or three families (17, 19, 22). Alignment of the amino acid sequences of the largest family of sigma factors identifies several regions that are highly conserved (7, 8, 17, 26). Two of these conserved regions, designated 2.4 and 4.2, have been shown in several sigma factors to interact with promoter DNA (13, 25, 27, 28, 35). However, the function of the most highly conserved region, region 2.1, is unknown. Sigma factors must be bound to core RNA polymerase in order to direct its use of specific promoters (1, 14, 15). Since all sigma factors probably bind to the same site on the core, the region of each sigma factor that interacts with the core is likely to be highly conserved. Therefore, Neidhardt and colleagues suggested that the most highly conserved region of sigma factors may be involved in core binding (20). Lesley and Burgess showed that peptides containing the sequences from a region in σ^{70} that overlaps with this region of highly conserved amino acids bind to core subunits of *Escherichia coli* RNA polymerase (16).

To determine the role of the most highly conserved region, we examined the effects of amino acid substitutions in σ^E from *Bacillus subtilis*. σ^E is a secondary sigma factor that is not required for growth; however, it is required for sporulation (12). Mutations in the structural gene for σ^E, *sigE*, were assayed for function by testing their ability to complement the sporulation phenotype of a *sigE* deletion strain. The mutant derivatives of σ^E encoded by the noncomplementing alleles of *sigE* were examined to determine whether the protein accumulated and to determine whether any were specifically affected in their ability to bind to *B. subtilis* core RNA polymerase. The mutant forms of σ^E also were assessed for their ability to bind *E. coli* core RNA polymerase. We identified two single amino acid substitutions in the most highly conserved region of σ^E that appeared to reduce the affinity of σ^E for the core subunits of RNA polymerase. These results suggest that this highly conserved region of sigma factors closely contacts a core subunit.

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MATERIALS AND METHODS

Bacterial strains. *B. subtilis* RS359 (*lon*::*cat*) and RS374 (*lon*::*kan*) were constructed by R. Schmidt and obtained from the R. Losick laboratory (24). We used the *E. coli* strains DH5α, GM33, and RY2504 (provided by G. Churchward). Other bacterial strains are listed in Table 1.

Mutagenesis. *sigE* mutations were made by site-directed mutagenesis using a modified mutagenesis procedure (10). pJ8905, a modified form of pJ8903 with a kanamycin resistance cassette inserted in the *Bgl*II site, was used in oligonucleotide-directed mutagenesis (31). pJ8905 was used to transform RY2504 (*dut ung*) to kanamycin resistance. A kanamycin-resistant transformant was grown in Luria broth (LB) supplemented with 40 μg of kanamycin per ml and 30 μg of chloramphenicol per ml to an optical density at 600 nm of 0.5. Phage M13KO7 (multiplicity of infection, 10) and uridine (0.25 μg/ml) then were added. The mixture was incubated for 1 h with vigorous shaking and then was diluted 25-fold into LB supplemented with kanamycin (70 μg/ml). Single-stranded DNA was isolated and annealed to the specific oligonucleotides shown in Table 2. The Sequenase (U.S. Biochemicals)-generated replicative form of pJ8905 was transformed into strain DH5α. Kanamycin-resistant colonies were grown, and the plasmid was isolated by using the CTAB minipreparation procedure (2). Plasmid sequences were determined by the dideoxy-terminated sequencing protocol with Sequenase (U.S. Biochemicals) using either primer 1.2 or primer 2.1 (Table 2).

Sporulation assays. The mutant *sigE* alleles were cloned with a *Pst*I cassette into vector pSI-1 (provided by Alan Grossman) for expression from promoter P*spac* in *B. subtilis*. GM33 transformants containing the recombinant pSI-1 mutant *sigE* plasmids were selected by growth on LB supplemented with chloramphenicol (10 μg/ml). The pSI-1 plasmids were isolated by the CTAB minipreparation procedure (2). The orientation of the *Pst*I cassette was determined by digestion with restriction endonucleases *Xba*I and *Sca*I (Boehringer Mannheim Biochemicals). The correctly oriented pSI-1 mutant *sigE* plasmids were used to transform EU8701 to chloramphenicol resistance (5 μg/ml). EU8701 strains harboring the mutant forms of *sigE* were grown in Difco sporulation medium (DSM) (23) supplemented with 5 μg of chloramphenicol per ml. Expression of the *sigE* allele was induced at an optical density at 600 nm of approximately 0.6 with isopropyl-β-D-thiogalactopyranoside (IPTG; 0.05 to 1 mM), and cultures were allowed to grow for 20 h. One milliliter of each culture was transferred to a glass tube and heated at 80°C for 10 min. The heated samples were then diluted and plated in order to calculate the number of heat-resistant spores per milliliter of culture.

Construction of *B. subtilis* strains containing integrational *sigE* plasmids. The mutant *sigE* alleles were subcloned into the integrational vector pUS19 (provided by W. G. Haldenwang) (Fig. 1). These integrational *sigE* plasmids then were used to transform *B. subtilis*. Spectinomycin-resistant transformants were selected by growth on LB supplemented with 100 μg of spectinomycin per ml. *B. subtilis*

TABLE 1. Bacterial strains used in this study

Species	Strain	Relevant genotype (source)
B. subtilis	JH642	Wild type (5)
	EU8701	JH642 Δ*sigE*::*erm* (12)
	EUMS9501	EU8701 (pSI-1 *sigEΔ61-3*)
	EUMS9502	JH642 *sigE*::*sigE68RC*
	EUMS9503	JH642 (pUS19 *sigE99VC*)
E. coli	DH5α	*recA1*
	EUMS9511	DH5α *PlacBS*–wild-type *sigE*
	EUMS9512	DH5α *PlacBS*–*sigEΔ61-3*

strains expressing the complementing alleles of *sigE* were screened by preparing chromosomal DNA, PCR amplification using primers IIGB5′ and IIIG.2 (Table 2 and Fig. 1), Wizard column purification, and *fmol* cycle sequencing (Promega) of the PCR fragment using either primer 1.2b or sigE.2 (Table 2). *B. subtilis* strains expressing the noncomplementing alleles of *sigE* were screened by their inability to sporulate on solid DSM supplemented with 100 μg of spectinomycin per ml. The expressed allele of *sigE* in these strains also was verified by *fmol* cycle sequencing.

Construction of gene conversion strains. The noncomplementing alleles of *sigE* were gene converted into the *B. subtilis* chromosome by transforming competent JH642 with the pSI-1 *sigE* plasmids and selecting on LB plates supplemented with 5 μg of chloramphenicol per ml. Single transformants were patched for three consecutive days on solid DSM and LB plates with no selection. Transformants that were unable to sporulate on DSM and chloramphenicol sensitive were chosen. The chromosomal *sigE* mutation was verified by *fmol* cycle sequencing.

Reversion analysis. Ethyl methanesulfonate mutagenesis was used to isolate sporulation-proficient (Spo^+) revertants of the *B. subtilis* strains expressing the noncomplementing *sigE* alleles. Revertants were selected by a procedure described earlier (10). Strains were grown to mid-logarithmic growth phase in LB with appropriate supplementation and plated onto solid DSM. A glass microfiber filter (Whatman) was placed in the center of each plate, and 200 μl of ethyl methanesulfonate was applied to each filter. The plates were incubated at 42°C for 48 h. The plates then were inverted over chloroform for 20 min to select for Spo^+ revertants. The plates were again incubated at 42°C, and revertants normally appeared within 2 days. Plasmid DNA was isolated from the *B. subtilis* revertant strains containing plasmid-encoded *sigE* and used to retransform EU8701. The Spo^+ phenotype of these transformants verified that the reversion of phenotype was due to an intragenic mutation. The reversion was identified by DNA sequencing. The original integrational pUS19 mutant *sigE* plasmid was used to transform the *B. subtilis* revertant strains containing chromosomally encoded *sigE*. The sporulation-deficient (Spo^-) phenotype of a percentage of these transformants verified that the phenotypic reversion was due to an intragenic mutation. Chromosomal DNA was prepared from these revertant strains, and the chromosomal *sigE* reversion was identified by *fmol* cycle sequencing.

TABLE 2. Oligonucleotides used for site-directed mutagenesis and primers used for PCR amplification and sequencing

Mutant allele or primer	Sequence (5′ to 3′)
Alleles	
sigEΔ61-3	CGCAAATTGCGTTCGGCGCGCGCCGCCTG
sigEΔ68-4	TTTACGGGCGATATACAAATTGCGTTCAA
sigE58AC	GAATGGCGCGACACGCCTGATCGCC
sigE62LC	TTGCGTTCAATACAAATGGCGCGCGC
sigE63IC	CAAATTGCGTTCACATAGAATGGCG
sigE68RC	CGACCAGACACAAATTGCG
sigE69LC	GATATATACGACACAACGCAAATTG
sigE70VC	CGATATATACACACAGACGCAAATT
sigE73IC	CAAATTTACGGGCACAATATACGACC
sigE74AC	CAAATTTACGACAGATATATACGAC
sigE75RC	CAAATTTACAGGCGATATA
sigE76KC	CGTATTTTCAAAACAACGGGCGATA
sigE77FC	GTATTTTCACATTTACGG
sigE81GC	CTATATTAATACACGTATTTTC
sigE85EC	CTGATTAAATCACATATATTAATTCC
sigE86DC	GCTGATTAAACACTCTATATTAAT
sigE87LC	CCGATGCTGATACAATCCTCTAT
sigE93IC	CTTTGATTAGACCACAGGTACCGATG
sigE94GC	GATTAGACAGATGGTACC
sigE95LC	CAGCTTTGATACAACCGATGGTACC
sigE97KC	GTATTAACAGCACAGATTAGACCG
sigE98AC	AATGTATTAACACATTTGATTAGAC
sigE99VC	GATTAAATGTATTACAAGCTTTGATTAG
Primers	
1.2	CCATTATCTAAAGATG
2.1	GAACGGATTTTGTTATTT
IIGB5′	CAGGGGAGTTGGTCAGC
IIIG.2	GCTGCCTAAACAGCTTTC
1.2b	GGAGCAGGTTTTGTTAATGAAGCTCCC
sigE.2	CACCGGGAGGCATAGGTAGCAAGC

β-Gal assays. *B. subtilis* strains were lysogenized with SPβ that contained the wild-type *spoIID* promoter fused to *lacZ* (3) and selected by growth on LB supplemented with chloramphenicol (5 μg/ml). Transductants were grown in appropriate liquid DSM. β-Galactosidase (β-Gal) assays were performed as described previously (12).

Preparation of bacterial lysates. *B. subtilis* strains were grown in appropriate liquid DSM. Forty-milliliter samples were harvested at the end of exponential-phase growth (t_0) and 2 and 4 h after t_0 (t_2 and t_4, respectively), and the cell pellets were stored at −20°C. Growth of the cultures continued overnight, and sporulation assays were performed. The cell pellets were gently thawed and resuspended in 3 ml of French press lysis buffer (10 mM Tris-HCl [pH 7.9], 0.2 M NaCl, 10 mM $MgCl_2$, 0.5 mM EDTA, 0.1 mM dithiothreitol, 10% glycerol, and 1 mM phenylmethylsulfonyl fluoride) (16). Two passages of French press lysis were performed to 20,000 lb/in^2 guage (SLM Aminco; SLM Instruments) at 4°C. To facilitate the gradient sedimentation procedure, Brij 58 (0.5%, vol/vol) was added and the mix was incubated at 4°C for 5 min. Cell debris was pelleted, and the supernatant was collected.

E. coli strains (DH5α with pJ8905 *sigE* alleles) were grown in liquid LB supplemented with kanamycin (40 μg/ml). The cultures were allowed to grow until stationary phase, and 40-ml samples were harvested. Lysis was performed as described above. The protein concentration of the lysates was determined by the Bio-Rad protein microassay.

Glycerol gradient sedimentation analysis. One milliliter (5.0 mg/ml) of lysate (t_4 sample) was layered onto a 10-ml 15 to 30% glycerol gradient in 10 mM Tris-HCl (pH 7.9)–0.2 M NaCl–10 mM $MgCl_2$–0.5 mM EDTA–0.1 mM dithiothreitol and centrifuged in an SW41 Ti rotor at 37,000 rpm for 21 h at 4°C (34). Fractions (0.5 ml) were collected from the bottom of the tube, subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), transferred to nitrocellulose, and probed with antibody to σ^E (30), σ^{70} (4), the α subunit of *E. coli* (provided by R. Burgess), or the β′ subunit of *B. subtilis* (provided by W. G. Haldenwang). The nitrocellulose was then incubated with an anti-mouse immunoglobulin G antibody conjugated with alkaline phosphatase. Nitro Blue Tetrazolium (Sigma) and 5-bromo-4-chloro-3-indolyl phosphate (BCIP; Sigma) were used to visualize the appropriate bands.

Estimation of protein distribution within gradients. Nitrocellulose filters were imaged and stored onto 3.25-in. floppy transfer disks with the Imagestore 5000 Gel Documentation System (UVP). The images were analyzed with Semper 6 plus GelBase Windows Software (UVP) to obtain raw densitometry data. SigmaPlot for Windows (Jandel Scientific) was used to create the graphical representations of the data.

RESULTS

Identification of single amino acid substitutions that affect σ^E activity. Alignment of the amino acid sequences of sigma factors from several species of bacteria has shown that region 2.1 has the highest proportion of conserved amino acid residues (17). We used a mutational approach to study the role of this region in sigma factors by examining the effects of amino acid substitutions for conserved amino acids in σ^E from *B. subtilis*. We anticipated that multiple amino acid substitutions or large deletions of highly conserved amino acids could significantly distort the structure of the protein, making it impossible to determine any specific direct effect of the mutations on σ^E function. On the other hand, single amino acid substitutions could have little effect on certain functions, for example, binding of σ to core RNA polymerase, because the affinity of σ for core RNA polymerase may be determined by a large number of interacting amino acyl residues. Nevertheless, we sought to identify single amino acid substitutions of conserved amino acids that disrupted σ^E function. We also examined the effects of small deletions that were very likely to affect σ function.

Site-directed mutagenesis using pJ8905 was used to generate

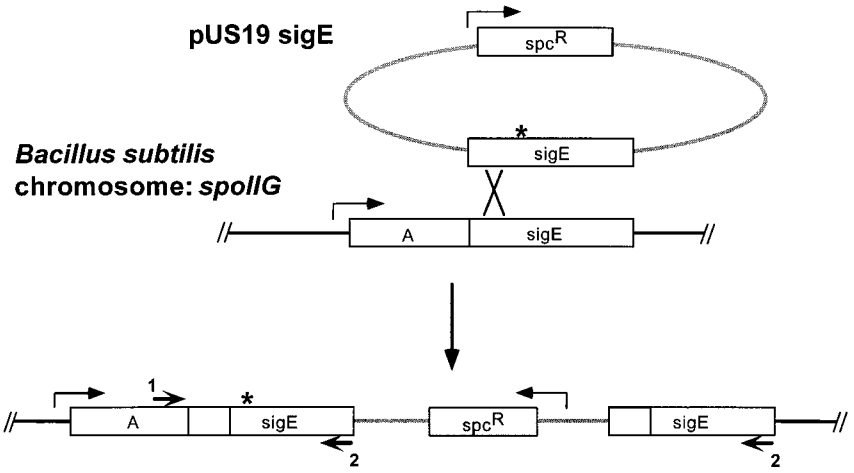

FIG. 1. Construction of integrational *sigE B. subtilis* strains. Different forms of the integrational plasmid pUS19 *sigE* were transformed into *B. subtilis* strains. Confirmation of the chromosomally expressed form of *sigE* was performed by PCR amplification with primers denoted by the arrows followed by *fmol* cycle sequencing. Arrow 1 is primer IIGB5′. Arrow 2 is primer IIG.2. *, a mutation.

two small deletions and 21 specific amino acid substitutions (Fig. 2). The *sigEΔ61-3* mutation deletes three amino acids (positions 61 to 63), two of which are highly conserved. The *sigEΔ68-4* mutation deletes four highly conserved amino acids (positions 68 to 71). The other mutations substitute cysteine for highly conserved amino acids located between positions 58 and 99 in σ^E. Cysteine was used because it is small and may be useful in future studies with cysteine-specific cross-linking agents.

Since σ^E is required for sporulation in *B. subtilis*, the effects of mutations can be assessed by testing the ability of cells expressing the mutant forms of *sigE* to sporulate. As an initial screen for mutations that affect σ^E activity, we determined the effects of plasmids expressing mutant alleles of *sigE* in an *sigE* deletion background. The sporulation assays showed that the mutant *sigE* alleles fell into two classes. The *sigE* mutant alleles in the first class were able to complement for sporulation activity to wild-type levels. The majority of the amino acid substitutions belonged to this class (Fig. 2). This group included *sigE58AC*, *sigE62LC*, *sigE63IC*, *sigE70VC*, *sigE73IC*, *sigE74AC*, *sigE75RC*, *sigE76KC*, *sigE77FC*, *sigE81GC*, *sigE85EC*, *sigE86DC*, *sigE87LC*, *sigE95LC*, *sigE98AC*, and *sigE99VC*. (The mutant *sigE* alleles are named by the number of the amino acid and the change at that position. For example, *sigE58AC* results in an alanine-to-cysteine substitution at amino acid position 58 in the full-length σ^E protein.) The mutant *sigE*

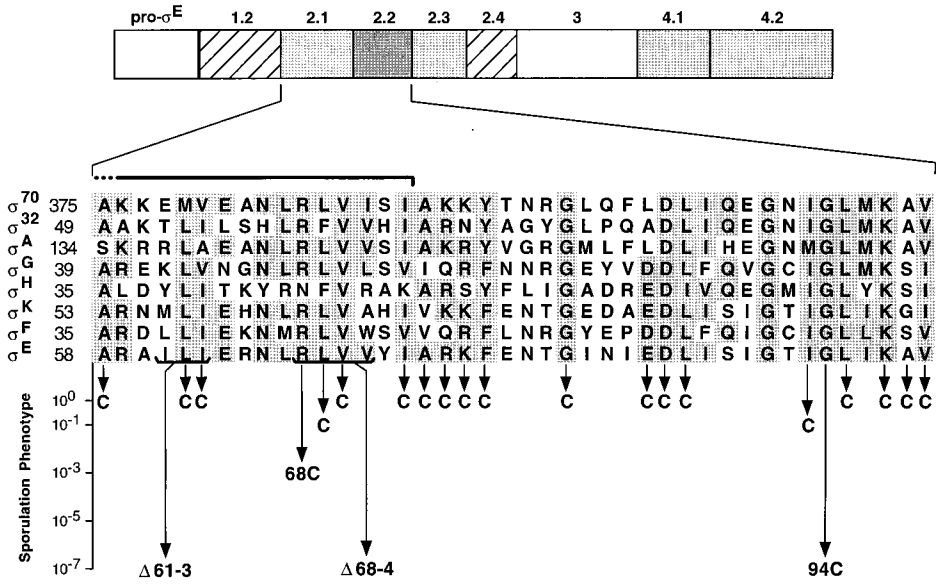

FIG. 2. Schematic representation of σ^E, sigma factor alignment, and sporulation phenotype results. The σ^E diagram is separated into specific regions as defined by amino acid homology and genetically determined functions (7, 8, 26). The degree of shading signifies the degree of conserved amino acid residues in the region. A portion of the Lonetto et al. (17) alignment of sigma factors from *E. coli* (σ^{70} and σ^{32}) and *B. subtilis* (σ^A through σ^E) is shown with conserved amino acids having a shaded background. The position in the sigma factor of the amino-terminal residue in the sequence is indicated by the number at the left of each sequence. The horizontal open bracket above the alignment indicates the peptide fragment studied by Lesley et al. (16). The effects of the *sigE* mutations on sporulation are shown with the arrows indicating the severity of the sporulation phenotype (10^0 indicates equal to wild-type [about 10^8 spores per ml], whereas 10^{-7} indicates fewer than 10 spores per ml).

TABLE 3. Reversion Analysis of Noncomplementing Alleles

sigE substitution	Mutation	Revertant	No. of spores/ml
Δ61-3	Δ 9 bp	None obtained	0
Δ68-4	Δ 12 bp	None obtained	0
94GC	GGT→TGT	GGT ("true")	3.6×10^8
		AGT (Ser)	3.5×10^6
68RC/97KC	CGT→TGT (68RC)/ AAA→TGT (97KC)	CGT (68RC reverted)/ TGT	2×10^8

alleles in the second class exhibited a reduced ability to complement for sporulation activity. Some of the mutant *sigE* alleles produced fewer than 50 spores per ml of culture, while other mutants resulted in intermediate levels of sporulation (Fig. 2).

We sought to isolate and characterize sporulation-proficient (Spo^+) revertants of the strains that contained defective alleles of *sigE*. Isolation of true revertants would demonstrate that the defect in σ^E activity was caused by the mutation in *sigE* that was produced by oligonucleotide-directed mutagenesis and not by a second undetected mutation. Identification of extragenic suppressors would also be informative. Ethyl methanesulfonate mutagenesis of Spo^- strains followed by chloroform selection was used to isolate revertants. The results of the reversion analysis are shown in Table 3.

Since the *sigE94GC* substitution resulted from only a single base pair change, the isolation of revertants was expected. Two kinds of revertants of *sigE94GC* were identified. Several true revertants, in which the original mutation was corrected to wild type, were isolated. Sporulation assays were performed on these revertants. They produced a wild-type level of spores (10^8 spores per ml). A second kind of revertant, in which the original base pair change was mutated to another base pair, was identified. This mutation resulted in a change of amino acid from a cysteine to a serine at the original position. This revertant was found to produce an intermediate number of spores (10^6 spores per ml). This reversion analysis provided further evidence that the substitution of a cysteine for glycine at position 94 of σ^E had produced a defective form of σ^E.

Another allele, *sigE97/68*, contained mutations that caused two amino acid substitutions, C for K at position 97 (K97C) and R68C. This double-mutant allele encoded a form of σ^E that was less active than the form containing the single R68C substitution (<50 and 10^5 spores per ml, respectively). A revertant of the *sigE97/68* was isolated. Sequencing of the revertant identified a true reversion of the base pair mutation that caused the R68C substitution. Evidently, the single K97C substitution had little effect on sporulation, but when combined with the R68C substitution, it enhanced the deleterious effect of the R68C substitution on σ^E function. No revertants of *sigEΔ61-3* or *sigEΔ68-4* were obtained. This was expected because these alleles contain small deletions.

Many of the mutant *sigE* alleles were introduced into their normal chromosomal positions either by gene conversion or by using the integrational plasmid pUS19. Linkage of the sporulation phenotype to the movement of the mutant allele to the chromosome would eliminate the possibility that other mutations outside of *sigE* affected the phenotype. Furthermore, the expression of the mutant *sigE* alleles in their normal chromosomal positions from the sporulation-induced *spoIIG* promoter is the best way to evaluate their effects on sporulation.

Several fully or partially complementing *sigE* alleles were introduced into the chromosome with the integrational plasmid, pUS19, including *sigE62LC*, *sigE69LC*, *sigE74AC*, *sigE75RC*, *sigE76KC*, *sigE77FC*, *sigE93IC*, *sigE95LC*, and *sigE99VC*. Since pUS19 cannot replicate in *B. subtilis*, transformation and selection for spectinomycin resistance resulted in the integration of the derivative pUS19 plasmids containing the mutant *sigE* alleles (Fig. 1). Two chromosomal copies of the *sigE* gene would result, with only one of them being expressed from the *spoIIG* promoter. Depending on the point of crossover, either the mutant *sigE* alleles or wild-type *sigE* would be expressed. PCR amplification and *fmol* cycle sequencing using primers IIGB5′ and IIIG.2 (Table 2) were used to determine which allele of *sigE* resided downstream from the *spoIIG* promoter. Sporulation assays were performed on the *B. subtilis* strains expressing the mutant *sigE* alleles from their normal chromosomal positions. As expected, all of the fully or partially complementing *sigE* alleles that were tested also were found to be fully or partially complementing, respectively, when expressed from the chromosome.

TABLE 4. Summary of core binding results and *spoIID-lacZ* expression

Allele	Core RNA polymerase in[a]:		β-Gal activity (Miller units)[b]
	B. subtilis	*E. coli*	
Wild-type *sigE*	B	B	233
sigEΔ61-3	N	N	2
sigE68RC	R	R	5
sigE94GC	—	N	3
sigE99VC	B	ND	248

[a] B, bound; N, not bound; R, reduced binding; —, no accumulation; ND, not determined.
[b] Sample harvested 3 h after the start of sporulation.

All of the second class of noncomplementing *sigE* mutants were introduced into the chromosome by gene conversion. Gene conversion of the mutant *sigE* alleles was performed by transforming *B. subtilis* with derivatives of the pSI-1 vector followed by passage of the transformants on nonselective media in order to cure strains of the plasmid. Screening of the transformants for a Spo^- phenotype resulted in the isolation of strains that had undergone gene conversion. The noncomplementing *sigE* mutants were found to be noncomplementing when expressed from their normal chromosomal positions (Fig. 2).

To examine the activity of RNA polymerase containing the mutant forms of σ^E on a specific promoter, we used *B. subtilis* strains that contain the σ^E-dependent promoter *spoIID* fused to *lacZ* (3). A summary of the β-Gal activity is shown in Table 4. The fully complementing forms of σ^E produced high levels of *spoIID* expression, as represented by the high levels of β-Gal activity. The mutant forms of σ^E encoded by *sigEΔ61-3*, *sigE68RC*, and *sigE94GC* were unable to direct transcription from the *spoIID* promoter.

Mutant forms of σ^E defective for binding to *B. subtilis* core RNA polymerase. In order to study the interaction of mutant forms of σ^E with core RNA polymerase, the glycerol gradient sedimentation procedure by Zhou et al. (34) was modified for use in *B. subtilis*. Glycerol gradient sedimentation will separate protein complexes so that a sigma factor bound to core RNA polymerase will sediment more rapidly in the gradient than a sigma factor that is not bound to core RNA polymerase. *E. coli* core RNA polymerase and molecular weight protein markers were sedimented through the glycerol gradient in order to standardize the procedure (data not shown).

Glycerol gradient sedimentation was performed with *B. subtilis* lysates expressing wild-type and mutant forms of σ^E. Most

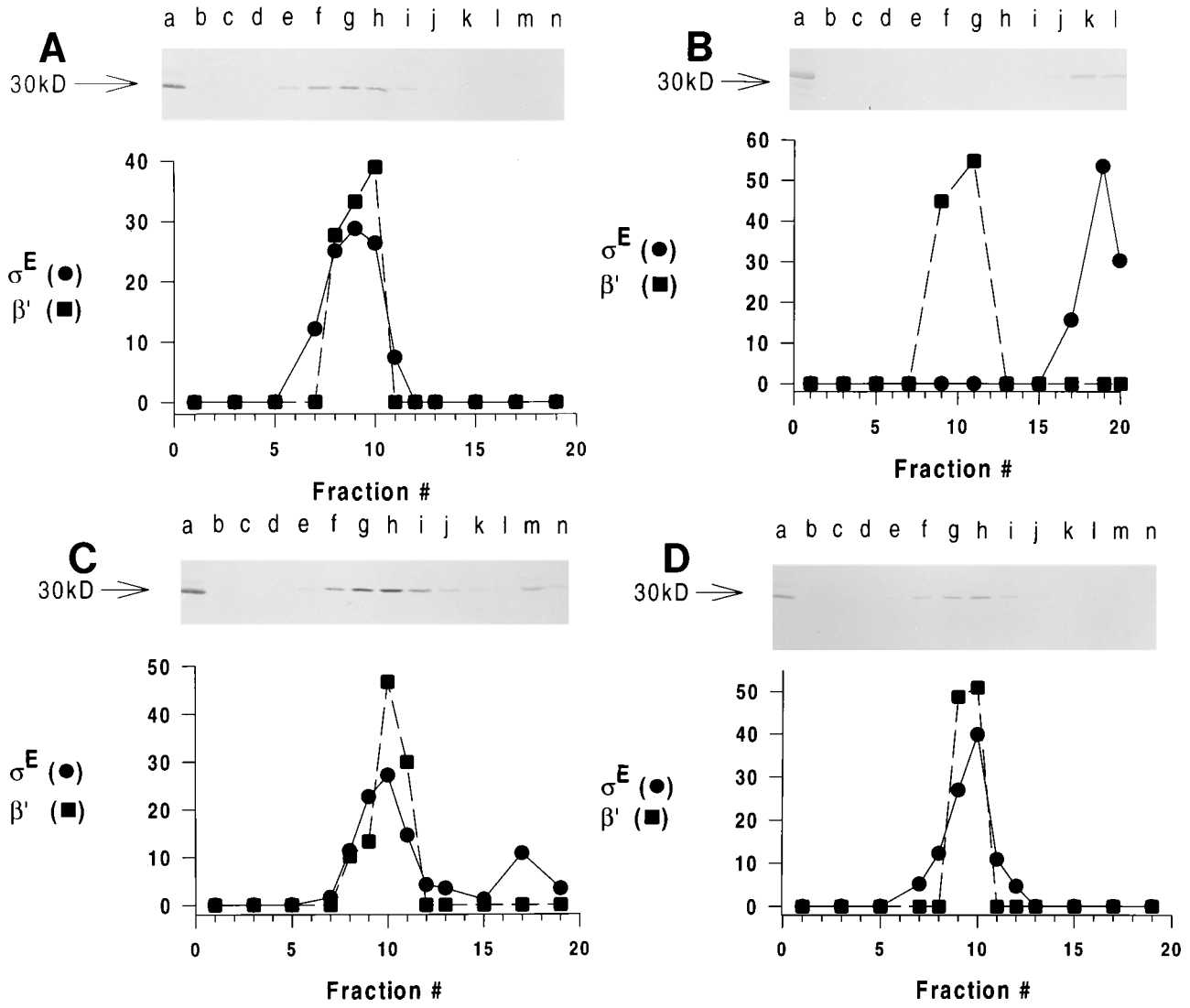

FIG. 3. Western blot analysis and graphical representations of fractions from the glycerol gradient sedimentation of *B. subtilis* lysates expressing different forms of σ^E. Control (lanes a) and gradient fraction samples (remaining lanes) were subjected to SDS-PAGE, transferred to nitrocellulose, and probed with a monoclonal antibody. Fractions are collected from the bottom of the gradient. The top panel in each part of the figure shows the blotted nitrocellulose filter probed with an anti-σ^E antibody. The bottom panel in each part of the figure shows the graphical representations of the individual lanes from the nitrocellulose shown in the top panel (●) and an identical nitrocellulose filter probed with a β′ antibody (■). The graphs show the percentage of the total immunodetected polymerase subunit in the gradient. (A) Lysate of JH642 (wild-type strain); (B) lysate of EUMS9501 ($\sigma^E\Delta 61$-3); (C) lysate of EUMS9502 (σ^E68RC); (D) lysate of EUMS9503 (σ^E99VC).

of the mutant forms of σ^E accumulated to about the same level as wild-type σ^E, except for a few (noted below) that accumulated to lower levels. Glycerol gradient sedimentation and Western blot (immunoblot) analysis of wild-type *B. subtilis* (JH642, Fig. 3A) showed that σ^E sedimented in the middle fractions of the gradient. σ^E and β′ were found in the same fractions of the gradient. This evidence supports the conclusion that wild-type σ^E was efficiently bound to core RNA polymerase. Glycerol gradient sedimentation and Western blot analysis of a *B. subtilis* strain expressing $\sigma^E\Delta 61$-3 from the pSI-1 plasmid (Fig. 3B) showed that this mutant form of σ^E sedimented at the top of the gradient. It was not found in the same fractions as β′; therefore, $\sigma^E\Delta 61$-3 was defective in binding to core RNA polymerase.

Analysis of a *B. subtilis* strain expressing the *sigE68RC* allele from the chromosome (Fig. 3C) showed that this mutant form of σ^E sedimented in the middle as well as the top of the gradient. Since it accumulated to about the same level as the wild-type σ^E, its affinity for core RNA polymerase must be less than that of wild-type σ^E. To determine whether the holoenzyme formed by the R68C-substituted σ^E was active, we tested the transcriptional activity of this holoenzyme in in vitro transcription reactions using each of the glycerol gradient fractions and a template containing the σ^E-dependent promoter *spoIID*. The holoenzyme containing the R68C-substituted σ^E, which sedimented into the middle of the gradient, was found to be as transcriptionally active as holoenzyme containing wild-type σ^E (data not shown).

Glycerol gradient sedimentation and Western blot analysis were also performed with a *B. subtilis* strain expressing the fully complementing mutation *sigE99VC* by plasmid integration into the chromosome (Fig. 3D). V99C-substituted σ^E sedimented into the middle of the gradient and was found in the same fractions as β′. This form of σ^E could fully complement for

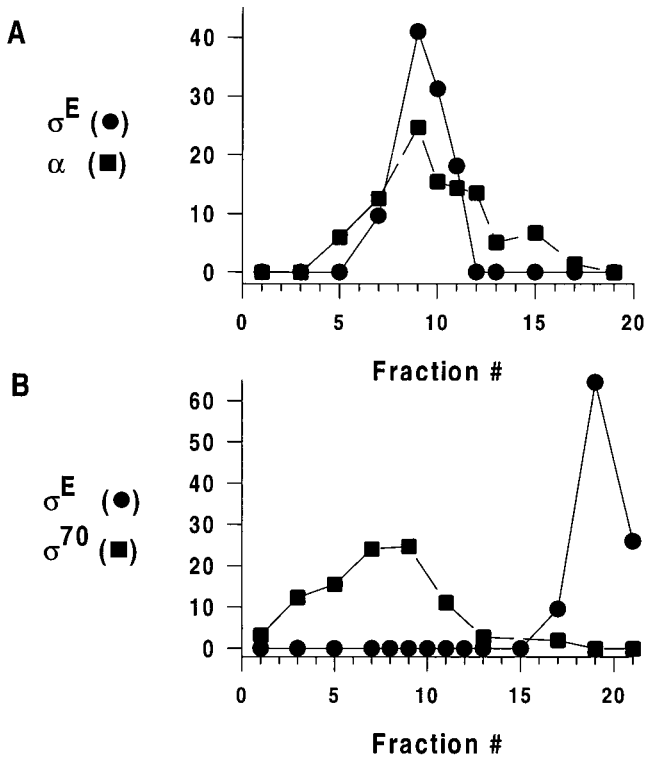

FIG. 4. Graphical representations of fractions from the glycerol gradient sedimentation of *E. coli* lysates expressing different forms of *B. subtilis* σ^E. Gradient fraction samples were subjected to SDS-PAGE, transferred to identical nitrocellulose filters, and probed with a monoclonal antibody to either σ^E from *B. subtilis*, σ^{70}, or α subunit from *E. coli*. Fractions were collected from the bottom of the gradient. The graphs show the percentage of the total immunodetected polymerase subunit in the gradient. (A) Lysate of EUMS9511 (wild-type σ^E); (B) lysate of EUMS9512 ($\sigma^E\Delta$61-3).

sporulation activity, so it was not surprising that it was efficiently bound to core RNA polymerase. A summary of the *B. subtilis* core RNA polymerase binding studies is shown in Table 4.

Western blot analysis showed that the products of three of the noncomplementing *sigE* alleles, *sigE*Δ*68-4*, *sigE94GC*, and *sigE*Δ*61-3*, did not accumulate when these alleles were located in the chromosome of an otherwise wild-type *B. subtilis* strain. A mutation in the gene for Lon protease (24) in strain RS359 or RS374 allowed accumulation of $\sigma^E\Delta$61-3 but not of $\sigma^E\Delta$68-4 or σ^E94GC (data not shown).

Mutant forms of σ^E also are defective in their ability to bind *E. coli* core RNA polymerase. Since some of the mutant alleles of σ^E failed to accumulate in *B. subtilis*, we examined their ability to accumulate in *E. coli* and bind *E. coli* core RNA polymerase. Glycerol gradient sedimentation analysis was performed on *E. coli* strains expressing wild-type and mutant forms of *B. subtilis* σ^E from the pJ8905 plasmid. Graphical representations of the gradient profiles are shown in Fig. 4. Sedimentation and immunoblot analysis of an *E. coli* lysate expressing wild-type σ^E showed that *B. subtilis* σ^E sedimented to the middle of the gradient (Fig. 4A). Nitrocellulose filters were probed with either *E. coli* α subunit or σ^{70} antibody in order to show the sedimentation of *E. coli* core RNA polymerase, since σ^{70} was expected to readily associate with the core. This analysis showed that both *E. coli* α subunit and σ^{70} were found in the same fractions as *B. subtilis* σ^E. This evidence supports the conclusion that *B. subtilis* σ^E was efficiently bound to *E. coli* core RNA polymerase.

Similar analysis of an *E. coli* lysate expressing the *sigE*Δ*61-3* mutant form revealed that $\sigma^E\Delta$61-3 was found in the top fractions of the gradient (Fig. 4B). As expected, *E. coli* σ^{70} sedimented into the middle of the gradient. Since $\sigma^E\Delta$61-3 is not found in the same fractions as *E. coli* σ^{70}, we conclude that it is not associated with *E. coli* core RNA polymerase.

σ^E94GC failed to accumulate in *B. subtilis* strains but accumulated to the same level as wild-type σ^E in *E. coli*. Glycerol gradient sedimentation and Western blot analysis of an *E. coli* strain expressing *sigE94GC* demonstrated that its product was found in the top fractions of the gradient (data not shown). Therefore, it was not associated with *E. coli* core RNA polymerase. A summary of the *E. coli* core RNA polymerase binding studies is shown in Table 4. Our results show that mutations in σ^E that affect core binding in *B. subtilis* also affect core binding in *E. coli*.

DISCUSSION

Several previous studies of mutations in σ^E (3, 9, 10, 27) (Fig. 2) and other sigma factors (6, 11, 25, 32, 33, 35) have been used to define the function of conserved regions in sigma factors. Genetic evidence showed that region 2.4 of σ^E interacts with the −10 region of its cognate promoters (3, 27). Substitution of an alanine for a methionine at position 124 in σ^E specifically suppressed the effect of an adenine-to-guanine transition at position −13 in the *spoIIID* promoter (27). Further experiments by Tatti et al. (29) have studied the effects of mutations in region 4.2 of σ^E on its ability to recognize mutant σ^E-dependent promoters. The substitution of an arginine for a glutamine at position 217 in σ^E suppressed the effect of a thymine-to-cytosine base pair change at position −31 in the *spoIIID* promoter. This result supports the model that region 4.2 of σ^E interacts with the −35 region of its cognate promoters. A study by Jones and Moran identified a mutant form of σ^E that was blocked in the transition between promoter binding and transcription initiation (9). This mutant form of σ^E was isolated as part of a mutational analysis conducted on regions 2.3 and 2.4. A mutant σ^E which had an arginine instead of a cysteine at position 117 was able to bind DNA but was unable to initiate transcription. This evidence suggested that sigma factors participate in steps of transcription initiation that occur after DNA binding, and this could be a function of region 2.3.

We used a mutational approach to study the role of the region between positions 58 and 99, which is the region in σ^E that is the most similar to other sigma factors. We expected that the most highly conserved amino acids would be necessary for function of the protein. Several of the amino acid substitutions as well as the two deletions showed effects on sigma activity. However, it was surprising that most of the amino acid substitutions in this highly conserved region of σ^E had no effect on sporulation and therefore had little or no effect on σ^E function.

Studies of σ^{70} in *E. coli* have implicated region 2.1 and an upstream region in the role of binding core RNA polymerase. Zhou et al. concluded that a mutant form of σ^{70} with a small deletion from amino acids 330 to 343 was defective in core binding since it suppressed the effect of a mutation in σ^{32} which affected its ability to bind to core RNA polymerase (34). The deletion in σ^{70} was in a region that is not conserved in other sigma factors. Lesley and Burgess generated σ^{70} peptide fragments and assessed their ability to bind to core RNA polymerase by gel filtration studies (16). A σ^{70} peptide fragment composed of amino acids 361 to 390 was sufficient to bind to

core RNA polymerase. In Fig. 2 the horizontal bar over the sigma alignment spans this peptide region. Other evidence showed that the heteromeric eukaryotic transcription factor RAP30/74 that has homology to regions 2.1 and 2.2 from bacterial sigma factors can bind *E. coli* RNA polymerase in a competitive manner with σ^{70} (18).

These studies suggested that the mutations in region 2 of σ^E affect binding of σ^E to core RNA polymerase. Our glycerol gradient binding assays demonstrated that $\sigma^E\Delta$61-3 expressed from a plasmid in both *B. subtilis* and *E. coli* was deficient in its ability to bind core RNA polymerase. In $\sigma^E\Delta$61-3 the deletion of three amino acids could have had a deleterious effect on the structure of σ^E. However, gross effects on the structure of σ^E seem unlikely since $\sigma^E\Delta$61-3 accumulated in both *E. coli* and *B. subtilis* when expressed from a plasmid. Also, in *lon*-deficient *B. subtilis* strains chromosomally expressed $\sigma^E\Delta$61-3 accumulated. In contrast, $\sigma^E\Delta$61-4 did not accumulate in *E. coli* or *lon*-deficient *B. subtilis*. Therefore, the deletion of four amino acids probably severely affected the overall structure of the protein.

Another mutant, σ^E94GC, did not accumulate in *B. subtilis* but did accumulate well in *E. coli*. Our analysis showed that σ^E94GC was deficient in its ability to bind *E. coli* core RNA polymerase. Reversion analysis on *sigE94GC* isolated true revertants as well as a *sigE94CS* revertant. Since cysteine and serine are of approximately equal sizes and charge densities, the *sigE94CS* revertant is interesting. A cysteine at this position is completely detrimental to the function of the protein, while a serine at the same position allows the protein to function weakly.

In previous studies active, processed σ^E not bound to core RNA polymerase has been found to be unstable and readily degraded (21). This may explain the difficulty that we had with accumulation of σ^E94GC and $\sigma^E\Delta$61-3 in *B. subtilis*. We believe that these two mutant forms of σ^E do not bind to or associate with the core subunits of RNA polymerase. If active σ^E not bound to the core is degraded in *B. subtilis*, then these two mutant forms of σ^E would be rapidly degraded. Perhaps since σ^E68RC binds core RNA polymerase better than σ^E94GC and $\sigma^E\Delta$61-3 do, the *sigE68RC* product is more stable in *B. subtilis*.

Our experiments showed that expression of *sigE68RC* from its normal chromosomal position in *B. subtilis* and from a plasmid in *E. coli* produced a form of σ^E with reduced affinity for core RNA polymerase. Since the R68C-substituted form of σ^E accumulated to wild-type levels, its structure is probably not grossly affected by the amino acid substitution. Furthermore, the holoenzyme that was formed by binding the R68C-substituted σ^E was active in vitro; therefore, the primary and possibly only defect of the sigma factor was its reduced affinity for core RNA polymerase. Since this amino acid substitution primarily affects the affinity of the sigma factor for core RNA polymerase and apparently does not cause large effects on sigma factor structure, this amino acid substitution identifies a position in σ^E that most likely is in close contact with a core subunit of the RNA polymerase holoenzyme.

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