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The response regulator RssB controls stability of the σ^S subunit of RNA polymerase in *Escherichia coli*

Andrea Muffler, Daniela Fischer, Shoshy Altuvia[1,2], Gisela Storz[1] and Regine Hengge-Aronis[3]

Department of Biology, University of Konstanz, 78434 Konstanz, Germany and [1]Cell Biology and Metabolism Branch, National Institute of Child Health and Human Development, National Institutes of Health, Bethesda, MD 20892, USA

[2]Present address: Department of Biotechnology and Molecular Genetics, The Hebrew University, Hadassah Medical School, 90101 Jerusalem, Israel

[3]Corresponding author

The *rpoS*-encoded σ^S subunit of RNA polymerase is a central regulator in a regulatory network that governs the expression of many stationary phase-induced and osmotically regulated genes in *Escherichia coli*. σ^S is itself induced under these conditions due to an increase in *rpoS* transcription (only in rich media) and *rpoS* translation as well as a stabilization of σ^S protein which in growing cells is subject to rapid turnover. We demonstrate here that a response regulator, RssB, plays a crucial role in the control of the cellular σ^S content. *rssB* null mutants exhibit nearly constitutively high levels of σ^S and are impaired in the post-transcriptional growth phase-related and osmotic regulation of σ^S. Whereas *rpoS* translational control is not affected, σ^S is stable in *rssB* mutants, indicating that RssB is essential for σ^S turnover. RssB contains a unique C-terminal output domain and is the first known response regulator involved in the control of protein turnover.

Keywords: osmoregulation/proteolysis/*rpoS*/sigma factor/stationary phase

Introduction

The σ^S subunit of RNA polymerase in *Escherichia coli*, which is encoded by the *rpoS* gene, is a regulator for most stationary phase-induced genes, and it is essential for the appearance of stationary phase-associated phenotypes such as ovoid cell morphology and multiple stress resistance (for recent reviews, see Hengge-Aronis, 1993a,b; Loewen and Hengge-Aronis, 1994). However, the action of σ^S is not restricted to stationary phase cells. σ^S is also required for hyperosmotic induction of numerous genes in growing cells, and therefore it is a global regulator in the osmotic control of gene expression in *E.coli* (Hengge-Aronis *et al.*, 1991, 1993). More than 40 genes or operons have already been identified as being regulated by σ^S (Loewen and Hengge-Aronis, 1994).

The cellular level of σ^S is strongly elevated during entry into stationary phase as well as upon osmotic upshift (Gentry *et al.*, 1993; Tanaka *et al.*, 1993; Lange and Hengge-Aronis, 1994). This regulation is exceedingly complex and involves transcriptional as well as post-transcriptional mechanisms. Whereas a gradual reduction in growth rate stimulates *rpoS* transcription (Mulvey *et al.*, 1990; Lange and Hengge-Aronis, 1991, 1994; Schellhorn and Stones, 1992), sudden starvation, for instance due to glucose exhaustion of the medium, hardly affects transcription, but triggers stabilization of σ^S, which in growing cells is a very unstable protein with a half-life between 1.5 and 2.5 min (Lange and Hengge-Aronis, 1994; Takayanagi *et al.*, 1994). In addition, translation of *rpoS* is stimulated 5-fold during late exponential phase, presumably by a cell density-dependent mechanism (Lange and Hengge-Aronis, 1994). The response to increased osmolarity is entirely post-transcriptional, involving increased translation of *rpoS* as well as a nearly complete stabilization of the σ^S protein (Lange and Hengge-Aronis, 1994; Muffler *et al.*, 1996).

The molecular mechanisms underlying these complex regulatory patterns are far from understood. So far, the only protein factor known to be involved in the regulation of σ^S is the histone-like protein H-NS. *hns* mutants strongly overexpress σ^S already during the exponential phase of growth, and, surprisingly, H-NS was found to act at the post-transcriptional level of σ^S control (Barth *et al.*, 1995; Yamashino *et al.*, 1995). Unfortunately, the direct mode of action of H-NS is unknown. In addition, UDP-glucose has been identified as a negatively acting signal molecule that affects a post-transcriptional mechanism in *rpoS* control (Böhringer *et al.*, 1995; A.Muffler and R.Hengge-Aronis, unpublished results).

The UDP-glucose pyrophosphorylase gene *galU* and *hns* are adjacent genes (Figure 1) located at 27.8 min of the physical map of the *E.coli* chromosome (Rudd, 1992). Here we report that an open reading frame (*orf37*) located just upstream of *galU* encodes a negative regulator of σ^S (hence the designation as *rssB*) and, like UDP-glucose and H-NS, affects the post-transcriptional level of σ^S control. Our data demonstrate that RssB plays an essential role in σ^S turnover. The N-terminal 110 amino acids of RssB exhibit strong sequence similarity to the receiver domain characteristic of the family of two-component response regulators. The C-terminal output domain of RssB, however, does not show significant similarity to any known proteins, consistent with RssB being the first known response regulator that is involved in regulated protein turnover.

Results

Isolation of mutations in rssB (orf37)

Mutations in the two adjacently located genes *galU* and *hns* result in strongly increased σ^S levels already during exponential growth (Barth *et al.*, 1995; Böhringer *et al.*, 1995; Yamashino *et al.*, 1995). The co-location of these

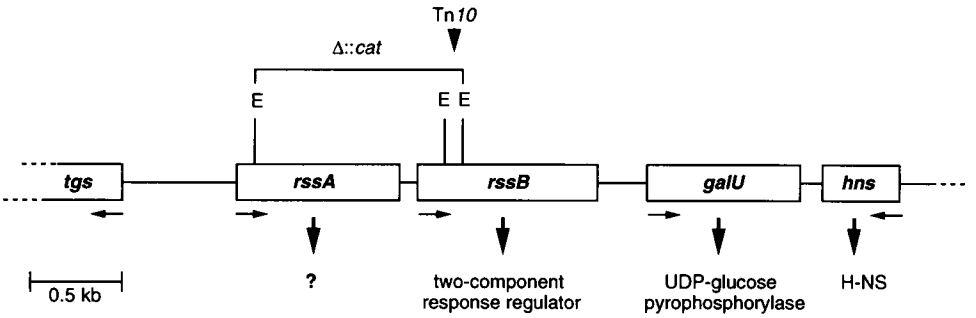

Fig. 1. Genetic organization of the *tgs–rssAB–galU–hns* region on the *E.coli* chromosome. The region shown is located at 27.8 min of the physical map of the chromosome (Rudd, 1992). The construction of the *rssB*::Tn*10* and Δ*rssAB*::*cat* insertion mutations is described in the text.

genes on the *E.coli* chromosome prompted us to investigate whether other genes in this totally sequenced but functionally not fully characterized chromosomal region are also involved in the regulation of σ^S.

Directly upstream of *galU*, there is an open reading frame (*orf37*, now termed *rssB*) that, by sequence criteria, encodes a two-component response regulator of hitherto unknown function (Bösl, 1993). A *rssB*::Tn*10* insertion was independently isolated in a study not further related to the work presented here. It was found as a mutation that affected the ability of a small regulatory RNA (OxyS) to repress the expression of an *acrH*::*lacZ* fusion (*acrH* encodes a protein of unknown function with similarity to transporters involved in metal resistance, nodulation or cell division). The characterization of the OxyS regulatory RNA, its role in gene expression and its target genes will be published separately (S.Altuvia and G.Storz, in preparation).

There is another open reading frame (*orf34*) of unknown function immediately upstream of *rssB*. The common direction of transcription, the close spacing and the absence of a transcriptional terminator between *orf34* and *rssB* (Bösl, 1993) indicate that the two genes constitute an operon (Figure 1). Preliminary results also indicate that the *orf34* gene product acts in the same pathway as RssB (see below). Therefore, *orf34* is referred to as *rssA* here. By making use of three *Eco*RV restriction sites present in this region, we isolated a chromosomal deletion (with the *cat* gene inserted), that eliminates most of *rssA* and the 5′ end of *rssB* until codon 85 and thus encompasses most of the region encoding the receiver domain of RssB (for the location of restriction sites and the details of isolation of the *rssB*::Tn*10* and Δ*rssAB*::*cat* mutations, see Figure 1 and Material and methods).

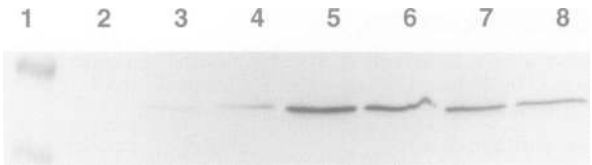

Fig. 2. *rssB* mutants exhibit increased cellular levels of σ^S during the exponential phase of growth. Strains MC4100 (*rssAB*$^+$; lanes 3 and 4), AM106 (*rssB*::Tn*10*; lanes 5 and 6) and RH158 (Δ*rssAB*::*cat*; lanes 7 and 8) were grown in M9 medium with 0.1% glucose, and samples were taken during the early (lanes 3, 5 and 7) and the late (lanes 4, 6 and 8) exponential phase (at optical densities of 0.2 and 0.8, respectively) and subject to immunoblot analysis with antibodies against σ^S. Strain RH90 was used as a *rpoS*-negative control (lane 2). Size standard proteins of 49 and 32 kDa are shown (lane 1).

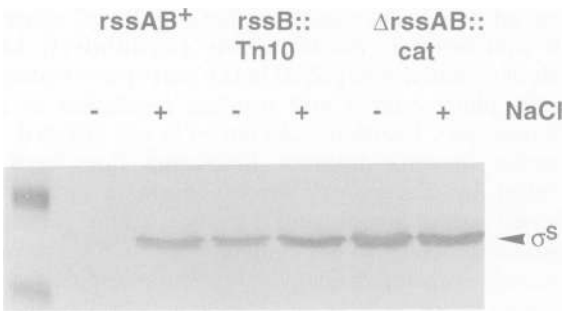

Fig. 3. Cellular σ^S levels in wild-type and *rssB* mutant strains before and after osmotic upshift. Strains MC4100 (*rssAB*$^+$), AM106 (*rssB*::Tn*10*) and RH158 (Δ*rssAB*::*cat*) were grown in M9 medium with 0.4% glycerol. At an OD_{578} of 0.3, the cultures were divided and 0.3 M NaCl was added to one of the aliquots. Thirty minutes after the addition of NaCl, samples were taken and subject to immunoblot analysis with antibodies against σ^S. Size standard proteins of 49 and 32 kDa are shown in the first lane at the left side of the blot.

rssB mutants exhibit strongly increased σ^S levels during exponential phase and are impaired in the osmotic induction of σ^S

In a wild-type strain growing in minimal glucose medium, σ^S levels are very low during early exponential phase. During the transition into stationary phase or in response to hyperosmotic shift, the cellular σ^S increases >10-fold (Gentry *et al.*, 1993; Tanaka *et al.*, 1993; Lange and Hengge-Aronis, 1994).

Throughout the exponential phase, the *rssB*::Tn*10* and Δ*rssAB*::*cat* mutants exhibited σ^S levels that were ~10 times higher than those observed even during late exponential phase for an otherwise isogenic *rssAB*$^+$ strain (Figure 2). These data indicate that the response regulator encoded by the *rssB* gene has an inhibitory function in the control of σ^S. RssB appears to be required to keep σ^S levels low during exponential phase growth.

Figure 3 shows σ^S levels before and after osmotic upshift (i.e. the addition of 0.3 M NaCl). Whereas a 14-fold induction of σ^S can be seen for a *rssAB*$^+$ strain, both the *rssB*::Tn*10* strain and the Δ*rssAB*::*cat* deletion mutant contained σ^S levels that in the absence of NaCl were already as high or even higher as those observed for the *rssAB*$^+$ strain only after osmotic upshift. Upon the addition of NaCl, the *rssB* mutants showed little or no further increase in σ^S levels. These data show that hyperosmotic

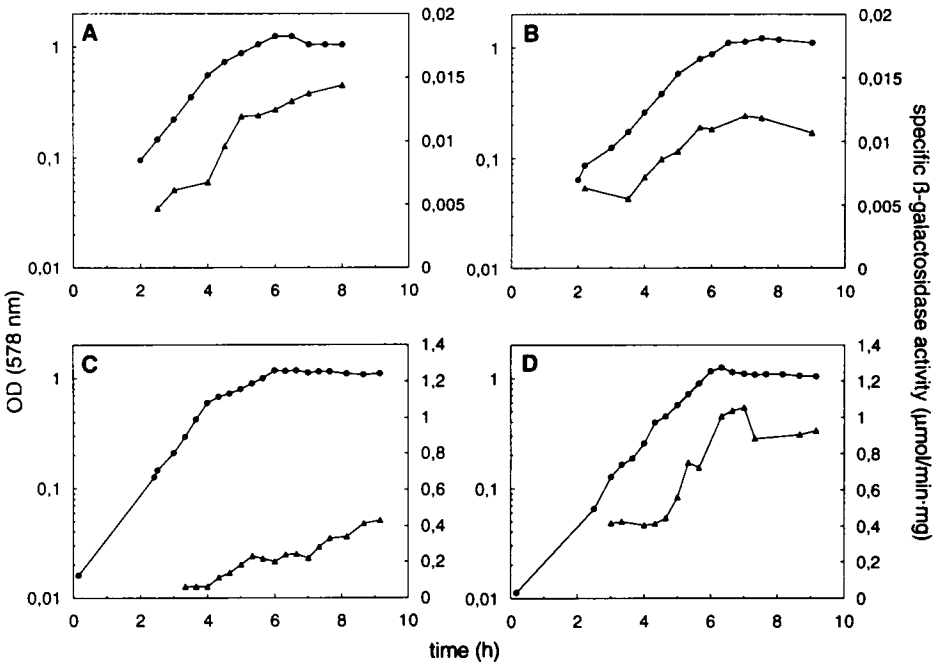

Fig. 4. The *rssB*::Tn*10* mutation affects the post-transcriptional regulation of *rpoS*. Strains carrying the transcriptional *rpoS742*::*lacZ* fusion (**A** and **B**), or the translational *rpoS742*::*lacZ* fusion (**C** and **D**) in either *rssAB*$^+$ (A and C) or *rssB*::Tn*10* (B and D) backgrounds were grown in M9 medium with 0.1% glucose. Optical densities (circles) and specific β-galactosidase activities (triangles) were determined along the growth curve.

induction of σ^S is impaired in the absence of the RssB response regulator.

An increased level of σ^S in the *rssB* mutants is also reflected in an increased expression of genes under the control of σ^S. Thus, *rssB* mutants exhibit higher exponential phase levels of β-galactosidase activity produced from *lacZ* fusions to σ^S-regulated genes such as *osmY*, *otsBA* and *dps* (data not shown).

RssB is involved in the post-transcriptional regulation of σ^S

In order to differentiate whether the *rssB* mutations affect a transcriptional or post-transcriptional mechanism in the control of σ^S expression, we tested the expression of various transcriptional and translational *rpoS*::*lacZ* fusions [located in single copy at the *att*(λ) site in a *rpoS*$^+$ background].

The expression pattern of a transcriptional *rpoS*::*lacZ* fusion inserted at nucleotide 742 within *rpoS* was not significantly changed by the *rssB*::Tn*10* mutation (Figure 4A and B). However, the expression of a translational fusion inserted at the same position within *rpoS* was clearly affected by the *rssB* mutation (Figure 4C and D). In the mutant background, this fusion exhibited at least 5-fold higher expression during the exponential phase. It still showed ~2-fold induction late in exponential phase but, in contrast to the otherwise isogenic *rssAB*$^+$ strain, induction did not continue in stationary phase (Figure 4D). Similar results were also obtained with the Δ*rssAB*::*cat* deletion mutant (data not shown).

From these results, we conclude that RssB is involved directly or indirectly in the post-transcriptional control of σ^S expression. This finding is remarkable since nearly all other known response regulators (with the exception of the CheB methylesterase and CheY) are DNA binding proteins involved in the transcriptional control of gene expression (Parkinson, 1993).

Mutations in rssB do not affect rpoS translation, but alter σ^S stability

The activity of the translational *rpoS742*::*lacZ* fusion (used in the experiment shown in Figure 4) reflects the regulation of *rpoS* translation as well as of σ^S stability. Like σ^S itself, the RpoS742::LacZ hybrid protein is degraded rapidly during exponential growth (Muffler *et al.*, 1996). We therefore used pulse–chase experiments to determine whether RssB is involved in *rpoS* translation or σ^S turnover.

The translation of *rpoS* is stimulated in response to high osmolarity or during the late exponential phase (Lange and Hengge-Aronis, 1994; Muffler *et al.*, 1996). Since transcript levels are not significantly changed under these conditions (Lange and Hengge-Aronis, 1994), increased rates of translation can be assayed by determining σ^S synthesis in pulse-labeling experiments (with pulse and chase times significantly shorter than σ^S half-life). Figure 5 demonstrates that the *rssB*::Tn*10* mutant and the otherwise isogenic *rssAB*$^+$ strain show a similar increase in σ^S synthesis in response to osmotic upshift (σ^S synthesis is stimulated 3.7-fold in the *rssB*$^+$ strain and 3.9-fold in the *rssB* mutant). In addition, the synthesis of σ^S is stimulated in both strains during the late exponential phase (data not shown). The RssB response regulator is thus not involved in the translational control of *rpoS*.

Pulse–chase experiments with extended chase times (Figure 6), however, demonstrated that the *rssB*::Tn*10* mutant is affected in σ^S turnover. σ^S half-life was determined to be >60 min. By contrast, the rapid turnover of σ^S in the wild-type strain is readily apparent (with a half-life of 1.35 min). After the onset of starvation, σ^S was

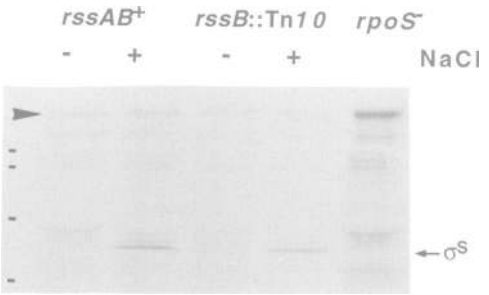

Fig. 5. Translational regulation of *rpoS* is not affected in the *rssB* mutant. Strains MC4100 (*rssAB*$^+$) and AM106 (*rssB*::Tn*10*) were grown in M9 medium with 0.4% glycerol. Samples were taken at optical densities of 0.3 and labeled with [^{35}S]methionine for 60 s, followed by a 30 s chase with non-radioactive methionine (for details, see Materials and methods). Immediately thereafter, 0.3 M NaCl was added to the cultures, followed by a similar labeling 10 min after the osmotic upshift. Labeled samples were subjected to immunoprecipitation and SDS–PAGE. Bands on the autoradiograph were quantitated in relation to an internal standard (the RpoS742::LacZ hybrid protein, indicated by an arrow head; see Materials and methods for details). The positions of size standard proteins are indicated by small dashes (106, 80, 49 and 32 kDa from top to bottom).

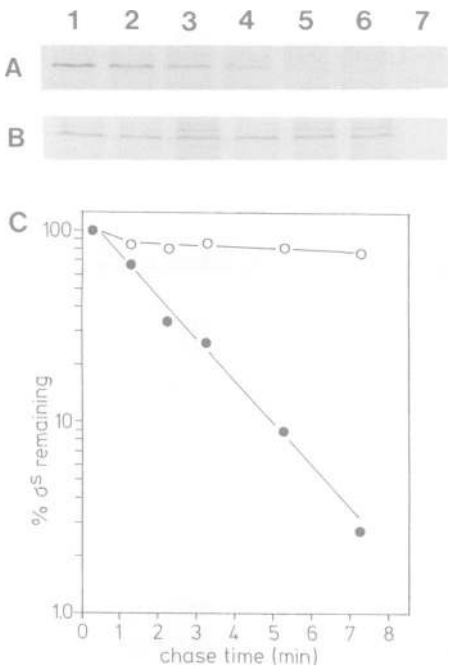

Fig. 6. σ^S turnover is abolished in the *rssB* mutant. Strains MC4100 (*rssAB*$^+$; **A**) and AM106 (*rssB*::Tn*10*; **B**) were grown in M9 medium with 0.1% glucose to an OD_{578} of 0.8, and labeled with [^{35}S]methionine as described in Materials and methods. The pulse time was 1 min, followed by chase times of 0.25, 1.25, 2.25, 3.25, 5.25 and 7.25 min (lanes 1–6). As a control, the *rpoS* mutant RH90 was used (lane 7). A densitometric quantification of the data in (A) and (B) is shown in (**C**) with open symbols for the *rssB* mutant AM106 and solid symbols for strain MC4100.

stable in both strains (data not shown). These results immediately explain the increased σ^S levels in *rssB* mutants (Figures 2 and 3). We conclude that the rapid turnover of σ^S in wild-type strains is dependent on the presence of the RssB response regulator.

We have shown recently that a translational fusion inserted at nucleotide 379 within *rpoS* produces a hybrid protein that, in contrast to RpoS742::LacZ, is not subject

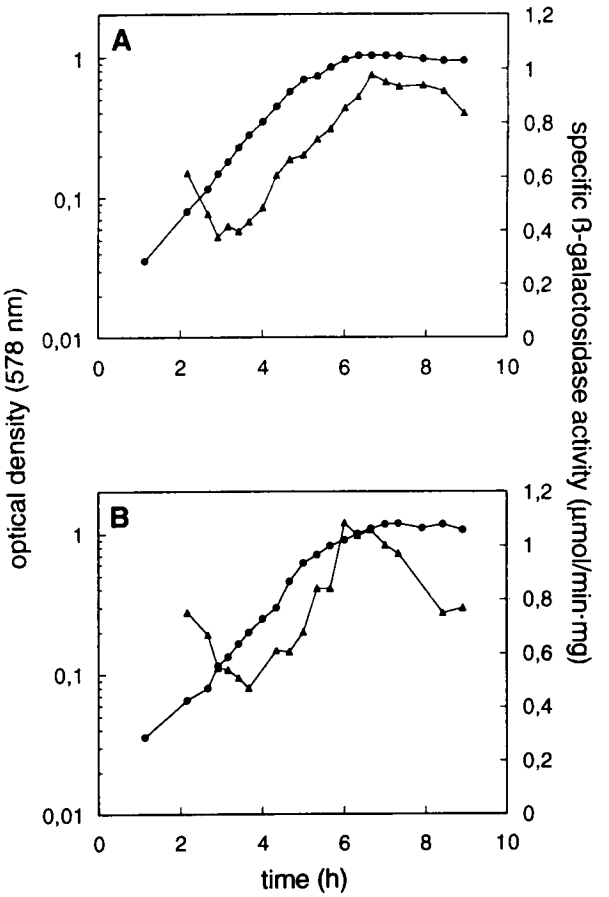

Fig. 7. A translational *lacZ* fusion containing only 379 nucleotides of *rpoS* is not subject to RssB-mediated control. The *rpoS379*::*lacZ*-carrying strains RO90 (*rssAB*$^+$) and AM108 (*rssB*::Tn*10*) were grown in M9 medium with 0.1% glucose. Optical densities (circles) and specific β-galactosidase activities (triangles) were determined along the growth curve.

to turnover, indicating that an element encoded between nucleotides 379 and 742 in *rpoS* is essential for σ^S degradation. Translational regulation, however, can be observed with *rpoS379*::*lacZ* (Muffler *et al.*, 1996). Figure 7 shows that the expression pattern of the translational *rpoS379*::*lacZ* fusion in the wild-type background was strikingly similar to that of the *rpoS742*::*lacZ* fusion in the *rssB* mutant background (compare with Figure 4D). Moreover, the expression of the *rpoS379*::*lacZ* fusion is not significantly affected by the *rssB*::Tn*10* mutation (Figure 7). This finding demonstrates that RssB acts exclusively on σ^S turnover and suggests that a region or an element encoded between nucleotides 379 and 742 of *rpoS* is required for RssB to exert its effect on σ^S degradation.

Discussion

σ^S induction is the result of a combination of increased rates of transcription and translation and a stabilization of σ^S protein, which in rapidly growing cells is a highly unstable protein (Loewen *et al.*, 1993; McCann *et al.*, 1993; Lange and Hengge-Aronis, 1994; Takayanagi *et al.*, 1994). *rpoS* translation is stimulated in response to high osmolarity or high cell density, whereas σ^S turnover is

inhibited in response to carbon starvation and also as a consequence of osmotic upshift (Lange and Hengge-Aronis, 1994; Muffler *et al.*, 1996). Neither the direct regulatory mechanisms nor the signal transduction pathways involved are understood at present.

In this study, we have identified a gene, *rssB*, that plays a crucial role in the post-transcriptional control of the cellular σ^S level. The '*rss*' designation stands for 'regulator of σ^S' and was chosen to be neutral with respect to the various inducing signals, since σ^S induction in response to starvation as well as to osmotic upshift was affected in *rssB* mutants. RssB is essential for σ^S turnover in growing *E.coli* cells. In a *rssB*::Tn*10* mutant, σ^S has a half-life of >60 min, which is in pronounced contrast to its rapid degradation (with a half-life of 1.35 min) in an otherwise isogenic *rssB*$^+$ strain (Figure 6).

rssB encodes a novel response regulator. This family of proteins is defined by the presence of an N-terminal receiver domain which is phosphorylated or dephosphorylated in response to signals transmitted by histidine sensor kinases, and which controls the activity of a C-terminal output domain. With the exception of the CheB methylesterase and the CheY protein involved in chemotaxis, the output domains of response regulators have been shown to act as transcription factors (Parkinson and Kofoid, 1992; Parkinson, 1993; Swanson *et al.*, 1994). By contrast, RssB is the first known response regulator involved in regulated proteolysis. Whereas the RssB protein contains a conventional receiver domain with highest similarity to the corresponding domains of NtrC and RcsC of *E.coli*, and with Asp58 as the putative phosphorylation site, its C-terminal output domain does not exhibit significant similarity to any other protein of known function. While this is consistent with its unprecedented role in protein degradation, it is in contrast to those response regulators that act as transcription factors. These response regulators fall into several subfamilies defined by homology in their C-terminal output domains that are responsible for DNA binding (Volz, 1995).

What might be the role of RssB in σ^S degradation? It is conceivable that RssB itself has proteolytic activity and thus directly degrades σ^S. There is, however, evidence that the ClpPX protease is involved in σ^S degradation (Schweder *et al.*, 1996). This makes it more likely that the response regulator RssB acts as a signal transducing factor, providing the link between the upper part of the signal transduction pathway(s) that responds to osmotic and starvation signals and the σ^S-degrading protease. RssB might be required for the protease to be active in general, or its role might be that of a chaperone-like factor that, under certain conditions, presents σ^S to the protease. The latter would be reminiscent of the role of the DnaK–DnaJ–GrpE complex in the control of σ^{32} stability (Straus *et al.*, 1990; Liberek *et al.*, 1992; Liberek and Georgopoulos, 1993; Nagai *et al.*, 1994). Interestingly, the degradation of σ^{32} requires the presence of a motif within σ^{32} (region C) (Nagai *et al.*, 1994) and, in a perhaps analogous way, a region encoded between nucleotides 379 and 742 within *rpoS* also seems to be necessary for σ^S turnover and for RssB to affect this turnover. Yet another possibility is that RssB controls the synthesis of the protease. However, in view of the extremely rapid inhibition of σ^S turnover in response to starvation and osmotic upshift, this seems unlikely (unless the protease is itself very unstable). Further experiments to distinguish between these possiblities are presently being undertaken.

How is RssB activity controlled in response to starvation and osmotic upshift? The presence of a typical N-terminal receiver domain in RssB suggests that phosphorylation and dephosphorylation of RssB are involved in this control. However, a cognate histidine kinase for RssB has not yet been identified. The genetic organization of the chromosome in the *rssB* region (Bösl, 1993) suggests that *rssB* (*orf37*) may be the second gene in an operon that also contains *rssA* (*orf34*). The RssA protein does not exhibit significant similarity to any other protein of known function. In particular, it is clearly not a two-component histidine sensor kinase. We have observed recently that a deficiency in *rssA* overcomes the detrimental effects of RssB overproduction. Moreover, a deficiency in *rssA* was found to affect the absolute levels of σ^S in the cell (our unpublished results). These preliminary data suggest that RssA is involved in the regulation of σ^S and that it acts in the same pathway as RssB, although its precise role remains to be clarified.

Downstream of *rssB*, the *galU* gene, which encodes UDP-glucose pyrophosphorylase, is located. Although the *rssB*::Tn*10* mutation is not polar on *galU* (in contrast to *galU* mutants, *rssB* mutants are not galactose sensitive; our unpublished results), there is no apparent terminator between *rssB* and *galU*, making some coordination of expression likely. We have demonstrated previously that UDP-glucose is a signal molecule with a negative role in the control of σ^S (Böhringer *et al.*, 1995). Moreover, UDP-glucose acts at the post-transcriptional level of σ^S regulation (A.Muffler and R.Hengge-Aronis, unpublished results). It may be that the activity of the RssB response regulator is modulated in response to changes in the intracellular level of UDP-glucose that may occur under certain environmental conditions. Further work to elucidate the roles of the different domains of the RssB response regulator as well as of the RssA protein and UDP-glucose in signal transduction and σ^S degradation is in progress.

Materials and methods

Bacterial strains and growth conditions

The bacterial strains used in this study are listed in Table I. Cultures were grown at 37°C under aeration in Luria-Bertani (LB) medium or minimal medium M9 (Miller, 1972) supplemented with 0.1% glucose or 0.4% glycerol as carbon sources. Antibiotics were added as recommended (Miller, 1972). Growth was monitored by measuring the optical density at 578 nm (OD_{578}).

Isolation of mutations in rssB

The *rssB*::Tn*10* mutation was isolated in a search for insertion mutants that relieved repression of an *acrH*::*lacZ* fusion mediated by overexpression of OxyS. A pool of random mini-Tn*10* insertions isolated in MC4100 as described (Kleckner *et al.*, 1991) was moved by P1 transduction into MC4100 carrying *acrH*::*lacZ*. Approximately 8000 transductants were pooled and transformed with a plasmid overexpressing the OxyS regulatory RNA. Several transformants that no longer showed repression by OxyS were isolated. One of these insertion mutants was chosen for further study, and a fragment carrying the left end of the mini-Tn*10* insertion and the flanking chromosomal DNA was subcloned and sequenced. Upon comparison with sequences in the database, the chromosomal sequence was found to correspond to genes identified as *hnr* (GenBank accession No. X66003) and *orf37* (Bösl, 1993).

For the isolation of the Δ*rssAB*::*cat* deletion–insertion mutation, the *rssAB* region was first cloned from a PCR fragment obtained from

Table I. Bacterial strains

Strain	Relevant genotype	Reference
MC4100	F⁻ *Δ(arg-lac)U169 araD139 rpsL150 ptsF25 flbB5301 rbsR deoC relA1*	Silhavy *et al.* (1984)
RH90	MC4100 *rpoS359*::Tn*10*	Lange and Hengge-Aronis (1991)
RO35	MC4100 [λRZ5: *rpoS70*::*lacZ*(hybr)]	Hengge-Aronis *et al.* (1993)
RO90	MC4100 [λRZ5: *rpoS379*::*lacZ*(hybr)]	R.Lange
RO91	MC4100 [λRZ5: *rpoS742*::*lacZ*(hybr)]	Lange and Hengge-Aronis (1994)
RO200	MC4100 (λRZ5: *rpoS742*::*lacZ*)	Lange and Hengge-Aronis (1994)
AM106	MC4100 *rssB*::Tn*10*	this study
AM107	RO35 *rssB*::Tn*10*	this study
AM108	RO90 *rssB*::Tn*10*	this study
AM109	RO91 *rssB*::Tn*10*	this study
AM110	RO200 *rssB*::Tn*10*	this study
RH158	MC4100 *ΔrssAB*::*cat*	this study
RH159	RO91 *ΔrssAB*::*cat*	this study
RH160	RO200 *ΔrssAB*::*cat*	this study
RH161	RO35 *ΔrssAB*::*cat*	this study
JCB433	MC4100 *recD1903*::miniTn*10* (Tet^r)	J.Bardwell

plasmid pGM10 as a template [pGM10 carries the region encompassing *tgs*, *rssAB* (*orf34-orf37*), *galU* and *hns* (May *et al.*, 1990)]. For PCR amplification, two primers (5′-CGTTGAATTCTTGAAGCTTTTTTA-CCACCTTCGG-3′ and 5′-CTGGTTCC-GGATCCCGCAACGGGG-3′) were used, that with a few mismatches for the introduction of *Eco*RI–*Hin*dIII and *Bam*HI restriction sites (underlined) are complementary to regions upstream and downstream of *rssAB*, respectively. The *Eco*RI–*Bam*HI-treated PCR fragment was cloned into pBR322. In the resulting plasmid, two adjacent *Eco*RV fragments carrying parts of the *rssAB* region (see Figure 1 for the location of restriction sites) were replaced by an *Eco*RV-treated PCR fragment obtained with pACYC184 as a template using two primers complementary to regions upstream and downstream of the *cat* gene (5′-ATACACGATATCAGTAAGTTGGC-3′ and 5′-CCAGCAATAGATATCAGCG-GC-3′, both with two mismatches to introduce the underlined *Eco*RV restriction sites). The *Eco*RI–*Bam*HI insert from the resulting *ΔrssAB*::*cat* plasmid was isolated and used for linear transformation by electroporation into the *recD* strain JCB433 selecting for chloramphenicol (Cm) resistance. A P1 lysate obtained from a Cm^r colony was used to transduce MC4100, again selecting for Cm resistance. The absence of the *ΔrssAB* (*Eco*RV) fragments as well as the correct insertion of the *cat* gene in the resulting strain were controlled by Southern blot analysis. The *rssB*::Tn*10* and *ΔrssAB*::*cat* mutations were introduced into various genetic backgrounds by P1 transduction as described (Miller, 1972).

While performing the above described procedure, we found that the *rssAB*-carrying PCR fragment derived from pGM10 carried a small insertion near the 5′ end of *rssB*. By the susequent *Eco*RV blip-out used to isolate the *ΔrssAB* deletion, this insertion was also excised. Finding an insertionally inactivated *rssB* gene on plasmid pGM10 is consistent with our observation that cells apparently do not tolerate *rssAB* cloned under the control of its own promoter on a standard medium copy number cloning vector (pBR322; our unpublished results).

DNA manipulations

For restriction digests, ligation, transformation and agarose gel electrophoresis, standard procedures were followed (Silhavy *et al.*, 1984; Sambrook *et al.*, 1989). Plasmid DNA preparations were performed with the JETstar and JETprep kits, for the recovery of DNA from agarose gels, the JETsorb kit was used (Genomed). Bacterial chromosomal DNA was prepared as described (Silhavy *et al.*, 1984). Southern transfer of DNA was performed by standard procedures (Sambrook *et al.*, 1989). For hybridization, the non-radioactive DNA labeling, hybridization and detection kit (Boehringer Mannheim) was used according to the directions given by the manufacturer.

SDS–PAGE and immunoblot analysis

Sample preparation for SDS–PAGE and immunoblot analysis were performed as described previously (Lange and Hengge-Aronis, 1994). Fifteen μg of total cellular protein was applied per lane. A polyclonal serum against σ^S, a goat anti-rabbit IgG alkaline phosphatase conjugate (Sigma) and a chromogenic substrate (BCIP/NBT; Boehringer Mannheim) were used for visualization of σ^S bands.

Pulse-labeling of cells and immunoprecipitation

The procedure used for pulse-labeling of cells with L-[^{35}S]methionine and immunoprecipitation of σ^S was described previously (Lange and Hengge-Aronis, 1994). Where necessary, the OD_{578} of the samples was adjusted by dilution with supernatant from the same cultures obtained by centrifugation immediately before taking the samples for pulse-labeling. For the determination of the rate of σ^S expression, pulse and chase times were 60 and 30 s, respectively. For the determination of σ^S half-life, the pulse time was 60 s, and chase times varied as mentioned in the respective figure legends. As a σ^S-deficient control, strain RH90 was used (labeled in exponential phase samples harvested at an OD_{578} between 0.5 and 0.7). For use as an internal control in the quantification of the σ^S bands, the *rpoS* mutant RH90 containing pRL44 (carrying the translational *rpoS742*::*lacZ* fusion) was grown in M9 with 0.4% glycerol, 0.3 M NaCl and ampicillin (50 μg/ml) and labeled with [^{35}S]methionine as described above (pulse and chase times were 90 and 60 s, respectively). Aliquots (50 μl) of the extracts prepared from these labeled cells were added to extracts (0.5 ml) from labeled cells to be assayed for σ^S expression. The RpoS742::LacZ hybrid protein is recognized by the polyclonal anti-σ^S serum.

β-Galactosidase assay

β-Galactosidase activity was assayed by use of *o*-nitrophenyl-β-D-galactopyranoside (ONPG) as a substrate and is reported as micromoles of *o*-nitrophenol per min per milligram of cellular protein (Miller, 1972).

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