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Sequential recognition of two distinct sites in σ^S by the proteolytic targeting factor RssB and ClpX

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σ^S (RpoS), the master regulator of the general stress response in *Escherichia coli*, is a model system for regulated proteolysis in bacteria. σ^S turnover requires ClpXP and the response regulator RssB, whose phosphorylated form exhibits high affinity for σ^S. Here, we demonstrate that recognition by the RssB/ClpXP system involves two distinct regions in σ^S. Region 2.5 of σ^S (a long α-helix) is sufficient for binding of phosphorylated RssB. However, this interaction alone is not sufficient to trigger proteolysis. A second region located in the N-terminal part of σ^S, which is exposed only upon RssB–σ^S interaction, serves as a binding site for the ClpX chaperone. Binding of the ClpX hexameric ring to σ^S-derived reporter proteins carrying the ClpX-binding site (but not the RssB-binding site) is also not sufficient to commit the protein to degradation. Our data indicate that RssB plays a second role in the initiation of σ^S proteolysis that goes beyond targeting of σ^S to ClpX, and suggest a model for the sequence of events in the initiation of σ^S proteolysis.

Keywords: protease/proteolysis/*rpoS*/σ factor/stress response

Introduction

Protein degradation is crucial for clearing cells of denatured, aggregated and/or incomplete polypeptides. In addition, it is increasingly recognized that proteolysis plays an important physiological role in global regulation. A number of key master regulators of stress responses, cell cycle control or developmental processes are conditionally unstable (for a recent overview see Jenal and Hengge-Aronis, 2003). A prominent example is the σ^S subunit of RNA polymerase in *Escherichia coli*, which functions as the master regulator of the general stress response. σ^S is synthesized and at the same time efficiently degraded in rapidly growing cells, which therefore contain little σ^S. However, σ^S proteolysis is inhibited in response to a number of different stress conditions, resulting in a rapid and strong increase in the cellular σ^S level (recently summarized in Hengge-Aronis, 2002b).

The proteases involved in regulated proteolysis are often complex multisubunit proteases such as ClpXP, ClpAP or Lon (Wickner *et al*., 1999). These complex molecular machines contain chaperone subunits or domains that are responsible for specific recognition, ATP hydrolysis-dependent unfolding and threading of the substrate into the proteolytic cavity (Horwich *et al*., 1999; Singh *et al*., 2000; Reid *et al*., 2001). Some proteolysis substrates carry recognition sites at their C-termini (Levchenko *et al*., 1995; Keiler *et al*., 1996; Flynn *et al*., 2001; Ishii and Amano, 2001), wherease others are recognized via N-terminally located determinants (Gonciarz-Swatek *et al*., 1999; Gonzalez *et al*., 2000; Hoskins *et al*., 2000a,b).

Some substrate proteins require additional specific recognition or targeting factors that increase specificity of recognition or even make recognition possible as the chaperone/protease system would otherwise not attack the substrate. In addition, specific recognition factors provide unique opportunities for regulation of proteolysis, since their substrate binding can be controlled, i.e. by modification. Thus, ClpXP-dependent degradation of σ^S requires the specific recognition factor RssB, which binds and targets σ^S to ClpXP, without being degraded itself (Muffler *et al*., 1996; Pratt and Silhavy, 1996; Becker *et al*., 1999; Klauck *et al*., 2001; Zhou *et al*., 2001). Here, recognition is tightly regulated, since RssB is a two-component response regulator, whose affinity for σ^S is dependent upon phosphorylation of its receiver domain (Becker *et al*., 1999; Klauck *et al*., 2001; Zhou *et al*., 2001). This allows the RssB/ClpXP system to integrate information from converging stress signal transduction pathways (Hengge-Aronis, 2002a).

An internal 'turnover element' in σ^S, and in particular a specific amino acid (K173), was shown to be essential for interaction with phosphorylated RssB and therefore for σ^S proteolysis (Becker *et al*., 1999). According to secondary structure prediction and the recently published crystal structures of bacterial RNA polymerase holoenzymes (Murakami *et al*., 2002; Vassylyev *et al*., 2002), K173 is located at the beginning of an α-helix that consists of region 2.5 and, together with the two following α-helices, constitutes domain 3 of σ^S. K173 is highly likely to be surface exposed in free σ^S as well as in the holoenzyme, consistent with its role in recognition by RssB (Becker *et al*., 1999) as well as in specific promoter recognition (Becker and Hengge-Aronis, 2001).

These features make σ^S a highly interesting model substrate for conditional proteolytic recognition. What is the extension of the RssB recognition site in σ^S that includes K173 as a crucial amino acid? Is RssB binding sufficient to trigger σ^S proteolysis? Is σ^S also specifically bound by ClpX and, if so, where is the ClpX-binding site? Is RssB binding a prerequisite for interaction of σ^S with ClpX? In the present study, we demonstrate that σ^S contains distinct binding sites for interaction with RssB

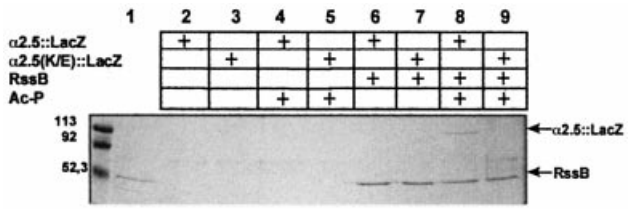

Fig. 1. A hybrid protein consisting of the α-helical region 2.5 of σ^S and β-galactosidase (α2.5::LacZ) binds to phosphorylated RssB. Direct interaction between RssB (carrying an S-TRX-His_6 tag, present in lanes 1 and 6–9) and either His_6-RpoS (lane 1) or crude cell extracts of strains overexpressing α2.5::LacZ or $\alpha2.5^{K173E}$::LacZ (lanes 2–9) was assayed by affinity chromatography on S-protein–agarose (as detailed in Materials and methods). Proteins adsorbed to S-protein–agarose were washed and eluted with sodium citrate (pH 2), separated by SDS–PAGE and visualized by immunoblotting. Numbers at the left indicate molecular masses (kDa) of size standard proteins.

and ClpX, and we present a model for the sequence of events in the initiation of σ^S degradation, which explains how the cell achieves tight regulation of σ^S proteolysis at the molecular level.

Results

Region 2.5 in σ^S is sufficient for RssB binding, but interaction with RssB is not sufficient to commit σ^S to proteolysis

Lys173 in σ^S, which is essential for σ^S proteolysis *in vivo* and for RssB binding *in vitro* (Becker *et al*., 1999), is part of the surface-exposed α-helix that comprises region 2.5 (α2.5). In order to test whether α2.5 is sufficient for interaction with RssB, we constructed a gene fusion that directs the expression of a hybrid protein containing α2.5 (H170–L189 in σ^S) linked to the N-terminus of β-galactosidase (α2.5::LacZ). As a control, a similar construct with a K173E exchange was used ($\alpha2.5^{K173E}$::LacZ; glutamate is the amino acid present at the corresponding position of the vegetative σ factor σ^{70}). When these hybrid proteins were overproduced from a *tac* promoter plasmid in an otherwise wild-type laboratory strain (MC4100), we observed that α2.5::LacZ, but not $\alpha2.5^{K173E}$::LacZ or LacZ alone, resulted in increased σ^S levels (data not shown), suggesting that α2.5 may interfere with σ^S proteolysis, probably by sequestering RssB.

Interaction with RssB was therefore tested *in vitro* by affinity chromatography ('pull-down') experiments (Becker *et al*., 1999; Klauck *et al*., 2001). Purified RssB (carrying an S-TRX tag) as well as cellular extracts from cells overexpressing α2.5::LacZ or $\alpha2.5^{K173E}$::LacZ were used (Figure 1). α2.5::LacZ, but not $\alpha2.5^{K173E}$::LacZ, co-eluted specifically with phosphorylated RssB, indicating specific binding via K173 in α2.5. This result indicates that α2.5 is sufficient for specific interaction with RssB. The immunoblot data mentioned above indicate that this interaction also takes place *in vivo*.

From this result arose the question of whether binding to RssB is sufficient to trigger degradation. In pulse–chase labeling experiments followed by immunoprecipitation (Figure 2), we observed that α2.5::LacZ is stable *in vivo*. In contrast, the hybrid protein RpoS742::LacZ (which carries the N-terminal 247 amino acids which include α2.5

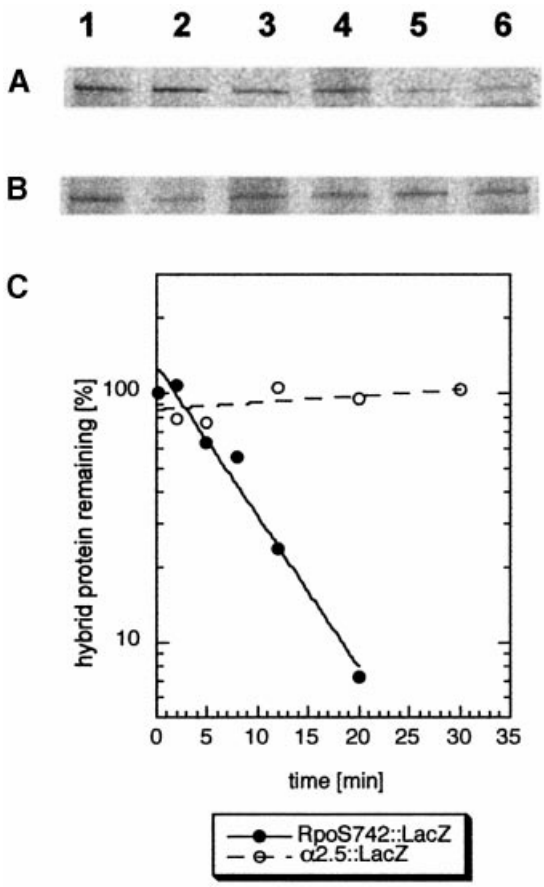

Fig. 2. Despite RssB binding, region α2.5 is not sufficient to trigger proteolysis. Cells expressing RpoS742::LacZ (**A**) or α2.5::LacZ (**B**) from pRH800 derivatives were grown in minimal medium (M9/0.1% glucose) to an OD_{578} of 0.6 and pulse-labeled with [^{35}S]methionine. The pulse time was 2 min; chase times varied between 2 and 30 min. For immunoprecipitation, a polyclonal serum against β-galactosidase was used. Quantification of the data shown in (A) and (B) is given in (**C**), with closed symbols for RpoS742::LacZ and open symbols for α2.5::LacZ.

of σ^S) is degraded with a similar half-life (3.8 min; Figure 2C) to σ^S itself (between 1.5 and 4 min, depending on the growth conditions; Lange and Hengge-Aronis, 1994; Becker *et al*., 1999; Pruteanu and Hengge-Aronis, 2002).

We conclude that region 2.5 in σ^S, i.e. the α2.5 helix, is sufficient for binding to the proteolytic recognition factor RssB. This interaction, however, is not sufficient to commit a protein to degradation. Thus, σ^S must contain at least one additional determinant that is essential for its proteolysis by the RssB/ClpXP system.

Analysis of $\sigma^{S(\Delta7–35)}$: identification of an N-terminal element essential for σ^S proteolysis

In the course of studies on IS element transposition (M.Noirclerc-Savoye and D.Schneider, in preparation), it was observed that the W3110-related *E.coli* K-12 strain 431 (Ghosal and Saedler, 1977; Raabe *et al*., 1988) exhibited relatively high levels of a σ^S protein of somewhat smaller size. Sequencing revealed a deletion of nucleotides 21–104 in the *rpoS* open reading frame (ORF), which results in an in-frame deletion of codons 7–35 [the resultant gene product was termed $\sigma^{S(\Delta7–35)}$].

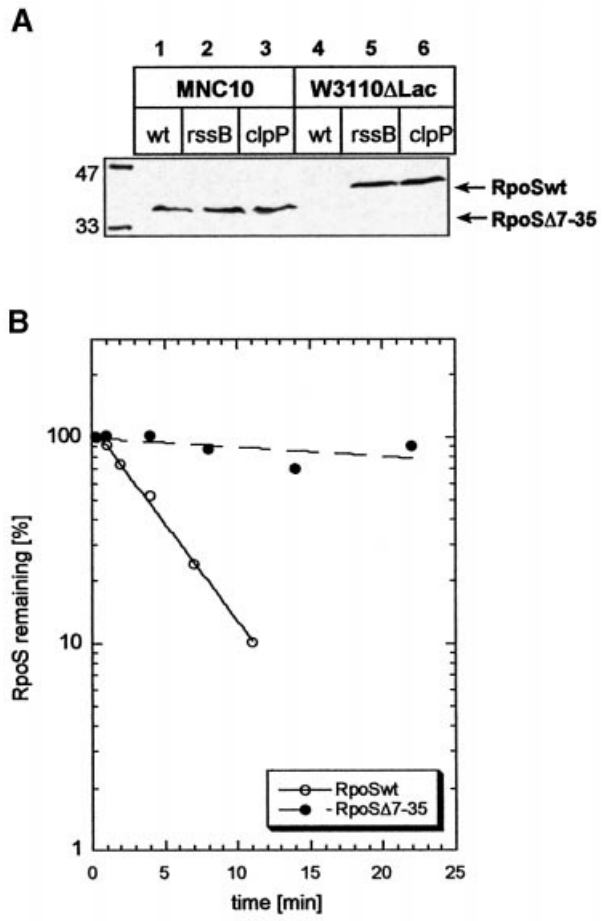

Fig. 3. $\sigma^{S(\Delta 7-35)}$ is present at increased cellular levels that are not altered by mutations affecting RssB or ClpP. (**A**) Cells of strain MNC10 (lanes 1–3), which carries the *rpoS*$^{(\Delta 7-35)}$ mutation, or W3110Δlac (lanes 4–6), which expresses wild-type σ^S, and *rssB* or *clpP* mutations as indicated were grown in LB. During mid-log phase (at an OD_{578} between 0.7 and 0.9), σ^S levels were assayed by immunoblot analysis. Numbers at the left indicate molecular masses (kDa) of size standard proteins. (**B**) Using the same conditions as those described in the legend to Figure 2, degradation of σ^S and $\sigma^{S(\Delta 7-35)}$ was assayed using strains W3110Δlac (open circles) and MNC10 (closed circles), respectively.

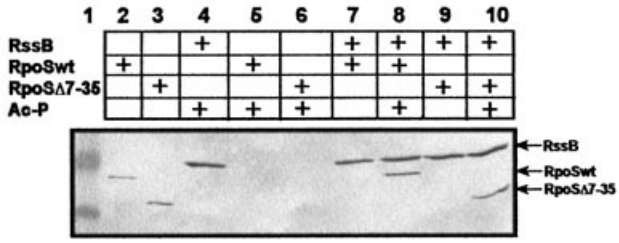

Fig. 4. $\sigma^{S(\Delta 7-35)}$ binds to phosphorylated RssB *in vitro*. Equimolar amounts (0.3 nmol) of S-TRX-His_6-RssB and His_6-σ^S or His_6-$\sigma^{S(\Delta 7-35)}$ were incubated at room temperature with or without 50 mM acetyl phosphate (Ac-P), and subject to affinity chromatography on S-protein agarose, separated by SDS–PAGE and visualized using Penta-His antibodies. Size standard proteins (49 and 32.5 kDa) are shown in lane 1. In lanes 2 and 3, His_6-σ^S and His_6-$\sigma^{S(\Delta 7-35)}$, respectively, were directly loaded on the gel.

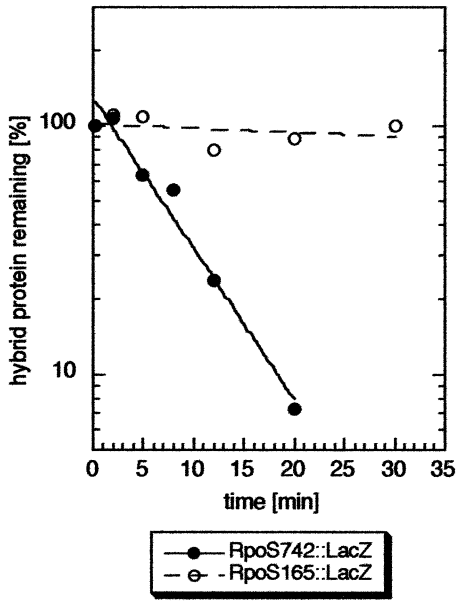

Fig. 5. RpoS165::LacZ is not degraded *in vivo*. Cells expressing RpoS742::LacZ (closed symbols) or RpoS165::LacZ (open symbols) from pRH800 derivatives were grown in M9/0.1% glucose to an OD_{578} of 0.6 and pulse-labeled with [^{35}S]methionine. Further treatment was as described in the legend to Figure 2.

High log phase levels of $\sigma^{S(\Delta 7-35)}$ were not increased further in *rssB* or *clpP* mutant backgrounds (Figure 3A). Moreover, pulse–chase labeling (Figure 3B) demonstrated that $\sigma^{S(\Delta 7-35)}$ has a largely increased half-life (~75 min), which is in pronounced contrast to the rapid proteolysis of wild-type σ^S, which in W3110 is very similar to the half-life in MC4100 (~3 min). These data suggested that there is a determinant close to the N-terminus of σ^S that is also important for σ^S degradation.

Complementation experiments indicated that $\sigma^{S(\Delta 7-35)}$ is as active in the initiation of transcription as wild-type σ^S. With the corresponding *rpoS* alleles subcloned onto pBAD18, basal expression levels of σ^S and $\sigma^{S(\Delta 7-35)}$ (which in the absence of inducer are comparable with expression levels observed with a chromosomal *rpoS* gene in a wild-type strain) not only complemented defective glycogen and catalase production in a *rpoS*::Tn*10* background, but resulted in expression levels of the σ^S-dependent genes *osmY* and *csiD*, which where proportionate to σ^S and $\sigma^{S(\Delta 7-35)}$ protein levels (data not shown). Moreover, RssB levels as determined by immunoblot analysis were similar in strains expressing either σ^S or $\sigma^{S(\Delta 7-35)}$ (data not shown). This indicates that the two variants of σ^S have similar specific activity in transcription initiation. This also rules out an indirect effect of the Δ7–35 mutation on σ^S proteolysis due to potentially reduced levels of RssB, whose expression is itself σ^S dependent (Ruiz *et al*., 2001; Pruteanu and Hengge-Aronis, 2002) and limiting for the cellular rate of σ^S degradation (Pruteanu and Hengge-Aronis, 2002).

In 'pull-down' experiments with purified $\sigma^{S(\Delta 7-35)}$ (Figure 4), we found that interaction with RssB was not affected by the Δ7–35 deletion in σ^S. This demonstrates that the N-terminal region affected does not contribute to RssB binding, and represents a second line of evidence indicating that RssB binding alone is not sufficient for σ^S proteolysis. We conclude that σ^S contains a second

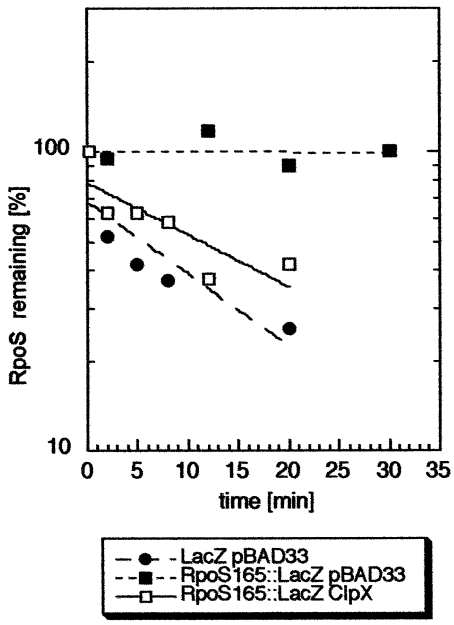

Fig. 6. RpoS165::LacZ interferes with σ^S proteolysis, and this interference can be suppressed by ClpX overexpression. MC4100Δ*(ara-leu)* derivatives were used that carried combinations of pRH800 and pBAD33 derivatives. From the pRH800 series, either wild-type β-galactosidase (LacZ) or RpoS165::LacZ were expressed (using IPTG as an inducer); from pBAD33, ClpX was overproduced (with arabinose as an inducer). Cells were grown in M9/0.4% glycerol. After induction with 0.2 mM IPTG and 0.001% arabinose (for one generation), cells were harvested at an OD_{578} of 0.6 and pulse-labeled with [^{35}S]methionine for 1 min. Immunoprecipitation of σ^S and further treatment were as described in the legend to Figure 2.

element involved in proteolysis, which is located close to the N-terminus and plays a role that is distinct from σ^S interaction with RssB.

The N-terminal region of σ^S contains an element that interacts with ClpX

In order to study the role in proteolysis of the N-terminal region of σ^S in more detail, a fusion protein was constructed that contains the N-terminal 55 amino acids of σ^S linked to β-galactosidase (RpoS165::LacZ). This length of the σ^S moiety was chosen because secondary structure prediction as well as the recently published RNA polymerase holoenzyme structures (Murakami *et al.*, 2002; Vassylyev *et al.*, 2002) indicated that this part of σ^S is unstructured, whereas a regular pattern of α-helices begins downstream of L55.

As expected, and unlike α2.5::LacZ, RpoS165::LacZ did not interact with RssB as tested by 'pull-down' affinity chromatography (data not shown). RpoS165::LacZ is also not degraded *in vivo* (Figure 5). However, just like α2.5::LacZ, overproduction of RpoS165::LacZ resulted in increased cellular σ^S levels (data not shown), suggesting that the N-terminal 55 amino acids of σ^S may also inhibit σ^S degradation *in trans*. Therefore, interference with σ^S degradation was directly monitored in pulse–chase experiments (Figure 6). In contrast to overproduction of wild-type β-galactosidase (LacZ), overproduction of RpoS165::LacZ completely inhibited the turnover of σ^S over the time tested. This finding suggested that RpoS165::LacZ binds and sequesters some factor essential

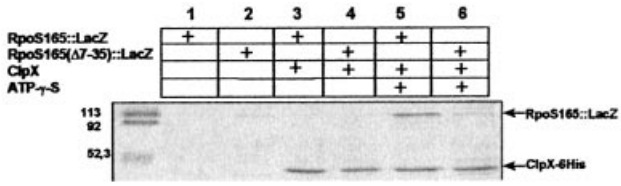

Fig. 7. RpoS165::LacZ binds to ClpX *in vitro*. Equimolar amounts of crude cell extracts of strains overexpressing either RpoS165::LacZ or RpoS165$^{\Delta 7-35}$::LacZ were incubated with 0.3 nmol ClpX in the presence or absence of ATP-γ-S and subject to co-immunoprecipitation, SDS–PAGE and protein staining as described in Materials and methods.

for σ^S proteolysis. Such sequestering should be suppressed by overproduction of this factor. As the interacting factor is not RssB (see above), an obvious candidate was ClpX. We therefore introduced a second plasmid, from which ClpX could be overproduced, into the strains used for the interference experiment. ClpX overproduction indeed suppressed inhibition of σ^S proteolysis by RpoS165::LacZ (Figure 6). The vector alone had no effect (Figure 6), and also ClpX overproduction did not affect σ^S degradation in a control strain that overexpressed wild-type LacZ instead of the hybrid protein (data not shown). These *in vivo* suppression data provide genetic evidence that a region within the N-terminal 55 amino acids of σ^S can interact with ClpX.

Interaction between RpoS165::LacZ and ClpX could also be demonstrated *in vitro* by co-immunoprecipitation experiments using purified ClpX and cellular extracts as a source of the hybrid proteins (Figure 7; co-immunoprecipitation was used, because in pull-down experiments as described above, the ClpX chaperone alone tended to exhibit too much unspecific interaction). Complexes were isolated using an antibody against His-tagged ClpX (this His tag does not interfere with ClpX function; for details, see Materials and methods). The interaction between RpoS165::LacZ and ClpX is specific for the σ^S-derived part of the hybrid protein, since it is lost as a consequence of the Δ7–35 deletion in RpoS165::LacZ. Moreover, interaction takes place only in the presence of ATP-γ-S, i.e. requires the formation of the native hexameric ClpX ring structure. From these *in vivo* and *in vitro* data, we conclude: (i) that the N-terminal 55 amino acids of σ^S contain a determinant that is recognized specifically by the hexameric ClpX chaperone ring; and (ii) that this recognition is not sufficient to commit at least the RpoS165::LacZ hybrid protein to proteolysis.

Sequence of events in the initiation of σ^S proteolysis and the role of RssB

The finding that RpoS165::LacZ is bound by ClpX *in vivo* (Figure 6) and *in vitro* (Figure 7), but is not degraded *in vivo* (Figure 5), is surprising. It means that there is some requirement beyond ClpX binding for the initiation of degradation, which is fulfilled by the larger hybrid protein RpoS742::LacZ, but not by RpoS165::LacZ. Either a third proteolytic determinant is required, at least part of which should be located between D56 and K173 in σ^S, or, RssB, which binds to RpoS742::LacZ, but not to RpoS165::LacZ, could play an active role in the initiation of proteolysis that goes beyond transfer of σ^S to ClpX.

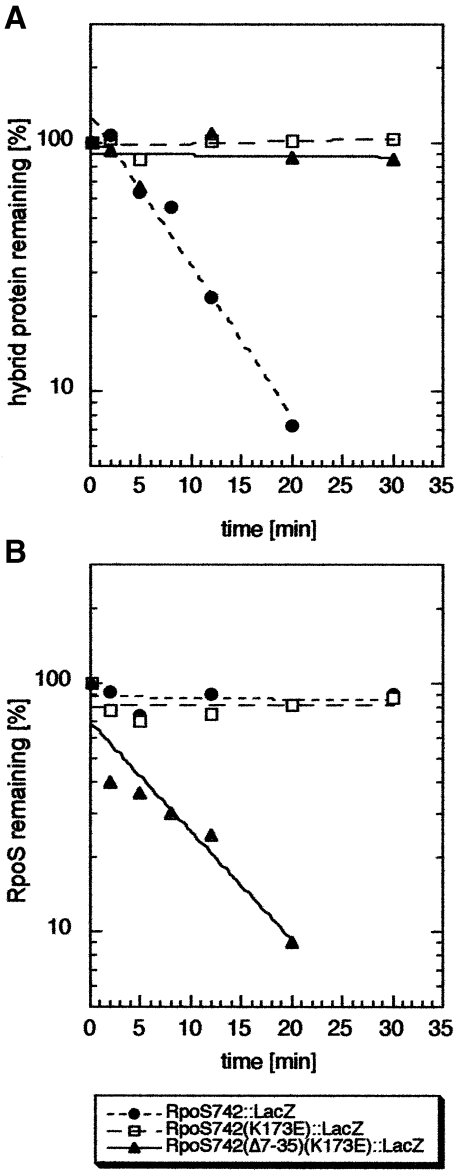

Fig. 8. RpoS742^{K173E}::LacZ, which is defective for RssB binding, still interacts with ClpX but is not degraded *in vivo*. MC4100 derivatives expressing RpoS742::LacZ, RpoS742^{K173E}::LacZ or RpoS742$^{\Delta 7\text{-}35+K173E}$::LacZ from pRH800 derivatives were grown in M9/0.1% glucose. For direct measurement of hybrid protein degradation (**A**), pulse-labeling was performed at an OD_{578} of 0.6, and a polyclonal antibody against β-galactosidase was used for immunoprecipitation. In order to test for interference with σ^S proteolysis by hybrid protein overproduction (**B**), 0.2 mM IPTG was added at an OD_{578} of 0.3, and cells were harvested, pulse-labeled and further treated as in (A), with the exception that the polyclonal antibody against σ^S was used for immunoprecipitation.

In order to distinguish between these two possibilities, we compared the long hybrid proteins RpoS742::LacZ, RpoS742^{K173E}::LacZ and RpoS742$^{(\Delta 7-35+K173E)}$::LacZ. All contain the σ^S region, in which a putative third proteolytic determinant should be located. Only RpoS742::LacZ, however, exhibited degradation *in vivo* (Figure 8A). Then, interference with σ^S proteolysis was assayed by pulse–chase experiments (Figure 8B). As expected, overproduction of RpoS742::LacZ, which contains binding sites for RssB as well as for ClpX, inhibits σ^S degradation

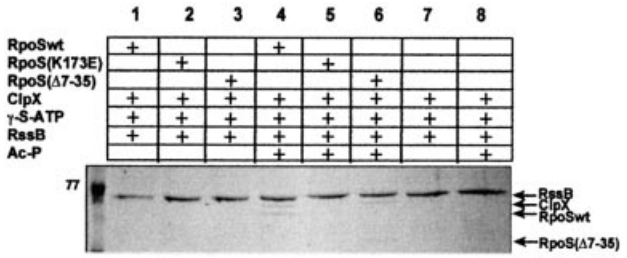

Fig. 9. Ternary complex formation between RssB, different variants of σ^S and ClpX. Interaction of these proteins was assayed *in vitro* by affinity chromatography as described in the legend to Figure 1 and Materials and methods (with the exception that ClpX-His_6 was also included). 77, molecular mass (in kDa) of a size standard protein.

completely. However, RpoS742^{K173E}::LacZ also interferes with σ^S turnover (Figure 8B). The latter interference is due to interaction with ClpX since it was not observed with RpoS742$^{(\Delta 7-35+K173E)}$::LacZ (Figure 8B). This means that even RpoS742^{K173E}::LacZ, which cannot bind to RssB, can interact with ClpX. However, this ClpX interaction remains as unproductive for proteolysis as the ClpX interaction of the shorter RpoS165::LacZ hybrid protein (see above), indicating that the missing component in the latter is not a third proteolytic determinant somewhere between D56 and K173. These data suggest that with these hybrid proteins, RssB is required for proteolysis, but its role is not to make the ClpX-binding site accessible. Rather, it may play a second active, not yet characterized role, e.g. in the initiation of denaturation or entry of σ^S and/or the σ^S-derived reporter proteins into the proteolytic cavity.

Earlier gel filtration data (Zhou *et al*., 2001) had indicated that, in contrast to the behavior observed here for RpoS::LacZ hybrid proteins, wild-type σ^S is not bound by ClpX. We could confirm this also in the experimental system used in the present study (co-immunoprecipitation, data not shown). Thus, the ClpX-binding site close to the N-terminus appears to be cryptic in full-size σ^S, but accessible in the various RpoS::LacZ hybrid proteins. Additional pull-down experiments, in which ternary complex formation between RssB, several variants of σ^S and ClpX was assayed, confirmed this conclusion (Figure 9) and even provided a mechanistic explanation. With wild-type σ^S, phosphorylated RssB (i.e. acetyl phosphate has to be present) and hexameric $ClpX_6$ (due to the presence of ATP-γ-S), ternary complex formation can be observed (Figure 9, lane 4); in the absence of any σ^S variant, ClpX does not interact with RssB (whether or not it is phosphorylated; Figure 9, lanes 7 and 8); this is also confirmed by the finding that in the presence of $\sigma^{S(K173E)}$, which cannot bind to RssB, ClpX is also not found in a complex with RssB (Figure 9, lane 5). Together with the observation (mentioned above) that native σ^S and ClpX do not interact, this means that binding to RssB is a prerequisite for interaction of σ^S with ClpX. This indicates that interaction with RssB triggers a change of conformation in σ^S that exposes the binding site for ClpX, which in the absence of RssB would be cryptic. The finding that the RpoS742$^{(K173E)}$::LacZ hybrid protein (lacking the C-terminal 25% of the σ^S sequence), which cannot bind to RssB, can interact with ClpX via its N-terminal site (Figure 8B) suggests that occlusion of the N-terminal

binding site for ClpX is due to interaction with a region in the C-terminal part of σ^S.

Finally, we also observed ternary complex formation for phosphorylated RssB, ClpX and $\sigma^{S(\Delta 7-35)}$ (Figure 9, lane 6). This indicates that at least in $\sigma^{S(\Delta 7-35)}$, additional ClpX-binding sites become accessible upon interaction with RssB. These sites could be just hydrophobic patches that become surface exposed upon the RssB-triggered change of conformation of $\sigma^{S(\Delta 7-35)}$. Alternatively, this change of conformation could expose a secondary specific ClpX interaction site, which may serve as a backup for recognition. The experiments with single amino acid mutations in the N-terminal site (see below) suggest such a secondary site in the C-terminal region of σ^S.

Identification of relevant amino acids in the N-terminal element for ClpX recognition

While this manuscript was under revision, K7 in σ^S was suggested to play a role in recognition by ClpX, because a lysine occurs in a comparable position close to the N-terminus in several other ClpXP substrates (e.g. Dps) and a K5D replacement in a Dps^{2-12}::Arc fusion protein eliminated its proteolysis (Flynn *et al*., 2003). In parallel, we had changed this K7 and the adjacent amino acids V8 and H9 as well as K28 to alanine (K28 provides the only positive charge in a long stretch of amino acids in σ^S, which in the closely related σ^{70} only contains negative charges). The effects of these point mutations on proteolysis were studied in otherwise wild-type σ^S as well as in the RpoS742::LacZ hybrid protein (Figure 10). The H9A exchange strongly affected proteolysis of both proteins. In contrast, and unlike predicted by Flynn *et al*. (2003), replacing K7 produced almost no effect. Interestingly, the defects observed for each mutation were more pronounced in the RpoS742::LacZ hybrid proteins (Figure 10A) than in the full-size σ^S proteins (Figure 10B). This indicates that the C-terminal region in σ^S may provide for residual recognition by ClpX, if the N-terminal binding site fails to operate adequately.

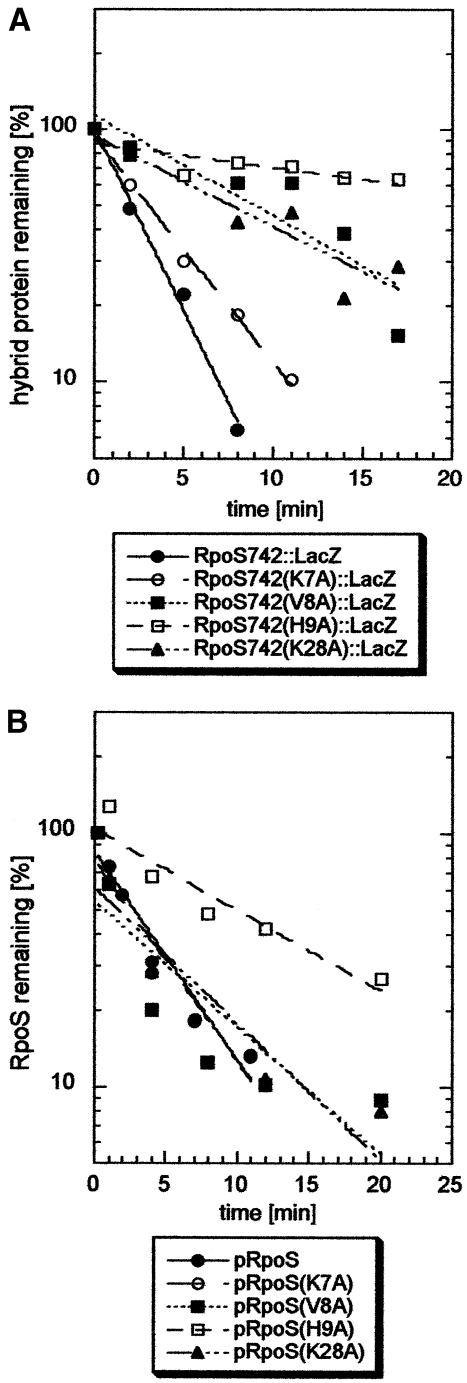

Fig. 10. Single amino acid exchanges in the N-terminal region affect proteolysis of the RpoS742::LacZ hybrid protein and of σ^S. In (**A**), derivatives of strain MC4100 expressing (from pRH800) the RpoS742::LacZ fusion protein (closed circles) or derivatives thereof with the K7A (open circles), V8A (closed squares), H9A (open squares) or K28A mutations (triangles) were grown in minimal medium and treated with tetracycline as described in Materials and methods. In (**B**), derivatives of the *rpoS* mutant RH90 expressing σ^S from pRH800 or derivatives thereof carrying the same mutations as above (with the same designations in the figure) were assayed in a similar way (with the exception that chloramphicol was used for terminating protein biosynthesis).

Discussion

σ^S has distinct binding sites for the recognition factor RssB and the chaperone ClpX

A single amino acid in σ^S (K173) has been known to be essential for σ^S degradation *in vivo* and for binding of the proteolytic recognition factor RssB *in vitro*, indicating that K173 is an essential part of the RssB-binding site in σ^S (Becker *et al*., 1999). Here, we demonstrate that the RssB-binding site is a single α-helix, i.e. region 2.5 of σ^S (α2.5). When N-terminally attached to β-galactosidase, i.e. a protein which in its wild-type form is not bound by RssB nor degraded *in vivo*, α2.5 allows binding to RssB *in vivo* (resulting in RssB sequestration and therefore inhibition of σ^S degradation *in trans*) and *in vitro* (Figure 1). Besides this binding site for RssB, σ^S has a second site that is essential for degradation. This determinant is located close to the N-terminus of σ^S (within the first 55 amino acids of σ^S), and is not involved in RssB binding, but is a site of interaction with hexameric $ClpX_6$ rings (Figures 3, 5, 7 and 8). Amino acids in this N-terminal recognition site for ClpX, which are important for proteolysis, are above all His9 and, to some minor extent, also Val8 and Lys28 (Figure 10). At first glance, our finding that $\sigma^{S(\Delta 7-35)}$ is defective for proteolysis seems at variance with a recent report (Rajkumari and Gowrishankar, 2002) that claimed that a σ^S variant with the first 50 amino acids deleted (and

replaced by a different sequence) exhibited normal proteolysis control. Unfortunately, this study did not measure proteolysis directly, e.g. by pulse–chase experiments, and used plasmid constructs throughout in such a way that known alterations of plasmid copy numbers in response to starvation and other stress conditions may equally explain apparently 'normal' regulation of σ^S levels.

The location of the ClpX-binding site close to the N-terminus of σ^S is consistent with other natural ClpXP substrates, which possess degradation determinants close to either the N- or C-terminus (Keiler *et al*., 1996; Gonciarz-Swatek *et al*., 1999; Gonzalez *et al*., 2000; Hoskins *et al*., 2000a,b; Flynn *et al*., 2001, 2003). A few other ClpXP substrates also have two separate determinants that contribute to degradation (Gonciarz-Swatek *et al*., 1999; Ryan *et al*., 2002). To our knowledge, however, σ^S is the only known ClpXP substrate where two distinct recognition sites are each essential for proteolysis and have been shown to interact with two separate but cooperating proteolysis-promoting factors. With CtrA, a global cell cycle regulator in *Caulobacter*, the situation may be similar, but a specific targeting factor besides ClpX has yet to be identified (Ryan *et al*., 2002).

Finally, our observation that point mutations in the N-terminal ClpX-binding site all have a more pronounced effect on proteolysis of RpoS::LacZ hybrid proteins (which lack the wild-type C-terminus) than of full-size σ^S suggests that the C-terminal part of σ^S may also contribute to recognition by ClpX. However, such a C-terminal site seems to be a minor 'back-up' site only that is not able to trigger ClpXP-mediated proteolysis on its own, since a deletion of the N-terminal ClpX recognition site (Δ7–35) strongly increases σ^S half-life (to ~75 min; Figure 3).

Neither binding of RssB alone nor of ClpX alone is sufficient to commit σ^S or a σ^S-derived reporter protein to degradation

We have shown here that α2.5 alone allows specific binding to RssB but that this interaction is not sufficient to trigger proteolysis of a reporter protein, i.e. β-galactosidase (Figure 2). This finding as well as the corresponding observation that $\sigma^{S(\Delta 7-35)}$ can interact with RssB but is not degraded (Figures 3B and 5) indicates that the presence of a ClpX-binding site, which we have localized close to the N-terminus of σ^S, is a prerequisite for RssB/ClpXP-dependent degradation. Thus, RssB is not a factor that extends the substrate spectrum for ClpXP by providing substrate binding to a protein, which by itself would not bind to ClpX. Interaction with ClpX of a specific site in the substrate protein remains a necessity, most probably for the initiation of substrate unfolding and threading of the unfolded substrate into the proteolytic cavity formed by the ClpP complex.

There is an interesting difference between the various RpoS::LacZ hybrid proteins and native full-size σ^S with respect to accessibility of the ClpX-binding site. In the fusion proteins, this binding site is exposed, which allows interaction with ClpX without RssB binding (Figures 7, 8 and 9). Wild-type σ^S, however, does not interact with ClpX alone. Only in the presence of phosphorylated RssB is a complex formed (Figure 9). Together with the observation that RssB and ClpX do not form a binary complex (Figure 9), this means that (i) σ^S is the 'bridging' component in the RssB–σ^S–ClpX complex; and (ii) the N-terminal ClpX-binding site is cryptic in native σ^S, probably due to shielding by sequences or a domain not present even in the longest fusion protein (RpoS742::LacZ, which contains the N-terminal 75% of the σ^S sequence), i.e. domain 4 of σ^S. In terms of molecular structure, this can easily be envisaged, since a σ^{70}-like σ factor consists of an unstructured N-terminal region followed by three domains that are connected to each other by flexible linkers (Campbell *et al*., 2002; Murakami *et al*., 2002; Vassylyev *et al*., 2002). It also indicates that such an intramolecular interaction cannot only prevent a σ factor from binding to DNA in the absence of RNAP core (by occluding regions 2.4/2.5 and 4.2) (Dombroski *et al*., 1993), but can also prevent it from being constitutively degraded (by occluding a recognition site in region 1). This intramolecular interaction makes RssB intervention a necessity for σ^S proteolysis, which establishes complex control over σ^S turnover due to regulation of the cellular level and phosphorylation state of RssB (Pruteanu and Hengge-Aronis, 2002). Sequestration of the RpoS742::LacZ hybrid protein by the ClpX complex may also explain why even strong overproduction of this fusion protein, which contains complete domains 2 and 3 of σ^S and therefore in principle should be able to interact with RNAP core, is not toxic for the cells.

The experiments with RpoS::LacZ hybrid proteins of various lengths also revealed that interaction with ClpX is not necessarily sufficient to commit these proteins to degradation, i.e. does not result in 'automatic' unfolding and threading into the ClpP complex of all substrates (although it seems sufficient for some substrates). σ^S and σ^S-derived hybrid proteins may have to overcome yet another hurdle before they definitely become degraded. What is this hypothetical hurdle mechanistically? RpoS742::LacZ is degraded by RssB/ClpXP, whereas $RpoS742^{K173E}$::LacZ, in which the RssB-binding site is mutated, is stable. Both hybrid proteins, however, bind to ClpX. Thus, successful degradation after ClpX binding correlates with the ability to interact with RssB, suggesting that RssB plays a second role that goes beyond initial interaction with σ^S and its transfer to ClpX.

It seems likely that RssB is a σ^S-specific proteolysis promoting factor (Zhou and Gottesman, 1998). This suggests that σ^S possesses some specific proteolysis-inhibiting feature not present in other ClpXP substrates that would have to be overcome by a second activity of RssB. In principle, this second action of RssB could affect σ^S, ClpX or ClpP. An interesting point to take into account may be that chaperones that cooperate with a processive protease obviously have to switch between two modes of substrate binding, i.e. (i) initial binding to a specific site of the substrate; and (ii) sequence-unspecific binding that allows sliding of an elongated substrate relative to the chaperone/enzyme. The switch between the first and second mode of binding should involve a specific binding site clearance event, which bears conceptual similarity to promoter clearance by RNA polymerase. If initial specific binding is relatively strong, clearance may become rare and perhaps even energy dependent. The putative RssB

dephosphorylation, i.e. hydrolysis of a high energy acyl phosphate bond, may not only result in RssB release from the RssB–σ^S–ClpXP complex, but may also be instrumental in this transition to the 'elongation mode' of the ClpXP machine with σ^S as a substrate. Future experiments with σ^S variants with different affinities for ClpX will have to test this hypothesis.

Conclusions: roles of RssB and ClpX and sequence of events in the initiation of σ^S proteolysis

In summary, we would like to propose a model that includes the following sequence of events in the initiation of σ^S degradation: (i) initially, σ^S is bound by RssB-P, which results in a change of conformation of σ^S that exposes the ClpX-binding site close to the N-terminus of σ^S; (ii) this leads to the formation of the RssB–σ^S–$ClpX_6$ complex, which is joined by $ClpP_{14}$; (iii) the initiation of σ^S degradation then requires a second activity of RssB (perhaps associated with RssB dephosphorylation), that may be essential for the initiation of σ^S denaturation and/or for disruption of the initial specific σ^S–ClpX interaction, i.e. the initiation of threading of σ^S into the ClpP proteolytic cavity. In principle, the interaction with the ClpP complex may turn the hexameric ClpX ring into a processive enzyme, but in the case of strongly bound σ^S there may be a need for assistance by RssB. The third step, which again requires RssB, may serve as a proofreading mechanism in the regulation of the initiation of σ^S proteolysis.

In conclusion, no other known prokaryotic proteolysis substrate is subject to such a complex multistep recognition process as described here for σ^S. In contrast to σ^S, however, other proteolysis model substrates are constitutively degraded. By rendering even more than one of these recognition steps dependent on the modification state of a specific recognition factor, the cells achieve precise regulation of σ^S recognition and therefore σ^S proteolysis by multiple signals.

Materials and methods

Bacterial strains and growth conditions

With the exception of strain MNC10 (identical to strain 431, which is a derivative of W3110) (Ghosal and Saedler, 1977; Raabe *et al*., 1988), all bacterial strains used in this study are derivatives of MC4100 (Silhavy *et al*, 1984). RH90 is a *rpoS359*::Tn*10* derivative of MC4100 (Lange and Hengge-Aronis, 1991). The strains carrying chromosomal *osmY::lacZ* and *csiD::lacZ* fusions have been described elsewhere (Becker and Hengge-Aronis, 2001; Germer *et al*., 2001). Cultures were grown at 37°C under aeration in Luria–Bertani (LB) medium or minimal medium M9 (Miller, 1972) supplemented with glucose or glycerol as a carbon source. Antibiotics were added as recommended (Miller, 1972). Growth was monitored by measuring the optical density at 578 nm (OD_{578}).

Cloning of rpoS::lacZ fusions

The multiple cloning site (MCS) of pJL28 (Lucht *et al*., 1994) (with a point mutation to eliminate the *Hin*dIII restriction site in the MCS) and the following promoterless *lacZ* gene (starting with the eighth codon of wild-type *lacZ*) was cloned into the isopropyl-β-D-thiogalactopyranoside (IPTG)-inducible vector pRH800 (Lange and Hengge-Aronis, 1994) (an *Eco*RI–*Hin*dIII-digested PCR fragment obtained with pJL28 as a template and the primers 5′-GATCGAATTCCCGGGGATCCGTCGACCTGCAGCCTAGCTTGCGATCCC-3′ and 5′-GATCAAGCTTATTATTATTTTTGACACCAGACCAACTGG-3′ were used, with underlined nucleotides indicating the *Eco*RI and *Hin*dIII restriction site, respectively; the second primer introduced two additional stop codons). This pRH800 derivate (pAS1) and PCR-amplified *rpoS* fragments of different lengths were digested with *Eco*RI and *Bam*HI and ligated, which resulted in the following plasmids: pα2.5::LacZ (containing codons 170–190 of the *rpoS* coding sequence immediately downstream of the initiator methionine and serine codons as present in wild-type *rpoS*); $p\alpha2.5^{K173E}$::LacZ (identical to pα2.5::LacZ except for a point mutation that leads to K173E); pRpoS165::LacZ (containing codons 1–55 of the *rpoS* coding sequence fused in-frame to the eighth codon of *lacZ*), $pRpoS165^{\Delta7-35}$::LacZ (identical to pRpoS165::lacZ except for the presence of the Δ7–35 deletion in the *rpoS* part) and pRpoS742::LacZ (containing codons 1–247 of the *rpoS* coding sequence fused in-frame to the eighth codon of *lacZ*). For PCRs, the following primer pairs were used (with *Eco*RI and *Bam*HI restriction sites, respectively, underlined in the primer sequence, and mutational exchanges in bold): pα2.5::LacZ, 5′-GAGAATTCTGGAGCCACCTTATGAGCCACATCGTAAAGGAGCTGAACGTTTAC-3′ and 5′-GATCGGATCCTCCAGCTTATGGGACAACTC-3′; $p\alpha2.5\Delta^{7-35}$::LacZ, 5′-GAGAATTCTGGAGCCACCTTATGAGCCACATCGTA**G**AGGAGCTGAACGTTTAC-3′ and the same downstream primer as for pα2.5::LacZ; 5′-GAGAATTCTGGAGCCACCTTATGAGTCAGAATACGC-3′ as the upstream primer combined with 5′-GAGATCGGATCCTCCAACACACCTGTGTGGCTC-3′ (pRpoS165::LacZ and $pRpoS165^{\Delta7-35}$::lacZ) or 5′-GAGATCGGATCCTCTTCCGGACCGTTCTC-3′ (pRpoS742::LacZ).

Site-directed mutagenesis in the 5′-terminal region of rpoS

Mutations in the N-terminal region of σ^S were introduced by a four-primer two-step PCR procedure described previosly (Becker and Hengge-Aronis, 2001). As 'internal' primers, 5′-GAATACGCTG**GC**AGTTCATGATTTAAATG-3′, 5′-GAACT**GC**CAGCGTATTCTGAC-3′ (K7**A**); 5′-ACGCTGAAAG**CA**CATGATTTAAATG-3′, 5′-CATTTAAATCATG**TG**CTTTCAGC-3′(V8**A**); 5′-CTGAAAGTT**GCA**GATTTAAATGAAGATGC-3′, 5′-CATTTAAATC**TGC**AACTTTCAGC-3′ (H9**A**); and 5′-TGACGAA**GCA**GCCTTAGTAG-3′, 5′-CTAAGGC**TGC**TTCGTCAAAAACC-3′ (K28**A**) were used. As external primers, 5′-GCGCCGACATCATAACGGTTC-3′ and 5′-CGCAACTCTCTACTGTTTCTCCATACCC-3′ served as upstream primers for *lacZ* fusions and *rpoS* mutants, respectively, and 5′-GCAAAATAAACTTCTTCTTCGG-3′ as a downstream primer. The *Eco*RI–*Eag*I-digested final PCR fragments were cloned into pRpoS742::LacZ or pRpoS18 (Becker *et al*., 1999), respectively, treated with the same restriction enzymes. For better expression levels, all pRpoS18-mutants were subcloned later into pRH800 (using *Eco*RI and *Hin*dIII).

Cloning of the clpX gene

For purification of ClpX protein, the *clpX* coding region was cloned into the pBAD33 vector (Guzman *et al*., 1995). A *Kpn*I–*Hin*dIII-digested PCR fragment obtained with MC4100 chromosomal DNA as a template and the primers 5′-GAGATCGGTACCAGGAGGAATTCACCATGACAGATAAACGCAAAGATGGCC-3′ and 5′-ACTAAAGCTTTCATTAATGATGATGATGATGGTGTTCACCAGATGCCTGTTGCGCTTCC-3′ were used (with underlined nucleotides indicating additions to or deviations from the wild-type sequence introduced in order to create *Kpn*I and *Hin*dIII restriction sites, respectively, a ribosomal-binding site in the first as well as a His_6 tag and an additional stop codon in the second primer). The gene product of the plasmid complemented a chromosomal *clpX1::kan* insertion (cellular levels of σ^S were assayed by immunoblot analysis).

Preparation of crude cell extracts

Overnight cultures of MC4100 derivatives carrying plasmids encoding various RpoS::LacZ hybrid proteins (see above) were grown overnight in LB with 100 μg/ml ampicillin, diluted 100-fold and grown at 30°C to an OD_{578} of 0.5–0.6. IPTG (1 mM) was then added. After 5 h, cells were harvested, resuspended in French press buffer (50 mM Tris–HCl pH 7.5, 50 mM KCl, 5 mM $MgCl_2$, 0.1 mM EDTA) and disrupted in a French pressure cell. The lysate was cleared by centrifugation (30 min at 16 000 r.p.m.). Hybrid protein concentrations were determined by using the SDS–gel electrophoresis calibration kit (Amersham).

Protein purification

RssB (carrying a S-TRX-His_6 tag) and His_6-σ^S were purified as previously described (Becker *et al*., 1999). For the purification of ClpX, the strain RH166 [MC4100 Δ*(ara-leu)7697*] carrying pClpX (see above) was grown overnight in LB medium with 30 μg/ml chloramphenicol, diluted 100-fold and grown again at 37°C to an OD_{578} of 0.5–0.6. Arabinose (0.005%) was then added. After 5 h, cells were harvested. All the following purification steps were carried out at 4°C. Cells were resuspended in French press buffer (50 mM NaH_2PO_4,

300 mM NaCl, 5 mM $MgCl_2$, 10 mM imidazole pH 8.0) and disrupted in a French pressure cell. The lysate was cleared by centrifugation (30 min at 16 000 r.p.m.) and loaded onto an Ni-NTA–agarose column (Qiagen) previously equilibrated in French press buffer. The column was washed with wash buffer (50 mM NaH_2PO_4, 300 mM NaCl, 5 mM $MgCl_2$, 20 mM imidazole pH 8.0) until the flow-through exhibited an absorption at 280 nm (A_{280}) of <0.02. The ClpX-His_6 protein was eluted with wash buffer containing 250 mM imidazole. The protein solution was dialyzed overnight against storage buffer [50 mM Tris pH 7.5, 250 mM NaCl, 10 mM $MgCl_2$, 50% glycerol, 0.1 mM EDTA, 1 mM dithiothreitol (DTT)] (Nguyen and Burgess, 1996).

SDS–PAGE and immunoblot analysis

Sample preparation for SDS–PAGE and immunoblot analysis were performed as described (Lange and Hengge-Aronis, 1994). A 12–15 µg aliquot 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.

Assays for protein degradation in vivo

Pulse labeling of cells with L-[^{35}S]methionine and immunoprecipitation of σ^S or RpoS::LacZ hybrid proteins was described previously (Lange and Hengge-Aronis, 1994). Cells were grown in either M9/0.1% glucose (for pAS1 derivates) or M9/0.4% glycerol (for pBAD33 derivates). For assaying interference with σ^S degradation, exponentially growing cells were induced with 0.2 mM IPTG (pAS1 derivates) or 0.001% arabinose (pBAD33 derivates) at an OD_{578} of 0.3. Cells were harvested at an OD_{578} of 0.6. For the determination of half-lives of σ^S and RpoS::LacZ hybrid proteins, the pulse time was 1 and 2 min, respectively. 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 immunoprecipitation, polyclonal sera against σ^S and β-galactosidase were used. Protein bands were quantified on a FLA2000G phospho/fluoroimager (Fuji Photo Film Co., Japan). The intensity of the bands representing σ^S and the hybrid proteins was calculated relative to the intensity of bands representing stable proteins that cross-reacted with the antisera used.

As a non-radioactive alternative way to assay protein degradation *in vivo*, 50 µg/ml tetracycline or 100 µg/ml chloramphenicol were added to cells grown as described above at an OD_{578} of 0.6. Samples were taken as indicated in the figure legends, and the protein of interest was visualized by SDS–PAGE and immunoblotting.

Protein–protein interaction assays

For affinity chromatography ('pull-down') assays, 50 µl of S-protein–agarose (Novagen) was equilibrated in binding buffer (20 mM Tris–HCl pH 7.5, 150 mM NaCl, 5 mM $MgCl_2$, 5 mM DTT). Equimolar amounts (0.3 nmol) of S-TRX-His_6-RssB, His_6-σ^S, ClpX-His_6 or crude cell extracts of strains overexpressing RpoS::LacZ hybrid proteins (corresponding to ~0.75 nmol hybrid protein), with or without 50 mM acetyl phosphate (Sigma), were incubated for 20 min at room temperature and treated as described (Becker *et al*., 1999). SDS–gels were either stained as described by Fairbank *et al*. (1971), or proteins were subject to immunoblot analysis by using polyclonal sera against σ^S, RssB and β-galactosidase, or a Penta-His antibody (Qiagen).

For co-immunoprecipitation experiments, reaction assays included 50 µl of interaction buffer (35 mM Tris–HCl pH 7.5, 25 mM NaCl, 150 mM KCl, 12 mM $MgCl_2$, 10% glycerol, 0.1 mM EDTA and 1 mM DTT), equimolar amounts of crude cell extracts of strains overexpressing various RpoS::LacZ hybrid proteins (~0.1 nmol) and 0.3 nmol ClpX-His6. Samples were incubated for 20 min at 37°C in the presence or absence of ATP-γ-S (inverting the test tube every few minutes) and then added to 55 µl of inactivated *Staphylococcus aureus* Cowan I cells (previously incubated with Penta-His antibody in excess for 1 h at room temperature). After incubation for 1 h at room temperature (inverting the test tube every few minutes), the cells were pelleted and the supernatant was removed. The cells were washed three times with 750 µl of interaction buffer also containing 0.1% NP-40 and once with 10 mM Tris–HCl pH 8.0. The pellet was resolved in 20 µl of SDS–PAGE sample buffer and boiled. After centrifugation, the supernatant was run on a 12% SDS–gel. Gels were Fairbanks stained.

β-galactosidase assay

β-galactosidase activity was assayed by use of *o*-nitrophenyl-β-D-galactopyranoside (ONPG) as a substrate and is reported as µmol of *o*-nitrophenol/min/mg of cellular protein (Miller, 1972).

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