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.

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exponentially in minimal medium, the half-life of $\sigma^S$ 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 $\sigma^S$ recognition factor, are essential for the process of $\sigma^S$ 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 $\sigma^S$ 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 $\sigma^S$ 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 $\sigma^S$, 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 $\sigma^S$ 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 GlcIa 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/$\alpha$-ketoglutarate [Senior 1975; Magasanik 1989] and PEP/pyruvate [Hogema et al. 1998]. The stringent response to amino acid shift-down relies 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 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 affects a multitude of physiological activities, including translation 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 $\sigma^S$, carbon-starvation induction of other genes of the $\sigma^S$-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 $\sigma^S$ 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, $\sigma^S$ 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 $\sigma^S$ levels both during growth and glucose starvation [Fig. 1F,G]. In addition, introduction of a mutated gene for 16S rRNA (on the plasmid [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 $\sigma^S$.

In this study, we demonstrate that the ribosome also acts as a sensor/signaling device contributing to the accumulation of $\sigma^S$ during carbon starvation. This accumulation of $\sigma^S$ 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 $\sigma^S$ accumulation and activation upon carbon starvation.

Results

$\sigma^S$ levels are regulated by translational accuracy

A link between translational fidelity and $\sigma^S$ 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 $\sigma^S$, carbon-starvation induction of other genes of the $\sigma^S$-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 $\sigma^S$ 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, $\sigma^S$ 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 $\sigma^S$ levels both during growth and glucose starvation [Fig. 1F,G]. In addition, introduction of a mutated gene for 16S rRNA (on the plasmid [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 $\sigma^S$. In this study, we demonstrate that the ribosome also acts as a sensor/signaling device contributing to the accumulation of $\sigma^S$ during carbon starvation. This accumulation of $\sigma^S$ 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 $\sigma^S$ accumulation and activation upon carbon starvation.

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Figure 1. Effect of translational fidelity on σ^5-dependent gene expression and σ^5 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 inhibits the rate of hydrogen peroxide in 1 min at 25°C. One unit of superoxide dismutase is defined as the amount of enzyme per milligram of total protein that degrades 1 µmol of superoxide dismutase (PF212), and full control; that is, including σ^5 stability-determining elements, including Lys173, of the σ^5-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^5 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^5 stability, antibodies against σ^5 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^5 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^5 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^5-dependent genes upon carbon starvation in the hyperaccurate mutant is caused by increased σ^5 proteolysis, we analyzed whether mutations in clpX could suppress the effect of rpsL141. ClpXP is an ATP-dependent protease responsible for σ^5 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^5. Note that the clpX mutation elevates σ^5 levels markedly in exponential-phase cells without a concomitant induction of katE (Fig. 2C). This is because σ^5-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^5, we found that the elevated levels of σ^5 in exponen-
in the wild type [DV206, closed squares] and the rpsD12 mutant [MBN27, open squares] in exponentially growing cells. [E] Effect of canavanine on $\sigma^5$ 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 $\sigma^5$ was determined by Western blotting. Above each lane, the time after the addition of chloramphenicol is shown. Below is the quantification of the $\sigma^5$ 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.

**Efforts of ribosomal fidelity on $\sigma^5$ stability in cells lacking and overproducing SprE**

ClpXP-dependent degradation of $\sigma^5$ 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 $\sigma^5$ 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 $\sigma^5$, since $\sigma^5$ is a positive feedback regulator of sprE [Ruiz et al. 2001]. However, the effect of the rpsL141 allele on $\sigma^5$ stability was totally abolished in cells lacking SprE [Fig. 3B]. We also tested whether increased accuracy affected $\sigma^5$ 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 $\sigma^5$ due to the elevated levels of SprE. Still, the rpsL141 mutation was able to further reduce $\sigma^5$ 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 $\sigma^5$ stability do not act via increased levels of SprE, but increased proofreading requires the presence of SprE to destabilize $\sigma^5$.

**Sequestration of ClpP stabilizes $\sigma^5$ upon glucose starvation**

To obtain hints toward a mechanistic explanation for the link between ribosomal proofreading and $\sigma^5$ stability, we looked for mutations that could suppress the low levels of $\sigma^5$-dependent gene expression in the rpsL141 mutant. As expected, the clpX mutation restored stationary-phase induction of $\sigma^5$-dependent genes and stabilized $\sigma^5$ both in exponential phase and during glucose starvation (see Fig. 2). Unexpectedly, however, a mutation in clpA also restored $\sigma^5$-dependent gene expression during glucose starvation [data not shown], which was the result of elevated $\sigma^5$ levels [Fig. 4A] and increased $\sigma^5$ 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 \( \sigma^5 \) stability in SprE-deficient SprE-overactive cells. (A) Levels of SprE in the wild-type [DV206] and rpsL141 mutant [AF1] 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 \( \sigma^5 \) in wild-type cells [DV206; closed squares], rpsL141 cells [AF1; open circles], sprE::tet cells [AF132; open squares], and rpsL141, sprE::tet cells [AF133; closed circles] during carbon starvation. Protein synthesis was inhibited by the addition of spectinomycin after 1 h of glucose starvation. Stability of \( \sigma^5 \) in exponentially growing cells (DV206; closed squares), rpsL141 clan (AF82; closed squares), and SprE-overactive cells carrying the rpsL141 allele (AF84). (D) Stability of \( \sigma^5 \) in SprE-overactive cells carrying the rpsL141 allele (AF84; 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 \( \sigma^5 \) is a specific substrate for ClpXP [Flynn et al. 2003]. Thus, stabilization of \( \sigma^5 \) by a clpA mutation in the rpsL141 background, presumably, cannot be due to the relief of ClpAP degradation of \( \sigma^5 \) itself. Note also that suppression by clpA, in contrast to clpX (Fig. 2), is conditional in the sense that \( \sigma^5 \) levels are only restored in glucose-starved cells [Fig. 4A]. We entertained the idea that the effect of rpsL141 on \( \sigma^5 \) 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 \( \sigma^5 \) degradation in the rpsL141 mutant, and \( \sigma^5 \) 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 \( \sigma^5 \) 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 \( \sigma^5 \) accumulation and decreased \( \sigma^5 \) 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 \( \sigma^5 \). Thus, the accumulation of \( \sigma^5 \) 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 \( \sigma^5 \) degradation, we tested the effects of overproducing a ClpXP substrate on \( \sigma^5 \) stability. Ectopic overproduction of an ssrA-tagged GFP resulted in stabilization of \( \sigma^5 \) 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 \( \sigma^5 \), which does not require SspB or ssrA tagging. Indeed, a knockout mutation of sspB markedly lowers \( \sigma^5 \) 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 \( \sigma^5 \) levels [Fig. 5E], demonstrating that the effect of the sspB::cam mutation is at the level of \( \sigma^5 \) stability rather than expression.

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Figure 4. Effects of a clpA deletion on \( \sigma^5 \) levels and stability in the rpsL141 mutant. (A) Levels of \( \sigma^5 \) in the wild-type [DV206], rpsL141 [AF1], rpsL141/clpA::kan [AF81], and clpA::kan [AF80] strains as indicated. Cells were sampled during exponential growth [1] and carbon starvation depicting the highest \( \sigma^5 \) levels reached [2]. (B) Stability of \( \sigma^5 \) in glucose-starved wild-type [DV206; closed squares], rpsL141 [AF1; open circles], and rpsL141/clpA::kan [AF81; 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.
Mistranslation induces RpoS accumulation

Figure 5. Effect of overproducing ClpP and a ClpPX substrate on σS accumulation and stability. (A) Levels of σS in the cells carrying the vector control (ÅF113), cells [ÅF125] carrying the uninduced [no IPTG] P~mc~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 σS levels reached (2). (B) Stability of σS in glucose-starved wild-type cells [DV206; closed squares], cells carrying the vector control [ÅF113; closed circles], cells carrying the uninduced [no IPTG] P~mc~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 σS in sprE::tet mutant cells carrying the uninduced [no IPTG] P~mc~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 σS in exponentially growing wild-type cells [closed squares], cells carrying the uninduced [no IPTG] P~mc~ssrA-gfp construct [ÅF142; open squares], and cells induced [+]IPTG for ssrA-gfp overexpression [open squares]. (E) A knockout mutation of sspB lowers σS levels in a SprE-dependent manner. Samples were taken in the exponential growth phase (LB). The presence [+I] 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 σS 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 σS, 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 σS 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 σS in stationary phase and found a reduced expression of σS-dependent genes (e.g., katE) and a reduced accumulation of σS (compared to aerobically starved cells) in cells starved for carbon anaerobically (Fig. 6A,B). In addition, the rpsL141 allele had no effect on σS-dependent gene expression or σS 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 σS 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 σS 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 σS levels since this shift allows oxidative modification of the accumulated pool of aberrant proteins to occur. Indeed, σS was rapidly, and transiently, accumulated during such a shift within a fraction of the generation time (Fig. 6D). Moreover, the accumulation of σS during such shifts in oxygen availability was reduced in cells carrying the
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**Discussion**

The σS 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 σS levels, the sensing–signaling device used by *E. coli* to trigger σS 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 σS 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; Wentzhel 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 σS. We suggest that such accumulation of σS 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 carboxylation [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 σS stabilization during carbon starvation (Fig. 7).

An important feature of the model is that one or several components of the σS degradation machinery must be limiting, at least during entry of cells into stationary phase. Indeed, overproduction of ClpP alone reduced σS accumulation and partly counteracted σS 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 σS in starved cells. In addition, decreased translational proofreading and overproduction of a ClpXP substrate stabilized σS already in exponential phase. Both elevated proofreading and ClpP overproduction required the pres-
elevation of SprE to destabilize $\sigma^5$, suggesting that the canonical SprE/ClpXP pathway achieves the degradation of the $\sigma$ factor under these conditions. In addition, the fact that $clpX$ and also $clpA$ deletions suppressed the instability of $\sigma^5$ 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 $\sigma^5$—for example, by elevated translation—will titrate out SprE and cause a drastic stabilization of $\sigma^5$ (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, $\sigma^5$ 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 $\sigma^5$ 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 $\sigma^5$ upon nitrogen starvation, whereas another mechanism works in parallel to ClpP titration during carbon starvation, giving rise to even higher levels of the $\sigma$ factor. This notion is consistent with the fact that there is residual induction of the rpoS regulon and accumulation of the $\sigma$ 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 $\sigma^5$ 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 $\sigma^5$ accumulation during phosphate starvation involves a novel protein, IraP, which interferes with SprE-dependent degradation of $\sigma^5$ during phosphate, but not carbon, starvation (Bougdour et al. 2006). In addition, increased translation of the rpoS transcript appears to be more important for $\sigma^5$ 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 $\sigma^5$ stability. Overproduction of $\sigma^5$ is difficult to achieve in exponentially growing cells (rich media—low levels of ppGpp) and in $\Delta{relA}\Delta{spoT}$ mutants (deficient in ppGpp), and we have noticed that $\sigma^5$ is unstable under such conditions despite the fact that overproduction ought to titrate out 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 $\sigma^5$ is not stabilized in this genetic background. This instability of $\sigma^5$ may be due to the fact that ppGpp is required for $\sigma^5$ to compete successfully for RNA polymerase (E) binding (Jishage et al. 2002). Thus, binding of $\sigma^5$ to E, which would protect the $\sigma$ factor from degradation, is another important aspect of regulating $\sigma^5$ stability and activity, and the involvement of ppGpp in this context provides an important hierarchy of physiological regulation. The requirement of $\sigma^5$ for ppGpp suggests that the $\sigma^5$ 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 $\sigma^5$ 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 $\sigma^5$ 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 $\sigma^5$ regulon. This may be the case during, for example, a shift from anaerobic to aerobic conditions. As seen in...
Figure 6, such a shift results in an immediate accumulation of σS. However, we found that the σS regulon genes are not induced during this shift (data not shown). This can be explained by the fact that this is a true upshift condition that does not elevate ppGpp levels. In fact, under such up-shift conditions, which primarily require housekeeping functions (Ere70), successful competition of σS 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 σS 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 σS 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-σS mouse monoclonal antibodies were from Neoclon, 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-D-thiogalactopyranoside (IPTG) were from Sigma. The chemiluminescence blotting substrate [ECL+] was obtained from Amershams Corp., and the Immobilon-P polyvinylidene 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 AF1 with a λ phase lysate harboring the appropriate construct. Monolysogeny was confirmed by PCR (Powell et al. 1994). The λΔ[katE-lacZ] construct was from Ohnuma et al. [2000], and the Δ[popS-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 AF80, AF81, AF82, AF84, AF132, and AF133, clpA319::kan [Katayama et al. 1988], rssA2::cam [strain from strain NR419, N. Ruiz], and sprE::tet [strain from strain NR253, N. Ruiz] were introduced into DV206 and AF1 by λI transduction. Strains MBN19 and MBN20 were similarly generated by transduction of ΔclpP1::kan [Katayama et al. 1988] into MBN7 and MBN8. Strain AF39 was constructed by transformation of AF1 with plasmid pBB535 [Takayama et al. 1982] and AF43 by transformation with pUC18 and pAF2.

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/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), carbenicillin (12.8 mg/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% CO2 and 95% N2 as described [Valadi et al. 2001]. To overproduce DnaK and DnaJ, 200 µM IPTG was added to exponentially growing cultures of strains AF38 and AF39 at an OD420 of 0.05 to induce expression from the plasmid pBB535, and to induce clpP expression from the plasmid pAF2, 50 µM IPTG was added at an OD420 of 0.1. To overproduce the ssrA-tagged GFP, 100 µM IPTG was added to exponentially growing cultures of strain AF142 at an OD420 of 0.05.

General methods

Pl transductions, plasmid transformations, and λ-aphage lysogeny were performed as described by Miller [1972] and Sambrook and Russell (2001). Protein extracts where prepared according to Sambrook and Russell (2001). σS, 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 σS, 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 A240nm of hydrogen peroxide as described previously [Gonzalez-Flecha et al. 1993]. One unit of
Table 1.  E. coli strains used in this work

| Designation | Sex, extrachromosomal markers | Genotype, chromosomal markers | Origin |
|-------------|-------------------------------|--------------------------------|--------|
| DV206       | F                            | λ− ilvC– rfb–50 rph–1 lacIZΔ[Mlt] | Vinella et al. 2005 |
| MC4100      | F                            | araD139A [argF-lac]U169 rpsL150 relA1 | Casadaban 1976 |
| CNP119      | F                            | MC4100 λ[pps750–lac]Z] | Sledjeski et al. 1996 |
| CNP217      | F                            | CNP119 sprE::cm | 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] | L.A. Isaksson |
| S12/U4      | F, proAB lacI [UGA]ZYA       | [As above] | L.A. Isaksson |
| Æ1i         | F                            | DV206 rpsL141 | Ballesteros et al. 2001 |
| MBN7        | F                            | DV206 λ[Kat–lac]Z] | This study |
| MBN8        | F                            | Æ1 λ[Kat–lac]Z] | This study |
| MBN19       | F                            | MBN7 ΔclpX1::kan | This study |
| MBN20       | F                            | MBN8 ΔclpX1::kan | This study |
| MBN27       | F                            | DV206 acrF::Tn10 rpsD12 | Fredriksson et al. 2006 |
| MBN31       | F                            | DV206 λ[pso–lacZ]OF | This study |
| MBN32       | F                            | Æ1 λ[pso–lacZ]OF | This study |
| MBN34       | F                            | DV206 λ[pso–lacZ]PF212 | This study |
| MBN35       | F                            | Æ1 λ[pso–lacZ]PF212 | This study |
| MBN37       | F                            | DV206 λ[pso–lacZ]PF977 | This study |
| MBN38       | F                            | Æ1 λ[pso–lacZ]PF977 | This study |
| Æ38         | F, pBB535                    | DV206 | Fredriksson et al. 2006 |
| Æ39         | F, pBB535                    | Æ1i | This study |
| Æ80         | F                            | DV206 clpA319::kan | This study |
| Æ81         | F                            | Æ1 clpA319::kan | This study |
| Æ82         | F                            | DV206 rssA2::Tncam | This study |
| Æ84         | F                            | Æ1 rssA2::Tncam | This study |
| Æ132        | F                            | DV206 sprE::tet | This study |
| Æ133        | F                            | Æ1 sprE::tet | This study |
| Æ134        | F, pUC18, pBB528             | Æ132 | This study |
| Æ135        | F, Æ1F2, pBB528              | Æ132 | This study |
| Æ138        | F, pUC18, pBB528             | DV206 | This study |
| Æ139        | F, Æ1F2, pBB528              |DV206 | This study |
| Æ142        | pGFP-ssrA, F, proAB lacI^n   | ZDM15::Tn10[tet]^[R] | This study |
| Æ143        | pGFP-ssrA, F, proAB lacI^n   | ZDM15::Tn10[tet]^[R] | This study |

| Plasmid | Gene construct | Resistance marker | Inducer | Origin |
|---------|----------------|-------------------|---------|--------|
| pUC18   | Vector         | Ampicillin        | IPTG    | Yanisch-Perron et al. 1985 |
| pÅF2    | pLacl–ClpP     | Ampicillin        | IPTG    | This study |
| pBB528  | lacI^[R]       | Chloramphenicol   | IPTG    | Tomoyasu et al. 2001 |
| pGFP-SsrA | gfp-ssrAu    | Ampicillin        | IPTG    | Dougan et al. 2003 |
| pBB535  | pA1/lacO, r–dnaKdnaf | 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

σ5 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].
Canavine 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 canavine 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 $\sigma^r$ 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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