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Stationary phase induction of RpoS in enteric bacteria

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Stationary Phase Induction of RpoS in Enteric Bacteria

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Abstract: Stationary Phase Induction of RpoS in Enteric Bacteria

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In enteric bacteria, stress adaptation is mediated by the RpoS protein, one of several sigma-factors that in association with RNA polymerase, collectively allow a tailored transcriptional response to environmental cues. Stress stimuli including low temperature, osmotic shock, and starvation all result in a substantial increase in RpoS abundance. Perhaps the most pronounced affect is observed during growth to stationary phase (SP) in rich medium. The mechanism of regulation depends on the specific signal, but may occur at the level of transcription, translation, protein activity or targeted proteolysis. In both *Escherichia coli* and *Salmonella enterica* cultured in rich undefined medium, the RpoS protein is barely detectable during exponential growth and increases >30-fold as cells enter SP. Under these conditions, SP induction depends on transcriptional and translational control with proteolysis affecting basal levels but not regulation per se. The transiently expressed Fis protein, whose abundance inversely correlates to that of RpoS, binds just upstream of the primary *rpoS* promoter and represses transcription nearly 10-fold specifically during exponential growth. SP induction at the translational level relies on a novel form of genetic control dependent on the 24 nucleotides preceding the *rpoS* initiation codon (ribosome-binding sequence, RBS). The RNA secondary structure of the *rpoS* RBS is necessary and sufficient for a nearly 10-fold translational increase during SP. Control at this level is not a result of differential transcript stability, nor does it involve the known *rpoS* regulators ppGpp, DksA, HU, Hfq or the small regulatory RNAs, DsrA and RprA. The environmental stimuli that trigger RBS-mediated SP induction of *rpoS* translation also remain unknown, but similar to transcriptional control, regulation is only seen in rich undefined media. Collectively, transcriptional repression by Fis and RBS-mediated

induction at the translational level account for approximately 95% of the overall SP induction of RpoS.

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It's strange how life guides you to different places, for instance, six years ago I would have guessed that I would currently be in a medical residency program. Instead, I am finishing up my foundation as a scientific investigator. I do not question my current position because it led me to the great classic geneticist Tom Elliott, figuratively, among the last of a dying breed. By always "telling me what I needed to hear" (often repeatedly) he has provided me with the understanding and confidence to succeed in research. Additionally, I appreciate the evening conversations with Nyles Charon and Dave Yelton and the technical support of Adam Goodwill.

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Literature Review

σ-factors and Directed Transcription

The ability to sense unfavorable growth environments and coordinate appropriate gene expression allows the survival of bacteria in nature. External stimuli adjust the abundance of global regulators that participate in signaling networks culminating in specific or general stress adaptation. Primary levels of regulatory input include transcription, translation, protein activity and protein degradation.

Transcriptional control is particularly important because the signal is amplified, each RNA molecule can generate thousands of protein molecules. In bacteria, RNA polymerase core enzyme (RNAP) catalyzes the synthesis of RNA using DNA as the reaction template. However, RNAP by itself is without direction and initiates transcription at aberrant sites including nicked or "open" regions of DNA (23). In *E. coli* and *S. enterica* one of seven additional factors, termed sigma (σ)-factors, bind RNAP (the complex is termed RNAP holoenzyme) and confer promoter recognition of specific groups of genes (termed regulons; 116). These σ-factors are listed in Table 1 along with their abundance during exponential growth, their RNAP dissociation constants, and the general function of the genes they regulate. Of particular importance are the two σ-factors that direct most gene expression under different growth conditions, RpoD and RpoS. RpoD is the most abundant σ-factor in dividing cells and directs RNAP to promoters of genes necessary for optimal growth. During this time, RpoS is barely detectable, but protein abundance dramatically increases in response to unfavorable growth conditions (i.e. starvation,

low pH, high osmolarity; reviewed in ref. 61). Through transcriptional control of a large regulon, RpoS orchestrates the general stress response in which there is a reversible transition from exponential growth to a non-dividing stress-resistant state called stationary phase (SP).

Table 1. The sigma factors of *E. coli*

σ-factor	**K_d(nM)** (RNAP-σ)	**Intracellular Concentration** (molecules/cell)	**Genes Activated**
σ^{70} (RpoD)	0.26	700	growth related/housekeeping
σ^{54} (RpoN)	1.55	110	nitrogen utilization
σ^{38} (RpoS)	4.26	<1	general stress
σ^{32} (RpoH)	1.24	<10	heat shock response
σ^{28} (RpoF)	0.74	370	flagellar synthesis; chemotaxis
σ^{24} (RpoE)	2.43	<10	extracytoplasmic/heat shock
σ^{FecI}	1.73	<1	ferric citrate transport

The sigma (σ)-factors of *E. coli* are listed with their RNAP dissociation constants (K_d) determined by core enzyme binding in mixed reconstitution experiments (90). The intracellular concentration of each factor during exponential growth is also presented along with the general function of each regulon (69, 70, 98, 99).

Regulation of σ-directed transcription has two primary components. First, σ-factors compete for a limited amount of RNAP, a process biased by their relative abundance and individual binding affinities for RNAP (Table 1; reviewed in ref. 124). Second, σ-factors must display promoter specificity; an enigmatic process that requires sequence elements at targeted promoters and often, additional transcriptional factors and physiological indicators (83, 99). Directed transcription by RpoS and RpoD is discussed below.

The competition model of σ-selectivity

The competition model for RNAP states that σ-factors compete for a limited amount of core RNAP and this in turn, contributes to targeted gene expression (42). During SP, RpoS-dependent expression of numerous genes occurs despite a 16-fold affinity deficit for RNAP compared to that of the housekeeping factor, RpoD (98). Experiments employing *lacZ* fusions to promoters expressed during SP determined that a mild overproduction of RpoD during SP eliminates RpoS-dependent gene expression; conversely, overproduction of RpoS reduces RpoD-dependent gene expression (42). These results correlate with RpoS abundance during different stages of growth; RpoS is low during expression of most RpoD-dependent promoters (exponential growth) and high during SP (70). Simplistically, the sizeable SP induction of RpoS abundance overcomes its affinity deficit for RNAP, resulting in RpoS-dependent gene expression (42).

Promoter Elements and σ-Selectivity

After a σ-factor successfully binds to core RNAP, it then confers promoter selectivity, a process, mainly defined by promoter specific elements. This is explicit in the conserved sequences of promoters individually recognized by the alternative σ-factors RpoN, RpoH, RpoF, RpoE or FecI (99). For example, RpoF-RNAP only transcribes genes involved in chemotaxis and the synthesis of flagella with promoter recognition defined by specific nucleotides (nt) of the -10 and -35 hexamers exhibited by RpoF-dependent promoters (83).

The recognition of specific promoters by RpoD and RpoS, however, is complicated by very similar consensus sequences and in general, is not completely understood (48). In search of a RpoS consensus sequence, regions preceding a core set of 140 RpoS-dependent genes induced regardless of the stress challenge, were analyzed *in silico* (178). The results identified conservation of an 11 nt sequence (5'-TCTATACTTAA-3') that strongly resembles an extended -10 promoter hexamer (61, 178). Additional RpoS-dependent promoters of genes expressed in response to a specific challenge exhibited no sequence similarities (178).

Tanaka et al. investigated RpoS and RpoD transcription specificity *in vitro* and defined three classes of promoters: (i) promoters recognized by RpoD or RpoS at equal efficiencies (e.g. *lacUV5* and *trp*), (ii) RpoD-dependent promoters (e.g. ribosomal and tRNA genes) and (iii) a promoter that prefers RpoS-RNAP holoenzyme (*fic*) (165). Alignment of promoters from each class did not identify any differences between class I and II promoters, while class III promoters generally lacked a typical -35 sequence (165). These results are consistent with the notion that promoter selectivity by RpoS may be achieved by degenerate RpoD promoter elements in conjunction with additional regulatory factors (48).

Intracellular ionic conditions and other physiological indicators also affect RpoD and RpoS promoter recognition (38, 76, 85). RpoS-dependent promoters expressed during SP were transcribed *in vitro* by both RpoS and RpoD (76). RpoD promiscuity was eliminated by the addition of high concentrations of glutamate salts, a condition reported to mimic intracellular ionic conditions under hyperosmotic stress (38, 76). Physiological indicators of SP physiology,

including trehalose, guanosine tetraphosphate (ppGpp), or a decrease in DNA superhelical density, also favor RpoS over RpoD in promoter selection antagonism (29, 71, 85, 86).

Global transcription factors that influence σ-selectivity at target promoters include the catabolite repressor protein (CRP), the integration host factor (IHF) and the leucine response protein (Lrp; 32). For example, both RpoD and RpoS initiate transcription at the *osmY* promoter (P_{osmY}) *in vitro,* but addition of the *in vivo* regulators CRP, IHF, and Lrp disproportionately repressed RpoD-directed transcription (32). Collectively, promoter recognition by RpoD and RpoS is influenced by many factors including promoter sequence elements, ionic conditions, trehalose concentrations, DNA topology, ppGpp, and protein regulators.

RpoS

Background

During the 1980's, the *rpoS* gene was identified by several labs for roles in sensitivity to near UV radiation (*nur*), regulation of hydroperoxidase HPII (*katF*), control of acid phosphatase activity (*appR*), and carbon starvation (*csi-2*; 61). Similar phenotypes of the mutants as well as genetic mapping confirmed that *nur*, *appR*, *katF*, *csi-2* were in fact different names for the same gene (90, 167). Mulvey and Loewen reported the sequence of this gene, originally as *katF* (for its role in catalase HPII synthesis), revealing an 1086 base pair (bp) open reading frame that encodes a 362 amino acid protein (112). The amino acid sequence demonstrates strong similarity to the housekeeping σ-factor RpoD, including the region required for binding RNAP.

Therefore, in 1991 Lange and Hengge-Aronis renamed it RpoS, after the RpoD-related protein family and its involvement in stress adaptation (90). Two years later, Tanaka et al. confirmed that RpoS is indeed a σ-factor for RNAP (165). In that work, RpoS –RNAP holoenzyme transcribed several RpoD-dependent promoters and one of them (*fic*) was favored by RpoS, suggesting altered promoter recognition specificity (165).

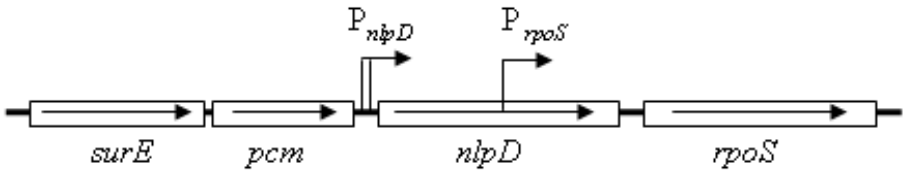

Figure 1. Genetic organization of *rpoS*

The *rpoS* region of the *E. coli* chromosome (minute 61.8 to 61.7) is depicted with straight arrows indicating transcriptional polarity. Promoters contributing to *rpoS* expression are shown with bent arrows (87, 126, 164).

The *rpoS* gene is highly conserved among the γ-branch of proteobacteria (Table 2) and its genetic organization is depicted in Figure 1. *rpoS* is located downstream of genes whose products are also involved in aspects of stress resistance: SurE (a novel phosphatase), Pcm (L-isoaspartyl methyltransferase), and NlpD (a lipoprotein with suggested hydrolytic functions in cell wall maintenance; 47, 65, 88, 94). Transcriptional induction of both *surE* and *pcm* occurs in response to stress in a RpoS-independent manner (95).

Table 2. Conservation of *rpoS*.

Bacteria	Nucleotide Conservation[a]
S. typhimurium	100%
S. typhi	99%
E. coli MG1655	92%
E. coli 0157:H7	92%
Shigella flexneri	92%
Enterobacter cloacae	89%
Kluyvera cryocrescens	84%
Serratia entomophila	83%
Erwinia carotovora	83%
Yersinia pestis	80%

[a] The percent nucleotide conservation of the *rpoS* coding sequence, relative to *S. enterica* Typhimurium, is shown.

RpoS function and regulon

RpoS has been deemed the "master regulator of the general stress response" due to its transcriptional control of over 10% of the *E. coli* genome in response to different types of stress (61, 67, 178). The gene products of the RpoS regulon have diverse functions under the general heading of stress survival (61, 178). Of these, transcriptional profiling has identified a core set of 140 genes activated in response to acid shock, osmotic shock, or growth to SP (178). In addition, hundreds of other RpoS-dependent genes are specifically expressed in response to a particular type of stress, allowing a tailored response (178). Stress stimuli that influence RpoS abundance, along with their reported levels of regulatory input, are listed in Table 3.

Table 3. Stimuli that increase RpoS protein abundance.

Stimulus	Level of Regulation	Reference
Slow growth rate	Transcription	(61)
	Translation	(34)
Growth to SP	Transcription	(88)
	Translation	(88)
Low temperature	Translation	(158)
High osmolarity	Translation	(111)
	Protein Stability	(111)
Carbon starvation	Protein Stability	(88, 130)
Low pH	Translation	(61)
	Protein Stability	(12)
Heat Shock	Protein Stability	(110)

Bacteria containing a mutation in *rpoS* exhibit increased sensitivity to a variety of stress conditions, most of which are encountered during mammalian infection (61). These challenges include single nutrient deprivation, outright starvation, oxidative stress, high osmolarity, acid stress, and DNA damage - all of which normally stimulate RpoS induction conferring resistance (41, 61, 106). The characteristic changes in morphology during stress, cells become smaller and spherical, are abolished in the *rpoS* mutant (89). In addition, mutants do not accumulate glycogen, the storage form of glucose, or the disaccharide trehalose, which normally accumulates during SP and plays an undefined role in stress survival (62, 86, 104).

RpoS and Salmonella Infection models

RpoS plays an essential role in virulence. Perhaps this is most explicit in a mouse model wherein infection by *Salmonella enterica* serovar Typhimurium results in a typhoid-like disease

that causes death within days (41). In this case, mice infected by *rpoS* mutants survived (41). This dramatic result is due to a combination of decreased colonization of the gut-associated lymphoid tissue and decreased expression of the RpoS-dependent virulence plasmid, *spv* (30, 41, 118, 180). In humans, *rpoS* mutants *of S. enterica* serovar Typhi do not cause disease. In fact, the live oral typhoid vaccine Ty21a is attenuated for virulence due primarily to a null mutation in *rpoS* (137).

In contrast, RpoS appears dispensable for *Salmonella* infection of either macrophages or epithelial cell cultures (118, 180). Infection of these cells by *S. enterica* Typhimurium causes a five to 10-fold increase in RpoS expression and RpoS-dependent reporter activity, including genes involved in virulence (31). However, *rpoS* mutants show wild type levels of attachment, invasion, and survival in the phagosomal compartment during infection of macrophages and intestinal epithelial cells (118, 180). Further investigations performed in mice demonstrate that RpoS is necessary for bacterial adherence of the Peyer's patches (118). These results demonstrate that current *in vitro* infection models do not reflect the importance of RpoS for pathogenesis *in vivo*.

Transcriptional regulation of *rpoS*

Analysis of *rpoS* transcription in *E. coli* by primer extension, and in *S. enterica* serovar Dublin by both primer extension and Northern blot, established that *rpoS* is transcribed from two distinct promoter regions (87, 126, 164). Two closely-spaced and relatively weak promoters

(collectively termed P_{nlpD}; Fig. 1) generate a bicistronic *nlpD-rpoS* message, while the major promoter (P_{rpoS}) is located approximately in the center of the *nlpD* coding sequence and generates a monocistronic *rpoS* transcript with a 5' untranslated leader region of 565 nt (87, 126). *E. coli* and *S. enterica* share identical -35 and -10 hexamers at P_{rpoS} with a 17 bp spacer, and initiate transcription at the same nt (87, 126).

Regulation of transcription from P_{rpoS} is a complex and poorly characterized phenomenon that is dependent upon the growth medium [i.e. Luria-Bertani (LB) vs. minimal medium]. When *E. coli* grows to SP in LB, *rpoS* transcriptional fusions demonstrate a five to10-fold increase in activity (88, 90). In contrast, no SP induction of transcription occurs when cells grow in minimal medium containing glucose despite a dramatic increase in RpoS abundance (187). The global regulators that influence *rpoS* transcription, CRP, ppGpp, and inorganic polyphosphate (poly-P) are discussed in the following sections. However, other conditions may also influence *rpoS* transcription, such as weak acids or homoserine lactone, although reports are often conflicting (61, 64, 154).

CRP-cAMP and *rpoS* transcription

Catabolite repression is a well established mechanism in most bacteria and some lower eukaryotes to allow the preferential use of the most energetically favorable carbon source (35, 80, 115). In *E. coli* and *S. enterica*, membrane-associated adenylate cyclase (encoded by the *cya* gene) catalyzes the reaction: ATP $\rightarrow$ cyclic AMP (cAMP) in response to low cellular

concentrations of catabolites (reviewed in ref. 115). Increased levels of cAMP result in binding to and activation of CRP, a homodimer that interacts with DNA near target promoters. CRP dually functions as a transcriptional activator and repressor, ensuring the timely expression of a large regulon (>100 genes) which includes other global regulators such as the factor for inversion stimulation, Fis (53).

Surrounding P_{rpoS} are two predicted CRP-cAMP binding sites (the consensus half-site is $TGTGAN_3$); one of these is centered at a classical upstream (with respect to the transcriptional start site) activation position, while the downstream location of the other site suggests a role in repression (24). *E. coli* strains harboring mutations in either *cya* or *crp* demonstrate a modest increase in *rpoS* transcription during exponential phase (88, 90). The exogenous addition of cAMP to the *cya* mutant culture restored P_{rpoS} activity to wild type levels (88). In addition, a *crr*-encoded EIIA(Glc) mutant, which lacks the soluble part of the phosphtransferase solute uptake system that activates adenylate cyclase, demonstrates elevated *rpoS* transcription (169). Collectively, these reports suggest CRP-cAMP mediated repression of P_{rpoS} during exponential phase. Additional unpublished results of Hengge-Aronis suggest that CRP-cAMP also activates P_{rpoS} expression during the transition to SP, possibly through binding to the downstream site (61).

ppGpp and *rpoS* transcription

Amino acid limitation or, more specifically, the failure to aminoacylate tRNAs results in pleiotropic physiological alterations that efficiently adjust gene expression to accommodate survival in the limited nutrient state. This cascade of events is generally termed the stringent response and is mediated by the alarmone ppGpp (115). As nutrients are depleted from the growth medium, increased levels of ppGpp fine-tune cellular physiology at the transcriptional level via interactions with RNAP holoenzyme (115). Sensibly, ribosomal RNA operons are among the genes inhibited by ppGpp, while genes necessary for amino acid biosynthesis and uptake are examples of those activated during the stringent response (115, 127).

ppGpp is synthesized by the ribosome-associated RelA protein, and to a lesser extent, by the cytosolic SpoT protein which also degrades ppGpp (27, 49). The RelA protein senses amino acid limitation by evaluating the ratio of charged / uncharged tRNA molecules at the acceptor position of the ribosome (139). If a threshold of uncharged tRNA molecules is achieved, RelA converts GTP + ATP (through the unstable intermediate pppGpp) or GDP into ppGpp (115). Consequently, the concentration of ppGpp indicates the cell's nutritional status and its basal levels vary inversely with growth rate (115).

A crystal structure of the RNAP-ppGpp complex shows that binding occurs near the catalytic center of RNAP (5). This interaction is thought to block incoming nucleoside triphosphates and thereby decrease the half-life of open transcriptional complexes at DNA promoters (9, 55). Regulation at this level is especially effective at promoters that exhibit short-lived open complexes including those of ribosomal RNA operons (9). Stabilization of the

ppGpp-RNAP interaction depends on DksA, a DNA-binding protein also implicated in post-transcriptional regulation of RpoS synthesis (22, 128).

ppGpp accumulation is often, if not always, accompanied by RpoS induction (22, 50, 87). Western blot experiments and both *rpoS* transcriptional and translational fusions demonstrate a dramatic reduction of RpoS expression in ppGpp deficient strains during both exponential growth and SP (50, 87). Consistently, artificial induction of ppGpp during exponential phase increased RpoS abundance approximately 50-fold (22). However, due to the pleiotropic nature of altered ppGpp levels, it is possible that its control of RpoS expression is indirect, and mediated via causal effects such as aberrant ribosomal RNA levels, decreased growth rate, or altered levels of inorganic polyphosphate (poly-P; 152).

Inorganic Phosphate and *rpoS* transcription

E. coli contain three types of inorganic phosphates, Pi, PPi and poly-P, the concentrations of which independently vary in accordance with cellular physiology (reviewed in ref. 115). During exponential growth their relative concentrations follow the scheme Pi >> PPi > poly-P (115). However during times of stress, polyphosphate kinase (encoded by *ppk*) utilizes the terminal phosphate of ATP to synthesize a long polymer of orthophosphate residues linked by high energy phosphoanhydride bonds (25). The importance of poly-P is emphasized by its ubiquitous nature in all forms of life examined (reviewed in ref. 182) and implicated functions

include an ATP substitute for adenylate kinases (129), a phosphate reservoir (25), and a role in the acquisition of competence (28).

E. coli and *S. typhimurium* mutants devoid of Ppk function are more susceptible to a variety of stresses and do not survive long periods of SP (77, 133). This is due, in part, to the influence of poly-P on *rpoS* transcription. *E. coli* cells lacking poly-P, due to gratuitous over expression of a yeast exophosphatase, are deficient in SP accumulation of RpoS (152). This result was primarily attributed to decreased *rpoS* transcription, although the mechanism remains largely unresolved (152). To complicate matters, over expression of poly-P does not affect ppGpp levels, although increased concentration of the latter results in a massive accumulation of poly-P (up to 1000-fold; 84). It is therefore possible that the influence of ppGpp on *rpoS* expression is mediated via elevated levels of poly-P.

Translational regulation of RpoS

Transcripts originating from P_{rpoS} carry a 565 nt 5' untranslated region (leader region) preceding the *rpoS* initiation codon whose sequence is conserved among enteric bacteria. In particular, 110 nt preceding the *rpoS* initiation codon, which includes a *cis*-acting antisense element that sequesters the *rpoS* ribosome-binding sequence (RBS), transiently act to decrease translation (61). The timely disruption of the inhibitory structure in response to particular stress stimuli induces *rpoS* synthesis (61). Genetic evidence and *in silico* structure predictions agree on a secondary structure for this region of the *rpoS* leader (Fig. 2, nt 454-565; 33, 101, 193).

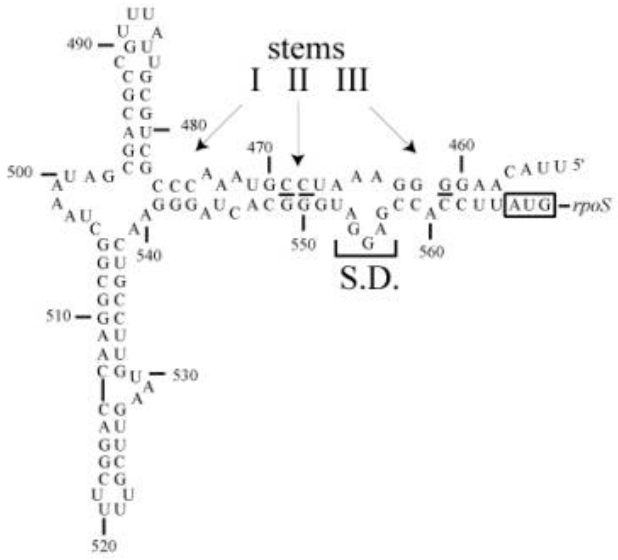

Figure 2. RNA Secondary Structure Prediction of the *rpoS* leader region.

A secondary structure prediction of 112 nucleotides of the *rpoS* leader region (21, 101). The numbering refers the nucleotide position of the *rpoS* transcript originating from P_{rpoS}. The *rpoS* initiation codon is outlined by a black box and the Shine-Dalgarno sequence is labeled S.D.. Three stems of the antisense element are designated I-III.

The physical structure(s) of the *rpoS* leader is not established conclusively, although a deletion analysis and site-directed mutations employing *rpoS-lacZ* translational fusions are consistent with an inhibitory role of the leading prediction (Fig. 2; 33, 101). A *rpoS* translational fusion containing both stems II and III (Fig. 2) demonstrated low basal activity (33). Elimination of these putative stems by a modest 5' deletion of only 23 nt significantly increased fusion activity (33). A more precise genetic approach showed that a mutation in either the top or bottom strand of stem II resulted in a similar five-fold increase in translational activity (Fig. 2, G469C or C549G; 21). Compensatory mutations at these positions, predicted to maintain stem II

pairing (Fig. 2, G469C and C549G), restored fusion activity to wild type levels (21). Mutations in either the top or bottom strands of stem III (Fig.2, G461C and C561G) increased *rpoS* translation although the compensatory mutations remained elevated in fusion activity compared to the wild type construct (21). The elevation in *rpoS-lac* activity in the presence of the individual stem mutations was comparable throughout the growth curve suggesting a similar role for the antisense element during exponential growth and SP.

Regulatory RNAs and RpoS synthesis

Genetic control mediated via small untranslated RNAs (sRNAs) is a rapidly expanding research area initially sparked by the anomaly of *rpoS* translation (54). This process is unique in that over expression of eight sRNAs are reported to influence RpoS translation, while no more than two sRNAs are reported to affect any other bacterial gene (54, 176). Two of the characterized sRNAs that affect RpoS translation, the thermoregulator DsrA and the membrane stress-induced RprA, are discussed in detail below. A list of the known sRNAs that influence RpoS translation are listed in Table 4 along with their inducing stimuli and affects on *rpoS* translation.

Table 4. Regulatory RNA that effect *rpoS* translation in *E. coli*

sRNA[a]	size (nucleotides)	Effect on *rpoS*	Stimuli	Reference
DsrA	85	+	low temperature	(158)
RprA	105	+	membrane stress	(100)
OxyS	109	-	hydrogen peroxide	(3)
RhyB	90	-	iron limitation	(105)
RhyA	45	+	?	(176)
RyhB	90	+	?	(176)
RydB	60	-	?	(176)
RyeE	86	-	?	(176)

[a] The regulatory RNAs (sRNAs) that influence *rpoS* translation are listed, along with their size and effect on *rpoS* translation. Stimuli that increase abundance of these sRNAs are also shown.

Additional evidence supporting the suggested structure of the *rpoS* leader region (Fig. 2) is the elegantly described interaction of the *rpoS* antisense element and non-translated regulatory RNA, DsrA (54, 91, 101). DsrA is an 85 nt sRNA predicted to form a structure with three stem-loops (157). Interestingly, two of the stem-loops activate different targets including *rpoS* translation and transcription of the capsular polysaccharide synthesis regulator, RcsA (157, 158). The DsrA molecule contains 21 nt of complementarity to the *rpoS* leader, including bases in the top strand of stems II and III (Fig. 2), and has been designated a thermoregulator of *rpoS* translation (134, 158). DsrA transcription is de-repressed at low temperatures (≤30° C) and the transcript is also stabilized six-fold (134). DsrA regulates *rpoS* by intermolecular RNA interactions with the antisense element of the leader region, a process mediated by the RNA-binding protein Hfq (92, 101). At low temperatures, *rpoS* translation increases in a DsrA-dependent manner while at higher temperatures a mutation in *dsrA* does not affect translational

activity (158). The DsrA-*rpoS* interaction is thought to alter the secondary structure of the *rpoS* antisense element in a manner that promotes ribosome access and subsequent translation (101, 158, 159).

Remarkably, another sRNA activates *rpoS* translation by targeting the same region of the *rpoS* leader as DsrA, stems II and III (Fig. 2; 100, 102). RprA is a 105 nt structured RNA which exhibits non-contiguous sequence complementarity to the antisense element of the *rpoS* transcript. RprA was identified in *E. coli* as a multi-copy suppressor of decreased *rpoS-lacZ* activity in a *dsrA* mutant background (100). In a wild type background, ectopic over expression of RprA increased *rpoS-lacZ* [pr] activity about six-fold (100).

Transcription of *rprA* is controlled by the phosphorelay system that regulates capsular polysaccharide synthesis genes, RcsB / RcsC, in which RcsC is a transmembrane sensor and RcsB is the response regulator (102). A mutation in *rcsB* eliminates the basal transcription of *rprA* while a mutation in *rcsC* increases expression 10-fold (102). This relatively large increase in RprA was not enough to increase the activity of a *rpoS* translational fusion (102). However, a 50-fold increase in *rprA* transcription, caused by a constituitive *rcsC* allele (*rcsC* 137), increased *rpoS* translation and RpoS abundance (102). A physiologically relevant role for activation of *rpoS* translation by RprA is awaiting description.

In addition to DsrA and RprA, at least six other sRNAs reportedly influence *rpoS* translation (3, 176). OxyS is a 109 nt sRNA that activates and represses translation of numerous proteins, including RpoS (3). OxyS expression is stimulated by hydrogen peroxide although the

mechanism by which it represses *rpoS* translation is unknown (188). A genome wide search for additional regulatory RNAs in *E. coli* suggested two candidates that increased *rpoS-lac* translational activity, *ryhA* and *rybB,* when expressed from a multi-copy plasmid (176). Particular foldings of these RNA molecules show complementarity to *rpoS* stems II and III (Fig. 2). Three modest repressors of *rpoS* translation were also suggested by this search *rydB*, *ryeE* and the iron responsive *ryhB* (105, 176). All of these predicted sRNAs (except for *rydB* which was not tested) bind Hfq and require further investigations to confirm physiologically relevant roles in the regulation of RpoS synthesis (176).

Hfq and RpoS synthesis

Hfq (also referred to as HF-1) is a 11.2 kDa protein and a pleiotropic regulator of diverse cellular functions. Hfq was first identified as a subunit of the RNA phage Qβ replicase (46) and subsequently found to associate with 30S ribosomal subunits (39). Over the past 25 years, the known roles of Hfq have expanded to include activities as a RNA chaperone (108), a modulator of RNA sensitivity to cellular ribonucleases (45, 168, 171), a stimulator of poly(A) tail elongation (58), and a post-transcriptional regulator of several RNAs including the *rpoS* transcript (20).

Hfq functions as a homo-hexameric ring (collectively termed Hfq) homologous to eukaryotic Sm proteins which are involved in pre-mRNA splicing and RNA degradation complexes (109, 143, 146, 190). Hfq has at least two RNA binding domains that prefer AU rich

sequences, positioned close to structured regions (19, 171, 190). It is thought that Hfq facilitates intermolecular RNA interactions through simultaneous binding of multiple transcripts (19).

Hfq mutants display a four to seven-fold reduction in RpoS synthesis during both exponential growth and SP, while SP induction of RpoS is not significantly altered (20). Co-immunoprecipitation studies using anti-Hfq recovered *rpoS* RNA from cell lysates and Hfq binds the *rpoS* leader region *in vitro* (93, 188). A 347 nt 5' deletion of the *rpoS* leader region abrogated Hfq control *in vivo* (33). In addition a point mutation within stem III also conferred Hfq independence (Fig. 2; 33). Together these results suggest that Hfq binds to an upstream region of the *rpoS* leader and contributes to basal translation. However, in conjunction with sRNAs, Hfq can also activate this process (21).

The exact mechanism by which Hfq influences *rpoS* synthesis is unknown. The nearly seven-fold reduction of *rpoS* translation in the *hfq* mutant background cannot be rationalized by the loss of function of the known Hfq-dependent sRNAs. It is possible that Hfq binding destabilizes an inhibitory leader conformation or occludes a RNase E cleavage site. Other theories suggest that Hfq facilitates the interaction of unidentified activating sRNA(s) or it recruits ribosomes to the *rpoS* message.

H-NS and RpoS synthesis

H-NS is a histone-like protein that primarily functions as a transcriptional repressor of >100 genes involved in environmental adaptation (63). As a homodimer H-NS binds specifically to curved regions of DNA, and is speculated to block transcription initiation at particular promoters (189). Several groups have demonstrated that H-NS also acts as a negative regulator of RpoS albeit by a post-transcriptional mechanism (10, 18, 34, 184). RpoS abundance is elevated in a *hns* mutant, an effect attributed to increased transcription and protein stabilization (18, 184). Despite the elevated level of RpoS in a *hns* mutant, SP induction of RpoS occurs normally (10).

DksA and RpoS synthesis

The DksA protein was first identified in *E. coli* as a multi-copy suppressor of the temperature sensitive growth and filamentation of a *dnaK* mutant (73). Since then, it has been implicated in a variety of cellular activities including cell division, the stringent response and *Salmonella* virulence (11, 22, 177). In 1999, it was demonstrated that *dksA* mutants failed to accumulate RpoS during SP (177). Analysis of *rpoS-lacZ* fusions in a *dksA* mutant attributed the deficit mainly to defective translation (15-fold) with only a minor two-fold decrease in transcription (177). Four years later, DksA was reported as a critical factor for activation of *rpoS*

translation by ppGpp (22). The mechanism by which DksA influences RpoS translation is unknown and intriguing given the role for DksA in transcriptional modulation of RNAP through its secondary channel (117).

RpoS stability

Another regulatory pathway limiting RpoS abundance in growing cells is proteolytic degradation involving the ATP-dependent ClpXP protease and a response regulator called MviA (in *S. enterica*) or SprE / RssB in *E. coli* (130, 147). In this pathway MviA is activated by poorly characterized stimuli, including carbon starvation, through phosphorylation on D58, which substantially increases its ability to bind to RpoS. The relevant kinase has not yet been found (34, 61). The binding event (dependent on K173 of RpoS) results in a sequestered non-functional RpoS molecule and thus modulates RpoS activity in itself (13, 192). The MviA-RpoS complex also interacts with the ClpXP protease, which then actively degrades RpoS, recycling MviA (111, 130). RpoS elevates transcription of the response regulator during SP, thus constituting an auto-regulatory loop in which the concentration of MviA is a limiting factor for the rate of RpoS degradation *in vivo* (132, 142).

Fis

Fis is a transiently expressed DNA binding and bending protein involved in diverse cellular functions including transcriptional activation and repression of a large regulon (44). Additionally, Fis plays integral roles in site-specific DNA recombination, stimulation of excision / integration of λ (6, 7), DNA-replication (43, 51), and transposition (179). In general, Fis

regulates cellular processes, including the expression of rRNA and tRNA, necessary for the transition to optimal growth, a time when Fis abundance peaks (119, 140).

The crystal structure of the 98 amino acid Fis protein displays 4 α-helical domains: A (residues 27-42), B (50-70), C (74-81), and D [85-94; (82)]. The N-terminal residues and both A helices of the homodimer are involved in stimulating site-specific DNA inversion (186). A truncated Fis protein, which is deleted for the N-terminal region, is defective in DNA inversion but maintains its ability to bind DNA and regulate gene expression as well as stimulate λ excision (79). Helices C and D of each Fis monomer constitute helix-turn-helix (H-T-H) binding motifs that are required for recognition of the Fis consensus sequence ATTGNTCAAAATTTGANCANT (60, 186). Due to the spacing of these H-T-H elements, binding to opposite DNA strands is accompanied by bending of the DNA molecule. Fis-induced alterations in global topology, in accordance with fluctuating physiological conditions, indirectly influences transcriptional regulation (113).

Regulation of Fis

Fis is under strict genetic control and protein abundance fluctuates dramatically in response to growth environment. Fis levels peak (60,000 dimers / cell) 90 minutes after dilution into fresh medium and decline throughout exponential phase to very low levels, that remain low during SP (2, 8). Regulation of Fis occurs at the transcriptional level and is not mediated by changes in mRNA stability (8, 125, 131). Activation of Fis transcription, which depends on the availability of particular nutrients (discussed below), occurs from a highly conserved promoter,

P_{fis}. Increased Fis abundance results in a feedback loop in which Fis represses its own transcription (121). In *E. coli* and *S. enterica*, a single Fis binding site just upstream of P_{fis} is necessary for the majority of the six-fold transcriptional increase in a *fis* mutant background (8, 174).

Activation of P_{fis} expression is dependent on the availability of the initiating nt of the *fis* transcript (174, 175). An investigation into growth phase regulation of P_{fis} revealed that a promoter sequence of 43 bp was sufficient for Fis activation (174). Specifically, growth phase dependent regulation at P_{fis} was attributed to a less preferred initiating nt, CTP, of the *fis* transcript (174). At this position, nt replacement with either ATP or GTP, resulted in high levels of *fis* mRNA during early SP, a time when P_{fis} transcription is normally shut-off (174). The sensitivity of P_{fis} to the concentration of CTP was clearly demonstrated by a 20-fold increase in activity upon the addition of excess CTP *in vitro* (175). It was also determined that the level of CTP in the growth medium directly correlates with P_{fis} expression (175). This form of transcriptional control is independent of ppGpp (8, 174). However, the *fis* promoter is also subject to repression by the stringent response, an effect dependent upon seven consecutive G-C bp immediately preceding the transcriptional start site (121).

Nucleoprotein complexes involving the global regulators CRP, Fis and IHF also control P_{fis} activity. In a *crp* mutant background, *fis* mRNA levels are abnormally elevated during late exponential phase (114). The effect of CRP on P_{fis} transcription is not well understood and complicated by coordinate control with the Fis protein (114). In the absence of Fis, CRP activates P_{fis} while CRP in conjunction with Fis, synergistically represses activity (114). Another

transiently expressed global transcription factor, IHF, whose abundance increases during exponential growth, is required for normal *fis* transcription (2). A mutation in *ihf* decreases P_{fis} activity nearly four-fold by an unknown mechanism (131). The crosstalk between the global regulators CRP, Fis, and IHF at P_{fis} awaits further characterization. However, it is possible that alterations in DNA topology, induced by these regulators upon binding, indirectly influences P_{fis} activity (145).

Functions of Fis

Inversion and Excision

The Fis protein was first identified as a host factor required for site-specific DNA inversion (72, 78). The G-segment of phage Mu encodes tail fiber genes, the orientation of which determines host specificity, flanked by inverted repeats. During replicative transposition, phage with both orientations of the G-segment are produced by an inversion event dependent upon Fis and the phage-encoded invertase, Gin (72). Fis serves a similar function in the invertasome during flagellar phase variation in which binding of Fis to an enhancer element is necessary for Hin-mediated recombination (72, 96). Fis also plays a crucial role in the Cin-mediated DNA inversion system of bacteriophage P1 (57).

The role of Fis was expanded to include partial regulation of the lysis versus lysogeny decision of phage λ (166). DNA excision is stimulated 20-fold *in vitro* by Fis binding to a

region of λ *attP* that overlaps a Xis binding site, a process that also requires the phage-encoded Xis protein. In the absence of Xis, Fis induces λ integration and lysogeny (7). Consistent with roles in chromosomal rearrangements, Fis also stimulates both Tn5 and IS50 transposition events in *E. coli* (179).

Transcriptional regulation by Fis

Over the last decade, Fis has been defined as a global regulator of transcription in both *S. enterica* and *E. coli*. Fis activates genes necessary for competitive replication, an effect that coincides with Fis abundance (Fis abundance peaks during initiation of exponential growth; 75). For instance, Fis binds several sites near the *oriC* promoter and is required for DNA replication of *oriC* minichromosomes (43, 51). Fis also strongly activates transcription of tRNA rRNA operons (17, 120). In the case of activation at *rrnB* P1, the relatively short-lived open transcriptional complex is stabilized by Fis (191). In this process, Fis binds to a site centered upstream of the transcriptional start site and interacts with the αCTD of RNAP (1). A similar mechanism of activation occurs at the *proP2* promoter and although at least four binding sites occur near this promoter only one, centered at -42, is necessary for most of the effect (107). Recently, DNA microarrays comparing wild type and *fis* mutant backgrounds of *S. enterica* serovar Typhimurium demonstrated a major role for Fis in virulence gene expression, including type III secretion factors (75). This is consistent with a 100-fold attenuation of virulence of a *fis* mutant in a mouse infection model (181).

Fis also acts as a transcriptional repressor of several global regulators including *gyrA,* and *gyrB*, which code for subunits of DNA gyrase, a topoisomerase that induces negative DNA supercoiling (74, 144). In this process, Fis binds near the -10 hexamers of the *gyrA* and *gyrB* promoters and blocks transcription initiation (144). A mutation in *fis* increases gyrase activity and in general, Fis abundance inversely correlates with the concentrations of GyrA and GyrB (144). The influence of Fis on DNA topology both directly and indirectly, via DNA gyrase, control, probably regulates gene expression in accordance with cellular physiology.

Regulation of Translational initiation

Translational regulation is a common genetic strategy that allows the timely induction / repression of protein synthesis or maintains the appropriate constitutive levels. Some authors speculate that control at this level allows a quicker response to a particular stress because it eliminates the time needed for transcription (61). In most cases, translational regulation depends on mRNA leader sequences either for sequence specific elements or structures that interact with *trans*-acting regulatory factors (protein or sRNAs; 16, 54).

Translation is divided into three stages; initiation, elongation and termination. Translation initiation is the process by which ribosomal subunits recognize a particular region of a RNA molecule, referred to as the Shine-Dalgarno sequence (SD), followed by ribosome assembly preceding initial peptide bond formation (56). The primary contact is thought to be specific intermolecular RNA interactions between the mRNA and the 16S rRNA in conjunction with non-specific protein-RNA interactions (36). The ribosome initiation complex is composed

of the ribosome, three initiating factors (IF1, IF2, and IF3), fMet-tRNA$_f^{Met}$, and of course, the template mRNA. Assembly of the ribosome initiation complex is reportedly stochastic (56). Subsequently, the ribosome moves along the RNA molecule and elongates the amino acid chain using residues of successive aminoacyl-tRNAs bound to the acceptor position of the ribosome via peptide-bonds. The polypeptide chain at the peptidyl site of the ribosome continues to grow until a stop codon is encountered and the polypeptide is released, followed by dissociation of the ribosomal subunits.

The composition, and at least partial function, of the ribosome changes as dividing cells enter SP. This transition is concomitant with a peak in the abundance of the ribosome modulation factor, RMF (173). RMF binds to the 50S subunit of the 70S ribosome and mediates 70S-70S ribosomal dimerization (172, 173). The resulting 100S dimer reportedly represents a storage form of the ribosome that is translationally inactive, possibly due to obstruction of the peptidyl-tRNA binding site (185). Upon subsequent culture in fresh medium, the 100S ribosomes dissociate back to the translationally active 70S ribosomes by an uncharacterized process (103, 173). Another protein transiently associated with the ribosome is the SP-induced ribosome-associated, SRA (68). During SP, there is a RpoS-dependent increase in SRA abundance and the ratio of SRA bound specifically to the 30S ribosomal subunit increases (68). The function of SRA is unknown. Due to the uncharacterized role of ribosomal composition in translational regulation, this review will focus specifically on the known factors that influence translation initiation: (i) the SD sequence and the spacer region between the SD sequence and the initiation codon, (ii) the initiation codon, (iii). RNA secondary structure and, (iv). the ribosomal protein S1.

Shine-Dalgarno sequence and Translational Regulation

The SD sequence is a short stretch of nt preceding the coding sequence of most genes that indicates the site of translation initiation (153). In this process, the canonical SD sequence AGGA, or a variant, base pairs with the anti-SD sequence, UCCU, of the 3' end of 16S rRNA (a component of the 30S ribosome along with 21 different proteins; 153). The free energy of this interaction reflects the efficiency of initiation; i.e. longer regions of complementarity are considered more attractive to ribosomes (97). For example, the SD sequence UAAGGAGG is four times more efficient in translation than AAGGA as measured using *lacZ* reporter fusions (136). However, it was also reported that extended SD / anti-SD interactions, beyond eight nt, actually decreased the activity of a reporter fusion possibly due to ribosome stalling at the clearance of the initiation stage (81). A study that examined the RBS of 124 mRNAs determined the average length of complementarity to be five nt and the minimum length was three nt. (161). Few reports have indicated that the presence of a SD sequence is not necessary for translation (151, 170, 183). Another study demonstrated translation of an unleadered RNA, a process dependent upon a SD-like sequence downstream of the initiating codon (151).

Due to the size and correct positioning of the ribosome at the initiation codon, the spacer region between the SD sequence and the initiation codon also determines translational efficiency (52). *In vivo* experiments indicate that the efficacy of translation does not vary much when the spacer is between the range of five to 13 nt and a spacing of nine nt is optimal (161). However,

in these experiments other variables exist, including altered primary sequences and RNA secondary structures, which complicate result interpretation.

Role of the initiation codon

Translation initiation also involves base-pairing between the start codon and the anticodon of the initiator tRNA, fMet-$tRNA_f^{Met}$. The preferred initiating codon, AUG, indicates the translational start of most translated RNAs, and is complementary to the anticodon of fMet-$tRNA_f^{Met}$, UAC. Less frequently, GUG, UUG and CUG serve as start codons in which case weaker base pairing leads to less ternary complex (mRNA, the 30S subunit and fMet-$tRNA_f^{Met}$) formation due to a decrease in overall complex stability (136). This is supported by *in vivo* analyses of reporter fusions that demonstrate decreased activity when GUG, UUG or CUG is substituted for AUG (59, 136, 170). However, the decrease in activity compared to that of AUG containing reporters varied substantially among different initiation codons and is naturally exploited to control gene expression (59, 136). In general, the activity of initiation codons follows the scheme: AUG > GUG > UUG > CUG (59, 136). The second codon has also been shown to affect the rate of translation initiation and highly expressed genes often display GCU at this position (40, 136).

Structured Ribosome Binding Regions

Translation initiation regions are often folded into complex secondary structures (e.g. *rpoS*; Fig. 2). An equilibrium exists such that at any given time a particular mRNA molecule may be partitioned between structured and non-structured conformations (37). This ratio is dependent upon the free energy of the different foldings (37). The theory that ribosomes are blocked by mRNA secondary structure predicts that stable foldings positively influence the folded:unfolded ratio and thus result in decreased initiation (37). This idea is at odds with a report suggesting that the 30S ribosomal subunit recognizes a particular folded mRNA structure (66). Either way, the secondary structures of the ribosome-binding region (which can include far upstream and downstream interactions; Fig. 2) have been reported to both enhance and inhibit translation initiation (discussed below).

Phage T4 gene 38 encodes a protein required for long tail fiber assembly whose synthesis is enhanced by its ribosome-binding region (52). In this case, a 20 nt stem-loop positions a distant SD sequence within five nt of the initiating codon facilitating translation initiation (52, 122). Mutations predicted to destabilize the structure resulted in decreased translation (52, 122). A similar mechanism of translational activation is reported for T4 gene 25 (122, 123).

Alternatively, structured regions that involve pairing of the SD sequence or the initiation codon result in decreased protein synthesis. Such is the case for the translation initiation region

of the coat gene of the bacteriophage MS2 (37). The mRNA structure of this region pairs the SD sequence with nt downstream of the initiation codon (in this case AUG; 156). Mutations predicted to stabilize this inhibitory structure resulted in decreased protein expression, while disruption of base pairing at the SD sequence had the opposite effect (37). An important conclusion of that study is that a strict correlation exists between translation efficiency and the stability of the structured ribosome-binding region (37). Based on the effects of nt substitutions on both protein expression and predicted secondary structure stability it was also suggested that ribosomes bind only single-stranded regions of RNA molecules (37). This model contradicts the notion that local single-stranded regions, for example the SD sequence or the initiation codon, facilitates translation initiation (148). In these experiments it is difficult to attribute the changes in protein expression solely to changes in the folding energies considering that the primary sequence and the predicted RNA structures are also altered.

Trans-acting Factors and Translational Regulation

The ribosomal protein S1 (encoded by *rpsA*) is the largest ribosomal protein and essential for viability (162). S1 interacts with the head, platform and body of the 30S subunit via intermolecular protein interactions late in assembly and its length is comparable to the longest dimension of the 30S subunit (149). The N-terminal domain is responsible for protein-protein interactions while the C-terminus contains four homologous repeats, the S1-motif, that constitute a RNA-binding domain (26, 162, 163). S1 plays an essential role in translation using promiscuous RNA interactions (with a preference for polyU, polyA and polyC nt sequences) to

promote binding of the ribosome to mRNA (16, 138, 160, 162, 163). Ribosomal associated S1-mRNA interactions occur anywhere from 22-145 nt upstream of the initiation codon, and purified S1 binds RNA *in vitro* (14-16, 135, 150).

Reports of actual translational regulation by S1 are scarce and the best described system is autogenous repression (14, 15, 141). Despite the absence of a canonical SD sequence, *rpsA* is actively translated and S1 repression occurs at this level (155). The proposed model relies on S1-mediated recognition and disruption of a phylogenetically conserved secondary structure of the *rpsA* mRNA leader (-90 to +20 with respect to the first nt of the initiation codon) that forms a non-contiguous SD sequence (15).

A recent report demonstrates that S1 binds to another leader region, the *rpoS* message in *Pseudomonas aeruginosa* (150). This interaction occurs specifically during exponential phase and depends on 78 nt upstream of the initiation codon however, the translational consequence of S1 binding to *rpoS* was not determined (150). These results suggest a possible role in regulation of *rpoS* translation, although the model is counterintuitive; S1 recruits ribosomes to the *rpoS* message during a time when translation is minimal.

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Role of ppGpp in *rpoS* Stationary Phase Regulation in *Escherichia coli*

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Abbreviation: ppGpp, guanosine 5'-diphosphate, 3'-diphosphate

Abstract

The bacterial sigma factor RpoS is strongly induced under a variety of stress conditions and during growth into stationary phase. Here, we use *rpoS-lac* fusions in *E. coli* to investigate control acting at the level of RpoS synthesis, which is especially evident when cells approach stationary phase in rich medium. Previous work has shown that the small molecule ppGpp is required for normal levels of RpoS in stationary phase. Despite the attraction of a model in which the ppGpp level controls stationary phase induction of RpoS, careful measurement of *rpoS-lac* expression in a mutant lacking ppGpp shows similar effects during both exponential growth and stationary phase: the main effect of ppGpp is on basal expression. In addition, a modest regulatory defect was associated with the mutant lacking ppGpp, delaying the time at which full expression is achieved by 2 to 3 hours. Deletion analysis showed that the defect in basal expression was distributed over several sequence elements, while the regulatory defect mapped to the region upstream of the *rpoS* ribosome-binding site (RBS) that contains a *cis*-acting antisense element. A number of other genes that have been suggested as regulators of *rpoS* were tested, including *dksA*, *dsrA*, *barA*, *ppkx*, and *hfq*. With the exception of the dksA mutant, which had a modest defect in Luria-Bertani medium, none of these mutants was defective for rpoS stationary-phase induction. Even a short *rpoS* segment starting at 24 nt upstream of the AUG initiation codon was sufficient to confer substantial stationary phase regulation, which was mainly posttranscriptional. The effect of RBS-proximal sequence was independent of all known *trans*-acting factors, including ppGpp.

Introduction

The *rpoS* gene encodes a sigma factor, σ^S or RpoS, which is required for expression of a large number of genes in response to various stresses, including nutrient limitation and osmotic challenge, and during growth into stationary phase (see references 15 and 21 for reviews). The *rpoS* gene has been found in a variety of Gram-negative bacteria and its function and regulation have been studied extensively in the enteric species *Escherichia coli* and *Salmonella enterica* serovar Typhimurium (here referred to as *S. enterica*). RpoS is also a virulence factor for *S. enterica* (13) and its expression is induced when these bacteria enter mammalian host cells (9). It is not clear how information about stress, nutrient limitation and host environment is used to control RpoS. Increased RpoS abundance has been reported to be regulated at many levels including transcription initiation and elongation (17,18,30,34), translation (19,22,24), and protein stability (19,31,35). RpoS protein activity is also regulated (32). No *in vitro* system that mimics any aspect of *in vivo* control of RpoS synthesis has been described.

Genetic analysis has led to the idea that some, perhaps most, regulation of RpoS synthesis occurs at the posttranscriptional level *via* an inhibitory mRNA secondary structure (7,20,23). An upstream antisense element has been localized through computer analysis of RNA folding and identification of compensatory mutations (7, our unpublished data); the antisense element can pair with the ribosome-binding site (RBS) region and inhibit *rpoS* translation. This proposed RNA structure is not yet supported by physical evidence. It is, however, strongly supported by genetic analysis of the DsrA RNA, a small untranslated RNA which acts as an anti-antisense RNA, increasing *rpoS* expression (23). DsrA RNA is important for expression of *rpoS* in *E. coli* at growth temperatures at or below 30°C

(33,39), but is not required in *S. enterica* (unpublished data). It is not yet clear whether the antisense element functions in other regulatory inputs to RpoS.

Mutations in more than 20 genes have been identified as affecting RpoS synthesis alone. Many of these "regulators" exhibit highly pleiotropic phenotypes, and it seems unlikely that most act directly on *rpoS* expression. Often, such mutants show changes in the shape of the growth curve even in rich medium. Thus, their effects on RpoS may be a secondary consequence of altered growth rates and early or prolonged entry into stationary phase. There are clearly strong selective forces both for RpoS activity (in early stationary phase) and against it (in both late stationary and exponential phase). Given these forces, it is more than a formal possibility that uncharacterized strain differences may influence the observed regulation. Known examples include the wild type *S. enterica* strain LT2, which is defective in the RpoS protein turnover mechanism 3,11); and the widely-used *E. coli* strain MC4100, which is a *relA* mutant, and is often used despite the reported role for ppGpp in RpoS regulation (14). Thus, even more than for most regulatory systems, the results observed may depend on which strain was used and how the cells were grown.

Here, we investigate the induction of RpoS that occurs in the wild type *E. coli* strain MG1655 as cells are grown to stationary phase in LB medium, usually at 37°C. This medium was chosen because the induction ratio (stationary phase expression / exponential phase expression) is particularly high under these conditions: ca. 35-fold as measured using an *rpoS-lac* protein fusion. Previous work showed that RpoS abundance is greatly reduced in a MG1655 Δ*relA* Δ*spoT* mutant which lacks ppGpp (14). (This genetic background is referred to below as ppGpp^{o} for convenience). Artificially increasing

ppGpp levels by synthesis of a truncated RelA protein also leads to a very large and rapid increase in RpoS abundance, while substantially increased RpoS abundance can also be observed in certain *spoT* mutants that have modestly elevated ppGpp (14).

Another study concluded that the main effect of ppGpp is on transcription elongation across the *rpoS* leader (17). This conclusion was based on apparently normal stationary phase and ppGpp-regulation of plasmid-borne *rpoS-lac* fusions (to codon 23 of *rpoS*) which had been deleted for the known *rpoS* promoters. However, the source of this low-level residual transcription was not identified. There is also an apparent conflict between this conclusion and experiments using ppGpp overproduction (8) which found that *rpoS* mRNA abundance is not elevated by ppGpp overproduction during exponential phase, pointing to translation control of *rpoS* by ppGpp.

To further investigate these questions, we employed a set of *lac*UV5 promoter substitution and deletion derivatives of *rpoS-lac*, which allow sensitive, quantitative measurement of *rpoS* expression in LB medium in different mutant backgrounds.

Materials and Methods.

Bacterial strains and construction. Strains used in this study for physiological experiments are derived from the wild type *E. coli* K-12 strain MG1655. The parental strain was CF7968, which is MG1655 that has been corrected to rph^+ (16) and deleted for *lacIZ*, obtained from M. Cashel. This *lac* deletion extends between *Mlu*I sites in *lacI* and *lacZ* and was constructed by D. Vinella. Many of the

lac fusions used in this work have been described previously (6, 7, 10). These fusions are placed in the *E. coli trp* operon as described (12). Phage P1 *vir* was used for transduction; P1 growth and transduction were carried out by standard methods (37).

Media and growth conditions. Bacteria were grown at 37°C (with one exception as noted below) in Luria-Bertani (LB) medium (37) and on nutrient agar plates containing 5 g of NaCl per liter, except where indicated. Minimal agar was prepared with NCE medium containing 0.2% glucose (4). Antibiotics were added to final concentrations in selective plates as follows: 20 μg of tetracycline hydrochloride/ml (10 μg / ml for minimal medium), 20 μg of chloramphenicol / ml, 50 μg of kanamycin sulfate / ml (except for *hfq* crosses, as noted below) and 30 μg of sodium ampicillin / ml (100 μg / ml when selecting for plasmids).

New mutations affecting putative trans-acting factors. New insertion mutations were made by the method of Yu et al (43) employing host strain DY330 (*E. coli* W3110 Δ*lacU169 gal490* [ΔcI857*ts* Δ(*cro-bioA*)]. Primers containing 20 nt of *tet* homology at the 3' end were used to amplify the *tetAR* genes from plasmid pWM7 (25). PCR was performed using Taq polymerase (Qiagen) as suggested by the manufacturer. Amplified DNA was purified by a QIAquik PCR purification kit (Qiagen); residual template DNA was then removed by digestion with *Dpn*I, which cuts specifically at methylated GATC sites, followed by repurification of the PCR product and elution in a volume equal to the original PCR reaction. Heat-induction and transformation of DY330 was as described (43) using 5 μl of DNA; transformants were selected at 30°C.

Primers for *tet* amplification were as follows (*tet* homology in upper case):

dksA atgcaagaagggcaaaaccgtaaaacatcgtccctgagtattctcgccatCTCTTGGGTTATCAAGAGGG,

ttagccagccatctgtttttcgcgaatttcagccagcgttttgcagtcgaACTCGACATCTTGGTTACCG;

barA ctttctcaatttaacagtgtgaccttaattgtcccataacgCTCTTGGGTTATCAAGAGGG,

ccagcgtcataaaaagccgattgctactcgacaagacatccattaACTCGACATCTTGGTTACCG;

ppkx ggtcaggaaaagctatacatcgaaaaagagctcagttggtCTCTTGGGTTATCAAGAGGG,

tcgtcggcccgcaaagtattaagcggcgatttctggtgtaACTCGACATCTTGGTTACCG.

The resulting deletion/insertion mutations result in loss of target gene sequence as follows: *dksA* (codons 18 through 136); *barA* (7 bp upstream of codon 1 through the termination codon); *ppkx* (codon 15 of *ppk* through codon 507 of *ppx*). Strains were checked for the insertion/deletion by PCR with flanking primers. Sequences of these primers are available on request. Only a small number of candidate insertions was checked for each gene knockout experiment; in every case, a PCR product of the predicted size was observed.

The same general method was used to substitute the *bla* (Amp^R) gene for Kan^R of certain *lac* fusions. The primers used have *bla* homology at their 3' ends (upper case) and the template was pBR322:

tcatgaacaataaaactgtctgcttacataaacagtaataTGAAGACGAAAGGGCCTCGTGATAC,

gcgtaatgctctgccagtgttacaaccaattaaccaattcTTACCAATGCTTAATCAGTGAGGCAC.

In contrast to the transformations used to construct *tet* insertions, for the *bla* substitutions it was found that only a minority of Amp^R transformants had lost the Kan^R marker as predicted for events of

the desired type (the frequency of the correct event ranged from 1-25%). However, the Amp^R marker of the desired class (Kan^S) showed 100% linkage to Lac^+ upon backcross.

Other mutations affecting trans-acting factors. The *relA* deletion used was from strain CF3032 (Δ*relA252*::*kan argA*::Tn*10*; 26). Since most of the *lac* fusions used here are marked with Kan^R, the Δ*relA* marker was introduced by co-transduction with *argA*::Tn*10*, selecting Tet^R. Transductants were screened for the Rel^- phenotype by testing sensitivity to SMGL (serine, methionine, glycine and leucine) on minimal glucose plates with tetracycline. When comparing wild type with $ppGpp^o$ (Δ*relA* Δ*spoT*) strains, the $relA^+$ $spoT^+$ control strains also carry the *argA*::Tn*10* (with one exception noted below). The *spoT* deletion used was from strain CF1693 (Δ*relA251*::*kan* Δ*spoT207*::*cat*; 42). The *dsrA* deletion used was from strain DDS724 (Δ*dsrA5* with linked Tn*10*), obtained from D. Sledjeski. This deletion is described in reference 39. The *dsrA* deletion was introduced by co-transduction with the linked Tn*10*. For comparison with Δ*dsrA* strains, wild type $dsrA^+$ strains also carry this linked Tn*10*. To construct strains for the epistasis test of *dsrA* and *relA spoT*, the Δ*relA252*::*kan* marker from CF3032 was introduced by linkage to *argA*::Tn*10* and then the Tn*10* was removed by subsequent transduction to Arg^+.

The *hfq* insertion used was from strain TX2822 (*hfq-1*::Ω-Km), obtained from M. Winkler (40). This insertion is at codon 41 of the 102 codon *hfq* gene. We encountered difficulty using Kan^R to select for transfer of *hfq-1*::Ω-Km. In fact, all the strains we obtained carrying *hfq-1*::Ω-Km grew very poorly when streaked out on either LB or NB agar containing 50 μg / ml kanamycin, showing a typical pattern

of colonies in the streak (suggesting suppression). This behavior is not understood. Growth was normal in the absence of kanamycin. To construct the needed strains, selection for transductants carrying *hfq-1*::Ω-Km was carried out on LB agar with kanamycin at 25 μg / ml at room temperature, and transductants were then purified on LB agar without kanamycin at 37°C. Successful introduction of the *hfq* insertion (and all other deletions) was confirmed by PCR.

***rpoS-lac* fusion.** We have previously described the detailed method used to make the *rpoS-lac* constructs employed for most of the experiments in the present work (6, 10). They use the general system originally designed by Simons et al. (38), as modified (12). The relevant gene segments include (in order) an upstream Kan^r element, tandem transcriptional terminators, and the promoter or regulatory sequence under investigation, followed by the *lac* operon. This assembly is placed in single copy in the bacterial chromosome (at *trp*); therefore all strains carry a wild-copy of *rpoS*. Most lac fusions used in this study have *lacZ* placed to form either an operon or protein fusion at the *Ea*gI site at codon 73 of *rpoS*. A different set of fusions, to codon 8 of *rpoS*, was used for the last set of experiments as described below.

Some constructs carried the native *rpoS* promoter (6). In the others, including the *Kpn*I construct as well as the numbered deletions, *rpoS-lac* is expressed from the *lac*UV5 promoter (*lac*UV5p) with a constant *lac*-derived leader of 36 nt plus several restriction sites, followed by different amounts of *rpoS* sequence; these constructs vary only in the extent of the deletion that removes *rpoS* sequence from the upstream side. The *lac*UV5p is derived from pRS476 (38); it includes only one of the cyclic AMP receptor protein (CRP) half-sites on the upstream side and the *lac* operator on the downstream side.

The reference *Kpn*I site construct (construct K), as well as the Δ1 and Δ2 constructs, were all previously described (10). The Δ3 and Δ4 deletions were made by PCR in exactly the same way as Δ1 and Δ2; the PCR amplified segments were re-sequenced to ensure that no unwanted changes had been introduced. These deletion endpoints for Δ2 through Δ4 are illustrated with respect to the sequence in Fig. 1. All deletion endpoints are numbered starting from the first transcribed nucleotide for transcripts initiated from the *rpoS* promoter. We have taken this transcript sequence to begin with GGGUGAACAG (the first G is nt 1; 17). The coordinates of the first base pair that is still present in each construct are as follows: construct K, nt 73; Δ1, nt 344; Δ2, nt 454; Δ3 nt 477; Δ4 nt 541. The *rpoS* ATG initiation codon is at nt 565.

Another construct in which *lac*UV5 drives *rpoS* expression starting from "+1" of *rpoS* (nt 1) was constructed by PCR on a *rpoS-lac* template with a *lac*-specific oligonucleotide together with the following oligonucleotide (the *lac*UV5 mutation in the promoter's -10 region is shown in bold; *rpoS* homology is shown by italics):

CGCGAATTCAGGCTTTACACTTTATGCTTCCGGCTCGTAT**AA**TGTGTGGAATT*GGGTGAACAGAGTGCTAACAAAA TG.*

Transcripts originating from *lac*UV5p in this construct are predicted to contain the 5' sequence AAUUGGGUGAACAGAGTGCTAACAAAATG, where the underlined nucleotides are derived from *rpoS* sequence. This construct does not include the *lac* operator. The PCR product was substituted as an *Eco*RI-*Kpn*I fragment in several steps, and this segment was sequenced to make sure that no unwanted mutations had been introduced by the PCR step. The strain with this fusion carried in the bacterial chromosome is TE8378.

Second method for making promoter fusions. We subsequently developed a convenient method for making constructs in which *rpoS* (or any gene) can be expressed from *lac*UV5p, by employing the lambda lysogenic strain background and technique of Yu et al (43). The general transformation method is the same as described in the section above on *tet* insertions.

For this purpose, we first placed *tetAR* upstream of *lac*UV5p, replacing Kan^R (38) in the standard Δ1 fusion by using the following two oligonucleotides:

ATAAAGTGTAAAGCCTGGGGTGCCTAATGAGTGAG*AATTCACTCGACATCTTGGTTACCG* (*tetR* homology in italic, *lac*UV5p homology elsewhere) and

TCATGAACAATAAAACTGTCTGCTTACATAAACAGTAATA*CTCTTGGGTTATCAAGAGGG* (*tetA* homology in italic, *kan* homology elewhere):

In the second step, the marked *lac*UV5 promoter was joined to each of the desired target sites by PCR amplifying ther *tetAR-lac*UV5p segment with an oligonucleotide which included appropriate *rpoS* homology attached 5' to the *lac*UV5-specific sequence: *AATTCCACACA*TT*AATACGAG*. As in the first step, on the upstream side the oligonucleotide for PCR was chosen so that the *tetAR-la*cUV5p substitution will replace the *kan* gene of the standard fusion. The resulting constructs are marked with Tet^r, join to *lac*UV5p directly to the desired target sequence, and do not include the *lac* operator. With this method, we made a new set of deletions extending to various positions directly upstream of the *rpoS* ATG initiation codon as described above in the text. Depending on the strain used for lambda *red*-mediated transcformation, the resulting constructs are *lac* [op] or [pr] fusions at codon 8 of *rpoS*. The full *la*cUV5p sequence is

GAATTCTCACTCATTAGGCACCCCAGGCTTTACACTTTATGCTTCCGGCTCGTATAATGTGTGGAATT.

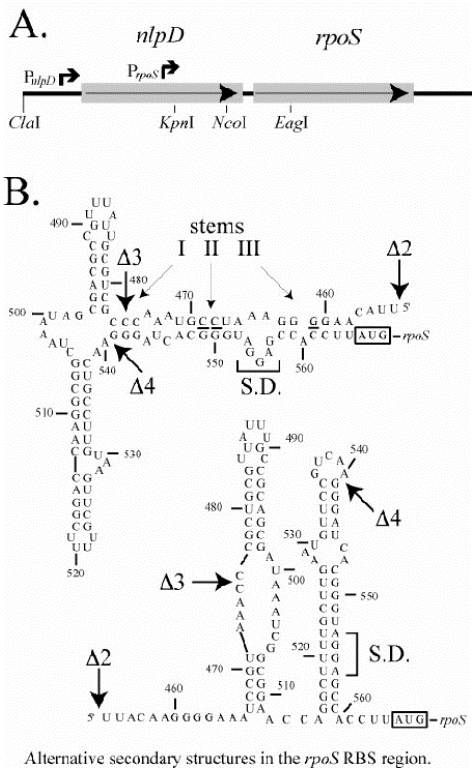
Constructs made by this method were confirmed genetically as Kan^s and physically by PCR with primers specific to *tetR* and *lac*, followed by DNA sequencing across the *lac*UV5p and the first 200 to 300 nt at the joint to *rpoS*. After verification, the fusion constructs were transduced into the CF7968 background by selecting Tet^r.

The *lac* fusions marked with Kan^r, as designed by Simons *et al.* (38), carry tandem insertions of a terminator between the drug resistance cassette and the site where test segments are joined to *lac*; terminators were not explicitly included in the fusions marked with Tet^r made by the new method. This was considered unlikely to be necessary because of the weak activity of the *tetR* promoter and its more than 20-fold dependence on tetracycline for induction. Indeed, assays of constructs grown in the presence or absence of tetracycline showed that induced transcription from *tetR* accounts for ≈10% of *lac* transcription from exponential phase cells; the contribution from uninduced transcription (i.e. our standard growth condition) is therefore negligible.

Assay of β-galactosidase. Cells were centrifuged and resuspended in Z-buffer (100 mM $NaPO_4$, pH 7.0, 10 mM KCl, 1 mM $MgSO_4$), then permeabilized by treatment with SDS and chloroform (27). The samples from exponential phase time points were concentrated before assay, to be approximately equal in density to samples from later times. Assays were performed in Z-buffer containing 50 mM β-mercaptoethanol by a kinetic method using a plate reader (Molecular Dynamics). Activities (OD_{420} per min) are normalized to actual cell density (OD_{650}) and were always compared to appropriate controls assayed at the same time. All β-galactosidase assays were performed within 3 hr of the time of sampling. The values shown are averages of at least three experiments with standard

deviations of less than 20%, except for the very low values from exponential phase for the ppGpp^o mutant, where the standard deviations were less than 30%. But we should point out that comparisons of the relative defect in stationary phase induction involve ratios of experimental values, with a corresponding increase in the uncertainty.

Figure 3. Partial Restriction map of the *nlpD* and *rpoS* open reading frames.



. Panel A shows a partial restriction map of the DNA encompassing the *nlpD* and *rpoS* open reading frames of *E. coli*, which consist of 321 and 330 codons, respectively. The function of *nlpD* (encoding a lipoprotein) is not related to that of *rpoS*. Arrows indicate the orientation of the two ORFs and the known promoters. The upstream promoter cluster (P_{nlpD}), which is not thought to be regulated, serves both *nlpD* and *rpoS*. The downstream promoter (P_{rpoS}), is regulated and serves only *rpoS*; its transcript includes an untranslated leader of 564 nt. The first nucleotide of the transcript from P_{rpoS}, (as specified in the text) is taken as the basis for numbering used here (nt 1). The numbers used previously by us (7,10) can be converted to this system by adding an offset of 341 nt Most *lac* fusions used in this study have *lacZ* placed to form either an operon or protein fusion at the *Eag*I site within *rpoS* (codon 73). Other fusions, to codon 8 of *rpoS*, are so indicated in the text. Panel B shows two possible secondary structures for RNA including the end of the *nlpD* coding sequence and the short intergenic region up to the AUG start codon of *rpoS*. The UAA stop codon terminating *nlpD* lies at nt 500-502. The top structure is that proposed previously by us (7), and includes an upstream antisense element with three stems that can pair with a complementary sequence within the RBS, directly upstream of the *rpoS* AUG start codon. The Shine-Dalgarno (S.D.) sequence complementary to 16S rRNA is also indicated. The *lac* UV5 promoter was used to drive expression of various constructs of two general types, as detailed in the text. The end-points of sequence derived from *nlpD-rpoS* included in these fusions can be described with reference to this figure as follows. Fusions for which the promoter is shown as "*rpoS*" include DNA starting from the *Cla*I site (panel A). Other fusions contain substitutions of the *lac*UV5 promoter followed by DNA starting from: nt 1 of the P_{rpoS} transcript, the *Kpn*I site, Δ1 (nt 344), Δ2 (nt 454),Δ3 (nt 477), or Δ4 (nt 541). For Δ2 through Δ4 included leader DNA sequences are shown in panel B.

Results

Expression of *rpoS-lac* along the growth curve. Growth of cells for β-galactosidase assay was at 37°C (with one exception noted below) in LB medium. Cultures were started by a 1:500 dilution starting from overnight cultures grown under the same conditions. In LB medium at 37°C the generation time of the wild type (*relA*$^+$ *spoT*$^+$) strain was 24 minutes, while the generation time of the otherwise isogenic ppGpp^o mutant (Δ*relA* Δ*spoT*) was 33 minutes (Fig. 4A). For each culture, the time at which stationary phase begins (designated S below and in data tables) was arbitrarily defined as 1 hour after the time at which the OD_{600} reached 0.5. This definition compensates for the slower growth rate of the ppGpp^o mutant; it also allows the times for stationary phase sampling to be fixed while the culture is still in exponential phase. The point on the growth curve at S is very close to the inflection point between lines for exponential and stationary phases.

In a preliminary experiment we found that wild type cells carrying the *rpoS-lac* [pr] fusion, taken at densities between OD_{600} = 0.01 and OD_{600} = 0.25, showed nearly the same activity for β-galactosidase (data not shown). So as to maximize recovery, OD_{600} = 0.25 was chosen as the reference density for exponential phase, before the increase characterizing the transition into stationary phase. Plots of β-galactosidase activity determined as cells achieved stationary phase are shown in Fig 4B. The times shown in this panel are slightly different than shown in panel A: the x-axis of the plot of the ppGpp^o mutant has been shifted to align it with wild type at OD_{600} = 0.5 (as well as S and subsequent points).

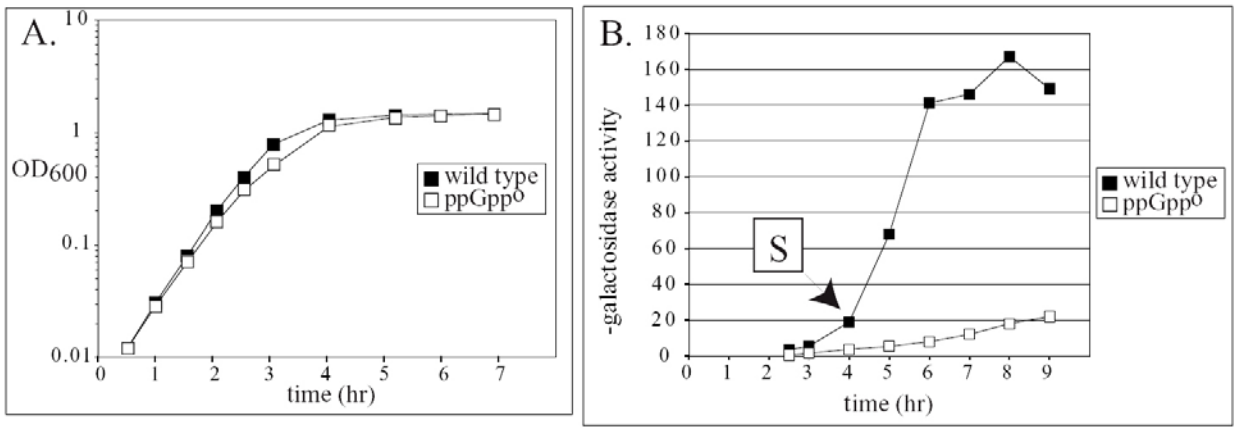


Figure 4. Expression of *rpoS-lac* [pr] as a function of growth phase.

A. Growth curve of the wild type strain carrying *rpoS-lac* [pr] (TE8197, filled squares) and its ppGpp° mutant derivative (TE8199, open squares) in LB medium at 37°C. As described in the text, stationary phase (S) was defined as one hour past the time at which the OD_{600} reached 0.5. In this experiment, for the wild type strain S = 3 hr 42 min, and for the ppGpp° strain S = 3 hr 54 min.
B. Cultures were grown as in panel A, and sampled at various times for assay of β-galactosidase. The first point for each curve corresponds to the time at which $OD_{600} = 0.25$. The x-axes of the plots have been shifted slightly to align the points corresponding to $OD_{600} = 0.5$ at the 4 hr mark. Each subsequent sample was taken at one hour intervals.

Our data are consistent with results reported in previous studies (14,17). The wild type strain with *rpoS-lac* [pr] shows low but significant activity during exponential phase (Table 5), which then rapidly increases during the approach to and in early stationary phase. Maximum activity is achieved by S+2 hr and is not increased by overnight growth (data not shown). The overall induction ratio is 35- to 40-fold. In contrast to wild type, the ppGpp° mutant has much lower expression of *rpoS-lac* at all times. The mutant shows about a 6-fold decrease compared to wild type even during exponential phase (Table 5), and the ratio of activity in the mutant compared to that in wild type is approximately the same during exponential phase and late stationary phase. It is only during early stationary phase that the ppGpp° mutant seems to be delayed in comparison to the increase seen in *rpoS-lac* expression for wild type. For

the mutant, there is a gradual three-fold increase in β-galactosidase activity seen at late times, between S+2 hr and S+5 hr (Fig. 4B).

Table 5. Effect of ppGpp on *rpoS-lac* and *lac* expression *in vivo* [a].

lac fusion			β-galactosidase activity (U)						Activity ratio, mutant/wild type		
			Wild type			Mutant (ppGpp^o)					
Promoter	reporter	type	E	S+3	S+3/E [b]	E	S+3	S+3/E	E	S+3	S+3/E
rpoS	*rpoS-lac*	Protein	3.4	120	35	0.54	5.1	9.4	0.16	0.04	0.27
rpoS	*rpoS-lac*	Operon	35	510	15	11	50	4.5	0.31	0.10	0.31
lacUV5	*rpoS-lac*	Protein	5.3	71	13	2.1	7.6	3.6	0.40	0.11	0.27
lacUV5	*rpoS-lac*	Operon	100	330	3.3	64	130	2.0	0.64	0.39	0.61
lacUV5	*rpoS-lac* (+1)	Protein	10	130	13	3.2	25	7.8	0.32	0.19	0.60
rpoS	*rpoS-lac* (codon 8)[b]	Protein	4	130	32	0.62	13	21	0.15	0.10	0.64
lacUV5	*lac*	Operon	120	160	1.3	81	80	0.99	0.67	0.50	0.74

[a] Exponential-phase (E) and stationary-phase (S + 3) samples are defined in text. Values are averages with a variation of <20%, with one exception noted in the text. The S + 3/E induction ratio was calculated as the activity of the S + 3 sample divided by that of the E sample.
[b] all other fusions were to codon 73 of *rpoS*.

Table 6. Role of the antisense element in stationary phase induction and ppGpp effects on *rpoS-lac*[a].

lac fusion		β-galactosidase activity (U)						Activity ratio, mutant / wild type		
		wild type			mutant (ppGpp^o)					
Promoter	Reporter	E	S+3	S+3/E	E	S+3	S+3/E	E	S+3	S+3/E
lacUV5	*rpoS-lac*[a]	5.7	82	14	2.1	6.7	3.2	0.37	0.08	0.22
	rpoS-lac, Δ1	4.7	65	14	1.8	7.5	4.2	0.38	0.12	0.30
	rpoS-lac, Δ2	3.1	34	11	1.6	5.7	3.6	0.52	0.17	0.32
	rpoS-lac, Δ3	13	73	5.6	8.4	62	7.4	0.65	0.85	1.3
	rpoS-lac, Δ4	8.3	40	4.8	5.2	30	5.8	0.63	0.75	1.2
rpoS	*rpoS-lac*	3.1	120	39	0.66	8.8	13	0.21	0.07	0.34
rpoS	*rpoS-lac* C469G	7.7	210	27	1.4	47	34	0.18	0.22	1.23
rpoS	*rpoS-lac* G549C	14	360	26	3.3	29	8.8	0.24	0.08	0.34
rpoS	*rpoS-lac* C469G G549C	6.5	170	26	1.4	7.9	5.6	0.22	0.05	0.22

[a] All fusions listed in this table are protein fusions. See Table 5, footnote [a], for definitions.

The defect in stationary phase *rpoS-lac* expression seen for the ppGpp^{o} mutant was previously reported by others (17). However, we also find a quantitatively similar exponential phase defect of *rpoS-lac* expression in the same mutant. We would distinguish between the defect in basal (or constitutive, non-regulated) expression and a true regulatory defect. The basal defect of the ppGpp^{o} mutant is about six-fold; while the regulatory defect for stationary phase induction seen at S+3 hr is approximately three-fold. These results indicate that stationary phase induction of *rpoS* is not simply due to increased ppGpp during stationary phase because it still occurs normally even in the complete absence of ppGpp. Accumulation of RpoS in the ppGpp^{o} mutant after overnight incubation in stationary phase is consistent with previous studies (14, 17). Of course, these experiments do not exclude the possibility that ppGpp might play a quantitatively larger regulatory role under other conditions.

Promoter substitution. Expression of *rpoS-lac* was measured from constructs in which the *lac*UV5 promoter was substituted for the native *rpoS* promoters (10). The *lac*UV5 promoter was chosen because its activity was previously reported to be completely independent of ppGpp (1, 2); also, its activity is only slightly increased during stationary phase (see the last entry of Table 5). When expression of *rpoS-lac* [pr] was driven by *lac*UV5p, a strong stationary phase induction still occurred (induction ratio of 13-fold; Table 5). The stationary phase induction was smaller by a factor of 3 than that seen with the native promoters, suggesting that the native promoters contribute to stationary phase induction to this extent. We also compared the effect of stationary phase on expression of *rpoS-lac* protein and operon fusions driven by either native or *lac*UV5 promoters. Combined, the results support the idea that stationary phase controls multiple stages in *rpoS* expression. (i) a role for translational

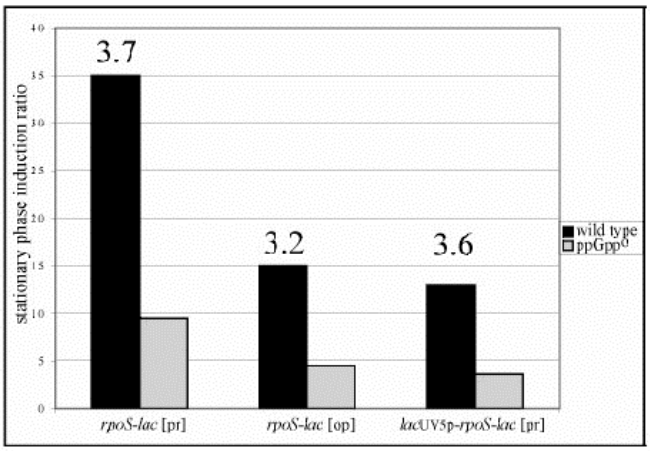
regulation is suggested by the larger induction ratio when [pr] fusions are compared with [op] fusions, whether driven by the native promoters or by *lac*UV5p; (ii) a role for the native *rpoS* promoters is suggested by the smaller induction ratio when *lac*UV5p is substituted in either [op] or [pr] fusion contexts; and (iii) a role for post-initiation transcriptional control is suggested by the residual stationary phase induction seen in the *lac*UV5p-*rpoS*-*lac* [op] construct.

Expression of *rpoS*-*lac* [pr] from the *lac*UV5 promoter was decreased significantly in the ppGpp^{o} mutant background (Table 5). The time course for both mutant and wild type (data not shown) was similar to that seen when the construct was driven by the native promoters (Fig 4B). Expression was lower for the mutant at all times, but with a gradual increase in expression in the mutant until S+5 hr. To detect the regulatory defect of the mutant, we chose S+3 hr as the time of sampling for stationary phase (Table 5). Selected stationary phase induction ratios calculated in Table 5 are plotted in Fig 5. It can be seen that the regulatory defect of the ppGpp^{o} mutant is similar for *rpoS*-*lac* protein and operon fusions, as well as for protein fusions whether driven by the native promoters or *lac*UV5p. This suggests that neither the promoter nor translation-level control are significant targets for the regulatory effect of ppGpp.

In contrast to its regulatory effects, the defect in basal expression of the ppGpp^{o} mutant seems to be distributed over several elements. For example, whether comparing exponential or stationary phase values, the biggest defect is shown by *rpoS*-*lac* [pr] driven by the native promoters. The relative defect is less for either a *lac*UV5 promoter substitution or an operon fusion. The former result is consistent with direct measurement of *rpoS* mRNA 5' ends (17). We also found that *lac*UV5 promoter activity

was somewhat lower in a ppGpp^{o} strain---as much as 2-fold lower in stationary phase (Table 5). This effect of ppGpp in the control, while small, makes the significance of other small effects uncertain (in particular, the effect of ppGpp in the *lac*UV5p-*rpoS-lac* [op] construct).

Figure 5. Nature of the ppGpp^{o} regulatory defect.



Various *rpoS-lac* fusion strains were grown and assayed for β-galactosidase as reported in Table 5. The stationary phase induction ratio (expression at S+3 divided by that at E1) is plotted in bar format for both mutant and ppGpp^{o} versions of each fusion. The ratio (value in wild type divided by that in ppGpp^{o} mutant) of the stationary phase induction ratios is shown above the bar for each wild type strain. The strains used are listed in Table 8.

The reference *lac*UV5p constructs were made using a convenient *Kpn*I site (10); this strategy removed 72 nt of *rpoS* leader sequence. Therefore an additional *lac*UV5p construct was made which includes all nucleotides of the native transcript (denoted +1); it is regulated identically to the reference construct in stationary phase, although it seems to be somewhat less sensitive to ppGpp during stationary phase. The significance of this difference is not understood at present. Finally, one fusion was tested in which *lac* is joined to *rpoS* at codon 8 (rather than to codon 73 as in all other *rpoS* fusions used to this

point). The codon 8 fusion (driven by the native *rpoS* promoters) was regulated normally by stationary phase and was highly responsive to ppGpp.

Deletion mutants. Deletion analysis was used to define the region(s) of the *rpoS* leader which are required for stationary phase induction and the response to wild type ppGpp levels (Table 6). Cell samples from wild type and ppGpp^{o} backgrounds were collected in both exponential and stationary phase, and stationary phase induction ratios calculated. For each construct, the promoter was *lac*UV5p. All constructs share a common transcribed leader of 60 nt (partially derived from *lac*); the only difference between constructs is the amount of *rpoS* sequence that is retained. The first entry in Table 6 shows the reference construct (substitution at the *Kpn*I site, construct K from reference 10). The deletions (Δ1 through Δ4) extend progressively closer to the *rpoS* ATG initiation codon, as shown in Fig 3. The Δ4 construct retains only 24 nt of *rpoS* sequence upstream of the *rpoS* ATG initiation codon.

There is no significant difference in either induction ratio or ppGpp response between the K construct and Δ1, and there is only a slight decrease in induction ratio as the deletion is extended in Δ2. However, a clear breakpoint can be seen between Δ2 and Δ3. It is striking that the two deletions that extend farther downstream (Δ3 and Δ4) lack the ppGpp-dependence shown by the reference construct and upstream deletions. Selected data from Table 6 highlighting the effect of ppGpp on induction ratio are shown in a bar plot in Fig. 6, panel A. The plot clearly shows that the Δ3 and Δ4 deletions retain substantial stationary phase induction but have lost the ppGpp response. The region between the Δ2 and Δ3 deletion endpoints required for this facet of the ppGpp response includes the upstream antisense element. There is also approximately a two-fold decrease in the stationary phase induction ratio

between Δ2 and Δ3. However, a substantial (five-fold) stationary phase induction is retained in both the Δ3 and Δ4 deletions, despite the fact that they do not respond to ppGpp.

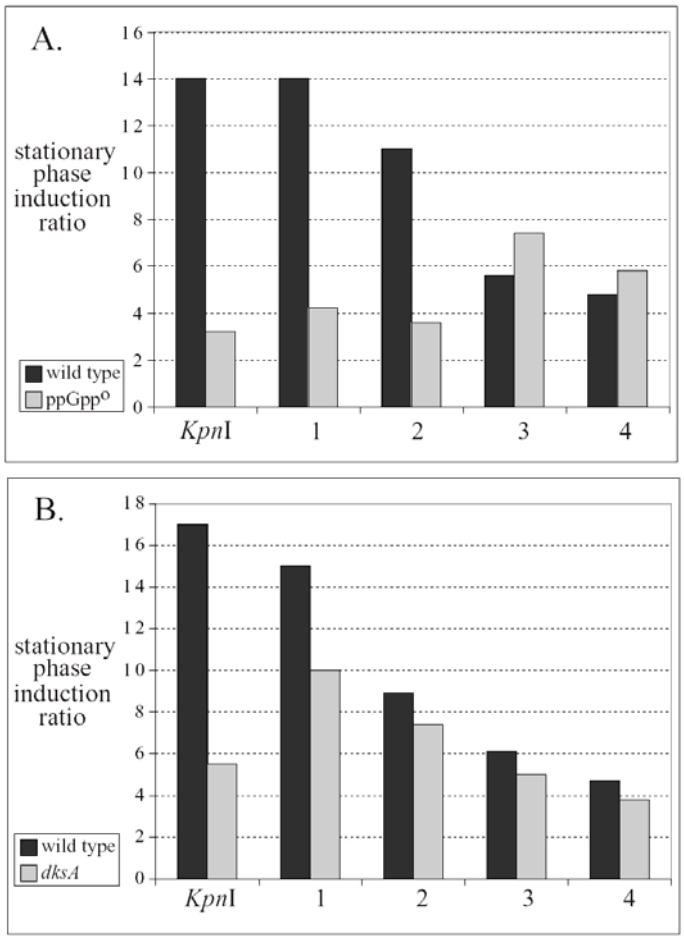


Figure 6. Deletion mapping of sequences required for ppGpp^{o} (A) and dksA (B) mutation effects on *rpoS* expression.

Various *rpoS-lac* fusion strains, all driven by the *lac*UV5 promoter but with varying amounts of the upstream *rpoS* leader, were assayed for β-galactosidase during exponential phase and stationary phase. Stationary phase induction ratios were calculated and plotted here. The underlying data for the ppGpp^{o} strain are from Table 6. The strains used are listed in Table 8.

In a previous study we described a number of mutations which affect the upstream antisense element or its complementary sequence within the RBS of *rpoS* mRNA, immediately upstream of the

rpoS ATG initiation codon (7). Two of these mutations were examined here: C469G and G549C, which alter the antisense (top) and sense (bottom) components of stem II respectively (see the legend to Fig. 3 for details). We chose these two mutations because, as tested in *S. enterica*, in the double mutant the resulting *rpoS-lac* expression phenotype is indistinguishable from wild type and very different from each single mutant, providing strong genetic support for the proposed inhibitory mRNA secondary structure. Furthermore, mutations at adjacent positions share these properties (unpublished data).

Here, it is striking that the C469G mutation allows high stationary phase expression and restores a normal induction ratio of *rpoS* in the ppGpp^{o} background (Table 6). This lesion clearly relieves most of the ppGpp requirement of *rpoS*. Furthermore, the C469G/G549C double mutant restores ppGpp dependence, which is consistent with the model that ppGpp-dependence can be counteracted by blocking interaction of the antisense element with the RBS. However, since the G549C single mutant should loosen the secondary structure as effectively as C469G, the model predicts that G549C should relieve the ppGpp requirement of *rpoS*, yet this prediction is not fulfilled.

Additionally, when comparing *E. coli* with *S. enterica*, the phenotype of the compensatory double mutant is not always as clear-cut in *E. coli*. Particularly in a wild type (*relA*$^{+}$ *spoT*$^{+}$) strain background, the C469G/G549C double mutant does not exhibit a fully wild type phenotype but rather is intermediate between C469G and wild type (Table 6): compare 120 units (wild type), 170 units (double mutant) with 210 units (C469G). On the other hand, at stationary phase in the ppGpp^{o} background, the phenotype of the double mutant is more convincingly like that of wild type: compare 8.8 units (wild type), 7.9 units (double mutant) with 47 units (C469G) and 29 units (G549C). The partial suppression

observed in the double mutant in a wild type background leaves some question whether the observed phenotypes result simply from a failure of antisense element interaction with the RBS. They might reflect an additional sequence-specific interaction. Nevertheless it is clear that C469G eliminates a requirement for ppGpp to observe normal stationary phase induction and the requirement for ppGpp is restored in the C469G/G549C double mutant.

Effect of dksA. A *dksA* insertion in *S. enterica* was reported to decrease stationary phase induction of an *rpoS-lac* protein fusion in supplemented minimal medium (41). In other work, we have found that a *dksA* deletion in *E. coli* almost completely blocks induction of *rpoS* by elevated ppGpp during exponential growth in LB medium (8). Therefore, we tested whether the effect of *dksA* on *rpoS-lac* [pr] expression exhibits the same sequence requirements as observed for the regulatory defect of ppGpp^{o}.

Expression of *rpoS-lac*[pr] in the Δ*dksA*::*tet* mutant was not detectably different from that in the wild type during exponential phase but was one-third to two-thirds of the wild type level in stationary phase,, measured either with the wild type fusion or with *lac*UV5p constructs (data not shown). The regulatory defect of *dksA*, as reflected in the stationary phase induction ratio, was larger for fusions carrying more upstream sequence (the *Kpn*I fusion in Fig. 6B). Only a small regulatory effect of *dksA* was seen for Δ1, and this was nearly completely lost for Δ2 and with reporters deleted for the antisense element (Δ3 and Δ4). This pattern is similar to that for *hfq*, where its effect on *rpoS* expression requires upstream sequences (10; see below), and differs from the requirements for ppGpp effects as described above.

We have found similar results for *dksA* in *S. enterica* in LB medium (unpublished data). A more substantial effect for *dksA* can be observed in *S. enterica* in minimal medium supplemented with amino acids (41, unpublished data); this difference may be related to the multiple auxotrophy of *dksA* mutants in both *E. coli* and *S. enterica*.

Other *trans*-acting factors. In addition to the above studies utilizing the ppGpp^{o} background (Δ*relA* Δ*spoT*) and a *dksA* mutation, we also examined the effects on stationary phase induction of *rpoS-lac* for mutations in four other genes. The mutations tested were newly constructed insertion/deletions of *barA* (29), or *ppkx* (36), and existing mutations affecting *dsrA* (deletion; 39), and *hfq* (Ω-Km insertion; 6, 28, 40).

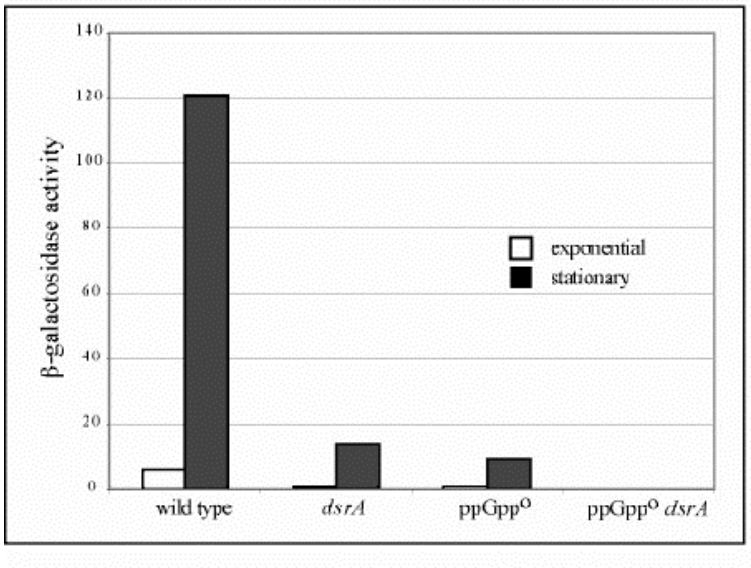


Figure 7. Additive effect of ppGpp^{o} and a *dsrA* mutation.
Activity of β-galactosidase from exponential (OD_{600} = 0.25) and stationary phase (S+3 hr) cultures are given. The strains used are listed in Table 8.

Previously, a *barA* insertion (of λp*lac*Mu53) in MC4100 was shown to reduce RpoS by Western blot and *rpoS* RNA by Northern blot (29). A *ppk ppx* mutant in the JM101 background was reported to have decreased HPII catalase (under RpoS control), and plasmid-encoded yeast PPX1 polyphosphatase interfered with *katE* and *rpoS* induction as measured by Western blot and *lac* operon fusion, consistent with positive regulation of *rpoS* by polyphosphate (36).

For the first two mutations (*barA* and *ppkx*), we found that the effects on *rpoS-lac* expression during growth in LB medium were small (data not shown). No significant effect of Δ*barA*::*tet* was observed for *katE-lac* [op] expression in either the MC4100 or MG1655 backgrounds; *rpoS-lac* [pr] expression was either unchanged (MC4100) or elevated (MG1655). The Δ*ppkx*::*tet* strain expressed *rpoS-lac* [pr] at about 75% of the wild type level in stationary phase, whereas the ppGpp^{o} derivative was about 10% of wild type in the same experiment. The Δ*ppkx*::*tet* mutant showed an *rpoS-lac* [pr] induction ratio of 26-fold, versus 34-fold for wild type. These small effects are difficult to interpret.

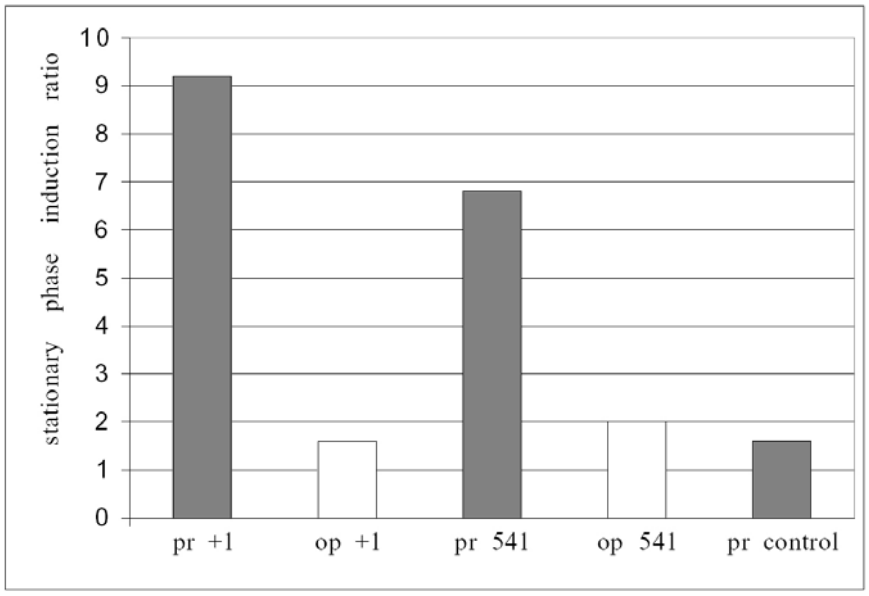
The *dsrA* gene encodes a small, untranslated RNA which is proposed to base pair with the upstream antisense element of *rpoS*, thereby freeing the RBS to be engaged by ribosomes for translation. Strong genetic evidence supports this model including the behavior of several sets of compensatory mutations (23). Sledjeski et al showed that in wild type cells RpoS abundance and *rpoS-lac* expression increase dramatically at lower growth temperatures. One major effect of the *dsrA* mutation is that this low temperature response is lost (39). At 37°C, there is no effect of a *dsrA* mutation on *rpoS-lac*

expression in either exponential or stationary phase, whether in the MC4100 background (33) or in MG1655 (our unpublished results). DsrA RNA is not required for stationary phase induction under these conditions.

In order to see the effect of loss of *dsrA*, cells were grown at 30°C. At this temperature, wild type cells with the standard fusion (construct A) showed a normal stationary phase induction (Fig. 7). Both the *dsrA* mutant and the ppGpp^{o} mutant showed a substantial reduction in *rpoS-lac* expression, but only modest effects were observed on the stationary phase induction ratio. A mutant lacking both *dsrA* and ppGpp showed a more severe phenotype in which *rpoS-lac* expression was almost completely absent. Because both the ppGpp^{o} and *dsrA* backgrounds show severe (10-fold) effects on *rpoS-lac*, we suggest that during growth at 30°C most *rpoS* expression requires both ppGpp and DsrA RNA (whether directly or indirectly). We interpret the additive effect of the combined mutations by suggesting that the two effectors act independently at least in part---ppGpp does not simply act by changing DsrA level or activity. However, we emphasize the finding that during growth at 37°C, *dsrA* function does not affect *rpoS* expression, and even at 30°C, *dsrA* mutants show a stationary phase induction ratio of about 23-fold.

We also compared the effect of Δ*dsrA* in Δ1 through Δ4 (data not shown). The model of DsrA acting as an anti-antisense RNA predicts that deletions of the antisense element should not respond to lack of DsrA. As predicted, the Δ*dsrA* deletion does not affect Δ3 and Δ4.

The last *trans*-acting regulator that we've investigated is *hfq*. We found that, as in *S. enterica* (10), the Δ2 construct does not respond to loss of *hfq* and the Δ1 construct shows only a modest effect (data not shown). Furthermore, the stationary phase induction ratio of an *hfq* mutant is very similar to the *hfq*$^{+}$ control, whether the fusion is *rpoS-lac* [pr] driven by the native promoters, or the Δ1 or Δ2 constructs, driven by *lac*UV5p (data not shown). Therefore, Hfq cannot be a mediator of stationary phase induction. Finally, many mutations identifying "regulators" of *rpoS* expression have been reported to have no effect in an *hfq* mutant background. This set includes: *hns* (28); *stpA*; *galU*, *pgi*, and *pgm* (5); and *oxyS* (44). Similarly, a *leuO* mutation makes no difference in the absence of *dsrA* (16a). Since stationary phase induction is normal in an *hfq* mutant, and these mutations presumably



pr 541 transcript AAUUGGGAUCACGGGUAGGAGCCACCUU **AUG**-*rpoS-lac*
pr control transcript AAUUUCACACAGGAAACAGCU **AUG**-*rpoS-lac*

affect RpoS secondarily through their effects on *hfq*, these factors cannot regulate stationary phase induction.

Figure 8. Stationary phase regulation requires the wild type *rpoS* RBS.

Various *rpoS-lac* fusion strains driven by the *lac*UV5 promoter were grown and assayed for β-galactosidase. The stationary phase induction ratio (expression at S+3 divided by that at E1) is plotted in bar format for protein (pr) or operon (op) versions of each fusion. Fusions marked +1 contain the entire 564 nt *rpoS* leader; those marked 541 contain only 24 nt upstream of the AUG start codon. The pr control transcript sequence is compared to the pr 541 transcript below the bar graph. In each fusion, codon 8 of the *rpoS* sequence is joined to *lac*. The strains used are listed in Table 8.

Stationary phase regulation localized to the *rpoS* RBS region. As shown in Table 6 and Fig. 6, a substantial stationary phase induction can be observed even with the Δ4 construct, driven by *lac*UV5 promoter, which contains only 24 nt upstream of the *rpoS* ATG initiation codon. All *lac* fusion constructs used so far have contained *rpoS* coding sequences extending to codon 73. We wondered whether this stationary phase response would be retained with *lac* fusions made to upstream sites, and whether operon fusions would show a difference from protein fusions. To make the new constructs, we used the λ*red* recombination method (43) to position the *lac*UV5 promoter precisely at various sites. We chose to retain the first 4 nt of the *lac* transcript (AAUU) in these fusions. Otherwise identical operon and protein fusion constructs were made by simply transforming the fusion fragment into a different host strain (see Materials and Methods for details). The results (Fig. 8) show that this aspect of the stationary phase response is seen with protein but not with operon fusions (indicating a translation-level defect) and does not require *rpoS* sequences downstream of codon 8. The fusion labeled pr control in Fig. 8, substitutes the *lac* RBS (not including operator sequences), for the *rpoS* RBS.

Discussion.

Ever since the discovery by Gentry et al that mutants lacking ppGpp are deficient in RpoS after growth to stationary phase in rich medium (14), a role for ppGpp in RpoS regulation has been an attractive unifying hypothesis. Thus, ppGpp control of RpoS might explain not only induction of RpoS during growth into stationary phase, but also growth rate regulation, induction by limitation for single nutrients and by other stresses such as challenge with high salt, and even induction during growth within

eukaryotic host cells. Here, the sensitivity obtained by using *lac* as a reporter for *rpoS* expression allows a clear demonstration that ppGpp^{o} mutants are just as defective during exponential as in late stationary phase and therefore, we suggest that ppGpp should be considered mainly as a basal and not a regulatory factor.

This conclusion should be qualified. First, ppGpp^{o} mutants do show a delay in the rate of increase in RpoS during stationary phase, so that relatively early (at the time we define as S+3 hr), there is a modest regulatory defect which is made up by a slow, late accumulation. Second, it is conceivable that ppGpp is regulatory in stationary phase in wild type cells, but a redundant mechanism operates in the ppGpp^{o} mutant. Also, the delay of the mutant in achieving stationary phase, and halting protein synthesis, could allow more time for RpoS to accumulate or might trigger some type of compensatory increase. Finally, a regulatory role for ppGpp under other conditions is not ruled out. Preliminary experiments indicate that osmotic shock can still induce *rpoS* normally even in a ppGpp^{o} host (unpublished data). But ppGpp control might explain the large difference between *rpoS* expression in rich and minimal medium. The complex nutritional defect of ppGpp^{o} mutants makes that idea difficult to test.

Analysis of *rpoS-lac* operon and protein fusions, as well as promoter substitutions and deletions, should allow us to suggest one or more mechanisms of ppGpp action in this system. The total effect is robust: expresssion of *rpoS-lac* [pr] is ca. 25-fold higher in wild type than for the ppGpp^{o} mutant at S+3 hr (Table 5). However, this large effect is distributed in small installments over several targets. The

basal increase in expression involves: (i) an effect lost when transcription is from the *lac*UV5 promoter (consistent with primer extension results in reference17), (ii) an effect seen with a protein fusion but not an operon fusion, and (iii) a target, probably transcription elongation, which is distinct from the first two. In contrast, the regulatory effect of ppGpp apparently involves the antisense element (Table 5 and Fig. 6) yet paradoxically, is still visible with an operon fusion and thus presumably involves transcription elongation (Fig. 5). The distributed nature of ppGpp's targets complicates analysis by combining several small, presumably multiplicative effects.

Another area of uncertainty involves the question whether high levels of ppGpp, achieved by inducing a truncated ribosome-independent RelA protein during exponential growth, alter *rpoS* expression in the same way as the effects seen here by use of a ppGpp^{o} mutant (14). Recent work shows that overproduction of ppGpp dramatically increases RpoS protein synthesis with little change in the amount of *rpoS* RNA (8). Our results, with only a small role for translational control, seem inconsistent with this. One resolution is to suppose that ppGpp overproduction affects *rpoS* by a different mechanism. In support of this interpretation, *dksA* function is required for ppGpp overproduction to induce *rpoS* (8), yet stationary phase regulation is normal in a *dksA* mutant. Furthermore, the sequences required for *dksA* to affect *rpoS* expression in our experiments are different than those required for ppGpp regulation (Fig. 6).

The most surprising conclusion from these experiments is that much of stationary phase regulation is retained in *lac*UV5p-driven fusions that retain only 24 nt upstream of the *rpoS* AUG initiation codon and an additional 7 codons downstream (Fig. 8). This effect of sequences close to the

ribosome binding site is independent of the antisense element, ppGpp, and all known trans-acting regulators of *rpoS* including *dsrA* and *dksA*.

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Fis regulates transcriptional induction of RpoS in *Salmonella enterica*

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Abstract

The sigma factor RpoS is known to regulate at least 60 genes in response to environmental sources of stress or during growth to stationary phase (SP). Accumulation of RpoS relies on integration of multiple genetic controls including regulation at the levels of transcription, translation, protein stability and protein activity. Growth to SP in rich medium results in a 50-fold induction of RpoS, although the mechanism of this regulation is not understood. Here we characterized the activity of promoters serving *rpoS* in *Salmonella enterica*, and report that regulation of transcription during growth into SP depends on Fis, a DNA-binding protein whose abundance is high during exponential growth and very low in SP. A *fis* mutant of *S. enterica* shows a nine-fold increase in expression from the major *rpoS* promoter (P_{rpoS}) during exponential growth, whereas expression during SP is unaffected. Increased transcription from P_{rpoS} in the absence of Fis eliminates the 10-fold transcriptional induction as cells enter SP. The mutant phenotype can be complemented by wild type *fis* carried on a single-copy plasmid. Fis regulation of *rpoS* requires the presence of a Fis site positioned at -50 with respect to P_{rpoS} and this site is bound by Fis *in vitro*. A model is presented in which Fis binding to this site allows repression of *rpoS* specifically during exponential growth, thus mediating transcriptional regulation of *rpoS*.

Introduction

Bacteria maintain intricate signaling networks that sense the environment and adjust cellular physiology accordingly. In *Salmonella enterica* (*S. enterica*) and *Escherichia coli* (*E. coli*), less favorable growth conditions (e.g. nutrient limitation, starvation, low temperature, or osmotic shock) initiate a general stress response by triggering the synthesis of the RNA polymerase sigma factor RpoS (σ^S). By directing RNA polymerase to promoters of specific genes involved in stress resistance, RpoS serves as the central regulator of the general protective response, also known as SP, and thus increases survival (28). The importance of RpoS to *S. enterica* pathogenesis is evident from a mouse model involving lethal infections where *rpoS* mutants are completely avirulent (22).

The complexity of RpoS regulation is illustrated by the variety of mechanisms reported so far in *E. coli*: transcription, translation, protein turnover and protein activity (28). One of the best characterized induction phenomena is regulation of RpoS translation at low temperature in rich medium (≤ 30°C). This stimulus increases transcription of a regulatory RNA, DsrA, which can pair with an upstream antisense element in the leader region of the *rpoS* transcript to relieve the antisense element's inhibition of *rpoS* translation (38). This process requires the Sm-like RNA-binding protein, Hfq, and results in activation of RpoS expression at a post-transcriptional step. Notably, *hfq* mutants show normal SP induction of RpoS in rich medium, both in *E. coli* (29) and *S. enterica* (our unpublished data).

Another regulatory pathway limiting RpoS abundance in growing cells is proteolytic degradation involving the ATP-dependent ClpXP protease and a response regulator called MviA (in *S. enterica*) or

SprE / RssB in *E. coli* (52, 62). In this pathway MviA is activated by an unknown stimulus through phosphorylation on D58, which substantially increases its ability to bind to RpoS. The relevant kinase has not yet been found (16, 28). The binding event (dependent on K173 of RpoS) results in a sequestered non-functional RpoS molecule and thus can modulate RpoS activity in itself (8, 75). The MviA-RpoS complex also interacts with the ClpXP protease, which then actively degrades RpoS, recycling MviA (44, 52). RpoS elevates transcription of the response regulator during SP, thus constituting an auto-regulatory loop in which the concentration of MviA is a limiting factor for the rate of RpoS degradation *in vivo* (53, 57).

Perhaps the most striking induction of RpoS is observed during growth to SP in rich medium, where the level of induction exceeds 30-fold, based on the activity of RpoS-responsive reporters and *rpoS-lac* fusions (28, our unpublished data). The transcriptional component of this induction ranges from 5-10 fold (28, 29). Expression levels are significantly lower in the absence of guanosine tetraphosphate during both growth and SP, but the actual induction ratio is nearly unchanged (29). The cyclic-AMP (cAMP) receptor protein, Crp, is also thought to be involved in *rpoS* transcriptional control yet the effect of the mutants is modest and interpretation is difficult due to the growth deficiency of the *crp* or *cya* (defective in adenylate cyclase) mutants in combination with growth-rate transcriptional control of *rpoS* (36, 37).

In this study we show that the Fis protein (factor for inversion stimulation) is involved in RpoS regulation during growth in rich medium. Fis is a DNA-binding and bending protein that was initially characterized for its stimulatory role in site-specific DNA recombination (31, 34). Fis has been implicated in many other processes such as stimulation of excision and integration of lambda (4, 5, 20,

67), DNA replication at *oriC* (24, 26, 58), transposition (70), invasion of HEp-2 cells (71) and transcriptional activation and repression of several genes including *hns, leuV, gyr, tyrT, proP, nuo, osmE,* and rRNA operons (21, 56, 33, 3, 72, 68, 12, 74).

Here we investigate *rpoS* promoter activity and demonstrate that Fis mutants have elevated expression of *rpoS* that is specific to exponential phase. This pattern of regulation is in good agreement with the known variation in Fis abundance in different phases of growth: Fis is undetectable in SP but is present at over 40,000 dimers per cell upon dilution into fresh medium (2, 6, 50). Based on these results and the requirement for specific sequences upstream of the major *rpoS* promoter, we present an intuitive model for transcriptional regulation of *rpoS* in which Fis binds to and represses transcription from P_{rpoS}.

Materials and Methods

Media and growth conditions.

Bacteria were grown at 37°C (except where noted) in various media: LB medium (63), LB medium containing 1x NCE minimal salts (buffered LB medium; 10), nutrient broth supplemented with 5 g/liter of NaCl (NB, Difco), and brain heart infusion (BHI, Difco). Liquid minimal medium was morpholinepropanesulfonic acid (MOPS) medium (48) as modified (11), supplemented with 0.2% glucose as the carbon and energy source. When indicated, minimal medium was supplemented with 1% casamino acids (CAA, Difco). Plates were prepared using nutrient agar (Difco). Antibiotics were added to final concentrations in selective media as follows: 100 μg of sodium ampicillin/ml, 20 μg of

chloramphenicol/ml, 50 μg of kanamycin sulfate/ml, and 20 μg of tetracycline hydrochloride/ml, except that the ampicillin concentration was reduced to 50 μg/ml for use with single copy plasmids. MacConkey lactose agar was prepared as described (43). X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) was used at 50 μg/ml.

Bacterial strains and construction. Most of the strains used in this study were derived from wild type *Salmonella enterica* LT2 (serovar Typhimurium); all strains are described in Table 8. We obtained strain LT2 from John Roth. Although this strain is the reference wild type, it has been shown to contain a nonfunctional *mviA* gene (V102G substitution) and is therefore defective in regulated RpoS turnover by ClpXP proteolysis (9, 16). LT2A is a derivative of LT2 whose only known difference is that it contains a functional *mviA* gene. LT2A was used to investigate RpoS proteolysis as indicated in the text (16). The phage P22 mutant HT105/1 *int-201* was used for transduction in *S. enterica* by standard methods (18), while P1 *vir* was used for transduction of fusions carried on the *E. coli* chromosome into *S. enterica* strain TE7304 (see below). Transductants inheriting *cya*::Tn*10* and *crp*::Tn*10* insertions were selected on NB plates containing tetracycline and supplemented with 0.2% glucose. The *crp** allele used in this study was originally isolated by Ailion *et al*. (1).

Construction of promoter fusions. The system used to construct promoter fusions relies on the cloning of PCR products amplified from *S. enterica* LT2 chromosomal DNA. In the Genbank sequence file AE008833.1 (40), the complement of the *nlpD* and *rpoS* sequences is given. Here, positions are indicated using coordinates from AE008833.1, including *nlpD* (bp 13178 -> 12045) and *rpoS* (bp 11982 > 10990), and coordinates are listed with the same polarity as the genes (higher to lower numbers).

DNA fragments were produced with flanking *Eco*RI (upstream) and *Bam*HI (downstream) sites and cloned into pRS551 to generate transcriptional *lacZ* fusions (19, 64). To this end we made four different P_{rpoS}-*lac* [op] fusions: construct A, bp 12764-12338 (TE8744); construct D, bp 12663-12467 (TE8971); construct E, bp 12663-12528 (TE8868); and construct F, bp 12586-12467 (TE8947). (All primer sequences are available upon request.) The P_{nlpD}-*lac* [op] fusion was generated in the same manner and contains the region encompassing bp 13471-13052. Recombinant plasmids were used to transform DH5α and the fusions confirmed by PCR and DNA sequencing. The fusions were recombined into the *E. coli* chromosome as described (19), then transduced into *S. enterica* using P1 *vir*. In *S. enterica*, they are located at the *put* locus in single copy.

Construction of deletion/insertions and point mutations. Most other constructs were made by direct transformation of *S. enterica* with different DNA segments amplified by PCR, utilizing the lambda *red* recombination system as provided on plasmid pKD46 (17). Exponential-phase recipient cells, growing at 30°C with selection for Amp^R, were induced by treatment with 0.2% arabinose for 1 hr before electroporation, after which transformants were plated and selected at 37°C.

To construct site-directed mutations in P_{rpoS}-*lac* [op], we developed a multi-step method. In order to prevent unwanted recombination events, a Cam^R cassette was inserted at the native *rpoS* locus (deleting bp 12720-12142). The Cam^R cassette was amplified using *rpoS*-specific primers extended to provide homology to *cat* at the 3' end as follows: TGCTTTTGCCGTTACGCACCAC (upstream) and GCCTCAGGCATTTGAGAAGCAC (downstream). The resulting insertion deleted the *rpoS* promoter region and gave loss of *rpoS* function as determined by absence of visible catalase activity (production of bubbles after spotting 5 μl of hydrogen peroxide on a patch of bacteria). In the second step, a *tetAR*

cassette (pWM7 as the template; 42) was inserted to delete the *rpoS* promoter of the P_{rpoS}-*lac* [op] fusion. Amplification used *rpoS*-specific primers extended to provide homology to *tet* at the 3' end as follows: CTCTTGGGTTATCAAGAGGG (*tetA*); ACTCGACATCTTGGTTACCG (*tetR*). The *tetAR cassette* is inserted at bp 12580-12555 of the P_{rpoS}-*lac* fusion in strain TE8864.

In the third step, a point mutation and an in-frame deletion mutation (both unmarked by drug resistance) were introduced by transformation of strain TE8864. PCR products were prepared containing either three point mutations or an in-frame deletion of a predicted Fis binding site. The upstream (mutagenic) primers used were GACCAGGTCTGCACCAAATGCCACGGTTGCAGTTGC and CACCCAGGCGGATGCAGCACAGCAAGGAGTTGTGACCAGG-Δ-GCAGTTGCGTCTCAACCAAC, together with a downstream primer from within *lacZ*. The desired transformants acquired the Fis site mutation and lost the *tetAR* insertion. These transformants also acquired a functional *rpoS* promoter; this allowed screening for them as Lac^+ Tet^S colonies that were confirmed by sequencing.

Isolation of *rpoS*::MudJ insertion and construction of promoter deletions. A large pool of MudJ insertions in LT2 was first generated by standard methods (30). A phage P22 lysate grown on this pool was then used to transduce strain TE8607 to Cys^+ Kan^R on minimal medium containing X-gal. Blue (Lac^+) transductants were then screened by testing patches of cells grown on NB Kan agar for catalase activity as described above. Putative *rpoS* mutants were purified and tested by PCR, then confirmed by DNA sequencing. The *rpoS1082*::MudJ insertion used in these experiments lies at codon 66 of *rpoS* (bp 11784; strain TE8737).

Several insertion / deletions were constructed in the TE8737 background using the *tetAR* cassette and the lambda *red* method. This method utilized strain TE8738, *rpoS*::MudJ containing pKD46 (17), as the target for transformation with PCR-generated *tetAR* inserts derived from pWM7. The linked MudJ and *tetAR* insertions were then backcrossed by P22 mediated transduction to *S. enterica* LT2 and the insertion joint was confirmed by DNA sequencing (see Table 8 for exact location of the *tetAR* insertions). To construct an in-frame deletion of the *rpoS* promoter region, we inserted *tetAR* to knock out P_{rpoS} in the context of the MudJ fusion (TE8913) and subsequently transformed the strain with pKD46 (TE8915). This strain served as the recipient for transformation with a PCR product carrying an in-frame deletion of the *rpoS* promoter (bp12582-12540). The deletion was generated by PCR using a 60-mer oligonucleotide with *rpoS* sequence interrupted by a 30 bp deletion including both the -35 and -10 hexamers of the *rpoS* promoter:

AGGAGTTGTGACCAGGTCTGCACAAAATTCCACCGTTGCA-Δ-

GAGGGCTCAGGTGAACAAAG, together with a downstream primer at bp 12301. Although deleted for the P_{rpoS} promoter, *lac* is expressed from P_{nlpD} in these strains, hence, transformants were screened for a subtle Lac^+ phenotype and confirmed as Tet^S and by DNA sequencing.

5'RACE reaction. RNA was isolated from wild type LT2 cells during exponential phase using a RNAeasy mini Kit (Qiagen). RNA served as the template for the 5' RACE reactions performed using the BD SMART RACE cDNA amplification kit as described by the manufacturer (BD Biosciences) with one exception. A *rpoS* specific primer was used in first strand cDNA synthesis, positioned at the initiation codon of *rpoS*. RACE reaction products were eluted from 1% agarose gels, using the NucleoTrap Gel extraction kit (BD Biosciences), T/A cloned into a pCR4-TOPO vector (Invitrogen) and subsequently used to transform TOP 10 Electrocomp cells (Invitrogen). Transformants were selected on

NB Kan plates containing X-gal (screening for a Lac^- phenotype). Plasmids were analyzed by PCR amplification of the insert followed by DNA sequencing.

Assay of β-galactosidase. Cells were centrifuged, resuspended in Z-buffer (100 mM $NaPO_4$ [pH 7.0], 10 mM KCl, 1 mM $MgSO_4$) and then permeabilized by treatment with sodium dodecyl sulfate (SDS) and chloroform (43). The samples from exponential-phase time points were concentrated before assay to be approximately equal in density to samples obtained at later times. For all experiments, exponential phase is defined as $OD_{600} = 0.25$. Assays were performed in Z-buffer containing 50 mM β-mercaptoethanol by a kinetic method using a plate reader (Molecular Dynamics). Activities (change in optical density at 420 nm [OD_{420}] per minute) were normalized to actual cell density (OD_{650}) and were always compared to appropriate controls assayed at the same time. All the β-galactosidase assays were performed within 1 hour of the time of sampling, during this interval cultures were kept on ice in Z buffer. The values shown are averages of at least 3 experiments with a standard deviation of less than 15% unless otherwise stated.

Immunological detection of proteins. For Western blots, cultures were grown as described in the text. Electrophoresis and protein transfer were as described previously (13, 16). After transfer to a Sequi-Blot PVDF membrane (BioRad), blots were blocked in non-fat milk and incubated in Phosphate Buffered Saline-Tween containing the anti-RpoS monoclonal antibody R12 (13), which is of the γ2a isotype. After 30 min. incubation, blots were washed twice in PBS-Tween, then incubated for 30 min in PBS-Tween containing biotinylated goat anti-mouse immunoglobin (Ig), and finally with streptavidin-conjugated horseradish peroxidase (both from Southern Biotechnology Associates). Detection was by

enhanced chemiluminescence (Amersham Biosciences).

Complementation test. TE8905 harbors pFis349 (71), which contains a 1.68 kb DNA fragment encoding the *orf1-fis* operon of *S. enterica* cloned into the single-copy plasmid pGS349 (32). Plasmid DNA isolated from an *E. coli* host was used to transform an r^-m^+ *S. enterica* strain (TE315) and subsequently, wild type (TE8744) and Δ*fis* (TE8764) strains containing the P_{rpoS}-*lac* [op] fusion. Cultures were grown in LB with ampicillin and assayed for β-galactosidase as described in text.

Gel-Shift assay. DNA target fragments were amplified from the *S enterica* chromosome using primers engineered to generate *Eco*RI and *Bam*HI restriction sites at opposite ends of the product. We made three different shift targets, designated A, B, and C, that correspond, respectively, to bp 12750-12604, bp 12643-12547, and bp 12574-12475 of the P_{rpoS} promoter. We also generated two mutant targets derived from target B, designated B* and B^{Δ}, using the same primers as for B but chromosomal templates isolated from strains TE8887 and TE8895. The PCR reactions were purified (Qiagen) and digested with *Eco*RI and *Bam*HI restriction endonucleases. The fragment ends were then labeled by incorporation of [α^{32}P]ATP using the Klenow fragment of DNA polymerase as described by the manufacturer (Promega), and purified using a PCR purification kit. Radiolabeled DNA fragments (ca. 15,000 cpm per reaction) were incubated with purified Fis protein (gift of R. Johnson), for 15 min at room temperature in buffer containing 20 mM Tris HCl [pH 7.5], 80 mM NaCl, 1 mM EDTA, 5% glycerol, and 2 ng of poly[d(I-C)]/μl. Binding reaction products were analyzed by electrophorhesis on a 8% native polyacrylamide gel as previously described (72). The gels were then dried and the radioactive DNA detected by autoradiography.

A.

Reporter	Background	β-galactosidase activity E	SP	SP/E
katE-lac [op]	*wt*	0.41	17	41
	clpX	1.9	75	39
	mviA	2.4	75	31

B.

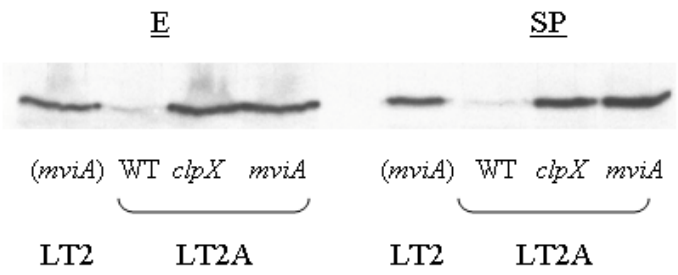

Figure 9. SP induction of RpoS is normal in turnover-defective mutants.

(A) LT2A strains harboring the RpoS reporter, *katE-lac* [op], and carrying the indicated mutations were sampled for β-galactosidase activity during exponential phase at $OD_{600} = 0.25$ (E), and after 24 hours of growth (SP). SP induction was calculated as the ratio of the SP activity to the exponential phase value (SP/E). (B) The indicated strains were probed for RpoS protein at time points E and SP by Western analysis. The gel for the exponential experiment was loaded with the lysate recovered from 10-fold more cells in order to visualize low concentrations of RpoS in the LT2A background. All strains were grown at 37°C in LB medium. Strains: LT2A *mviA* (TE6851); LT2A *clpX* (TE6850).

Results

SP induction of RpoS in *S. enterica* is normal even in the absence of regulated proteolysis.

Studies of Schweder (62) and Zgurskaya (73) suggest that in starving *E. coli* cells RpoS abundance increases mainly as a result of increased protein stability. Another *E. coli* study reports RpoS induction in the absence of an intact RpoS degradation pathway (52). We investigated the role of this pathway in the induction of RpoS for *S. enterica* grown to SP in rich medium (LB). A *katE-lac* [op] fusion was used as a reporter of RpoS activity (13, 45). Expression of the *katE-lac* fusion was measured both in a

wild type LT2A background and in *clpX* and *mviA* mutants defective in regulated turnover of RpoS. [The *mviA* gene is the *S. enterica* ortholog of *E. coli rssB* / *sprE* (9)].

During exponential growth (OD_{600} = 0.25), expression of *katE-lac* in both the *clpX* and *mviA* mutants was approximately five-fold higher than in the wild type (Fig. 9A). This result is consistent with the idea that MviA and ClpXP function together to degrade RpoS during exponential growth (16, 44, 52). A quantitatively similar increase in *katE-lac* expression in the mutant backgrounds was also observed after 24 hours of growth (defined as SP). This result suggests that the MviA and ClpXP pathway for RpoS degradation functions at a similar level during both exponential growth and at SP in LB medium. The normal SP induction ratio for *katE-lac* expression in the *mviA* and *clpX* mutants indicates that this proteolytic pathway does not regulate SP induction of RpoS (ratio shown as SP/E, Fig.9A). Somewhat higher *katE-lac* activity in the *mviA* mutant compared to the *clpX* mutant is consistent with a role for MviA to sequester RpoS even if proteolysis is blocked (8, 75).

To confirm this result, the abundance of RpoS was determined by Western blot analysis of cells in both exponential growth and SP (10-fold more material was loaded for exponential cells to allow visualization of RpoS in wild type). Both *clpX* and *mviA* mutations resulted in a marked increase in RpoS abundance compared to wild type LT2A. The increased amount of RpoS in the mutants appeared similar to the amount of RpoS detected in the LT2 strain, which is naturally defective in *mviA* function. The relative increase in RpoS observed in the mutant backgrounds for cells in exponential growth is apparently the same as during SP (Fig.9B). The combined *lac* fusion and Western blot results indicate that in *S. enterica*, the MviA/ClpXP turnover pathway does not mediate the SP induction of RpoS in LB medium.

Promoters contributing to *rpoS* expression in *S. enterica*. We next considered transcriptional regulation of *rpoS*. Studies in *E. coli* (28, 29, 60) indicate that regulation of *rpoS* at the transcriptional level may be particularly important when cells grow to SP in rich medium, in contrast to the post-transcriptional mechanisms mediating response to osmotic shock, carbon starvation or low temperature. We previously demonstrated a 15-fold induction of an *rpoS-lac* transcriptional fusion, when the *rpoS* gene was derived from *E. coli*, and studied both in an *E. coli* (29) and an *S. enterica* host (13).

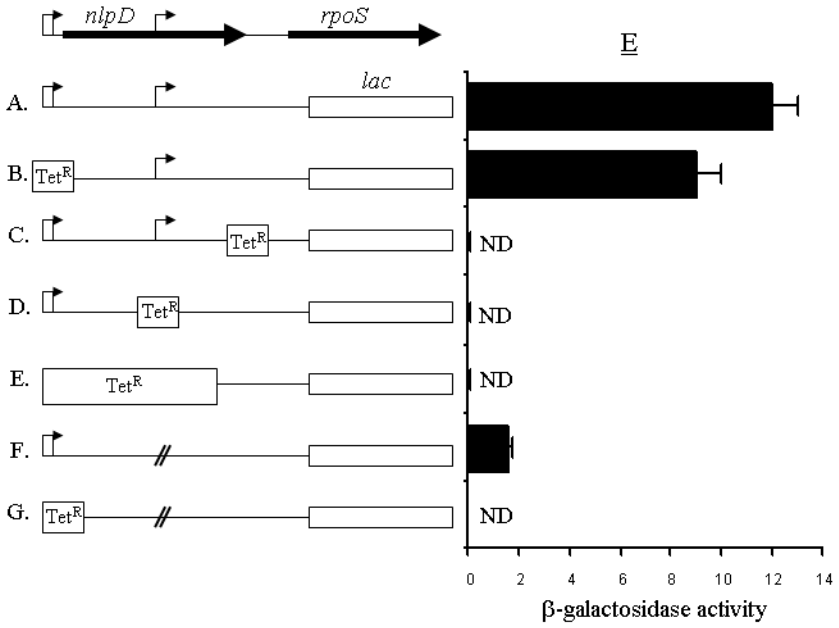

Figure 10. Promoter activity analyzed using *rpoS*::MudJ as a reporter.
The top line depicts the genetic organization of the *rpoS* region, with long horizontal arrows showing gene and transcriptional polarity, and small bent arrows indicating the promoters contributing to *rpoS* expression. Construct A contains the *lac* fusion formed by MudJ insertion at codon 66 of *rpoS*, and is otherwise wild type. Constructs B to E contain the same *rpoS*::MudJ insertion as construct A and in addition, contain insertions of a tetracycline resistance cassette (Tet^R) accompanied by deletions (insertion / deletions). Construct F is identical to construct A except it contains an in-frame deletion (represented by slanted lines) of the P_{rpoS} promoter. Construct G is like construct F but also contains the insertion / deletion from construct B. The MudJ element is not drawn to scale. Construction details are given in Materials and Methods and precise insertion sites are given in Table 8. Shown next to each fusion is the β-galactosidase activity as determined at OD_{600}=0.25 (E) in cultures grown at 37°C in rich medium. [N.D.(not detected).] Strain numbers for these constructs are as follows: A, TE8737; B, TE8901; C, TE8907; D, TE8913; E, TE8914; F, TE8925; and G, TE8937.

Analysis of *rpoS* transcription in *E. coli* by primer extension, and in *Salmonella dublin* by both primer extension and Northern blot, established that *rpoS* is transcribed from two distinct promoter regions (35, 51, 66). Two closely-spaced and relatively weak promoters (P_{nlpD}) generate a bicistronic *nlpD-rpoS* message, while the major promoter (P_{rpoS}) is located approximately in the center of the *nlpD* gene and generates a monocistronic *rpoS* transcript with a long untranslated leader region of 566 nucleotides (35, 51). *E. coli* and *S. dublin* share identical -35 and -10 hexamers for P_{rpoS} with a 17 bp spacer, and initiate transcription at the same nucleotide. This region of *S. enterica* is identical to *S. dublin* and therefore it is likely that the defined P_{rpoS} promoter of *E. coli* and *S. dublin* is conserved in *S. enterica*. We investigated the transcriptional start of P_{rpoS} in *S. enterica* using RACE cDNA amplification. Total cellular RNA harvested during exponential phase served as template in a reverse transcription reaction that exhibits terminal transferase activity, adding 3-5 residues to the 3' end of the first strand cDNA. These residues anneal to an oligonucleotide that serves as an extended template for reverse transcriptase thus generating a complete cDNA copy of the original RNA with known sequence at the end. The first strand cDNA is then used directly in a 5' RACE PCR reaction to generate double stranded cDNA products. Three cDNA products were observed using a primer positioned at the initation codon of *rpoS* and these corresponded to the predicted sizes of transcripts from P_{nlpD} and P_{rpoS} (data not shown). The product representing P_{rpoS} was cloned and sequenced. The results positioned the first base of the transcript 566 nucleotides upstream of the *rpoS* coding region at the identical initiating nucleotide of P_{rpoS} in *E. coli* and *S. dublin*.

Genetic analysis of the region upstream of *rpoS* in *S. enterica* suggests a similar pattern of transcriptional control compared to *S. dublin* and *E. coli*. We first isolated an insertion of the *lac* fusion-forming transposon MudJ in the *rpoS* gene, forming a transcriptional fusion of *rpoS* to *lac* (at codon 66

of *rpoS*), to use as a reporter of *in vivo* transcriptional regulation (Fig. 10). Expression of the *rpoS*::MudJ fusion was analyzed during exponential phase in combination with insertion / deletion mutations constructed with a *tet* cassette predicted to affect the promoters serving *rpoS* or a deletion mutation of P_{rpoS} (Fig. 10). All constructs were placed at the native *rpoS* locus in the bacterial chromosome.

Insertion of *tet* downstream of P_{rpoS} or an insertion of *tet* which also makes a small deletion encompassing P_{rpoS}, eliminated detectable activity of the *rpoS*::MudJ reporter (Fig. 10, constructs C and D, lower limit of detection is 0.8 U). In contrast, an insertion / deletion of the P_{nlpD} promoter region but retaining P_{rpoS} showed relatively high (≈ 75%) expression of the parental *rpoS*::MudJ (construct B). In a further test, a precise in-frame deletion of 30 bp including the conserved -35 and -10 hexamers of the P_{rpoS} promoter was constructed (Fig. 10, construct F). This deletion reduces expression of *rpoS*::MudJ to ≈ 15% of wild type. Similar to *rpoS* transcription in *E. coli*, we conclude that in *S. enterica*, P_{rpoS} is the major *rpoS* promoter and P_{nlpD} plays a minor role.

Activity of the major *rpoS* promoter, P_{rpoS}. Since most transcription of *rpoS* originates from P_{rpoS}, we characterized this promoter in isolation by using a *lac* fusion system described previously (19), in which the fusion is transferred to the *S. enterica* chromosome at the *put* locus. The fusion employed, P_{rpoS} -*lac* [op] (strain TE8744), includes 426 bp encompassing P_{rpoS}, from -209 to +217 with respect to the transcriptional start site. Activity of β-galactosidase was determined during exponential growth and in SP for cultures grown at 37°C in different media (Table 7). Expression of the P_{rpoS} –*lac* [op] fusion increased 8-fold in SP during growth in two different rich media, which is consistent with results obtained from *E. coli* (28).

Table 7. Activity of P_{rpoS} in different media

Growth Medium	β-galactosidase activity[a]		
	E	SP	SP/E
LB	31	230	7.6
Buffered LB	28	262	9.3
Buffered LB + glucose	28	208	7.4
BHI	31	232	7.4
Minimal glucose	124	191	1.5
Minimal glucose + casamino acids	109	203	1.8

[a] exponential phase (E) and stationary phase (SP) are defined in text. Values are averages with a variation of <17%. Stationary phase induction is defined as SP/E.

Both carbon availability and the production of weak acids have been shown to affect *rpoS* transcription in *E. coli* (37, 46, 60), and we hypothesized that these stimuli might be involved in SP induction of *S. enterica rpoS*. However, when activity and SP induction of P_{rpoS} -*lac* [op] were assayed in buffered LB medium and buffered LB supplemented with 0.2% glucose the results were very similar to those observed in LB and brain heart infusion (BHI) medium. This suggests that neither pH changes nor lack of a suitable carbon source are responsible for SP induction. When strain TE8744 was grown in minimal medium (either with or without casamino acids), *rpoS-lac* expression increased approximately 3.5-fold over expression in LB. This increase in expression was specific to exponential growth in minimal medium. As a result, SP induction of *rpoS* transcription was much reduced. This result might be explained by growth-rate regulation of *rpoS* transcription, however, our previous analysis of *rpoS* growth rate regulation in *S. enterica* indicated that it is mainly at a post-transcriptional level (16).

No auto-transcriptional role of *rpoS*. The transcriptional start of P_{rpoS} is preceded by a typical

sigma 70 RNA polymerase-dependent promoter consensus sequence (TTGCGT-17 nt spacer-TATTCT). To examine whether RpoS contributes to its own transcription, we investigated the activity of several promoters as well as P_{rpoS} in both wild type and *rpoS* mutant backgrounds. Strains harboring P_{rpoS} -*lac* [op] or a P_{nlpD} -*lac* [op] fusion, 419 nucleotides encompassing the *nlpD* promoter region (-264 to +154), in either a wild type or *rpoS* background, were grown at 37°C in LB medium for 24 hours and assayed for β-galactosidase activity (Fig. 11). A small increase in the expression of P_{nlpD} and P_{rpoS} was evident in the mutant background, rather than the decrease predicted by a model involving self-transcription. As a positive control, we used the *katE-lac* [op] fusion and observed a 95% reduction in *katE-lac* activity in the *rpoS* mutant (13, 59). A *lac*UV5-*lac* [op] fusion was used as a negative control. Expression of this fusion demonstrated a 15-20% increase in the absence of RpoS, similar to that seen with the P_{nlpD} and P_{rpoS} fusions. These results confirm that RpoS is not involved in autoregulation during SP. The increased expression of sigma 70 promoters in the absence of RpoS is consistent with competition of sigma factors for RNA polymerase (23).

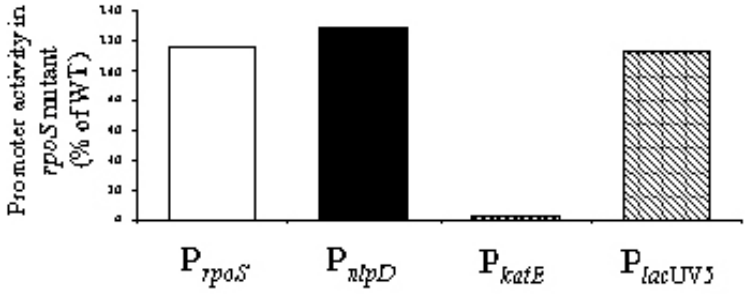

Figure 11. Testing autotranscription of *rpoS*.

LT2 strains harboring transcriptional *lacZ* fusions, expressed from the indicated promoters, in either a wild type or *rpoS* mutant background, were grown at 37°C in LB medium for 24 hours and β-galactosidase activity was determined. Promoter activity in the *rpoS* mutant is plotted as a percentage of the activity in the corresponding wild type strain. Strains were as follows: P_{rpoS} (TE8744, TE8758), P_{nlpD} (TE8698, TE8761), P_{katE} (TE6153, TE8760), P_{lacUV5} (TE6676, TE8759).

A.

-175 GTATAGCCTGAATGTAGGGCAAACTTTGCAAGTTGGTAACGCATCCGGTA

-125 CGCCAATTACTGGCGGAAATGCGATCACCCAGGCGGATGCAGCACAGCAA

-75 TGAGTTGTGACCAGGTCTGCACAAAATTCCACCGTTGCAGTTGCGTCTCA

-25 ACCAACAATTACGTATTCTGAGGGCTCAGGTGAACAAAGTGCTAACAAAA

+26 TGTTGCCAAACAACAAGCCTGCTGGGACGGTTGTCACAG

B.

Fis binding consensus ATTGNTCAAAATTTGANCANT

Fis site	Position	Sequence	Bit score
I.	-173 to -153	ATAGCCTGAATGTAGGGCAAA	5.9
II.	-117 to -97	ACTGGCGGAAATGCGATCACC	6.1
III.	-60 to -40	TCTGCACAAAATTCCACCGTT	10.9
IV.	+4 to +24	GGTGAACAAAGTGCTAACAAA	4.1
V.	+14 to 34	TGCTAACAAAATGTTGCCAAA	4.1

Figure 12. Analysis of the sequence near P_{rpoS}.

(A) The sequence of the P_{rpoS} promoter region is shown (bp 12764-12338 of AE008833.1). Predicted Fis binding sites are underlined, and arrows designate putative CRP-binding half-sites. The numbering is relative to the transcriptional start site (labeled +1). (B) The consensus sequence for Fis protein binding is given as well as the sequences of predicted Fis binding sites near P_{rpoS}, individually designated by Roman numerals. The column labeled bit score represents the similarity of each putative site to a collection of known Fis binding sites as determined by information analysis (described in the text). Five of the most conserved bp in the consensus are marked with an underline (where present in each sequence), and the asterisk marks the axis of rotational symmetry for the Fis consensus sequence.

Computational analysis of the *rpoS* promoter. To further explore the transcriptional regulation of *rpoS*, we used the DNA-motif search engine available at http://arep.med.harvard.edu/ecoli_matrices/ to recognize potential protein binding sites in the region of the P_{rpoS} promoter (Fig. 12; 55). This program utilizes the known, characterized binding sites of 59 transcriptional regulators to predict putative binding

sites throughout the entire *E. coli* chromosome. Results suggested putative binding sites for a large number of regulators including CytR, CRP, DnaA, FarR, Fis, FNR, HNS, IHF, GlpR, Lrp, MalT, MetJ, MetR, NarL, OmpR, SoxS and TyrR. Of these, only CRP has been reported as a regulator of *rpoS* transcription (36, 37). We constructed *tet* insertion / deletions in *cytR*, *fnr* and *dps*. No role for these three genes in the control of *rpoS* transcription was indicated, based on equivalent activity of P_{rpoS} -*lac* [op] in the wild type and mutant backgrounds (data not shown). The roles of Fis and CRP were further investigated.

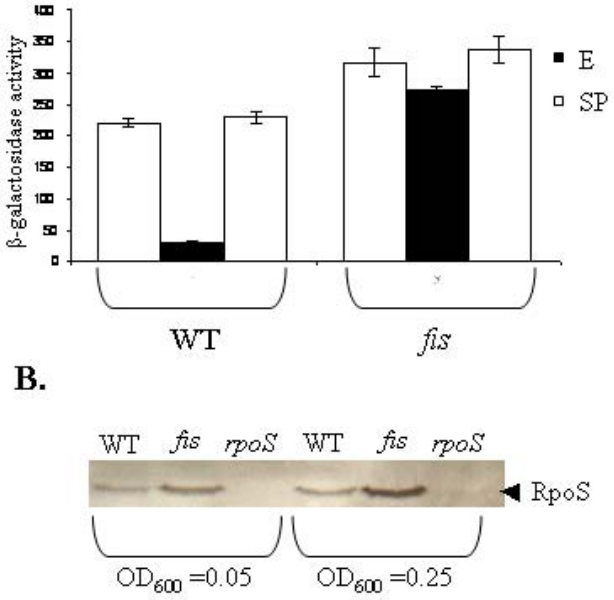

Figure 13. RpoS protein is elevated in a *fis* null mutant.

(A) Wild type and *fis* mutant strains carrying P_{rpoS}-lac [op] (TE8744 and TE8764 respectively) were grown to SP and the β-galactosidase activity determined; the same cultures were diluted into pre-warmed fresh LB medium to allow exponential growth into SP, and the activity determined for both E and SP. (B) Wild type (TE6285) and *fis* mutant (TE8768) strains were analyzed for RpoS protein at two exponential phase time points as described in Materials and Methods.

P_{rpoS} expression and RpoS protein level are elevated in a *fis* mutant. One of the most convincing (and intriguing) potential binding sites was a strong Fis site centered at bp -50 with respect to the transcriptional start. Repression of *rpoS* transcription by Fis was an attractive hypothesis because the amount of Fis varies substantially at different points along the growth curve: Fis is abundant during exponential phase when RpoS is at a low level, whereas the Fis level drops sharply in SP as RpoS is induced (2, 6). To investigate the role of Fis in *rpoS* regulation, we tested the effect of a *fis* insertion / deletion (49) on the activity of P_{rpoS} -*lac* [op]. During exponential phase, P_{rpoS} -*lac* expression was 9-fold higher in the *fis* mutant than in wild type (Fig. 13A). This large increase was evident throughout exponential phase, yet there was little difference from wild type during SP. Since the negative effect of Fis is restricted to exponential phase, this finding supports a role for Fis as negative regulator of *rpoS* at the transcriptional level. The activity of P_{rpoS} -*lac* [op] in both wild type and *fis* mutant backgrounds did not change even after extended growth in exponential phase, achieved by three repeated dilutions of dividing cells into pre-warmed fresh LB medium, (data not shown).

Western blot analysis was used to determine the abundance of RpoS protein in the *fis* mutant. Samples for Western analysis were taken two generations after dilution (OD_{600} = 0.05) and near mid-exponential phase, OD_{600}= 0.2 (Fig. 13B). At both exponential time points RpoS protein abundance appeared significantly higher in the *fis* mutant (3-4 fold as measured by densitometry). RpoS protein observed in the *fis* mutant after 24 hours of growth, a time when Fis levels are at a minimum, was indistinguishable from wild type (data not shown). We also observed increased exponential phase expression of the RpoS-dependent dependent reporters *katE-lac* [op] (three-fold) and *proV-lac* [op] (four-fold) in the *fis* mutant, consistent with the Western blot analysis of RpoS protein. Again, the

specificity of the *fis* effect to exponential phase defines a regulatory role.

Complementation of the *fis* mutation. To confirm that the increase in *rpoS* transcription in the *fis* mutant was due to the absence of Fis, exponential phase activity of P_{rpoS} -*lac* [op] was measured in wild type and *fis* mutant backgrounds harboring either the single-copy *fis* expression plasmid, pFis349, or the empty vector control pGS349 (32, 71). The nine-fold elevation of *rpoS* transcription in the *fis* mutant was completely eliminated by pFis349 (Fig. 14). The presence of pFis349 in the wild type strain slightly decreased P_{rpoS} -*lac* activity, consistent with the idea that Fis represses *rpoS* transcription. The wild type and *fis* strains containing the control plasmid exhibited similar activities compared to the plasmid-free strains.

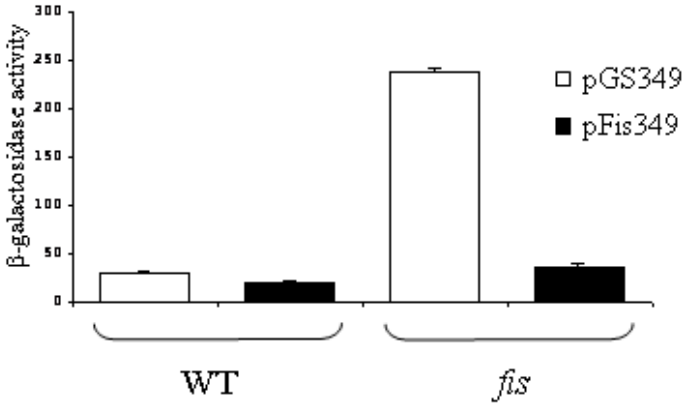

Figure 14. Complementation of the *fis* mutant.

LT2 wild type and *fis* mutant strains containing the P_{rpoS}-lac [op] fusion, and also harboring pFis349 (*fis*$^+$; TE8916, TE8917) or its vector control pGS349 (TE8911, TE8912) were grown at 37°C in LB medium containing ampicillin. Activity of β-galactosidase was determined for cultures grown to OD_{600} = 0.25.

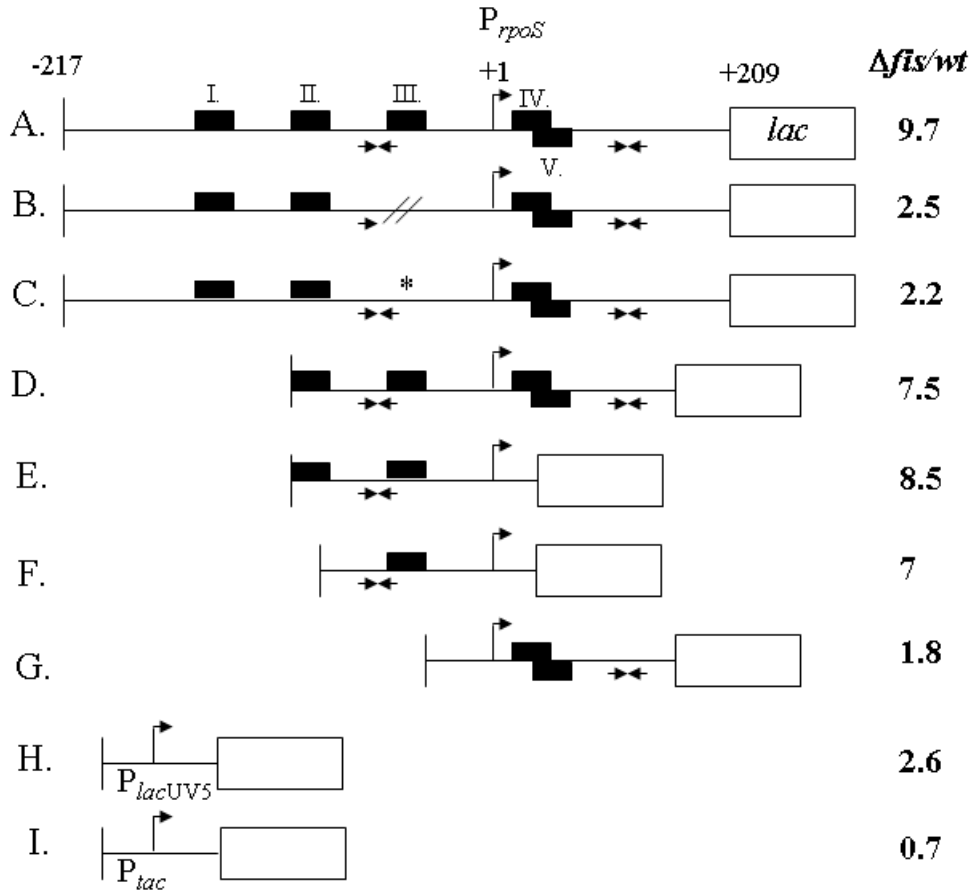

Figure 15. Transcriptional regulation of *rpoS* by Fis depends on Fis site III.

Construct A represents the full-length, wild type P_{rpoS}-*lac* [op] fusion. Predicted Fis binding sites (labeled I to V) are represented by black boxes, and predicted CRP half-sites are shown using straight arrows. The bent arrow represents the transcriptional start. Construct B is a derivative of construct A carrying an in-frame deletion of the high-scoring Fis site III. Construct C is identical to construct A except for a set of 3 point mutations (represented by an asterisk) altering conserved nucleotides of Fis site III. In constructs D, E, F, and G additional segments of this region are deleted as shown. Constructs H and I are control transcriptional fusions driven by either the P_{lacUV5} or P_{tac} promoter. These constructs were assayed during exponential phase growth in LB medium, in wild type and *fis* mutant backgrounds. Results are plotted as the ratio of activity in the Δ*fis* strain to the activity observed in wild type. Wild type and *fis* mutant strains are, respectively, as follows: A, (TE8744, TE8764); B, (TE8899, TE8900); C, (TE8887, TE8888); D, (TE8971, TE8972); E, (TE8868, TE8869); F, (TE9083, TE9096); G, (TE8947, TE8949); H, (TE6676, TE8766); I, (TE6675, TE8948).

Elevation of P_{rpoS} activity in a *fis* mutant depends on a predicted Fis binding site. Fis-binding DNA sequences have been analyzed by using information analysis (27). In this method, known binding sites are first aligned based on highly conserved nucleotides. The nucleotide distribution at each position within the alignment is then used to derive a weight matrix according to classic information theory (61, 65). To determine the quantitative "goodness" of a candidate site, the relevant entries for each position in the weight matrix are summed. If a particular position were completely conserved within the known sites, a correct match in the candidate site would contribute 2 bits to its score. Characterized Fis sites have total scores that range from 2.5 to 15.7, and the well-studied ones in *hin* proximal to the *hixL* site have scores of 8-9 (27), while total scores for random sequence average 0. Information analysis has been shown to accurately predict new Fis DNA binding sites (27).

Software to perform the calculations is available (http://www.lecb.ncifcrf.gov/~toms/delila.html) but we chose to implement these relatively simple computations as a Python script (unpublished data). Our analysis predicted a single high-scoring Fis binding site centered at position –50 with respect to the P_{rpoS} start site (bit score of 10.9; TCTGCACAAAATTCCACCGTT, Fig. 12; Fis site III in Fig. 15). Only 10 out of 60 characterized Fis sites have a higher score. Weaker Fis sites near P_{rpoS} were also predicted (scores from 4.1, Fig. 12B). In fact, we found 83 sites with scores equal to or greater than *rpoS* Fis site III within the first 10^6 bp of the *E. coli* genomic sequence. Nevertheless, since Fis is an abundant DNA binding protein at its peak levels (up to 100,000 monomers per cell), it is possible that most predicted sites are actually bound by Fis protein during exponential growth.

The contribution of Fis site III to *rpoS* regulation was determined by constructing mutant derivatives of the standard P_{rpoS} -*lac* [op] fusion (Fig. 15, construct A). This construct displays over a nine-fold elevation during exponential growth in the *fis* mutant, compared to a wild type background. Construct B contains a deletion of Fis site III (in frame for *nlpD*, Δ -60 to -40) in which half of a putative CRP binding site, which overlaps Fis site III, was also deleted (Fig. 12 and 15). This construct was nearly blind to the effect of the *fis* mutation and demonstrated a ratio of activity (*fis* mutant / wild type) similar to the P_{lacUV5}-*lac* control (Fig. 15). Construct C is identical to construct A except for three point mutations at critical base pairs in Fis-site III [A(-53)G, T(-48)G, C(-43)G; bit score of -3.3) that do not alter the predicted CRP site. This fusion also failed to show elevated expression of P_{rpoS} in the *fis* mutant. Both of the Fis site III mutations (constructs B and C) confer a modest defect in expression in the *fis* mutant, as compared to construct A (1.5 and 2.4 fold decrease, respectively), perhaps because these mutations also affect basal promoter activity slightly. All fusions that retained the intact Fis site III were subject to control by Fis (Fig. 15 constructs A, D, E and F). Conversely, constructs in which Fis site III was altered or deleted (Fig. 15 constructs B, C and G) were independent of regulation by Fis. We used the P_{tac} promoter as a second control in addition to P_{lacUV5}; its activity was not increased and in fact was faintly depressed in the *fis* background.

Fis protein binds to the P_{rpoS} region. Next, we characterized Fis binding in the P_{rpoS} promoter region using gel-shift analysis. All binding reactions were performed in the presence of the non-specific competitor DNA, poly [d(I-C)]. As a control for these studies, we first demonstrated binding of purified Fis protein (a gift from R. Johnson) to the *E. coli* $proP_2$ promoter region over the concentration range reported by Xu and Johnson (72).

PCR products corresponding to three adjacent regions near P_{rpoS} (Fig. 16, fragments A, B, and C) were used as binding targets. Fis bound to each of the fragments, notably with apparent affinities that reflected the score for the predicted Fis site(s) carried on each fragment. Fragment B includes Fis site III required for the *in vivo* effect of Fis on *rpoS* (Fig. 15). This fragment exhibited binding at a low concentration of Fis (32 nM) and greater than 90% of the DNA target was in the bound form at 325 nM Fis. In marked contrast, fragments A and C demonstrate 15% or less than 1%, respectively, of bound target at the same Fis concentration. To further define the contribution of Fis site III, fragment B* was generated from a template carrying the three point mutations in site III that block Fis regulation *in vivo*. At a higher Fis concentration, in which the wild type B target was essentially all (98%) in the bound form, the mutant target B* was predominantly unbound. The B^{Δ} target that has a 21 bp deletion of Fis site III also lost the ability to bind Fis. These results suggest that Fis acts directly as a repressor of the P_{rpoS} promoter.

Testing the interaction of CRP and Fis in transcriptional regulation of *rpoS*. In *E. coli*, cAMP-CRP is reported as a negative regulator of *rpoS* transcription during exponential phase while during entry to SP, the complex may activate transcription (28, 39). A motif search of the P_{rpoS} promoter region by the method of Schneider *et al.* (61) confirmed two putative CRP binding sites that were also previously predicted in *E. coli* (37). The higher-scoring of the two sites is centered at -63.5 and actually overlaps Fis site III (Fig. 12). This placement suggests a potential relationship between Fis and CRP in the regulation of *rpoS* transcription. Coordinate transcriptional regulation between Fis and CRP has been reported for several systems (14, 72).

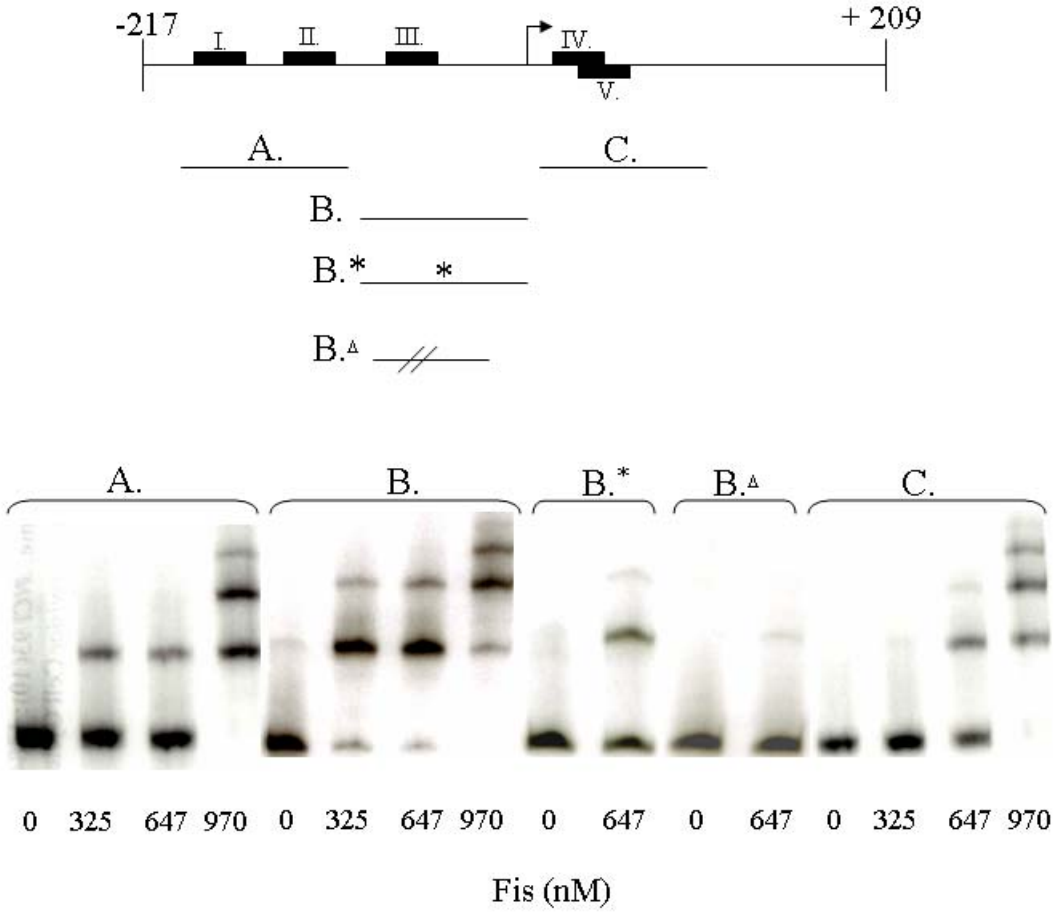

Figure 16. Fis binds several DNA sites near P_{rpoS} *in vitro*.

Electrophoretic mobility shift assay analysis of Fis protein binding to the P_{rpoS} region. A map of the region is depicted at the top with black boxes representing predicted Fis binding sites (labeled I-V). PCR was used to generate targets for shift assays which are labeled A through C. For B* and B^{Δ}, respectively, the asterisk and the slanted lines represent either a set of 3 point mutations altering Fis site III or a deletion of that site. Radiolabeled fragments were incubated with increasing concentrations of purified Fis protein and analyzed by electrophoresis on a native polyacrylamide gel as described previously (72).

To investigate the role of CRP, we measured expression of P_{rpoS} -*lac* [op] during exponential growth in strains bearing the indicated mutations (Fig. 17). These experiments were performed at 37 °C in buffered LB medium supplemented with 0.2% glucose to minimize any growth deficiency of the *cya* and *crp* mutants. The *cya* and *crp* mutants both demonstrated a three-fold increase in *rpoS* transcription during exponential phase. When a mutant *crp** gene encoding a constitutively active form of CRP was introduced into the *cya* mutant background, wild type expression was restored. In the *fis crp* double mutant, only a slight increase in P_{rpoS} -*lac* [op] expression was observed compared to the *fis* single mutant. Furthermore, in the *fis crp* double mutant P_{rpoS} -*lac* [op] expression was substantially elevated compared to the *crp* single mutant. These results are consistent with a model in which most Fis regulation of *rpoS* is independent of CRP function, and is not mediated through, for example, competitive binding of the two regulators at the overlapping Fis III and CRP sites.

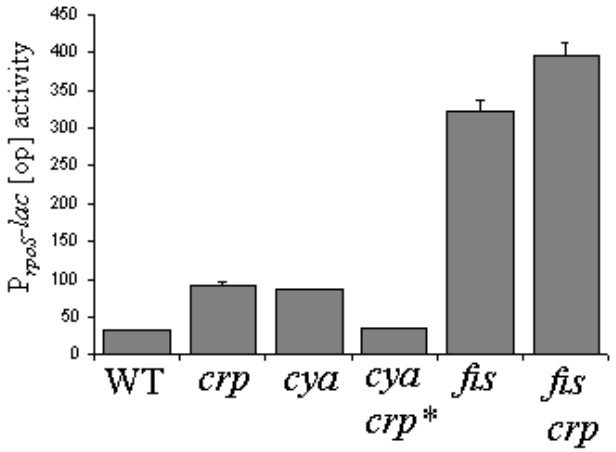

Figure 17. CRP and cAMP influence *rpoS* transcription.

P_{rpoS}-lac [op] activity was assayed during exponential phase (OD_{600} = 0.25) in strains containing the indicated mutations. Growth was at 37°C in buffered LB supplemented with 0.2% glucose. The *crp** allele encodes a constitutively active form of the CRP protein (independent of cAMP).

Discussion

The sigma factor RpoS has been described as the "master regulator of the general stress response" (28). It is noteworthy that this transcriptional regulatory protein is itself up-regulated in response to a number of different stresses, by pathways that act on diverse targets including transcription, translation, protein stability and protein activity. The SP induction of RpoS in rich medium is a dramatic effect, whether observed by Western blot of RpoS protein (25) or in a number of studies employing *rpoS-lac* fusions including our own work, where we estimate the magnitude of the response at 30-fold (28, 29, unpublished data). What seems surprising is that various stimuli induce RpoS by such different mechanisms. Starvation for carbon seems to involve mainly stabilization of the protein against attack by the ClpXP protease (36, 66, 73). Osmotic shock involves both protein stabilization (44) and a post-transcriptional effect dependent mainly on DsrA RNA and the Hfq protein. This pathway does not lead to an increase in the amount of DsrA (38). In contrast, low temperature leads to increased synthesis of DsrA RNA and Hfq-dependent activation of RpoS translation (54). Finally, SP induction of RpoS in rich medium involves both transcriptional and post-transcriptional components (28, 29).

We initially eliminated the ClpXP-MviA degradation pathway as a regulator of SP induction of RpoS in rich medium (Fig. 9). This conclusion is consistent with the results of Pratt et al. (52) who demonstrated increased RpoS during both exponential and SP in a *sprE* mutant of *E. coli* grown in LB. However, during growth of *E. coli* in minimal medium, the RpoS protein has a short half-life (1 min) and upon osmotic challenge, starvation or switch to an acidic pH,

protein stabilization is responsible for RpoS induction (7, 44, 73). These reports are consistent with only a small transcriptional induction of P_{rpoS} during growth to SP in minimal medium (Table 7). In rich media (LB and BHI) control of SP induction of RpoS is exerted at the transcriptional and translational level while in minimal medium proteolysis seems to play the major role.

Consistent with previous reports, *rpoS* is transcribed from two promoter regions P_{nlpD} and P_{rpoS} (35, 51). P_{nlpD} is a minor contributor during both growth and SP and does not exhibit SP induction beyond the P_{lacUV5} control (Fig. 10, data not shown). P_{rpoS} is the major *rpoS* promoter during growth and SP, when increased transcriptional activity coincides with elevated RpoS. Three lines of evidence suggest that σ^{70} recognizes P_{rpoS} *in vivo*: (i) the presence of an apparent σ^{70} promoter sequence (Fig. 12); (ii) RpoS does not contribute to expression from its own promoter (Fig. 11); (iii) RNA polymerase holoenzyme (σ^{70}) transcribes this promoter *in vitro* (data not shown).

The elevation of P_{rpoS} activity in the *fis* mutant during exponential phase eliminated SP transcriptional induction, an effect that can be totally complemented by plasmid-encoded Fis. The standard P_{rpoS} fusion (Fig. 15, construct A) contains 5 predicted Fis binding sites. Fis binding was demonstrated to at least 3 sites (Fig. 16) suggesting a nucleoprotein complex forms near P_{rpoS} *in vivo*. However only Fis site III, positioned at -50, was required for the regulatory effect. The importance of a single Fis binding site near transcriptional start sites has been reported for two other promoters, P_{fis} and $P2_{proP.}$ (50, 72). In each case Fis binds to several positions although nearly all regulation is conferred by a single Fis binding site centered at -42

(P_{fis}) and -41 ($P2_{proP}$) from the transcriptional start sites (41, 50, 72). Transcriptional regulation by Fis at P_{fis} causes repression, possibly by blocking RNA polymerase interactions at the -35 region. During growth, the *fis* mutant displayed a nine-fold elevation of P_{rpoS} activity although the increase of RpoS protein was estimated at three to four-fold (Fig. 13). This suggests that additional (post-transcriptional) regulation may prevent some of the expected increase in protein levels, thereby making both *rpoS* transcription and translation rate limiting.

Concerning the role of CRP-cAMP in the regulation of P_{rpoS}, we suggest that it functions as a repressor although its effect is modest (two to three-fold). The relationship between CRP and Fis appears to be one of Fis epistasis. The *fis crp* double mutant does not display an additive effect on P_{rpoS} activity but there is only a slight increase over the large effect of the *fis* single mutant. From this it seems that Fis regulation does not require CRP and that full CRP regulation is hindered in the absence of Fis. Further experimentation is necessary to define the role of CRP in the regulation of *rpoS* transcription.

In this study, we document a role for the DNA-binding protein, Fis, as a negative regulatory element for RpoS, acting at the transcriptional level. This model is intuitive, because Fis abundance varies inversely with RpoS. Synthesis of Fis is under transcriptional control and Fis abundance varies dramatically from undetectable in SP to over 40,000 dimers per cell upon dilution into fresh medium (2, 6, 50). In *E. coli*, Fis displays auto-regulation in which Fis protein competes with RNA polymerase for binding to the *fis* promoter thus repressing its own transcription (6). This auto-regulatory effect is less pronounced in *S. enterica* (50). In both organisms growth-phase expression of *fis* is thought to occur by a mechanism apparently

involving a non-optimal -35 to -10 region and specific base pairs near the transcriptional start site (69). It has been demonstrated that normal regulation is also dependent upon CRP and *in vitro* results suggest *fis* promoter activation in the absence of Fis, while Fis and CRP act synergistically as transcriptional repressors (47).

We believe that Fis probably acts directly as a repressor given that a specific site positioned at -50 is necessary for complete repression and that this site is specifically bound *in vitro*. Fis activates transcription of rRNA and many other genes including some involved in replication, so it is conceivable that Fis also works indirectly. Further investigations into the regulation of Fis expression would provide a greater understanding of the interplay of global regulators in physiological adaptation.

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Stationary Phase Regulation of RpoS synthesis in *Escherichia coli*

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Abstract

In enteric bacteria, adaptation to a number of different stresses is mediated by the RpoS protein, one of several sigma-factors that collectively allow a tailored transcriptional response to environmental cues. Stress stimuli including low temperature, osmotic shock, nutrient limitation, and growth to stationary phase (SP), all result in a substantial increase in RpoS abundance and activity. The mechanism of regulation depends on the specific signal, but may occur at the level of transcription, translation, protein activity or targeted proteolysis. In both *Escherichia coli* and *Salmonella enterica*, SP induction of RpoS in rich medium is >30-fold and includes effects on both transcription and translation but not protein turnover. Recently, we showed that SP control of *rpoS* transcription in *S. enterica* involves repression of the major *rpoS* promoter by the global transcription factor Fis during exponential-phase. Working primarily in *E. coli*, we now show that 24 nucleotides of the *rpoS* ribosome-binding sequence (RBS) are necessary and sufficient for the nearly 10-fold increase in *rpoS* translation as cells grow to SP. Genetic evidence supports a model in which the paired structure of the RBS enforces regulation. This regulation is conserved between *E. coli* and *S. enterica*. When combined with a *fis* mutation, substitution of the *rpoS* RBS sequence by the *lacZ* RBS eliminates nearly all SP induction of RpoS.

Introduction

Bacteria maintain intricate signaling networks that sense the environment and adjust cellular physiology accordingly. In *Salmonella enterica* and *Escherichia coli*, unfavorable growth conditions (including nutrient limitation, outright starvation, low temperature, osmotic shock, and other stresses) initiate a generalized stress response by triggering increased abundance of the RNA polymerase sigma factor RpoS (σ^S; reviewed in reference 16). In association with RNA polymerase, RpoS directs transcription of as much as 10% of the *E. coli* genome, including genes necessary for stress resistance and virulence (13, 48). RpoS thereby serves as the central regulator of the general protective response (16).

The transition to SP is accompanied by morphological and physiological changes resulting in a non-dividing and multiple-stress resistant state. Growth into stationary phase (SP) in rich media, such as Luria-Bertani (LB), leads to a dramatic increase of > 30-fold in RpoS abundance (14, 17, 18, 28, 33, 39). In recent work, we characterized transcriptional regulation of *rpoS* in *S. enterica* as cells enter SP (18). The mechanism involves Fis, a DNA-binding protein which acts globally as a transcription factor. Fis is itself growth-phase regulated in an inverse relationship to RpoS: the Fis protein is undetectable in SP but rapidly increases to a level of more than 40,000 dimers per cell upon dilution into fresh medium (1, 3, 35). A strong Fis-binding site near the major *rpoS* promoter (P_{rpoS}), is required for this regulation. Fis likely binds to this site specifically during exponential growth, resulting in repression of *rpoS* transcription (18). As cells enter SP, Fis disappears, and *rpoS* transcription increases nearly 10-fold (1, 18).

The *rpoS* transcript contains a 565 nucleotide 5' untranslated region (the *rpoS* leader; 16, 43). This sequence includes an antisense element [leader nucleotides (nt) 461 to 478] that can pair with the *rpoS* RBS to inhibit translation, presumably by blocking ribosome access (8). The antisense element is the reported target of three regulatory RNAs which are thought to alter conformation of the RBS to an "open" position, increasing translation (22, 23, 25, 31, 38, reviewed in ref. 15). The best-characterized example of regulation of *rpoS* translation occurs at low temperature and relies on the direct pairing of the antisense element with the 85-nucleotide regulatory RNA, DsrA (42). This interaction activates *rpoS* translation five to 10-fold and is mediated by the RNA-binding protein Hfq (25, 38).

In the present study, we show that 24 nucleotides of the *rpoS* RBS are necessary and sufficient for a nearly 10-fold increase in *rpoS* translation as cells grow to SP. Genetic evidence supports a model in which the secondary structure of the RBS is required for regulation. Substitution of this sequence with the RBS of *lacZ*, in a *fis* mutant background, virtually eliminates SP induction of RpoS.

Materials and Methods

Bacterial strains and construction. Most strains used in this study are derived from the wild type *E. coli* K-12 strain MG1655 (Table 8). The parental strain was CF7968, which is MG1655 that has been corrected to *rph*$^{+}$ (20) and deleted for *lacIZ*, obtained from M. Cashel. Phage P1 *vir* was used for transduction in *E. coli* by standard methods (41). The *katE-lac* [op] (operon) fusion used in this work

has been described previously and is used as a reporter of RpoS activity (7, 9, 18). All fusions in *E. coli* are located in single-copy in the *trp* region of the bacterial chromosome as described previously (12).

We also investigated the behavior of particular *rpoS-lacZ* constructs in *Salmonella enterica* serovar Typhimurium. The parental strain was LT2, obtained from J. Roth, or LT2A (10, 18). To this end, constructs in *E. coli* were transduced into a *galE* mutant of *S. enterica* by using P1 *vir* as described previously (12). The phage P22 mutant HT105 / 1 *int-201* was then used for transduction in *S. enterica* by standard methods (11). All fusions in *S. enterica* are located in single-copy at the *putPA* locus (12).

Media and growth conditions. Bacteria were grown at 37°C in LB medium (41) and on nutrient agar (NB) plates containing 5 g of NaCl per liter, except where indicated. Minimal agar was prepared with NCE medium containing 0.2% glucose (5). Liquid minimal medium was morpholinepropanesulfonic acid (MOPS) medium (34) as modified (6), supplemented with 0.2% glucose as the carbon and energy source. Antibiotics were added to final concentrations in selective media as follows: 20 μg of chloramphenicol/ml, 50 μg of kanamycin sulfate/ml, and 20 μg of tetracycline hydrochloride/ml. X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) was used at 50 μg/ml.

Fusion construction. We took advantage of our previously described method for making *lac* transcriptional and translational constructs in which a region of interest is inserted between the promoter $P_{lac}UV5$ and the *lacZYA* genes (17). This method relies on the high efficiency λ Red recombination system of Yu et al (49). A chromosomal template that has the *tetAR* cassette immediately upstream of

P_{lac}UV5 was amplified by PCR, using the following primer design (all primer elements are listed in order from 5' to 3'): (i) a common upstream 60-mer that contains 40 nt of *kan* homology to mediate upstream recombination followed by 20 nucleotides of *tetA* priming sequence; (ii) construct-specific downstream 80-mers contained 40 nucleotides of either *lac* or *rpoS* homology for downstream recombination followed by a variable region of interest preceding priming homology within the *lac*UV5 promoter. The resulting PCR products have the following structure: *kan-tetAR*-P_{lac}UV5-region of interest-*rpoS* codons 1-8 or, alternatively, *lacZ* coding sequence. Products with downstream homology to *rpoS* were used to transform TE8402 or TE9277 to generate translational or transcriptional fusions respectively. When recombination directly to the *lacZ* coding sequence was desired, strain TE9059 served as the recipient. Transformants were selected on NB plates containing tetracycline. The region extending from upstream of P_{lac}UV5 through the downstream recombination site (*lacZ* codon 22) was sequenced. All fusions were then backcrossed into *E. coli* MG1655 Δ*lacIZ*. The sequences for primers used in this study are available upon request.

Unselected chromosomal mutations.

To replace the *rpoS* RBS with that of *lacZ* in the *rpoS* native context (i.e. at the *rpoS* locus), a strain with λ Red was utilized that carries the *katE-lac* [op] fusion. First, 24 nt surrounding the *rpoS* RBS (5'-3'), GGGATCACGGGTAGGAGCCACCTT, were substituted with *tetAR*. Transformants had a Lac- phenotype on NB plates containing tetracycline and X-gal. Next, a 188 bp PCR product was generated from a wild type template using an upstream *rpoS* primer (position 410 of the *rpoS* transcript) and a downstream primer that contained (i) 40 nt of downstream homology to allow recombination downstream of the ATG initiation codon of

rpoS; (ii) 17 nt of the *lacZ* RBS (AATTTCACACAGGAAACAGCT), and (iii) 19 nt of priming homology to the *rpoS* leader (nt 541-522). This PCR product was used to transform the TetR strain constructed in the first step followed by dilution into fresh medium for overnight growth. Cultures were then diluted and plated on NB containing kanamycin and X-gal. The phenotype of desired transformants was Lac+; these were recovered at a frequency of >10-4, and the insertion was confirmed by DNA sequencing. This unmarked substitution was then backcrossed into a MG1655 background by using a recipient strain which contains the *katE-lac* [op] fusion, Δ*rpoS::cat* (CamR), and also Δ*cysC::tetAR*. The *cysC* locus is ≈ 6 kb from *rpoS* and tightly linked to it by P1 transduction. Cys+ Lac+ transductants were selected on minimal medium plates containing X-gal and the *lacZ* RBS substitution in the *rpoS* leader was confirmed again by sequencing.

Typically, our *lac* [op] constructs contain a RNase III processing site (7, 24), which insulates *lacZ* expression from variations due to differences in upstream sequences. For the experiments described here, this property is not desirable. A similar non-selective transformation method to that described above was employed to eliminate the processing site from a strain containing the *kan*-P_{lacUV5}-*rpoS* (codon 8) transcriptional fusion (TE8403). Briefly, the *cat* gene was used to make an insertion-deletion with loss of 76 bp including the RNase III cleavage site, located in the 170 nt spacer region between *rpoS* codon 8 and the *lacZ* RBS. The desired transformants were Lac^- (TE9274). Next, a PCR product was generated with the following structure: *rpoS* codons 6-8 followed by 31 nucleotides of the spacer region, the *lacZ* RBS and 144 nt of the *lacZ* coding sequence. Transformation of TE9274 with this PCR product followed by screening for Lac^+, resulted in a strain that is deleted for the processing

site and sensitive to chloramphenicol. The relevant region was confirmed by DNA sequencing (TE9277).

The *fis* gene of *E. coli* was deleted and substituted with *cat* using standard methods (18). Regions surrounding the sites of recombination, *fis* codon 22 and immediately following the *fis* termination codon, were confirmed by DNA sequencing.

Assay of β-galactosidase. Cells were centrifuged and resuspended in Z-buffer (100 mM $NaPO_4$ [pH 7.0], 10 mM KCl, 1 mM $MgSO_4$) and then permeabilized by treatment with sodium dodecyl sulfate (SDS) and chloroform (29). The samples from exponential-phase were concentrated before assay to be approximately equal in density to samples obtained from later times. For all experiments, exponential-phase is defined as OD_{600} = 0.25 and SP is 24 hours after inoculation. Assays were performed in Z-buffer containing 50 mM β-mercaptoethanol by a kinetic method using a plate reader (Molecular Dynamics). In all experiments, β-galactosidase activity (change in OD_{420} per minute) was normalized to cell density (OD_{650}) and was always compared to appropriate controls assayed at the same time. The values shown are averages of at least four experiments with a standard deviation of <17%, unless otherwise stated.

Immunological detection of proteins. For Western blots, cultures were grown as described in the text. Electrophoresis and protein transfer were as described previously (7, 10). After transfer to a Sequi-Blot polyvinylidene difluoride membrane (BioRad), blots were blocked in 5% non-fat milk and incubated in phosphate buffered saline (PBS)-Tween (0.05%) containing the anti-RpoS monoclonal

antibody R12 (7). After 60 min of incubation, blots were washed in PBS-Tween, incubated for 60 min in PBS-Tween containing biotinylated goat anti-mouse immunoglobin, and finally incubated in PBS-Tween containing streptavidin-conjugated horseradish peroxidase (Southern Biotechnology Associates). Detection was by enhanced chemiluminescence (Amersham Biosciences).

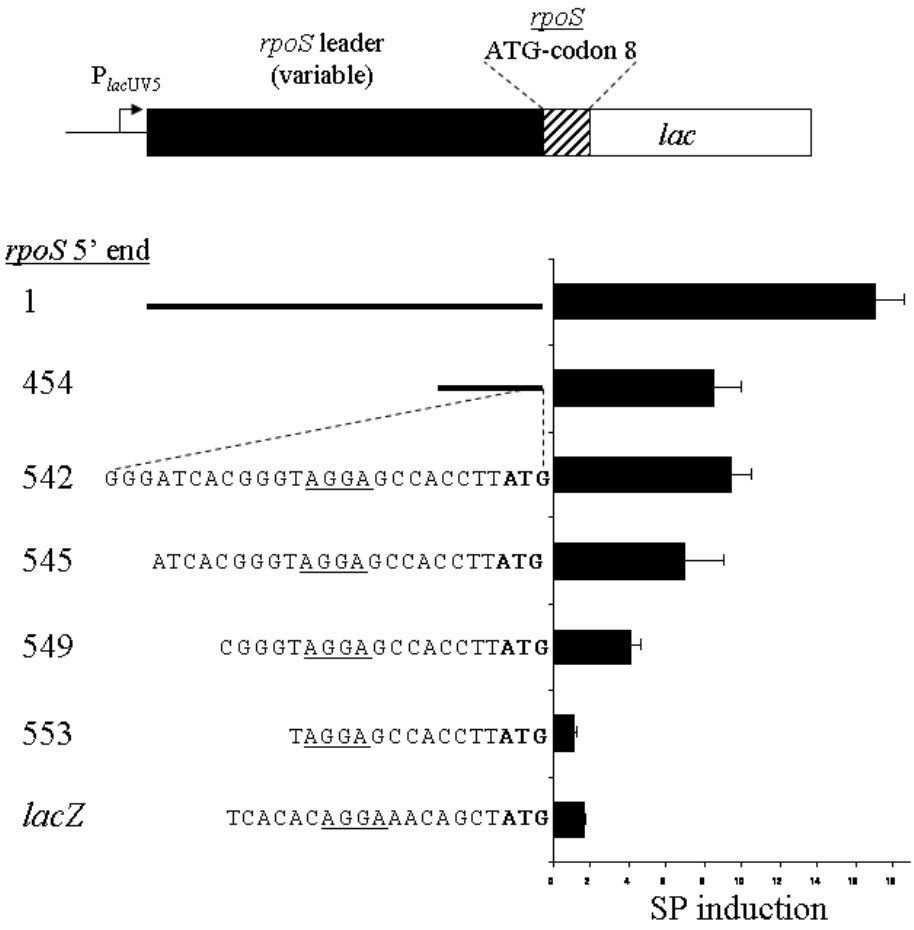

Figure 18. Stationary phase induction of *rpoS* translation relies on the ribosome-binding sequence.

The top line depicts the general fusion context. The *lac*UV5 promoter drives expression of a variable segment of the *rpoS* 5' untranslated region (*rpoS* leader) followed by the first 8 codons of *rpoS*. At this position, the *lacZ* gene is joined to *rpoS* to form a translational fusion. Six different *rpoS* constructs are shown, labeled with the position of the variable upstream end of the segment. Next to each construct is the stationary phase (SP) induction ratio, obtained by dividing SP activity by the exponential-phase activity. The variable region of constructs 1 and 454 fusions are shown as black lines due to their longer size, while the broken lines indicate a magnification of *rpoS* ribosome-binding sequence. The RBS of *lacZ* served as a control and is labeled *lacZ*.

Results

The *rpoS* RBS regulates SP induction of translation. Growth of *E. coli* or *S. enterica* into SP in rich medium (LB) results in a >30-fold increase in expression of *rpoS-lac* [pr] (protein) and an even greater increase in RpoS activity as demonstrated by *katE-lac* [op] (17, 18). As shown by others (21, 28, 33, 39, reviewed in ref 13) and confirmed by us for both *E. coli* and *S. enterica* (17, 18) this increase has components of both transcriptional and post-transcriptional regulation. SP induction in LB is independent of the response regulator RssB/SprE/MviA as well as the energy-dependent protease ClpXP, which together regulate RpoS abundance under other conditions (18, 27, 32, 36, 40). SP induction is also independent of the Hfq protein and likewise, the regulatory DsrA RNA (17).

A striking result from previous analysis of *rpoS* translation in *E. coli* was that most of the SP induction (at 37°C in LB) is maintained when most of the 565 nt *rpoS* leader region is deleted (17), including the antisense element that binds DsrA (25). Nearly 10-fold induction was observed for a *rpoS-lac* [pr] fusion expressed from $P_{lac}UV5$, that contains only 48 nt of *rpoS* - 24 nt of the RBS, which includes the Shine-Dalgarno (S.D.) sequence (GGGATCACGGGTAGGAGCCACCTT**ATG**), followed by the first eight codons of the gene (third construct in Fig. 18, labeled 542; construct I in Fig. 19).

To further define the sequence required for translational regulation of SP induction, three additional constructs with further deletions of the *rpoS* leader were analyzed (Fig. 18). Cultures were

assayed for β-galactosidase activity during exponential-phase (OD_{600} = 0.25) and after 24 hours of growth (SP) in LB medium at 37°C. The SP induction ratio (SP activity / exponential-phase activity) for each construct is reported in Fig. 18.

The construct bearing the entire 565 nt leader region of *rpoS*, was induced 17-fold as cells grew to SP (Fig. 18, labeled 1, and Fig. 19, construct G). Constructs with sequential 5' leader deletions to within 21 nt of the *rpoS* initiation codon each maintained about half of this regulation, with induction ratios of seven-fold to nine-fold (Fig. 18, constructs 454, 542 and 545). Removal of an additional four nt decreased induction to an intermediate value of four-fold (Fig. 18, construct 549), while SP regulation was completely eliminated in a construct retaining only 13 nt of the *rpoS* leader (Fig. 18, construct 553). As a negative control for these experiments, 21 nt of the *lacZ* RBS in the same fusion context (and maintaining the first eight codons of *rpoS*), showed a minimal 1.6-fold increase during SP (Fig. 18, *lacZ*).

Conservation of *rpoS* RBS-mediated induction. The sequence of the *rpoS* RBS is completely conserved among several species of enteric bacteria including several strains of *E. coli* (including MG1655 and O157:H7), serovars of *S. enterica* (including Typhi and Typhimurium), *Shigella flexneri* and *Enterobacter cloacae*. To determine if RBS-mediated SP induction is specific to *E. coli*, we investigated the activity of construct 542 in *S. enterica* serovar Typhimurium. As cells grew into SP, the activity of construct 542 increased 10-fold in contrast to the 1.5-fold SP induction of the *lacZ* RBS (data not shown). The nearly identical induction ratios obtained with *E. coli* MG1655 and *S. enterica* serovar Typhimurium (LT2A and LT2) suggest a conserved regulatory mechanism.

	P_{lacUV5}	Ribosome Binding Sequence Bold = *rpoS* white = *lacZ*	β-galactosidase activity SP	 SP/E
Hybrid constructs				
A.	TATAATGTGTGGAATT**GGGATCACGGGTAGGAGCCACCTTATG**		8	68	8.5
B.	TATAATGTGTGGAATT**GGGATCACGGGTAGGAGAACAGCTATG**		20	156	7.8
C.	TATAATGTGTGGAATT**GGGATCACGGGTAGGA-AACAGCTATG**		8.2	51	6.1
D.	TATAATGTGTGGAATT**GGGATCACGGGTAGGAGAACAGCTATG**		2.9	30	10
E.	TATAATGTGTGGAATT**------TCACACAGGAGCCACCTTATG**		395	883	2.2
F.	TATAATGTGTGGAATT**------TCACACAGGA-AACAGCTATG**		45	70	1.6
Full-length constructs (1-565)					
G.	TATAATGTGTGGAATT//**GATCACGGGTAGGAGCCACCTTATG**		9.6	169	17
H.	TATAATGTGTGGAATT//**TCAATCACACAGGA-AACAGCTATG**		67	103	1.5
Leader deletion constructs (542-565)					
I.	TATAATGTGTGGAATT**GGGATCACGGGTAGGAGCCACCTTATG**		11	105	9.4
J.	TATAATGTGTGGAATT**GGGATCAC**CC**GTAGGAGCCACCTTATG**		26	79	3
K.	TATAATGTGTGGAATT**GGGATCAC**CC**GTAGGAGCCAC**GG**TATG**		4.7	69	15
L.	TATAATGTGTGGAATT**GGG-**TTCCACC**AGGAG**TGGGCACT**ATG**		1.0	13	13

Figure 19. β-galactosidase activity of various ribosome-binding sequences.

The β-galactosidase activity of various ribosome-binding sequences (RBS), in a translational *lacZ* fusion context, was determined during exponential growth (E) and during stationary phase (SP). These values were used to determine the SP induction (SP / E) of the listed constructs. All fusions are expressed from the *lac*UV5 promoter (P_{lacUV5}) and a partial promoter sequence, including the -10 hexamer is underlined. The fusions are grouped into three categories: (i) hybrid constructs which contain nucleotides from both the *rpoS* RBS and *lacZ* RBS, shown in bold black font and white font, respectively; (ii) full-length constructs having the entire *rpoS* leader region (nucleotides 1-565), in this case a double slash mark symbolizes nt 1-541 of the native *rpoS* transcript which are not shown; (iii) leader deletion constructs which are based on the *rpoS* RBS (nucleotides 542-565) with mutations affecting the RBS shown in normal font (not bold); Each RBS is linked either to the first eight codons of *rpoS* and then to *lacZ*, shown by ATG nucleotides in bold, or directly to the *lacZ* coding sequence (ATG in white). All strains were grown at 37°C in LB medium.

the β-galactosidase activity of a construct that has just the 24 nt of the *rpoS* RBS preceding native *lacZ* (Fig. 19, construct A). This sequence maintained an SP induction ratio of 8.5-fold, similar to the induction shown by construct 542 (Fig. 18; Fig. 19, construct I). Together with the deletion results, this clearly demonstrates that the *rpoS* RBS is necessary and sufficient for the nearly nine-fold increase in translation after cells enter SP.

To investigate the regulatory role of the 24 nt *rpoS* RBS in the context of the entire *rpoS* leader, a fusion was constructed in which these bases were substituted by 21 nt of the *lacZ* RBS (Fig. 19, construct H). In this case, SP induction decreased to that of *lacZ*, 1.6-fold, compared to the 17-fold induction of the native leader region (Fig. 19, compare constructs G and H).

The 24 nt RBS of *rpoS* includes a five base S.D. sequence near its center (AGGAG), bounded by 12 bases upstream and seven bases downstream. The *lacZ* RBS also consists of a nearly centered AGGA bordered by upstream and downstream sequence elements. We exchanged the upstream and downstream elements, to construct a panel of *rpoS*/*lacZ* RBS in the fusion contexts mentioned above (Fig. 19). In construct B, the upstream segment is from *rpoS* and the downstream segment is substituted from *lacZ*; construct C is identical except that it has the shorter *lacZ* S.D. sequence (AGGA). Both hybrid RBSs demonstrated significant SP induction similar to results obtained with the wild type *rpoS* RBS (Fig. 19, compare constructs A, B and C). Substitution of the downstream *lacZ* element is also without effect on SP induction in the context of a fusion bearing the first 8 codons of *rpoS* (construct D).

A quite different result is observed for substitution of the upstream element. Construct E contains the upstream segment from *lacZ* and the downstream segment from *rpoS* (Fig. 19). This hybrid

RBS construct is unique among all tested herein in that its relative activity is extremely high (Fig. 19). This result appears to be due to increased translation because a corresponding transcriptional fusion demonstrates the same activity of all other transcriptional fusions tested (data not shown). Importantly, construct E is not regulated during growth to SP. This result was confirmed for the same hybrid RBS expressed from a mutant P_{lacUV5} (T→A at −12), which is reduced 100-fold in overall transcriptional activity (data not shown). Thus, the bases directly upstream from the S.D. sequence (*rpoS* nt 542-557) seem to be required for SP induction.

Testing the role of potential *trans*-regulators in SP induction. We considered the possibility that *trans*-acting factors recognize the *rpoS* RBS and repress activity during exponential-phase or activate translation during SP. Three genes, whose products have been implicated in control of *rpoS* translation, were investigated including DksA, the transiently expressed subunit of the DNA-binding HU dimer, and the RNA-binding protein Hfq (2, 7, 31, 47). Normal SP induction of construct 542 occurred in a *dksA*, *hupB* or *hfq* mutant background (data not shown). Additionally, it seems unlikely that induction is mediated by a small regulatory RNA, since most are dependent on Hfq for action (38).

We investigated whether known variations in ribosome composition as cells grow to SP confer an attraction for the *rpoS* RBS. The genes encoding four transiently expressed, ribosome-associated proteins YfiA, YhbH, Sra and Rmf (19, 26, 45) were individually inactivated and the SP induction of construct 542 was determined in the mutant backgrounds. In each case, SP regulation was not significantly different than wild type (data not shown).

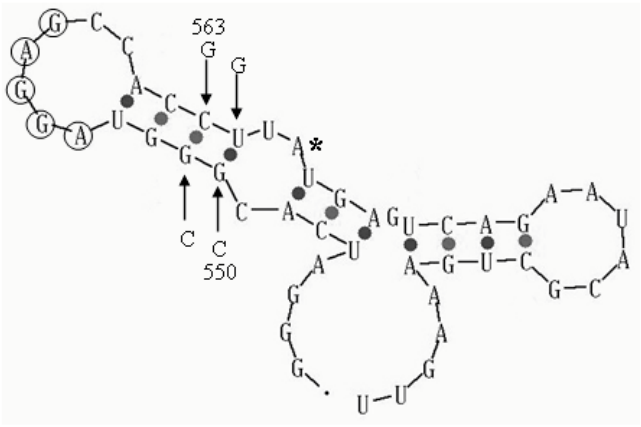

Figure 20. Predicted RNA secondary structure of the *rpoS* ribosome-binding region.

Mfold was used to predict the RNA secondary structure of a 48 nucleotide region of the *rpoS* transcript: 24 nucleotides directly preceding the *rpoS* start codon (labeled with an asterisk) extending to *rpoS* codon 8 (50). The nt of the extended Shine-Dalgarno sequence of *rpoS* are individually circled. Arrows indicate positions of directed mutations, the substituted nt are also shown, as described in the text

Secondary structure and SP induction. Another model posits that the secondary structure of the *rpoS* RBS acts directly as a regulatory signal. The thermodynamically favored secondary structure of construct 542 (48 nt of *rpoS*) was predicted using the mfold algorithm of M. Zuker (Fig. 20; 50). In this structure, the *rpoS* S.D. sequence (AGGAG) is positioned within a single-stranded loop flanked by a 4 bp stem. Disruption of the stem with a targeted double mutation eliminated most of the SP induction observed for the wild type *rpoS* RBS (Fig. 19 construct J; Fig. 20, G550C/G551C). A construct containing two compensatory mutations (Fig. 19, construct K; Fig. 20, G550C/G551C/C563G/T564G), which restore the predicted structure of the wild type RBS, was also investigated. In this case, SP induction was restored and slightly elevated compared to wild type (Fig. 19, compare constructs I, J, and K).

To test this model further, another construct was made which reversed the sequence of the *rpoS* RBS while maintaining the extended S.D. sequence (Fig. 19, construct L). Remarkably, the reversed

RBS demonstrated significant SP induction, with a ratio even higher than the wild type *rpoS* RBS (Fig. 19, constructs L and I). This result, along with those from the directed-mutation analyses, strongly supports the idea that the structure of the RBS, and not primary sequence, functions as a regulatory signal.

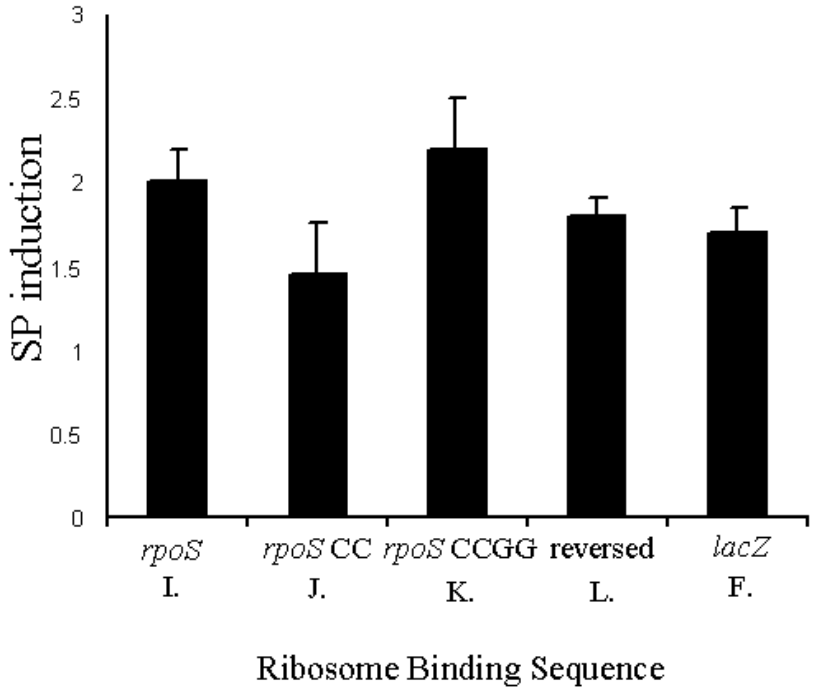

Figure 21. Stationary phase regulation of various ribosome-binding sequences at the transcriptional level.

The stationary phase (SP) induction ratios of various transcriptional fusions containing the indicated ribosome-binding sequences were investigated. A description of the ribosome-binding sequences is given under each column and the actual sequences correspond with the letter designations of Fig. 19. The β-galactosidase activity of each construct was determined during exponential growth (E) and SP, and the SP induction ratio (SP/E) is shown.

RNA stability and SP induction. To investigate the possibility that differential transcript stability mediates SP induction of *rpoS* synthesis, we constructed several strains with RBS variants in a transcriptional fusion context. All transcriptional fusions investigated demonstrated a low 1.5 to 2-fold SP induction ratio, similar to the *lacZ* control, regardless of translational regulation (Fig. 21). This small increase in transcriptional activity does not account for the large differences in translational induction seen among the various RBSs, indicating that altered RNA stability does not regulate SP induction.

Regulation at the native *rpoS* locus.

SP induction of RpoS in LB occurs at both the transcriptional and translational levels, while targeted proteolysis influences RpoS abundance but not regulation per se (18). To determine if the RBS of *rpoS* has a role in SP regulation in the native context, we replaced the 24 nt sequence with the RBS of *lacZ* (Fig. 22A). In this background RpoS activity, as measured by the *katE-lac* [op] reporter, increased nearly three-fold, specifically during exponential-phase, thereby reducing SP induction (Fig. 22B). This result is supported by significantly increased RpoS protein abundance during exponential-phase (Fig. 22C, WT vs. *lacZ* RBS).

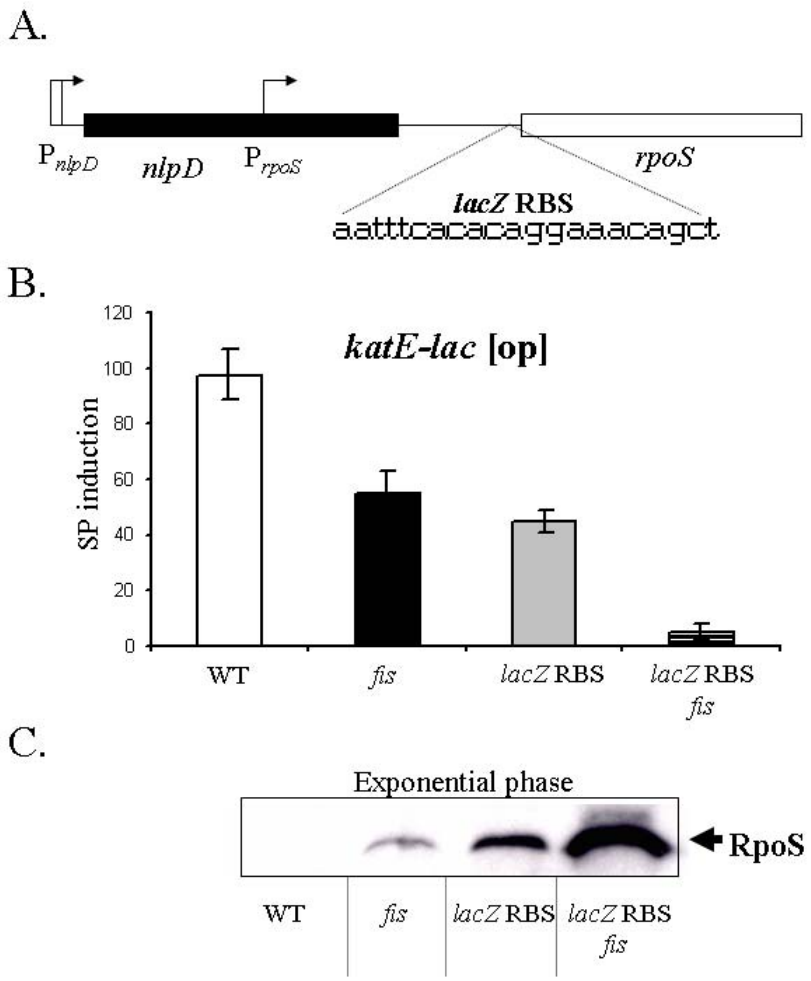

Figure 22. SP regulation of RpoS depends on the *rpoS* RBS and Fis.

The 24 nucleotides preceding the *rpoS* initiation codon were replaced with the depicted *lacZ* RBS in the native *rpoS* context (A). RpoS activity during exponential growth and stationary phase was measured in a wild type background, a *fis* deletion mutant (*fis*) and in a background that contains the replacement shown in (A) with or without a *fis* deletion (*lacZ* RBS and *lacZ* RBS *fis* respectively, B). (C) Western analysis of RpoS protein during exponential growth in the indicated backgrounds.

In *S. enterica* serovar Typhimurium, Fis controls SP induction of *rpoS* transcription by binding near P_{rpoS} and repressing activity during exponential-phase (18). We investigated Fis regulation of RpoS in *E. coli* by determining the SP induction of *katE-lac* [op] in a *fis* mutant background. Due to increased expression during exponential-phase, SP induction of RpoS decreased two-fold in the *fis* mutant (Fig. 22B), an effect that correlates with a direct measure of RpoS abundance (Fig. 22C).

Finally, the SP induction of RpoS was determined in a *E. coli* background that is defective in both transcriptional control (*fis* mutant) and translational control (*rpoS* RBS replaced with that of *lacZ*). In this context, the near 100-fold SP induction of *katE-lac* [op] decreased to five-fold. This result was due to a large increase in RpoS protein during exponential-phase (Fig. 22B and 22C, compare WT vs. *lacZ* RBS *fis*).

Discussion

Regulation of RpoS in the enterics is remarkable for its diversity---both in the signals that increase the level of the protein, as well as the mechanisms by which the increase is achieved. Examples are known where regulation occurs at the level of transcription, translation, targeted proteolysis, and protein activity, as well as combinations of these (reviewed in16). Perhaps the most dramatic difference in RpoS abundance occurs between exponential-phase and SP in rich medium and is maintained by transcriptional and translational control (17, 18). Although this can be described as an induction or

increase, the effects of regulatory mutants suggest that much of the control is negative, acting to restrict expression during exponential-phase.

In this study, we explore SP control of RpoS at the post-transcriptional level. Remarkably, 24 nt of the *rpoS* RBS are both necessary and sufficient for a nearly nine-fold increase in translation during SP (Fig. 19). These nucleotides are highly conserved among several enteric bacteria, and the induction phenomenon occurs in *S. enterica* as well. Genetic evidence supports a model in which the paired structure of the *rpoS* RBS enforces this control (Fig. 19). However, the precise mechanism resulting in SP induction remains unclear. It is not a result of differential transcript stability (Fig. 21) or control by the reported *rpoS* regulators DksA or the HU dimer. Also, the RNA-binding protein Hfq is not involved and presumably, neither are small RNAs. A simple model would be that the *rpoS* RBS is more attractive to ribosomes during SP. If this true, then it is not mediated by known proteins associated with the ribosome specifically during SP.

The environmental stimulus that triggers RBS-mediated SP induction of *rpoS* translation also remains unknown, but similar to transcriptional control, regulation is only seen in rich undefined media, including LB and its individual components tryptone or yeast extract (18, data not shown). No RBS-mediated SP induction occurs in minimal medium containing different carbon sources even when supplemented with amino acids and or putrescine, a polyamine reported to stimulate *rpoS* translation (44, data not shown). In these cases, fusion activity (Fig. 18, construct 542) is already high during exponential growth (data not shown). This media-dependent differential regulation of *rpoS* expression is not due to altered growth rates.

SP induction of RpoS in rich medium depends on regulation of both transcription and translation (17, 18). During exponential growth, Fis protein binds to a site near P_{rpoS} and blocks transcription (18). As cells grow into SP, Fis abundance is drastically reduced and expression from P_{rpoS} is released from Fis repression (1, 18). At the translational level, an unknown regulator acts on the structure of the *rpoS* RBS and represses synthesis during exponential growth or activates it during SP (Fig. 19 and Fig. 22). Collectively, these regulatory targets account for approximately 95% of the overall SP induction of RpoS.

Acknowledgements

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Table 8. Bacterial strains.

Strain	Genotype or description
E. coli	
DH5α	K-12 F$^-$ λ$^-$ *endA1 hsdR17*($r_K^- m_K^+$) *supE44 thi-1 recA1 gyrA96* (Nal^r) *relA1* Δ(*lacZYA-argF*)U169 (ϕ80*dlacZ*ΔM15)
TE1400	K-12 F$^-$ λ$^-$ *araD139* Δ*lacX74 galU galK* $r_K^- m_K^+$ Str^R
BW26678	*lacI*^{Q} *rrnBTL4* Δ*lacZ*(WJ16) *hsdR514* Δ*araBAD*(AH33) Δ*rhaBAD*(LD78) / pKD46 [pSC101*rep* (Ts) Amp^R *araC*$^+$ P_{BAD}-λ*red*]
CF1693	Δ*relA251*:: *kan* Δ*spoT207*::*cat*
CF3032	*argA*::Tn*10* Δ*relA252*::*kan*
CF7968	MG1655 Δ(*lacIZ*) *rph*$^+$
DDS724	MC4100 *cpsB-lac* Δ*dsrA5 zed*::Tn*10d*-Tet
DY330	W3110 *lacU169* [λ cI857*ts* Δ(*cro-bioA*)]
TE8184	CF7968 *trpDC700*::*putPA1303*:: *kan-rpoS-lac* [pr] [b]
TE8197	CF7968 *argA*::Tn*10 trpDC700*::*putPA1303*:: *kan-rpoS-lac* [pr]
TE8199	TE8197 Δ*relA252*::*kan* Δ*spoT207*::*cat*
TE8222	CF7968 *argA*::Tn*10 trpDC700*::*putPA1303*:: *kan-rpoS-lac* [op] [a]
TE8224	TE8222 Δ*relA252*::*kan* Δ*spoT207*::*cat*
TE8226	CF7968 *argA*::Tn*10 trpDC700*::*putPA1303*:: *kan-lac*UV5*p-rpoS-lac* [pr]
TE8228	TE8226 Δ*relA252*::*kan* Δ*spoT207*::*cat*
TE8230	CF7968 *argA*::Tn*10 trpDC700*::*putPA1303*:: *kan-lac*UV5*p-rpoS-lac* [op]
TE8232	TE8230 Δ*relA252*::*kan* Δ*spoT207*::*cat*
TE8260	TE8184 Δ*barA*::*tet*
TE8263	CF7968 *argA*::Tn*10 trpDC700*::*putPA1303*:: *kan-lac*UV5*p-lac* [op]
TE8265	TE8230 Δ*relA252*::*kan* Δ*spoT207*::*cat*
TE8378	CF7968 *argA*::Tn*10 trpDC700*::*putPA1303*:: *kan- lac*UV5*p -rpoS-lac* [pr] (+1)
TE8383	TE8378 Δ*relA252*::*kan* Δ*spoT207*::*cat*
TE8380	CF7968 *argA*::Tn*10 trpDC700*::*putPA1303*:: *kan-rpoS-lac* [pr] (codon 8)
TE8382	TE8380 Δ*relA252*::*kan* Δ*spoT207*::*cat*
TE8267	CF7968 *argA*::Tn*10 trpDC700*::*putPA1303*:: *kan-lac*UV5*p-rpoS-lac* [pr] (Δ1)
TE8269	TE8267 Δ*relA252*::*kan* Δ*spoT207*::*cat*
TE8271	CF7968 *argA*::Tn*10 trpDC700*::*putPA1303*:: *kan-lac*UV5*p-rpoS-lac* [pr] (Δ2)
TE8273	TE8271 Δ*relA252*::*kan* Δ*spoT207*::*cat*
TE8344	CF7968 *argA*::Tn*10 trpDC700*::*putPA1303*:: *kan-lac*UV5*p-rpoS-lac* [pr] (Δ3)
TE8340	TE8344 Δ*relA252*::*kan* Δ*spoT207*::*cat*
TE8345	CF7968 *argA*::Tn*10 trpDC700*::*putPA1303*:: *kan -lac*UV5*p-rpoS-lac* [pr] (Δ4)
TE8341	TE8345 Δ*relA252*::*kan* Δ*spoT207*::*cat*
TE8275	CF7968 *argA*::Tn*10 trpDC700*::*putPA1303*:: *kan -rpoS-lac* [pr] (C469G)
TE8277	TE8275 Δ*relA252*::*kan* Δ*spoT207*::*cat*
TE8279	CF7968 *argA*::Tn*10 trpDC700*::*putPA1303*:: *kan -rpoS-lac* [pr] (G549C)

TE8281 TE8279 Δ*relA252*::*kan* Δ*spoT207*::*cat*
TE8283 CF7968 *argA*::Tn*10 trpDC700*::*putPA1303*::*kan-rpoS-lac* [pr] (C469G, G549C)
TE8285 TE8283 Δ*relA252*::*kan* Δ*spoT207*::*cat*
TE8266 CF7968 *trpDC700*::*putPA1303*:: *kan-lac*UV5*p-rpoS-lac* [pr] (Δ1)
TE8316 CF7968 *trpDC700*::*putPA1303*:: *kan-rpoS-lac*[pr]*dsrA*$^{+}$ *zed*::Tn*10d*-Tet
TE8317 CF7968 *trpDC700*::*putPA1303*:: *kan-rpoS-lac*[pr]Δ*dsrA5 zed*::Tn*10d*-Tet
TE8318 TE8316 Δ*relA252*::*kan* Δ*spoT207*::*cat*
TE8319 TE8317 Δ*relA252*::*kan* Δ*spoT207*::*cat*
TE8372 TE8266 Δ*dksA*::*tet*
TE8270 CF7968 *trpDC700*::*putPA1303*:: *kan-lac*UV5*p-rpoS-lac* [pr] (Δ2)
TE8373 TE8270 Δ*dksA*::*tet*
TE8314 CF7968 *trpDC700*::*putPA1303*:: *kan-lac*UV5*p-rpoS-lac* [pr] (Δ3)
TE8374 TE8314 Δ*dksA*::*tet*
TE8315 CF7968 *trpDC700*::*putPA1303*:: *kan-lac*UV5*p-rpoS-lac* [pr] (Δ4)
TE8375 TE8315 Δ*dksA*::*tet*
TE8363 CF7968 *trpDC700*::*putPA1303*::*bla-rpoS-lac* [pr]
TE8377 TE8363 *hfq-1*::Ω-Km
TE8387 CF7968 *trpDC700*::*putPA1303*::*bla-lac*UV5*p-rpoS-lac* [pr] (Δ1)
TE8388 TE8387 *hfq-1*::Ω-Km
TE8391 CF7968 *trpDC700*::*putPA1303*::*bla-lac*UV5*p-rpoS-lac* [pr] (Δ2)
TE8392 TE8391 *hfq-1*::Ω-Km
TE8402 DY330 *trpDC700*::*putPA1303*:: *kan -rpoS* (*Cla*I, codon 8)-*lac* [pr]
TE8403 DY330 *trpDC700*::*putPA1303*:: *kan -rpoS* (*Cla*I, codon 8)-RNAse III site-*lac* [op]
TE8405 TE8184 Δ*ppkx*::*tet*
TE8419 CF7968 *trpDC700*::*putPA1303*::*tet-lac*UV5*p-rpoS-lac* [pr] (+1, codon 8)
TE8420 CF7968 *trpDC700*::*putPA1303*::*tet-lac*UV5*p-rpoS-lac* [pr] (541, codon 8)
TE8421 CF7968 *trpDC700*::*putPA1303*::*tet-lac*UV5*p-rpoS-lac* [op] (+1, codon 8)
TE8422 CF7968 *trpDC700*::*putPA1303*::*tet-lac*UV5*p-rpoS-lac* [op] (541, codon 8)
TE8439 CF7968 *trpDC700*::*putPA1303*::*tetAR-lac*UV5p-*rpoS* (454, codon 8)-*lac* [pr]
TE8448 CF7968 *trpDC700*::*putPA1303*::*tet-lac*UV5*p-lac*RBS-*rpoS-lac* [pr] (565, codon 8)
TE8483 CF7968 *trpDC700*::*putPA1303*::*tetAR-lac*UV5p-*rpoS* (541, 558)-*lac* (AACAGCT)-*rpoS* (ATG, codon 8)-*lac* [pr]
TE8520 CF7968 *trpDC700*::*putPA1303*::*tetAR-lac*UV5p-(TCACAC)-*rpoS* (554, codon8)-*lac* [pr]
TE8999 CF7968 *trpDC700*::*putPA1303*::*tetAR-lac*UV5p-*rpoS* (548, codon 8)-*lac* [pr]
TE9024 CF7968 *trpDC700*::*putPA1303*::*tetAR-lac*UV5p-*rpoS* (541, codon 8; G550C, G551C)-*lac* [pr]
TE9030 CF7968 *trpDC700*::*putPA1303*::*tetAR-lac*UV5p-*rpoS* (544, codon 8)-*lac* [pr]
TE9036 CF7968 *trpDC700*::*putPA1303*::*tetAR-lac*UV5p-*rpoS* (552, codon 8)-*lac* [pr]
TE9059 DY330 *trpDC700::putPA1303*::*kan*-P_{lacUV5}-*lac* [op]
TE9042 CF7968 *trpDC700*::*putPA1303*::*tetAR-lac*UV5p-*rpoS* (+1, 542)-(TCACACAGGAACAGCT)-*rpoS* (ATG, codon 8)-*lac* [pr]
TE9145 CF7968 *trpDC700*::*putPA1303*::*kan -katE-lac* [op]
TE9146 CF7968 *trpDC700*::*putPA1303*::*tetAR-lac*UV5p-*rpoS* (541, 565)-*lac* (ATG) [pr]

TE9200 CF7968 *trpDC700*::*putPA1303*::*tetAR-lac*UV5p-*rpoS* (541, codon 8; G550C, G551C, C563G, T564G)-*lac* [pr]
TE9218 CF7968 *trpDC700*::*putPA1303*::*tetAR-lac*UV5p-*rpoS* (542, 558)-(AACAGCT)-*lac* [op]
TE9249 CF7968 *trpDC700*::*putPA1303*::*tetAR-lac*UV5p-*rpoS* (542, 557)-(AACAGCT)-*lac* [op]
TE9251 CF7968 *trpDC700*::*putPA1303*::*tetAR-lac*UV5p-GGGTTCCACCAGGAGTGGGCACT-*lac* [pr]
TE9268 CF7968 *trpDC700*::*putPA1303*::*tetAR-lac*UV5p-ACCACCCTGAACAGGATCAGGGCA-*lac* [pr]
TE9270 CF7968 *trpDC700*::*putPA1303*::*tetAR-lac*UV5p-ATCGATTGAGAGGATTTGA-*lac* [op]
TE9274 DY330 *trpDC700*::*putPA1303*:: *kan-rpoS* (ClaI, codon 8)-*cat-lac* [op]
TE9277 DY330 *trpDC700*::*putPA1303*:: *kan-rpoS* (ClaI, codon 8)-*lac* [op]
TE9284 CF7968 *trpDC700*::*putPA1303*:: *kan-katE-lac* [op] *rpoS*(540)-AATTTCACACAGGAAACAGCT-*rpoS*(ATG)
TE9291 CF7968 *trpDC700*::*putPA1303*::*tetAR-lac*UV5p-ACCACCCTGAACAGGATCAGGGTA-*lac* [op]
TE9300 CF7968 *trpDC700*::*putPA1303*::*tetAR-lac*UV5p-*rpoS* (542, codon 8)-*lac* [op]
TE9301 CF7968 *trpDC700*::*putPA1303*::*tetAR-lac*UV5p-*lac* (TCACACAGGAACAGCT)-*rpoS* (ATG, codon 8)-*lac* [op]
TE9302 CF7968 *trpDC700*::*putPA1303*:: *kan-katE-lac* [op] *fis*::*cat*
TE9303 CF7968 *trpDC700*::*putPA1303*:: *kan-katE-lac* [op] *rpoS*(541)-AATTTCACACAGGAAACAGCT-*rpoS*(ATG) *fis*::*cat*
TE9304 CF7968 *trpDC700::putPA1303::tetAR-lac*UV5p-GGGTTCCACCAGGAGTGGGCACT-*lac* [op]
TE9305 CF7968 *trpDC700::putPA1303::tetAR-lac*UV5p-*rpoS* (541, codon 8; G550C, G551C, C563G, T564G)-*lac* [op]
TE9312 CF7968 *trpDC700::putPA1303::tetAR-lac*UV5p-*rpoS* (541, codon 8; G550C, G551C)-*lac* [op]
TE9335 CF7968 *trpDC700::putPA1303::tetAR-lac*UV5p-*rpoS* (542, codon 8; G550C, G551C)-*lac* [op]

S. enterica

LT2 wild type (*mviA* V102G)
LT2A LT2 *mviA*$^{+}$
TH2285 *fis-3*::*cat*
TE315 TR5877 = *hsdL6 hsdSA29* (r_{LT}^{-} m_{LT}^{+} r_{S}^{-} m_{S}^{+}) *metA22 metE551 ilv-452 trpB2 xyl-404 rpsL120* (Str^{R}) H1-b H2-e,n,x (Fels2^{-}) *nml*
TE6153 *putPA1303*::*kan-katE-lac* [op]
TE6675 *putPA1303*::*kan*-P_{tac}-*lac* [op]
TE6676 *putPA1303*::*kan*-P_{lacUV5}-*lac* [op]
TE6756 LT2A *putPA1303*::*kan-katE-lac* [op]
TE6850 LT2A *putPA1303*::*kan-katE-lac* [op] *clpX1*::Tn*10d*-Cam

TE6851	LT2A *putPA1303*::*ka -katE-lac* [op] *mviA22*::Tn*10d*-Cam
TE7304	TE315 *galE putPA1303*:: *kan*::*cat-lac*
TE8536	*putPA1303*::*kan-katE-lac* [op] / pKD46
TE8607	Δ*cysC*::*tetAR*
TE8698	*putPA1303*::*kan*-P_{nlpD}-*lac* [op] (13475-13053)
TE8737	*rpoS1082*::MudJ (codon 66)
TE8738	*rpoS1082*::MudJ (codon 66) / pKD46
TE8744	*putPA1303*::*kan*-P_{rpoS}-*lac* [op] (12765-12338)
TE8754	*putPA1303*::*kan*-P_{rpoS}-*lac* [op] (12765-12338) [op] *cya*::Tn*10*
TE8755	*putPA1303*::*kan*-P_{rpoS}-*lac* [op] (12765-12338) *cya*::Tn*10* *zhc-3729*::Tn*10d*-Cam *crp*-661*
TE8758	*putPA1303*::*kan*-P_{rpoS}-*lac* [op] (12765-12338) *rpoS1071*::Tn*10d*-Cam
TE8759	*putPA1303*::*kan*-P_{lacUV5}-*lac* [op] *rpoS1071*::Tn*10d*-Cam
TE8760	*putPA1303*::*kan-katE-lac* [op] *rpoS1071*::Tn*10d*-Cam
TE8761	*putPA1303*::*kan*-P_{nlpD}-*lac* [op] (13475-13053) *rpoS1071*::Tn*10d*-Cam
TE8764	*putPA1303*::*kan*-P_{rpoS} *-lac* [op] (12765-12338) *fis-3*::*cat*
TE8766	*putPA1303*::*kan*-P_{lacUV5}-*lac* [op] *fis-3*::*cat*
TE8767	*putPA1303*::*kan*-P_{rpoS}-*lac* [op] (12765-12338) *crp773*::Tn*10*
TE8768	LT2 *fis-3*::*cat*
TE8770	*putPA1303*:: *kan*-P_{rpoS}-*lac* [op] (12765-12338) *crp773*::Tn*10* *fis-3*::*cat*
TE8776	*putPA1303*::*kan*-P_{rpoS}-*lac* [op] (12765-12338) *nlpD*::Cam (12720)
TE8787	*putPA1303*::*kan*-P_{rpoS}-*lac* [op] (12765-12338) *nlpD*::Cam (12720) / pKD46
TE8864	*putPA1303*::*kan*-[P_{rpoS}::*tet*]-*lac* [op] (*lac* fusion contains bp 12765-12338, *tetAR* deletes 12580- 12555) *nlpD*:: *cat* (12720)
TE8867	TE8864 / pKD46
TE8868	*putPA1303*::*kan*-P_{rpoS}-*lac* [op] (12663-12553)
TE8869	*putPA1303*::*kan*-P_{rpoS}-*lac* [op] (12663-12553) *fis-3*::*cat*
TE8887	*putPA1303*::*kan*-P_{rpoS}*-*lac* [op] (12765-12338) (* carries A12601C T12596G C12591G)
TE8888	TE8887 *fis-3*::*cat*
TE8895	*putPA1303*::*kan*-P_{rpoS}Δ-*lac* [op] (12765-12338, Δ removes bp 12608-12588)
TE8900	TE8895 *fis-3*::*cat*
TE8903	*rpoS1082*::MudJ (codon 66) Δ*nlpD*::*tetAR* (13360-13003)
TE8990	*rpoS1082*::MudJ (codon 66) Δ*nlpD*::*tetAR* (12522-12454)
TE8911	*putPA1303*::*kan*-P_{rpoS}-*lac* [op] (12765-12338) / pGS349
TE8912	*putPA1303*::*kan*-P_{rpoS}-*lac* [op] (12765-12338) *fis-3*::*cat* / pGS349
TE8991	*rpoS1082*::MudJ (codon 66) Δ*nlpD*::*tetAR* (12584-12555)
TE8992	*rpoS1082*::MudJ (codon 66) Δ*nlpD*::*tetAR* (13360-12555)
TE8915	*rpoS1082*::MudJ (codon 66) Δ*nlpD*::*tetAR* (12584-12555) / pKD46
TE8916	*putPA1303*::*kan*-P_{rpoS}-*lac* [op] (12765-12338) / pFis349
TE8917	*putPA1303*::*kan*-P_{rpoS}-*lac* [op] (12765-12338) *fis-3*::*cat* / pFis349

TE8919	P_{rpoS}::*tetAR*(12580-12555)-*lac* [op] *nlpD*::*cat* (12720) / pKD46
TE8925	TE8737 ΔP_{rpoS} (12584-12555)
TE8993	TE8925 *nlpD*::*tetAR* (13360-13003)
TE8947	*putPA1303*::*kan*-P_{rpoS}-*lac* [op] (12587-12477)
TE8948	*putPA1303*::P_{tac}-*lac* [op] *fis-3*::*cat*
TE8949	*putPA1303*::*kan*-P_{rpoS}-*lac* [op] (12587-12477) *fis-3*::*cat*
TE8971	*putPA1303*::*kan*-P_{rpoS}-*lac* [op] (12663-12477)
TE8972	*putPA1303*::*kan*-P_{rpoS}-*lac* [op] (12663-12477) *fis-3*::*cat*
TE9083	*putPA1303*::*kan*-P_{rpoS}-*lac* [op] (12654-12477)
TE9096	*putPA1303*::*kan*-P_{rpoS}-*lac* [op] (12654-12477) *fis-3*::*cat*
TE9195	LT2A *putPA1303*::*tetAR*-*lac*UV5p-*rpoS* (542, codon 8)-*lac* [pr]
TE9196	LT2 *putPA1303*::*tetAR*-*lac*UV5p-*rpoS* (542, codon 8)-*lac* [pr]
TE9294	LT2A *putPA1303*::*tetAR*-*lac*UV5p-*lac* (TCACACAGGAACAGCT)-*rpoS* (ATG, codon 8)-*lac* [pr]
TE9295	LT2 *putPA1303*::*tetAR*-*lac*UV5p-*lac* (TCACACAGGAACAGCT)-*rpoS* (ATG, codon 8)-*lac* [pr]

All numbering in parenthesis is in base pairs (unless otherwise indicated) relative to the first nt of the transcript originating from P_{rpoS}. In this description, the first nt of the *rpoS* transcript corresponds to 2866139 of GenBank AE000111 for *E. coli* and nt 12589 of GenBank AE00833.1 for *S. enterica* serovar Typhimurium (nt upstream of the transcriptional start directly correlate with the nt designation under the given accession number). The extent of the *rpoS* sequence in the fusions of TE8267, TE8271, TE8344, TE8345, TE8387, TE8391 is described in the text (Chapter 2). In some cases strains were previously constructed and are noted in the text. Str^r, streptomycin resistance; Nal^r, nalidixic acid resitance.

[a] [op], operon (transcriptional) fusion

[b] [pr], protein (translational) fusion

General Discussion

"Regulation of RpoS itself is arguably the most complicated system in bacteria"- M. Cashel (3). Individual and combined environmental cues adjust RpoS abundance at every primary level of regulation with genetic anomalies existing at the translational level (chapter 4; 5, 6). Despite over a decade of experimentation by nearly a dozen labs, only two well established stimulus → response pathways have been elucidated: (i) carbon starvation results in RpoS induction through inhibition of ClpXP/SprE-mediated proteolysis (9, 16, 21) and (ii) low temperature induces *rpoS* translation through intermolecular RNA interactions between the *rpoS* leader and DsrA (12). Herein, we describe a third stimulus → response pathway; SP induction of RpoS in rich medium.

Initially, we investigated the unifying hypothesis that ppGpp regulates SP induction of RpoS in *E. coli* (8). This was an attractive model due to three previous results: (i) conditions that induce ppGpp also induce RpoS (14), (ii) mutants deficient for ppGpp are dramatically decreased in RpoS abundance (4) and (iii) artificial overproduction of ppGpp results in RpoS induction (3). Our analysis demonstrated that SP induction occurs normally in the absence of ppGpp, although basal expression was decreased six-fold (8). In these experiments overall induction of *rpoS*, at both the transcriptional and translational levels was 35-fold. Surprisingly, regulation at the transcriptional level appeared to play a larger role in SP induction (15-fold) which prompted further investigations (7, 8).

Several other important conclusions were drawn from this work including the intriguing result that SP induction of *rpoS* translation does not rely on the antisense element of the leader region (8). However, this element is necessary for adjustment of *rpoS* synthesis by at least two sRNAs and Hfq (11, 12). It is likely that the role of the antisense element is to maintain low levels of synthesis during optimal conditions and regulators open the structure to increase translation regardless of growth phase. This interpretation may be applicable to DsrA, Hfq and ppGpp, all of which influence *rpoS* translation and require the *rpoS* leader for action (8). In the respective mutant backgrounds, *rpoS* translation was defective although SP induction was virtually unchanged (8).

Next, we focused on transcriptional regulation of *rpoS*, which at that time, was basically uncharacterized due to a general focus on translational models of control (6). A sequence analysis of the P_{rpoS} region suggested putative binding sites for 17 transcriptional regulators including Fis(7). Transcriptional repression by Fis is a coherent model because an inverse correlation exists between Fis and RpoS abundance (1, 7). In addition, they are induced by opposite stimuli, fresh medium (Fis) and spent medium (RpoS; 2, 6). It is likely that Fis acts directly as a repressor given the site necessary for regulation *in vivo* is specifically bound *in vitro* (7). However, Fis pleiotropy in the mutant background could indirectly contribute to regulatory effects on P_{rpoS} activity.

If Fis regulates RpoS then what regulates Fis? The generic answer is dilution into fresh medium which results in a massive induction making Fis the most abundant nucleoid-associated protein within the cell (1, 2, 15). Fis is autoregulatory and increased Fis abundance results in

transcriptional repression and levels decrease as a function of cell division (2). Activation of P_{fis} transription is tightly coupled to nutritional status including the availability of CTP, the first nt of the *fis* trancript (19). Additionally, P_{fis} activity is repressed by unfavorable growth conditions via the stringent response (20). Thus it is possible that decreased *rpoS* transcription in a ppGpp mutant background is due to increased Fis repression. Further investigations into the regulation of Fis expression would provide a greater understanding of the interplay of global regulators in physiological adaptation.

Our investigations regarding SP induction of RpoS at the translational level exposed a novel form of regulation (Chapter 4). Genetic dissection of the *rpoS* leader clearly demonstrates that the RBS mediates nearly 10-fold induction in synthesis as cells enter SP. The importance of these nt in regulation was also shown at the native *rpoS* locus. The *rpoS* RBS is highly conserved among enteric bacteria and the induction phenomenon occurs in *S. enterica* as well.

The mechanism of translation induction remains elusive and is not a result of differential transcript stability, Hfq-dependent sRNAs, and protein regulators that recognize a primary sequence motif (Chapter 4). Genetic evidence heavily favors a model in which the structure of the *rpoS* RBS signals regulation. The structural elements of the RBS necessary for SP regulation are currently unclear, although a direct correlation was made between regulation and a single-stranded SD sequence in the predicted secondary structure (data not shown). This theory is supported by the nature of the SD in the characterized RBS structures of *cbiA* (single-stranded) and *rpoH* (partially double-stranded) (13, 17). Of these RBSs, only *cbiA* signals SP induction, a result that suggests control at this level is a general phenomenon (data not shown).

Another shocking result of our translational analysis was that *lac/rpoS* hybrid RBSs varied in translational efficiency up to 100-fold (Chapter 4). This was not a result of transcript stabilization (data not shown). The large translational variation in our engineered RBS is also not due to a variable spacer region, between the SD sequence and the initiation codon, or an extended RBS. However, we did show that the five nt SD sequence (AGGAG) is expressed approximately three-fold higher than AGGA in a similar context consistent with earlier reports (10, 18). These results demonstrate that slight nt adjustments in a RBS can dramatically affect translational efficiency and regulation.

SP regulation of RpoS induction in LB is not regulated by ClpXP-SprE proteolysis (7, 16). Although, mutants defective in protein turnover, demonstrate a three-fold increase in RpoS abundance and activity (7). It seems energetically unfavorable to make excess RpoS protein and then actively degrade it unless a quick response to an unknown stimulus is necessary. This may be the case for increases RpoS in response to carbon starvation.

SP induction of RpoS in rich medium- The model

SP induction of RpoS in rich medium depends on regulation of both transcription and translation. During exponential growth, Fis molecules bind several sites near P_{rpoS} and block transcription (7). As cells grow into SP, Fis abundance is low and P_{rpoS} expression increases (1, 7). At the translational level, the structure of the *rpoS* RBS signals SP induction by an uncharacterized

mechanism (Chapter 4). Collectively, these regulatory targets account for approximately 95% of the overall SP induction of RpoS.

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