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SprE Levels Are Growth Phase Regulated in a σ^S-Dependent Manner at the Level of Translation

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SprE regulates σ^S levels in response to nutrient availability by promoting ClpXP-mediated degradation. Paradoxically, we observe that SprE is similarly regulated, accumulating preferentially upon starvation. This regulation of SprE levels is σ^S dependent, altering SprE synthesis at the level of translation. Thus, we demonstrate that SprE and σ^S function within a regulatory feedback loop.

The *Escherichia coli* starvation response is largely dependent on the activity of the alternative primary sigma factor σ^S, encoded by the *rpoS* gene (5, 6, 9). Since the physiological adaptations of *E. coli* growing under starvation conditions are quite dramatic and require a major shift in gene expression (7), the commitment to initiate the starvation response is tightly regulated. Under conditions of nutrient sufficiency, σ^S is rapidly degraded by the ClpXP protease (13, 17). However, once nutrients become limiting for growth, degradation ceases and there is a dramatic increase in σ^S levels. This regulation of σ^S stability in response to nutrient availability is dependent on the two-component response regulator SprE, also termed RssB, which promotes ClpXP-mediated degradation of σ^S (10, 12). SprE specifically promotes σ^S degradation without influencing the degradation of other ClpXP substrates (18). More recently, SprE has been shown to physically bind σ^S in vitro (1), and through this interaction SprE promotes the specific degradation of σ^S by ClpXP.

What remains unclear is the molecular nature of the signal(s) that regulates SprE activity in response to nutrient availability. Based on homology with other response regulators, it is likely that SprE activity is modulated by phosphorylation at the conserved aspartic acid residue D58 within the N-terminal receiver domain of SprE. Consistent with this hypothesis, it was observed in vitro that phosphorylated SprE was more efficient at binding σ^S than unphosphorylated SprE (1). Thus far, acetyl phosphate is the only reported source of phosphate for SprE (2). The Δ(*ackA pta*) mutant, which does not synthesize acetyl phosphate, has approximately 2.5-fold higher levels of σ^S than the wild type during exponential growth (2). However, increased stabilization of σ^S in response to starvation in the Δ(*ackA pta*) mutants indicates that there is still significant regulation of SprE activity in the absence of acetyl phosphate (2).

A constitutive allele of *sprE*, *sprE19*::*cam*, which results from insertion of a Tn*cam* element 22 bp upstream of the *sprE* open reading frame, has been described (12). This constitutive allele, which alters the expression level of *sprE*, promotes degradation of σ^S irrespective of growth phase and any phosphorylation signal(s) that may regulate SprE activity. This suggested to us that there might be important growth phase regulation of *sprE* expression, which is overcome by the *sprE19*::*cam* allele. Experiments reported here directly test the hypothesis that SprE levels are responsive to the bacterial growth phase.

SprE levels are growth phase regulated in a σ^S-dependent manner. Strains used in this study are listed in Table 1. To better understand the mechanism(s) behind growth phase regulation of SprE activity, we tested whether SprE levels varied in a growth phase-dependent manner with the idea that decreased levels during stationary phase could account in part for the decreased SprE activity observed. Therefore, we assayed SprE levels throughout the growth curve by Western blot analysis (Fig. 1a). In contrast to our expectation, we observed that SprE levels were minimal during exponential growth and increased dramatically as bacteria entered into stationary phase. In fact, we were unable to reliably detect SprE during mid-exponential phase because protein levels were so low. SprE levels were approximately threefold higher in the gain-of-function *sprE19*::*cam* mutant than in the wild type during both exponential (data not shown) and stationary phases (Fig. 1b). However, SprE levels in the *sprE19*::*cam* mutant still exhibited greater than 10-fold induction under starvation conditions (data not shown), suggesting that growth phase regulation was independent of *sprE* transcription.

We thought it possible that SprE was degraded concomitantly with σ^S in vivo, thereby accounting for the growth phase expression pattern we observed. To test this, we assayed SprE levels by Western blotting in both *rpoS* and *clpXP* null backgrounds. If the decreased amount of SprE observed during exponential growth was dependent on σ^S degradation, we would expect an increased amount of SprE in the absence of σ^S or ClpXP. As observed with the wild type, however, SprE is

TABLE 1. Bacterial strains

Strain	Genotype
MC4100[a]	F⁻ *araD139* Δ(*argF-lac*)*U169 rpsL150 relA1 flb5301 ptsF25 deoC1 thiA1*
KEG423	MC4100 *sprE*::Tn*10*
KEG424	MC4100 *sprE19*::*cam*
KEG425	MC4100 *rpoS*::*kan*
KEG426	MC4100 *clpP*::*cam clpX*::*kan*
KEG428	KEG425 *clpP*::*cam clpX*::*kan*
KEG452	MC4100 λ*sprE112'-lacZ*⁺
KEG453	KEG425 λ*sprE112'-lacZ*⁺
KEG500	MC4100 pKEG4
KEG501	KEG425 pKEG4
KEG504	MC4100 λ*sprE984'-'lacZ*
KEG505	KEG425 λ*sprE984'-'lacZ*
KEG512	MC4100 λ*sprE114'-'lacZ*
KEG513	KEG425 λ*sprE114'-'lacZ*

[a] See reference 3.

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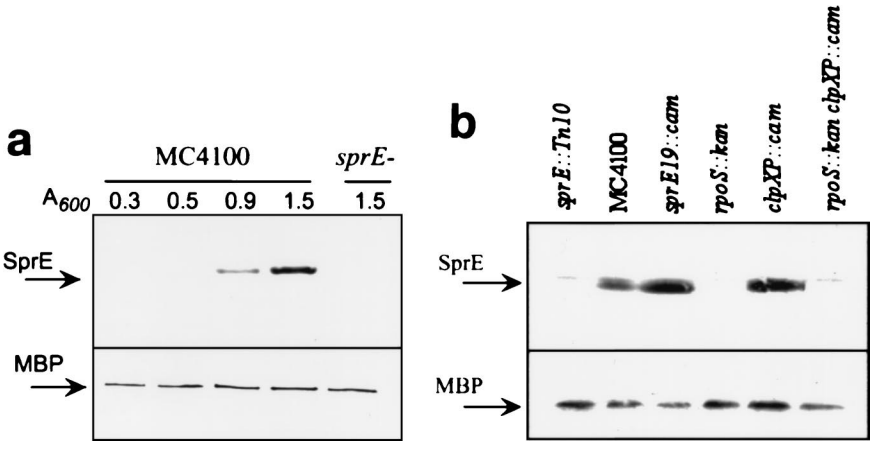

FIG. 1. Growth phase regulation of SprE as determined by Western blot analysis. Arrows, SprE and maltose-binding protein (MBP; internal loading control). Each strain was grown in LB broth (14) at 37°C with aeration, and 1-ml samples were taken at the indicated A_{600}. Cells were pelleted and resuspended in a volume (in milliliters) of loading buffer (14) equal to $A_{600}/5$. The resulting whole-cell lysate was used for sodium dodecyl sulfate–12% polyacrylamide gel electrophoresis (8), followed by Western blot analysis (16) with anti-SprE polyclonal antiserum used at a dilution of 1 μl per ml of blocking solution. Horseradish peroxidase-linked goat anti-rabbit secondary antibody (Amersham) was used at a dilution of 1 μl per 8 ml of blocking solution. The membrane was subsequently stripped and reprobed with anti-MBP polyclonal antiserum used at a dilution of 1 μl per 5 ml of blocking solution. Protein band intensities were analyzed with ImageQuant, version 5.0. (A) MC4100 (wild-type) growth curve with KEG423 (*sprE*::Tn*10*) as a negative control for SprE; (B) MC4100 and the indicated mutant derivatives grown to stationary phase at an A_{600} of ~2.0.

nearly undetectable during exponential growth in the *clpXP* mutant (data not shown), which constitutively accumulates σ^S. In addition, the *clpXP* null mutation did not significantly alter stationary-phase levels of SprE (Fig. 1b).

In contrast, we observed a significant decrease in SprE levels during stationary phase in the *rpoS* null mutant (Fig. 1b). This decreased level of SprE was equivalent to that observed during exponential growth in the wild type, conditions in which σ^S activity was diminished through rapid ClpXP-mediated degradation. Additionally, the decreased SprE observed in the *rpoS* null mutant was not reversed in an *rpoS clpXP* triple mutant, demonstrating that this *rpoS*-dependent decrease did not result from ClpXP-mediated degradation (Fig. 1b).

The above results demonstrated that SprE was growth phase regulated such that upon starvation protein levels increased dramatically. While σ^S was necessary for the observed stationary-phase accumulation of SprE, σ^S alone was insufficient to increase SprE levels during exponential growth, as revealed by the *clpXP* null mutant. This suggested that an additional factor(s), induced upon starvation, acted in concert with σ^S to mediate growth phase regulation of SprE.

The *sprE112′-lacZ*$^+$ transcription fusion is not regulated by σ^S. Since SprE levels varied throughout the growth curve in a σ^S-dependent manner, we constructed an *sprE112′-lacZ*$^+$ transcription fusion (Fig. 2) to test whether this was the result of transcriptional regulation. This seemed an unlikely mechanism, as noted above, since the constitutive *sprE19*::*cam* allele was also subject to growth phase regulation. However, we wanted to test this more directly since little was known about *sprE* transcription. The pKEG3 fusion construct was recombined with λRZ5, and the recombinant λ*sprE112′-lacZ*$^+$ phage was lysogenized into MC4100 at the *att* site.

The expression level of λ*sprE112′-lacZ*$^+$ was determined in a β-galactosidase assay during both exponential and stationary phases in *rpoS*$^+$ and *rpoS* null backgrounds. We observed no alteration in λ*sprE112′-lacZ*$^+$ expression upon introduction of the *rpoS* null allele in comparison to the wild type in either Luria-Bertani (LB) media (Table 2) or M63 minimal media with 0.2% glucose (data not shown). Importantly, *sprE* transcription did increase 25-fold upon starvation, although in a manner independent of σ^S activity. While these results demonstrated significant growth phase regulation of *sprE* transcription, they further supported the conclusion that SprE protein levels increased upon starvation by a σ^S-dependent mechanism that functions posttranscriptionally.

SprE is subject to posttranscriptional σ^S-dependent growth phase regulation. The above results indicated that SprE levels were regulated posttranscriptionally by σ^S, at the level of either translation or protein stability. To test this directly, we constructed a set of protein fusions between the open reading frame of *sprE* and that of *lacZ*. The *sprE114′-′lacZ* translation

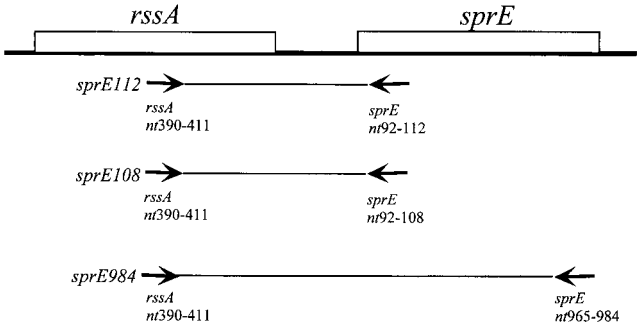

FIG. 2. Construction of *sprE* fusions. The putative regulatory sequences required for *sprE* regulation were amplified by PCR from the MC4100 chromosome, with *Taq* polymerase (United States Biochemical Corp.) and the primers *rssA390* (CTTGCTATTCGCGCATCATGC) and *sprE112* (CCACCAGTACCG TTGTCG). The resulting PCR fragment was polished with *Pfu* polymerase (Stratagene) and blunt end ligated into *Sma*I-digested pRS415 (15) with T4 ligase (New England Biolabs). The resulting plasmid, pKEG3, contained a *sprE112′-lacZ*$^+$ transcription fusion. The pKEG3 construct was recombined in vivo with λRZ5 (11), and the recombinant Lac$^+$ Amp^r phage was integrated at the *att* site in MC4100. The *sprE114* PCR fragment was amplified using primers *rssA390* and *sprE114* (TCCCCCGGGAGCCGCCAGTACCGTTGTCG), followed by *Sma*I digestion and *Pfu* polymerase polishing. The *sprE984* PCR fragment was amplified using primers *rssA390* and *sprE1014* (CGTTTGCTCATTCTGC), followed by *Acc*I digestion (New England Biolabs) and mung bean nuclease polishing (New England Biolabs). These blunt-ended PCR products were then ligated into the *Sma*I-digested pRS414 vector (15). The resulting fusions were recombined into the chromosome as described above. nt, nucleotides.

TABLE 2. β-Galactosidase activity of *sprE′-lacZ* fusions

Fusion	β-Galactosidase activity[a] of culture with indicated *rpoS* background at:			
	Exponential phase		Stationary phase	
	rpoS$^+$	*rpoS* null	*rpoS*$^+$	*rpoS* null
sprE112′-lacZ$^+$	6.5 ± 2	5.2 ± 1	164 ± 26	203 ± 16
sprE114′-′lacZ	0.5 ± 0.2	0.2 ± 0.1	16 ± 1	4.6 ± 0.6
sprE984′-′lacZ	1.2 ± 0.3	0.4 ± 0.2	23 ± 3	4.5 ± 0.3

[a] These data, in Miller units, reflect the averages ± standard deviations of four separate cultures and were independently reproduced at least three times. The cultures were grown in LB media at 37°C. Stationary-phase cultures were harvested after 24 h of growth at an A_{600} of ~2.5. Exponential-phase cultures were derived from a 1:100 subculture of the stationary-phase samples and were harvested once they reached an A_{600} of ~0.3.

fusion is analogous to the previous *sprE112′-lacZ*$^+$ transcription fusion, with an additional 2 bp of the *sprE* open reading frame to allow an in-frame fusion with *lacZ* (Fig. 2). The *sprE984′-′lacZ* translation fusion contains nearly the entire open reading frame of *sprE*, the intent being to include all potential *cis*-acting regulatory sites (Fig. 2). The resulting pKEG5 and pKEG6 fusion constructs were recombined with λRZ5 and introduced into the chromosome at the *att* site.

Expression of both translation fusions was determined by a β-galactosidase assay from cultures grown in LB media during exponential and stationary phases in an *rpoS*$^+$ and *rpoS* null background (Table 2). Stationary-phase activities of both SprE114′-′LacZ and SprE984′-′LacZ were highly dependent on σ^S, such that the wild type possessed approximately fivefold greater activity than the *rpoS* null mutant (Table 2). In contrast, there was no significant difference between the wild type and the *rpoS* null mutant during exponential growth (Table 2).

Analogous σ^S-dependent growth phase regulation of each translation fusion was observed in M63 minimal media containing 0.2% glucose (data not shown). In fact, the difference in levels of λ*sprE114′-′lacZ* and λ*sprE984′-′lacZ* expression was significant enough to distinguish the wild type, which could form single colonies on M63 minimal 0.2% lactose agar, from the *rpoS* null mutant, which was unable to grow on the same medium.

These data demonstrate that σ^S is necessary for promoting high levels of SprE114′-′LacZ and SprE984′-′LacZ (Table 2) during stationary phase. Since the λ*sprE114′-′lacZ* translation fusion, but not the λ*sprE112′-′lacZ*$^+$ transcription fusion, is sensitive to σ^S-dependent growth phase regulation, we conclude that SprE levels are posttranscriptionally regulated by σ^S. However, since the *rpoS* null mutant allows nearly 10-fold induction of SprE114′-′LacZ and SprE984′-′LacZ upon starvation, there is also a σ^S-independent means of increasing levels of SprE during stationary phase. This likely reflects regulation of *sprE* transcription, since the λ*sprE112′-lacZ*$^+$ fusion is strongly induced in a σ^S-independent manner. Interestingly, our observation that σ^S is not sufficient to induce high levels of SprE during exponential growth in the *clpXP* null mutant may reflect a requirement for increased transcription of *sprE*.

SprE translation is regulated by σ^S. Based on the Lac phenotypes described in the previous section, it was clear that at least some of the σ^S-dependent *cis*-acting sites were present in the λ*sprE984′-′lacZ* protein fusion. As discussed above, it appeared likely that σ^S-dependent regulation was mediated through effects on either posttranscriptional synthesis of SprE or SprE protein stability. We assayed SprE984′-′LacZ in LB media throughout the growth curve by Western blot analysis and obtained results analogous to those shown for native SprE (data not shown). However, SprE984′-′LacZ and native SprE, expressed from the chromosome in M63 minimal media with 0.2% glucose, were undetectable by [S^{35}]methionine incorporation and immunoprecipitation with our antibodies (data not shown). For this reason, we probed the mechanism behind σ^S-dependent regulation of SprE984′-′LacZ with strains containing the medium-copy-number plasmid pKEG4. We assayed SprE984′-′LacZ levels throughout the growth curve in *rpoS*$^+$ and *rpoS* null strains by Western blotting and found that the fusion protein expressed from pKEG4 was regulated in a σ^S-dependent manner (data not shown). Therefore, the information necessary for σ^S-dependent regulation of SprE was present in this protein fusion and functioned within the plasmid construct.

In order to distinguish between regulation of SprE at the levels of translation and protein stability, we performed a pulse-chase analysis of SprE984′-′LacZ during stationary phase (Fig. 3). SprE984′-′LacZ synthesized 3 min postchase was stable for at least 30 min in the wild-type strain. The same degree of SprE984′-′LacZ stability was observed in the *rpoS* null mutant; however, the overall amount of protein synthesized was decreased significantly (Fig. 3). SprE984′-′LacZ was also stable for up to 30 min during exponential growth in the wild type (data not shown). Thus, once SprE984′-′LacZ had been synthesized, it was quite stable regardless of growth phase or the *rpoS* allele present. This clearly demonstrates that σ^S promotes synthesis of SprE during stationary phase.

Conclusions. Our results demonstrate σ^S-dependent posttranscriptional regulation of SprE synthesis during the growth cycle. At present, the mechanism of this growth phase-dependent regulation of SprE remains unknown. However, both SprE′-′LacZ protein fusions were similarly regulated by σ^S, suggesting that all the information necessary for this growth phase regulation is present in the shorter SprE114′-′LacZ fusion. Since σ^S regulates promoter recognition and transcription initiation of core RNA polymerase, it likely alters SprE translation indirectly through the regulated expression of a small regulatory RNA or an RNA-binding protein. While σ^S is required to regulate SprE translation, it is not sufficient when overexpressed during exponential growth, so another factor,

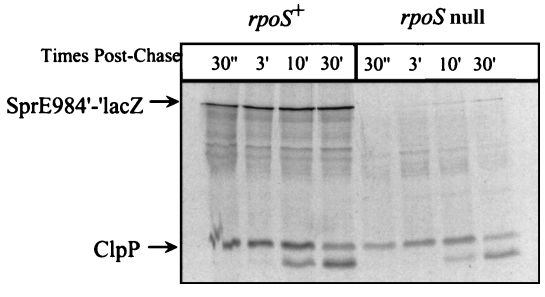

FIG. 3. Growth phase regulation of SprE984′-′LacZ synthesis. Stationary-phase synthesis and stability of SprE984′-′LacZ were determined by pulse-chase analysis (16) performed on stationary-phase (A_{600} = 1.5) cultures of KEG500 (MC4100 pKEG4) and KEG501 (KEG500 *rpoS*::*kan*). The strains were grown in M63 minimal media containing 0.2% glucose plus 25 μg of ampicillin/liter at 37°C with aeration. Three milliliters of cell culture was labeled with 100 μCi of [^{35}S]methionine/ml for 2 min, followed by addition of 3 ml of minimal medium containing 0.2% glucose plus 0.8% cold methionine. One-milliliter samples were taken at the indicated times postchase, and total protein was precipitated with trichloroacetic acid. The labeled whole-cell lysate was added to 1 ml of immunoprecipitation buffer containing 4 μl of LacZ antiserum and 1 μl of ClpP antiserum. The immunoprecipitated proteins were pelleted and resuspended in 40 μl of protein sample buffer. Subsequently, the sample was boiled, and 12 μl was analyzed by sodium dodecyl sulfate–12% polyacrylamide gel electrophoresis and autoradiography (4).

whose concentration also responds to nutrient availability, must be involved. As noted above, we suspect that this unknown factor acts at the level of transcription. The precise nature of the starvation signal that promotes SprE translation is also unclear. We observe translational regulation of SprE in both rich LB broth and M63 minimal medium with glucose, but whether it occurs under other growth conditions remains to be determined.

Interestingly, SprE levels are quite low during logarithmic growth; however, they are clearly sufficient to promote ClpXP-mediated σ^S degradation. In contrast, SprE accumulates during stationary phase but is not competent to promote σ^S degradation. Thus, the regulation of SprE levels is secondary to the growth phase regulation of SprE activity with regard to ClpXP-mediated degradation of σ^S. However, our observation that a mechanism for increasing the intracellular pool of SprE under starvation conditions exists suggests that this accumulation could be important for a rapid transition from stationary phase to exponential growth once nutrients become available by providing a large pool of SprE receptive to activating signals. This regulation could also provide a feedback mechanism for reducing σ^S levels during exponential growth after transient induction by stresses such as heat or osmotic shock, which are known to lead to elevated levels of σ^S (5).

Alternatively, SprE could function to regulate the stability of additional target proteins in a growth phase-dependent manner. In this way, the absolute amount of SprE could play a regulatory role through differential affinity for the various target proteins. The maintenance of an appropriate amount of SprE appears to be quite important for *E. coli*, since overexpression of this protein results in dramatic growth defects and loss of viability (our unpublished observation). This decreased viability is not relieved by loss of σ^S, consistent with the idea that there might be other regulatory targets of SprE with important physiological roles during growth.

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