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Increased RNA polymerase availability directs resources towards growth at the expense of maintenance

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Nutritionally induced changes in RNA polymerase availability have been hypothesized to be an evolutionary primeval mechanism for regulation of gene expression and several contrasting models have been proposed to explain how such 'passive' regulation might occur. We demonstrate here that ectopically elevating *Escherichia coli* RNA polymerase ($E\sigma^{70}$) levels causes an increased expression and promoter occupancy of ribosomal genes at the expense of stress-defense genes and amino acid biosynthetic operons. Phenotypically, cells overproducing $E\sigma^{70}$ favours growth and reproduction at the expense of motility and damage protection; a response reminiscent of cells with no or diminished levels of the alarmone guanosine tetraphosphate (ppGpp). Consistently, we show that cells lacking ppGpp displayed markedly elevated levels of free $E\sigma^{70}$ compared with wild-type cells and that the repression of ribosomal RNA expression and reduced growth rate of mutants with constitutively elevated levels of ppGpp can be suppressed by overproducing $E\sigma^{70}$. We conclude that ppGpp modulates the levels of free $E\sigma^{70}$ and that this is an integral part of the alarmone's means of regulating a trade-off between growth and maintenance.

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Introduction

The most pervasive means of altering the relative abundance of transcripts in the cell is to control the efficiency at which the RNA polymerase initiates transcription at different promoters. In *Escherichia coli*, RNA polymerase consists of the core enzyme (E) containing the $\alpha_2\beta\beta'\omega$ subunits, and one of the seven possible sigma (σ) factors conferring promoter specificity to E by making sequence-specific contacts to DNA (Gross *et al*, 1992). The majority of genes (housekeeping genes) expressed during exponential growth of *E. coli* require σ^{70} (encoded by *rpoD*) for transcription initiation. This sigma factor directs E to genes encoding proliferation-related activities, including the genes of the protein synthesizing system (PSS; i.e. those encoding the ribosomes, tRNAs and factors required for translation). In addition, a large number of maintenance and stress-defense genes induced upon growth limitations are also dependent on the $E\sigma^{70}$ holoenzyme. Directing $E\sigma^{70}$ to appropriate promoters (or inhibiting promoter contacts) under changing conditions is to a large part accomplished by specific activators and repressors. However, $E\sigma^{70}$-dependent genes have been argued to be subjected to differential control also by changes in $E\sigma^{70}$ availability, a so-called passive regulation of gene expression.

Ole Maaløe was perhaps first in presenting a model for passive control of gene expression inspired by the conviction that the massive alterations of gene expression needed during drastically changing growth conditions, such as nutrient limitations, required a more robust control than those afforded by specific repression, de-repression, and activator circuits (Maaløe, 1979). This type of gene regulation might have been operational before the evolution of specific regulatory factors and maintained some of its importance in representing a regulatory baseline control on top of which more specific and pronounced effects can be achieved with activators and repressors. Maaløe suggested, for example, that the synthesis of the PSS is regulated by $E\sigma^{70}$ availability, which changes with changing growth conditions (Bremer and Dennis, 1996). Although details of Maaløe's original model has been found wanting, the assumption that individual promoters compete with each other for a limiting amount of free $E\sigma^{70}$ has been confirmed (Shepherd *et al*, 2001; Jishage *et al*, 2002; Bremer *et al*, 2003; Magnusson *et al*, 2003; Grigorova *et al*, 2006).

Several authors have presented updated models for passive regulation and endorsed passive control as an integral part of stringent control by the nucleotide guanosine tetraphosphate (ppGpp). Cells of *E. coli* elicit a swift down-regulation of the PSS during amino acid starvation—a response called the stringent response—and ppGpp, acting together with the protein DksA, is the effector molecule of this response (reviewed in Potrykus and Cashel, 2008). The nucleotide ppGpp is produced by RelA and/or SpoT. This occurs not only in response to amino acid limitation but also upon starvation for many different kinds of nutrients and in circumstances restricting growth (Cashel *et al*, 1996). A key feature in one passive model of gene regulation, referred to

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here as the 'saturation model', is that altered availability of free $E\sigma^{70}$ affects different promoters differently based on the fact that promoters display diverse saturation properties (Jensen and Pedersen, 1990). For example, a reduction in $E\sigma^{70}$ availability is argued to specifically reduce expression from rRNA promoters and other promoters of the PSS as it is reasonable to assume that genes whose products (ribosomes in this case) are in high demand will possess promoters exhibiting high maximal velocity. Indeed, no other promoters seem to initiate transcription with the same high frequency as *rrn* promoters (estimated to have up to 90 initiations per minute per *rrn* gene at fast growth (Dennis *et al*, 2004)) and thus, despite an excess of about 2000 mRNA promoters, the small number of rRNA promoters are responsible for >50% of all transcripts made during exponential growth (Wagner, 2000). As *rrn* promoters are not saturated with $E\sigma^{70}$ under most growth conditions, their activities are argued to increase with increasing growth rates because of increasing concentrations of free $E\sigma^{70}$ (Jensen and Pedersen, 1990; Dennis *et al*, 2004). In contrast to rRNA genes (and other PSS genes), σ^{70}-dependent promoters that are easily saturated, such as promoters driving stress-defense genes, would, in the saturation model, be repressed in relative terms, upon an increased pool size of $E\sigma^{70}$.

In contrast to the 'saturation model' described above, an alternative model points to promoter affinity as the key feature behind differential control by alterations in the concentration of $E\sigma^{70}$ (Zhou and Jin, 1998; Barker *et al*, 2001a, b), referred to here as the 'Affinity Model'. This model argues for an increased, rather than decreased, availability of free $E\sigma^{70}$ during slow growth and a stringent response. Such an increase in $E\sigma^{70}$ concentration is suggested to be a result of $E\sigma^{70}$ falling off rRNA promoters because of decreased open complex stability elicited by ppGpp (Gaal *et al*, 1997; Barker *et al*, 2001b), whose concentration is inversely dependent on growth rate and is drastically elevated during nutrient exhaustion (Cashel, 1969; Ryals *et al*, 1982; Zacharias *et al*, 1989; Teich *et al*, 1999). As a consequence, promoters with relatively poor ability to recruit $E\sigma^{70}$—in the affinity model argued to be those requiring ppGpp for their induction (e.g. stress-defense genes and amino acid biosynthetic operons)—are suggested to be induced upon growth limitation and elevated ppGpp levels because of the increased availability of $E\sigma^{70}$. Although it has been argued that the data supporting this model place considerable constraints on models invoking hypothetical factors that might increase amino acid promoter activity in a ppGpp-dependent manner (Barker *et al*, 2001a), it was later shown that DksA is indeed such a factor (Brown *et al*, 2002; Paul *et al*, 2004a, 2005). Hence, the passive models for gene regulation need to be considered as working in concert with, or in parallel to, direct and active control by ppGpp/DksA.

Both models of passive regulation suggest that alterations in the availability of free $E\sigma^{70}$ may re-direct transcriptional resources between growth-related activities (PSS) and maintenance/stress defenses but the predictions of the models are conflicting in almost every instance possible (Figure 1). For example, whereas the affinity model predicts that increased $E\sigma^{70}$ concentration would lead to an increased expression of maintenance and amino acid biosynthetic genes at the expense of PSS genes (i.e. a stringent-like pattern of gene expression) the saturation model predicts the exact opposite (i.e. a relaxed response, a phenotype linked to ppGpp deficiency). The affinity model argues for a direct correlation between ppGpp concentration and the pool size of free $E\sigma^{70}$, whereas the saturation model predicts an inverse correlation. In addition, overproduction of functional $E\sigma^{70}$ is in the saturation model envisaged to suppress the stringent phenotype of mutant cells with constitutively elevated levels of ppGpp, whereas the affinity model expects this to be ineffective or counterproductive. In this work, we report on a set of experiments designed to test these contrasting predictions experimentally.

Results

Overproduction of $E\sigma^{70}$ elevates expression of PSS genes

To test the effect of increasing $E\sigma^{70}$ holoenzyme availability, we used two plasmids encoding the subunits of core RNA polymerase (E) (i.e. the α, β, β′ subunits) and the housekeeping sigma factor σ^{70} under the control of P*tac* promoters (Figure 2A) (Dykxhoorn *et al*, 1996). Western blot analysis confirmed that all $E\sigma^{70}$ subunit proteins were overproduced ~2–3-fold during exponential growth by the addition of isopropyl-β-D-thiogalactopyranoside (IPTG) (Figure 2B). Further analysis showed that the two-fold increase was kept in stationary phase (data not shown). To asses whether this increase in subunits also resulted in an increased level of $E\sigma^{70}$ complexes, we measured the amount of σ^{70} that co-purified with the core enzyme using an affinity column with antibodies against β′. The over-expression of all subunits resulted in a more than two-fold increase of $E\sigma^{70}$ complexes, whereas overproduction of α and σ^{70} did not (Figure 2C). It

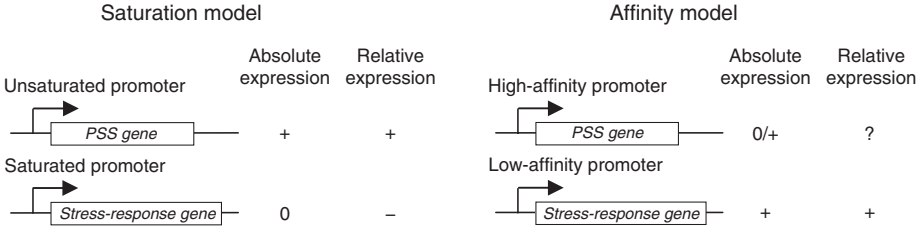

Figure 1 The predictions from the two models of passive regulation upon increase in $E\sigma^{70}$ availability. In the saturation model, an increase in $E\sigma^{70}$ availability would increase (+) absolute expression from unsaturated promoters, whereas saturated promoters will not be affected (0). A saturated promoter would in this case be relatively repressed (−). In contrast, the affinity model argues that an increase in $E\sigma^{70}$ availability would not benefit a high-affinity promoter (0/+), only promoters with low affinity would have an increase in absolute expression (+). Thus, in relative terms, a low-affinity promoter is predicted to have an increase in relative expression (+), whereas no prediction can be made for high-affinity promoters (?). The two models then make opposite predictions for relative expression from stress response genes (saturated, low affinity) upon an increase in $E\sigma^{70}$.

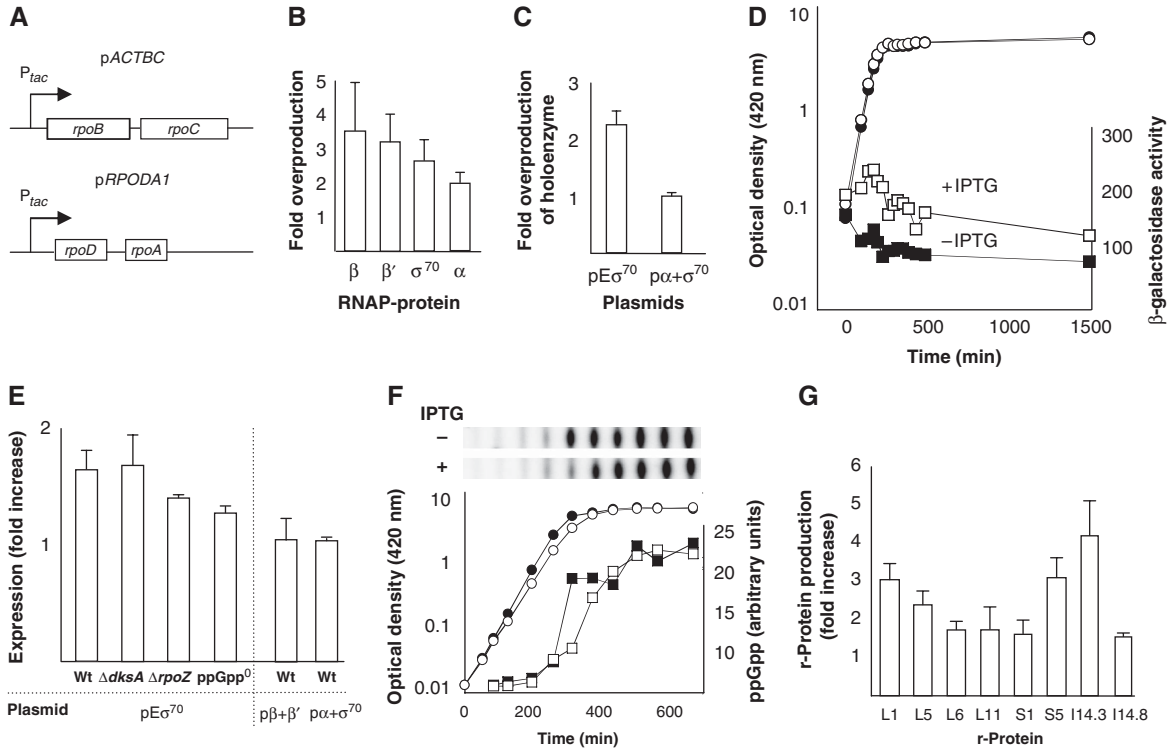

Figure 2 Overproduction of Eσ^{70} elevates expression from PSS genes. (**A**) Expression vectors; p*ACTBC* and p*RPODA1*, expressing β + β′ and σ^{70} + α, respectively, under the control of a P*tac* promoter (Dykxhoorn *et al*, 1996). (**B**) Quantification of Eσ^{70} subunit overproduction. Samples were taken in exponential growth phase. (**C**) Quantification of σ^{70} subunits co-purified with β′ (core enzyme) in a strain containing expression vectors p*ACTBC* and p*RPODA1* (pEσ^{70}) and in a strain containing only p*RPODA1* (pσ^{70} + α). (**D**) Growth (circles) and expression from the *rrnB*P1$_{-41-+50}$ promoter (squares) with (open symbols; 1 mM IPTG) and without (closed symbols; no IPTG) overproduction of Eσ^{70}. (**E**) Fold increase in expression from the *rrnB*P1$_{-41-+50}$ promoter upon Eσ^{70}, β + β′, or σ^{70} + α overproduction. The first four bars from the left show the effect of overproducing Eσ^{70} in wild-type (Wt), Δ*dksA*, Δ*rpoZ*, and ppGpp^0 cells as indicated. The last two bars shows overproduction of only β and β′ subunits (pβ + β′) and of only σ^{70} and α subunits (pσ^{70} + α) in wild-type (Wt) cells. (**F**) The effects of Eσ^{70} overproduction on the levels of ppGpp. Growth (circles) and ppGpp levels (squares) with (open symbols; 1 mM IPTG) and without (closed symbols; no IPTG) overproduction of Eσ^{70}. The autoradiograms of the thin-layer chromatography (TLC) plates are shown in the upper panel. (**G**) The effects of Eσ^{70} overproduction on ribosomal protein production as detected on 2D gels. The bars indicate the difference in relative production of the particular protein with (1 mM IPTG) compared with without (no IPTG) Eσ^{70} overproduction.

should also be noted that this overproduction has but a marginal effect on growth (Figure 2D).

We first examined the effect of Eσ^{70} overproduction on *rrnB*P1 promoter activity, which is under negative control by ppGpp (Cashel *et al*, 1996; Paul *et al*, 2004b). Expression from both promoter constructs, *rrnB*P1$_{-41-+50}$-*lacZ* (Figure 2D) and *rrnB*P1$_{-51-+50}$-*lacZ* (not shown), was increased about two-fold as a result of Eσ^{70} overproduction. This increase was observed both during growth and stationary phase (Figure 2D). Overproduction of only β and β′, or of only α and σ^{70} did not result in increased expression from *rrnB*P1 (Figure 2E), demonstrating that the effect on expression is a specific effect of increased Eσ^{70} concentration rather than of overproduction *per se*.

Earlier *in vitro* experiments suggested that the E subunit ω is required for Eσ^{70} sensitivity to ppGpp, but that this requirement is relieved in the presence of DksA (whose activity is independent of ω) (Vrentas *et al*, 2005). In line with the fact that ω seems of minor importance for Eσ^{70} function when DksA is present in the cell, we found an increased expression from *rrnB*P1 upon overproduction of Eσ^{70} in mutants lacking ω (Δ*rpoZ*) (Figure 2E).

As the *rrn* genes are negatively regulated by ppGpp, we tested whether the elevated *rrnB*P1 activity observed upon Eσ^{70} overproduction was a result of reduced levels of ppGpp. This was not the case as the concentration of ppGpp was not significantly altered during either growth or starvation by overproducing Eσ^{70} (Figure 2F). We also found that overproduction of Eσ^{70} effectively increased *rrnB*P1 expression in cells lacking DksA (Figure 2E), a protein influencing the regulatory activities of ppGpp (Paul *et al*, 2004a; Magnusson *et al*, 2007). As an additional control, Eσ^{70} was also overproduced in a ppGpp^0 background and despite the fact that the lack of ppGpp itself elevates expression from *rrnB*P1, Eσ^{70} overproduction resulted in a further 1.3-fold increased expression (Figure 2E). Thus, we conclude that overproduction of Eσ^{70} does not change the ppGpp levels and that it elevates expression from *rrnB*P1 independent of both DksA and ppGpp.

Non-equilibrium pH 2D polyacrylamide gel electrophoresis (NEPHGE) was used to examine the pattern of ribosomal protein (r-protein) production upon Eσ^{70} overproduction. The r-proteins analysed are shown on a standard NEPHGE gel (Supplementary Figure S1). Pulse labelling demonstrated that the rates of synthesis of all r-proteins analysed were markedly higher in the cells overproducing Eσ^{70} (Figure 2G) ranging from 1.5- to 4-fold induction. The data suggest that production of the PSS is elevated upon

increased availability of $E\sigma^{70}$, in line with predictions of the saturation model.

Overproduction of $E\sigma^{70}$ attenuates expression of stress-defense genes and amino acid biosynthetic operons

We next tested the effect of $E\sigma^{70}$ overproduction on the expression of the $E\sigma^{70}$-dependent *usp* genes (Nystrom and Neidhardt, 1996; Gustavsson *et al*, 2002; Nachin *et al*, 2005), which, in contrast to genes of the PSS, are positively regulated by ppGpp (Kvint *et al*, 2000; Gustavsson *et al*, 2002). The expression from all the *usp*-promoters tested, *uspA/uspD/uspF* (Figure 3A and C), and *uspC/uspE* (not shown), were markedly attenuated upon $E\sigma^{70}$ overproduction. The expressions from promoters of amino acid biosynthetic genes, like promoters of the *usp* genes, also require ppGpp. Similar to the *usp* promoters, the expression from both *livJ* and *thrABC* promoters (Figure 3C) were negatively affected by $E\sigma^{70}$ overproduction. As a control, we overproduced only β and β′ or only α and σ^{70}, which confirmed that the decreased induction of the *uspA* promoter is specific to $E\sigma^{70}$ overproduction (Figure 3B).

In addition, we confirmed that $E\sigma^{70}$ overproduction decreased the induction also in mutants lacking ω (Δ*rpoZ*) (Figure 3B) and that a Δ*rpoZ* strain, in the absence of $E\sigma^{70}$ overproduction plasmids, do not exhibit altered *uspA* promoter activity (Supplementary Figure S2). Further, overproduction of ω cannot restore *uspA* expression in cells lacking either ppGpp or DksA (Supplementary Figure S2). Thus, we conclude that the effects of overproducing $E\sigma^{70}$ are not because of the absence of simultaneous overproduction of the ω subunit.

Repression of σ^{70}-dependent stress-defense genes and amino acid biosynthetic operons upon ectopic elevation of $E\sigma^{70}$ levels is in accord with the saturation model (Jensen and Pedersen, 1990; Dennis *et al*, 2004), which argues that such genes are driven by promoters that are much more easily saturated than *rrn* promoters and would not, relative to *rrn*, benefit from increased concentrations of free $E\sigma^{70}$. In other words, the apparent repression of promoters like P*uspA* upon $E\sigma^{70}$ overproduction is predicted to be a relative rather than absolute phenomenon and because of the fact that *rrn* promoters, in comparison, have a superior capacity to accommodate high levels of $E\sigma^{70}$. We tested this notion by chromatin immunoprecipitation (ChIP) using antibodies against β′ and σ^{70}. This analysis demonstrated that $E\sigma^{70}$ overproduction *in vivo* resulted in an 8- and 11-fold increase in the ratio of promoter occupancy at *rrn* and *uspA* promoters, for β′ and σ^{70}, respectively (Figure 3D). In contrast,

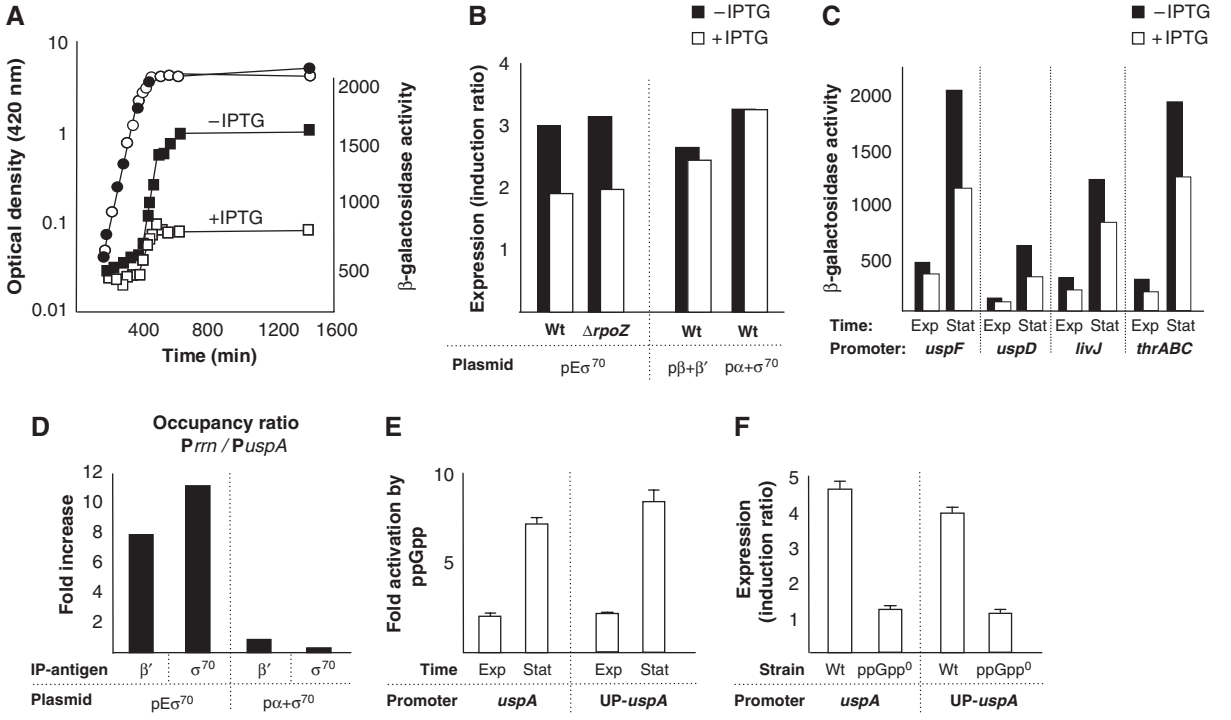

Figure 3 Expression of stress-defense genes and amino acid biosynthetic operons is attenuated upon $E\sigma^{70}$ overproduction. (**A**) Growth (circles) and expression from the P*uspA* promoter (squares) with (open symbols; 1 mM IPTG) and without (closed symbols; no IPTG) overproduction of $E\sigma^{70}$. (**B**) Induction ratios (stationary phase promoter activity/exponential growth promoter activity) for the *uspA* promoter with (open bars; + 1 mM IPTG) and without (closed bars; -IPTG) overproduction of $E\sigma^{70}$, β + β′, or σ^{70} + α. The first four bars from the left show the effect of overproducing $E\sigma^{70}$ ($pE\sigma^{70}$) in wild-type (Wt) and Δ*rpoZ* cells as indicated. The last four bars shows overproduction of only β and β′ subunits (pβ + β′) and of only σ^{70} and α subunits ($p\sigma^{70}+\alpha$) in wild-type (Wt) cells. (**C**) Expression from P*uspF*, P*uspD*, P*livJ*, and P*thrABC* promoters during exponential growth (Exp) and in stationary phase (Stat) with (open bars; + 1 mM IPTG) and without (closed bars; -IPTG) overproduction of $E\sigma^{70}$ (**D**) DNA isolated from formaldehyde-fixed cells with (1 mM IPTG) and without (no IPTG) overproduction of $E\sigma^{70}$ ($pE\sigma^{70}$) or σ^{70} and α ($p\sigma^{70}+\alpha$) alone was subjected to immunoprecipitation using antibodies against either the β′ or the σ^{70} subunit of $E\sigma^{70}$. PCR-based amplification of co-precipitated DNA was done using specific primers to the *uspA* promoter or to the *rrn* promoters. The $E\sigma^{70}$ promoter occupancy ratio (P*rrn*/P*uspA*) of cells not subjected to IPTG was set to 1 and all other values are expressed relative to that. Representative data are shown. (**E**, **F**) Effect on ppGpp-dependent expression from a *uspA* promoter upon addition of an UP element. (E) Fold induction of the P*uspA* and UP-P*uspA* promoters by ppGpp (wild-type promoter activity/$ppGpp^0$ promoter activity) during exponential growth (Exp) and in stationary phase (Stat). (F) Induction ratios (stationary phase promoter activity/exponential growth promoter activity) for the P*uspA* and UP-P*uspA* promoters in wild-type (Wt) and $ppGpp^0$ strains.

overproduction of only α and σ^{70} did not significantly alter the occupancy ratio, in line with the lack of effect on expression from these promoters (Figures 2E and 3B).

In contrast to the saturation model, the affinity model predicts that $E\sigma^{70}$ overproduction would benefit promoters positively regulated by ppGpp, such as the P*uspA* and P*thrABC* tested here, as the defining characteristics of such promoters, according to this model, is their poor ability to recruit $E\sigma^{70}$. It has been reported that P*argI*, a promoter that is positively regulated by ppGpp, becomes less dependent on ppGpp by inserting an UP element just upstream the −35 hexamer and thereby increasing the promoter-$E\sigma^{70}$ association rate, supporting the affinity model (Barker *et al*, 2001a). We constructed a derivative of the *uspA* promoter fused to the UP element (see Materials and methods) and tested to what extent this diminished the promoters' requirement for ppGpp. The fold activation by ppGpp in both exponential and stationary phase was similar for the wt P*uspA* and the UP-P*uspA* promoters (Figure 3E) and the induction of the two promoters upon glucose starvation was the same in both the wt and $ppGpp^0$ (Δ*relA* Δ*spoT*) genetic backgrounds (Figure 3F). Thus, we conclude that the ppGpp-dependent expression from a *uspA* promoter is insensitive to addition of an UP-element to the promoter, suggesting that it is insensitive to increased $E\sigma^{70}$ affinity.

Overproduction of $E\sigma^{70}$ creates a phenocopy of relaxed cells

The results presented suggest that cells with increased levels of $E\sigma^{70}$ holoenzyme exhibit a 'relaxed' phenotype, that of cells lacking ppGpp, with respect to gene expression of stringently regulated promoters. We, therefore, tested whether strains with elevated $E\sigma^{70}$ levels exhibited any other of the phenotypes associated with a relaxed response. Indeed, we observed that cells overproducing $E\sigma^{70}$ were, similar to a $ppGpp^0$ strain (Magnusson *et al*, 2007), defective in motility on 0.3% agar plates (Figure 4).

As a $ppGpp^0$ mutant and an $E\sigma^{70}$ overproducing strain seem to direct its transcriptional resources predominantly to growth and the production of the PSS at the expense of stress-defense systems, we hypothesized that such cells may show signs of diminished maintenance activities and, as a consequence, elevated damage to its cellular constituents. Protein carbonylation serves as a general diagnostic marker of a diminished performance of maintenance systems and increases as a consequence of, for example, diminished oxidative stress defenses, decreased translational proof-reading, reduced proteolytic activities, or mutations in chaperone genes (Dukan *et al*, 2000; Ballesteros *et al*, 2001; Fredriksson *et al*, 2006). As shown in Figure 5A and B, protein carbonyl levels were markedly higher in the relaxed $ppGpp^0$ strain during both exponential growth and upon growth arrest. Similarly, $E\sigma^{70}$ overproduction elevated carbonylation during both growth and growth arrest (Figure 5C and D), suggesting, again, that $E\sigma^{70}$ overproduction mimics a relaxed phenotype. However, $E\sigma^{70}$ overproduction did not result in amino acid auxotrophy (not shown), a phenotype seen in $ppGpp^0$ strains (Xiao *et al*, 1991).

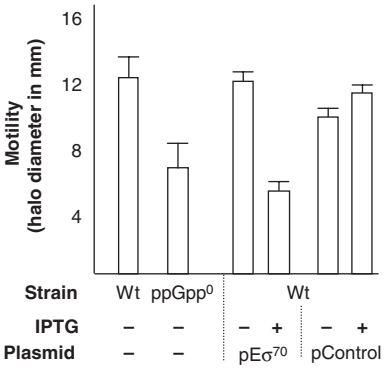

Figure 4 Overproduction of $E\sigma^{70}$ decreases motility, a phenocopy of a relaxed cell. Motility measured as the diameter of the halo formed after 24 h incubation on soft agar plates of wild-type (Wt), $ppGpp^0$, and a $E\sigma^{70}$ overproduction strain and vector control strain with and without addition of 1 mM IPTG.

The pool size of free $E\sigma^{70}$ is elevated in cells lacking ppGpp

As overproduction of $E\sigma^{70}$ mimics a relaxed response, we asked whether cells lacking ppGpp display an elevated concentration of free $E\sigma^{70}$ and whether such elevation is, in fact, an integral part of the relaxed response. To measure the levels of cytoplasmic free $E\sigma^{70}$, a minicell approach was used (Rogerson and Stone, 1974; Shepherd *et al*, 2001). A strain with an inactivated *minB* locus allows septum formation at the cell poles resulting in the possibility to form either two normal (large) cells or one chromosome-free minicell and a filamentous-like cell (de Boer *et al*, 1988). The $E\sigma^{70}$ content in the minicells will represent free $E\sigma^{70}$ as they are devoid of DNA. Large cells and chromosome-free minicells from wild-

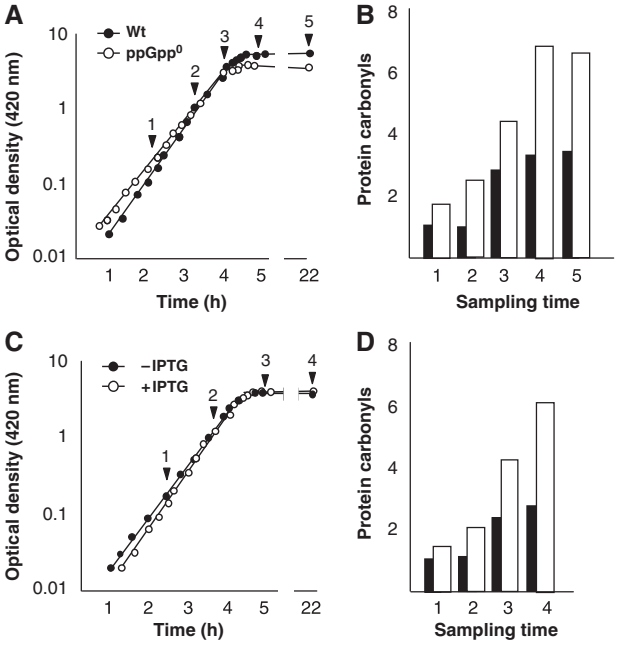

Figure 5 Overproduction of $E\sigma^{70}$ increases protein carbonylation, a phenocopy of a relaxed cell. (**A**) Growth of wild-type (closed symbols) and $ppGpp^0$ (open symbols) cells. The arrows indicate where samples for protein carbonylation measurements were taken. (**B**) Quantified carbonylation levels of wild-type (closed bars) and $ppGpp^0$ (open bars) cells. (**C**) Growth of a wild-type strain containing the $E\sigma^{70}$ overproduction plasmids with (open symbols) or without 1 mM IPTG (closed symbols). The arrows indicate where samples for protein carbonylation measurements were taken. (**D**) Quantified carbonylation levels, with (open bars) and without (closed bars), overproduction of $E\sigma^{70}$.

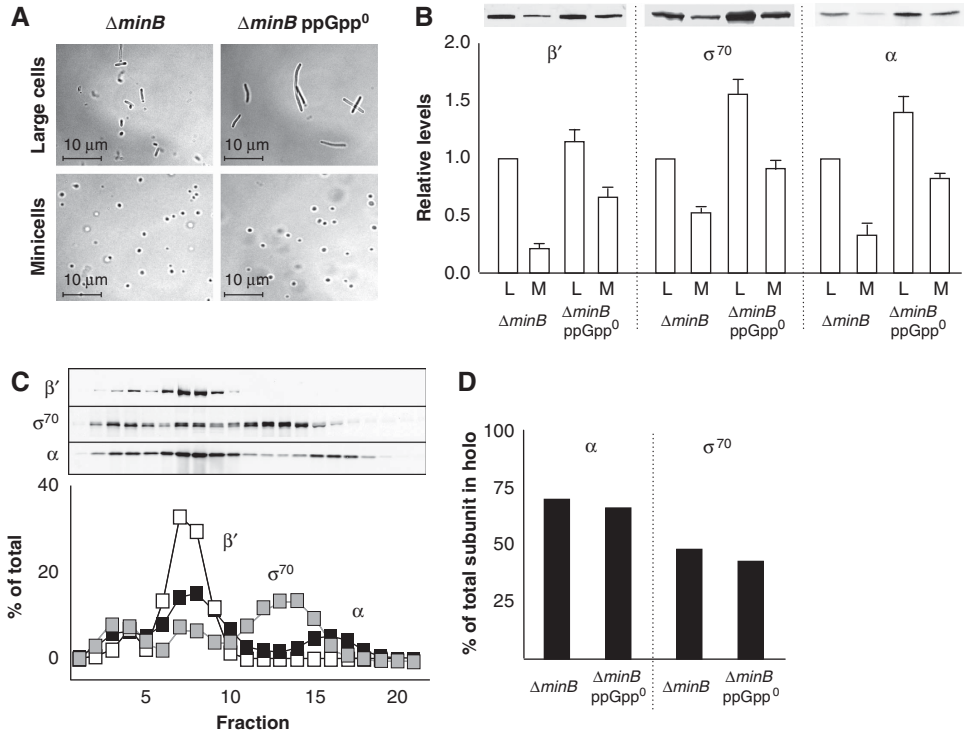

Figure 6 A ppGpp^0 strain displays elevated levels of free Eσ^{70}. (**A**) A typical image of a 1000 × magnification of isolated large cell and minicell fractions from Δ*minB* and Δ*minB* ppGpp^0 strains. (**B**) Relative levels of Eσ^{70} subunits β′ (left panel), σ^{70} (centre panel), and α (right panel) in large cells (L) and minicells (M) from Δ*minB* and Δ*minB* ppGpp^0 strains. Upper panel shows representative blots of β′, σ^{70}, and α, respectively. Equal protein concentration was loaded. (**C**) Western blot and the corresponding quantifications of gel-filtrated fractions from the Δ*minB* ppGpp^0 minicell lysate. (**D**) The relative amount of total subunit that co-elutes with β′ (holo) in Δ*minB* and Δ*minB* ppGpp^0 cells.

type (Δ*minB*) and ppGpp^0 (Δ*minB* Δ*relA* Δ*spoT*) were isolated (Figure 6A) and assayed for their content of Eσ^{70}. The concentration of free Eσ^{70}, as estimated from measurements of α, β′, and σ^{70} levels, were clearly higher in the ppGpp^0 cell (~60% of total Eσ^{70} was free) compared with the wild-type cell (~30% of total Eσ^{70} was free), demonstrating that relaxed cells indeed have elevated concentrations of free Eσ^{70} (Figure 6B).

To ascertain that the increased concentration of Eσ^{70} subunits in ppGpp^0 cells represented increased levels of free holoenzyme, rather than free subunits, we fractionated crude cell extracts by gel filtration and the content of α, β′, and σ^{70} subunits in each fraction were quantified (one example, Δ*minB* ppGpp^0 minicells, is shown in Figure 6C). This analysis demonstrated that there was only a minor difference between wild-type and ppGpp^0 cells in the relative amount of α and σ^{70} bound to E (Figure 6D). However, the somewhat lower fractions of bound α and σ^{70} in the ppGpp^0 strain corresponds well with the fact that the increase in total levels of these subunits, compared with wild type, is higher than the increase of β′ (Figure 6B). Thus, the increased concentrations of α, β′, and σ^{70} subunits in the absence of ppGpp reflect a higher level of free Eσ^{70} holoenzyme, which seems to be limited by the concentration of β′ (and probably β).

Overproduction of Eσ^{70} counteracts the effect of elevating ppGpp

If, as predicted by the saturation model, stringency is partly accomplished by a reduction in free Eσ^{70} levels, an overproduction of Eσ^{70} should to some extent repress the stringent response. To test this possibility, we analysed expression from *rrn*, *uspA*, and *thrAB* promoters in a *spoT201* mutant strain exhibiting constitutively elevated levels of ppGpp because of a diminished ppGpp hydrolysis activity of the mutated SpoT protein. The *spoT201* allele results in 2.5-fold increase in ppGpp concentration and mutants carrying this allele display the mildest growth rate reduction of all mutant *spoT* alleles that have been examined (Sarubbi *et al*, 1988). In line with earlier studies of this strain (Sarubbi *et al*, 1988), the *spoT201* allele represses expression from the *rrnB*P1 promoter (Figure 7A). Interestingly, this repression could be suppressed by overproducing Eσ^{70} in the *spoT201* strain (Figure 7A), indicating that the reduced expression of *rrn* genes in cells with elevated ppGpp levels is, indeed, at least partly because of limitation in Eσ^{70} availability. In fact, such a limitation in Eσ^{70} seems to be partly responsible for the reduced growth rate of the s*poT201* mutant, as Eσ^{70} overproduction greatly shortened the generation time of the cells (from 105 to 70 min; Figure 7B). As seen in Figure 7C, introducing the s*poT201* allele elevates both *uspA* and *thrABC* promoter activity and overproducing Eσ^{70} totally suppressed this up-regulation of *uspA* and *thrABC* (Figure 7D). Taken together, the data demonstrate that elevating Eσ^{70} counteracts stringency and argues for limitations in free Eσ^{70} being an integral regulatory component of a stringent response of *E. coli*.

Discussion

We demonstrate here that a two-fold overproduction of Eσ^{70} elevates the production of PSS at the expense of stress-defense genes (*usps*) and amino acid biosynthetic

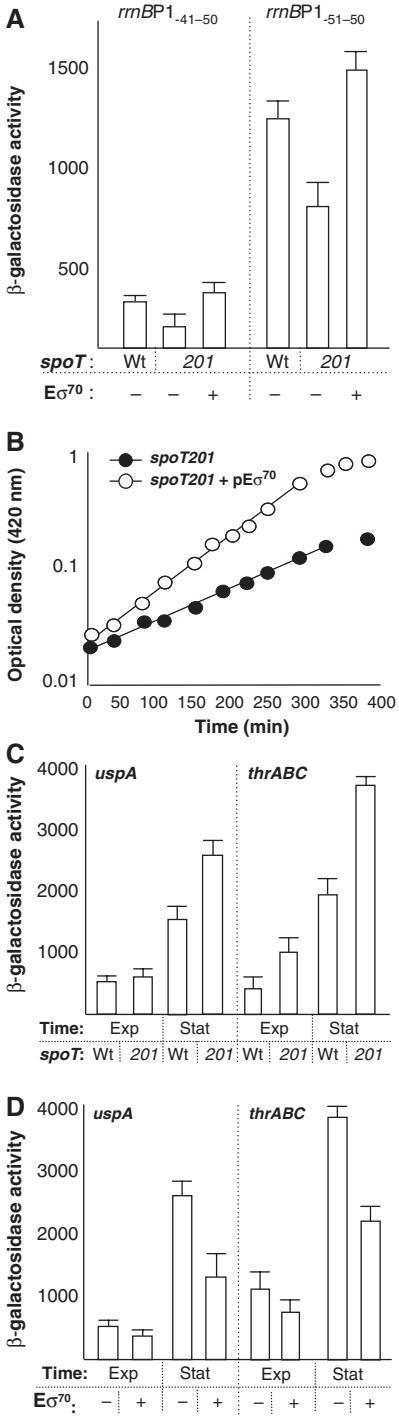

Figure 7 Overproduction of $E\sigma^{70}$ suppresses the effect of elevated ppGpp levels. (**A**) Gene expression from P*rrnB* $P1_{-41-50}$ and P*rrnB* $P1_{-51-50}$ during exponential growth in wild type (wt) and a strain with *spoT201* (201) with or without overproduction of $E\sigma^{70}$. (**B**) Growth rate of *spoT201* cells without (closed circles; no IPTG) and with (open circles; 1 mM IPTG) overproduction of $E\sigma^{70}$. (**C**) Expression from P*uspA* and P*thrABC* during exponential growth (Exp) and stationary phase (Stat) in wild-type (Wt) and *spoT201* (201) cells. (**D**) Expression from P*uspA* and P*thrABC* during exponential growth (Exp) and stationary phase (Stat) in the *spoT201* strain with (1 mM IPTG) and without (no IPTG) overproduction of $E\sigma^{70}$.

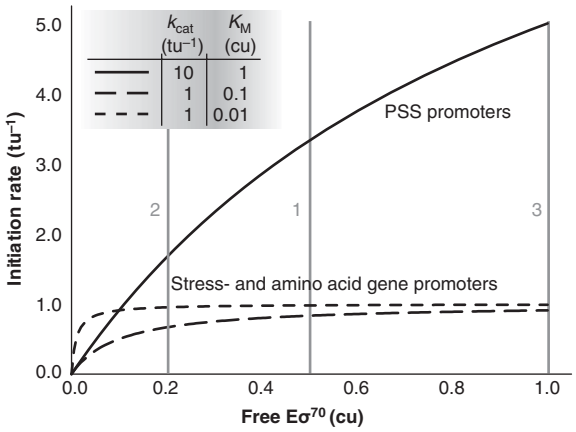

Figure 8 Illustration of the mechanism of passive regulation by the saturation model. The transcription initiation rate in arbitrary time units^{-1} (tu^{-1}) is plotted as a function of $E\sigma^{70}$ in arbitrary concentration units (cu), using the classic Michaelis–Menten expression, but with the $E\sigma^{70}$ as the 'substrate' and the promoter as the 'enzyme':

$$\text{initiation rate} = \frac{k_{\text{cat}} \cdot [E\sigma^{70}]}{[E\sigma^{70}] + K_{\text{M}}}$$

The figure illustrates how promoter-$E\sigma^{70}$ interactions, which results in different maximal initiation rates (k_{cat}) or half promoter saturation concentrations (K_{M}), leads to different responses to changes in the concentration of free $E\sigma^{70}$. Assume, for example, that the concentration of free $E\sigma^{70}$ is 0.5 cu in wild-type cells at exponential growth (grey line 1), and that it decreases to 0.2 cu in stationary phase (grey line 2) or increases to 1.0 cu by over-expression of $E\sigma^{70}$ from the plasmids (grey line 3). The initiation rate for PSS promoters (with high k_{cat} and high K_{M}) depends strongly on the concentration of free $E\sigma^{70}$. In contrast, the initiation rates from stress and amino acid biosynthesis genes (with low k_{cat} and low K_{M}) show only minor changes or remain unaltered at higher concentration of $E\sigma^{70}$, but this results in a decreased expression relative to the total transcription in the cell.

operons—a response typical of cells with a diminished ability to make ppGpp. These results are in line with the saturation model (Jensen and Pedersen, 1990; Dennis *et al*, 2004), which argues that PSS genes possess unsaturated promoters exhibiting high maximal initiation velocity, whereas promoters of, for example, stress-defense genes (*usp*s) and amino acid biosynthetic operons are often saturated (or close to saturated) with $E\sigma^{70}$ and exhibit lower maximal initiation velocity (Figure 8). Thus, the PSS promoters are limited by the rate of $E\sigma^{70}$ recruitment and, in contrast to saturated promoters, become sensitive to the concentration of free $E\sigma^{70}$. Elevated levels of $E\sigma^{70}$ would, therefore, be expected to specifically boost the expression from *rrn*-promoters. The fact that $E\sigma^{70}$ overproduction indeed caused a marked elevation of promoter occupancy and transcription from *rrn*, demonstrates that *rrn*-promoters do not work at their maximal capacity even during exponential growth in rich medium. This is in line also with data from the Squires laboratory, which has demonstrated that the expression from individual *rrn* operons can increase to compensate for the deletion of other *rrn* operons (Condon *et al*, 1992, 1993, 1995). It is likely that the *rrnB*P1-*lacZ* reporter constructs underestimate the initiation capacity of a native *rrnB*P1, because these constructs lack the exceptionally fast elongation rate of natural *rrn* genes (Dennis *et al*, 2009). Thus, the clearing rate of the *rrnB*P1-*lacZ* might be limited by the transcription rate and queuing rather than intrinsic promoter properties.

In view of the fact that elevating the pool size of $E\sigma^{70}$ favours functions related to growth at the expense of

maintenance and that reducing $E\sigma^{70}$ levels has the opposite effect (Magnusson *et al*, 2003), one might ask whether alterations in the pool size of free $E\sigma^{70}$ comprise a normal, physiologically relevant, control mechanism for allocating resources between growth and maintenance activities in *E. coli*. If so, it is expected that the levels of free, and transcription available, $E\sigma^{70}$ should rise with the quality of the growth medium and this has been argued to be the case (Jensen and Pedersen, 1990; Bremer and Dennis, 1996). Specifically, Bremer *et al* (2003) derived a theory to calculate free $E\sigma^{70}$ concentrations from the concentrations of total RNA polymerase and promoters in a model system with estimated Michaelis–Menten constants for the transcription initiation on different promoter (compare with Figure 8). According to such calculations, the concentration of free $E\sigma^{70}$ is about 0.4 and 1.2 μM at growth rates corresponding to 1.0 and 2.5 doublings/h, respectively, that is, the free $E\sigma^{70}$ concentration increases with increasing growth rates. Using a minicell approach, we report here that cells lacking ppGpp displayed a two-fold higher concentration of free $E\sigma^{70}$ compared with wild-type cells. Thus, we believe that growth medium regulation of free $E\sigma^{70}$ levels requires ppGpp and that this is an integral part of this alarmone's means of regulating gene expression. In line with this, we found that in cells with constitutively elevated ppGpp levels, the expression levels, from both positively and negatively regulated promoters, could be restored, and the slow growth rate could be improved by providing them with more $E\sigma^{70}$. The data strongly suggest that the nutritional quality of the environment is regulating the growth rate of cells by altering the availability of free $E\sigma^{70}$ and that this control is influenced by the alarmone ppGpp.

How, then, does the nutritional quality affect $E\sigma^{70}$ availability mechanistically through alterations in ppGpp levels? As ppGpp is known to increase transcriptional pausing and reduce the rate of transcription elongation (Kingston *et al*, 1981; Sorensen *et al*, 1994; Krohn and Wagner, 1996), it has been suggested that the alarmone sequesters $E\sigma^{70}$ in the elongating stage—more so the poorer the media (Jensen and Pedersen, 1990). However, it should be noted that the reduction in transcription elongation has been argued to be too small to effectively reduce the concentration of free $E\sigma^{70}$ (Bremer and Ehrenberg, 1995). Another mechanism for reducing $E\sigma^{70}$ availability may be linked to the role of ppGpp as a master regulator of sigma factor competition as seen by data demonstrating that alternative sigma factors; σ^S, σ^{32}, and σ^{54}, compete significantly better against σ^{70} in the presence of ppGpp both *in vivo* and *in vitro* (Jishage *et al*, 2002; Laurie *et al*, 2003). In addition, the fraction of σ^{70} bound to E is increased at the expense of both σ^S and σ^{32} in cells lacking ppGpp (Jishage *et al*, 2002) and elevated ppGpp levels have been shown to reduce the fraction of σ^{70} bound to E *in vivo* (Hernandez and Cashel, 1995). Thus, ppGpp seems to prime the $E\sigma^{70}$ in accordance with environmental signals such that the transcriptional apparatus will be primarily occupied with transcription of σ^{70}-dependent housekeeping genes as long as the ppGpp levels are low, which signals that the nutritional status of the environment is favourable for growth (reviewed in Nystrom, 2004). In addition, it has been demonstrated that σ^{70} occasionally remains attached to E during transcriptional elongation (Bar-Nahum and Nudler, 2001; Mukhopadhyay *et al*, 2001) and that the fraction of σ^{70} that remains attached increases during nutritional stress (Bar-Nahum and Nudler, 2001). Although it is unknown whether this increase in elongating $E\sigma^{70}$ complexes is a consequence of increased ppGpp levels, it has been suggested that it promotes pausing at promoter-like sequences in the coding region (Mooney and Landick, 2003), and it is reasonable to assume that this reduces the availability of free $E\sigma^{70}$. Surprisingly, a recent theoretical study (Klumpp and Hwa, 2008) suggests that neither of the suggested effects of ppGpp can result in a substantial decrease of the concentration of free $E\sigma^{70}$. However, the reason for this result is that their model propose that a large majority of all $E\sigma^{70}$ is non-specifically bound to DNA at all growth rates (Klumpp and Hwa, 2008), which thereby creates a large pool of non-transcribing $E\sigma^{70}$ that masks any change in the concentration of free $E\sigma^{70}$. To have such a large fraction of the $E\sigma^{70}$ inactivated by non-specific binding is remarkable from an evolutionary perspective and, as demonstrated experimentally here using the minicell approach, the pool size of free $E\sigma^{70}$ is at least two-fold higher in cells lacking ppGpp.

The regulatory design of higher organisms is proposed to comprise a trade-off between activities dedicated to growth and those devoted to homeostasis. The trade-off is, in the disposable soma hypothesis, argued to be a consequence of limited resources in any one organism and is regulated by the nutritional quality of the environment (Kirkwood, 1977). Trade-offs between reproduction and survival may not be restricted to organisms with a soma distinct from the germ line and has been suggested to be part of the intrinsic regulatory design also of unicellular prokaryotes (Nystrom, 2004). We propose that nutritionally induced alterations in $E\sigma^{70}$ availability elicit such a trade-off between proliferation and maintenance and that this trade-off is regulated by the alarmone ppGpp. However, it should be noted that a stringent down-regulation of rRNA genes can occur in the absence of ppGpp accumulation (and transcription factors) by any condition effectively sequestering and/or reducing the availability of free $E\sigma^{70}$. Indeed, pyrimidine limitation, reducing transcription elongation rates, was shown to cause a phenocopy of a stringent response in the absence of ppGpp accumulation (Vogel *et al*, 1991) and a genetic approach to reduce cellular $E\sigma^{70}$ availability is likewise effective in repressing rRNA expression both in the presence and absence of ppGpp (Magnusson *et al*, 2003). This passive regulation in response to limitations in building blocks might represent an ancient regulatory baseline control in the cell on top of which more specific and pronounced effects could be achieved with the evolution of specific effector molecules, activators, and repressors.

Materials and methods

Bacterial strains and growth conditions

The *E. coli* strains used in this work are listed in Supplementary Table S1 (Supplementary data). Transformation of the $E\sigma^{70}$ overproduction plasmids (Dykxhoorn *et al*, 1996) was performed by standard methods. The *spoT201* (Sarubbi *et al*, 1988) allele and Δ*minB*::kan cassette (de Boer *et al*, 1989) were transduced into the different strains by standard P1 transduction. The presence of the *spoT201* allele was confirmed by sequencing. The UP-P*uspA* promoter was constructed such that the −60 to −39 part of the *rrnB*P1 promoter UP element (Ross *et al*, 1993) was fused into the same position upstream of the *uspA* promoter. All promoter-*lacZ* constructs in this work were incorporated into bacteriophage λRS45

and integrated in the *E. coli* chromosomal λ *att* site as described earlier (Farewell *et al*, 1996). Cultures were grown in Erlenmeyer flasks in M9-defined medium supplemented with a limiting concentration of glucose (0.08%) and all the amino acids in excess (Wanner *et al*, 1977) and thiamine (10 μM) at 37°C. Carbenicillin (50 μg/ml) or/and chloramphenicol (30 μg/ml) was added in all experiments with strains containing plasmids. For the ppGpp measurement, the cells were grown in MOPS buffered media with low phosphate (0.33 mM) supplemented with a limiting concentration of glucose (0.08%) and all the amino acids in excess and thiamine (10 μM). Minicell strains were grown at 37°C in MOPS buffered Luria-Bertani (LB) medium supplemented with 0.1% glucose and 50 μg/ml kanamycin as described in Shepherd *et al* (2001). Large cells and minicells were isolated using sucrose gradient centrifugation according to Shepherd *et al* (2001).

Measurement of cellular components

Relative β-galactosidase levels were determined as has been described (Miller, 1972) with modifications (Albertson and Nystrom, 1994). The β-galactosidase activity is expressed as:

$$\beta\text{-gal activity} = \frac{1000 \cdot \mathrm{OD}_{420}^{\mathrm{reaction}}}{\mathrm{OD}_{420}^{\mathrm{culture}} \cdot \text{Reaction time (min)} \cdot \text{Sample volume (ml)}}$$

Western blot analysis of $E\sigma^{70}$ subunit levels was performed with primary antibodies from NeoClone as described (Jishage and Ishihama, 1995). IRDye 800CW-labelled goat anti-mouse immunoglobulin G antibodies (LI-COR Biosciences) were used for detection and blots were analysed with the Odyssey infrared imaging system and software (LI-COR Biosciences).

Estimating holoenzyme content using affinity chromatography

Cultures were grown as described above in the presence or absence of 1 mM IPTG. The cells were harvested at OD 1 by centrifugation and freezing the pellet in −80°C. Pellets were dissolved in lysis buffer (50 mM Tris–HCl [pH 8.1 at 4°C], 0.1 mM EDTA, 0.5 M NaCl, 1 mM DTT, 5 μg/ml DNaseI, 0.1 mM PMSF and 10% glycerol) and cells were disrupted using a Freezer/Mill 6870 (Spex Sampleprep). The cell lysate was cleared by centrifugation, aliquoted, and freezed in −80°C. Cell lysate containing 100 μg total proteins were diluted in 10 times 50 mM Tris–HCl [pH 8.1 at 4°C], 0.1 mM EDTA, before adding to a column packed with 100 μl Softag 1 resin (Neoclone). Affinity purification was performed according to the manufacturer and the content of the eluate was quantified by western blot analysis as described above.

Determination of ppGpp levels

Cultures were continuously labelled for at least two generations before sampling with 75 μCi/ml of [^{32}P]orthophosphate (Amersham Biosciences, Uppsala, Sweden) in low-phosphate (0.33 mM) MOPS medium. Measurements were essentially done according to Gentry *et al* (1993). In short, samples (100 μl) were mixed with 20 μl cold 13 M formic acid and immediately frozen in dry ice. Nucleotides were extracted by three freeze-thaw cycles and cell debris was removed by centrifugation. Subsequently, the supernatant was added onto thin-layer chromatography (TLC) plates (Polygram CEL 300 PEI, Machery-Nagel, Düren, Germany). Levels of ppGpp were quantified using a phosphoimager (Personal FX, Bio-Rad) and the images were analysed using the QuantityOne software (Bio-Rad).

Resolution of proteins by 2D polyacrylamide gel electrophoresis

Samples were taken in stationary phase and 1 ml of the culture was mixed with 10 μl of [^{35}S]-methionine (10 mCi/ml, 1000 Ci/mmol, Amersham Biosciences) for pulse labelling. Incorporation was allowed to proceed for 10 m at 37°C followed by a chase (3 min) with excess of non-radioactive methionine (50 μl, 0.2 M). The samples were processed for resolution on 2D polyacrylamide gels according to O'Farrell (1978) with modifications (O'Farrell, 1978). Electrophoresis in the first dimension was carried out between pH 3.5 and pH 10 and the gels were run according to the NEPHGE protocol (non-equilibrium pH gel electrophoresis). The second dimension was run on 11.5% polyacrylamide gels. Radio-labelled proteins were detected in a phosphoimager (Personal FX, Bio-Rad) and the images were analysed using the PDQuest 6.2 software (Bio-Rad). Alphanumeric (A-N) designations and/or protein names were assigned to protein spots after matching them to the reference 2D images of the gene–protein database of *E. coli* (VanBogelen *et al*, 1992) or after identification using mass spectrometry.

ChIP assays and real-time PCR analysis

ChIP assays were performed essentially as described earlier (Lin and Grossman, 1998). Cells were grown in M9-defined media described above, and formaldehyde was added to a final concentration of 1% at an optical density of 0.7 (420 nm). After 20 min of incubation, glycine was added to a final concentration of 0.5 M, and cells were harvested by centrifugation and washed twice with Tris-buffered saline (pH 7.5). Cells were resuspended in 1000 μl of lysis buffer (10 mM Tris at pH 8.0, 20% sucrose, 50 mM NaCl, 10 mM EDTA, 10 mg/ml lysozyme) and incubated at 37°C for 30 min. A measure of 500 μl of IP buffer (50 mM HEPES-KOH at pH 7.5, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 0.1% sodium deoxycholate, 0.1% SDS) and 1 mM PMSF were added to the cell extract. DNA was sheared by sonication to an average size of ~500–1000 bp. Insoluble cellular material was removed by centrifugation for 10 min, and the supernatant was transferred to a fresh tube. An aliquot of this supernatant were kept for use as the 'input' sample. Proteins were immunoprecipitated by diluting a fraction of the cross-linked cell extract with IP buffer to a final volume of 500 μl. This was then incubated with 20 μl of Protein A-Sepharose beads (Amersham-Pharmacia) and either RNAP β′ subunit mouse monoclonal (NeoClone, W0001) or RNAP σ^{70} subunit mouse monoclonal (NeoClone, W0004) for at least 4 h at 4°C with gentle mixing. Samples were then washed twice with IP buffer, twice with IP buffer + 500 mM NaCl, and once with wash buffer (10 mM Tris–HCl at pH 8.0, 250 mM LiCl, 1 mM EDTA, 0.5% Nonidet-P40, 0.5% sodium deoxycholate). Immunoprecipitated complexes were eluted by incubation of beads with 100 μl 2 × elution buffer (100 mM Tris–HCl at pH 7.8, 10 mM EDTA, 1% SDS) at RT for 30 min. Cross-links, of the eluates and the corresponding 'input' samples, were reversed by incubation for 6 h at 65°C in elution buffer + 100 μl NaCl solution containing 0.2 mg/ml Proteinase K. DNA was purified using a PCR purification kit (Qiagen). Co-precipitated DNA was analysed in triplicate by quantitative PCR using the Bio-Rad iQ5 detection system (Bio-Rad). Primer sequences are listed in Supplementary Table S2 (Supplementary data).

Motility assays

Bacterial cells were picked from colonies grown on LB agar plates and inoculated onto low agar LB plates (0.3%). The plates were incubated at room temperature for 24 h and motility was assessed quantitatively by measuring the diameter of the circular halo formed by the growing motile bacteria cells.

Measurement of protein carbonylation

Analysis of carbonylated proteins was performed using the chemical and immunological reagents of the OxyBlot Oxidized Protein Detection Kit. The carbonyl groups in the protein side chains were derivatized to 2,4-dinitrophenylhydrazone by reaction with 2,4-dinitrophenylhydrazine. The derivatized proteins were analysed immunochemically on 1D western blots onto polyvinylidene difluoride membranes as described earlier (Fredriksson *et al*, 2006).

Determination of α, β′, and σ^{70} as free subunits or in the holoenzyme

Isolated large cells and minicells were resuspended in reconstitution buffer (R-buffer) (10 mM Tris–HCl [pH 7.6 at 4°C], 0.1 mM DTT, 0.1 mM EDTA, 200 mM NaCl, and 5% glycerol (Jishage *et al*, 2002). The cells were disrupted using French press (Spectronic Instruments) and the cell debris was removed by centrifugation. The clear supernatant was precipitated with 50% ammonium sulphate and the precipitate was again resuspended in R-buffer and applied to a Superdex 200 PC 3.2/30 column (GE Healthcare) connected to a Smart chromatography system (Pharmacia LKB). Fractions of 50 μl were collected and their content was quantified by western blot analysis as described above.

Supplementary data

Supplementary data are available at *The EMBO Journal* Online (http://www.embojournal.org).

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Conflict of interest

The authors declare that they have no conflict of interest.

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