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Decline in ribosomal fidelity contributes to the accumulation and stabilization of the master stress response regulator σ^S upon carbon starvation

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The σ^S subunit of RNA polymerase is a master regulator of *Escherichia coli* that retards cellular senescence and bestows cells with general stress protective functions during growth arrest. We show that mutations and drugs triggering translational errors elevate σ^S levels and stability. Furthermore, mutations enhancing translational fidelity attenuate induction of the *rpoS* regulon and prevent stabilization of σ^S upon carbon starvation. Destabilization of σ^S by increased proofreading requires the presence of the σ^S recognition factor SprE (RssB) and the ClpXP protease. The data further suggest that σ^S becomes stabilized upon starvation as a result of ClpP sequestration and this sequestration is enhanced by oxidative modifications of aberrant proteins produced by erroneous translation. ClpP overproduction counteracted starvation-induced stabilization of σ^S, whereas overproduction of a ClpXP substrate (ssrA-tagged GFP) stabilized σ^S in exponentially growing cells. We present a model for the sequence of events leading to the accumulation and activation of σ^S upon carbon starvation, which are linked to alterations in both ribosomal fidelity and efficiency.

[*Keywords*: *Escherichia coli*; stationary phase; RpoS; SprE; *rpsL*; ClpP; protein oxidation]

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Unicellular and multicellular organisms harbor genetic networks that sense the quality of the environment and boost the individual's maintenance functions when growth conditions become suboptimal (Larsen 1993; Dukan and Nyström 1998, 1999; Hekimi and Guarente 2003; Hsu et al. 2003; Libina et al. 2003). Examples of such regulatory networks are the RAS/TOR pathways, the FOXO forkhead transcription factor family working in concert with insulin/insulin like signaling (Rohde et al. 2001; Libina et al. 2003; Hwangbo et al. 2004; Schmelzle et al. 2004; Kloting and Bluher 2005), and, in Gram-negative prokaryotes, the σ factor σ^S regulon. The σ^S, FOXO, and RAS/TOR regulatory systems are functionally analogous; they all respond to starvation (e.g., dietary restriction); they are required to mount general stress protection; and they are longevity determinants (Larsen 1993; Marchler et al. 1993; Lange and Hengge-Aronis 1994; Johnson et al. 2000; Weber et al. 2005; Powers et al. 2006). It is not clear which individual genes of these regulons are most important in slowing down senescence, but oxidative stress defense has been argued to be a key feature of all of them (Eisenstark et al. 1996; Dukan and Nyström 1998; Hlavata et al. 2003; Heeren et al. 2004; Kondo et al. 2005). In addition, several virulence traits are regulated by σ^S, and enteric bacteria lacking this σ factor are less pathogenic (Heiskanen et al. 1994; Webb et al. 1999). In fact, up to 10% of all *Escherichia coli* genes are directly or indirectly controlled by σ^S, indicating that this σ factor controls an exceptionally large network of genes (Weber et al. 2005).

An almost baffling number of *cis*-regulatory determinants and *trans*-acting regulatory factors involved in σ^S regulation have been identified (Hengge-Aronis 2002). For example, cAMP-CRP, GlcIIa, BarA, polyphosphate, homoserine lactone, acetate, and the NADH/NAD ratio have been shown to affect *rpoS* transcription, whereas DnaK, DksA, Hfq, HU, StpA, LeuO, and several small regulatory RNAs control *rpoS* translation (Hengge-Aronis 2002; Repoila et al. 2003). Yet, the key process responsible for the accumulation of σ^S during carbon depletion is regulated σ^S proteolysis. In cells growing

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exponentially in minimal medium, the half-life of σ^S is ~1 min, but the protein is rapidly and drastically stabilized upon carbon starvation of cells (Hengge-Aronis 2002). The protease ClpXP and the two-component orphan response regulator SprE (RssB), a specific σ^S recognition factor, are essential for the process of σ^S degradation (Muffler et al. 1996; Pratt and Silhavy 1996; Becker et al. 1999; Moreno et al. 2000; Mandel and Silhavy 2005). The affinity of SprE for σ^S is modulated, in vitro, by phosphorylation of the SprE receiver domain (Moreno et al. 2000; Klauck et al. 2001; Mandel and Silhavy 2005). Thus, it is tempting to speculate that stabilization of σ^S during starvation is a result of dephosphorylation of the SprE response regulator, and it has been proposed that the ArcB two-component sensor might be involved in such a mechanism (Mika and Hengge 2005). However, mutations (SprED58A) in the conserved phosphorylation site of SprE only affect basal levels of σ^S, whereas its accumulation and increased stability upon starvation/stationary phase remain normal (Peterson et al. 2004; Bougdour et al. 2006). Thus, it appears that the sensing/signaling device used by *E. coli* to stabilize σ^S upon carbon starvation operates, to a large extent, independently of SprE phosphorylation/dephosphorylation.

Nutrient limitation can be sensed by a variety of mechanisms, the most direct being performed by proteins associated with nutrient uptake systems, such as GlcIIa and PhoU, which sense/measure the presence/concentration of glucose and phosphate, respectively (Wanner 1993; Meadow et al. 2006). Depletion of ammonium or non-PTS carbon sources, on the other hand, are sensed by systems measuring the ratio of key metabolites, such as glutamine/α-ketoglutarate (Senior 1975; Magasanik 1989) and PEP/pyruvate (Hogema et al. 1998). The stringent response to amino acid shift-down relies instead on the ribosomes as sensors of amino acid deficiency (Cashel et al. 1996). Specifically, an uncharged tRNA finding its way into the A-site of the ribosome activates ppGpp production via the ribosome-associated ppGpp synthase I (PSI; RelA) (Cashel et al. 1996). Under a variety of other stress conditions, ppGpp is produced by the ppGpp synthase II (PSII) encoded by *spoT* (Cashel et al. 1996). The exact sensing–signaling pathway responsible for SpoT activation is not known, but fatty acid metabolism and interactions with the acyl carrier protein appear essential for SpoT activity under some conditions (Battesti and Bouveret 2006). The alarmone ppGpp is the effector molecule of the stringent response, which, in concert with the RNAP-binding protein DksA, affects a plethora of physiological activities, the main target being transcription (Paul et al. 2004b). In addition to its role in repressing superfluous rRNA synthesis during starvation (Cashel et al. 1996; Paul et al. 2004a; Magnusson et al. 2005), ppGpp also acts as a positive effector of gene expression, and σ^S-dependent genes require this nucleotide for their induction during starvation (Gentry et al. 1993; Kvint et al. 2000). One reason for the ppGpp-dependency of σ^S-regulated genes is that σ^S competes more successfully with the housekeeping σ factor σ^{70} when the RNA polymerase is programmed with ppGpp (Jishage et al. 2002). Thus, the ribosome, via ppGpp, can act as a sensor/signaling component, which regulates a switch toward maintenance functions during nutrient depletion by affecting the activity (competitiveness) of σ^S.

In this study, we demonstrate that the ribosome also acts as a sensor/signaling device contributing to the accumulation of σ^S during carbon starvation. This accumulation of σ^S is linked to the fidelity of the ribosome rather than ppGpp production and acts via production of aberrant and oxidized proteins sequestering the ClpP protease. We present a model for the physiological sequence of events leading to σ^S accumulation and activation upon carbon starvation.

Results

σ^S levels are regulated by translational accuracy

A link between translational fidelity and σ^S regulation was serendipitously discovered during our analysis of protein oxidation in stationary-phase cells. Specifically, previous experiments have demonstrated that stasis-induced, deleterious oxidative modifications of proteins can be reduced by the *rpsL141* allele, which increases translational accuracy (Ballesteros et al. 2001; Fredriksson et al. 2006). This allele, encoding a mutant ribosomal protein S12, has been argued to reduce protein oxidation by mitigating the production of aberrant proteins, since aberrant proteins are intrinsically sensitive targets of oxidative attack (Dukan et al. 2000). Another possibility is that the levels and/or activities of oxidant defense systems are elevated in the *rpsL* mutant. To analyze this, superoxide dismutase (SOD) and catalase activity (CAT) were determined in wild-type and *rpsL141* mutant strains during growth and glucose starvation-induced growth arrest. While SOD activity was similar in both strains (Fig. 1A,C), CAT activity was significantly lower in the *rpsL* mutant (Fig. 1A,B). Intrigued by the unexpected reduction of CAT activity, specifically during glucose starvation, we wondered whether expression of the *katE* gene, which encodes the starvation-induced catalase II, was affected by the *rpsL141* mutation. As seen in Figure 1D, *katE* expression was markedly less induced in the *rpsL141* mutant than in the wild-type strain upon glucose starvation. Since *katE* is regulated by σ^S, carbon-starvation induction of other genes of the σ^S-dependent regulon (*bolA* and *uspB*) was analyzed, demonstrating a poor induction also of these genes during carbon starvation (data not shown). In addition, Western blot analysis revealed that the accumulation of σ^S upon starvation was less pronounced in the *rpsL141* mutant (Fig. 1E). To further test if ribosomal proofreading is a key process regulating the RpoS regulon, σ^S levels were determined in an *rpsD12* mutant. The *rpsD12* allele encodes a mutant ribosomal protein, S4, which reduces ribosomal proofreading (Ballesteros et al. 2001; Fredriksson et al. 2006). This allele elevated σ^S levels both during growth and glucose starvation (Fig. 1F,G). In addition, introduction of a mutated gene for 16S rRNA (on the plasmid

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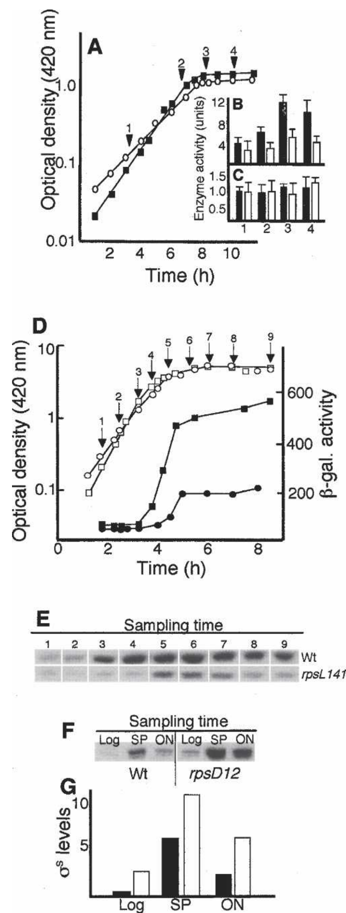

Figure 1. Effect of translational fidelity on σ^S-dependent gene expression and σ^S levels during growth and glucose starvation. (*A*) Growth of the wild-type (Wt/Δ14; closed squares) and the *rpsL141* mutant (S12/Δ14; open circles) strains under aerobic conditions. The arrows and numbers indicate the time at which samples were taken for catalase activity (*B*) and superoxide dismutase activity (*C*). Catalase and superoxide dismutase activities were determined and expressed as described in Materials and Methods. One unit of catalase is defined as the amount of enzyme per milligram of total protein that degrades 1 µmol of hydrogen peroxide in 1 min at 25°C (Dukan et al. 2000). One unit of superoxide dismutase is defined as the amount of enzyme per milligram of total protein that inhibits the rate of cytochrome *c* reduction by 50% at 25°C. Filled bars represent the wild type and open bars represent the *rpsL141* mutant. (*D*) Growth (open symbols) and *katE* promoter activity (closed symbols) in the wild type (MBN7; squares) and *rpsL141* mutant (MBN8; circles). (*E*) Levels of σ^S in the wild type (DV206) and *rpsL141* mutant (ÅF1), as indicated, during growth and during carbon starvation. The sampling times are indicated in *D*. (*F*) Levels of σ^S in the wild type (DV206) and *rpsD12* mutant (MBN27) during growth and glucose starvation. Samples were taken in exponential growth (Log), early glucose starvation, depicting the highest σ^S levels reached (SP), and cultures starved overnight (ON). (*G*) Quantitative representation, using the Image Gauge software, of the data shown in *F*. (Filled bars) Wild type; (unfilled bars) *rpsD12* mutant. All experiments were repeated at least three times. Representative results are shown.

pKK726G), which when incorporated into the ribosome renders it prone to errors (Prescott and Dahlberg 1990), elevated expression of the σ^S regulon (data not shown). The results demonstrate that σ^S levels correlate directly to changes in the proofreading capacity of the translational apparatus.

The heat-shock chaperone DnaK has been implicated as a positive regulator of σ^S upon entry of cells into stationary phase (Rockabrand et al. 1998). Since the heat-shock regulon is negatively affected by the *rpsL141* mutation (Ballesteros et al. 2001; Fredriksson et al. 2006), it is possible that the low levels of σ^S are a consequence of reduced induction of *dnaK* in the mutant. However, ectopic overproduction of DnaK failed to counteract the low levels of σ^S in the *rpsL141* mutant (data not shown).

Translational fidelity affects σ^S stability

To determine at which level translational proofreading affects σ^S levels, a series of *lacZ* fusion constructs was used as reporters of *rpoS* transcription (RO200), transcription and translation (PF212), and full control; that is, including σ^S stability-determining elements, including Lys173, of the σ^S-β-galactosidase fusion protein (PF977). As shown in Figure 2A, the effect of reduced translational errors was most clearly seen when the reporter construct included elements controlling σ^S stability; the β-galactosidase activity obtained from PF977 were more than sixfold lower in the *rpsL141* mutant compared with the wild type (Fig. 2A). To confirm that increased translational accuracy affects σ^S stability, antibodies against σ^S were used to measure the half-life of the σ factor after a total block of protein synthesis with spectinomycin or chloramphenicol. Western blot analysis of extracts from glucose-starved cells revealed that the rate of σ^S degradation is very much increased by the *rpsL141* mutation (Fig. 2B). (Note that the *rpsl141* mutant was not more sensitive to the protein synthesis inhibitors used, spectinomycin and chloramphenicol, than the wild-type strain.) In addition, the half-life of σ^S in exponentially growing wild-type and *rpsL141* cells, when mistranslation is relatively low in both strains, was similar (between 1 and 2 min) (data not shown). To further ascertain that the poor induction of σ^S-dependent genes upon carbon starvation in the hyperaccurate mutant is caused by increased σ^S proteolysis, we analyzed whether mutations in *clpX* could suppress the effect of *rpsL141*. ClpXP is an ATP-dependent protease responsible for σ^S degradation (Schweder et al. 1996). As depicted in Figure 2C, deletion of *clpX* suppressed the poor induction of *katE–lacZ* in the *rpsL141* mutant strain, confirming that increased proofreading acts on the RpoS regulon by affecting degradation of σ^S. Note that the *clpX* mutation elevates σ^S levels markedly in exponential-phase cells without a concomitant induction of *katE* (Fig. 2C). This is because σ^S-dependent genes require elevated levels of ppGpp for their full induction (Kvint et al. 2000; Jishage et al. 2002).

In support of mistranslation controlling the stability of σ^S, we found that the elevated levels of σ^S in exponen-

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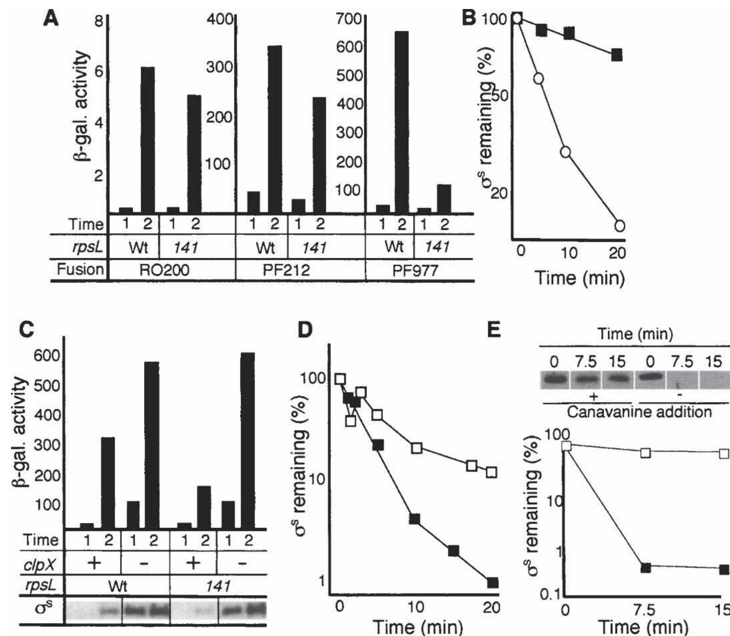

Figure 2. Effect of increased translational fidelity on σ^S production and stability. (*A*) The effects of the *rpsL141* allele on *rpoS* transcription (strains MBN31/32: Wt/*rpsL*; fusion RO200), *rpoS* transcription/translation (strains MBN34/35: Wt/*rpsL*; fusion PF212), and *rpoS* transcription/translation and σ^S stability (strains MBN37/38: Wt/*rpsL*; fusion PF977). Sampling times are for exponentially growing cells (*1*) and glucose-starved cells depicting the highest β-galactosidase activity reached from the different *lacZ* fusions (*2*). (*B*) Stability of σ^S in the wild type (DV206; closed squares) and the *rpsL141* mutant (ÅF1; open circles) in glucose-starved cells. Protein synthesis was inhibited by the addition of spectinomycin after 1 h of glucose starvation, and σ^S levels were determined by Western blot analysis and quantified using Image Gauge software. (*C*) The effect of a *clpX* deletion on *katE* expression and σ^S levels in the wild-type and *rpsL141* strains. The β-galactosidase activity is from the *katE–lacZ* fusion, and the levels of σ^S (Western blots) are depicted at the *bottom* of the graph for the respective mutants; strains: MBN7 (Wt), MBN19 (Wt, *clpX1*::*kan*), MBN8 (*rpsL141*), MBN20 (*rpsL141, clpX1*::*kan*). Sampling times are for exponentially growing cells (*1*) and glucose-starved cells depicting the highest σ^S levels reached (*2*). (*D*) Stability of σ^S in the wild type (DV206; closed squares) and the *rpsD12* mutant (MBN27; open squares) in exponentially growing cells. (*E*) Effect of canavanine on σ^S levels and stability. A wild-type culture was grown to early exponential phase in LB and divided into two cultures, with one half receiving canavanine (12.8 mg/mL) and the other half receiving only LB. Forty-five minutes later, protein synthesis was inhibited by the addition of chloramphenicol, and the half-life of σ^S was determined by Western blotting. *Above* each lane, the time after the addition of chloramphenicol is shown. *Below* is the quantification of the σ^S levels, with closed squares representing no canavanine addition and open squares representing canavanine addition. All experiments were repeated at least three times. Representative results are shown.

tially growing *rpsD12* mutants (Fig. 1G) were accompanied by increased σ^S stability (Fig. 2D). Also, the addition of canavanine to an exponentially growing culture stabilized σ^S (Fig. 2E). Canavanine is an analog of arginine in which the terminal methylene group has been replaced by oxygen. When incorporated into proteins, it causes misfolding (Miersch et al. 2000). Thus both genetically and chemically induced mistranslation enhanced σ^S stability.

Effects of ribosomal fidelity on σ^S stability in cells lacking and overproducing SprE

ClpXP-dependent degradation of σ^S is facilitated by the recognition factor SprE (Muffler et al. 1996; Pratt and Silhavy 1996; Becker et al. 1999; Mandel and Silhavy 2005), and we wondered whether increased translational accuracy might destabilize σ^S by elevating the levels of this recognition factor. This was not the case. Instead, Western analysis revealed that the levels of SprE were lowered by the *rpsL141* mutation (Fig. 3A), which is consistent with lower levels of σ^S, since σ^S is a positive feedback regulator of *sprE* (Ruiz et al. 2001). However, the effect of the *rpsL141* allele on σ^S stability was totally abolished in cells lacking SprE (Fig. 3B). We also tested whether increased accuracy affected σ^S levels in cells overproducing SprE. For this purpose, the *rssA2*::*TnCam* mutant was used in which *sprE* transcription is constitutively overexpressed from the *cam* promoter (Ruiz et al. 2001). This mutant exhibits reduced levels and decreased stability of σ^S due to the elevated levels of SprE. Still, the *rpsL141* mutation was able to further reduce σ^S levels in the *rssA2*::*TnCam* mutant (Fig. 3C) and rendered the half-life of the protein even shorter (Fig. 3D). Thus, the effects of translational proofreading on σ^S stability do not act via increased levels of SprE, but increased proofreading requires the presence of SprE to destabilize σ^S.

Sequestration of ClpP stabilizes σ^S upon glucose starvation

To obtain hints toward a mechanistic explanation for the link between ribosomal proofreading and σ^S stability, we looked for mutations that could suppress the low levels of σ^S-dependent gene expression in the *rpsL141* mutant. As expected, the *clpX* mutation restored stationary-phase induction of σ^S-dependent genes and stabilized σ^S both in exponential phase and during glucose starvation (see Fig. 2). Unexpectedly, however, a mutation in *clpA* also restored σ^S-dependent gene expression during glucose starvation (data not shown), which was the result of elevated σ^S levels (Fig. 4A) and increased σ^S stability (Fig. 4B) in the *rpsL141* mutant background. Both ClpAP and ClpXP catalyze ATP-dependent unfolding and proteolysis. Their substrates generally contain recognition sig-

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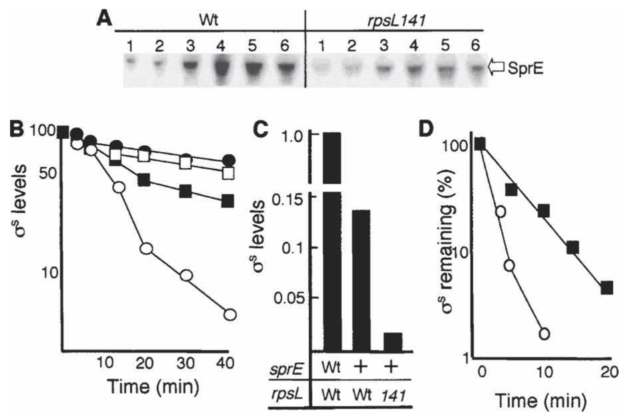

Figure 3. Effect of increased translational fidelity on σ^S stability in SprE-deficient SprE-overactive cells. (*A*) Levels of SprE in the wild-type (DV206) and *rpsL141* mutant (ÅF1) strains during growth and glucose starvation. Sampling points were exponential growth (*1*), transition phase (*2*), glucose starvation-induced stationary phase (30 min to 3 h) (*3–5*), and overnight glucose-starved cultures (*6*). (*B*) Stability of σ^S in wild-type cells (DV206; closed squares), *rpsL141* cells (ÅF1; open circles), *sprE*::*tet* cells (ÅF132; open squares), and *rpsL141*, *sprE*::*tet* cells (ÅF133; closed circles) during carbon starvation. Protein synthesis was inhibited by the addition of spectinomycin after 1 h of glucose starvation. (*C*) Levels of σ^S in wild-type cells (DV206), SprE-overactive cells (ÅF82), and SprE-overactive cells carrying the *rpsL141* allele (ÅF84). (*D*) Stability of σ^S in SprE-overactive cells (ÅF82; closed squares) and SprE-overactive cells carrying the *rpsL141* allele (ÅF84; open circles) in glucose-starved cells. Protein synthesis was inhibited by the addition of spectinomycin after 1 h of glucose starvation. All protein levels were determined by Western blot analysis and quantified using Image Gauge software. All experiments were repeated at least three times. Representative results are shown.

nals (~10 amino acids) at the N or C terminus, but σ^S is a specific substrate for ClpXP (Flynn et al. 2003). Thus, stabilization of σ^S by a *clpA* mutation in the *rpsL141* background, presumably, cannot be due to the relief of ClpAP degradation of σ^S itself. Note also that suppression by *clpA*, in contrast to *clpX* (Fig. 2), is conditional in the sense that σ^S levels are only restored in glucose-starved cells (Fig. 4A). We entertained the idea that the effect of *rpsL141* on σ^S stability and its suppression by *clpA* are both features linked to the pool size of aberrant proteins. The *rpsL141* mutation is known to reduce the production of aberrant and oxidatively modified proteins. If such proteins are targets for ClpXP and ClpAP, then more of the ClpXP protease will be available for σ^S degradation in the *rpsL141* mutant, and σ^S would be destabilized. However, a *clpA* mutation would increase the pool size of aberrant proteins in the *rpsL141* strain, and if ClpAP and ClpXP, to some extent, share aberrant substrates, ClpXP would be increasingly occupied with such substrates, and σ^S would be stabilized. There are two critical notions included in this reasoning; first, that ClpAP and ClpXP to some degree recognize similar substrates (this has previously been shown) (Flynn et al. 2003), and second, that ClpXP or one of its individual components is limited in the cell. To approach the latter notion, we tested whether ectopic overproduction of ClpX or ClpP counteracted σ^S accumulation and decreased σ^S stability in glucose-starved cells. We found that overproduction of ClpP alone was enough to cause such effects (Fig. 5A,B). ClpP overproduction, like the *rpsL141* mutation (Fig. 3B), required the presence of SprE (Fig. 5C) to destabilize σ^S. Thus, the accumulation of σ^S upon entry of cells into stationary phase must be due, at least in part, to limitation in ClpP availability. It should be noted also that ClpP levels do not change during starvation (Schweder et al. 1996; Mandel and Silhavy 2005).

To further approach the possibility of ClpP being limiting for σ^S degradation, we tested the effects of overproducing a ClpXP substrate on σ^S stability. Ectopic overproduction of an ssrA-tagged GFP resulted in stabilization of σ^S in exponentially growing cells (Fig. 5D). We also tested the effects of mutating the *sspB* gene. SspB is an adaptor protein that facilitates the ClpXP-mediated degradation of ssrA-tagged truncated proteins (Levchenko et al. 2000; Flynn et al. 2004). The C-terminal region of SspB has been shown to be the site of ClpX binding and is very similar to the C-terminal region of SprE (Dougan et al. 2003). We argued that SspB deficiency might lead to more ClpXP being available for degradation of σ^S, which does not require SspB or ssrA tagging. Indeed, a knockout mutation of *sspB* markedly lowers σ^S levels (Fig. 5E). This effect of an *sspB* mutation was dependent on the presence of SprE; in the *sprE*::*tet* background, the presence of the *sspB*::*cam* allele did not affect σ^S levels (Fig. 5E), demonstrating that the effect of the *sspB*::*cam* mutation is at the level of σ^S stability rather than expression.

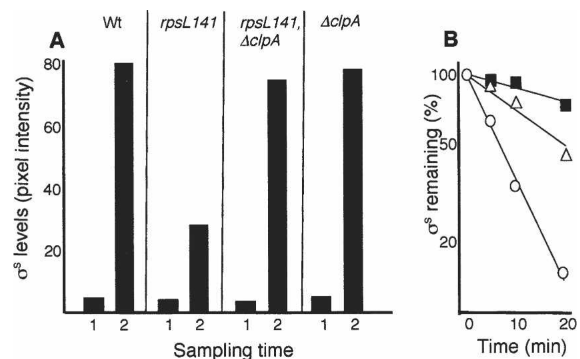

Figure 4. Effects of a *clpA* deletion on σ^S levels and stability in the *rpsL141* mutant. (*A*) Levels of σ^S in the wild-type (DV206), *rspL141* (ÅF1), *rpsL141*/*clpA*::*kan* (ÅF81), and *clpA*::*kan* (ÅF80) strains as indicated. Cells were sampled during exponential growth (*1*) and carbon starvation depicting the highest σ^S levels reached (*2*). (*B*) Stability of σ^S in glucose-starved wild-type (DV206; closed squares), *rpsL141* (ÅF1; open circles), and *rpsL141*/*clpA*::*kan* (ÅF81; open triangles) cells. Protein synthesis was inhibited by the addition of spectinomycin after 1 h of glucose starvation. All protein levels were determined by Western blot analysis and quantified using Image Gauge software. All experiments were repeated at least three times. Representative results are shown.

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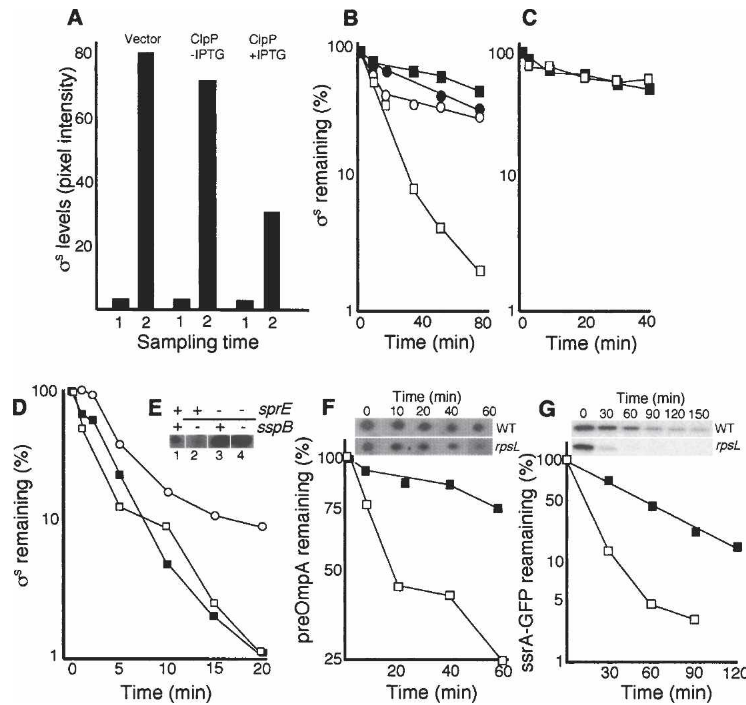

Figure 5. Effect of overproducing ClpP and a ClpPX substrate on σ^S accumulation and stability. (*A*) Levels of σ^S in the cells carrying the vector control (ÅF113), cells (ÅF125) carrying the uninduced (no IPTG) P_{lac}–*clpP* construct, and cells (ÅF125) induced (+IPTG) for *clpP* overexpression, as indicated. Sampling times are in exponential growth (*1*) and glucose starvation depicting the highest σ^S levels reached (*2*). (*B*) Stability of σ^S in glucose-starved wild-type cells (DV206; closed squares), cells carrying the vector control (ÅF113; closed circles), cells carrying the uninduced (no IPTG) P_{lac}–*clpP* construct (ÅF125; open circles), and cells induced (+IPTG) for *clpP* overexpression (ÅF125; open squares). Protein synthesis was inhibited by the addition of spectinomycin after 1 h of glucose starvation. (*C*) Stability of σ^S in *sprE*::*tet* mutant cells carrying the uninduced (no IPTG) P_{lac}–*clpP* construct (ÅF135; open squares), and cells induced (+IPTG) for *clpP* overexpression (closed squares). Protein synthesis was inhibited by the addition of spectinomycin after 1 h of glucose starvation. (*D*) Stability of σ^S in exponentially growing wild-type cells (closed squares), cells carrying the uninduced (no IPTG) P_{lac}–*ssrA-gfp* construct (ÅF142; open squares), and cells induced (+IPTG) for *ssrA-gfp* overexpression (open circles). (*E*) A knockout mutation of *sspB* lowers σ^S levels in a SprE-dependent manner. Samples were taken in the exponential growth phase (LB). The presence (+) and absence (−) of the *sprE* and *sspB* genes are indicated on *top* of the Western blot. The strains used are CNP119 (lane *1*), CNP217 (lane *2*), CNP153 (lane *3*), and CNP218 (lane *4*). All protein levels were determined by Western blot analysis and quantified using Image Gauge software. (*F*) Stability of the preOmpA in wild-type (DV206; closed squares) and *rpsL141* mutant cells (ÅF1; open squares) during carbon starvation (1 h). *Inset* shows the preOmpA spots on two-dimensional gels after inhibition of protein synthesis in the wild type and the *rpsL141* mutant. (*G*) Stability of the ssrA-GFP fusion protein in wild type (ÅF142; closed squares) and the *rpsL141* mutant (ÅF143; open squares) during carbon starvation (1 h). *Inset* shows the ssrA-GFP protein on Western blots in the wild type and *rpsL141* mutant after inhibition of protein synthesis. All experiments were repeated at least three times. Representative results are shown.

If more ClpP is available for σ^S degradation in the *rpsL141* mutant because this mutant produces fewer aberrant proteins, then other ClpXP/ClpAP substrates may exhibit a similar decreased stability. We tested the stability of the preOmpA (i.e., OmpA with the signal sequence), reported to be a substrate for both ClpXP and ClpAP (Flynn et al. 2003), in the wild type and *rpsL141* mutant upon carbon starvation and found that preOmpA, like σ^S, is less stable in the *rpsL141* background (Fig. 5F). Likewise, the stability of the ssrA-GFP fusion was markedly reduced in the *rpsL141* mutant (Fig. 5G).

The effect of ribosomal fidelity on σ^S accumulation requires oxidative conditions

Translational frameshifting (Barak et al. 1996; Wenthzel et al. 1998; Fredriksson et al. 2006), missense errors (O'Farrell 1978), and stop codon readthrough (Ballesteros et al. 2001) increase immediately upon carbon starvation of *E. coli* cells. Since aberrant proteins are more susceptible to oxidation than native ones, this sudden increase in mistranslation results in increased levels of oxidatively modified proteins (Ballesteros et al. 2001; Fredriksson et al. 2006). The *rpsL141* mutant retains its translational fidelity during stasis, and protein oxidation is drastically attenuated in the early stages of stasis in the cells carrying this allele (Ballesteros et al. 2001). We approached the question of whether such oxidative modification of mistranslated proteins is important for the accumulation of σ^S in stationary phase and found a reduced expression of σ^S-dependent genes (e.g., *katE*) and a reduced accumulation of σ^S (compared to aerobically starved cells) in cells starved for carbon anaerobically (Fig. 6A,B). In addition, the *rpsL141* allele had no effect on σ^S-dependent gene expression or σ^S accumulation in anaerobically starved cells (Fig. 6A,B). As shown previously (Fredriksson et al. 2006), we found that mistranslation occurs more frequently in anaerobically cultivated and starved cells and that this mistranslation is almost totally blocked by the *rpsl141* allele (Fig. 6C). Yet, the production of aberrant proteins is not "sensed" by the cells, with respect to the σ^S system, in the absence of oxygen. However, anaerobically cultivated cells would carry a relatively high load of aberrant proteins that could act as potential "inducers" of σ^S accumulation once they become oxidized. Thus, a shift from anaerobic conditions to aerobic conditions (a true up-shift condition) could cause an instantaneous elevation of σ^S levels since this shift allows oxidative modification of the accumulated pool of aberrant proteins to occur. Indeed, σ^S was rapidly, and transiently, accumulated during such a shift within a fraction of the generation time (Fig. 6D). Moreover, the accumulation of σ^S during such shifts in oxygen availability was reduced in cells carrying the

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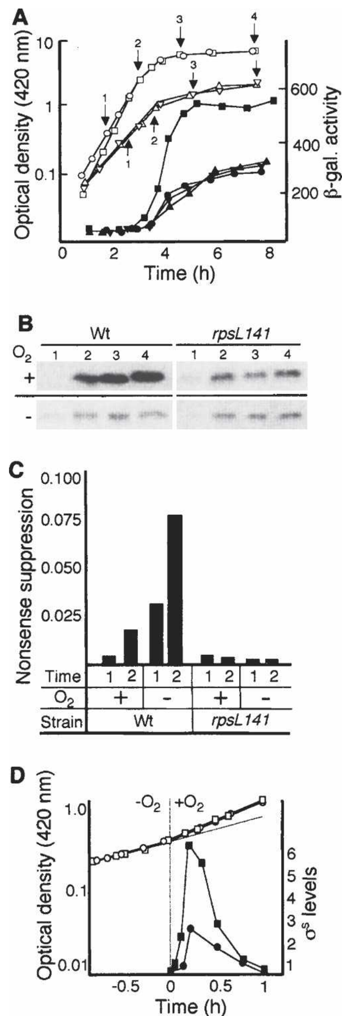

Figure 6. Effects of oxygen on σ^S activity and levels. (*A*) Growth (open symbols) and *katE* expression (closed symbols) in wild-type (Wt/Δ14; ▲□ and ▲△) and *rpsL141* (S12/Δ14; ●○ and ▼▽) cells grown and carbon-starved aerobically (squares and circles) and anaerobically (triangles). Arrows indicate sampling times for analysis of σ^S levels. (*B*) Levels of σ^S in wild-type (Wt/Δ14) and *rpsL141* (S12/Δ14) cells growing aerobically (+) and anaerobically (–) as indicated. The sampling times 1–4 are those indicated in *A*. (*C*) Mistranslation, (nonsense suppression) in wild-type (Wt/U4 to Wt/Δ14) and *rpsL141* (S12/U4 to S12/Δ14) cells growing and starving aerobically (+) and anaerobically (–) as indicated. Mistranslation was determined as described in Materials and Methods. A value of 0.01 indicates that one out of 100 transcripts generates a full-length protein due to nonsense readthrough. Sampling times are in exponential growth (*1*) and glucose starvation depicting the highest level of mistranslation reached (*2*). (*D*) Growth (open symbols) and levels of σ^S (closed symbols) in wild-type (Wt/Δ14; squares) and *rpsL141* (*rpsL141*; circles) cells upon a shift from aerobic to anaerobic conditions as indicated. The dotted line denotes the preshift growth rate of the wild-type culture. σ^S levels were determined by Western blot analysis and quantified using Image Gauge software. All experiments were repeated at least three times. Representative results are shown.

rpsL141 allele (Fig. 6D), demonstrating that the effect observed is intimately coupled to the pool size of aberrant proteins.

Discussion

The σ^S regulon is rapidly induced as cells experience starvation and its member genes are required for cells to remain viable under starvation-induced growth arrest. Despite the fact that a large number of *trans*- and *cis*-regulatory components have been identified as important in regulating σ^S levels, the sensing–signaling device used by *E. coli* to trigger σ^S accumulation upon starvation has not been fully deciphered (Hengge-Aronis 2002). Based on the result presented here, we present a model for the physiological sequence of events contributing to σ^S accumulation and activation during carbon starvation.

An immediate consequence of carbon starvation and amino acid shift-downs is a reduction in the pool size of charged tRNAs. This diminished availability of amino acyl-tRNAs leads to increased mistranslation; for example, misincorporation of erroneous amino acids, translational frameshifting, and stop-codon readthrough (O'Farrell 1978; Barak et al. 1996; Wenthzel et al. 1998; Ballesteros et al. 2001). The rapid increase in the levels of oxidatively modified proteins upon starvation is a direct consequence of this reduction in translational fidelity because the aberrant protein isoforms produced exhibit increased susceptibility to oxidative attack (Ballesteros et al. 2001; Fredriksson et al. 2006). We show here that the sudden drop in translational fidelity upon carbon starvation is a key event also in the accumulation of σ^S. We suggest that such accumulation of σ^S is the consequence of a protease titration mechanism in which the surge in the pool size of aberrant proteins upon carbon starvation sequesters the ClpP protease. It has been shown previously that ClpP is required for degradation of both misfolded, puromycyl-containing proteins (Thomsen et al. 2002) and proteins damaged by oxidative carbonylation (Nair et al. 2003). It appears that the oxidatively modified species of the aberrant proteins are more efficient in titrating the ClpP protease (Figs. 6, 7). However, it is also possible that increased ribosome stalling and ssrA-tagging of truncated peptides contribute to σ^S stabilization during carbon starvation (Fig. 7).

An important feature of the model is that one or several components of the σ^S degradation machinery must be limiting, at least during entry of cells into stationary phase. Indeed, overproduction of ClpP alone reduced σ^S accumulation and partly counteracted σ^S stabilization in early stationary phase (Fig. 7), suggesting that ClpP denotes such a limiting component. In line with the model, mutations that reduce translational errors, omission of oxygen, and ClpP overproduction are all conditions that reduce the accumulation and stabilization of σ^S in starved cells. In addition, decreased translational proofreading and overproduction of a ClpXP substrate stabilized σ^S already in exponential phase. Both elevated proofreading and ClpP overproduction required the pres-

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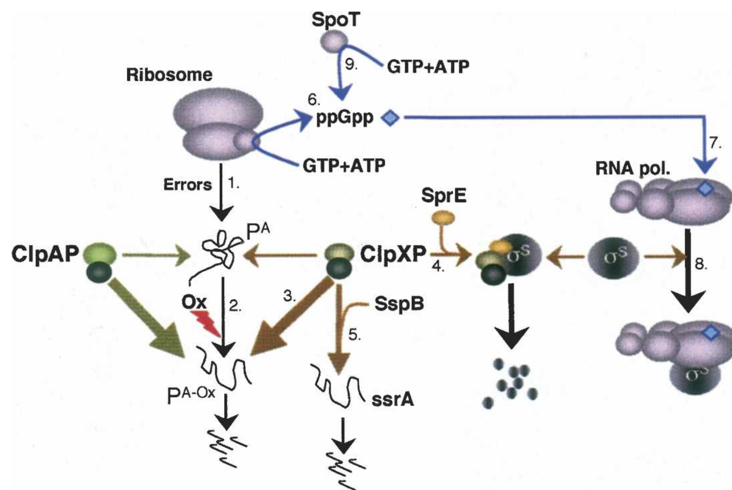

Figure 7. Schematic representation of the model for σ^S stabilization upon starvation. (*1*) Translational errors increase as an immediate response to, for example, amino acid and carbon starvation resulting in the production of erroneous and misfolded proteins σ^S. (*2*) The aberrant proteins are susceptible to oxidative modifications, which cause further structural deviations of the proteins. (*3*) The aberrant, especially oxidatively modified, species of the mistranslated proteins are substrates for the ClpAP and ClpXP proteases, which as a consequence of the reduced fidelity of the translational apparatus becomes increasingly occupied in the management of aberrant polypeptides. (*4*) Thus, less ClpXP is available for SprE-dependent degradation of σ^S, and even a marginal elevation of σ^S levels caused by this ClpXP sequestration might titrate out SprE and further stabilize σ^S upon these conditions. The limiting factor of the proteolytic system may be ClpP, since ClpP overproduction greatly diminished σ^S accumulation. (*5*) Elevated levels of SsrA-tagged peptides generated on stalled ribosomes may also sequester ClpXP and stabilize σ^S. Another consequence of starving ribosomes is the RelA-dependent production of ppGpp (*6*), which binds to RNA polymerase (*7*) and enhances the ability of σ^S to compete for core binding (*8*). During other starvation and stress conditions, σ^S competition is favored by ppGpp produced from SpoT.

ence of SprE to destabilize σ^S, suggesting that the canonical SprE/ClpXP pathway achieves the degradation of the σ factor under these conditions. In addition, the fact that *clpX* and also *clpA* deletions suppressed the instability of σ^S in glucose-starved *rpsL141* mutants suggests that the ClpAP and ClpXP to some extent are occupied with the same aberrant substrates in carbon-starved cells (Fig. 7). It has been proposed that SprE is limiting in vivo and that a marginal increase in the cellular concentration of σ^S—for example, by elevated translation—will titrate out SprE and cause a drastic stabilization of σ^S (Pruteanu and Hengge-Aronis 2002). We suggest that sequestration of ClpP upon starvation-induced mistranslation might be an additional physiologically relevant event that titrates the SprE recognition factor during carbon starvation. In this scenario, σ^S is stabilized by two sequential titration events, titration of ClpP followed by SprE.

Nitrogen starvation has been shown to cause a similar increased mistranslation and elevated levels of protein carbonyls as carbon starvation (Ballesteros et al. 2001), but σ^S does not reach the same high concentration during nitrogen as carbon starvation (Mandel and Silhavy 2005). Possibly, mistranslation/protein oxidation and ClpP titration may account for most of the stabilization of σ^S upon nitrogen starvation, whereas another mechanism works in parallel to ClpP titration during carbon starvation, giving rise to even higher levels of the σ factor. This notion is consistent with the fact that there is residual induction of the *rpoS* regulon and accumulation of the σ factor in the *rpsL141* mutant upon carbon starvation (Fig. 1D,E). In contrast to carbon and nitrogen starvation, translational errors and protein oxidation do not increase significantly during phosphate starvation (Ballesteros et al. 2001). Thus, the stabilization of σ^S upon phosphate depletion is expected to be accomplished by a mechanism other than titration of ClpP by aberrant proteins. Interestingly, it has recently been shown that σ^S accumulation during phosphate starvation involves a novel protein, IraP, which interferes with SprE-dependent degradation of σ^S during phosphate, but not carbon, starvation (Bougdour et al. 2006). In addition, increased translation of the *rpoS* transcript appears to be more important for σ^S accumulation during phosphate starvation than carbon starvation (Mandel and Silhavy 2005).

Experiments with strains lacking the alarmone ppGpp suggest that there are more components than SprE of importance in regulating σ^S stability. Overproduction of σ^S is difficult to achieve in exponentially growing cells (rich media—low levels of ppGpp) and in Δ*relA* Δ*spoT* mutants (deficient in ppGpp), and we have noticed that σ^S is unstable under such conditions despite the fact that overproduction ought to titrate the SprE factor. In addition, cells lacking ppGpp display increased mistranslation and levels of carbonylated proteins (M. Ballesteros, L. Magnusson, and T. Nyström, in prep.), yet σ^S is not stabilized in this genetic background. This instability of σ^S may be due to the fact that ppGpp is required for σ^S to compete successfully for RNA polymerase (E) binding (Jishage et al. 2002). Thus, binding of σ^S to E, which would protect the σ factor from degradation, is another important aspect of regulating σ^S stability and activity, and the involvement of ppGpp in this context provides an important hierarchy of physiological regulation. The requirement of σ^S for ppGpp suggests that the σ^S regulon can only be efficiently induced under suboptimal growth conditions, which elevate the production of this nucleotide. In fact, we do not know of any condition that triggers expression of σ^S regulon genes without a concomitant increase in ppGpp levels. The requirement for ppGpp may thus be an important checkpoint control such that elevated levels of σ^S will not automatically trigger the regulon if the cell senses that its physiological status (low ppGpp) does not call for the functions encoded by the σ^S regulon. This may be the case during, for example, a shift from anaerobic to aerobic conditions. As seen in

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Figure 6, such a shift results in an immediate accumulation of σ^S. However, we found that the σ^S regulon genes are not induced during this shift (data not shown). This can be explained by the fact that this is a true up-shift condition that does not elevate ppGpp levels. In fact, under such up-shift conditions, which primarily require housekeeping functions ($E\sigma^{70}$), successful competition of σ^S for E binding would reduce the fitness of the cells.

In summary, decreased ribosomal fidelity generating aberrant and oxidized proteins that sequester the ClpP protease are key events contributing to the stabilization of the σ^S transcription factor upon carbon starvation. Future analysis may clarify whether specific aberrant substrates may act as specific carbon starvation "sensors" in the sense that they sequester the ClpP protease upon entry of cells into stationary phase or if σ^S stabilization is due to a more general and nonspecific effect of mistranslation of bulk proteins.

Materials and methods

Chemicals and reagents

Anti-DnaK mouse monoclonal antibodies were from Stressgen Bioreagents (Biosite), anti-σ^S mouse monoclonal antibodies were from Neoclone, and SprE antibodies were a gift from N. Ruiz. GFP antibodies were purchased from Roche. Anti-mouse IgG peroxidase conjugates, 2-nitrophenyl β-D-thiogalactoside (ONPG), and isopropyl β-thiogalactopyranoside (IPTG) were from Sigma. The chemiluminscence blotting substrate (ECL^+) was obtained from Amersham Corp., and the Immobilon-P polyvinyldene difluoride (PVDF) membrane was from Millipore. Plasmid DNA was purified by using Qiagen columns (Qiagen, Inc.) or a Wizard minipreparation kit (Promega, Inc.). The Gene-Clean Kit used for isolation of DNA fragments was from Bio 101, Inc. All chemicals and reagents were used according to instructions provided by the manufacturer.

Bacterial strains, plasmids, and growth conditions

The *E. coli* K-12 strains and plasmids used in this study are listed in Table 1. *LacZ* fusion reporter strains MBN7, MBN8, MBN31, MBN32, MBN34, MBN35, MBN37, and MBN38 were constructed by infection of DV206 and ÅF1 with a λ phage lysate harboring the appropriate construct. Monolysogeny was confirmed by PCR (Powell et al. 1994). The λΦ(*katE–lacZ*) construct was from Ohnuma et al. (2000), and the λΦ(*rpoS–lacZ*) constructs were from strains RO200 (OF fusion) (Lange and Hengge-Aronis 1994), CU264 (PF212, transcriptional fusion) (Ueguchi et al. 2001), and CU263 (PF977, translational fusion) (Ueguchi et al. 2001). To generate strains ÅF80, ÅF81, ÅF82, ÅF84, ÅF132, and ÅF133, *clpA319*::*kan* (Katayama et al. 1988), *rssA2*::*cam* (from strain NR419; N. Ruiz), and *sprE*::*tet* (from strain NR253; N. Ruiz) were introduced into DV206 and ÅF1 by P1 transduction. Strains MBN19 and MBN20 were similarly generated by transduction of Δ*clpX1*::*kan* (Katayama et al. 1988) into MBN7 and MBN8. Strain ÅF39 was constructed by transformation of ÅF1 with plasmid pBB535 (Tomoyasu et al. 2001) and ÅF142 and ÅF 143 by transformation of DV206 with plasmid pGFP-ssrA (a kind gift from B. Bukau) (Dougan et al. 2003). The F′*lacI*^{q} was introduced via mating with strain XL-1 Blue. The cloned *clpP* was confirmed by sequencing plasmid pÅF2. A *clpP* under control of the IPTG-inducible P_{lac} was constructed by PCR amplification of the *clpP* gene, cleavage with SacI and KpnI, and cloning into the SacI–KpnI fragment of pUC18. Strains ÅF134, ÅF135, ÅF138, and ÅF139 were constructed by transformation of DV206 and ÅF132 with plasmid pBB528 followed by transformation with pUC18 and pÅF2.

Cultures were grown aerobically or anaerobically at 37°C in Erlenmeyer flasks in a rotary shaker in liquid Luria-Bertani (LB) or minimal M9 defined medium (Miller 1972). For glucose-starvation experiments, the defined M9 medium was used with reduced glucose concentrations (typically 0.05%) such that glucose was the first nutrient to become depleted, upon which the cells entered carbon starvation. When indicated, the M9 media was supplemented with thiamine (10 µM), all 20 amino acids in excess, and glucose (0.1% or 0.4% for anaerobic/aerobic shift experiments). When appropriate, the media were supplemented with kanamycin (50 µg/mL), chloramphenicol (30 µg/mL), rifampicin (150 µg/mL), tetracycline (20 µg/mL), canavanine (12.8 mg/mL), carbenicillin (100 µg/mL), and/or IPTG at concentrations indicated for each experiment. Anaerobic conditions were achieved by constant bubbling of the cultures with a gas mixture consisting of 5% CO_2 and 95% N_2 as described (Valadi et al. 2001). To overproduce DnaK and DnaJ, 200 µM IPTG was added to exponentially growing cultures of strains ÅF38 and ÅF39 at an OD_{420} of 0.05 to induce expression from the plasmid pBB535, and to induce *clpP* expression from the plasmid pÅF2, 50 µM IPTG was added at an OD_{420} of 0.1. To overproduce the ssrA-tagged GFP, 100 µM IPTG was added to exponentially growing cultures of strain ÅF142 at an OD_{420} of 0.05.

General methods

P1 transductions, plasmid transformations, and λ-phage lysogeny were performed as described by Miller (1972) and Sambrook and Russell (2001). Protein extracts where prepared according to Sambrook and Russell (2001). σ^S, DnaK, and SprE levels were determined by gel electrophoresis and immunoblotting according to standard procedures using 11.5% SDS–polyacrylamide gels and mouse monoclonal antibodies directed toward σ^S, or mouse monoclonal antibodies directed toward DnaK. For detection, the ECL-plus blotting kit was used with horseradish peroxidase-conjugated anti-mouse IgG as secondary antibody. Blots were subsequently exposed in the Fuji Film Image Reader LAS-1000 Pro. For quantitative analyses of the blots, the Image Gauge 3.46, Science Lab 99 software was used. Measurements of β-galactosidase activity from *lacZ*–gene reporter constructs were performed as described (Miller 1972) with modifications (Albertson and Nyström 1994). All experiments were repeated several times to ensure reproducibility, and the variation was <10%.

Mistranslation assay

Nonsense suppression was determined by measuring a stop codon readthrough in a *lacI–lacZ* fusion as described in Andersson et al. (1982). The frequency of nonsense suppression was calculated based on the β-galactosidase produced by the wild-type allele (transcribed from the same promoter) under the same conditions. Thus, a value of 0.01 indicates that one out of 100 transcripts generates a full-length protein due to nonsense readthrough. Both alleles were carried on F′ factors in the wild-type strain and the *rpsL141* mutant.

Catalase activity

Catalase activity in bacterial extracts was determined by measuring the decrease in the A_{240nm} of hydrogen peroxide as described previously (Gonzalez-Flecha et al. 1993). One unit of

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Table 1. *E. coli strains used in this work*

Designation	Sex, extrachromosomal markers	Genotype, chromosomal markers	Origin
DV206	F⁻	λ⁻ *ilvG- rfb*-50 *rph*-1 *lacIZ*Δ(*Mlu*)	Vinella et al. 2005
MC4100	F⁻	*araD139*Δ (*argF*-lac)*U169 rpsL150 relA1 flbB5301 deoC1 ptsF25 rbsR*	Casadaban 1976
CNP119	F⁻	MC4100 λϕ(*rpoS750–lacZ*)	Sledjeski et al. 1996
CNP217	F⁻	CNP119 *sspB*::*cam*	This study
CNP153	F⁻	CNP119 *sprE*::*tet*	This study
CNP218	F⁻	CNP217 *sprE*::*tet*	This study
Wt/Δ14	F′ *proAB lacIZYA*	*ara argE* Δ(*lac proAB*) *gyrA thi*	L.A. Isaksson
Wt/U4	F′ *proAB lacI* (UGA)*ZYA*	(As above)	L.A. Isaksson
S12/Δ14	F′ *proAB lacIZYA*	(As above) *rpsL141*	L.A. Isaksson
S12/U4	F′ *proAB lacI* (UGA)*ZYA*	(As above)	L.A. Isaksson
ÅF1	F⁻	DV206 *rpsL141*	Ballesteros et al. 2001
MBN7	F⁻	DV206 λΦ(*KatE–lacZ*)	This study
MBN8	F⁻	ÅF1 λΦ(*KatE–lacZ*)	This study
MBN19	F⁻	MBN7 Δ*clpX1*::*Kan*	This study
MBN20	F⁻	MBN8 Δ*clpX1*::*Kan*	This study
MBN27	F⁻	DV206 *acrF*::Tn10 *rpsD12*	Fredriksson et al. 2006
MBN31	F⁻	DV206 λΦ(*rpoS–lacZ*) OF	This study
MBN32	F⁻	ÅF1 λΦ(*rpoS–lacZ*) OF	This study
MBN34	F⁻	DV206 λΦ(*rpoS–lacZ*) PF212	This study
MBN35	F⁻	ÅF1 λΦ(*rpoS–lacZ*) PF212	This study
MBN37	F⁻	DV206 λΦ(*rpoS–lacZ*) PF977	This study
MBN38	F⁻	ÅF1 λΦ(*rpoS–lacZ*) PF977	This study
ÅF38	F⁻, pBB535	DV206	Fredriksson et al. 2006
ÅF39	F⁻, pBB535	ÅF1	This study
ÅF80	F⁻	DV206 *clpA319*::*kan*	This study
ÅF81	F⁻	ÅF1 *clpA319*::*kan*	This study
ÅF82	F⁻	DV206 *rssA2*::*Tncam*	This study
ÅF84	F⁻	ÅF1 *rssA2*::*Tncam*	This study
ÅF132	F⁻	DV206 *sprE*::*tet*	This study
ÅF133	F⁻	ÅF1 *sprE*::*tet*	This study
ÅF134	F⁻, pUC18, pBB528	ÅF132	This study
ÅF135	F⁻, pÅF2, pBB528	ÅF132	This study
ÅF138	F⁻, pUC18, pBB528	DV206	This study
ÅF139	F⁻, pÅF2, pBB528	DV206	This study
ÅF142	pGFP-ssrA, F′ *proAB lacI*^{q} ZDM15::Tn10(*tet*^{R})	DV206	This study
ÅF143	pGFP-ssrA, F′ *proAB lacI*^{q} ZDM15::Tn10(*tet*^{R})	ÅF142 *rpsL141*	This work

Plasmid	Gene construct	Resistance marker	Inducer	Origin
pUC18	Vector	Ampicillin	IPTG	Yanisch-Perron et al. 1985
pÅF2	P_{lac}–*ClpP*	Ampicillin	IPTG	This study
pBB528	*lacI*^{q}	Chloramphenicol	IPTG	Tomoyasu et al. 2001
pGFP-SsrA	*gfp-ssrAu*	Ampicillin	IPTG	Dougan et al. 2003
pBB535	$P_{A1/lacO\text{-}1}$–*dnaKdnaJ*	Spectinomycin	IPTG	Tomoyasu et al. 2001

catalase is defined as the amount of enzyme that degrades 1 µmol of hydrogen peroxide in 1 min at 25°C (Dukan et al. 2000).

Superoxide dismutase activity

Superoxide dismutase activity was assayed using the xantine oxidase/cytochrome *c* method (Imlay and Fridovich 1991). One unit of superoxide dismutase is defined as the amount of enzyme that inhibits the rate of cytochrome *c* reduction by 50% at 25°C.

Protein stability

σ^S stability measurements were performed as described (Zhou and Gottesman 1998). Briefly, cells were grown exponentially at 37°C. After 1 h of glucose starvation, protein synthesis was blocked by addition of spectinomycin (400 µg/mL) or chloramphenicol (30 µg/mL), and samples were withdrawn at indicated times and resuspended in SDS gel loading buffer, and subjected to SDS-PAGE and quantitative Western blotting as described above. The stability of the ssrA-GFP fusion was analyzed in a similar fashion using antibodies directed against GFP. The ssrA-GFP fusion was not overproduced in this experiment to avoid titration of ClpP. The stability of preOmpA (OmpA with signal sequence) was analyzed, after inhibition of protein synthesis, using two-dimensional gel electrophoresis, and preOmpA was identified on the gels using the gene–protein database (VanBogelen et al. 1997).

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Canavanine exposure

A wild-type (MC4100) culture was grown to early exponential phase in LB and divided into two cultures, with one half receiving 12.8 mg/mL canavanine and the other half receiving only LB. Forty-five minutes later, protein synthesis was inhibited by the addition of chloramphenicol, and the half-life of σ^S was determined by Western blotting as described above.

Anaerobic/aerobic shifts

Cells were grown in M9 media supplemented with thiamine and amino acids in Erlenmeyer flasks anaerobically as described above. The exponentially growing cultures were shifted to aerobic conditions by pouring them into prewarmed Erlenmeyer flasks and aerated by rotary shaking at 240 rpm. Immediately before (sample "zero") and after the shift, samples were removed at the indicated times and precipitated with 10% trichloroacetic acid. The precipitates were washed with cold 80% acetone, resuspended in SDS loading buffer, and subjected to quantitative Western blotting.

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