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Signal Transduction and Regulatory Mechanisms Involved in Control of the σ^S (RpoS) Subunit of RNA Polymerase

Regine Hengge-Aronis*

Institut für Biologie, Mikrobiologie, Freie Universität Berlin, 14195 Berlin, Germany

* Mailing address: Institut für Biologie, Mikrobiologie, Freie Universität Berlin, Königin-Luise-Str. 12-16, 14195 Berlin, Germany. Phone: (49)-30-838-53119. Fax: (49)-30-838-53118. E-mail: Rhenggea @zedat.fu-berlin.de.

INTRODUCTION

σ^S, or RpoS, is a sigma subunit of RNA polymerase in *Escherichia coli* that is induced and can partially replace the vegetative sigma factor σ^{70} (RpoD) under many stress conditions. As a consequence, transcription of numerous σ^S-dependent genes is activated (for reviews, see references 75, 77, 79, 124, and 125). Consistent with the multiple functions of the σ^S regulon, the *rpoS* gene was discovered independently and named accordingly by several groups (recently summarized in references 79 and 105). It was identified as a gene involved in near-UV resistance (*nuv*) (216); as a regulator for the *katE*-encoded catalase HPII (*katF*) (126, 186), exonuclease III (*xthA*) [186], and acidic phosphatase (*appR*) (211); and, finally, as a starvation-inducible gene encoding a central regulator for stationary phase-inducible genes (*csi-2*) (113). Only then was it recognized that all the previous studies had described alleles of the same gene (113, 212), which codes for a sigma factor (152, 156, 209). Because of its crucial role in stationary phase or under stress conditions, the name σ^S or RpoS was proposed (113). In addition, the term σ^{38} is used sometimes (although the molecular mass of σ^S deviates from 38 kDa in various species and even in some *E. coli* strains). The *rpoS* gene has also been identified in other enteric and related bacteria. At present, it seems that σ^S occurs in the γ branch of the proteobacteria, i.e., in a group of gram-negative bacteria that includes many species with special importance for humans because of their pathogenic or beneficial potential. With minor variations, the general function of σ^S in these bacteria appears to be similar to that in *E. coli* (summarized in reference 79).

In more recent studies, it was demonstrated that σ^S and σ^S-dependent genes not only are induced in stationary phase but actually respond to many different stress conditions (76, 82, 121, 144, 148). Therefore, σ^S is now seen as the master regulator of the general stress response, which is triggered by many different stress signals, is often (though not always) accompanied by a reduction or cessation of growth, and provides the cells with the ability to survive the actual stress as well as additional stresses not yet encountered (cross-protection). This is in pronounced contrast to specific stress responses, which are triggered by a single stress signal and result in the induction of proteins that allow cells to cope with this specific stress situation only. While specific stress responses tend to eliminate the stress agent and/or to mediate repair of cellular damage that has already occurred, the general stress response renders cells broadly stress resistant in such a way that damage is avoided rather than needing to be repaired (for a recent review of different bacterial stress responses, see reference 203).

The major function of the general stress response is thus preventive, which is clearly reflected in the σ^S-dependent multiple stress resistance observed with starved or otherwise stressed cells (82, 113, 136) (for a recent review of the general stress response that also includes physiological aspects, see reference 79). Accordingly, the majority of the more than 70 σ^S-dependent genes known so far confer resistance against oxidative stress, near-UV irradiation, potentially lethal heat shocks, hyperosmolarity, acidic pH, ethanol, and probably other stresses yet to be identified. Additional σ^S-controlled gene products generate changes in the cell envelope and over-

all morphology (stressed *E. coli* cells tend to become smaller and ovoid). Metabolism is also affected by σ^S-controlled genes, consistent with σ^S being important under conditions where cells switch from a metabolism directed toward maximal growth to a maintenance metabolism. σ^S also controls genes mediating programmed cell death in stationary phase, which may increase the chances for survival for a bacterial population under extreme stress by sacrificing a fraction of the population in order to provide nutrients for the remaining surviving cells (22). Finally, a number of virulence genes in pathogenic enteric bacteria have been found to be under σ^S control, consistent with the notion that host organisms provide stressful environments for invading pathogens (recently summarized in reference 80). However, even though numerous σ^S-dependent genes have been identified (see references 77, 79, and 125 for recent compilations), many more such genes will probably be found in the future. Moreover, the functions of the genes known so far are incompletely understood. Even after more than 10 years of intensive research on σ^S, much remains to be learned about the physiology of the σ^S-mediated response. The same is true for the regulatory interdependencies within the large regulatory network directed by σ^S. By contrast, the basic mechanisms of regulation of σ^S itself are now reasonably well understood and are the subject of this review (for recent minireviews on the same subject, see references 81 and 125). Since by far most of the relevant work has been done with *E. coli* and *Salmonella*, the systems referred to in this review are those described for these enteric bacteria if not otherwise mentioned. Whereas the physiological function of σ^S is comparable in all species where it has been discovered to date, there are significant differences between enteric bacteria and pseudomonads in the regulation of σ^S that are outlined specifically.

THE PROBLEM OF MULTIPLE STRESS SIGNAL INTEGRATION

Complex and physiologically far-reaching bacterial responses often use a single master regulator at the interface of upstream signal processing and downstream regulatory mechanisms. In the general stress response of *E. coli*, σ^S plays the role of this top-level master regulator. Other examples of such central regulators are σ^B in the general stress response of various gram-positive bacteria (172) and the response regulator Spo0A in sporulation initiation of *Bacillus subtilis* (202). The master regulators serve as the decisive information processing units, which connect complex signaling networks with downstream regulatory cascades or networks that ultimately control the expression of numerous structural genes associated with a response. These regulatory networks exhibit a hierarchical and modular structure; i.e., they can be subdivided into lower-level smaller modules that are under the control of secondary regulators, which also allow specific signal input at such lower and more confined levels. A master regulator may also commit the cell to a certain complex developmental program, with specific temporal and spatial control being exerted by secondary regulalors.

Depending on the type of master regulator (sigma factor, two-component response regulator, etc.) as well as on whether its cellular level or its activity (or both) is the decisive param-
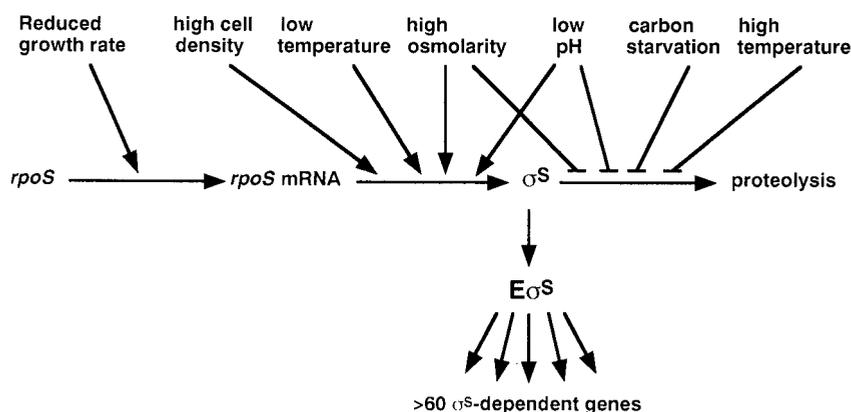
FIG. 1. Various levels of σ^S regulation are differentially affected by various stress conditions. An increase of the cellular σ^S level can be obtained either by stimulating σ^S synthesis at the levels of *rpoS* transcription or *rpoS* mRNA translation or by inhibiting σ^S proteolysis (which under nonstress conditions is extraordinarily rapid). The most rapid and strongest reaction can be achieved by a combination of these processes (as observed, e.g., on hyperosmotic or pH shifts). For further details, see the text.

eter, signal transduction and integration upstream of the central regulator can be very different. In the general stress response of *E. coli*, the decisive parameter is the cellular level of σ^S (however, as described below, there is now some initial evidence that σ^S may also be subject to some sort of activity control). σ^S levels increase in response to starvation for carbon, nitrogen, or phosphate sources as well as for amino acids. This leads to entry into stationary phase, i.e., a complete cessation of growth, but σ^S can also be induced by a partial reduction of the growth rate (64, 89, 92, 113, 114, 157, 210). Additional inducing conditions are hyperosmolarity (148), nonoptimally high (144) or low (199) temperature, acidic pH (18, 121), high cell density (114), and probably other environmental stress situations.

How can so many different stress signals be integrated toward a single parameter, i.e., the intracellular σ^S concentration? Do they generate a common intracellular signal? Initially it seemed that a reduction or cessation of growth might be such a signal. However, σ^S is also induced in late exponential phase (provided that a certain cell density is reached) without any change of growth rate (114). In response to the classical heat shock procedure (i.e., a shift from 30 to 42°C), σ^S is induced even though growth is accelerated (144). On the basis of more recent studies, it has become clear that the concept of a unifying intracellular σ^S-inducing signal cannot be correct, simply because different environmental signals affect different levels and therefore completely different processes in the regulation of σ^S.

Regulation of σ^S occurs at nearly every theoretically possible level (Fig. 1). *rpoS* transcription is stimulated by controlled downshifts in growth rate in a chemostat (157, 210) as well as by continuous reduction in growth rate which results in an inversely correlated increase in *rpoS* transcription (5- to 10-fold) (113, 114). By contrast, abrupt cessation of growth, as for example, in response to sudden glucose starvation, only weakly increases *rpoS* transcription (less than twofold) (113, 114). *rpoS* translation, i.e., the rate of translation of already existing *rpoS* mRNA, is stimulated (i) by high osmolarity (hyperosmotic shift rapidly activates translation more than fivefold, but also continuous growth at high osmolarity has clear effects) (148), (ii)

during growth at moderately low temperatures (e.g., at 20°C) (199), (iii) on reaching a certain cell density (approximately 1 × 10^8 to 2 × 10^8 cells ml^{-1}) during growth in minimal glucose medium (114), and (iv) in response to a pH downshift from pH 7 to pH 5 in rich medium (G. Kampmann and R. Hengge-Aronis, unpublished data).

Besides this multifaceted regulation of σ^S synthesis, there is also control of σ^S degradation. In cells growing on minimal medium, σ^S (which is produced at a low but measurable rate) is degraded with a half-life between 1 min and a few minutes (114, 144, 148, 208). However, in response to stresses such as starvation (114, 208), shift to hyperosmolarity (148), the classical heat shock (144), or pH downshift to pH 5 (18), σ^S proteolysis is considerably reduced or even completely inhibited. As a consequence, σ^S rapidly accumulates in the cell. The kinetics of this stabilization can be very rapid (on hyperosmotic shift, σ^S is strongly stabilized within a few minutes [148]) or can take somewhat more time (as, e.g., after heat shock [144]). This again indicates that even in cases where the same level of σ^S regulation is affected by different stress conditions, the regulatory mechanisms involved are likely to be different.

In recent years, considerable progress has been made in identifying (i) *cis*-acting regulatory regions at the DNA, mRNA, or protein levels; (ii) *trans*-acting factors such as regulatory proteins or regulatory RNAs; and (iii) additional large or small molecules that modulate σ^S regulation at these different levels. In general, the closer to the *rpoS* gene, its mRNA, or the σ^S protein itself these factors act, the better understood is their molecular mechanism of action. By contrast, the upper parts of the corresponding signal transduction pathways have remained more elusive. It is, however, already clear that these pathways do not operate independently and in parallel until they finally converge to influence, e.g., *rpoS* mRNA secondary structure or the activity of a specific σ^S recognition factor for proteolysis. Rather, these pathways are highly interconnected, such that specific stress conditions can influence the cellular σ^S concentration by multiple mechanisms. Moreover, specific components of these pathways also control each other. Thus, σ^S is controlled by a complex signal transduction network whose redundancy, additiveness, and internal feedback regu-

A

```
              nlpDp1+p2        rpoSp
   pcm            nlpD                    rpoS
          (B)          (C)
   ── 500 bp
```

B

```
                                    . -35   .
GCTTAAAACGTGAGGAAATACCTGGATTTTTCCTGGTTATTTTGCCGCAG
(pcm)
    -10        +1(p1)   -35
GTCAGCGTATCGTGAACATCTTTTCCAGTGTTCAGTAGGGTGCCTTGCAC

     -10           +1(p2)
GGTAATTATGTCACTGGTTATTAACCAATTTTTCCTGGGGGATAAATGAG
                                          (nlpD)
```

C

```
CCAATCACTGGCGGAAATGCCATTACCCAGGCCGACGCAGCAGAGCAAGG

CRP box I:
AAATGTGATCTAGATCACATTT
|||||| ||  ||  ||||||||
AGTTGTGATCAAGCCTGCACAAAATTCCACCGTTGCTGTTGCGTCGCAAC
                                 -35

          -10      +1(rpoSp)
CGACAATTACGTATTCTGAGTCTTCGGGTGAACAGAGTGCTAACAAAATG

               CRP box II:
               AAATGTGATCTAGATCACATTT
               || |||||| |  | ||||||
TTGCCGAACAACAAGCCAACTGCGACCACGGTCACAGCGCCTGTAACGGT
```
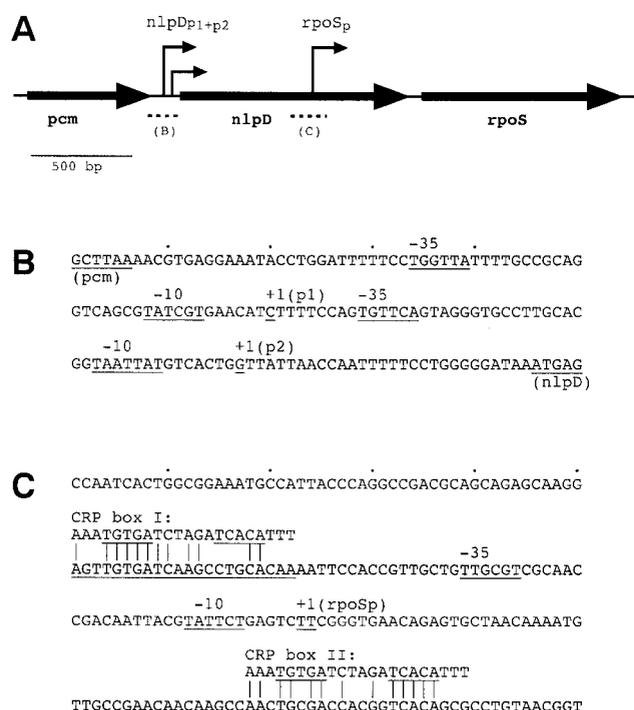
FIG. 2. Transcriptional control regions upstream of the *rpoS* gene. (A) The *nlpD-rpoS* operon is located at 61.76 min on the *E. coli* chromosome, where it is trancribed in counterclockwise direction. (B) The operon promoters (*nlpD*p1 and *nlpD*p2) contribute to basal expression of *rpoS* but are not regulated by growth rate or growth phase (115). (C) The major *rpoS* promoter (*rpoS*p) is located within the *nlpD* gene, is flanked by two putative cAMP-CRP binding sites (CRP box I and II), and is subject to stationary-phase induction when cells are grown on rich medium (112). Broken lines in panel A indicate the relative positions of the sequences shown in panels B and C.

latory loops are crucial for its admirable signal-integrative power (and probably its nonlinear behavior) but at the same time have made life difficult for researchers trying to elucidate these pathways.

REGULATION OF *rpoS* TRANSCRIPTION

Soon after σ^S had been recognized as a stationary-phase regulator that was itself induced in stationary phase (113), it became clear that much of its regulation was due to posttranscriptional mechanisms (114, 127, 135). Although σ^S protein levels are very low in exponentially growing cells, relatively high levels of *rpoS* mRNA are present and do not seem to change in response to several stresses that actually result in strongly elevated σ^S protein levels. Therefore, attention has focused on the control of *rpoS* translation and σ^S proteolysis (see below) whereas *rpoS* transcription has remained insufficiently characterized. Nevertheless, transcriptional regulation of *rpoS* occurs, e.g., during the gradual decrease in growth rate when cells grow in rich medium and finally enter stationary phase. Under these conditions, *rpoS* transcription is activated approximately 5- to 10-fold (113, 114, 135, 153, 190, 208).

Promoters Contributing to *rpoS* Transcription

Several promoters are involved in *rpoS* transcription (Fig. 2).

Two transcripts can be detected by Northern analysis (8). Polycistronic *nlpD-rpoS* mRNA originates from two closely spaced promoters (*nlpD*p1 and *nlpD*p2) upstream of the *nlpD* gene (112, 115), which encodes a lipoprotein of unknown function (87, 115). Another promoter (*rpoS*p) is located within the *nlpD* gene and produces a monocistronic *rpoS* mRNA with an unusually long nontranslated 5′ region of 567 nucleotides (112, 208). Studies with transcriptional fusions that included a 5′ deletion analysis indicated that this transcript is the major *rpoS* mRNA (112). Moreover, *rpoS*p accounts for activation of transcription in Luria broth-grown cells during entry into stationary phase (112, 208). The NlpD protein is not stationary phase induced, which indicates that the *nlpD* promoters are not growth phase regulated (115).

In other enteric bacteria, *Vibrionaceae* members, and pseudomonads studied so far, the *nlpD* and *rpoS* genes are always linked, which suggests similar transcriptional regulation to that in *E. coli*. Downstream of *rpoS*, however, variations are quite common and even occur between different *E. coli* strains (28, 37, 83). However, *nlpD* can occur alone in bacteria that do not possess an *rpoS* gene, such as *Haemophilus influenzae* (55).

Trans-Acting Factors Controlling *rpoS* Transcription

cAMP-CRP and EIIA(Glc). In strains carrying mutations in *cya* (encoding adenylate cyclase) or *crp* (encoding the cyclic AMP [cAMP] receptor protein [CRP], also called the catabolite activator protein), σ^S levels and the activities of transcriptional *rpoS*::*lacZ* fusions are already high in exponential phase, indicating that cAMP-CRP is a negative regulator of *rpoS* transcription. In the *cya* mutant, this phenotype can be reversed by the addition of external cAMP (113, 114). However, the mode of action of cAMP-CRP in *rpoS* transcriptional control seems to depend on the growth phase. Recent evidence indicates that during entry into stationary phase, cAMP-CRP positively controls *rpoS* transcription (F. Scheller and R. Hengge-Aronis, unpublished results). This may resolve an apparent contradiction between the above-mentioned observation of high log-phase levels of σ^S in *cya* or *crp* mutants (114) and the finding that certain *rpoS*::*lacZ* fusions show reduced expression in a *cya* background (135).

Two putative cAMP-CRP binding sites are present upstream and downstream of *rpoS*p (Fig. 2), and the role of these potential binding sites is currently under investigation. Whereas the location of the upstream cAMP-CRP box is similar to that in the *lac* promoter and corresponds to a classical activator position at a class I promoter (31), the location of the second cAMP-CRP box downstream of the transcriptional start site may suggest an inhibitory action. In addition, cAMP-CRP may also have an indirect effect on *rpoS* expression, since *cya* or *crp* mutants exhibit a reduced growth rate, which in turn can affect *rpoS* transcription, as mentioned above.

Adenylate cyclase activity is positively modulated by the *crr*-encoded EIIA(Glc), which is the soluble part of the glucose-specific EII component of the phosphotransferase system for solute uptake. Consistent with this, a *crr* mutation results in elevated σ^S levels during exponential phase, which reflects increased *rpoS* transcription as well as increased *rpoS* translation. The former can be suppressed by cAMP addition, indicating that EIIA(Glc) affects *rpoS* transcription through its

modulation of adenylate cyclase activity (217). Phenotypically, the *crr* mutant thus seems to mimic the log-phase behavior of the *cya* mutant.

Polyamines stimulate adenylate cyclase expression at the level of translational initiation, and at the same time they lead to 2.3- and 4-fold increases of the cellular levels of σ^S and σ^{28}, respectively (235). This polyamine-induced upregulation of σ^S may mimic the positive effect of cAMP-CRP on *rpoS* transcription during entry into stationary phase.

The GacS-GacA two-component and Las-Rhl quorum-sensing systems in pseudomonads. GacA is a two-component response regulator in various *Pseudomonas* species that has long been known to positively affect the production of secondary products such as antibiotics, toxins, and lytic exoenzymes during entry into stationary phase (117, 123, 178). The cognate histidine sensor kinase is the GacS (LemA) protein (85, 178). What is actually sensed by GacS has not been clarified. Homologs of GacA are present in *Salmonella* (SirA, which controls certain virulence genes [93]) and *E. coli* (YecB or UvrY [see below]). The GacS-GacA two-component system is at the top of a regulatory cascade that controls the LasI-LasR quorum-sensing system, which in turn regulates a second quorum-sensing system, RhlI-RhlR (110, 167, 176). These quorum-sensing systems are crucial for the control of virulence factors, exoenzymes, and stress-protective proteins as well as for the formation of biofilms (62, 74, 111, 159, 232); for summaries of quorum-sensing systems, see references 61 and 231).

Mutations in *gacA* or *gacS* also result in a more than 80% reduction of σ^S levels and equally reduced expression of a transcriptional *rpoS*::*lacZ* fusion specifically during transition into stationary phase (227). Whether this regulation of *rpoS* by the Gac system is direct or indirect has not been demonstrated. There are also conflicting data about how *rpoS* is linked to the Las-Rhl cascade. An earlier study had indicated that *rpoS* is under positive control of both the Las and Rhl systems (110). More recently, however, *rpoS* was found not to be affected by *rhl* mutations; in contrast, *rhlI* is upregulated in *rpoS* mutants, indicating that σ^S is a negative regulator of the Rhl system (228). This fits with the observation that pyocyanin and pyoverdin are overproduced in *rpoS* mutants (205), since these virulence-associated factors are under positive control of the Rhl system (27).

In contrast to the situation in *Pseudomonas,* the GacA homolog YecB (UvrY) in *E. coli* appears not to be involved in the control of *rpoS* expression. Even though YecB overproduction stimulated the expression of transcriptional and translational *rpoS*::*lacZ* fusions and resulted in a twofold-higher level of σ^S specifically during transition into stationary phase, a *yecB*::*cat* knockout mutation did not alter the expression of these *rpoS*::*lacZ* fusion, nor were σ^S levels affected (Kampmann and Hengge-Aronis, unpublished). While σ^S plays similar physiological roles in *E. coli* and *Pseudomonas* (188, 205), differential control by YecB-GacA may reflect the different environmental conditions characteristic of the natural habitats of these bacteria.

BarA, a histidine sensor kinase in search of a response regulator. The *E. coli* homolog of GacS is a hybrid sensor kinase called BarA (40% identity and 59% overall similarity at the amino acid level to *P. aeruginosa* GacS). BarA was previously found as a multicopy suppressor of an *envZ* mutation;

i.e., when present at high levels, BarA is able to cross-phosphorylate the response regulator OmpR and thereby activate porin synthesis (88, 154). Under the name AirS, BarA has also been identified as a virulence factor in uropathogenic *E. coli* (241). There is evidence that BarA plays a positive role in *rpoS* expression. A strain with a *lacZ* insertion in the chromosomal copy of *barA,* which was originally isolated as a hydrogen peroxide-sensitive mutant, exhibits reduced levels of σ^S (150, 151). In the mutant, *rpoS* mRNA levels were reduced during exponential phase but were normal in stationary phase. Therefore, BarA was suggested as a positive regulator of *rpoS* transcription (150). By specifically affecting *rpoS* mRNA levels in exponential phase, this control may determine the range within which σ^S levels can be modulated by posttranscriptional control mechanisms in response to various stress conditions.

The homology to the GacS-GacA system, as well as recent biochemical data (166), suggests that BarA is a cognate sensor kinase for YecB. Therefore, it seems surprising that BarA but not YecB (see above) is involved in σ^S control. However, it is possible that BarA acts on more than one response regulator with an unknown target response regulator being involved in *rpoS* control. BarA (GacS) belongs to the complex "built-in phosphorelay" sensor kinases in which sensor, transmitter, receiver, and histidine-containing phosphotransfer domains are combined in a single polypeptide chain. In view of their multiple interactions, phosphorelay components seem especially adequate for establishing such phosphotransfer networks.

PsrA in pseudomonads: a TetR-like regulator. A search for insertional mutations that downregulated the expression of a transcriptional *rpoS*::*lacZ* fusion in *P. putida* yielded a mutant defective in a gene termed *psrA* (for "*Pseudomonas* sigma regulator"), which encodes a TetR repressor-like regulatory protein. *psrA* is required for increased *rpoS* transcription during entry into stationary phase and is negatively autoregulated (104). Whether this control of *rpoS* is direct or indirect is currently unknown.

Role for Polyphosphate in *rpoS* Regulation

Inorganic polyphosphate occurs in most microorganisms and often accumulates in stationary phase or under other stress conditions (106, 107). In *E. coli,* the actual polyphosphate level is the result of a balance between synthesis (catalyzed by polyphosphate kinase, encoded by *ppk*) and degradation (catalyzed by exopolyphosphatases, encoded by *ppx* and *gppA*) (106). Polyphosphate accumulation is positively affected by the "alarmone" guanosine 3',5'-bispyrophosphate (ppGpp; see below), which seems to inhibit the *ppx*-encoded exopolyphosphatase (108). Polyphosphate stimulates Lon-mediated degradation of ribosomal proteins; i.e., it may be crucial for gaining access to intracellular amino acid pools under conditions of sudden carbon or amino acid starvation (109).

Polyphosphate-free *ppk* mutants are multiple stress sensitive and impaired in stationary phase survival (36, 175). Consistent with these phenotypes, σ^S levels as well as the expression of a transcriptional *rpoS*::*lacZ* fusion are reduced in a strain that overproduces yeast exopolyphosphatase and is therefore depleted of polyphosphate (195). These findings indicate that polyphosphate somehow stimulates *rpoS* transcription and thereby contributes to stationary-phase induction of *rpoS*

(which in rich media is partly due to increased *rpoS* transcription). However, polyphosphate fails to stimulate *rpoS* transcription in vitro and therefore may exert an indirect influence in vivo (195).

Small Molecules That Influence *rpoS* Transcription

ppGpp. ppGpp levels in *E. coli* strongly increase in response to amino acid limitation (triggering the stringent response) or starvation for carbon, nitrogen, and phosphorus sources. Amino acid limitation causes a rise in the cellular level of uncharged tRNA, which is sensed by the ribosome-associated RelA protein (ppGpp synthase I). Under other starvation conditions, ppGpp synthesis is mediated by SpoT (ppGpp synthase II). SpoT is also the degrading enzyme. Only *relA spoT* double mutants are completely devoid of ppGpp (32).

Such ppGpp-free mutants contain strongly reduced σ^S levels. Glucose and phosphate starvation, but not amino acid limitation, still induce σ^S in these mutants (albeit to lower levels than in the wild type). On the other hand, σ^S accumulation can be triggered by artificially stimulating ppGpp accumulation (64).

ppGpp affects *rpoS* transcription, as demonstrated with transcriptional *rpoS*::*lacZ* fusions (112). However, ppGpp does not seem to specifically target the promoters involved in *rpoS* transcription, since a transcriptional *rpoS*::*lacZ* fusion construct, in which these natural promoters were deleted (and basal expression was due to vector-dependent transcriptional readthrough activity), exhibited similarly reduced expression as the promoter-carrying construct in a ppGpp-free genetic background. It was therefore proposed that in the case of *rpoS*, ppGpp may affect transcriptional elongation or transcript stability rather than transcriptional initiation (112). In the absence of ppGpp, starvation may result in an uncoupling of transcription and translation, which may lead to increased premature termination, as demonstrated for *lacZ* mRNA (52, 219, 220).

It is also unclear whether this ppGpp effect is direct or indirect. An increase in the cellular ppGpp content results in the accumulation of polyphosphate, which also stimulates *rpoS* transcription by an unknown mechanism (see above). It is therefore conceivable that ppGpp acts indirectly via polyphosphate. The finding that a polyphosphate-depleted strain is not impaired in ppGpp accumulation but contains strongly reduced σ^S levels is consistent with such an indirect mode of action (195). Clearly more research is required to elucidate these relationships at the molecular level.

Is *rpoS* expression controlled by quorum sensing? Sometimes high cell density in a bacterial population turns out to be the inducing signal for "stationary phase-inducible" genes. The classical "quorum-sensing" system is the *lux* system in *Vibrio fischeri*, where a membrane-permeable acylated homoserine lactone (acylated HSL, the "autoinducer") is produced by LuxI and accumulates in the medium. Beyond a certain threshold concentration, the autoinducer binds to and activates LuxR, which stimulates the expression of the *luxI* and *luxR* genes themselves and the luciferase structural genes (60, 61, 187). Numerous bacterial species contain homologs of the LuxI-LuxR pair, which control a wide variety of output functions (recently summarized in reference 231). Other types of quorum-sensing systems use different kinds of inducing molecules;

e.g., gram-positive species use small peptides in general. A hallmark of all these systems is their inducibility on addition of conditioned medium, i.e., spent supernatant obtained from a culture grown to relatively high cell density, which contains the inducing molecule in sufficient concentration.

With respect to *rpoS* induction by conditioned medium, conflicting data have been reported. Such induction (approximately fourfold) was observed for a transcriptional *rpoS*::*lacZ* fusion, and acetate was proposed as the inducing agent (190). With a different *rpoS*::*lacZ* operon fusion present in multiple copies, fourfold induction was also found with conditioned Luria broth medium (153), but when the same fusion was present in single copy in the chromosome, induction by spent medium was reduced to a mere 1.6-fold (197). In another study, a transcriptional *rpoS*::*lacZ* fusion was found to be completely unaffected by conditioned medium (63). With a set of single-copy transcriptional and translational *rpoS*::*lacZ* fusions (114), very little if any induction was obtained, no matter whether conditioned rich or minimal medium was used, and even spent medium freshly prepared in parallel with the induction experiments (to compensate for potential instability of a putative inducer) had little effect on *rpoS* expression levels (D. Traulsen and R. Hengge-Aronis, unpublished results). It therefore seems that quorum sensing mediated by some excreted medium component does not play a significant role in the regulation of *rpoS* in *E. coli*. Therefore, translational induction of *rpoS* beyond a certain cell density (114) may also be connected to some metabolic alterations rather than to quorum sensing mediated by an excreted substance (see below). The finding that *rpoS* itself is probably not or is only weakly controlled by quorum sensing does not preclude certain σ^S-transcribed genes from being subject to such regulation, which may affect the promoters of these genes directly (11, 197, 206).

The *E. coli* genome sequence (23) does not reveal obvious homologs of genes encoding known acyl-HSL synthases of the LuxI and AinS families (17, 61, 66). There is, however, a LuxR-related protein, SdiA, which may respond to an unidentified acyl-HSL (63, 197). Expression of SdiA itself, as well as the activity of a known target promoter, *ftsQ*p2, responds negatively to conditioned (*E. coli*) medium, which may mean that the potential of *E. coli* to respond to an acyl-HSL via SdiA (and thereby activate cell division genes) is downregulated in stationary phase. *rpoS*, however, does not seem to be under the control of SdiA (63).

Homoserine lactone and homocysteine thiolactone. Nonacylated HSL has been implicated in *rpoS* control in *E. coli*. It was reported that a *thrA metL lysC* mutant, which is deficient early in the branched pathway that leads to biosynthesis of lysine, methionine, threonine, and isoleucine, had reduced σ^S levels, which apparently could be suppressed by exogenously adding HSL (at concentrations up to 1 mM). This suppression was weaker in the presence of multiple copies of RspA. Such overexpression also reduced stationary-phase expression of an *rpoS*::*lacZ* fusion (which apparently was a transcriptional fusion). Therefore, it was hypothesized that HSL is an inducer for *rpoS* expression and that RspA may be involved in the degradation of HSL (86). At the time this work was published, this seemed to be in line with quorum-sensing studies that demonstrated the role of acylated HSLs in gene regulation. However, it is now known that free HSL is not the precursor

for acylated HSLs that serve as autoinducers in LuxI-LuxR-like systems (72, 165) but, rather, plays the role of an intracellular metabolite. Moreover, there is good evidence against quorum sensing affecting *rpoS* regulation (see above).

More recently it was found that HSL (up to 1 mM) added to wild-type *E. coli* did not induce *rpoS* (197), and the RspA overproduction effect on *rpoS* is now considered nonphysiological (67). Nevertheless, the idea that RspA may degrade HSL is consistent with the recent observation that RspA-overproducing strains indeed seem to have increased homoserine and decreased HSL levels (U. Sauer, personal communication). Unexpectedly, these strains actually show elevated σ^S levels, which would not seem consistent with HSL being an inducer for *rpoS* (226).

Another recent study (67) implicates specifically the methionine biosynthesis pathway in σ^S control. A *metE* mutant, which is deficient for conversion of homocysteine to methionine and therefore accumulates homocysteine thiolactone (HCTL), exhibits increased σ^S levels. Moreover, an *asd* mutant, which is deficient earlier in methionine biosynthesis (and therefore also in homocysteine and HCTL formation), shows decreased σ^S levels, and this phenotype could be suppressed by exogenous HCTL (1 mM) (67). During entry into stationary phase in minimal glucose medium, a 2.5-fold accumulation of HCTL was observed in wild-type cells (67). Under these conditions, however, there is little if any activation of *rpoS* transcription, but σ^S accumulation is due to posttranscriptional control (114; also see below). Unfortunately, HCTL effects were demonstrated by assaying for σ^S protein only, and so the level of control affected remains open to speculation (67).

In summary, these studies (67, 86) have demonstrated that amino acid biosynthetic pathways, in particular the branch that leads to methionine (with HCTL as the putative effector), can influence the expression of *rpoS*. It has not been clarified, however, whether this effect is at the level of *rpoS* transcription, and the underlying molecular mechanism remains unknown.

Acetate and other weak acids. In an early study that reported the induction of a transcriptional *rpoS*::*lacZ* fusion in spent culture supernatant, the fermentation product acetate (used at 40 mM) was found to activate *rpoS* expression (190). In another study, however, acetate did not have an effect on *rpoS* expression (153). The studies agreed that benzoate (10 or 25 mM) has an inducing effect, and it was concluded that this may be so in general for weak acids with a pKa of 4.8 to 4.9 (40 mM propionate was also found to be effective) (153, 190). However, a translational *rpoS*::*lacZ* fusion did not show this induction by weak acids (190). One has to take into account, however, that these studies were performed before the advent of limitless precise PCR cloning, and therefore reporter gene fusions had to be constructed in rather complicated ways and often remained incompletely characterized. To finally settle the issue of weak acids in *rpoS* control, these experiments would have to be repeated and expanded with the precisely characterized systems available today.

Recent genome-wide analyses have shown that addition of acetate to buffered medium results in the activation of various σ^S-dependent genes and proteins, but unfortunately the cellular concentration and regulation of σ^S itself were not studied under these conditions (7, 100). Similar conditions resulted in increased synthesis of σ^S in *Salmonella,* but the underlying control mechanisms seemed at least in part posttranscriptional (39).

Cellular NADH-to-NAD$^+$ ratio. Experiments with a transcriptional *rpoS*::*luxAB* fusion in a *nuoG* mutant background (which is defective in a subunit of NADH dehydrogenase) suggested that a high NADH-to-NAD$^+$ ratio somehow downregulates *rpoS* transcription. Consistent with this proposal, *rpoS* transcription is low under oxygen-limited (microaerobic) growth conditions, where NADH levels should increase due to the scarcity of oxygen as an electron acceptor for respiration (194). The mechanistic basis of this effect is unclear.

REGULATION OF *rpoS* TRANSLATION

Initial evidence for posttranscriptional regulation of *rpoS* was provided by clearly different patterns of expression of transcriptional and translational *rpoS*::*lacZ* fusions (114, 127, 135). Translational *rpoS*::*lacZ* fusions can actually reflect regulation of *rpoS* translation as well as of σ^S proteolysis (114, 148, 192). The latter can be excluded by using translational fusions that contain fewer than 173 N-terminal codons of *rpoS,* since an essential proteolytic recognition element is located at and around K173 in σ^S (20; also see below).

Translation of *rpoS* mRNA is stimulated by a shift to hyperosmolarity (114, 148), by low temperature (199), by a shift to acidic pH (pH 5; Kampmann and Hengge-Aronis, unpublished), or during late exponential phase when a growing culture reaches a certain cell density (114). After the onset of carbon starvation, i.e., on entry into stationary phase, *rpoS* translation is reduced again, and further increases in σ^S levels are then due to inhibition of σ^S degradation, as described below (114).

Role of *rpoS* mRNA Secondary Structure

There are two species of *rpoS* mRNA of clearly different lengths (the locations of relevant promoters are given in Fig. 2). Polycistronic *nlpD-rpoS* mRNA can have two different 5′ ends since there are two closely spaced promoters upstream of *nlpD* (115). Monocistronic *rpoS* mRNA originates from *rpoS*p within the *nlpD* gene and contains an unusually long nontranslated 5′ region of 567 nucleotides (112, 208). This leader sequence is functionally important, since 5′ deletions in it reduce *rpoS* expression (38).

Even under conditions where σ^S protein is hardly detectable, cells produce fair amounts of *rpoS* mRNA, which seems to remain constant under the translation-inducing conditions mentioned above (8, 147). It is generally believed that control of the rate of translation of already existing complete *rpoS* mRNA is based on an mRNA secondary structure in which the translational initiation region (TIR) is based paired and therefore not sufficiently accessible to ribosomes (under noninducing conditions). Certain stress signals are hypothesized to trigger changes in this mRNA secondary structure that allow more frequent translational initiation. However, the actual appearance of this *rpoS* mRNA structure is still largely a matter of speculation.

Theoretical predictions generated with the MFOLD computer program (using complete or partial *rpoS* mRNA se-

quences) indicate that approximately 340 nucleotides at the 5′ end of *rpoS* mRNA fold into an very stable and complex cruciform-type structure (Traulsen and Hengge-Aronis, unpublished). Further downstream, the putative structures are somewhat less stable and the TIR has the potential to fold into two energetically almost equivalent principal structures. One is characterized by a large hairpin that includes the Shine-Dalgarno sequence. There is genetic evidence against this structure playing a role in *rpoS* translational control (S. Bouché and R. Hengge-Aronis, unpublished results). In the second putative structure, the region around the Shine-Dalgarno sequence is partially base paired to an "internal antisense" region located further upstream, with a relatively long and probably internally structured intervening sequence. There are, however, several theoretical possibilities for the exact location of the "internal upstream antisense" region (Traulsen and Hengge-Aronis, unpublished). Several variations of this second theoretical structure have been published (30, 38, 120, 131), and it is generally believed that this structure may come close to the in vivo reality under noninducing conditions. The only preliminary experimental evidence that such an "internal upstream antisense" structure is in principle correct is provided by two different complementary double point mutations, which showed wild-type expression levels (although one double mutant altered the regulatory pattern, in particular Hfq dependency [see below]) (30, 38). However, the exact details of the in vivo *rpoS* mRNA secondary structure still await experimental clarification.

In fact, the problem of the correct in vivo *rpoS* mRNA secondary structure is more complex than, e.g., in the related case of *rpoH* mRNA, which encodes the heat shock sigma factor σ^{32}.*rpoH* mRNA folds into a translationally incompetent secondary structure also involving an internal antisense element, which opens up upon heat shock, resulting in a directly temperature-triggered translational induction of *rpoH* (summarized in reference 236). This process does not involve any regulatory proteins (143), and the experimentally demonstrated *rpoH* mRNA secondary structure is the one theoretically predicted (142). In *rpoS* mRNA, however, several proteins and small regulatory RNAs are positively or negatively involved in translational control, and at least some of these can directly bind to *rpoS* mRNA in vitro (see below). Therefore, theoretical calculations or in vitro structural probing based on *rpoS* mRNA alone is likely to yield incorrect or at least incomplete results. It seems that the only way of settling the issue of the correct *rpoS* mRNA secondary structure and its dynamics may be in vivo structural probing. Wild-type strains under different conditions as well as various mutants with *cis*- or *trans*-regulatory defects in *rpoS* translation will have to be tested in such experiments. However, in view of the technical difficulties of such an endeavor, especially with a large mRNA with complex and semistable secondary structure, it is not surprising that such data have yet to be reported for *rpoS* mRNA.

trans-Acting Factors Involved in *rpoS* Translation

The RNA binding protein Hfq (HF-I). More than 30 years ago, the Hfq protein was identified as a host factor (host factor I [HF-I]) essential for replication of phage Qβ RNA (56, 57).

Hfq acts as an accessory component of Qβ replicase that binds to several sites in Qβ RNA including the 3′ end (14, 139, 193). Hfq is required for initiating replication specifically of the Qβ RNA plus strand, probaby by affecting the secondary structure at its 3′ end (191). The role of the ribosome-associated (45) Hfq protein in *E. coli* physiology, however, remained enigmatic until an *hfq* mutant was observed to have a very pleiotropic phenotype (214), which resembles the phenotype of an *rpoS* mutant (147). This led to the discovery that Hfq is required for efficient *rpoS* translation (29, 146). While this can explain the pleiotropy of *hfq* mutants, Hfq also has physiological functions that are independent of σ^S (147). In particular, it stimulates the degradation of *ompA, miaA, mutS,* and its own mRNA (215, 221, 222). Hfq is a 11.2-kDa oligomer-forming protein (57). While this review was under revision, Hfq was reported to form hexameric rings homologous to eukaryotic Sm and Lsm proteins, which occur in the spliceosome and play various roles in mRNA processing (140, 240).

The molecular function of Hfq in *rpoS* translation is still relatively speculative. Epistasis experiments, where *hfq* mutations were combined to other mutations or overproduction constructs that affect *rpoS* translation indicated that Hfq is probably directly involved in translation initiation; i.e., it acts close to or at the level of *rpoS* mRNA (130, 146, 200, 217, 238). *rpoS* mRNA coimmunoprecipitates with Hfq in cellular extracts (238). Hfq also binds with high affinity to several sites in a large 5′ fragment of *rpoS* mRNA synthesized in vitro, which is predicted to fold into the same secondary structure as the wild-type mRNA (Traulsen and Hengge-Aronis, unpublished). A 5′ deletion analysis of *rpoS* mRNA indicated that regions relatively far upstream of the TIR are important for translational stimulation by Hfq (38). Thus, Hfq binds *rpoS* mRNA, just as it is able to bind Qβ RNA. However, Hfq does not show similarity to RNA helicases. This makes an active processive unfolding activity unlikely. Alternatively, by binding to a few crucial positions of *rpoS* mRNA, Hfq may affect the equilibrium between possible alternative secondary structures that are differentially productive for translational initiation. Thus, Hfq may stabilize a semistable *rpoS* mRNA secondary structure, which can easily open up when some additional stimulating factor is induced or activated (e.g., HU protein or DsrA-RNA, [Fig. 3; see below]). Yet another possibility is that Hfq does not necessarily affect *rpoS* mRNA secondary structure (although this would not be excluded) but acts like a "platform" bound to *rpoS* mRNA that recruits additional factors involved in *rpoS* translational control. The finding that single potentially base-pair-disrupting point mutations in the TIR or in the region likely to be base paired to the TIR result in increased *rpoS* translation and reduced Hfq dependence (30) appears consistent with both of these putative mechanisms of Hfq action.

It was reported that Hfq can also bind to DsrA RNA (200), which is a small regulatory RNA partially complementary to *rpoS* mRNA that stimulates *rpoS* translation above all at low temperature (see below for details). Therefore, it was suggested that Hfq may influence DsrA action by forming an active DsrA-Hfq complex and/or by altering DsrA structure (200). However, at 37°C an *hfq* mutation reduces *rpoS* translation much more than a *dsrA* mutation does (146, 199), indicating that Hfq does not act exclusively through DsrA. A hypothetical model consistent with all data available would be
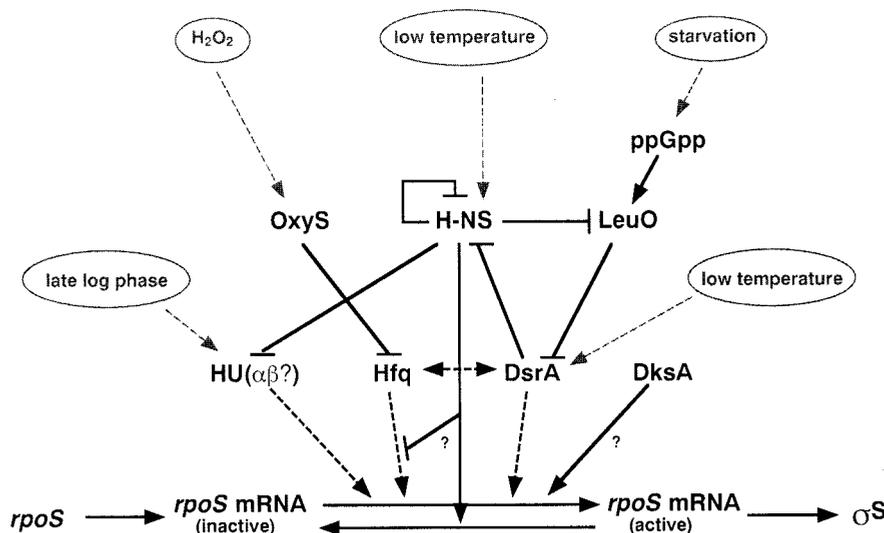
FIG. 3. The *rpoS* translational control network. *rpoS* mRNA is thought to occur in at least two different conformations, one being a more closed structure with the translation initiation region base paired to an upstream internal antisense element, and the other being a more open and translationally competent structure. The translation-stimulating factors Hfq, HU, and DsrA RNA can bind to *rpoS* mRNA (indicated by broken heavy lines) and together probably drive it into the translationally competent structure. The other components shown are likely to act more indirectly (for further details, see the text).

that Hfq bound to *rpoS* mRNA recruits DsrA into a ternary complex (in which secondary structures of *rpoS* mRNA and/or of DsrA could also be altered) and thereby facilitate translational stimulation by DsrA. In this complex, several Hfq molecules or oligomers bound to different sites on *rpoS* mRNA could be present. During revision of this review, ternary-complex formation with Hfq was also reported for *flhA* mRNA and OxyS RNA (240) as well as for *galK* mRNA and spot 42 RNA (140). Both studies came to the conclusion that the role of Hfq (and perhaps of Sm and LSm proteins in general) is to facilitate specific RNA-RNA interaction. Thus, Hfq could stimulate any process dependent on such RNA-RNA interactions.

If so, stress signal input into *rpoS* translational control would not necessarily be via a control of the activity or the level of Hfq itself. In fact, *hfq* mutants show overall reduced activity of translational *rpoS::lacZ* fusions or rates of σ^S synthesis (as measured in pulse-labeling experiments), but regulation by stress signals, e.g., by hyperosmotic shift, is not abolished (146). This suggests that stress signals affect the cellular concentrations or activities of the specifically translation-activating or inhibiting components (e.g., DsrA and OxyS) that can join the *rpoS* mRNA-Hfq complex. These components indeed exhibit pronounced regulation (see below).

HU: a nucleoid protein that also stimulates *rpoS* translation. Protein HU is a major protein component of the bacterial nucleoid. It affects overall nucleoid structure and topology but also participates in specific gene regulation, DNA recombination, and DNA repair (155). In addition, HU is required for optimal survival during prolonged starvation (35). In members of the *Enterobacteriaceae* and *Vibrionaceae,* two homologous subunits (HUα and HUβ [encoded by *hupA* and *hupB,* respectively]) contribute to the formation of active HU protein (158). During growth, the HUα2 homodimer is abundant, whereas during late exponential phase, HUβ is induced and HUαβ heterodimers are formed in *E. coli* (35). An HU-deficient *hu-*

pAB double mutant exhibits strongly reduced σ^S levels because of reduced *rpoS* translation (12).

In vitro, HU binds with high affinity to a small *rpoS* mRNA fragment (150 nucleotides covering the TIR and the upstream antisense region probably base paired to the TIR) (12) as well as to a larger fragment (covering more than 700 nucleotides starting from the original mRNA 5′ end) that also binds Hfq (Traulsen and Hengge-Aronis, unpublished). As a DNA binding protein, HU has a strong preference for nicked or cruciform DNA (94). Thus, HU may preferentially recognize secondary-structure elements, such as pronounced bends or kinks, which also occur in RNA secondary structure. HU may directly alter the *rpoS* mRNA secondary structure, but it is unknown how this effects relates to that of Hfq or of other components that affect *rpoS* translation (Fig. 3).

Since the induction of HUβ (which is under the negative control of the nucleoid protein FIS [34]) correlates with stimulation of *rpoS* translation during late exponential growth phase, and specifically since the HUαβ heterodimer is required for stationary phase survival (35), it is tempting to speculate that the heterodimer is the form of HU involved in *rpoS* translation. However, this hypothesis has yet to be tested experimentally. Nevertheless, phylogenetically, the occurrence of an HUαβ heterodimer correlates with the occurrence of σ^S (with the exception of the *Pseudomonas* group, but regulation of σ^S is significantly different in several aspects in this group).

H-NS and StpA: histone-like proteins acting as RNA chaperones? H-NS is an abundant histone-like protein with functions in nucleoid organization as well as in gene regulation, where in nearly all cases it acts as a repressor or silencer that can form large nucleoprotein complexes. StpA is a closely related paralog of H-NS with similar properties (although it seems more efficient as an RNA chaperone). Just as with HUα and HUβ, homo- as well as heterooligomers are formed by H-NS and StpA (for reviews, see references 9, 43, and 230).

H-NS-deficient mutants exhibit strongly increased σ^S levels, which in exponential phase are already similar to those reached by the wild-type only in stationary phase or under other stress conditions (15, 234). In these *hns* mutants, the rate of *rpoS* translation is enhanced and proteolysis of σ^S is strongly reduced or even abolished (234). The slow growth and genetic instability typical of *hns* mutants are at least partially connected to these abnormally high σ^S levels, since they can be suppressed by mutations in *rpoS* (15).

Mechanistically, it is still unclear how H-NS downregulates *rpoS* translation, but there are a number of possibilities for direct or indirect influences (Fig. 3). Since H-NS can bind to RNA (although high-affinity specific binding has not yet been demonstrated [40, 48]), it may directly interact with *rpoS* mRNA and affect its secondary structure, perhaps in a transient way as an RNA chaperone. H-NS may also counteract the effects of positive regulators of *rpoS* translation such as Hfq and/or HU. These possibilities are not mutually exclusive, since the positively acting factors and H-NS may have opposite effects on the equilibrium between two *rpoS* mRNA conformations that can be translated with different efficiencies (Fig. 3). Consistent with H-NS counteracting Hfq, H-NS deficiency has no effect on *rpoS* translation in a *hfq* mutant background (146). H-NS and HU in general seem to play antagonistic roles, e.g., in determining DNA supercoiling (44) or in the expression of certain genes such as *ompF* (41, 164). Thus, it is also possible that H-NS inhibits *rpoS* translation by affecting the cellular level of HU or by directly counteracting the stimulatory effect of HU on *rpoS* translation.

Another candidate for promoting *rpoS* translation is the H-NS homolog StpA. Several studies have shown that StpA levels are significantly lower than H-NS levels (201, 239), although one report gives approximately equal numbers of H-NS and StpA molecules per cell (10). It seems clear, however, that H-NS and StpA regulate each other negatively at the level of transcription. Therefore, an *hns* mutant should have an increased cellular concentration of StpA (201, 239). Moreover, StpA is upregulated after a hyperosmotic shift (58). Thus, increased StpA levels appear to correlate with increased *rpoS* translation. Since StpA can act as a RNA chaperone (40), it was tempting to speculate that it may stimulate *rpoS* translation. However, high-log-phase levels of σ^S in an *hns* mutant were not suppressed by introducing an *stpA* mutation, and also osmotic induction of *rpoS* translation was normal in a *stpA* mutant (Bouché and Hengge-Aronis, unpublished). Therefore, under these conditions, StpA does not seem to play a role in *rpoS* translation. This, however, does not exclude a potential involvement of StpA under different conditions or in other genetic backgrounds.

Role of small regulatory RNAs in *rpoS* translation: DsrA, OxyS, and RprA. Several small regulatory RNAs with important fine-tuning functions in complex regulatory circuits have been identified in *E. coli* (summarized in reference 1), and three very recently published studies suggest that small regulatory RNAs in *E. coli* are much more common and significant than previously thought (6, 179, 224). *rpoS* translation seems to be an especially prominent target for such regulation, with three regulatory RNAs having been found so far. While DsrA and RprA promote *rpoS* translation, OxyS has an inhibitory function.

DsrA was originally identified as a multicopy suppressor of H-NS-mediated silencing of the *rcsA* gene in *E. coli* (198) and was then found to be essential for increased *rpoS* translation at low temperature (199). DsrA is a stable 87-nucleotide RNA that folds into a three-stem-loop structure (119, 131). A region covering most of stem-loop 1 and the following single-stranded part of DsrA is complementary to an upstream "antisense element" in *rpoS* mRNA that is assumed to base pair with the TIR region, suggesting that DsrA functions by an "anti-antisense" mechanism that disrupts intramolecular basepairing in *rpoS* mRNA (119, 120, 131). DsrA plays only a minor role for *rpoS* translation in cells grown at 37 or 42°C yet becomes the major stimulating factor at 30°C and especially at 20°C (199). The basis of low-temperature translational induction of σ^S is the clearly enhanced transcription of *dsrA* as well as a sixfold-increased stability of DsrA at low temperature (177). DsrA and Hfq were recently reported to interact specifically, and Hfq was suggested to stabilize DsrA as well as to alter its secondary structure in a way that promotes association with *rpoS* mRNA (200). Whether the formation of such a binary complex facilitates DsrA action on *rpoS* mRNA or whether Hfq already bound to *rpoS* mRNA (as described above) recruits DsrA, the result is likely to be a ternary complex (see also above). Hfq may affect the secondary structure of both RNAs such that they optimally interact, and with all partners involved interacting with each other, the complex is probably relatively stable. As a result, the formation of an "open" conformation at the TIR of *rpoS* mRNA that allows ribosome entry would be facilitated (Fig. 3).

Besides *rpoS* mRNA, DsrA has at least one other target, *hns* mRNA. While DsrA was initially thought to act like a conventional antisense RNA interfering with *hns* translation initiation (120), it now seems likely that a region corresponding to unfolded stem-loop 2 of DsrA forms a coaxial stack with two regions in *hns* mRNA. Negative regulation of *hns* expression is a consequence of more efficient degradation of *hns* mRNA within this complex (118). DsrA is predicted to form similar complexes with *argR* and *ilvIH* mRNAs, but an involvement of DsrA in the regulation of these genes has not yet been demonstrated (118).

Multifunctionality exerted by different regions may be common in small regulatory RNAs, since it has also been observed for OxyS. OxyS is a 109-nucleotide regulatory RNA that folds into a similar secondary structure to that of DsrA. As a member of the OxyR regulon, OxyS is induced by oxidative stress (hydrogen peroxide) and acts as a pleiotropic regulator (2). Small regions located in loops 1 and 3 of OxyS control translation of *fhlA* (which encodes a transcriptional activator) by forming a "kissing complex" with two sites of *fhlA* mRNA, one of which contains the Shine-Dalgarno sequence (3, 5). By contrast, the rather long A-rich single-stranded region between stem-loops 2 and 3 of OxyS is involved in negative regulation of *rpoS* translation, although this part of OxyS does not show significant sequence complementarity to *rpoS* mRNA. Coimmunoprecipitation experiments indicate that OxyS binds to Hfq protein. Thus, OxyS may sequester Hfq or form a translationally incompetent ternary complex with Hfq and *rpoS* mRNA (238) (Fig. 3). OxyS-mediated translational repression of *rpoS* may be a fine-tuning mechanism to avoid redundant overinduction of oxidative-stress protective genes (*katG, gorA,*

and *dps*) that are under dual positive control of OxyR/σ^{70} and σ^S. It may also prevent the uneconomical induction of the large multifunctional σ^S regulon under conditions where the cell has to cope with oxidative stress only, i.e., a situation that can be managed by the stress-specific OxyR-mediated response alone.

The third small regulatory RNA involved in *rpoS* translational control, RprA, was found as a multicopy suppressor for a *dsrA* mutation (130). In the *dsrA* mutant background, an *rprA* null mutation also reduces hyperosmotic stimulation of *rpoS* translation. However, in the presence of DsrA, neither RprA overproduction nor its knockout seems to affect *rpoS* expression. Thus, RprA clearly has the potential to stimulate *rpoS* translation, but the physiological conditions under which this becomes relevant are unknown. The *rprA* promoter is under positive control of RcsB, a response regulator that activates capsule synthesis (unpublished evidence mentioned in reference 130). RprA exhibits some sequence complementarity to the upstream "antisense" element that basepairs with the TIR of *rpoS* mRNA and may thus act similarly to DsrA (M. Majdalani and S. Gottesman, personal communication).

The LysR-like regulator LeuO: a repressor for *dsrA* expression. LeuO is a LysR-like regulator (189), which is strongly repressed by H-NS in growing *E. coli* cells (101). Overproduction of LeuO (either from a multicopy plasmid or in a mutant that carries a Tn *10* transposon immediately upstream of *leuO* with p$_{out}$ of the transposase gene reading into *leuO*) reduces *rpoS* translation, especially at low temperature. This effect is entirely dependent on the presence of DsrA, and LeuO was shown to repress *dsrA* transcription (101). This regulation is direct since LeuO binding sites have recently been identified in the *dsrA* promoter region (177). A *leuO* knockout mutation, however, does not affect increased *rpoS* translation during late exponential phase or in response to high osmolarity or low temperature. This is not entirely surprising, since under these conditions, *leuO* expression is repressed or even "silenced" by H-NS (101). However, during entry into stationary phase, *leuO* is induced in a ppGpp-dependent manner (50). This ppGpp-mediated activation may be indirect, since *leuO* expression is subject to a "promoter relay" activation mechanism that involves the surrounding *ilvIH* and *leuABCD* operons (33, 51). As a consequence, LeuO probably downregulates DsrA in stationary phase. While this may alter the expression of other targets of DsrA, the σ^S level is not affected (E. Klauck and R. Hengge-Aronis, unpublished results), probably because other σ^S-inducing mechanisms compensate for the reduced levels of DsrA. When all these results are taken together, the physiological role of LeuO is far from clear. However, a hint may come from the "cryptic" *bgl* operon, where LeuO can antagonize H-NS-mediated (and under certain conditions also σ^S-dependent) "silencing" (160, 218). Interestingly, the *bgl* operon becomes expressed in a mammalian host (97). It is thus conceivable that LeuO plays an important regulatory role in a host environment.

DnaK and DksA: a link to heat shock and chaperones. The heat shock chaperone DnaK, as well as a protein termed DksA (originally identified as a DnaK suppressor [95]), has been implicated in *rpoS* translation. A *dnaK* mutant exhibits a stationary-phase-specific multiple-stress-sensitive phenotype very similar to that observed for *rpoS* mutants (180, 181). This correlates with reduced σ^S levels in starving *dnaK* mutant cells

(144, 181). Part of this effect is due to reduced *rpoS* translation, since it can also be seen with RpoS::LacZ hybrid proteins that are not subject to proteolysis. The mechanism behind this effect remains unknown, but the overproduction of the heat shock sigma factor σ^{32} in the *dnaK* mutant does not play a role, since a suppressor mutation that reduces the σ^{32} level and/or activity does not suppress the *dnaK* effect on σ^S (144).

DksA is a putative zinc binding protein with similarity to the transcriptional activator TraR (59) and other prokaryotic and eukaryotic regulators (103). The basis of *dnaK* suppression by multiple copies of *dksA* is still unclear, but it was suggested that production of some stress response factors might be involved (16). This is consistent with the more recent finding that *dksA* mutations in *Salmonella* exhibit reduced σ^S induction in stationary phase and after a shift to acidic pH. Work with *rpoS*::*lacZ* translational fusions indicated that DksA affects *rpoS* translation by some not yet characterized mechanism (225). In *P. aeruginosa,* overexpression of DksA inhibits the expression of *rhlI, rhlAB,* and *lasB* (26). This would be in line with a repressing effect of σ^S on the *rhl* system (228). However, additional data suggest that this effect of DksA overproduction is not due to upregulation of σ^S alone (26).

EIIA(Glc): a link to the carbon source and energy supply. A *crr* mutant, which is defective in the glucose-specific PTS component EIIA(Glc), contains strongly elevated σ^S levels. Both transcriptional and posttranscriptional effects contribute to this phenotype (217). Higher expression of a transcriptional *rpoS*::*lacZ* fusion is fully suppressed by cAMP addition, indicating that the effect reflects stimulation of adenylate cyclase by EIIA(Glc) and negative control of *rpoS* transcription by cAMP-CRP (see above). However, high σ^S levels and increased activity of a translational *rpoS*::*lacZ* fusion are not fully suppressed by cAMP addition, nor does this effect of *crr* disruption disappear in a *rssB* mutant background, where σ^S is not degraded. Thus, EIIA(Glc) obviously downregulates *rpoS* translation by some uncharacterized and perhaps indirect mechanism. Moreover, the phosphorylated form of EIIA(Glc) is required for this activity. However, external addition of glucose, which is known to drastically decrease the level of phosphorylated EIIA(Glc) (207), does not result in σ^S induction (217). In the absence of phosphotransferase system-mediated glucose uptake, however, phosphorylation of EIIA(Glc) reflects the intracellular phosphoenolpyruvate-to-pyruvate ratio (84). Negative regulation of σ^S by EIIA(Glc) may thus be a function of this ratio, which depends on the nature of the carbon source and the energy supply in general (217).

The cold shock domain proteins CspC and CspE. CspC and CspE belong to the CspA cold shock protein family in *E. coli,* although they are expressed at 37°C and are not temperature regulated (169). Overproduction of these two RNA binding proteins strongly stabilizes and thereby increases the cellular level of *rpoS* mRNA. Whether this is a direct or indirect effect is currently unknown. Such high *rpoS* mRNA levels are assumed to translate into higher σ^S levels, since the σ^S-dependent genes *osmY, dps, proP,* and *katG* are significantly activated. Conversely, a *cspC cspE* double mutant exhibits reduced osmotic induction of *osmY* and *dps* (168). Unfortunately, *rpoS* mRNA levels were not determined in the osmotic shift experiment, and in general the rates of σ^S synthesis and the cellular σ^S level were not monitored directly in this CspC-CspE study.

It was previously reported that a shift to high osmolarity does not increase the *rpoS* mRNA level (146), which would not be consistent with *rpoS* mRNA-stabilizing factors playing a major role in osmotic regulation of *rpoS*. Therefore, it is possible that the CspC and CspE effects on *osmY* and *dps* expression are direct and do not always reflect the regulation of *rpoS* (168).

A Small Molecule That Influences *rpoS* Translation: UDP-Glucose

UDP-glucose has been implicated in σ^S regulation, since several mutants with defects in central carbon metabolism that result in UDP-glucose deficiency exhibit increased σ^S levels during exponential growth (24). These defects can be in phosphoglucose isomerase (encoded by *pgi*), with the mutant growing on fructose, as well as phosphoglucomutase (*pgm*) or UDP-glucose pyrophosphorylase (*galU*), with the latter two mutants growing on glucose. Glucose and galactose given in trace amounts to the *pgi* and *pgm* mutants, respectively, rapidly replenish the internal UDP-glucose pool and in parallele result in a rapid decrease of σ^S levels (24). More recent work with transcriptional and translational *rpoS*::*lacZ* fusions and direct pulse-chase measurements of σ^S synthesis and degradation indicate that UDP-glucose specifically affects *rpoS* translation. Moreover, enhanced σ^S levels in a *galU* mutation are observed only with an intact *hfq* gene, which suggests that UDP-glucose directly or indirectly interferes with Hfq function in *rpoS* translation (A. Muffler and R. Hengge-Aronis, unpublished results). However, the molecular mechanism of UDP-glucose action has yet to be clarified, and it is also unknown whether the cellular UDP-glucose level changes in response to any stress signals that affect *rpoS* translation.

rpoS Translational Control Network and Stress Signal Input

When all the regulatory factors involved in *rpoS* translation are considered together, a highly intertwined network characterized by positive and negative feedback regulation emerges (Fig. 3). The regulatory output of this network under different physiological conditions is difficult to predict, especially when changing environmental conditions affect the cellular levels of indirectly acting and multiply connected components such as H-NS or LeuO. DsrA is obviously a central player, since it affects the two global regulators σ^S and H-NS, with the latter in turn downregulating σ^S. Thus, DsrA seems to have a dual positive effect on *rpoS* translation, one direct and the other indirect via H-NS. DsrA, H-NS, and LeuO also seem to form a negative feedback loop (Fig. 3). The physiological function of this regulatory loop, i.e., its behavior and consequences for *rpoS* translation when external stress signals affect the level of single components in the loop, is currently not clear.

The complexity of the *rpoS* translational control network makes it difficult to define stress signal input. The only σ^S-inducing condition, for which the underlying signal transduction mechanism seems pretty straightforward, is a shift to low temperature (around 20°C). This treatment clearly induces DsrA RNA (177), which in turn has a direct positive effect on *rpoS* translation as described above. Whether low-temperature induction of H-NS (116) plays any role in *rpoS* regulation is unclear. Starvation induction of LeuO (50) may be relevant

only at low temperature, since LeuO acts by repressing DsrA RNA (101). So far, however, different stress conditions have not been studied in combination. Late-exponential-phase induction of *rpoS* translation (114) correlates with the induction of HUβ (35), but whether this reflects a causal relationship is a matter of speculation. Finally, the intracellular signal that is triggered by osmotic upshift and stimulates *rpoS* translation more than fivefold within a few minutes (148) is completely elusive.

As complex as the translational control network may be, it is even further interconnected to the networks that control *rpoS* transcription and σ^S stability. If ppGpp stimulates LeuO expression under starvation conditions (50), it may at the same time be a strong positive regulator of *rpoS* transcription (see above) and a negative regulator of *rpoS* translation (Fig. 3). The physiological function of this multiple role of ppGpp is currently not clear. Its purpose may be to avoid nonappropriate overexpression of σ^S under conditions of combined stresses, e.g., in response to starvation at low temperature. Also, EIIA(Glc) affects transcription (by controlling adenylate cyclase activity) as well as *rpoS* translation. H-NS, on the other hand, represses *rpoS* translation and at the same time keeps σ^S protein levels down by somehow stimulating σ^S turnover (see below). At present, it still seems appropriate and helpful to treat the different levels of σ^S control as separate "regulatory modules." In the somewhat longer run, however, their interconnection will have to be taken into account.

REGULATION OF σ^S PROTEOLYSIS

rpoS transcription as well as translation can be stimulated under certain stress conditions, but even in cells that grow in the relative absence of stress, there is a certain basal rate of σ^S synthesis. However, the cellular σ^S level remains low because of rapid degradation (114, 208). During growth in rich medium, *rpoS* transcription is very low and the steady-state σ^S level is usually at or below the limit of detection, which makes quantitative analyses of σ^S synthesis and proteolysis difficult. During growth in minimal medium, however, rates of σ^S synthesis and turnover can be determined by pulse-chase experiments and σ^S levels can be quantified by immunoblot analysis. Under these conditions, the σ^S half-life is between 1 min and several minutes (depending on the carbon source) (114, 144, 148, 192, 208). This rapid turnover sets the stage for various stress conditions affecting σ^S levels by modulating the rate of σ^S proteolysis. In general, it seems that relatively threatening stress conditions tend to affect σ^S degradation, maybe because this allows the most rapid reaction. These stresses include sudden carbon starvation (114, 208), osmotic upshift (148), and shift to acidic pH (18), which result in σ^S stabilization within a few minutes. On the other hand, the classical heat shock procedure, i.e., a shift from 30 to 42°C, results in a more moderate increase in σ^S half-life, which takes approximately 20 min to develop (144).

σ^S Degradation by the Complex ATP-Dependent ClpXP Protease

The ClpXP protease is responsible for σ^S degradation. ClpXP is a barrel-shaped processive protease consisting of two

six-subunit rings of the ATP-hydrolyzing ClpX chaperone, which play the role of substrate-discriminating and unfolding gatekeepers to the inner proteolytic chamber formed by two seven-subunit rings of ClpP (69, 99, 223). Mutations in *clpP* as well as in *clpX* result in stabilization of σ^S (192). Since the *clpP* and *clpX* genes constitute an operon (68, 133), the *clpP* phenotype could in principle have been due to polarity on *clpX*, but the inability to suppress σ^S stability in the *clpP* mutant by providing *clpX* in *trans* confirmed that the entire ClpXP complex is required for σ^S proteolysis (Muffler and Hengge-Aronis, unpublished). Recently, it has been possible to reconstitute σ^S degradation in vitro, and these experiments have defined ClpXP as well as a specific recognition factor (see below) as essential and sufficient for the basic process of σ^S proteolysis (243). σ^S degradation by ClpXP is complete; i.e., no stable degradation products have been observed.

The Response Regulator RssB: a σ^S Recognition Factor with Phosphorylation-Modulated Affinity

In contrast to other ClpXP substrates, σ^S cannot be recognized by ClpXP alone, as demonstrated both in vivo and in vitro (145, 243). Rather, a specific recognition factor, the RssB protein (also termed SprE, MviA, or ExpM in different bacterial species), is required (4, 18, 145, 170). A mutation in *rssB* results in the stabilization of σ^S (and also of otherwise unstable RpoS::LacZ hybrid proteins) and therefore in elevated σ^S levels in exponential phase (145, 170). RssB belongs to the two-component response regulator family of proteins, whose activity is modulated by phosphorylation of a conserved aspartyl residue in the N-terminal receiver domain (D58 in RssB). In vitro experiments have shown that phosphorylated RssB directly interacts with σ^S (20). Phosphorylation as well as σ^S binding in vitro is lost with RssB variants, in which D58 is replaced by other amino acids, consistent with strains carrying the same mutations exhibiting high levels of stable σ^S (21, 25, 102). RssB is essential for σ^S degradation in vitro (243) and may be specific for σ^S, since turnover of another ClpXP substrate, λO protein, does not depend on RssB (242, 243). In conclusion, the response regulator RssB is an essential, specific, and direct σ^S recognition factor, whose affinity for σ^S and therefore whose activity in σ^S proteolysis are modulated by phosphorylation of its receiver domain.

Like most response regulators, RssB consists of at least two domains, the N-terminal receiver and a C-terminal output domain (the latter could also be more than a single domain). The unique role of RssB in proteolysis is reflected in a unique output domain(s) without similarity to any other protein of known function. In certain response regulators, the output domain alone is mechanistically responsible for the molecular function (most often in transcription initiation), with the receiver domain imposing regulation by phosphorylation-modulated intramolecular inhibition (42, 70). In other cases, phosphorylation of the receiver domain actively contributes to the output function, e.g., by stimulating oligomerization (53, 233) or by exposing an interactive surface in the receiver itself (138, 237). RssB belongs to the latter class, since the isolated N- and C-terminal domains of RssB are functionally inactive in vitro and in vivo; i.e., the N-terminal receiver domain plays an active and positive role in RssB function (102). The molecular details of the RssB-σ^S interaction remain to be elucidated, but there is evidence that RssB, unlike many other response regulators, does not dimerize or oligomerize on phosphorylation and/or σ^S binding and that the RssB-σ^S complex exhibits 1:1 stoichiometry (102).

The cellular concentration of RssB (which in growing cells is around the limit of detection) is the limiting factor for the rate of σ^S proteolysis in vivo. This means that RssB can be titrated by increased σ^S synthesis (174). This mechanism can be exploited for stress-induced stabilization of σ^S (see below). On the other hand, cells have to continuously adjust the expression of RssB to σ^S in order to maintain σ^S proteolysis during growth despite controlled or accidental variations in the rate of σ^S synthesis. This is achieved by a homeostatic feedback coupling that is provided by *rssB* transcription being dependent on σ^S (185; Pruteanu and Hengge-Aronis, submitted). These two reports, however, do not agree on the location of the σ^S-dependent promoter, since Ruiz et al. (185) invoke a promoter just upstream of *rssB*, which was not found by Pruteanu and Hengge-Aronis (174), who provide evidence that *rssB* transcription is driven exclusively from the σ^S-controlled *rssAB* operon promoter. σ^S control of *rssB* expression also results in indirect negative autoregulation of *rpoS* as well as of *rssB*, since σ^S stimulates the expression of a factor, RssB, that initiates σ^S disappearance (174).

The Turnover Element: the RssB Binding Site within σ^S

Unlike many other proteolysis substrates, which feature recognition sequences or elements at or close to the N or C termini (96, 229), σ^S was found to contain a "turnover element" somewhere in the middle of its sequence. Initial evidence for such a proteolysis-promoting element came from the analysis of RpoS::LacZ hybrid proteins carrying N-terminal σ^S fragments of different lengths. Whereas relatively short hybrid proteins were stable and yielded high β-galactosidase activities in log phase, extending the σ^S part beyond a certain region resulted in hybrid proteins that were subject to the same regulated turnover as σ^S itself and yielded low β-galactosidase activities (148, 192). These studies roughly mapped the turnover element somewhere in or downstream of region 2.4 (which is involved in recognition of the −10 promoter element). Consistent with σ^S and σ^{70} recognizing the same −10 consensus (19, 47, 81), there is extreme amino acid similarity of these two sigmas up to the end of region 2.4. Just beyond this point, however, the sequences diverge. Reasoning that only σ^S is unstable and therefore should contain the turnover element, a number of amino acids in this region of σ^S, which clearly differ from those in σ^{70}, were replaced by the latter ones. This identified K173 as an absolutely crucial amino acid for σ^S proteolysis. A single point mutation, K173E, eliminates rapid σ^S proteolysis (20). Single mutations in E174 or V177 also enhance the σ^S half-life two- and threefold, respectively (20). In conclusion, K173 is a core amino acid of the turnover element. Moreover, K173 is also crucial for promoter recognition in the extended −10 part of a promoter (specifically of a C in position −13) (19), and this part of σ^S or σ^{70} is now termed region 2.5 (13, 19).

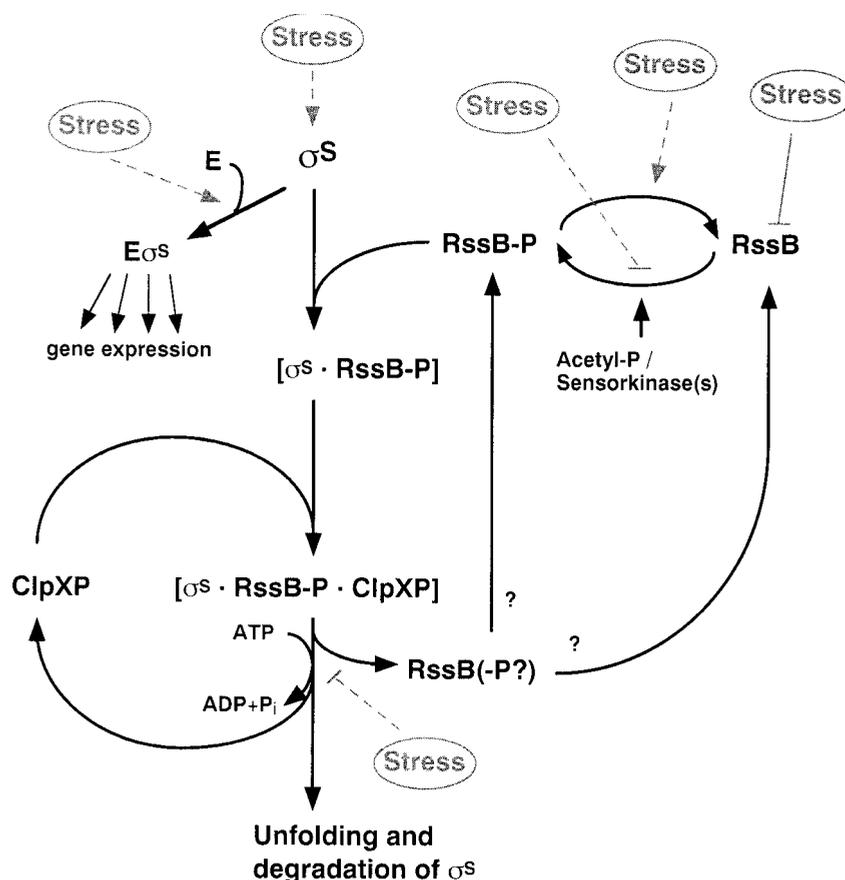In vitro experiments with the σ$^{S(K173E)}$ variant demonstrated

FIG. 4. Role of RssB-ClpXP and putative signal input in the σ^S recognition and degradation pathway. The response regulator RssB is an essential, specific, and direct σ^S recognition factor. RssB delivers σ^S to the ClpXP protease, where σ^S is unfolded and completely degraded whereas RssB is released. σ^S binding requires RssB phosphorylation, but it is unclear whether the catalytic cycle of RssB involves obligatory dephosphorylation during release and subsequent rephosphorylation. Stress signals may affect (i) the phosphorylation of RssB and therefore RssB-σ^S complex formation; (ii) the cellular level of RssB (which in growing cells is rate limiting for σ^S proteolysis); (iii) the synthesis of σ^S such that RssB becomes titrated on σ^S overproduction; (iv) σ^S association with RNA polymerase core enzyme, which protects against binding by RssB; and (v) the function of the ClpXP protease itself (see the text for details). However, the molecular details of the stress signal input pathways involved are still largely unknown.

that K173 is essential for interaction with RssB. In other words, the turnover element around K173 represents the binding site for RssB (or an essential part thereof) (20). Unfortunately, there is no experimentally determined structural information for region 2.5 of sigma factors (a known partial structure of σ^{70} ends with region 2.4 [132]), but the sequence between V172 and K188 is strongly predicted to be in an α-helical conformation. The double role of K173 in RssB binding and in interaction with the extended −10 promoter region also means that K173 must be surface exposed, no matter whether σ^S is in the RNA polymerase complex or not. In an initial attempt to estimate the extension of the binding site for RssB, the amino acids predicted to form the α-helix in region 2.5 were N-terminally fused to β-galactosidase and were found to be sufficient for RssB binding in vivo as well as in vitro (A. Stüdemann, E. Klauck, and R. Hengge-Aronis, unpublished results). This means that a relatively small part of σ^S, most probably just one α-helix, is sufficient for interaction with RssB, whereas on the other side, the entire RssB protein (or at least more than a single domain) is required for binding. This situation is reminiscent of protein binding by the DnaK chaperone, where a

small target sequence with one crucial amino acid in the substrate protein is bound in a pocket formed by DnaK (134, 184, 244). One may speculate that a comparable mechanism operates for σ^S binding to RssB, with formation of a high-affinity binding cavity in RssB being dependent on phosphorylation of its receiver domain.

Initiation of σ^S Proteolysis: the RssB Cycle

Once bound to RssB, σ^S is transferred to the ClpXP protease, where, like other Clp protease substrates, it is unfolded and completely degraded by an ATP hydrolysis-dependent mechanism (Fig. 4). A ternary complex between σ^S, RssB, and ClpX and a quaternary complex also involving ClpP have been observed in vitro (243). RssB is then released from the complex, as indicated by in vitro as well as in vivo data (102, 243). Thus, RssB is recycled and plays a catalytic role in the initiation of σ^S degradation. Taking into account a σ^S half-life of 1.5 min (114), the cellular σ^S-to-RssB ratio of approximately 20:1 (21), and the fact that RssB remains a monomer in the σ^S-RssB complex (102), it has been estimated that a single mol-

ecule of RssB can initiate the degradation of at least six or seven molecules of σ^S per minute (in cells growing in minimal glucose medium). The real number may actually be somewhat higher, since this estimation was based on the degradation of fully synthesized σ^S molecules only (as visible in pulse-chase and immunoprecipitation experiments). In addition, however, nascent σ^S polypeptide chains can probably enter the degradation pathway as soon as they are long enough to contain the recognition site for RssB (102).

So far, it is unknown whether RssB is dephosphorylated during its catalytic cycle. Dephosphorylation could be a convenient mechanism for RssB release. Contact with ClpXP may stimulate an RssB autophosphatase activity, which in purified RssB alone would be cryptic (spontaneous in vitro dephosphorylation occurs with a half-life of more than 1 h) (102). Alternatively, σ^S may lose its affinity for RssB during its unfolding and initial transfer into ClpP, which would alter the conformation of σ^S in and around region 2.5, whose integrity is required for RssB interaction (20). Dephosphorylation of RssB during its catalytic cycle would imply rephosphorylation as an obligatory part of this cycle (Fig. 4), which has interesting regulatory implications (see below).

Signal Integration in the Control of σ^S Proteolysis

Defining the linkages between stress signal transduction pathways and the σ^S recognition and degradation pathway remains a challenge for future studies, mainly because these signal transduction pathways themthelves have yet to be elucidated. However, it is becoming apparant that the basic RssB-ClpXP system has the potential to act like a multiple-signal-integration machinery. Theoretically, this system provides a wide range of possibilities for downregulating the rate of σ^S degradation by stress signal transduction pathways (Fig. 4). In the presence of preliminary data only, the existence of these mechanims remains speculative at present, but the following theoretical discussion may provide useful hypotheses for future work.

With RssB being a response regulator, it is reasonable to expect that some stresses will affect the phosphorylation state of RssB. Unfortunately, this could not yet be demonstrated directly in vivo, since the cellular RssB level is at the limit of (and sometimes below) detection in immunoblot experiments (21, 141). So far, no cognate sensor kinase for RssB has been identified and the *E. coli* genome sequence does not provide any obvious candidate. Acetyl phosphate seems to contribute to RssB phosphorylation, since acetyl phosphate-free *pta-ackA* mutants exhibit longer σ^S half-lifes; however, since σ^S proteolysis is not completely abolished in these mutants, at least one additional phosphoryl donor for RssB is likely to exist (25). It therefore seems possible that RssB is phosphorylated by "cross talk" from other sensor kinases, consistent with phosphorylated RssB (and therefore rapid σ^S recognition and degradation) representing the "default" state of the system in the absence of stress. Environmental stress would then trigger some mechanism that actively dephosphorylates RssB. However, a specific RssB phosphatase (or a sensor kinase that switches to RssB phosphatase activity) still awaits identification.

An interesting variation on this theme becomes possible if RssB is obligatorily dephosphorylated during its release from the complex with σ^S and ClpXP (Fig. 4). In this case, a specific stress-activated phosphatase may be dispensable, and the entire regulation of phosphorylation and dephosphorylation of RssB could be mediated by one (or several) cross-talking sensor kinases that would then have to be inhibited by certain stresses. However, signal input flexibility and precision would certainly be higher if the system actively controls phosphorylation as well as dephosphorylation, which could be differentially targeted by different stress conditions.

In cells growing in minimal medium, there is a finely tuned balance between σ^S synthesis and proteolysis. There is evidence that the cellular level of RssB is the rate-limiting factor for σ^S proteolysis in vivo (174). Accordingly, a sudden strong increase in σ^S synthesis results in σ^S stabilization because of titration of RssB. This is observed on artificial induction of σ^S synthesis (174), or on osmotic upshift or pH downshift, where the rate of σ^S synthesis increases severalfold within a few minutes (146, 148; Kampmann and Hengge-Aronis, unpublished). Therefore, osmotically triggered or pH-triggered stabilization of σ^S may in part be a passive consequence of the stimulation of *rpoS* mRNA translation (see above).

With RssB being limiting for σ^S proteolysis, it is theoretically also possible that some sort of stress may result in a reduction of RssB levels rather than of RssB activity. However, starvation leads to a moderate increase in the cellular RssB concentration (21, 185; Pruteanu and Hengge-Aronis, submitted), whereas osmotic upshift has no effect (21). Also, for some other stresses known to affect σ^S proteolysis, alterations in RssB levels were not observed (M. Pruteanu and R. Hengge-Aronis, unpublished results).

Association of σ^S with RssB or RNA polymerase core enzyme seems mutually exclusive. Core enzyme protects σ^S against degradation in vitro and, at equimolar concentrations with RssB, can even actively displace RssB from the σ^S-RssB complex (243). This suggests that any factors that in vivo may disfavor σ^{70} in its competition with σ^S for core polymerase or that may somehow directly stimulate σ^S holoenzyme formation would also contribute to σ^S stabilization. Such σ^S-activating and -stabilizing factors have not yet been unequivocally identified, but there are reasons to postulate their existence (see below).

Finally, there is indirect in vivo evidence that a σ^S-RssB complex is still formed in carbon-starved cells (21), which indicates that under these conditions, inhibition of some activity in the unfolding and degradation pathway downstream from σ^S-RssB binding contributes to stabilization of σ^S. In that respect, it may be relevant that the ClpX level is likely to be reduced due to growth stage-specific *clpPX* mRNA processing in stationary-phase cells (122).

In summary, this extraordinary potential for multiple signal integration in the RssB-ClpXP system can explain why so many different stress signals can finally result in the same phenomenon, i.e., σ^S stabilization. In the future, it will have to be worked out which stresses act by which of the mechanisms outlined above. An increased flexibility and fine-adaptive power would be achieved if certain stresses used different combinations of these mechanisms.

Additional Factors with Uncharacterized Molecular Functions in σ^S Turnover

It is obvious from the previous section that signal integration in the control of σ^S recognition and degradation is highly complex and that therefore probably several, if not many, components involved are still missing from our picture. Unfortunately, however, it may be predicted that in a system that integrates many signal input pathways (which may also be interconnected!), a mutation in a single signal-transducing component probably produces a minor or even no phenotype unless the mutant is tested under very specific conditions. This may explain why mutant searches using screens that reflect σ^S degradation have not yielded mutations in novel genes with clear-cut phenotypes other than in *rssB, clpP,* or *clpX* (49; F. Reindl, E. Kampmann, and R. Hengge-Aronis, unpublished results). However, there is circumstantial evidence that certain genes somehow contribute to the control of σ^S proteolysis.

RssA. The *rssA* gene is located upstream of *rssB,* and the two genes constitute an operon with a single promoter upstream of *rssA* (174). The N-terminal part of RssA belongs to a family of putative serine esterases of unclear physiological functions that have been conserved from bacteria to humans (128). RssA deficiency, as well as overproduction, was observed to have minor but reproducible effects on the cellular σ^S levels (G. Kampmann, M. Marquardt, and R. Hengge-Aronis, unpublished results). So far, it is not clear whether RssA acts directly or indirectly and what its actual biochemical function is. RssA may be involved in RssB dephosphorylation under some conditions, but alternative explanations are at present not excluded.

The histone-like protein H-NS. *hns* mutants exhibit abnormally high σ^S levels in exponential phase (15, 234). Although H-NS is known as an abundant nucleoid-associated protein that represses or even silences the transcription of numerous genes (230), its effect in the control of σ^S is posttranscriptional, with *rpoS* translation being stimulated and σ^S proteolysis being strongly reduced (15, 234). It seems likely that H-NS indirectly affects σ^S degradation by controlling the expression of some other regulatory factor. The stability of σ^S in the *hns* mutant is certainly not due to a lack of RssB; rather, the cellular RssB level even seems slightly increased in the mutant (102), consistent with σ^S control of *rssB* expression (174, 185). Alternatively, H-NS may regulate some component involved in the control of RssB activity, such as, phosphorylation, but the component(s) still awaits identification.

The LysR homolog LrhA. In stationary-phase cells, the outer membrane porin OmpF is downregulated by a σ^S-dependent mechanism (170). Overexpression of LrhA, a regulator of the LysR family with hitherto unknown function, was found to suppress this phenotype by reducing σ^S levels, whereas an *lrhA* null mutation had the opposite phenotype. Epistasis experiments with *rssB* mutants have shown that LrhA affects σ^S proteolysis. The *lrhA* effect was still observed in a strain in which *rssB* was expressed from a nonnative promoter. Therefore, it was speculated that LrhA somehow affects the activity of RssB, i.e., its phosphorylation state (65). However, LrhA belongs to a family of transcriptional regulators, and therefore it is likely to control the expression level of some other factor, which may then play a direct role in of RssB activation. Since stress-induced alterations in the rate of σ^S proteolysis are too rapid to involve transcriptional induction of some factor, which in turn affects RssB activity, LrhA is unlikely to be part of a stress signal transduction pathway that controls σ^S proteolysis. A homolog of LrhA, HexA, is known in *Erwinia carotovora,* where it controls genes involved in exoenzyme synthesis, plant virulence, and motility (73, 149).

The DnaK chaperone. In stationary phase, *dnaK* mutants show reduced σ^S content and exhibit a pleiotropic phenotype very similar to that of an *rpoS* mutant (144, 180, 181). As outlined above, part of this effect is due to reduced *rpoS* translation. In addition, however, various lines of evidence indicate that *dnaK* mutants are partially defective for σ^S stabilization in starved cells (144, 181). However, the mechanistic link between the DnaK chaperone and the σ^S recognition and degradation system (Fig. 4) has not been identified. It is interesting that the DnaK system plays opposite roles in controlling the proteolysis of σ^S and of the heat shock sigma factor σ^{32}: whereas DnaK stabilizes σ^S, it is crucial for the degradation of σ^{32}. DnaK may also play a role in heat shock stabilization of σ^S, which is a relatively slow process (taking up to 20 min) that more or less correlates with the accumulation of the heat shock protein DnaK (144). By contrast, the extremely rapid and transient heat shock stabilization of σ^{32} is due to titration of the DnaK chaperone by suddenly accumulating denatured proteins (236).

It is possible that DnaK plays a role in starvation sensing. Sudden carbon source starvation is almost immediately followed by a strong reduction in overall protein biosynthesis and consequently in reduced levels of newly synthesized but not yet natively folded polypeptides, some of which are DnaK substrates. Thus, more DnaK may be available to somehow protect σ^S from degradation (144). At present it is unknown whether only DnaK or the entire DnaK chaperone machine (also including DnaJ and GrpE) is involved in σ^S protection from proteolysis.

A Small Molecule That Affects σ^S Proteolysis: Acetyl Phosphate

During growth on glucose as a carbon source, acetyl phosphate is produced by phosphotransacetylase (encoded by the *pta* gene) from acetyl coenzyme A. Acetate kinase (*ackA*) then uses acetyl phosphate and ADP to produce ATP and acetate, which is excreted (46, 173). An acetyl phosphate-free *pta-ackA* mutant exhibits an approximately twofold-increased σ^S half-life, and since acetyl phosphate is an excellent phosphoryl donor for RssB in vitro, a similar in vivo function has been postulated (25). With a few exceptions (98, 173, 196), this effect is in contrast to findings with most other response regulators, where in vivo phosphotransfer from acetyl phosphate usually cannot be detected unless the cognate sensor kinase (which in the absence of its specific stimulus often acts as the response regulator phosphatase) is eliminated by mutation (137). Thus, for most response regulators, phosphorylation with acetyl phosphate is possible in vitro but does not play a physiological role. For σ^S proteolysis, however, it seems to be physiologically relevant. Nevertheless, acetyl phosphate cannot be the only phosphodonor for RssB, because σ^S turnover is not completely abolished in the *pta-ackA* mutant. Also, the observation that the *pta-ackA* mutation has a minor (and under some conditions

no) effect on the σ^S level (25, 39) indicates that acetyl phosphate plays a rather subtle role in the combination of all the influences that together determine the actual σ^S level (in practice this means that in order to see such an effect, it is not sufficient to measure the σ^S level, an all-integrative parameter, but that σ^S degradation has to be directly assayed, e.g., by pulse-chase labeling).

Recently, the effects of acetate addition (at neutral pH) on genome-wide gene expression have been investigated. More than two dozen acetate-inducible genes (7) or proteins (100) have been identified, many of which are σ^S controlled. This effect is specific for acetate since formate produced largely opposite effects (100). In one of these studies, acetyl phosphate was excluded as the direct inducer, since a *pta-ackA* mutant exhibited constitutively high levels of the same proteins. Therefore, a high acetyl coenzyme A level was proposed to be the inducing signal (100). Unfortunately, σ^S itself and its different levels of control were not studied in those acetate-treated cells (see above for discussion of a putative effect of acetate on *rpoS* transcription).

REGULATION OF σ^S ACTIVITY

σ^S activity in transcriptional initiation requires its association with RNA polymerase core enzyme. However, of all the sigma factors of *E. coli,* σ^S is the one with the lowest affinity for the core enzyme in vitro (129). Moreover, even in stationary phase, the cellular level of σ^S does not exceed approximately one-third of the cellular level of σ^70 (89, 92). Given these basic data and the general competion of sigma factors for core, one wonders how σ^S can recruit RNA polymerase core to any significant extent in vivo and activate the expression of genes at all. It may be that the putative anti-σ^70 factor Rsd (90, 91) shifts the balance somewhat in favor of σ^S, but, given the relatively low cellular level of Rsd (91), this effect cannot be expected to quantitatively eliminate σ^70 activity. Moreover, some stationary-phase-induced genes are expressed by σ^70-containing RNA polymerase (77). It is conceivable that covalent modification (163) or ppGpp binding (213) of core polymerase may improve the interaction with σ^S. In addition, it is tempting to speculate that at least under stress conditions, where σ^S is induced, some unidentified factor(s) may exist that stimulates its interaction with core polymerase.

In Vivo Evidence for Regulation of σ^S Activity

The *crl* gene product stimulates the expression of curli fimbriae (162), which are involved in cell-cell aggregation (182, 183) and adhesion to eukaryotic cells (71, 162). Synthesis of curli (with the subunits encoded by *csgAB*) is also dependent on σ^S (8, 161). More recently, it was found that the role of Crl is not curli specific but that Crl has a stimulatory effect on the expression of a number of σ^S-activated genes. Also, for negative effects of σ^S (e.g., on the expression of OmpF or in a not further clarified negative autoregulation of σ^S itself), Crl seems to play a synergistic role (171). However, Crl does not downregulate σ^S itself (if anything, σ^S levels increase in a *crl* mutant, because the above-mentioned negative feedback in σ^S control is relieved). Moreover, Crl does not seem to be a DNA binding

protein since it does not contain any known DNA binding motif, nor could (nonspecific) binding to DNA cellulose be observed. Therefore, it was proposed that Crl may activate σ^S, perhaps by modulating the σ^S association with RNA polymerase core (171). This is certainly an attractive hypothesis, but a direct demonstration of such a function is still missing. If Crl stimulates σ^S-core interaction in stationary phase, it could be expected to directly interact with σ^S and/or core, and it should also contribute to σ^S stabilization (since association with core protects σ^S from RssB binding, as outlined above), but these specific hypotheses have not yet been tested.

There are specific conditions where high cellular levels of σ^S do not result in high expression of σ^S-dependent genes, i.e., where σ^S levels and activities do not appear to correlate. This seems to be the case when σ^S is artificially overproduced during exponential growth (e.g., from an isopropyl-β-D-thiogalactopyranoside [IPTG]-inducible promoter [R. Lange and R. Hengge-Aronis, unpublished results]). Another such situation is provided by the classic glucose-lactose diauxie experiment (54). σ^S is degraded with a half-life of 2 min during the first growth phase on glucose. During the lag phase, σ^S is completely stabilized, resulting in the accumulation of σ^S as well as mRNA of the σ^S-dependent *osmY* gene. When the cells then start to grow on lactose, the σ^S half-life remains relatively high (more than 20 min) and σ^S levels therefore decrease only slowly. However, no *osmY* mRNA can be detected during the end of the lag phase and during growth on lactose (54). This observation is formally reminiscent of the inactivation of σ^32 during temperature downshift, where σ^32 levels decrease much more slowly than expression levels of σ^32-dependent heat shock genes (204).

It is tempting to speculate that under such conditions, some factor(s) necessary for σ^S activation may be missing or inactive (or some inactivating factor may be abundant). However, such effects have to be interpreted with caution, because some σ^S-dependent genes require additional regulatory factors besides σ^S (78), which may not be present or active under the specific conditions studied.

In summary, there is initial although not conclusive evidence that activity of σ^S, i.e., probably σ^S association with RNA polymerase core in competition with other sigma factors, is regulated. At present, it can only be speculated that core enzyme modification (e.g., by ppGpp binding) or additional proteins (e.g., Crl) could be involved. One of the problems, however, is to experimentally distinguish this activation of σ^S from activation of σ^S-dependent transcription by some conventional regulatory mechanism. A clear answer probably requires in vitro transcription experiments that would allow the effects on sigma factor competition to be separated from "normal" activation of transcription.

The Response Regulator RssB Can Act Like an Anti-Sigma Factor for σ^S

In wild-type cells, RssB binding to σ^S is the first step in σ^S delivery to the ClpXP protease. However, in *clp* mutants as well as in stationary-phase cells engineered to contain a slightly increased RssB-to-σ^S ratio, RssB binding to σ^S results in σ^S inhibition as a transcription initiation factor; i.e., reduced expression of σ^S-dependent genes can be observed (21, 141, 242).

This suggested that in the absence of ClpXP, or under conditions where σ^S degradation is inhibited at the protease level, RssB can in principle act like an anti-sigma factor, e.g., by interfering with σ^S-core polymerase association (21). Recent in vitro data indicate that the association of σ^S with RssB is indeed mutually exclusive with σ^S-core association (243).

Thus, in principle, RssB has the potential to function as an anti-sigma factor for σ^S. Are there any conditions where this is physiologically relevant? Under the best-studied conditions (growth in minimal medium with various carbon sources), any binding of σ^S to RssB results in rapid degradation of σ^S. Moreover, RssB is present at clearly substoichiometric concentrations (21). However, two scenarios where σ^S inhibition by RssB could be physiologically relevant are at least conceivable (21). First, earlier in evolution, RssB may have been a stress-regulated anti-σ^S factor (originally produced in stoichiometric amounts with σ^S) before it was recruited by the proteolysis machinery to serve as a specific recognition factor with a catalytic function. Second, there may be unidentified conditions where (i) significant levels of σ^S are present, (ii) RssB may be upregulated, and (iii) ClpXP may be less active or downregulated. Although RssB is moderately upregulated in stationary phase (21, 174, 185), further studies of *rssB* regulation have so far not produced evidence that there are any strongly RssB-inducing conditions under which RssB could function as an anti-σ^S factor (Pruteanu and Hengge-Aronis, unpublished). Thus, the evolutionary scenario of a change in RssB function from anti-σ^S factor to σ^S proteolysis recognition factor seems more likely.

CONCLUSIONS AND PERSPECTIVES

With an ever-increasing number of factors that contribute to *rpoS* transcription and translation as well as to σ^S proteolysis, σ^S now appears to be an *E. coli* protein with one of the most complex regulation systems. Nevertheless, a relatively clear picture of the basic regulatory mechanisms, at least in post-transcriptional regulation, has emerged recently. The basic control of *rpoS* translation uses *rpoS* mRNA secondary structure, the Hfq and HU proteins, and small RNAs such as the DsrA mRNA. The core σ^S degradation machinery clearly consists of the ClpXP protease and the phosphorylation-modulated σ^S recognition factor RssB. Beyond this, however, numerous questions have yet to be answered.

Above all, the way in which the multiple signals that control σ^S are integrated remains largely unexplored. Translational control of *rpoS* involves a plethora of components (Fig. 3), but what are their molecular functions and interplay? How does late log phase, high osmolarity, or shift to acid pH affect the rate of *rpoS* translation? In σ^S proteolysis, the way in which the RssB-ClpXP system functions allows us to predict the overall functions of the "missing" components that have yet to be identified (Fig. 4). These include factors that, in response to certain stress conditions, affect (i) RssB phosphorylation, (ii) ClpXP activity in general or its ability to specifically degrade σ^S, or (iii) σ^S association with core RNA polymerase. The latter also indicates a link between the control of σ^S proteolysis and activity. In general, the different levels of σ^S control do not operate independently from each other, but components like H-NS or EIIA(G1c), which affect more than one level of control, may play a coordinating role.

There is growing evidence for complex connections between σ^S regulation and other regulatory circuits. These include a linkage to oxidative stress that operates via OxyS RNA; to the CRP regulon, catabolite repression, and inducer exclusion that uses cAMP-CRP and EIIA(Glc); or to the heat shock response which involves the DnaK chaperone. These connections are certainly relevant under multiple simultaneous stress conditions, which is probably a more natural situation than the carefully controlled single-stress situations usually studied in the laboratory.

With all the currently available information taken together, we appear to be approaching a situation where the σ^S regulatory network is becoming so complex that quantitative (mathematical) analysis and simulation may become helpful in really understanding its inherent overall potential and actual behavior under different conditions. With many regulatory components and their basic biochemical functions now identified, such analysis seems feasible. Since σ^S is connected to many other crucial regulatory modules in the cell, it may even provide a good starting point for a future quantitative analysis of the entire cellular regulatory network.

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