Please cite this article in press as: Shachrai et al., Cost of Unneeded Proteins in *E. coli* Is Reduced after Several Generations in Exponential Growth, Molecular Cell (2010), doi:10.1016/j.molcel.2010.04.015

Molecular Cell
Article

Cost of Unneeded Proteins in *E. coli* Is Reduced after Several Generations in Exponential Growth

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DOI 10.1016/j.molcel.2010.04.015

SUMMARY

When *E. coli* cells express unneeded protein, they grow more slowly. Such penalty to fitness associated with making proteins is called protein cost. Protein cost is an important component in the cost-benefit tradeoffs that underlie the evolution of protein circuits, but its origins are still poorly understood. Here, we ask how the protein cost varies during the exponential growth phase of *E. coli*. We find that cells growing exponentially following an upshift from overnight culture show a large cost when producing unneeded proteins. However, after several generations, while still in exponential growth, the cells enter a phase where cost is much reduced despite vigorous unneeded protein production. We find that this reduced-cost phase depends on the ppGpp system, which adjusts the amount of ribosomes in the cell and does not occur after a downshift from rich to poor medium. These findings suggest that protein cost is a transient phenomenon that happens upon an upshift in conditions and that cost is reduced when ribosomes and other cellular systems have increased to their appropriate steady-state level in the new condition.

INTRODUCTION

Expression of unneeded proteins in *E. coli* has long been known to reduce the cell's growth rate (Novick and Weiner, 1957; Horiuchi et al., 1962; Andrews and Hegeman, 1976; Koch, 1988; Nguyen et al., 1989; Dong et al., 1995; Dekel et al., 2005). This effect is known as protein cost or burden. The cost, defined as the fractional reduction in growth rate, is thought to be due to limited cell resources, such as ribosomal capacity (Maaløe and Kjeldgaard, 1966; Vind et al., 1993). In this picture, production of unneeded proteins diverts resources from making other beneficial proteins.

Protein cost is a fundamental aspect of evolutionary selection: Cost and benefit of a protein contribute to its selected expression level, as has been demonstrated in experiments on the *lac* operon of *E. coli* (Dekel and Alon, 2005; Alon, 2006), and can affect which regulatory circuits are selected in given conditions (Dekel et al., 2005; Babu and Aravind 2006; Camas et al., 2006; Zaslaver et al., 2006b; Kalisky et al., 2007; Tănase-Nicola and ten Wolde, 2008). Understanding protein cost is also important for improving the use of cells to produce desired proteins.

An early measurement of protein cost by Novick and Weiner (1957) was performed by inducing the *lac* operon of *E. coli* using a gratuitous inducer in a chemostat. Since the medium had no lactose, induction of the *lac* proteins brought no benefit and led to a reduction in growth rate of about 5%. This reduction was attributed to cellular resources that were funneled into the *lac* operon products and were thus unavailable for the synthesis of useful proteins. Following studies measured protein cost in other growth media (Andrews and Hegeman, 1976; Koch, 1983) and in early exponential growth in batch cultures (Nguyen et al., 1989; Dong et al., 1995). These measurements showed that the cost as a function of the levels of the unneeded protein is not a linear function, but rather grows faster than linear (Dong et al., 1995; Dekel et al., 2005). As the concentration of unneeded protein approaches about 30% of the total protein in *E. coli*, growth stops (Dong et al., 1995, 1996). A study of the sources of protein cost in *E. coli* suggested that cost originates in the production process and not in the products (Stoebel et al., 2008). Cost was also recently measured in yeast (Lang et al., 2009).

Here, we study protein cost by means of accurate, automated measurements of the growth rate of *E. coli* cells producing green fluorescent protein (GFP, an unneeded protein). We find that protein cost is high in early exponential growth. After a few generations, however, while still in exponential growth, cells enter a phase in which cost is markedly reduced. The phase of reduced protein cost is dependent on the ppGpp ribosomal control system and shows reduced ribosomal RNA promoter activity. A mathematical model suggests that as cells transition from slow growth to a more rapid exponential growth, ribosomal capacity is limiting (Vind et al., 1993), but after several generations, balanced growth is reached where ribosomal capacity is no longer the only limiting factor for growth (Maaløe and Kjeldgaard, 1966). As expected in such a mechanism, we find that the reduced-cost phase is found immediately upon downshift, going from exponential growth in a rich to a poor medium. This study

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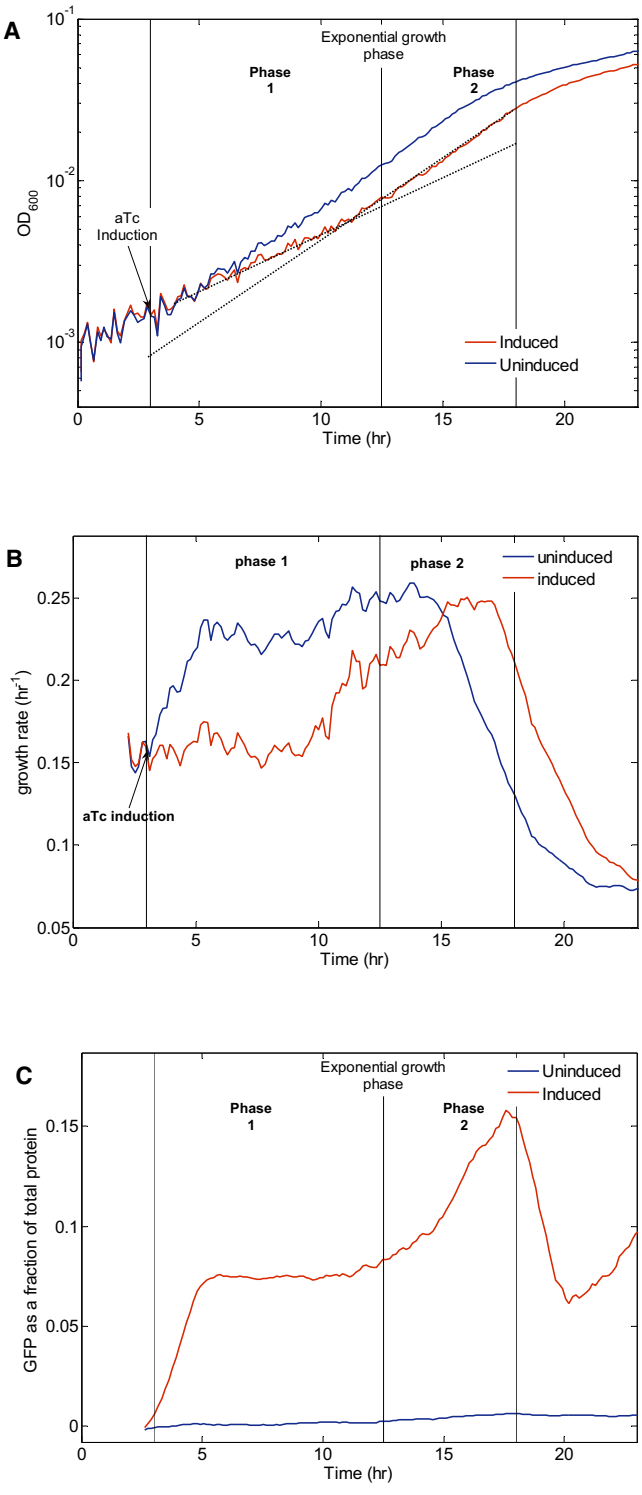

Figure 1. Bacteria that Express Unneeded Proteins Show Two Phases of Exponential Growth

(A) OD of cells with control vector (unburdened bacteria, blue) compared to cells that produce GFP (burdened bacteria, red) as a function of time. Time t = 0 indicates the time the bacteria were transferred into a fresh medium.

(B) Measured growth rate of the unburdened (blue) compared to the burdened (red) bacteria. An increase in growth rate is observed when the bacteria enter into the second growth phase. The midpoint of this increase is at about 12.5 hr and is defined operationally as the boundary between the phases.

(C) Measured GFP production rate of the unburdened (blue) and burdened (red) bacteria. GFP production is high during the first growth phase and further increases as the bacteria enters into the second growth phase.

extends our understanding of the interplay between growth control and protein burden. We suggest that protein cost is a transient phenomenon that happens upon an upshift in conditions and that cost is reduced when ribosomes and other cellular systems have increased to their appropriate steady-state levels in the new condition.

RESULTS

System for Measuring Protein Cost

As the unneeded protein, we use GFPmut3, a rapidly folding GFP variant that is optimized for bacteria and has minimal toxicity (Cormack et al., 1996). To measure protein cost, we expressed GFP from the *tet* promoter on a high-copy plasmid. To study different levels of GFP expression, we constructed several plasmids, identical except for point mutations in the ribosomal binding sites (RBSs) of the *gfp* gene. Induction employed saturating amount of the inducer aTc (20 nM). We find that the inducer has no measurable effect on growth (Figure S1A).

The bacteria were grown overnight in a defined medium, M9 + 0.2% glycerol, and diluted into fresh medium. Cell density (OD) and GFP fluorescence were measured every 8 min in a robotic system in 96-well plates. The induced strain was compared to a strain with the same plasmid but without aTc in alternating wells on the 96-well plate. The growth rate was evaluated at each time point by the logarithmic temporal derivative of the OD, g = d(log(OD))/dt. This method allowed a day-day error in the measurement of the growth rate difference between strains of about 1% (Figure S1B) (Dekel et al., 2005). Cost was defined as the reduction in growth rate of cells expressing GFP relative to the growth rate of uninduced cells.

High Protein Cost Is Found for Several Generations in Exponential Growth and Is Then Reduced

We followed bacterial growth rate following 1:500 dilution from overnight culture into fresh minimal medium (Figure 1A). During the first three generations of exponential growth, cells showed a substantial reduction in growth rate when expressing GFP (Figures 1B and 1C). This cost increased with the level of GFP production, reaching a 35% ± 5% relative reduction in growth when GFP reached about 15% of the cells' total protein (see also Figure S1C).

We found that after approximately three generations, the cells continued their exponential growth and increased their GFP production but, surprisingly, reduced the cost of GFP production to a low level, below the detection of our assay. One can thus divide exponential growth into two phases: phase 1 is the early stage of exponential growth with high cost, and phase 2 of exponential growth has low or undetectable cost. To operationally define the demarcation point between the phases, we used the halfway transition point in growth rate, t = 12.5 hr in the present case.

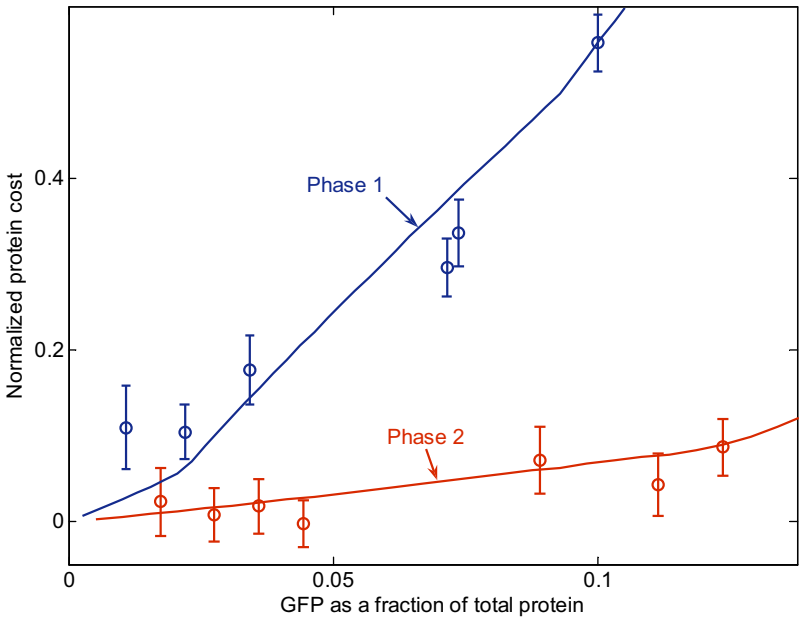

Figure 2. Protein Cost Is High during the First Phase and Very Low during the Second Phase of Growth

Shown is the reduction in growth rate as a function of GFP production rate during the first growth phase (first three generations, blue line) and during the second growth phase (red line). Circles are data from plasmids with variant ribosomal binding sites (RBSs) in the GFP gene (error bars are standard errors from 48 parallel measurements in 96-well plates). Solid lines are the mathematical model results (Experimental Procedures). The kinks in the solid lines result from the switching of the limiting factor from ribosomal to nonribosomal factors (Equation 8, Experimental Procedures).

We repeated this experiment in cells transformed with five variants of the plasmid that had mutated RBSs and thus produced GFP at different rates (Figures S1D–S1G show the measurements for all variants), ranging from about 5% to 15% of total cell protein. The variants followed the same pattern, showing two phases: the first generations of exponential growth show a sizable cost of GFP production (phase 1), and then the cost reduces to near zero (phase 2). The amount of GFP produced has a slight effect on the duration of phase 1: Phase 1 lasts about two generations in the variant with lowest expression and three generations in the variant with highest expression.

We find that even the uninduced strain shows a small increase in growth rate after two cell generations (Figure 1B), suggesting that the two phases exist even in the absence of unneeded protein production. To observe the subtle transition between phases without unneeded protein production requires the present high-accuracy assay, because the growth rate in phase 1 without unneeded protein production is very close (2%–4%) to the growth rate in phase 2.

As a further test, we measured the cost of expressing the *lac* operon from its endogenous promoter under the induction of the nonmetabolizable inducer IPTG (Figure S1H). In this experiment, no lactose is present so that the *lac* proteins do not confer a growth advantage and may be considered as unneeded proteins. We find the two cost phases, similar to those found with GFP expression. This indicates that the two phases are not specifically related to GFP.

The cost in phase 1 increases with GFP amounts (Figure 2). The blue points show the cost in phase 1, and the red points show the reduced cost during phase 2.

Similar results were found when induction of GFP was performed by adding the inducer aTc at different time points to the culture (Figure 3). Induction in the first three generations led to a large cost (Figures 3A and 3B), whereas induction in later generations led to a reduced cost (Figures 3C–3F). This further supports the observation that the two phases occur even without unneeded protein induction.

To further study the reduced-cost phase, we diluted the cells to a larger extent, 1:5000. Here, OD is below our detection threshold in the first three generations. By the time that enough generations have occurred so that the OD reaches the detection threshold (OD = 10^{-3}), we find that cost is reduced to zero to within experimental error.

Growth in defined medium supplemented by amino acids, M9C, which is richer than the medium used above (M9 that has no amino acids), resulted in the same effects. Here, it takes more generations (six) to reach phase 2, the exponential growth phase with reduced cost (Figure S3). During the first six generations, the cost is considerable, and then cost is reduced below the detection threshold while cells still grow exponentially and produce large amounts of GFP (Figure 4). We find that the reduced-cost phase also persists in serial dilution experiments that keep the cells growing exponentially by repeated dilutions of exponential cultures into fresh medium (Figure S3). This indicates that the exponential growth phase with reduced cost (phase 2) is not linked to proximity to stationary phase. We also find that as the growth medium is supplemented with increasing concentration of amino acids, growth rate increases while cost in the first phase is reduced (Figures S2B and S2C).

Reduced Cost Is Found Immediately after Downshift from Rich to Poor Medium

We also compared an upshift experiment, in which cells were grown exponentially in poor medium (M9) and then shifted to richer medium (M9C) (Figure S3D), to the reverse (downshift) experiment, in which cells were shifted from exponential growth in M9C to M9 (Figure S3C). We find that immediately upon upshift, the cells show phase 1, whereas immediately after downshift, they show phase 2. This suggests that the cost is a transient phenomenon associated with upshift but not downshift in conditions.

Ribosomal Gene Expression Is High in Phase 1 and Reduces to a Low Steady Level in Phase 2

The cost of protein production is thought to be linked with ribosomes (Maaløe and Kjeldgaard, 1966; Vind et al., 1993; Stoebel

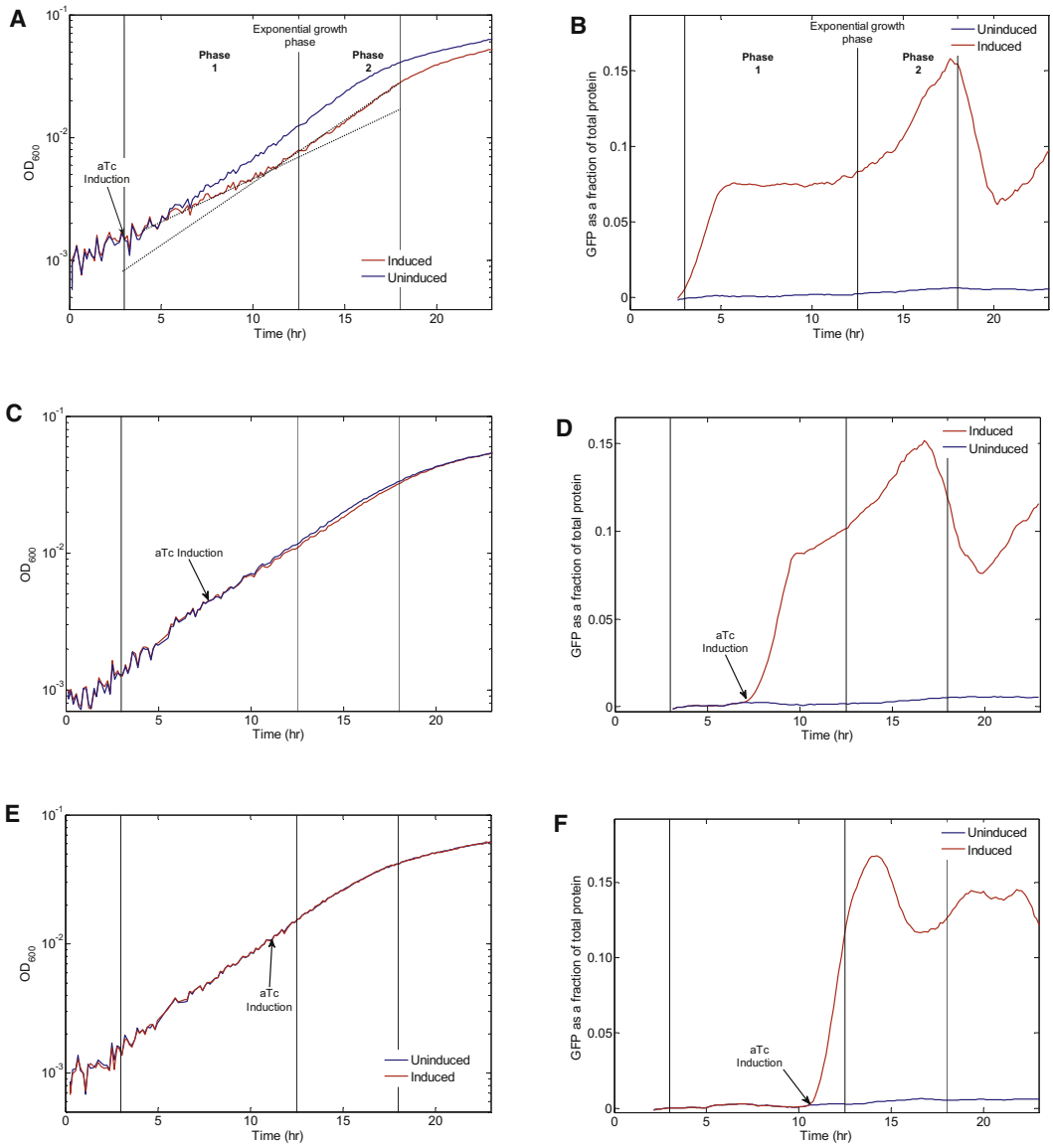

Figure 3. Induction of Unneeded Proteins at Different Times during Exponential Growth Does Not Affect Entry into the Second Growth Phase

(A–F) Shown are the OD curves (A, C, and E) and the corresponding GFP production curves (B, D, and F) of unburdened bacteria (blue line) and burdened bacteria (red line). (A) and (B) are for induction during start of growth (same as Figures 1A and 1B). At this early induction, the cost is high in the first growth phase and reduces during the second growth phase. (C) and (D) show induction in the middle of the first growth phase. Induction at this stage reduces growth mildly. Growth recovers during the second growth phase. Induction during the second growth phase (E and F) has no observable cost, despite high production of GFP.

et al., 2008). We thus considered the role of ribosomes in the cost-reduction phenomenon. We began with measuring ribosomal gene expression in our system using a GFP reporter library (Zaslaver et al., 2006a). We measured the activity of four ribosomal RNA promoters—*rrnA*, *rrnB*, *rrnC*, and *rrnH*—at high temporal resolution. Promoter activity was normalized to a σ_{70} promoter (Kaplan et al., 2008), which represents the regulation of nonribosomal promoters in growing cells. The mean activity during the second phase was then normalized to 1.

We find that ribosomal gene expression is high in phase 1 and decreases to a lower constant value in phase 2 (Figure 5). This is consistent with a picture in which ribosomes limit growth in the first generations after transfer from stationary phase. In this phase, therefore, ribosomal promoters are highly active to supply more ribosomes. Ribosome production is then reduced (after three generations in M9 + glycerol medium) to the level that supports balanced exponential growth.

The Reduced-Cost Phase Depends on the ppGpp Control System

We further tested the role of ribosomal regulation in the cost phenomenon by focusing on the primary mode of ribosomal

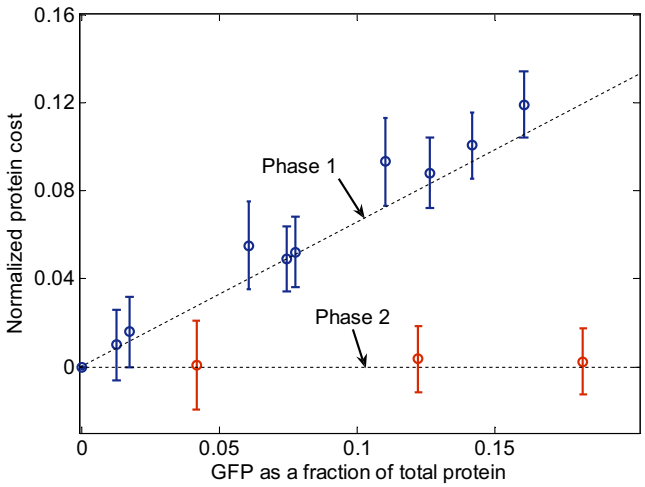

Figure 4. High- and Low-Cost Phases Are Also Found in Rich-Defined Medium

Shown is the cost of protein production in defined medium supplemented with amino acids (M9C) as a function of unneeded GFP production rate. Cost is high during the first growth phase and reduces during the second growth phase. Blue circles are for the protein production cost during the first growth phase. Red circles are for the protein production cost during the second (low-cost) growth phase. To reach the second growth phase, growth was prolonged by means of a serial transfer step of exponentially growing cells to fresh medium (see also Figure S3). Error bars are standard errors from 48 parallel measurements in 96-well plates.

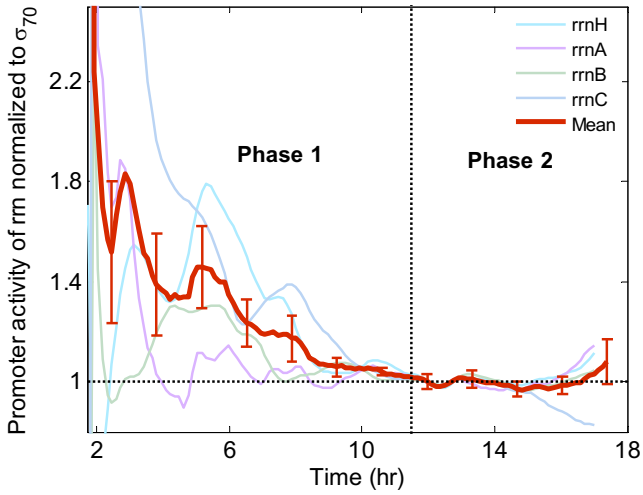

Figure 5. Ribosomal RNA Promoter Activity Is High during the First Growth Phase and Approaches a Low, Constant Level during the Second Phase

Shown are the mean and individual responses of four ribosomal RNA promoter activities (*rrnA*, *rrnB*, *rrnC*, and *rrnH*), normalized by the promoter activity of a σ_{70} promoter, as a function of time. The promoter mean activity during the second (low-cost) phase was also normalized to 1. The error bars are the standard error of the four promoters from three independent repeats.

gene control, the ppGpp system (Paul et al., 2004; Dennis et al., 2004). ppGpp is a small molecule that represses the activity of ribosomal promoters (Cashel and Gallant, 1969; Cashel and Kalbacher, 1970). ppGpp is synthesized by the enzyme RelA in response to amino acid deprivation ("stringent response") (Cashel et al., 1996) and by SpoT in response to submaximal ribosomal activity (Hernandez and Bremer, 1991; Bremer and Dennis, 2008). ppGpp is also hydrolyzed by SpoT (Laffler and Gallant, 1974; An et al., 1979). In Δ*spoT* mutants, ppGpp is not degraded, and ribosomal concentration cannot be adjusted at different growth rates (Bremer and Dennis, 2008). In Δ*relA* mutants, ppGpp can still be produced and degraded by SpoT, and ribosomes are able to adjust their concentration to some extent in response to poor-nutrient medium and suboptimal growth rate (Bremer and Dennis, 2008).

We find that in a strain deleted for *spoT*, the reduced-cost phase (phase 2) is abolished (Figures 6A and S4). Cells grow exponentially and show high cost throughout exponential growth (Figures 6A and 6B), even when high dilution is used. In *ΔrelA* mutants, in contrast, two phases are seen with high and low costs. The low-cost phase, however, displays higher cost than in the wild-type (Figures 6C and 6D). These results indicate that the ppGpp system is required for growth with reduced cost.

A Model of Ribosomal Regulation Suggests that Reduced Cost Occurs When Ribosomes Are Not the Only Limiting Factor for Cell Growth

What is the mechanism that allows protein production with a low cost? To understand this, we studied a mathematical model based on and extending the models suggested by Marr (1991) and Bremer and Ehrenberg (1995). The present model describes the partitioning of resources between ribosomal and nonribosomal promoters.

We describe the model briefly; a full description is given in the Experimental Procedures. The model describes the feedback between ribosomal and nonribosomal protein production, governed by ppGpp, by means of coupled differential equations for the concentration of ribosomes, nonribosomal protein, free amino acids, and ppGpp. At high ppGpp levels, transcription is directed to nonribosomal promoters, whereas low ppGpp levels direct transcription resources to ribosomal promoters. Maximal ribosome production is thus reached when the level of ppGpp is zero. The cell's growth rate is modeled as the minimum of two potential limiting factors: ribosomes and nonribosomal proteins that perform essential functions.

Figure 7 shows a typical simulation of the model. At time zero, just after bacteria are transferred from stationary phase to fresh medium, the bacteria start with a low level of ribosomes and with a low ppGpp level (Figures 7A and 7B). At this stage, ribosomal production is at a maximal rate, and the growth rate is limited by the number of ribosomes (Figure 7C). After 3.5 hr in the simulation, the number of ribosomes increases to a level in which they are not the only limiting factor. Growth limitation is shifted to the nonribosomal proteins (Figure 7C). At this point, ppGpp level increases (since there are excess ribosomes leading to stalled ribosomes), and ribosomal production is reduced. ppGpp level increases until the number of ribosomes reduces to a level in which their number is again limiting (time 7 hr in the simulation). As a result, the level of ppGpp goes down again, and so on. This results in damped oscillations of ribosome levels that approach a value that may be called the "balanced-growth level of

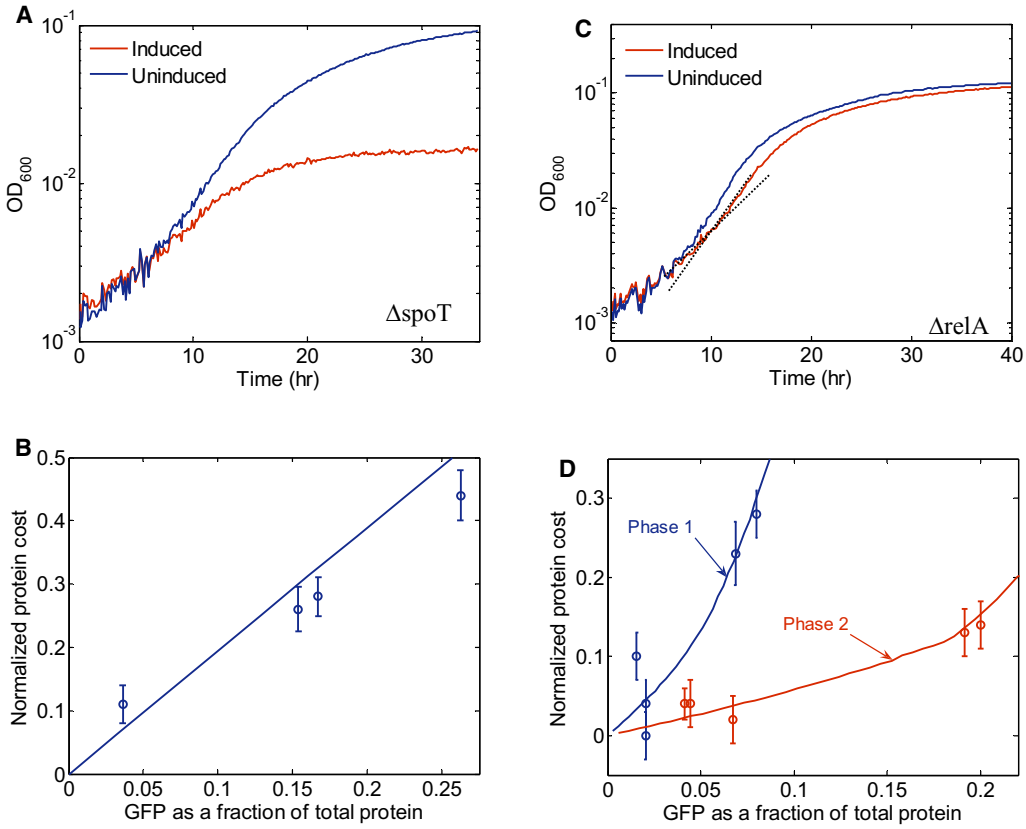

Figure 6. Deletion of ppGpp Synthesizing/Degrading Enzyme SpoT Abolishes the Second Growth Phase, Whereas Deletion of the ppGpp Synthesizing Enzyme RelA Affects Second Phase Mildly

(A and B) Shown are OD curve (A) and the cost of protein production (B) of a strain deleted for *spoT*. The mutant strain does not display the second (low-cost) phase.

(C) A mutant deleted for *relA* shows the two growth phases in its OD curve.

(D) The *ΔrelA* mutant shows reduction of unneeded protein cost in the second phase, but the cost is not as low as in the wild-type. Circles are measurements and solid lines are the results of the model. Error bars are standard errors from 48 parallel measurements in 96-well plates.

ribosomes." The limiting factor oscillates between ribosomes and nonribosomal protein, approaching a state in which both are equally limiting (balanced growth) (Figure 7C).

The cost is captured in this model by adding production of unneeded proteins. This reduces the number of ribosomes available for essential protein production and results in reduced growth rate. In this calculation, unneeded protein production leads to a higher number of ribosomes and a longer amount of time needed to reach balanced growth. Plotting the cells' growth curves predicted by the model results in a growth curve with two phases, as observed in the experiment (compare Figure 7D to Figure 1A).

Figure 7E shows the predicted cost as a function of time, comparing growth with unneeded protein production to growth without unneeded protein production. As observed in the experiment (Figure 1), cost is greatly reduced when cells reach a balance between ribosomal and nonribosomal promoters.

Thus, to understand reduced cost, one can look at the system as if growth limitation in balanced growth rapidly switches between ribosomes and nonribosomal proteins. Therefore, ribosomes limit growth only part of the time. Since protein cost is associated with ribosomes, it is high in the first generations when ribosomes are limiting and lower in later generations where ribosomes are limiting only part of the time.

This model explains also why high cost (phase 1) is observed upon upshift from exponential growth in a poor medium to a rich medium but not upon the corresponding downshift experiment from rich to poor medium. After downshift, ribosomes adjust to lower levels appropriate for their new low steady-state growth rate. As a result, ribosomes are not limiting after downshift, and phase 1 is not observed. After upshift, conversely, ribosomes must increase in order to reach their new high steady-state level, and phase 1 occurs.

DISCUSSION

We studied the cost of unneeded protein production in *E. coli*. We find that cost is high in exponential growth after inoculation from stationary phase. However, after a few generations, cells enter a phase of exponential growth with strong GFP production but greatly reduced cost. The reduced-cost phase persists throughout serial dilutions that keep cells in exponential growth

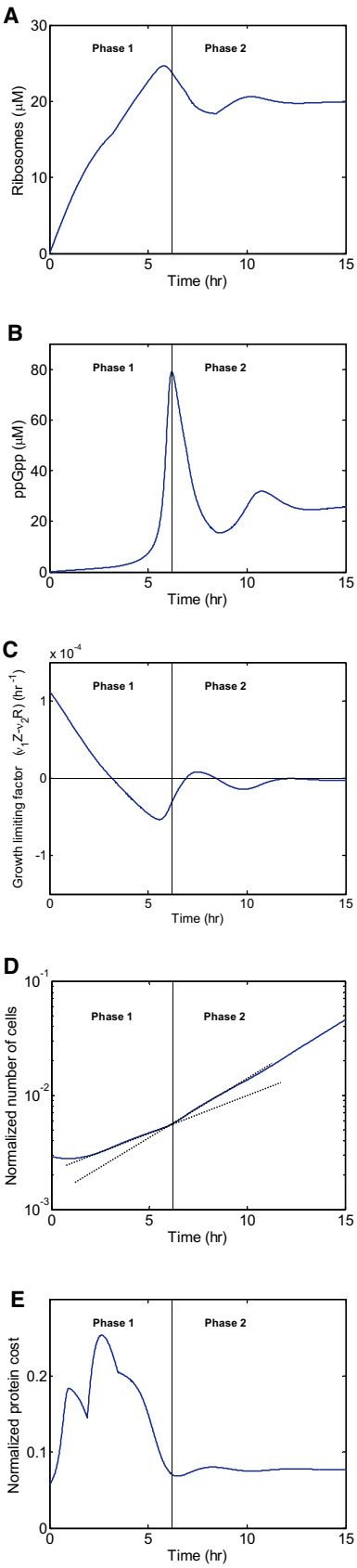

Figure 7. A Model of ppGpp Regulation Captures the Two Phases of Protein Cost

(A) Calculated mean ribosome concentration as a function of time increases in the first growth phase and balances during the second growth phase.

(B) Calculated ppGpp concentration is low during the first phase and high during the second phase.

(C) The limiting factor during growth. Positive value is for ribosomal limitation of growth, and negative value is for nonribosomal limitation of growth. The growth begins with ribosomal limitation and enters balanced growth in which growth limitation switches between ribosomal and nonribosomal limitation.

(D) Calculated number of bacteria as a function of time. Shown is the increase in growth rate as bacteria enter the balanced growth phase.

(E) Calculated cost of unneeded protein production. The calculated cost is high during the first growth phase and reduces during the second (balanced and low-cost) growth phase.

and depends on the ppGpp ribosomal control system. Modeling suggests that high cost corresponds to early stages in which ribosomes limit growth rate, whereas low cost results from approaching balanced growth in which ribosomes and nonribosomal proteins are both limiting.

This study defines two phases of exponential growth. The first phase (phase 1) is a transient phase that occurs upon an upshift in conditions, in which cost is high and growth is not balanced. The second phase can be described as balanced growth and shows much-reduced cost. The transition between the two phases is smooth. In contrast to upshift experiments, upon downshift in conditions in which cells move from exponential growth in a rich medium to poorer one, phase 2 seems to be reached immediately without phase 1.

Our findings further show that the cost is generally higher the lower the cell's growth rate (compare Figure 1 to Figure S3; see also Figures S2B and S2C). Furthermore, phase 1 seems to last longer in rich media than in poor media (compare Figure 1 and Figure S3). The present model suggests that this is due to the higher number of ribosomes needed in the balanced-growth phase (phase 2) in conditions of higher growth rate, because the steady-state ribosome number measured in balanced growth increases with growth rate (Bremer and Dennis, 1996).

The present experiments also point to a role for the ppGpp system in the reduced-cost phase. The reduced-cost phase is abolished in *spoT* mutants and is correlated with a low, constant ribosomal RNA promoter activity. The present model of ribosomal control suggests that high cost results from the transient phase where ribosomes are vigorously produced, because they are the main limiting factor for growth. Unneeded proteins in this phase reduce ribosomal capacity and thus reduce growth (see also Vind et al., 1993). In contrast, as cells reach a balanced level of ribosomes, the action of nonribosomal proteins begins to be limiting, together with ribosomes. In this phase, unneeded protein production has a smaller effect on growth (low cost), because ribosomes are no longer the only limiting factor.

One may also suggest alternative hypotheses for the origin of the reduced-cost phase. One possibility is that after a few generations in exponential growth, bacteria begin to produce proteins that bring no benefit currently but can be useful for future environments (Tagkopoulos et al., 2008; Mitchell et al., 2009). In this case, unneeded proteins may come at the expense of

currently nonbeneficial proteins and have reduced impact on the cell's growth. Another alternative is that the limiting factor in the first phase is not ribosomes but other factors, such as chaperones or other cellular machinery, perhaps also regulated by ppGpp. It is possible that proteins or mRNAs compete for such factors, leading to cost. Future work can test these possibilities experimentally.

The present study also helps us understand the biological step in protein production that is associated with cost. Elegant work by Dykhuizen and colleagues on the *lac* system (Stoebel et al., 2008) indicated that protein cost in *E. coli* is due to the process of protein production, including transcription and translation, rather than in limitations on amino acids or on detrimental activity of the unneeded proteins. Our findings support the view of Stoebel et al. (2008), suggesting that the source of protein cost lies in the process of production rather than in the product accumulation (the latter includes effects of deleterious interactions with other proteins and of taking up space in the cell). In the present data, although there is more GFP accumulation (more fluorescence per cell) during phase 2 compared to phase 1, the cost reduces, while growth rate and GFP expression increase. This suggests that accumulation effects are not the main factor in determining cost (see also Figure S2A).

In summary, this study shows that exponentially growing *E. coli* display large protein cost in a transient phase upon a shift from stationary phase and then, after a few generations, enter a phase with vigorous unneeded protein production but low cost. The origin of this low-cost phase is linked with ribosomal regulation and the ppGpp system. Since, in the natural environment, bacteria such as *E. coli* shift conditions often, cost might be a ubiquitous factor in their natural selection. The phases of protein cost and their origin may thus be relevant for understanding the cost-benefit tradeoffs of protein circuits.

EXPERIMENTAL PROCEDURES

Strains and Media

E. coli MG1655 (U106; *E. coli* Genetic Stock Center, Yale University; New Haven, CT) was used. Experiments were carried out in M9-defined media consisting of M9 salts (0.6% Na_2HPO_4, 0.3% KH_2PO_4, 0.05% NaCl, 0.01% NH_4Cl, 1 mM $MgSO_4$, and 0.1 mM $CaCl_2$). M9 media were supplemented with 0.2% glycerol as the sole carbon source. For amino acid media (M9C), M9 media were supplemented with 0.05% casamino acids. Bacteria were transformed with the high-copy plasmid pZE21-gfp (Lutz and Bujard, 1997) (ColE1 origin, kanamycin resistance), which bears GFPmut3 under a PLtetO-1 promoter. GFPmut3 becomes fluorescent in less than 5 min and has very low toxicity and negligible degradation (Cormack et al., 1996). As a control, we used the same vector with no aTc induction. Identical results are found with a control vector with no GFP gene. Different expression levels of GFP were obtained by point mutations in the RBS of the *gfp* gene (strains U397–U402, QuikChange II Site-Directed Mutagenesis Kit [Stratagene; La Jolla, CA]). The point mutations were confirmed by sequencing of the resulting vector. The *tetR* gene was introduced into the chromosome of these strains by P1 transduction from U182.

Sequence of RBS Mutant Variants

RBS of variant number 1 (U400): 5′- CTGACCGAATTCATTAAAGAGGAG AAATGTACCGCATGC
RBS of variant number 2 (U398): 5′- CTGACCGAATTCATTAAAGATTTG AAATACCGCATGC
RBS of variant number 3 (U402): 5′- CTGACCGAATTCATTAAAGAGGAA AATGTACCGCATGC
RBS of variant number 4 (U399): 5′- CTGACCGAATTCATTAAAGATTTGA AAGGTACCGCATGC
RBS of variant number 5 (U403): 5′- CTGACCGAATTCATTAAAGAGTAGA AAGGTACCGCATGC

For analysis of ribosomal promoter activity, we used our previously described fluorescent reporter library (Zaslaver et al., 2006a). The promoters analyzed are of *rrnA*, *rrnB*, *rrnC*, and *rrnH* and a pure σ_{70} promoter (HV13) (Kaplan et al., 2008).

Construction of Deletion Mutants

Deletion strains were constructed by replacing the entire deleted ORF from start to stop codon with a kanamycin antibiotic cassette (PKD4), followed by elimination of the kanamycin resistance using FLP recombinase following the method of Datsenko and Wanner (2000). Genomic deletion was verified by PCR and sequencing. Primers for deleting *spoT* (strain U404):

5′ TTACCGCTATTGCTGAAGGTCGTCGTTAATCACAAAGCGGGTCGCC CTTG GTGTAGGCTGGAGCTGCTTC 3′
5′ CGTGCATAACGTGTTGGGTTCATAAAACATTAATTTCGGTTTCGGGT GAC CATATGAATATCCTCCTTAG 3′

Primers for deleting *relA* (strain U403):

5′ TTGCGATTTGCCGATTTCGGCAGGTCTGGTCCCTAAAGGAGAGGAC GATG GTGTAGGCTGGAGCTGCTTC 3′
5′ CGTCGGTTGCACGGGAGTTAGGCCGAAATTTGCTCGTATCTACAAT GTAG CATATGAATATCCTCCTTAG 3′

Growth Conditions and Measurements

Bacteria were inoculated from frozen stocks into M9 + glycerol or M9C medium, supplemented by 50 μg/ml kanamycin, and grown overnight. Identical results were found upon plating cells from frozen stocks followed by overnight growth. Bacteria were diluted 1:500 or 1:5000 into fresh medium as indicated. Measurements were conducted by a robotic liquid handler (Freedom EVO, Tecan; Männedorf, Switzerland). 96-well plates (Nunc; Roskilde, Denmark) were prepared with 150 μl media with bacteria. Wells were then covered with 100 μl mineral oil (Sigma; St. Louis) to prevent evaporation (Ronen et al., 2002; Zaslaver et al., 2004) and transferred into a robotic incubator. Bacterial cultures were grown in an automated incubator with shaking at 30°C, which resulted in a growth rate of 0.15–0.25 hr^{-1}. We used 30°C rather than 37°C because the lower growth rates at 30°C resulted in higher costs in phase 1, which reduced measurement errors. Qualitatively similar results with the two phases were observed also in 37°C (Figure S1I). After about 3 hr (or at times indicated), 48 wells were inoculated with 20 nM anhydrotetracycline (aTc) in a 96-well plate in a checkerboard pattern (Dekel et al., 2005) to achieve full induction of GFP, while the other half was left with no aTc serving as a control. Bacteria were then measured for OD and GFP for about 20 hr by a robotic system (Infinite F200, Tecan) at a resolution of 8 min. For serial dilution experiments, bacteria were diluted 1:25 into a new plate with fresh media every 9 hr by the robotic liquid handler. aTc was checked for toxicity using bacteria containing a plasmid with no GFP and was found to have no effect on growth rate (Figure S1A).

Growth Rate and Expression Analysis

Growth rate was calculated as the temporal derivative of the OD curves, $\alpha = d\,ln(OD)/dt$. The exponential growth rate difference between the two strains (induced versus uninduced) was measured by comparing 48 wells of each strain grown in a checkerboard pattern. Growth rate differences were determined and averaged between neighboring wells, yielding an accuracy of about 0.8%. The cost is the fractional reduction in growth rate relative to the uninduced strain: $cost = \alpha_{induced} - \alpha_{uninduced}/\alpha_{uninduced}$. Promoter activity was calculated by PA = (dGFP/dt)/OD (Ronen et al., 2002). Promoter activity was converted to GFP production rate as a fraction of total protein production rate. This was calculated as the number of amino acids invested in GFP out of

the total amino acids in proteins in the cell at the specific growth rate measured (Bremer and Dennis, 1996) (see Supplemental Information). Number of GFP molecules per fluorescence unit was calibrated using recombinant GFP standards of known concentration in solution (Zotal; Tel Aviv). Number of bacteria per OD unit was calculated using a standard OD curve (Bremer and Dennis, 1996; Sezonov et al., 2007).

Number of Generations in Phase 1

The time of transition between phase 1 and phase 2 was defined as the midpoint of the transition between low and high growth rates. The number of generations in each phase corresponds to the number of doublings of OD observed. For example, in the M9 data of Figure 1, the growth at phase 1 starts with $OD_{600} = 8 \times 10^{-4}$ (OD at time of aTc addition) and ends, at the end of phase 1 at 12.5 hr, with $OD_{600} = 6 \times 10^{-3}$, a factor of 7.5, which corresponds to $\log_2(7.5) = 2.9$ generations.

A Simple Model of Resource Allocation Explains the Correction of Growth Rate

We employed a mathematical model by Marr (Marr, 1991) to describe ribosomal regulation. We extended this model by including nonribosomal proteins and by modeling bacterial growth rate. The rate of change of ribosome concentration R is given by their synthesis rate minus their dilution rate (Marr, 1991):

$$\frac{dR}{dt} = k_{rm} \cdot r \cdot g_{rm} - \alpha \cdot R, \quad (1)$$

where $k_{rm} = 1.5\ s^{-1}$ (Marr, 1991; Dennis et al., 2009) is the rate of transcription of ribosomal genes and $g_{rm} = 0.025\ \mu M$ is the concentration of *rrn* genes, which is constant at the growth rates measured in the present study (Marr, 1991). α is the specific exponential growth rate, and r represents the allocation of transcription resources between ribosomal and nonribosomal genes. The resource allocation r is a function of ppGpp, denoted G, and is well described by a Hill function with a Hill coefficient of 2 (Marr, 1991; Dennis et al., 2004):

$$r = \frac{K_g^2}{K_g^2 + G^2}, \quad (2)$$

where $K_g = 40\ \mu M$ (Bremer and Dennis, 1996). ppGpp is produced when ribosomal activity is submaximal or when the A-compartment of the ribosome is filled with an uncharged tRNA. Production of ppGpp is thus given by the rate equation (Marr, 1991)

$$\frac{dG}{dt} = k_1 \cdot R \cdot \left[\frac{\frac{U}{K_u}}{1 + \frac{U}{K_u} + \frac{C}{K_c}} \right] - k_2 \cdot G, \quad (3)$$

where $k_1 = 1\ s^{-1}$ (Marr, 1991) is the specific rate of ppGpp formation and $k_2 = 0.035\ s^{-1}$ (Cashel et al., 1996) is the rate of ppGpp degradation. U and C are the uncharged and charged tRNA concentrations, respectively. $\frac{U}{K_u}/\left[1 + \frac{U}{K_u} + \frac{C}{K_c}\right]$ is the fraction of stalled ribosomes, where $K_u = 10\ \mu M$ (Marr, 1991) is the dissociation constant of uncharged tRNA from the ribosome, and $K_c = 2.75\ \mu M$ (Marr, 1991) is the dissociation constant of charged tRNA.

To grow, cells also need nonribosomal proteins, which we denote Z. The total allocation of transcription resources to these proteins, given by $1 - r$, increases with ppGpp. Their rate of change is given by a balance between production and dilution by cell growth (we assume that the proteins are stable):

$$\frac{dZ}{dt} = k_p \cdot R \cdot (1 - r) \cdot \left[\frac{\frac{C}{K_c}}{1 + \frac{U}{K_u} + \frac{C}{K_c}} \right] - \alpha \cdot Z, \quad (4)$$

where k_p is the maximal rate of peptide synthesis, about $22\ s^{-1}$ in rapidly growing bacteria, and reduces to about $11\ s^{-1}$ in slow-growing bacteria (Bremer and Dennis, 2008; Dennis et al., 2009).

We also model free amino acid pools A, needed for protein synthesis. The rate of amino acid formation (or uptake) depends on the environment in which the bacteria grow, and the rate of protein formation in ribosomes is the main source of amino acid utilization:

$$\frac{dA}{dt} = V_A - k_p \cdot R \cdot \left[\frac{\frac{C}{K_c}}{1 + \frac{U}{K_u} + \frac{C}{K_c}} \right], \quad (5)$$

where V_A is the environmentally dependent rate of amino acid synthesis/uptake, and the second term is the ribosomal rate of protein synthesis. We neglected the feedback inhibition of amino acid production because we assume that in the present conditions, amino acid levels are too low for significant inhibition. To estimate the fraction of stalled ribosomes, one needs to model the formation of charged tRNA, C. For this purpose, we use a Michaelis-Menten term that increases with free amino acids:

$$C = T \frac{A}{K_a + A}, \quad (6)$$

where T is the total tRNA concentration $T = U + C$. Measurements show that the molar ratio between T and ribosomes is independent of growth rate (Bremer et al., 1987):

$$T = C_r R, \quad (7)$$

where $C_r = 0.25$.

Finally, we model bacterial growth rate by considering two potentially limiting factors: ribosomal capacity to produce protein that is proportional to R (Bremer and Ehrenberg, 1995) and the concentration of nonribosomal protein that supplies the building blocks and conditions essential for cell growth, proportional to Z. The bacterial growth rate is thus modeled as the minimum of these two terms:

$$\alpha = \min(\nu_1 R, \nu_2 Z), \quad (8)$$

where ν_1 and ν_2 are proportion factors. The model was solved numerically using MATLAB. Initial conditions after dilution of cells from stationary phase are $R = 1\ \mu M$, $G = 0$, $Z = 0$, and $A = 0$.

To model protein cost, we introduced to the model a reduction in the number of ribosomes that are engaged in the production of needed proteins. When unneeded proteins are made at a rate that engages R_u ribosomes, R in Equation 4 is replaced by $R_{eff} = R - R_u$.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, Supplemental References, and four figures and can be found with this article online at doi:10.1016/j.molcel.2010.04.015.

ACKNOWLEDGMENTS

This work was supported by the Kahn Family Foundation.

Received: June 4, 2009
Revised: January 7, 2010
Accepted: April 2, 2010
Published online: April 29, 2010

REFERENCES

Alon, U. (2006). An Introduction to Systems Biology: Design Principles of Biological Circuits (Boca Raton, FL: Chapman & Hall/CRC).

An, G., Justesen, J., Watson, R.J., and Friesen, J.D. (1979). Cloning the spoT gene of Escherichia coli: identification of the spoT gene product. J. Bacteriol. *137*, 1100–1110.

Andrews, K.J., and Hegeman, G.D. (1976). Selective disadvantage of nonfunctional protein synthesis in Escherichia coli. J. Mol. Evol. *8*, 317–328.

Babu, M.M., and Aravind, L. (2006). Adaptive evolution by optimizing expression levels in different environments. Trends Microbiol. *14*, 11–14.

Bremer, H., and Dennis, P. (1996). Modulation of chemical composition and other parameters of the cell by growth rate. In Escherichia coli and Salmonella: Cellular and Molecular Biology, F.C. Neidhardt, ed. (Washington, D.C.: American Society for Microbiology), pp. 1553–1569.

Bremer, H., and Dennis, P. (2008). Feedback control of ribosome function in Escherichia coli. Biochimie *90*, 493–499.

Bremer, H., and Ehrenberg, M. (1995). Guanosine tetraphosphate as a global regulator of bacterial RNA synthesis: a model involving RNA polymerase pausing and queuing. Biochim. Biophys. Acta *1262*, 15–36.

Bremer, H., Baracchini, E., Little, R., and Ryals, J. (1987). Control of RNA synthesis in bacteria. In Genetics of Translation: New Approaches, M.F. Tuite, M. Picard, and M. Bolotin-Fukuhara, eds. (New York: Springer-Verlag), p. 14.

Camas, F.M., Blázquez, J., and Poyatos, J.F. (2006). Autogenous and nonautogenous control of response in a genetic network. Proc. Natl. Acad. Sci. USA *103*, 12718–12723.

Cashel, M., and Gallant, J. (1969). Two compounds implicated in the function of the RC gene of Escherichia coli. Nature *221*, 838–841.

Cashel, M., and Kalbacher, B. (1970). The control of ribonucleic acid synthesis in Escherichia coli. V. Characterization of a nucleotide associated with the stringent response. J. Biol. Chem. *245*, 2309–2318.

Cashel, M., Gentry, D.R., Hernandez, V.H., and Vinella, D. (1996). The stringent response. In Escherichia coli and Salmonella: Cellular and Molecular Biology, F.C. Neidhardt, ed. (Washington, D.C.: American Society for Microbiology), pp. 1458–1496.

Cormack, B.P., Valdivia, R.H., and Falkow, S. (1996). FACS-optimized mutants of the green fluorescent protein (GFP). Gene *173*, 33–38.

Datsenko, K.A., and Wanner, B.L. (2000). One-step inactivation of chromosomal genes in Escherichia coli K-12 using PCR products. Proc. Natl. Acad. Sci. USA *97*, 6640–6645.

Dekel, E., and Alon, U. (2005). Optimality and evolutionary tuning of the expression level of a protein. Nature *436*, 588–592.

Dekel, E., Mangan, S., and Alon, U. (2005). Environmental selection of the feedforward loop circuit in gene-regulation networks. Phys. Biol. *2*, 81–88.

Dennis, P.P., Ehrenberg, M., and Bremer, H. (2004). Control of rRNA synthesis in Escherichia coli: a systems biology approach. Microbiol. Mol. Biol. Rev. *68*, 639–668.

Dennis, P.P., Ehrenberg, M., Fange, D., and Bremer, H. (2009). Varying rate of RNA chain elongation during rrn transcription in Escherichia coli. J. Bacteriol. *191*, 3740–3746.

Dong, H., Nilsson, L., and Kurland, C.G. (1995). Gratuitous overexpression of genes in Escherichia coli leads to growth inhibition and ribosome destruction. J. Bacteriol. *177*, 1497–1504.

Dong, H., Nilsson, L., and Kurland, C.G. (1996). Co-variation of tRNA abundance and codon usage in Escherichia coli at different growth rates. J. Mol. Biol. *260*, 649–663.

Hernandez, V.J., and Bremer, H. (1991). Escherichia coli ppGpp synthetase II activity requires spoT. J. Biol. Chem. *266*, 5991–5999.

Horiuchi, T., Tomizawa, J.I., and Novick, A. (1962). Isolation and properties of bacteria capable of high rates of beta-galactosidase synthesis. Biochim. Biophys. Acta *22*, 152–163.

Kalisky, T., Dekel, E., and Alon, U. (2007). Cost-benefit theory and optimal design of gene regulation functions. Phys. Biol. *4*, 229–245.

Kaplan, S., Bren, A., Zaslaver, A., Dekel, E., and Alon, U. (2008). Diverse two-dimensional input functions control bacterial sugar genes. Mol. Cell *29*, 786–792.

Koch, A.L. (1983). The protein burden of lac operon products. J. Mol. Evol. *19*, 455–462.

Koch, A.L. (1988). Why can't a cell grow infinitely fast? Can. J. Microbiol. *34*, 421–426.

Laffler, T., and Gallant, J. (1974). spoT, a new genetic locus involved in the stringent response in E. coli. Cell *1*, 27–30.

Lang, G.I., Murray, A.W., and Botstein, D. (2009). The cost of gene expression underlies a fitness trade-off in yeast. Proc. Natl. Acad. Sci. USA *106*, 5755–5760.

Lutz, R., and Bujard, H. (1997). Independent and tight regulation of transcriptional units in Escherichia coli via the LacR/O, the TetR/O and AraC/I1-I2 regulatory elements. Nucleic Acids Res. *25*, 1203–1210.

Maaløe, O., and Kjeldgaard, N.O. (1966). Control of macromolecular synthesis: a study of DNA, RNA and protein synthesis in bacteria (New York: Benjamin, Inc.).

Marr, A.G. (1991). Growth rate of Escherichia coli. Microbiol. Rev. *55*, 316–333.

Mitchell, A., Romano, G.H., Groisman, B., Yona, A., Dekel, E., Kupiec, M., Dahan, O., and Pilpel, Y. (2009). Adaptive prediction of environmental changes by microorganisms. Nature *460*, 220–224.

Nguyen, T.N., Phan, Q.G., Duong, L.P., Bertrand, K.P., and Lenski, R.E. (1989). Effects of carriage and expression of the Tn10 tetracycline-resistance operon on the fitness of Escherichia coli K12. Mol. Biol. Evol. *6*, 213–225.

Novick, A., and Weiner, M. (1957). Enzyme induction as an all-or-none phenomenon. Proc. Natl. Acad. Sci. USA *43*, 553–566.

Paul, B.J., Ross, W., Gaal, T., and Gourse, R.L. (2004). rRNA transcription in Escherichia coli. Annu. Rev. Genet. *38*, 749–770.

Ronen, M., Rosenberg, R., Shraiman, B.I., and Alon, U. (2002). Assigning numbers to the arrows: parameterizing a gene regulation network by using accurate expression kinetics. Proc. Natl. Acad. Sci. USA *99*, 10555–10560.

Sezonov, G., Joseleau-Petit, D., and D'Ari, R. (2007). Escherichia coli physiology in Luria-Bertani broth. J. Bacteriol. *189*, 8746–8749.

Stoebel, D.M., Dean, A.M., and Dykhuizen, D.E. (2008). The cost of expression of Escherichia coli lac operon proteins is in the process, not in the products. Genetics *178*, 1653–1660.

Tagkopoulos, I., Liu, Y.C., and Tavazoie, S. (2008). Predictive behavior within microbial genetic networks. Science *320*, 1313–1317.

Tănase-Nicola, S., and ten Wolde, P.R. (2008). Regulatory control and the costs and benefits of biochemical noise. PLoS Comput. Biol. *4*, e1000125.

Vind, J., Sørensen, M.A., Rasmussen, M.D., and Pedersen, S. (1993). Synthesis of proteins in Escherichia coli is limited by the concentration of free ribosomes. Expression from reporter genes does not always reflect functional mRNA levels. J. Mol. Biol. *231*, 678–688.

Zaslaver, A., Mayo, A.E., Rosenberg, R., Bashkin, P., Sberro, H., Tsalyuk, M., Surette, M.G., and Alon, U. (2004). Just-in-time transcription program in metabolic pathways. Nat. Genet. *36*, 486–491.

Zaslaver, A., Bren, A., Ronen, M., Itzkovitz, S., Kikoin, I., Shavit, S., Liebermeister, W., Surette, M.G., and Alon, U. (2006a). A comprehensive library of fluorescent transcriptional reporters for Escherichia coli. Nat. Methods *3*, 623–628.

Zaslaver, A., Mayo, A., Ronen, M., and Alon, U. (2006b). Optimal gene partition into operons correlates with gene functional order. Phys. Biol. *3*, 183–189.