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Comparison of Repressor and Transcriptional Attenuator Systems for Control of Amino Acid Biosynthetic Operons

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In bacteria, expression from amino acid biosynthetic operons is transcriptionally controlled by two main mechanisms with principally different modes of action. When the supply of an amino acid is in excess over demand, its concentration will be high and when the supply is deficient the amino acid concentration will be low. In repressor control, such concentration variations in amino acid pools are used to regulate expression from the corresponding amino acid synthetic operon; a high concentration activates and a low concentration inactivates repressor binding to the operator site on DNA so that initiation of transcription is down or up-regulated, respectively. Excess or deficient supply of an amino acid also speeds or slows, respectively, the rate by which the ribosome translates mRNA base triplets encoding this amino acid. In attenuation of transcription, it is the rate by which the ribosome translates such ''own'' codons in the leader of an amino acid biosynthetic operon that decides whether the RNA polymerase will continue into the operon, or whether transcription will be aborted (attenuated). If the ribosome rate is fast (excess synthesis of amino acid), transcription will be terminated and if the rate is slow (deficient amino acid supply) transcription will continue and produce more messenger RNAs.

Repressor and attenuation control systems have been modelled mathematically so that their behaviour in living cells can be predicted and their system properties compared. It is found that both types of control systems are unexpectedly sensitive when they operate in the cytoplasm of bacteria. In the repressor case, this is because amino acid concentrations are hypersensitive to imbalances between supply and demand. In the attenuation case, the reason is that the rate by which ribosomes translate own codons is hypersensitive to the rate by which the controlled amino acid is synthesised.

Both repressor and attenuation mechanisms attain close to Boolean properties *in vivo*: gene expression is either fully on or fully off except in a small interval around the point where supply and demand of an amino acid are perfectly balanced.

Our results suggest that repressors have significantly better intracellular performance than attenuator mechanisms. The reason for this is that repressor, but not attenuator, mechanisms can regulate expression from biosynthetic operons also when transfer RNAs are fully charged with amino acids so that the ribosomes work with maximal speed.

Keywords: attenuation; aminoacylation; control of gene expression; repressor control; Boolean control

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Introduction

The two major principles for transcriptional regulation of amino acid biosynthetic operons in *Escherichia coli* and *Salmonella typhimurium* are clas-

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sical repressor control on the one hand[1] and ribosome-mediated attenuation of transcription[2] on the other. These two control systems use two fundamentally different strategies to adjust the supply of newly synthesised amino acids to the current demand. The major scientific aim of this study is to explore the physiological consequences of their different modes of action. For this, we model mathematically the two control systems in isolation and then in the context of logarithmically growing bacterial cells.

In repressor control, it is the level of an amino acid pool that regulates expression from the corresponding biosynthetic operon. In attenuation control, the regulatory signal is instead the speed by which ribosomes read base triplets encoding the amino acid that is synthesised by the enzymes expressed from the controlled operon. This means, for instance, that increased expression from an operon controlled by attenuation requires a significantly reduced level of the corresponding aminoacyl-tRNA. Repressors, in contrast, can regulate gene expression up or down also when transfer RNAs are fully charged with amino acids and the ribosomes move with full speed.

A brief inventory of how amino acid synthetic operons are controlled reveals that repressor and attenuation mechanisms are used to about the same extent in both *E. coli* and *S. typhimurium*.

For instance, repressors are involved in control of the *arg*,[3] *trp*,[4] *lysA*[5] and *metBL*[6] operons as well as of the *asp*[7] (activator) and *tyr*[8] regulons. The *trp*,[9] *his*,[10,11] *leu*,[12] *thr*,[13] *ilvGMEDA*,[14] *ilvBN*,[15] and *pheST*[16] operons along with the *pheA* gene[17] are controlled by attenuation.[18,19] For the *his, pheA, ilvGMEDA* and the *thr* operons, attenuation appears to be the sole operon-specific regulatory mechanism. The *trp* operon is regulated by a combination of attenuation and repressor control.[2,20] For other operons involved in amino acid synthesis, like the *ala, gln, serA, B, C, glyA*,[21] *gcv*[22] and *cys*[23] operons, the control mechanisms are more complex. Interestingly, production of proline synthetic enzymes appears to lack a specific control system.[24]

One important property of intracellular control systems is how sensitively they respond to changes in a signal. If, to give an example, the concentration of an amino acid pool is taken as a signal and the rate of initiation of transcription of an amino acid synthetic operon is the response, then it is relevant to know if a twofold increase in the pool size leads to a twofold or, say, a 20-fold reduction in promoter activity. In the first case the response is ''soft'', and in the second it is ''sharp''. A useful measure of sensitivity in control is the sensitivity amplification,[25] sometimes referred to as logarithmic gain[26] For small changes in signals and responses, it is defined as the relative increase in the response divided by the relative increase in the signal. In the example above, the sensitivity amplification would be -10 ($-20/2$), if we disregard the fact that the definition is strictly valid only for small perturbations. If the absolute value of the sensitivity amplification is much larger than 1, then the mechanism is often called ultrasensitive.[25] It has been shown that ultrasensitivity is crucial for efficient copy number control of important plasmids,[27] and we have reasons to believe that the same is true for control of gene expression in general. We have characterised the sensitivity of repressor control and attenuation of transcription, and used mathematical modelling to estimate this important parameter in the cytoplasm of exponentially growing bacteria.

In general, the sensitivity of cooperative repressor control increases with the number of subunits of the repressor. A new result of this work is that the sensitivity of attenuation mechanisms can, in principle, increase without limit, if the number of elongation steps over which the ribosome speed is measured is allowed to increase indefinitely. When these two control systems are evaluated under *in vivo* conditions, several remarkable and unexpected results emerge.

Firstly, the sensitivity of repressor control is enhanced dramatically in the bacterial cell due to the very sharp responses in amino acid pools to even very small imbalances between supply and demand. Secondly, the sensitivity of attenuation is significantly enhanced *in vivo*. This is because the time for ribosomes to translate regulatory codons in the leader sequence of the mRNA in the attenuator is hypersensitive to the rate of synthesis of an amino acid.

The most surprising finding is, however, that ribosome-dependent attenuation appears to be principally inferior to repressor systems. The reason is that attenuation, in contrast to repressor control, has very low sensitivity when amino acid biosynthesis is just sufficient or in excess over demand. This implies that attenuation mechanisms can regulate gene expression only when ribosomes are partially starved for an aminoacyl-tRNA. Such starvation is believed to impair growth-rate and reduce the fitness of bacterial populations in two different ways. When ribosomes are slowed, this leads to a proportional decrease in growth-rate.[28] Furthermore, when the level of an aminoacyl-tRNA drops in the cell, the amino acid substitution errors in protein synthesis may increase drastically,[29] which will further reduce the growth-rate.[30]

Results

Transcriptional attenuation

In ribosome-mediated transcriptional attenuation, it is the outcome of a race between an RNA polymerase transcribing the leader of a regulated mRNA and a ribosome translating this leader that determines the level of expression.

A scheme for attenuation control of initiation of transcription that includes a conditional pause site for RNA polymerase in the mRNA leader for the

regulated operon as suggested by Yanofski and coworkers[18,31–33] is shown in Figure 1. The leader sequence contains, starting from the 5′-end, the start signal AUG for mRNA translation followed by region I in which there are m ''own'' codons for the amino acid that is synthesised by the enzymes in the controlled operon[2] as well as other codons. Region I is followed by region II of the leader and then by a strong pause site for the RNA polymerase. Further downstream, there are n transcriptional steps subdivided into regions III and IV of the leader. When the RNA polymerase has reached the pause site, it stops and remains there until a ribosome starts melting the hairpin structure formed by regions I and II.[32,33] When the polymerase resumes transcription, it moves forward in synchrony with the ribosome. This synchronization, which is critical for the sensitivity of the mechanism, was not considered in earlier mathematical modelling[34–36] of transcriptional attenuation. If the ribosome is slow in translating the own codons of region I due to deficient amino acid synthesis, it will remain in the ''control region'' of the leader when the RNA polymerase finishes transcription of region IV (Figure 1). In this case the II:III, but not the III:IV, hairpin is formed and the RNA polymerase will continue into the open reading frames of the operon. When, in contrast, the amino acid synthetic activity of the enzymes encoded by the operon is sufficient or in excess, the ribosome will move fast over the own codons and be in region II when the polymerase finishes transcribing region IV. In this case, the hairpin III:IV, which signals rho-independent termination of transcription, will be formed rather than II:III, so that transcription is aborted (attenuated) before the RNA polymerase starts transcribing the coding sequences of the operon.

Time zero is defined as the time when the RNA polymerase resumes transcription from its pausing state under the influence of an approaching ribosome. The probability, Q, that transcription is continued from the leader into the open reading frame of the operon is determined by the probability, $R(t)$, that the ribosome is in the control region, when the RNA polymerase finishes transcribing region IV (Figure 1). Q is given by the integral:

$$Q = \int_0^\infty R(t)p(n,t)\mathrm{d}t \qquad (1)$$

$p(n,t)$ is the rate by which the polymerase leaves the nth base in regions III and IV (Figure 1), which is the same as the probability density for the time that the polymerase spends transcribing bases 1 to n in regions III and IV (Methods of Computation). The number n is defined operationally as the number of bases transcribed when the RNA polymerase moves from its pause site to a position that, when region III is single-stranded, allows formation of the III:IV structure. For simplicity, it will be assumed that the m control codons in the leader of the operon dominate the ribosome's translation time, that they are all own codons, and that translation of each is characterised by an exponentially

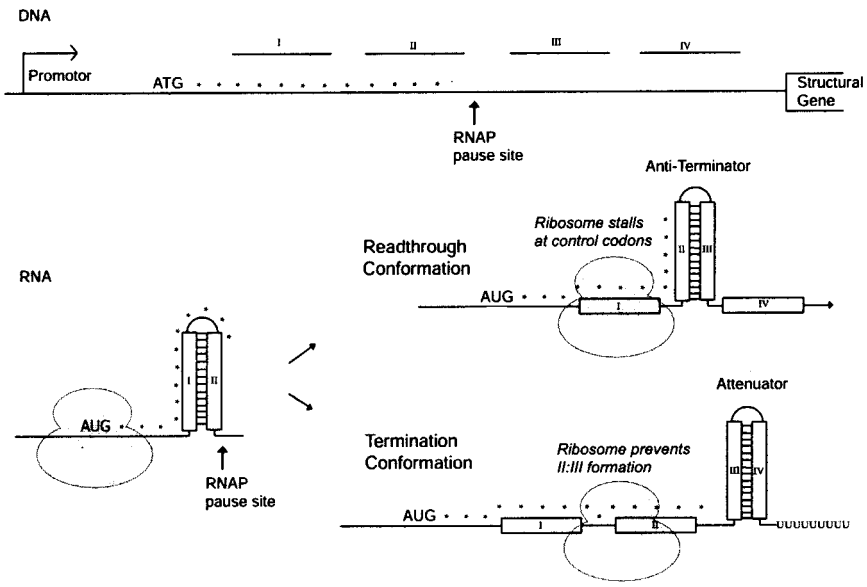

Figure 1. Scheme for attenuation control. Top: The DNA leader sequence encoding a leader peptide of varying length depending on the place of the stop codon and four regions that can form mutually exclusive secondary RNA structures. Bottom left: The ribosome initiates translation of the RNA leader, while the RNAP stalls at its pause site. When the ribosome starts to melt the I:II hairpin, the RNAP resumes transcription. Bottom right, lower: when translation is fast the ribosome prevents formation of the II:III hairpin, thereby allowing the transcriptional terminator III:IV to form and transcription terminates. Bottom right, upper: when translation is slow the II:III hairpin forms, thereby preventing formation of the III:IV terminator and transcription continues into the open reading frames of the operon.

distributed time with average $1/k$. It will also be assumed that transcription of the n bases in regions III and IV occurs in n identical steps, each characterised by an exponentially distributed time with average $1/q$. Exponentially distributed step-times for ribosomes and RNA polymerases means that both translation of a codon and transcription of a base have one rate-limiting step and can be described by a single rate constant, k or q, respectively. With these simplifications, the probability $R(t)$ is given by:

$$R(t) = \sum_{i=1}^{m} Po(i,kt) \tag{2}$$

The probability density $p(n,t)$ is given by:

$$P(n,t) = qPo(n,qt) \tag{3}$$

where:

$$Po(j,\lambda) = \frac{\lambda^j}{j!}e^{-\lambda} \tag{4}$$

is the probability to sample j events of a Poisson-distributed variable with expectation value λ (Methods of Computation). Figure 2(a) shows the response $r = Q$ to the signal s, which in this case is defined as the rate constant k for translation of own codons in the control region (Figure 1). Two attenuation mechanisms are considered with different numbers of steps for the ribosome (m) and RNA polymerase (n). Figure 2(b) shows how the sensitivity amplification, $a_{rs} = a_{Qk}$, of these mechanisms varies as a function of the signal parameter k. a_{rs} is defined as:[26]

$$a_{rs} = \frac{d\log r}{d\log s} = \frac{s}{r}\frac{dr}{ds} \tag{5}$$

In molecular control analysis,[37–39] a_{rs} is, depending on context, called the control coefficient or the elasticity, and in biochemical systems theory the parameter sensitivity.[26,40]

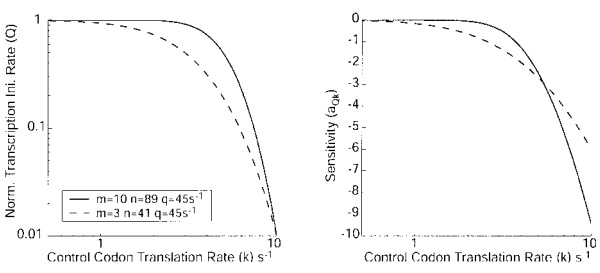

Figure 2. Signal-response and sensitivity in attenuation control. (a) Response (probability Q of successful transcription initiation) to signal (ribosome step rate, k) for m control codons, where m is 3 or 10. The number, n, of bases in regions III and IV was adjusted to make $Q = 0.01$ when $k = k_{max} = 10\ s^{-1}$. (b) The sensitivity, a_{Qk}, (equation (5)) as function of the step rate k.

Figure 2 shows that a mechanism with $m = 10$ own codons has larger sensitivity amplitude, $|a_{Qk}|$, than when $m = 3$, and that mechanisms with high m and n values can combine high sensitivity with expression levels Q close to saturation. This is in contrast to the exponential sensitivity amplification suggested for copy number control of plasmid ColE1, where the sensitivity amplitude becomes very small when Q approaches 1.[25,27] Neither of the attenuation mechanisms in Figure 2 can regulate beyond the point $k = 10\ s^{-1}$, since this corresponds to the maximal rate of translation of codons.

We studied more systematically how sensitivity in attenuation control depends on the number of translation (m) and transcription (n) steps in the mechanism and on the value of the response function Q. Two cases were considered. In the first, the number m of ribosome steps was varied from 1 to higher values with a single, rate-limiting transcription step ($n = 1$). This corresponds to a situation where the motion of the RNAP from base 1 to n is dominated by a single, long-lived pause site. In the second case, the number of rate-limiting steps for translation and transcription were set equal ($m = n$) and varied from m, $n = 1$ to high values. The results (Figure 3) show that, for given values of m and n, $|a_{Qk}|$ increases with decreasing expression level Q. They also reveal that for mechanisms with $n = 1$ and a fixed expression level Q, the sensitivity amplitude increases asymptotically with increasing m to a maximal value $|a_{Qk}| = -\log Q$. This corresponds to exponential sensitivity amplification.[27,41,42] Another new result

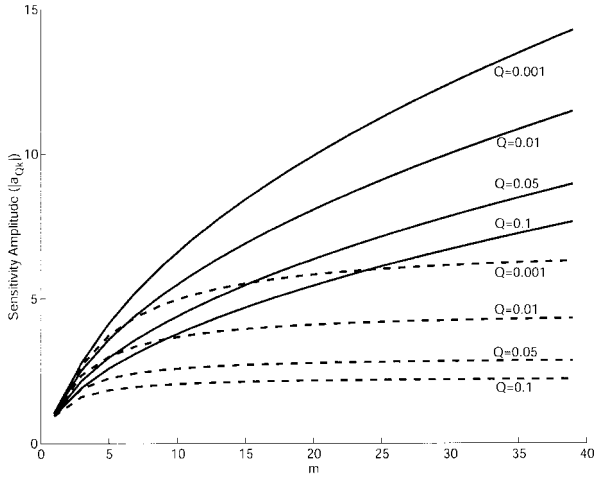

Figure 3. Sensitivity for attenuation with different numbers of control codons and transcribed bases. The sensitivity amplitude, $|a_{Qk}|$, for the response Q to variation in the signal k for different numbers of control codons (m) and transcribed bases (n). $|a_{Qk}|$ was calculated at $k = 10\ s^{-1}$. Continuous lines, $n = m$. Broken lines, $n = 1$. The step rate, q, of the RNA polymerase was adjusted to obtain the Q values that characterize the different curves in the Figure.

is that when m and n increase in tandem, $|a_{Qk}|$ increases without limit for any value of $Q < 1$, so that a very high sensitivity amplitude can be obtained also near saturation of the response, where Q is close to 1.

Repressor control of initiation of transcription

When a repressor that controls expression from an amino acid biosynthetic operon binds to the amino acid that is produced by the enzymes encoded by this operon, the repressor's DNA-binding conformation is favoured so that initiation of transcription is blocked. In this way, large amino acid pools will tend to decrease and small pools will tend to increase the expression from amino acid biosynthetic operons.

We have used a simple, cooperative model for control of amino acid biosynthetic operons as suggested by Savageau.[26] The repressor has m identical subunits, one DNA-binding and one DNA-inert conformation, and there is one binding site for repressor on DNA. In the absence of amino acids, the DNA-inert conformation dominates and increasing amino acid concentration increases the probability that the repressor is in its DNA binding state (Methods of Computation). With K as the dissociation constant for binding of repressor to operator and R_0 as the total repressor concentration, the probability, Q, that the operator is free so that initiation of transcription can take place, is given by:

$$Q = \left(1 + \frac{(K_0 R_0/K)(1 + x/K_2)^m}{(1 + x/K_1)^m + K_0(1 + x/K_2)^m}\right)^{-1} \qquad (6)$$

Here, x is the amino acid concentration, K_1 and K_2 are, respectively, the dissociation constants for amino acid binding to the DNA-inert and DNA-binding repressor states. K_0 is the equilibrium ratio between DNA-binding and DNA-inert repressor states in the absence of the amino acid. In the derivation of equation (6) it was assumed that the fraction of bound repressors is small, so that the total and free repressor concentrations can be put equal. The response, $r = Q$, to the signal, $s = x$, is shown in Figure 4(a) for $m = 1$, 2, 4 and 6 subunits, and the corresponding sensitivity a_{Qx} in Figure 4(b). As expected, the maximal value of $|a_{Qx}|$ increases with increasing number of subunits.

So far, we have discussed the sensitivity of attenuation and repressor control using the ribosome step rate k at own codons as signal in the former and the amino acid concentration x as signal in the latter case. However, in bacterial cells it is the biosynthetic activity of a metabolic pathway, rather than a ribosome step time or an amino acid pool concentration, that is controlled. The physiologically relevant choice of signal is therefore in both cases the total activity of the enzymes that produce the amino acid. Characterisation of repressor systems and attenuation mechanisms under *in vivo* conditions requires global models[30] for growing cells.

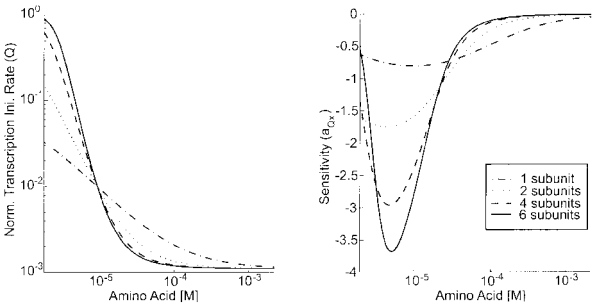

Figure 4. Signal-response and sensitivity for repressor control. (a) Response (probability Q that the operator is free) to signal (concentration x of amino acid) calculated from equation (9) for repressors with one, two, four and six subunits. Parameters (Table 1) were adjusted to make $Q = 0.001$ when $x \to \infty$ and $Q = 0.01$ when $x = 10^{-5}$. (b) The sensitivity , a_{Qx}, for response in Q to variation in signal x.

Characterisation of attenuation and repressor control *in vivo*

In order to predict response functions and sensitivities *in vivo* it is necessary to relate the probability Q, that determines the level of gene expression for attenuation control according to equation (1) and for repressor control according to equation (6), to the concentration [E] of the enzyme system that synthesises the regulated amino acid. This we do by modelling n enzyme systems that produce n different types of amino acids, which are activated in n aminoacylation reactions and consumed by ribosomes in the synthesis of new proteins (Figure 5). We define a signal variable, s, as the total activity of an amino acid biosynthetic pathway normalised to the maximal rate of consumption of this amino acid by ribosomes when it is supplied in excess:

$$s = \frac{k_E[E]}{[R] f k_{max}} \qquad (7)$$

[R] is the concentration of ribosomes in protein elongation phase, k_E is the rate constant for amino acid synthesis per unit of the enzyme system, f is the frequency by which the amino acid is incorporated in proteins and k_{max} is the maximal rate of protein elongation. A steady-state analysis (equations (M5) to (M11)), has been carried out based on the scheme in Figure 5. Figure 6 shows how the concentration, x, of the amino acid pool and the charging level of the corresponding tRNA vary when s increases from low to high values. The concentration x depends linearly on s when the enzyme concentration is limiting. This is

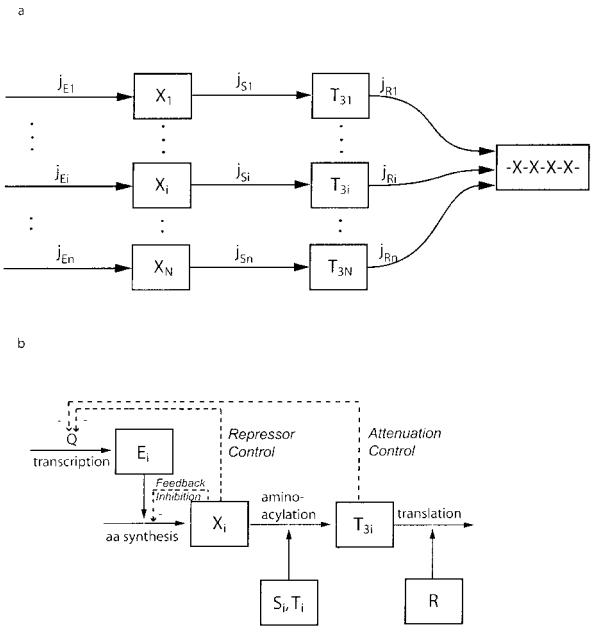

Figure 5. Global scheme for synthesis of amino acids, aminoacylation of tRNAs and protein synthesis. (a) An amino acid X_i (concentration x_i) is synthesised with rate j_{Ei} by an enzyme system (E_i, concentration $[E_i]$), aminoacylated with rate j_{Si} by an aminoacyl-tRNA synthetase (S_i, concentration $[S_i]$). Ternary complexes (T_{3i}, concentration $[T_{3i}]$) formed between aminoacyl-tRNA, EF-Tu and GTP, are consumed by ribosomes (R, concentration [R]) with rate j_{Ri}, where $i = 1,2,\ldots n$. (b) Control scheme: the rate of transcription (proportional to Q) of the biosynthetic enzymes (E_i) are controlled by a repressor system sensing the amino acid (X_i) concentration and/or a ribosome-mediated attenuation mechanism directly dependent on the availability of ternary complexes (T_{3i}). The synthesis of amino acid is also regulated by direct feedback inhibition of the activity of the biosynthetic enzymes.

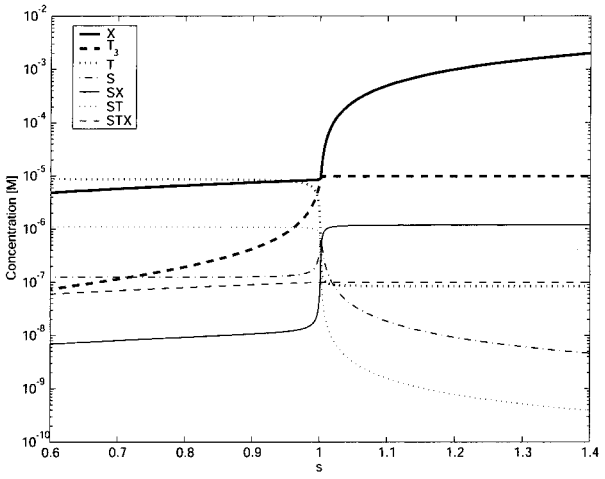

Figure 6. Responses of amino acid pools and ternary complex concentrations to changes in the intracellular rate of synthesis of amino acids. Steady-state concentrations of amino acid (x), aminoacyl-tRNA synthetase free ([S]), bound to amino acid only ([SX]), bound to tRNA only ([ST]), bound to amino acid and tRNA ([STX]), free tRNA [T] and free ternary complex [T_3] were calculated and plotted as functions of the signal s (equation (7)). Parameter values are given in Table 1.

because the rate of aminoacylation under those conditions must be proportional to x and equal to the rate of synthesis of free amino acid, which is proportional to s. When the inflow into the amino acid pool approaches the maximally possible outflow, i.e when. $s = 1$, x increases rapidly for a small relative increase in s. At very high values of x there is, again, a linear relation between s and x, when amino acid synthesis is significantly reduced by feed-back inhibition[38] (equation (M6)), or when dilution of the amino acid pool by volume growth becomes larger than amino acid consumption by ribosomes. The fraction of aminoacylated tRNA displays a sharp dependence on s, going from a very small value to near 100 % over an extremely narrow interval close to $s = 1$. The response in x to variation in s near $s = 1$ becomes sharper when the capacity of the aminoacylation reaction increases (Figure 7). When the rate of amino acid synthesis is below the demand from the ribosome activity in the cell, it is essentially only the association rate constant for amino acid binding to a synthetase and the synthetase concentration that determine the exit rate from the amino acid pool. When, in contrast, the rate of amino acid synthesis is in excess over demand, the outflow from the pool is of zero order, independent of synthetase kinetics and pool size (Figure 7).

The intracellular response, Q, of expression from an amino acid synthetic operon to changes in intracellular signal, s, is shown in Figure 8(a) for the two attenuation mechanisms displayed in Figure 2 and the four repressor systems shown in Figure 4. Figure 8(a) reveals, firstly, that Q varies in response to s very differently from the responses of repressor (Figure 2(a)) and attenuation (Figure 4(a)) mechanisms to variations in amino acid concentration or ribosomal step time, respectively. Secondly, the attenuation mechanism reduces gene expression sharply at a lower value of s than the repressor system. Thirdly, Q remains virtually constant at a low level when s increases further in the attenuation case but not for repressors, where Q can continue to decrease. These fundamental differences between repressor and attenuation control are remarkably insensitive to parameter variations, and are robust system properties of rapidly growing cells.

Quantitative information about the sensitivity amplification, a_{Qs}, of the control systems is given in Figure 8(b). When s is larger than 1, the sensitivity of attenuation control is virtually zero. This means that attenuation mechanisms are ''blind'' and cannot regulate amino acid synthesis when it is in excess. The reason is that the rate constant k for

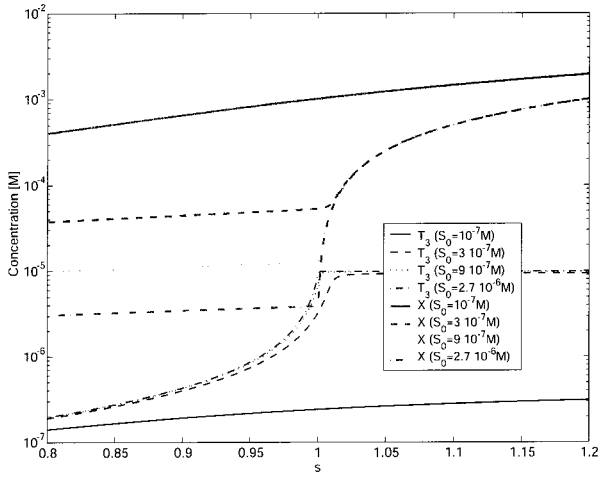

Figure 7. Responses of amino acid pools and ternary complex concentrations to changes in the intracellular rate of synthesis of amino acids for different concentrations of aminoacyl-tRNA synthetases. The stationary concentrations of amino acid (x) and aminoacyl-tRNA ($[T_3]$) are plotted as functions of the signal s (equation (7)) for different total concentrations, $[S_0]$, of aminoacyl-tRNA synthetases.

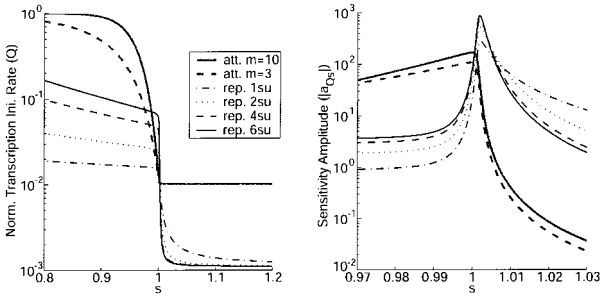

Figure 8. Control characteristics in the global context the whole cell. (a) Control curves for the repressor and attenuation mechanisms in Figures 2 and 4, with the probability Q for gene expression plotted as function of the normalised signal, s (equation (7)). (b) The sensitivity amplitudes, $|a_{Qs}|$, for the curves in (a) plotted as functions of the signal s.

translation of control codons is almost exactly equal to its largest value k_{max} just above the point where amino acid synthesis matches the maximal influx into protein synthesis. For repressors, the sensitivity remains relatively high also when s is larger than 1. This is because amino acid pools, in contrast to protein elongation rates, respond to changes in the rate of amino acid synthesis also when it is in excess over demand. The switch-like saturation of the rate of protein elongation when the signal parameter s approaches 1 arises as soon as the aminoacylation reaction has sufficient over-capacity in relation to the demand for aminoacyl-tRNA in protein synthesis. This over-capacity of synthetases in the bacterial cell is in accord with experimental data,[43] and assures that our conclusions regarding repressor control and attenuation are robust and depend little on kinetic details.

Another striking feature is that the sensitivity amplitudes are much higher for repressor and attenuation mechanisms in the intracellular context. This can be seen by comparing data in Figure 9(b) with results in Figure 2, where the ribosomal rate of translating own codons is used as the input signal, or with data in Figure 4, where an amino acid concentration is the signal. Since all parameters for the attenuation and repressor parts of the control are identical, this means that a relative variation in s must lead to a larger relative variation in both the amino acid pool, x, for repressor control and in the ribosomal step time, k, for attenuation. To inspect this phenomenon more closely we partition the sensitivity amplification, a_{Qs} in the repressor case as:

$$a_{Qs} = a_{Qx}a_{xs} \tag{8a}$$

The sensitivity amplification a_{xs} describes how sensitively the amino acid pool responds to changes in s, and all three sensitivities in equation (8a) are shown in Figure 9(a). For attenuation, the corresponding factorisation is:

$$a_{Qs} = a_{Qk}a_{ks} \tag{8b}$$

The sensitivity amplification a_{ks} describes how sensitively the ribosome's step-rate in translating own codons responds to changes in the rate of synthesis of the controlled amino acid. Relations like those in equation (8) are valid for all signal-response cascades and follow directly from the chain rule for differentiating functions.

The results in Figure 9 show that for certain values of s, $|a_{xs}|$ and $|a_{ks}|$ can take values much larger than 1. In the repressor case, the explanation is that amino acid pools in growing bacteria are very sensitive to imbalances between in and out fluxes, as illustrated in Figures 6 and 7. The pool sizes are normally much smaller than the intracellular amounts of the corresponding amino acids in proteins. This means that the fraction of inflow necessary to double the pool of free amino acid during a generation time is very much smaller than the outflow required in order to double the cell's protein pool in the same time interval. In this way, a small relative change in s can induce a very large relative change in x. In fact, amino acid pools in bacteria have properties reminiscent of zero-order kinetics, as originally suggested for modification-demodification reactions catalysed by enzymes operating close to saturation.[44] The high value of $|a_{ks}|$ that appears in attenuation is due to another type of sensitivity amplification. To demonstrate the principle, we will consider an idealised case where the inflow into an amino acid pool, determined by $k_E[E]$, exactly matches the flow of this amino acid into nascent proteins (Methods of Computation):

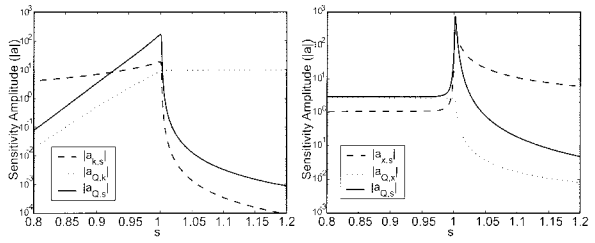

Figure 9. Partitioning of amplification factors. (a) Sensitivity amplitudes $|a_{Qs}|$, $|a_{Qk}|$ and $|a_{ks}|$ for attenuation control plotted as functions of the signal s (equation (7)), illustrating how a_{Qs} is composed of contributions from a_{Qk} and a_{ks}. (b) Sensitivity amplitudes $|a_{Qs}|$, $|a_{Qx}|$ and $|a_{xs}|$ for repressor control plotted as functions of the signal s, illustrating how a_{Qs} depends on contributions from a_{Qx} and a_{xs}.

$$k_E[E] = f[R]v = f[R]\frac{1}{\frac{f}{k} + \frac{1-f}{k_{max}}} \quad (9)$$

v is the average rate of translation of all types of codons. Own codons for the controlled amino acid are read with variable rate k and all other codons with the same, maximal rate k_{max}. The frequency of the controlled amino acid in intracellular proteins is f and the frequency of all other amino acids lumped together is $1-f$. From equation (9) it follows that:

$$a_{ks} = \frac{d \ln k}{d \ln [E]} = 1 + \frac{k}{k_{max}} \frac{1-f}{f} \quad (10)$$

For values of k close to k_{max} and with $f = 1/20$, the sensitivity amplitude for the response in the step rate k for reading own codons to changes in s is close to 20. This type of sensitivity amplification has, to our knowledge, never been described, and can be explained as follows. When amino acid synthesis is rate-limiting ($s < 1$), the total rate of protein synthesis is proportional to s. A certain relative change $\Delta s/s$ is therefore equal to the relative change $\Delta v/v$ in the average ribosome velocity v. Since, however, the fraction of time that ribosomes spend translating a particular codon is about $1/20$, the relative change in this latter time must be about 20 times larger than the relative change in total translation time ($1/v$) (Figure 9, Methods of Computation).

Discussion

Mathematical modelling has been used to predict the behaviour of transcriptional control mechanisms with repressors and attenuators for amino acid biosynthetic operons in growing bacteria. The analysis suggests that, for both types of control systems, the responses in gene expression to variations in the cell's capacity to synthesise amino acids is markedly switch-like (Figure 8(a)). Gene expression changes from fully on to low values over a very small interval of signal, giving the control systems a distinctly Boolean character. This is underscored by the results in Figure 8(b), which show that the sensitivity amplitudes peak at extremely high values, and then decay rapidly as the signal changes up or down. The high sensitivity and the Boolean character of control originate from cascades of sensitivity amplification. In the attenuation case, the sensitivity in gene expression to variations in the rate by which ribosomes translate own codons is amplified by the sensitive response in ribosomal step time at own codons to the rate of synthesis of an amino acid (Figure 9(a)). In the repressor case, a modest sensitivity in the response of gene expression to variations in the amino acid pool is amplified by a high sensitivity in pool size to variations in amino acid synthesis rate (Figure 9(b)). By extensive variations of parameter values, we have found that both repressor and attenuation systems always have their most sensitive responses close to the point where the inflow into an amino acid pool just matches the maximally possible outflow. This is a robust system property, which is virtually independent of where the flow matching occurs. This means that both types of control systems will have similar characteristics independent of growth conditions, and extends the validity of the present analysis to a large range of environmental conditions and enzymatic activities.

This work shows that control of gene expression by ribosome-mediated transcriptional attenuation mechanisms has severe limitations, since they are sensitive only when the charging level of a transfer RNA is so low that the protein elongation rate is slowed significantly. This is in contrast to repressor mechanisms, which respond to amino acid pools so that gene expression can be tuned also when the supply of amino acids is in excess over demand (Figure 8(b)). This difference between the two control mechanisms suggests that, in general, repressor systems are better control modules than attenuation mechanisms in the sense that they will lead to higher fitness values for rapidly growing organisms.

To illustrate this, consider two bacterial populations that are identical in all respects except that one uses repressor control to regulate expression from amino acid biosynthetic operons and the other uses ribosome-dependent attenuation of transcription. To discuss a simple case, assume further that both populations grow logarithmically in constant and identical external environments. Since repressors can regulate also when amino acid supply is in small excess, the population that uses repressor control can keep the charging levels of its transfer RNAs near 100% and its ribosomes elongating with full speed all the time. When the capacity of an amino acid synthetic pathway becomes dangerously low due to volume expansion by growth, the resulting reduction in the

amino acid concentration can signal enhanced initiation of transcription of the operon early enough so that new enzymes will appear before the charging levels of the corresponding transfer RNAs start to drop. In the population with attenuation control, in contrast, initiation of transcription from the biosynthetic operon cannot respond until there is sufficient reduction in the charging levels of the corresponding transfer RNAs to slow the rate of translation of own codons in the leader of the operon significantly (Figure 1). Since there is always a delay between initiation of transcription and the synthesis of new, active enzymes, the charging levels will continue to drop and the average rate of codon translation will continue to decrease until the level of the amino acid synthetic enzymes has been restored. The repressor system can, in other words, ''foresee'' a coming shortage in aminoacyl-tRNA levels and react before the ribosomes are slowed, but the attenuation mechanism cannot. As a consequence of this, the population of bacteria using repressors can sustain a higher growth-rate than the population using attenuators. We have verified these qualitative arguments by simulating the dynamics of the control systems in growing bacteria, including a time-delay between initiation of transcription and the emergence of newly synthesized protein. The detailed results of these computations will be presented elsewhere.

This apparent deficiency in attenuation control is further accentuated by other phenotypic consequences of a drop in tRNA charging levels.[29] Amino acid substitution errors in nascent proteins are directly proportional to the ratio between non-cognate and cognate ternary complexes. This means that if the charging level for a transfer RNA that is cognate for a certain codon is very low, but the charging level for an efficiently competing near-cognate transfer RNA is not, the error in the reading of this codon can easily go up by orders of magnitude. Since the responses of attenuation systems depend on significantly reduced charging levels of transfer RNAs, this type of mechanism will increase the frequency of missense errors in protein synthesis and intermittently induce the stringent response.[45] Both these phenomena will further reduce the growth-rate of the population. In conclusion, bacteria that use attenuation as the only control for expression of amino acid biosynthetic operons appear to be at a population genetic disadvantage relative to those that use repressor control.

One way to improve attenuation control would be to make the probability to form the anti-terminator in the mRNA-leader (Figure 1) depend on the amino acid pool size, rather than on the ribosomal step time. Indeed, such a scheme has been implemented by *Bacillus subtilis*, where expression from the *trp* operon is regulated by a protein-mediated attenuation mechanism. This senses the amino acid concentration and not the rate of translating own codons.[46,47] An interesting possibility is that *B. subtilis* originally used ribosome-mediated attenuation control for this operon and later managed to evolve a better mechanism with greater fitness.

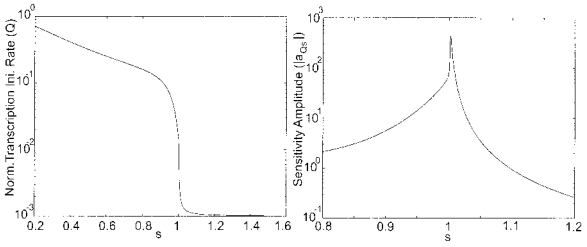

Figure 10. Combined attenuation-repressor control systems. (a) Control curve for a mechanism with both repressor (two subunits, $K_1 = 2.49 \times 10^{-5}$ M) and ribosome-mediated attenuation (three translation and 26 transcription steps). The normalised rate, Q, of gene expression plotted as function of the signal s (equation (7)). (b) The sensitivity amplitude, $|a_{Qs}|$, for the curve in (a) plotted as function of the signal s.

Another way to improve the performance of ribosome-mediated attenuation mechanisms is to combine them with repressor control and preferentially in such a way that expression from the attenuator starts to increase only when the repressor system is fully derepressed. Such combinations of control systems would considerably increase the dynamic range of control, allow regulation both below and above balanced rate of synthesis of amino acids (see Figure 10), and would be particularly useful when the repressor has moderate cooperativity. Such a combined mechanism controls expression from the *trp*-operon in *E. coli*,[48] but in many other cases, as for the *his*-,[8,9] *leu*-,[10] *thr*-[11] and *ilvGMEDA*[12] operons, attenuation appears to operate alone or in conjunction with global control systems.[49–51] This could suggest that *E. coli* in some cases may not have been able to evolve control mechanisms that, from a population genetic perspective, are optimal. However, a more likely alternative is that there exist hitherto unidentified feedback systems that complement attenuation and confer optimal control for fast growth in ways that are unknown at the present time.

Methods of Computation

Stochastic treatment of attenuation with ribosome-released RNA polymerase

The probability, Q, that the RNA polymerase (RNAP) continues into the open reading frames of an attenuation controlled operon is given by the probability that the ribosome blocks the I:II structure in the leader so that the II:III antiterminator, rather than the III:IV terminator, is formed just when the RNAP finishes transcription of the base in position n counted from the pause site (Figure 1). Q can be written:

$$Q = \int_0^\infty p(n,t) \sum_{\substack{j \in \text{Control} \\ \text{Codons}}} R_l(j,t)\mathrm{d}t \qquad \text{(M1)}$$

$p(n,t)\mathrm{d}t$ is the probability that the RNAP passes step n in the leader between time t and $t + \mathrm{d}t$, so that $p(n,t)$ is the probability density of the time that the RNAP spends transcribing bases 1 to n. The number n is defined in Results. We assume, for simplicity, that the rate of incorporating each base is dominated by one, rate-limiting step with the same rate constant q. Then the movement of the RNAP is a simple Poisson process where the residence time at each transcription step is exponentially distributed with average $1/q$ and $p(n,t)$ is given by:

$$\begin{aligned} p(n,t) &= -\frac{\mathrm{d}}{\mathrm{d}t}\sum_{l=1}^{n} Po(l,qt) = qPo(n,qt) \\ &= q\mathrm{e}^{-qt}\frac{(qt)^n}{n!} \end{aligned} \tag{M2}$$

$R_l(j,t)$ is the probability that the ribosome is at codon j at time t after release of RNAP from the pause (Figure 1), and l is the total number of codons in the leader sequence. To simplify, we assume that translation of a codon j is dominated by a rate-limiting step with rate constant k_j. $R_l(j,t)$ is then defined by the system of master equations:

$$\begin{aligned} \frac{\mathrm{d}R_l(1,t)}{\mathrm{d}t} &= -k_1 R_l(1,t) \\ \frac{\mathrm{d}R_l(j,t)}{\mathrm{d}t} &= k_{j-1}R_l(j-1,t) - k_j R_l(j,t); \\ j &= 2,3,\ldots l \end{aligned} \tag{M3}$$

To simplify further, we assume that all l codons in equation (M3) are own codons for the controlled amino acid ($l = m$), that they all are in the control region and that all rate constants for codon translation are equal ($k_i = k$). Then, also the movement of the ribosome is a simple Poisson process and the solution to (M3) is:

$$R_l(j,t) = R_m(j,t) = \frac{(kt)^j}{j!}\mathrm{e}^{-kt};\ j = 1,2\ldots m \tag{M4}$$

When all steps are equal for the movements of ribosome and RNAP, as assumed here, the standard deviation in the time to complete a random walk normalised to the average time decreases as the inverse of the square-root of the number of steps. When the number m of own codons and the number n of transcribed bases increase, both the time for the ribosome to leave the mth control codon and the time for RNAP to reach base n become distributed more narrowly. A similar decrease in the relative standard deviation with increasing number of steps in the random walk will occur also when the rate constants differ between different steps, provided that the process is not dominated by one, or a few, very slow steps. This narrowing of the distributions for the times used by ribosomes and RNAPs to complete their random walks is the reason why the maximal sensitivity amplitude $|a_{Qk}|$ introduced just below equation (5) in Results increases with increasing m and n-values. If there are other than own codons in the control region, the sensitivity amplitude decreases with the fraction of own codons, since the other codons make the transition time for the ribosome ''noisier'', without contributing to the regulation. The responses (Q) and sensitivities (a_{Qk} and a_{Qs}) for attenuation control in Figures 2 and 8, respectively, were calculated from equation (M1) assuming n identical transcription steps, m identical own control codons and no other codon.

The possibility that ribosomes reach a stop codon, terminate and dissociate from the leader, thereby causing readthrough of the attenuator,[52] has not been considered here. This phenomenon, which reduces the sensitivity of attenuation, determines the basal expression level of the *trp* operon.[53] The possibility of super attenuation[20] has also been disregarded here, and both these complications will be discussed in a different context elsewhere.

Global scheme for synthesis of amino acids, aminoacylation and protein synthseis

Our scheme for protein synthesis is shown in Figure 5 and definitions of parameters are given in the corresponding legend. In steady state, the following relations hold for a particular amino acid X_i, where the index i is dropped for convenience:

$$\begin{aligned} j_\mathrm{E} &= j_\mathrm{S} + \mu x \\ j_\mathrm{S} &= j_\mathrm{R} + \mu[\mathrm{T}_3] \approx j_\mathrm{R} \end{aligned} \tag{M5}$$

The total rate, j_E, of synthesis of an amino acid is equal to the rate of consumption, j_S, of this amino acid by aminoacylation plus dilution due to exponential growth (μx). The flow, j_S, is equal to the rate of consumption of amino acids by ribosomes, j_R, plus the rate of dilution of ternary complex ($\mu[\mathrm{T}_3]$). The latter term can be neglected, since ternary complex is bounded above by the constant total concentration of a tRNA isoacceptor.

j_E is determined by the enzyme concentration [E], the efficiency k_E of E and feedback inhibition with inhibition constant K_P, according to:

$$j_\mathrm{E} = \frac{k_\mathrm{E}[\mathrm{E}]}{1 + x/K_P} \tag{M6}$$

j_S is determined by total synthetase concentration $[\mathrm{S}_0]$, association rate constant k_0 for binding of X to S, dissociation constant K_S for tRNA binding to S, concentration [T] of free deacylated tRNA and rate constant k_2 for aminoacylation and release of aminoacyl-tRNA, according to:

$$j_\mathrm{S} = \frac{x[\mathrm{S}_0]k_2}{\frac{k_2}{k_0} + x\left(1 + \frac{K_\mathrm{S}}{[\mathrm{T}]}\right)} \tag{M7}$$

This type of expression follows from all schemes where the ATP concentration is high enough to saturate the synthetase, tRNA equilibrates rapidly with synthetase and the aminoacyl-adenylate is in stable complex with the enzyme. This means that equation (M7), in spite of its simplicity, is very general and accounts accurately for aminoacylation *in vivo*. Recently, a similar expression was used by Chassagnole *et al.*[54] to describe control of the threonine synthesis pathway. However, the detailed interpretation of the parameters is slightly different, since they assume rapid equilibrium between synthetase and amino acid, while we assume binding and formation of an aminoacyl-adenylate in stable complex with the synthetase on the pathway to aminoacylation of tRNA according to Fersht & Kaethner.[55] This difference in mechanistic interpretation of the aminoacylation is of no concern for the present analysis, since both expressions have equivalent mathematical structures.

The rate of consumption (j_R) of a particular amino acid in protein synthesis is determined by its frequency f ($=f_i$) of occurrence in cellular proteins, the ribosome concentration [R] and the average ribosomal velocity v (Figure 5):

$$j_R = f[R]v = \frac{f[R]}{\sum_{j=1}^{n} \frac{f_j}{k_{Rj}}\left(1 + \frac{K_{Rj}}{[T_{3j}]}\right)} \quad \text{(M8)}$$

K_{Rj} and k_{Rj} are K_m and k_{cat} values, respectively, for individual codons. Equation (M8) follows from the facts that the average rate, v, of protein synthesis per ribosome is the inverse of the average time, τ, to translate all types of codons and this time is given by:

$$\tau = \sum_j f_j \tau_j$$

where

$$\tau_j = \frac{1}{k_{Rj}}\left(1 + \frac{K_{Rj}}{[T_{3j}]}\right)$$

Here, τ_j, is the average time to translate a codon of type j. The effective association rate constant for the binding of a ternary complex, T_{3j}, to the ribosome is the ratio k_{Rj}/K_{Rj}. The parameter k_{Rj} is the inverse of the time it takes to hydrolyse GTP on EF-Tu, execute peptidyl-transfer, translocate peptidyl-tRNA from A to P-site by the action of elongation factor EF-G and dissociate EF-G from the post-translocation ribosomal complex. The validity of equation (M8) depends only on the assumption that the compound parameters k_{Rj} and K_{Rj} do not display significant variation. For clarity, we will now assume that all codons have the same Michaelis-Menten parameters ($K_{Rj} = K_R$, $k_{Rj} = k_R$), that all codons except one are translated with maximal speed (k_{max}) and neglect the marginal difference (due to different maximal ternary complex concentrations) between k_R and the maximal rate, k_{max}, of translating codons ($k_R = k_{max}$). This gives:

$$j_R = f[R]v = \frac{f[R]}{\frac{1-f}{k_{max}} + \frac{f}{k}} = \frac{f[R]k_{max}}{1 + \frac{fK_R}{[T_3]}} \quad \text{(M9)}$$

The ribosomal step rate k is defined from:

$$\frac{1}{k} = \frac{1}{k_{max}}\left(1 + \frac{K_R}{[T_3]}\right) \quad \text{(M10)}$$

A situation where all codons except one are translated with maximal rate arises as soon as the synthesis of one or several amino acids is insufficient: the pathway where the synthesis normalised to demand is the smallest will become limiting, while all other pathways will automatically synthesise amino acids in excess over the current demand.

The exponential growth rate μ is, by definition, determined by the total rate of protein synthesis per volume $v[R]$, divided by the density ρ_0 of amino acids in intracellular proteins:

$$\mu = \frac{[R]v}{\rho_0} \quad \text{(M11)}$$

The steady-state equations were solved numerically (Newton-Gauss algorithm) with the parameter values in Table 1 and taking into account that the sum of the concentrations of free tRNA, tRNA on synthetase and tRNA in ternary complex is constant. The sensitivities $a_{Qx} = (d\ln Q/d\ln x)$, $a_{Qk} = (d\ln Q/d\ln k)$ and $a_{Qs} = (d\ln Q/d\ln s) = (d\ln Q/d\ln[E])$, and $a_{xs} = (d\ln x/d\ln s) = (d\ln x/d\ln[E])$ and $a_{ks} = (d\ln k/d\ln s) = (d\ln k/d\ln[E])$ in Figures 2, 4, 8 and 9 were derived by approximating derivatives with difference ratios and by applying the chain-rule to the relevant equations.

Our approach to global modelling is reminiscent of the ''top-down'' approach described by Fell,[38] since we have lumped all the consecutive and branched reactions that constitute a biochemical pathway responsible for the synthesis of an amino acid into one block. This procedure has been analysed carefully and found to be methodologically sound.[38] In the language of metabolic

Table 1. Model parameters

	Value	Reference
$[S_{tot}]$ (M)	1.3×10^{-6}	Neidhardt[56]
$[tRNA^i_{tot}]$ (M)	1×10^{-5}	Dong *et al*[57]
[R] (M)	2×10^{-5}	Bremer[58]
k_2 (s^{-1})	100 s^{-1}	Calc. from Neidhardt[56]
K_S (M)	10^{-6}	Holler[59]
K_R (M)	1×10^{-6}	Diaz[60]
K_P (M)	5×10^{-3}	
k_{max} (s^{-1})	10 s^{-1}	
F	0.05	
k_0 (M^{-1} S^{-1})	1×10^{6}	
ρ_0 (M)	2	Calc. from Churchward *et al*.[61]
Repressor control parameters		
$[R_0]$ (M)	1×10^{-7}	Lissens[62]
K (M)	1.0×10^{-10}	Glansdorff[63]
K_1/K_2	1000	

M	1	2	4	6
K_0	0.01	10^{-5}	10^{-11}	10^{-17}
K_1 (M)	9.90×10^{-4} M	8.62×10^{-5}	2.10×10^{-5} M	1.12×10^{-5} M

control analysis, a simplified version of Figure 1 could be written:

$$\xrightarrow{\text{block 1}} X \xrightarrow{\text{block 2}} T_3 \xrightarrow{\text{block 3}} \text{proteins}$$

Here, block 1 is the metabolic pathway for amino acid synthesis, block 2 is the aminoacylation reaction and block 3 is protein synthesis carried out by ribosomes. However, we have used the extensive biochemical knowledge that is available concerning both aminoacylation and protein synthesis to make the modelling of these two steps kinetically correct at a level of detail that is appropriate for the purpose of the present analysis.

Transcriptional control models

The probability Q that an operator is free is, in general, given by:

$$Q = \frac{1}{1 + [R_A]/K} = \frac{1}{1 + (R_0/K)P_A(x)} \qquad \text{(M12)}$$

K is the dissociation constant for the binding of an active repressor with free concentration $[R_A]$ to the operator. R_0 is the total concentration of free repressor and $P_A(x)$ is the probability that a free repressor is active at a free ligand concentration x. For a repressor that is driven from active to inactive form with increasing x in a cooperative fashion as described in Results, $P_A(x)$ is given by:

$$P_A(x) = \frac{K_0(1 + x/K_2)^m}{(1 + x/K_1)^m + K_0(1 + x/K_2)^m} \qquad \text{(M13)}$$

Equations (M12) and (M13) lead directly to equation (6) in Results.

It may be emphasized that attenuation implies competition between two energy-driven stochastic processes (translation and transcription), while repressor binding is an equilibrium phenomenon. The reason why equations (1) and (6) in Results appear different is thus not because a stochastic approach has been used for attenuation and an enzyme kinetic approach for repressor binding. Stochastic approaches have been used in both cases, and the difference between equations (1) and (6) is a consequence of the fundamentally different kinetic properties of the two control systems that they describe.

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