Cell

Growth Rate-Dependent Global Effects on Gene Expression in Bacteria

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DOI 10.1016/j.cell.2009.12.001

SUMMARY

Bacterial gene expression depends not only on specific regulatory mechanisms, but also on bacterial growth, because important global parameters such as the abundance of RNA polymerases and ribosomes are all growth-rate dependent. Understanding of these global effects is necessary for a quantitative understanding of gene regulation and for the design of synthetic genetic circuits. We find that the observed growth-rate dependence of constitutive gene expression can be explained by a simple model using the measured growth-rate dependence of the relevant cellular parameters. More complex growth dependencies for genetic circuits involving activators, repressors, and feedback control were analyzed and verified experimentally with synthetic circuits. Additional results suggest a feedback mechanism mediated by general growth-dependent effects that does not require explicit gene regulation if the expressed protein affects cell growth. This mechanism can lead to growth bistability and promote the acquisition of important physiological functions such as antibiotic resistance and tolerance (persistence).

INTRODUCTION

With the emergence of a ''system-level'' focus in biology, there has been an increasing emphasis on characterizing gene expression and its regulation in a quantitative fashion (Bintu et al., 2005; Golding et al., 2005; Hasty et al., 2002; Kaplan et al., 2008; Kuhlman et al., 2007). Quantitative and semiquantitative studies have generated new concepts regarding the organization and the dynamic properties of gene regulatory networks, including, e.g., stability of control, robustness of the networks, and stochastic heterogeneity of populations (Elowitz et al., 2002; Rao et al., 2002; Shen-Orr et al., 2002), and have led to the design of synthetic genetic circuits (Andrianantoandro et al., 2006; Atkinson et al., 2003; Elowitz and Leibler, 2000; Gardner et al., 2000; Guido et al., 2006). One complication in the quantitative studies of genetic circuits is that these circuits are always coupled to the physiological state of the cell, which, for example, affects the machinery of transcription and translation. As long as the state of the cell remains unchanged, this dependence does not affect the quantification of gene regulation. However, changes in gene expression often reflect changes in the environment, which also affect the state of the cell. In that case, the coupling of gene expression to the global state of the cell cannot be ignored. We show in this study that this coupling generates an unappreciated layer of physiologically important global effects on gene expression. We focus on gene expression in bacteria in balanced exponential growth, for which the effects of environment on the state of the cell are reflected first and foremost by the growth rate.

The growth rate of bacteria can vary wildly, depending on the type or amount of nutrients available in the growth medium. For example, the doubling time of *E. coli* in exponential batch culture growth ranges easily between ~20 min and several hours. Many parameters of the cells such as their macromolecular composition and the cell size are strongly dependent on the growth conditions (Maaløe, 1979; Neidhardt et al., 1990; Schaechter et al., 1958). For *E. coli* and *Salmonella*, in which this dependence has been quantitatively characterized, the results can be expressed as a dependence on *growth rate* rather than on the specific growth media themselves: growth experiments with many different media have shown that media that support the same growth rate produce cells with the same macromolecular composition (Maaløe, 1979; Neidhardt et al., 1990; Schaechter et al., 1958). Many parameters of the cell have therefore been characterized quantitatively as functions of the growth rate for *E. coli* (Bremer and Dennis, 1996).

Many of these growth rate-dependent parameters, e.g., gene and plasmid copy numbers and the abundance of RNA polymerases and ribosomes (Bremer and Dennis, 1996), are known to affect gene expression. Changes in gene expression, which are often accompanied by a change of the growth rate, thus result from a combination of gene regulation and intrinsic global effects due to growth rate. Any quantitative understanding of gene expression therefore requires an understanding of these global effects. Indeed, expression of a large number of proteins

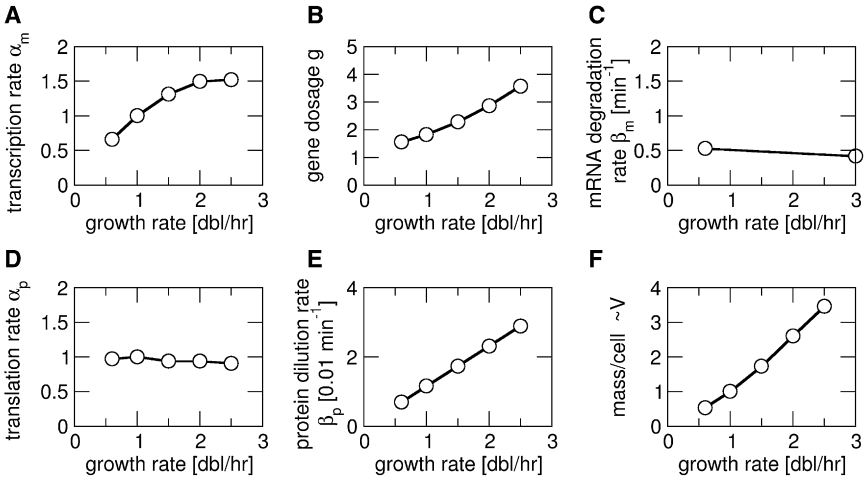

Figure 1. Growth-Rate Dependence of Global Cellular Parameters Affecting Gene Expression

Transcription rate per gene (A), gene copy number per cell (B), mRNA degradation rate (C), translation rate per mRNA molecule (D), protein dilution rate due to growth (E), and cell mass (F), used as a measure for the cell volume *V*, as functions of the growth rate. All parameters are for constitutively expressed (unregulated) genes. For a description how the data was collected from the literature and for references, see the main text and Table S1.

is known to exhibit different types of growth-rate dependences (Pedersen et al., 1978). Growth rate-dependent regulation is most notable for the transcription of ribosomal RNA (Haugen et al., 2008), but is also known for ribosomal proteins, where it relies largely on posttranscriptional regulation (Keener and Nomura, 1996), as well as for several nonribosomal proteins, where it is based on transcriptional mechanisms that appear to be different from the control of ribosomal RNA (Chiaramello and Zyskind, 1989; Husnain and Thomas, 2008). In contrast to these instances of specific growth rate-dependent regulation, the global effects addressed here are expected to affect all genes. Their interplay with specific mechanisms of gene regulation can lead to rather complex behaviors, and it is possible that they play a role in some of the known examples for specific growth rate-dependent regulation.

An obvious starting point to study global growth rate-dependent effects on gene expression is the growth-rate dependence of the expression of an unregulated (constitutively expressed) gene. Indeed, several studies have shown that the expression of a constitutively expressed gene is growth-rate dependent (Liang et al., 1999a; Wanner et al., 1977; Willumsen, 1975). We will show that the observed dependence can be quantitatively explained by a simple model using the known growth-rate dependencies of the key cellular parameters without invoking any adjustable free parameters.

We then expand our model to investigate the effect of growth rate on regulated genes and simple genetic circuits to address the following questions: How is the growth-rate dependence of gene expression affected by positive or negative regulation? How should a gene be regulated to exhibit a growth rate-independent protein concentration? Is the qualitative behavior of a circuit the same at different growth rates? Answers to these questions may also help in the design on synthetic genetic circuits in order to obtain robust performance over a wide range of growth conditions. Experimental results are presented to validate key predictions of the model using simple synthetic genetic circuits.

Finally, we explore cases with global feedback mediated by growth rate-dependent effects: in these situations, there is not only an effect of growth rate on gene expression, but the expression level of a protein also has an effect on the growth rate. Circuits of this type can lead to growth bistability. We discuss possible roles these effects may play in metabolic control, antibiotic resistance, and tolerance (persistence).

RESULTS

Growth-Rate Dependence of Global Cellular Parameters

We start by considering the growth-rate dependence of unregulated (constitutive) gene expression, which has been reported experimentally for *E. coli* in several cases (Liang et al., 1999a; Wanner et al., 1977; Willumsen, 1975). From a bottom-up perspective, it is not clear whether the concentration of a constitutively expressed protein should be expected to increase or decrease at faster growth. On the one hand, faster dilution of the protein by faster growth should reduce its concentration, but on the other hand, transcription rates are known to be increased at faster growth, as well (Liang et al., 1999a). To predict the growth-rate dependence of a constitutively expressed protein, we used a simple model of gene expression and searched the literature for the growth-rate dependence of all relevant parameters (Figure 1 and Table S1 available online). In our model, the expression level of a protein depends on six parameters, the cellular copy number of the gene (g), the transcription rate per copy of the gene (α_m), the mRNA degradation rate (β_m), the translation rate per mRNA (α_p), the protein degradation rate (β_m), and the cell volume (V), all of which may have a dependence on the growth rate (μ). These parameters determine the numbers of mRNA and protein molecules per cell, $M = g\alpha_m/\beta_m$ and $P = g\alpha_m\alpha_p/(\beta_m\ \beta_p)$, as well as the corresponding concentrations, $m = M/V$ and $p = P/V$. The quantity of main interest is the resulting concentration of the protein, which is given by

$$p = g\alpha_m\alpha_p/(\beta_m\beta_p V). \quad (1)$$

The growth-rate dependence of the transcription rate per gene has been characterized for several constitutive promoters (Liang et al., 1999a). They were found to exhibit the same dependence, increasing at slow growth and saturating at fast growth (Figure 1A). This growth-rate dependence is believed to reflect the availability of RNA polymerase in the cell (Klumpp and Hwa, 2008; Liang et al., 1999a).

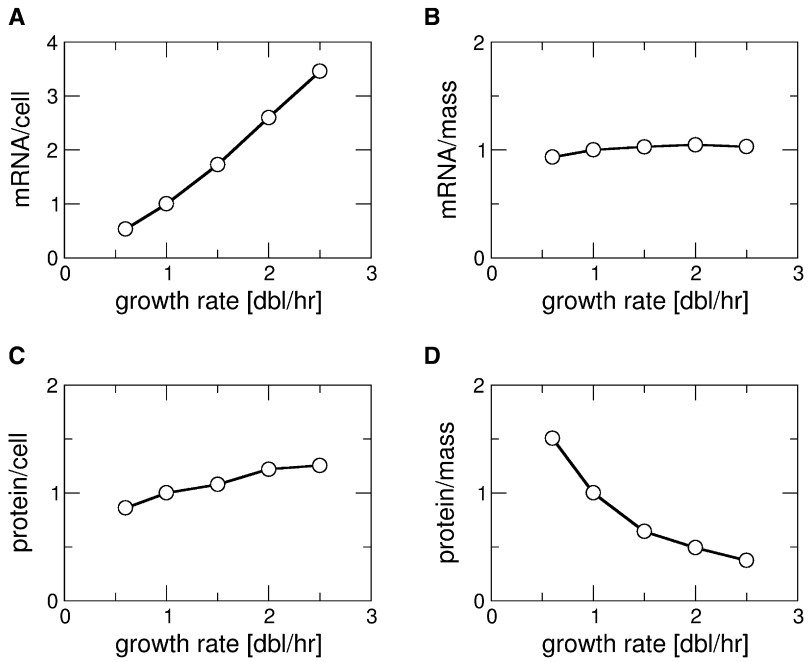

Figure 2. Calculated Growth-Rate Dependence of Constitutive Gene Expression

Expression level of a constitutively expressed gene as characterized by the number of mRNA transcripts of that gene per cell (A), its mRNA concentration (B), its number of protein molecules per cell (C), and the protein concentration (D), calculated from the growth-rate dependence of the parameters shown in Figure 1. All curves are normalized to their value at one doubling per hour.

The gene copy number per cell is determined by the dynamics of DNA replication and cell division and has been well characterized (Cooper and Helmstetter, 1968). It is growth-rate dependent, because at different growth rates a gene is replicated at different time points in the cell division cycle. At fast growth the gene copy number is further increased due to overlapping rounds of DNA replication. The growth-rate dependence of the gene copy number is calculated using the Cooper-Helmstetter relation (see Table S1) and is plotted in Figure 1B for a position halfway between the origin and terminus of replication.

The degradation rate of mRNA appears to be rather independent of growth rate, as indicated by studies of the stability of total cellular mRNA (Coffman et al., 1971; Pato and von Meyenburg, 1970) and of specific transcripts such as lacZ (shown in Figure 1C), bla, and lpp, which all had almost the same lifetime at different growth rates (Liang et al., 1999b; Nilsson et al., 1984). Furthermore, a genome-wide study (Bernstein et al., 2002) found that the lifetimes of most transcripts differed by less than 2-fold between growth in minimal and rich medium, with no obvious correlation between the two conditions. The independence of mRNA stability to growth rate may be attributed to the autoregulation of RNase E (Jain et al., 2002) (see the Supplemental Data).

The growth-rate dependence of the translation rate α_p has been determined for the lacZ transcript and was found to be approximately constant over a range of growth rates from 0.6 to three doublings per hour (Liang et al., 2000), as shown in Figure 1D. This finding is surprising, given that the cellular concentration of ribosomes increases strongly with increasing growth rate (Bremer and Dennis, 1996), and is discussed further in the Supplemental Data. Here, we take the finding for lacZ as typical and assume the translation rate to be growth-rate independent. Finally, if our protein of interest is stable, it is not degraded, but rather diluted out by cell growth and division, so that β_p is given by the growth rate μ through $\beta_p = \mu \ln 2$ (Figure 1E).

To predict protein and mRNA concentrations, we also need the growth-rate dependence of the cell volume. As a measure of cell volume we use cell mass, which is easily measured by optical density and is commonly used to express measured concentrations. Several studies have shown that cellular mass and volume exhibit the same growth-rate dependence (Donachie and Robinson, 1987; Nanninga and Woldringh, 1985), increasing strongly with increasing growth rate as shown in Figure 1E (data from Bremer and Dennis, 1996) (see also the detailed discussion in the Supplemental Data). An alternative normalization for concentrations is per total cellular protein rather than per cell mass. While the two are roughly equivalent, the total cellular protein concentration (per mass) increases slightly at slower growth, so that this normalization leads to a slightly weaker growth-rate dependence (see below).

Growth-Rate Dependence of Constitutive Gene Expression

From the growth-rate dependence of the global parameters (Figure 1), we calculated by Equation 1 the predicted growth-rate dependence of the protein and mRNA expression levels for a constitutively expressed gene (Figures 2A–2D). Our results predict that the number of transcripts of such a gene per cell is strongly increased at faster growth (Figure 2A), while the concentration of transcripts is rather independent of growth rate (Figure 2B). Likewise, the protein copy number per cell is increased at faster growth (Figure 2C), although less than the number of transcripts, and the protein concentration is decreased at faster growth (Figure 2D). The decrease of the protein concentration despite an increase of its molecule number per cell reflects the strongly increased cell volume at fast growth.

In Figures 3A and 3B, we compare the calculated growth-rate dependence of the concentrations of constitutively expressed proteins with available experimental data. These data are derived by different labs from various *E. coli* strains, for various genes expressed constitutively because their regulation has been inactivated (by deletion of the regulators or mutations of the operator sites), and for a synthetic promoter-reporter system constructed for this study (green squares). Data in Figure 3A show concentrations obtained by normalization to cell mass, while data in Figure 3B are normalized to total protein. In general, our calculated growth-rate dependence (red) agrees very well

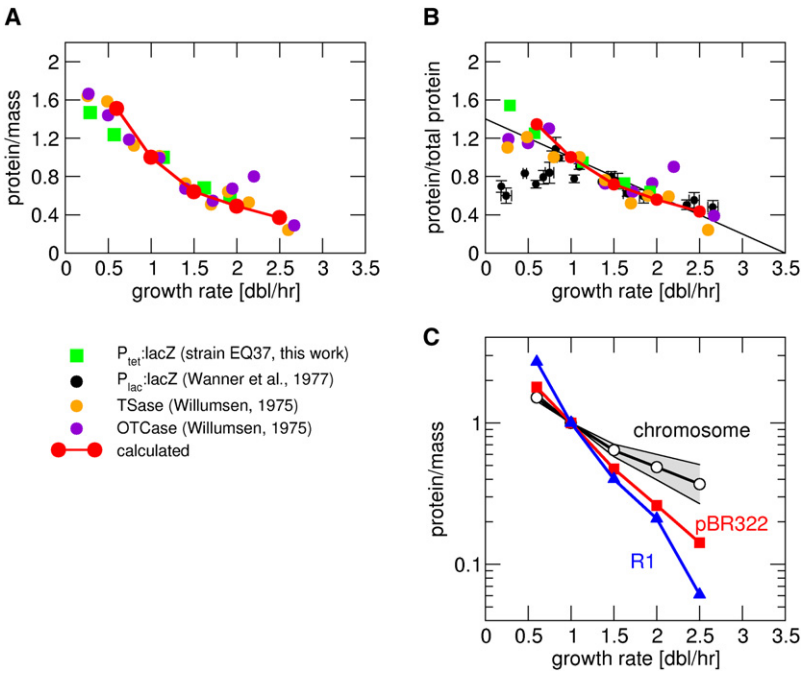

Figure 3. Growth-Rate Dependence of Constitutively Expressed Genes on the Chromosome and Plasmid

(A and B) The calculated growth-rate dependence of the concentration of a protein encoded by a constitutively expressed gene (red) is compared to experimental data on constitutively expressed genes in various *E. coli* strains. The orange and purple dots are derived from the activities of tryptophan synthase and ornithine transcarbamylase respectively, from a strain in which their respective regulators were deleted (Willumsen, 1975). The black dots are derived from LacZ expressed from the mutant LacL1 type promoters (Wanner et al., 1977). The green squares are from this work, with LacZ expressed from the synthetic $P_{LTet\text{-}O1}$ promoter in strain EQ37, which contains no TetR. Data in (A) are normalized to total mass as measured by optical density, and data in (B) to total protein. All data are plotted relative to their expression levels in cells grown at one doubling per hour.

(C) Comparison of the calculated protein concentration for genes on the chromosome (shaded gray area; the black line indicates the curve from Figure 3A), on plasmids pBR322 (red) and R1 (blue).

with the data. Data normalized to total protein, however, show a significant spread for very slow growth. The origin of this spread is not clear. We also note that the experimental data for constitutively expressed protein per total protein are well approximated by a linear relation (black line in Figure 3B). According to this relation, the concentration of such a protein would become zero for a (hypothetical) growth rate of ~3.5 doublings per hour, slightly higher than the highest growth rate that can be attained. The origin of this linear relation is discussed elsewhere (M. Scott, C.W. Gundersen, E. Mateescu, Z.Z., and T.H., unpublished data).

The gene copy number per cell depends on the position on the chromosome (Bremer and Dennis, 1996; Cooper and Helmstetter, 1968). Figure 3C indicates the range of growth-rate dependencies for different chromosomal positions. The two boundaries of the gray area indicate the strongest and the weakest growth-rate dependence for chromosomal genes, obtained for genes close to the origin and terminus of DNA replication, respectively. For genes on a plasmid, the gene copy number is given by the plasmid copy number and will in general be different from those for chromosomal genes. For plasmid pBR322, the copy number per cell is slightly increased at faster growth, but much less than for chromosomal genes (Lin-Chao and Bremer, 1986). The copy number of plasmid R1 even decreases at faster growth (Engberg and Nordström, 1975) (see also Table S1). In both cases, the protein concentration for a plasmid-encoded constitutively expressed gene is predicted to follow a much stronger growth-rate dependence than that for chromosomal genes (Figure 3C).

Simple Regulatory Elements: Activation and Repression

Regulated genes are directly affected by growth rate in the same way as constitutively expressed genes; in addition, they are affected by the growth-rate dependence of their regulator concentrations. For example, the expression of a protein E that is negatively regulated by a constitutively expressed repressor R (Figure 4A) is affected by a reduced expression at faster growth because of the direct growth effects discussed above (Figure 3), and a weakened repression at faster growth because the growth-rate dependence of the repressor concentration itself. The balance of these two effects is analyzed by expanding the above model of growth-rate dependence to include basic features of gene regulation (Bintu et al., 2005) (see the Supplemental Data). The compounded effect is predicted to depend mainly on the cooperativity of repression, but also on the strength of repression (strengths of the promoter driving the repressor relative to the repression threshold). An example is illustrated in Figure 4A for strong repression, with a weak, inverse growth-rate dependence (red squares) for noncooperative repression (Hill coefficient n = 1), and a strong, positive growth-rate dependence (red triangles) for cooperative repression. Weaker repression results in weaker growth-rate dependence also in the cooperative case (see the Supplemental Data).

For positive regulation by a constitutively expressed activator A (Figure 4B), the two effects of growth both tend to decrease the expression level of the target protein E, so that in this case, the target is predicted to always exhibit a stronger inverse growth-rate dependence (green) than a constitutive gene (black).

The above predictions were tested with several synthetic genetic circuits (Table S2) expressing LacZ reporter in strains derived from *E. coli* MG1655. LacZ activity was assayed during exponential growth in a variety of media that provided a range of growth rates. In strain EQ38, a constitutively expressed repressor (TetR) controls LacZ expression through the synthetic TetR-dependent $P_{LTet\text{-}O1}$ promoter (Lutz and Bujard, 1997). Its LacZ expression (red dots in Figure 4D) is seen to have a weaker

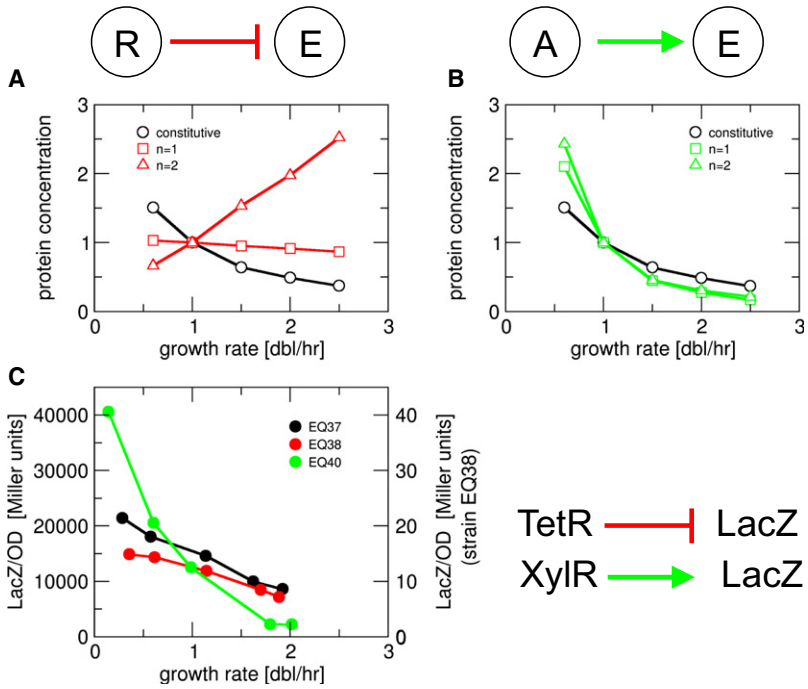

Figure 4. Growth-Rate Dependence of Simple Negative and Positive Regulation

(A and B) Concentration of a protein under negative regulation by a constitutively expressed repressor (A) and positive regulation by a constitutively expressed activator (B). The two plots are generated, respectively, by Equations S9 and S11 in the Supplemental Data, for noncooperative (Hill coefficient n = 1, squares) and cooperative regulation (n = 2, triangles). Black symbols show the concentration of constitutively expressed protein. The parameters used for the plots are r_1/K = 10 (A) and a_1/K = 0.1 and f = 100 (B).

(C) Experimental data for the concentrations of LacZ reporter under constitutive expression (strain EQ37: P_{LtetO1}-*lacZ*, no *tetR*, black), repression (strain EQ38: P_{con}-*tetR*, $P_{LTet\text{-}O1}$-*lacZ*, red), and activation (strain EQ40: $P_{Llac\text{-}O1}$-*dnxylR*, Pu-*lacZ*, no *lacI*, green), showing weaker growth-rate dependence under repression and stronger growth-rate dependence under activation as compared to the constitutive case.

growth-rate dependence than the cogenic strain (EQ37) not containing *tetR* (black). In strain EQ40, a constitutively expressed activator (dnXylR) controls LacZ expression through the Pu promoter derived from the TOL plasmid of *Pseudomonas putida* (Pérez-Martín and de Lorenzo, 1996). A stronger growth-rate dependence (green) than for constitutive expression is seen for this system. The experimental results are in good semiquantitative agreement with the predictions (compare like color curves in Figure 4). Detailed quantitative comparisons require quantitative knowledge of the promoter characteristics (e.g., cooperativity and repression threshold) and will be pursued elsewhere.

Homeostatic Circuits

Although simple repression can result in rather weak growth-rate dependence (Figure 4C, red dots), significant growth-rate dependence of LacZ expression is seen for strain EQ38 (Figure 5A, red symbols) in the presence of cl-Tc, an inducer of TetR which itself hardly affected growth (Figure S1). The inducer dependence is complex due to a variety of factors, including the inducer-TetR interaction and inducer transport, and is beyond the scope of this study. Here, we ask how a gene should be regulated to obtain constant protein concentration over a wide range of growth rates even in the presence of complex growth

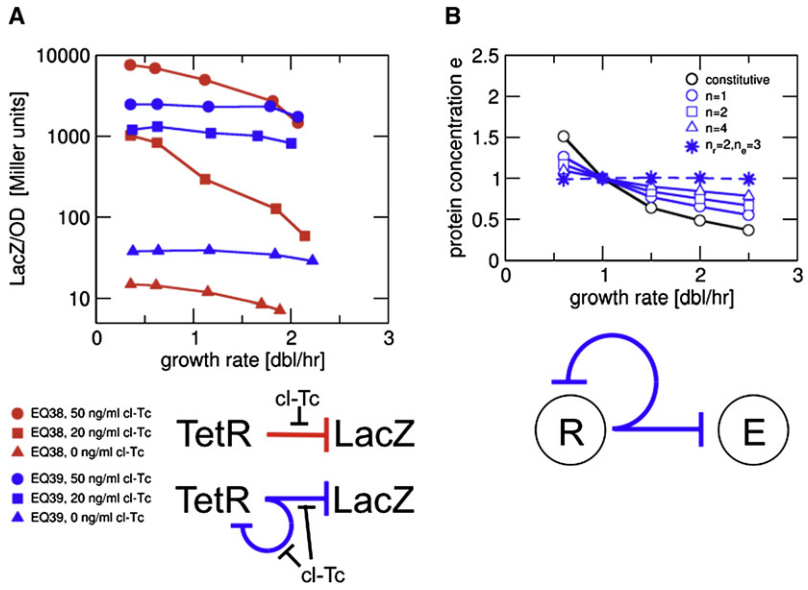

Figure 5. Growth-Rate Dependence of Genetic Circuits with Negative Autoregulation

(A) Experimental data for growth-rate dependence of simple repression (EQ38: P_{con}-*tetR*, $P_{LTet\text{-}O1}$-*lacZ*, red symbols), and autorepression (EQ39: $P_{LTet\text{-}O1}$-*tetR*, $P_{LTet\text{-}O1}$-*lacZ*, blue symbols) in the presence of the inducer cl-Tc (circles, 50 ng/ml; squares, 20 ng/ml; triangles, no inducer). For simple repression, induction results in significant growth-rate dependence. Autorepression exhibits growth rate-independent LacZ concentration, which is nevertheless tunable by the inducer level.

(B) Our model predicts weak growth-rate dependence for a protein E controlled by an autoregulated repressor R. If E and R are driven by the same promoter (solid lines, from Equation S14, with r_1/K_r = 10), weaker growth-rate dependence is obtained by increasing cooperativity (larger Hill coefficient n). Independence of growth rate is predicted for E and R driven by different promoters, whose respective Hill coefficients for repression (n_e and n_r) satisfy $n_e = n_r+1$ (dashed line, from Equation S17, with r_1/K_r = 10 and r_1/K_e = 10).

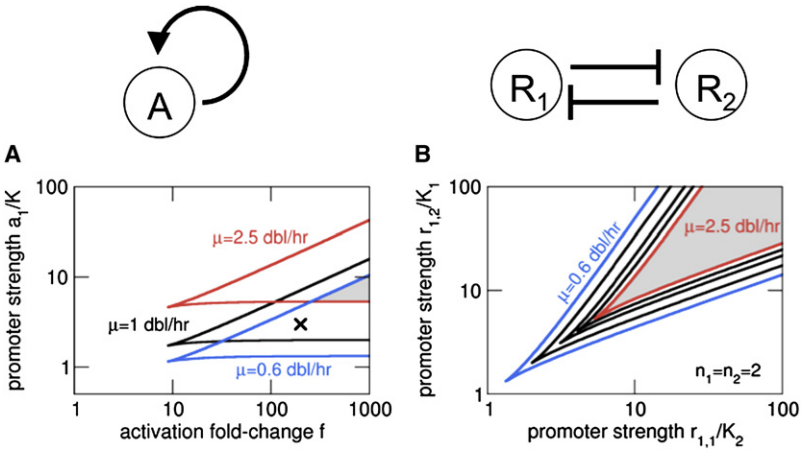

Figure 6. Effects of Growth Rate on Bistable Genetic Circuits

The parameter ranges for bistability at different growth rates are plotted for the autoactivator (A) and the toggle switch (B). The lines describe boundaries of the bistable regime, obtained from linear stability analysis of Equation S18 (A) and Equations S19 and S20 (B). The gray areas indicate the parameter range for which bistability is obtained over the full range of growth rates considered here (0.6–2.5 doublings per hour).

rate-dependent effects. A longstanding candidate (Sompayrac and Maaloe, 1973) is negative autoregulation, a well-known mechanism for homeostasis (Savageau, 1974). Using our model to investigate the expression of a target protein E controlled by a negative autoregulator R, we find very weak growth-rate dependence, in particular for highly cooperative repression (large *n*; see the solid blue curves in Figure 5B for some examples where both R and E are regulated by the same promoter). If the two genes are expressed by different promoters, it is in principle possible to fine-tune the cooperativity of repression to achieve complete independence to growth effects (dashed blue curve in Figure 5B; see also the discussion in the Supplemental Data).

The behavior of the negative feedback regulated circuit was tested by replacing the promoter driving *tetR* in strain EQ38 by the $P_{LTet\text{-}O1}$ promoter. The resulting strain EQ39 exhibited much reduced growth-rate dependence in LacZ expression (Figure 5A, blue symbols) with or without induction, yet the expression levels clearly depended on the inducer levels. This result shows that negative autoregulation can indeed allow the cell to tune enzyme levels in a growth rate-independent manner.

Bistable Circuits

As an example for more complex genetic circuits, we consider bistable circuits, where for some range of the circuit parameters genetically identical cells in a population can exhibit different behaviors, e.g., with a high expression level of a reporter gene in one subpopulation and a low expression level in the other subpopulation. Two such circuit designs have been described in the synthetic biology literature: (1) a single gene controlled by positive autoregulation (Atkinson et al., 2003; Isaacs et al., 2003) and (2) a ''toggle switch'' system consisting of two genes which repress each other (Gardner et al., 2000).

The autoactivating circuit is known to exhibit bistability provided that autoactivation is cooperative with a Hill coefficient n > 1 (Atkinson et al., 2003; Isaacs et al., 2003). In Figure 6A, we plot the bistable regimes obtained from our model for different growth rates in the space of two key parameters for this circuit, the promoter strength (a_1/K), and the maximal fold-activation (*f*) of the promoter, for n = 2. The bistable regime for each growth rate is contained within the wedge defined by the pair of lines with corresponding color. The results show that the circuit can exhibit qualitatively different behavior at different growth rates: a circuit with parameter values marked by the cross in Figure 6A will exhibit bistability, i.e., a mixed population of bacteria with either a high or a low level of activator expression, at slow growth (both at 1 and 0.6 doublings per hour) but not at fast growth, where the whole population goes to the low state. The parameter range where bistability persists over a wider range of growth rates can be quite narrow (shaded gray area in Figure 6A), although it can be expanded within the model by either an increased fold activation *f* or higher cooperativity.

For the toggle switch system based on the mutual repression of two genes, the bistable regime predicted by the model depends on the strengths of the two promoters, as plotted in Figure 6B. We see that the bistable regime for fast growth, although smaller, is entirely contained within the regime for slow growth. Thus, unlike the autoactivator case, the toggle switch circuit exhibiting bistability at fast growth is also expected to exhibit bistability at slow growth. Figure 6B actually pertains to a case where the repressors can provide arbitrarily large degree of repression if expressed high enough. The alternative case with a finite basal expression level produces qualitatively similar behavior as described in the Supplemental Data. Consequently, we expect it to be easier to maintain bistability at different growth rates for a toggle switch circuit than an autoactivating circuit.

Feedback through Expression-Dependent Growth Rate

So far, we have considered growth rate-dependent effects on genetic circuits, assuming that the output of the circuits would not affect the growth of the cells. While this situation can be mimicked by synthetic circuits expressing moderate amounts of reporter proteins, we note that in many cases, the expression of a target protein will also affect cell growth, if the protein is, e.g., toxic to the cell (inhibiting growth) or relieves a metabolic ''bottleneck'' (stimulating growth). These cases represent examples of positive and negative feedback through an expression level-dependent variation of the growth rate, which in turn affects the expression level.

Let us consider the slowdown of growth due to expression of a toxin (an example is HipA; see the Discussion), which we model by a noncooperative Hill function of the toxin concentration *p* with threshold p_μ (Figure 7A). For constitutive expression of the toxin, the growth inhibition is expected to increase toxin expression because of the generic growth rate-dependent effect discussed above (Figure 3). This increase will slow down growth

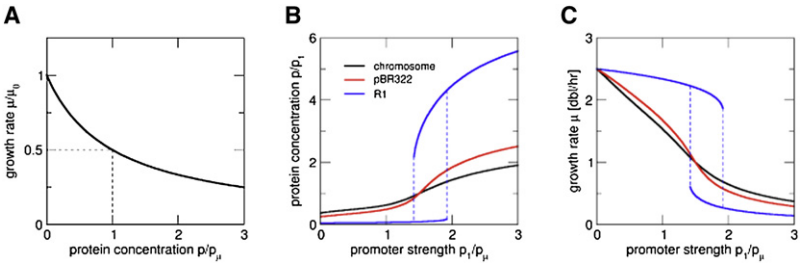

Figure 7. Feedback through Growth

(A) Growth inhibition due to expression of a toxin is described by a Hill function of the toxin concentration p, characterized by a threshold concentration (p_μ) for which the growth rate is reduced to half the maximal growth rate (μ_0).

(B and C) Toxin concentration (normalized to its concentration at one doubling per hour, p_1) (B) and growth rate (C) as functions of the promoter strength (characterized by p_1 and normalized to p_μ), obtained from Equation S22. Growth inhibition results in nonlinear increase of the toxin concentration, which is steeper for a gene on a pBR322 plasmid (red) than for a gene on the chromosome (black). For a gene on plasmid R1 (blue) with its strong growth-rate dependence (Figure 3C), there is a region of bistability with two branches, one with high toxin concentration and slow growth and the other with low toxin concentration and fast growth.

further, resulting in positive feedback. Quantitatively, our model predicts a nonlinear relation between the steady-state concentration p and the promoter strength (Figure 7B). Figure 7C depicts the corresponding predictions on the growth rate. The nonlinearity is seen to be the weakest for a chromosomally encoded toxin (black line) and increases progressively for a toxin gene on the pBR322 plasmid (red line) and on the R1 plasmid (blue line). According to the model, the increasing nonlinearity results from the increasing dependence of constitutive expression on growth rate for the three systems (Figure 3C). Note that the blue lines in Figures 7B and 7C admit two possible solutions for a range of promoter strengths. This implies occurrence of growth bistability, i.e., the coexistence of two genetically identical subpopulations with different levels of toxin expression and hence different growth rates, merely due to a constitutively expressed gene on the R1 plasmid if its promoter strength falls between the two dashed lines. We note that growth-mediated feedback may also work in conjunction with regulatory feedback; see the Supplemental Data for an example.

DISCUSSION

Genetic circuits are unavoidably coupled to the physiological state of the cell, which is reflected in global cellular parameters such as cell size, gene copy numbers, and the abundance of RNA polymerases and ribosomes, all of which change when the cell state changes. In this study, we have taken the classic result that these global parameters depend on growth media primarily through the growth rate as an empirical fact (Maaløe, 1979; Schaechter et al., 1958) and explored its consequences by building a simple, parameter-free model of constitutive gene expression that is completely determined by the cellular parameters measured meticulously by different labs in the past three decades. The validity of this approach is established through the excellent agreement on the growth rate dependence of constitutive gene expression generated by the model and the experimental results obtained by different labs using different strains of *E. coli* grown in different media, with different reporter genes expressed by different promoters (Figure 3A and 3B). We note that the growth rate-dependent effects studied in this work pertain to "nutrient-limited" growth and should not be applied to growth modulation by, e.g., translational inhibition, which results in very different ribosome abundance (Harvey and Koch, 1980), or osmotic stress, which changes the mass-volume relationship (Cayley and Record, 2004). We also do not exclude the possibility that certain nutrients may not obey the trends discussed here, because of, e.g., growth-inhibiting effects by toxic intermediate metabolites. Nevertheless, the very robust correlation between growth rate and the cellular parameters documented over the past five decades (Bremer and Dennis, 1996; Maaløe, 1979; Schaechter et al., 1958) lead us to believe that deductions based on these correlations will be equally robust.

The importance of these global growth-dependent effects is illustrated first and foremost by the variable expression level of a constitutively expressed gene: in that case, the resulting protein concentration can differ up to 10-fold between growth in rich and poor medium (Figures 2 and 3). The predictions of our model for this quantity are in excellent agreement with measured protein concentrations (Figure 3). Other predictions remain to be tested, including the different growth-rate dependence for the expression of genes encoded on the chromosome versus plasmid (Figure 3C) and the qualitatively different (i.e., opposing) growth-rate dependence of mRNA or protein levels, measured per cell or per cellular mass (Figure 2).

Growth-rate dependencies become more complex when gene regulation and feedback are involved. Expansion of our basic model gives precise predictions on the growth-rate dependence of regulated genes, by simple repression, activation, as well as including feedback loops. Particularly noteworthy features among these results are the amplification of growth-rate dependence for genes expressed by activators and the suppression of growth-rate dependence for genes controlled by autorepressors. Some of these predictions have been validated at a semi-quantitative level by our experiments with synthetic circuits. Other predictions regarding more complex feedback loops, including regulatory feedback and growth-dependent feedback due to the effect of expressed genes on growth, remain to be tested.

Practical Consequences: Interpretation of Data, Circuit Modeling, and Design

Different Measures of Gene Expression Have Different Growth-Rate Dependence

The existence of global effects on gene expression that reflect the physiological state of the cell has several practical consequences for the analysis of experimental gene expression data in both large-scale expression profiles and studies of individual circuits, for the design of synthetic genetic circuits, and for circuit

modeling. First, our results predict that different measures of the level of gene expression such as the transcription rates, mRNA concentration, and protein concentration exhibit different growth-rate dependence (Figures 1 and 2). While these quantities may be considered as equivalent measures of the "expression level" at a fixed growth rate, they are generally not equivalent when data with different growth rates are compared.

Changes in Gene Expression Need Not Reflect Regulation

Second, as even the concentration of a protein product of an unregulated gene is growth-rate dependent, care has to be taken when interpreting data using the expression of a reporter protein. An increased or decreased concentration of the reporter protein in one condition compared to another (e.g., two different growth media, with and without induction, wild-type versus mutant, etc.) does not necessarily imply the existence of specific regulation, if the growth rate changed between the conditions. The discrepancy will be particularly pronounced for reporter genes on plasmids, where the global growth rate-dependent effects are very strong (Figure 3C). The effects of growth rate and of regulation can however be disentangled with the results presented here. In particular, this is important when the change in protein concentration is only a few fold, i.e., of the same order of magnitude as the growth rate-dependent effects. Large fold changes in protein concentration, on the other hand, are very unlikely to be solely due to growth rate and will therefore typically indicate regulation, but even then growth-rate dependence needs to be taken into account for a quantitative interpretation. Finally, we mention that mRNA abundance is in principle a good candidate measure of gene expression, as mRNA/mass hardly exhibited any growth-rate dependence for constitutive expression (Figure 2B). However, quantification of mRNA (e.g., by qPCR) involves comparison to a "standard," often taken to be the 16S ribosomal RNA. The latter unfortunately has perhaps the strongest growth-rate dependences known (Bremer and Dennis, 1996). Thus care should be taken in the interpretation of these results.

Growth-Rate Effects Can Change the Qualitative Behavior of Genetic Circuits and Need to be Included in Circuit Design

The growth-rate dependence of gene expression is also an aspect to be taken into account in the design of synthetic genetic circuits. One question of interest is how to design a circuit to make the concentration of an enzyme independent of growth rate (or at least approximately so). We showed that this may be achieved by negative autoregulation (Figure 5), a strategy which is widely used in bacteria, in particular in the regulation of biosynthetic operons (Shen-Orr et al., 2002; Thieffry et al., 1998).

Furthermore, even the qualitative behavior of more complex circuits may be different at different growth rate, as shown by the example of bistability (Figure 6). In some cases, the growth-rate dependence may be desirable, e.g., to have a switch responding to growth status. In many cases, however, one may be more interested in achieving the same circuit functionality under a wide range of growth conditions, which will impose constraints on the circuit design. For example, in the case of bistability, the toggle switch system will be preferable compared to the autoactivator system.

Growth Effects Are Not Accurately Described by Protein Dilution Only

Growth rate-dependent effects are not accurately captured by "dilution models" that include only the effect of protein dilution, as is often done in circuit modeling (e.g., Narang and Pilyugin, 2008). Such models are incompatible with the observation that other parameters of gene expression are also growth-rate dependent; they result in approximately correct predictions for the growth-rate dependence of protein concentrations (for growth rates >0.6 doublings per hour), but not for the amount of protein per cell (Figures S2A and S2B). At slower growth, dilution models strongly overestimate growth rate-dependent effects (Figure S2A); they therefore tend to overemphasize growth-dependent effects such as growth bistability (Figure S2C). Finally, we expect dilution-only models to be especially problematic for plasmid-encoded genes that can exhibit very strong growth-rate dependencies (Figure 3C) and for translation-limited growth (M. Scott, C.W. Gundersen, E. Mateescu, Z.Z., and T.H., unpublished data).

Possible Physiological Roles of Growth Feedback

Feedback through Growth-Dependent Gene Expression May Be an Integral Part of Metabolic Control

Feedback through growth should also play an important role in natural regulatory processes. For example, if growth is limited by the concentration of one specific protein (e.g., because it imports or synthesizes an essential metabolite), there is a built-in negative feedback since a decrease in the concentration of this protein would lead to a slowdown of growth which would in turn increase the protein concentration (Figure 3), even if the protein is constitutively expressed. A bottleneck enzyme is indeed expected to be effectively constitutive, since it should be expressed at the maximal level, i.e., fully activated or fully derepressed, according to well-designed metabolic control mechanisms. This built-in negative feedback can dampen harmful effects of fluctuations in enzyme levels on growth and compensate against fluctuations in external nutrient levels.

Positive Feedback through Growth Can Lead to Growth Bistability

If a protein is "toxic," i.e., if a high concentration of the protein has a detrimental effect on growth, then expression of this protein will lead to decreased growth that will further increase its concentration, resulting effectively in a positive feedback even in the absence of specific regulation. Such positive feedback can lead to bistability, i.e., heterogenous subpopulation with different degree of gene expression in genetically identical cells. Here, the bistability is reflected not only in gene expression but also in the (very) different growth rates of the subpopulations expressing or not expressing the toxic protein. In Figure 7, we described an example where this growth bistability is expected to occur for an unregulated gene encoded on a plasmid whose copy number strongly depends on the growth rate.

Growth Bistability May Underlie Persistence

Growth bistability may be a mechanism underlying the phenomenon of persistence, i.e., the tolerance of bactericidal antibiotics in a subpopulation due to an epigenetic (nonmutational) mechanism. Single-cell experiments have shown that persistent cells grow slowly and may switch stochastically back to normal

growth (Balaban et al., 2004). One known mechanism for slow growth in persistent cells depends on the expression of the toxin HipA (Keren et al., 2004). These observations suggest that persistence may be linked to growth bistability due to a feedback loop through expression level-dependent growth reduction. Of particular interest here is that HipA can induce persistence when expressed constitutively on a high-copy number plasmid (Korch and Hill, 2006). Our analysis suggests that this may be an effect of a strong growth-rate dependence of plasmid copy number (Figures 7B and 7C), which leads to a strong growth rate-dependent expression of the plasmid-encoded gene, without the need of invoking any hypothetical cooperative growth inhibition mechanisms, as has been proposed recently (Lou et al., 2008).

Similar type of growth bistability is expected to arise from growth stimulation by the constitutive expression of antibiotic resistance genes in the presence of antibiotics (M. Scott, C.W. Gundersen, E. Mateescu, Z.Z., and T.H., unpublished data). The abrupt increase of the growth rate, and thus of bacterial fitness, in the bistable region may provide a driving force promoting the rapid evolution of antibiotic resistance (Walsh, 2000) without the need for elaborate regulation. Nontrivial feedback effects obtained in the absence of genetic regulation is in fact a common theme in all of the growth-dependent effects discussed above. We suggest that these effects may provide an evolutionary expedient mechanism for the development of a rudimentary molecular network for which nonlinear feedback effects are essential. Once a bare network is in place, more elaborate regulatory mechanisms (which are more difficult to arise evolutionarily) may be acquired step-by-step later to fine-tune the system.

EXPERIMENTAL PROCEDURES

Model for Gene Expression

The expression of a gene is modeled by two equations for the mRNA and protein copy number per cell, as described in detail in the Supplemental Data. The growth-rate dependence of all parameters is taken from the literature as described in the Results and summarized in Table S1. Most of the data consist of values for the parameters at a few different growth rates. These were interpolated where necessary to obtain a complete parameter set for the same set of growth rates. The gene copy number for chromosomal genes was calculated according to the Cooper-Helmstetter model for DNA replication (Bremer and Dennis, 1996; Cooper and Helmstetter, 1968).

Model for Regulation

A detailed description of the circuits studied here is given in the Supplemental Data. In brief, the direct growth-rate dependence of a regulated gene, which is the same as for unregulated genes, is described by a function $F(\mu)$, given by the concentration of a constitutively expressed protein at a growth rate μ, normalized by its concentration at one doubling per hour. The concentration of an unregulated gene is then given by $p = p_1 F(\mu)$, where p_1 is the concentration at one doubling per hour. Gene regulation modulates this expression for the protein concentration (e) by a Hill-type regulation function, $e = e_1 F(\mu) R(r)$ for repression and $e = e_1 F(\mu) A(a)$ for activation (Bintu et al., 2005). Here, e_1 is the concentration of the protein at growth rate of one doubling per hour, taken at maximal expression (i.e., for full activation or in the absence of repression); r and a are the concentrations of the repressor and activator, respectively. The parameters of the regulation function (R or A), the fold change f, the Hill coefficient n, and the threshold K, which reflect physical properties of the regulatory elements, are taken to be independent of growth rate for simple regulatory elements.

Experimental Measurement of Growth Rate-Dependent Gene Expression

All the strains used were derived from *E. coli* K12 strain MG1655, and the detailed information for making each derivative strain is described in the Supplemental Data. The strains and oligonucleotides used are listed in Tables S2 and S3, respectively.

To culture the strains at different growth rates, five defined media were used. They are derived from M63 minimal medium (Miller, 1972) and rich defined medium (RDM) (Neidhardt et al., 1974), supplemented by either glycerol or glucose at 0.5% (w/v) as the primary carbon source. These five media are (1) RDM + glucose, (2) RDM + glycerol, (3) M63 + NH_4Cl (20 mM) + glycerol + casamino acids (0.2%), (4) M63+ NH_4Cl (20 mM) + glycerol, and (5) M63 + glycerol + glycine (20 mM, the sole nitrogen source). The resulting growth rates range from 0.3 to 2.4 doublings per hour (Figure S1).

For gene expression measurements, experimental strains were first cultured in LB, and then in one of the above five media, in glass tubes with vigorous shaking at 37°C. The inducer chlorotetracycline was added at 20 or 50 ng/ml to some media. After a round of preculture growth (five to seven doublings) and another two to three doublings in the experimental culture, samples were taken for measurements of OD_{600} (0.1 to 1), total protein amount, and β-galactosidase activities at four points. Growth rates and β-galactosidase expression levels were determined by linear regression as detailed in the Supplemental Data.

SUPPLEMENTAL DATA

Supplemental Data include Supplemental Results, Supplemental Experimental Procedures, five figures, and three tables and can be found with this article online at http://www.cell.com/supplemental/S0092-8674(09)01505-0.

ACKNOWLEDGMENTS

We are grateful to Hans Bremer, Patrick Dennis, Mans Ehrenberg, Peter Geiduschek, Richard Gourse, Carl Gunderson, Po-han Lee, Eduard Mateescu, Atul Narang, Moselio Schaechter, Matthew Scott, Arbel Tadmor, and Lingchong You for discussions during the course of this work. This work was supported by the National Science Foundation through the Center for Theoretical Biological Physics (grant PHY-0822283) and through grant MCB0746581, by the National Institutes of Health through grant RO1GM77298, and by a fellowship from Deutsche Forschungsgemeinschaft (grants KL818/1-1 and 1-2, to S.K.).

Received: April 7, 2009
Revised: September 1, 2009
Accepted: December 1, 2009
Published: December 24, 2009

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