Design Principles for Regulator Gene Expression in a Repressible Gene Circuit

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We consider the design of a type of repressible gene circuit that is common in bacteria. In this type of circuit, a regulator protein acts to coordinate the repression of effector genes when a signal molecule with which it interacts is present. The regulator protein can also independently influence the expression of its own gene, such that regulator gene expression is repressible (like effector genes), constitutive, or inducible. Thus, a signal-directed change in the activity of the regulator protein can result in one of three patterns of coupled regulator and effector gene expression: direct coupling, in which regulator and effector gene expression change in the same direction; uncoupling, in which regulator gene expression remains constant while effector gene expression changes; or inverse coupling, in which regulator and effector gene expression change in opposite directions. We have investigated the functional consequences of each form of coupling using a mathematical model to compare alternative circuits on the basis of engineering-inspired criteria for functional effectiveness. The results depend on whether the regulator protein acts as a repressor or activator of transcription at the promoters of effector genes. In the case of repressor control of effector gene expression, direct coupling is optimal among the three forms of coupling, whereas in the case of activator control, inverse coupling is optimal. Results also depend on the sensitivity of effector gene expression to changes in the level of a signal molecule; the optimal form of coupling can be physically realized only for circuits with sufficiently small sensitivity. These theoretical results provide a rationale for autoregulation of regulator genes in repressible gene circuits and lead to testable predictions, which we have compared with data available in the literature and electronic databases.

Keywords: gene regulation; repression; design principles; mathematical model; autoregulation

Introduction

Changes in bacterial gene expression in response to a signal are often mediated by the product of a regulator gene that coordinately regulates the expression of a set of effector genes. The regulator gene encodes a signal-responsive protein, such as the repressor of the tryptophan (trp) operon in Escherichia coli,¹ that acts as an activator or repressor of transcription of the effector genes, and the effector genes encode enzymes and/or other types of effector molecules (e.g. flagellar proteins). The effector genes in many well-studied systems encode metabolic enzymes that are members of the same cellular pathway/system. When a signal molecule interacts with the regulator protein, the activity of the regulator protein is modified and effector gene expression changes.

The genes and gene products involved in the response to a signal are what make up a genetic regulatory circuit. Circuits can be classified into two types, repressible or inducible, based on the qualitative response to a signal. In a repressible circuit, an increase in the level of a signal molecule leads to a decrease in effector gene expression. For example, an increase in the cellular availability of

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tryptophan, which interacts with the Trp repressor, causes a decrease in expression of the Trp-regulated trp operon in E. coli, which encodes genes required for tryptophan biosynthesis.\(^2\) The opposite occurs in an inducible circuit, the classical example of which is the lactose (lac) system in E. coli.\(^3\) The signal molecule in many well-studied systems is a metabolite that is a substrate and/or product of enzymes encoded by the co-regulated effector genes.

Circuits can also be classified based on the pattern of coupled regulator and effector gene expression. The regulator protein, independent of its function at the promoters of effector genes, can influence transcription of its own gene, a phenomenon that is called autoregulation.\(^4\) Thus, in response to a signal, three patterns of coupled changes in regulator and effector gene expression are possible: direct coupling, uncoupling, and inverse coupling. In a directly coupled circuit, changes in regulator and effector gene expression are coordinate. In an uncoupled circuit, regulator gene expression remains constant while effector gene expression changes. In an inversely coupled circuit, changes in regulator and effector gene expression are opposite. An example of a directly coupled circuit is the tryptophan (trp) system in E. coli,\(^7\) in which the level of regulator protein is repressible, just as the level of enzymes is repressible. An example of an uncoupled circuit is the asparagine (asn) system in E. coli.\(^8\) Unfortunately, an example of an inversely coupled circuit seems to be unavailable among repressible systems. However, inverse coupling has been documented among inducible systems,\(^6\) so we expect that this form of coupling is also relevant for repressible systems, at least as a formal possibility.

Faced with this diversity of circuit design, the following question arises.\(^10\) How do we explain the evolution of directly coupled, uncoupled, and inversely coupled circuits for repression? It is not immediately obvious why the level of regulator protein is repressible in some systems, constitutive in others, and possibly inducible in yet others, especially because the different types of circuits tend to have similar physiological functions. For example, the trp and asn circuits each spare the biosynthesis of an amino acid when the amino acid is available in the environment.\(^2\) Here, we address this question by comparing the functional capabilities of directly coupled, uncoupled, and inversely coupled circuits for control of repressible gene expression.

The present work extends the results of previous comparisons of direct coupling, uncoupling and inverse coupling in inducible gene circuits.\(^6\) Although the possible forms of coupling in repressible and inducible circuits are the same, the two types of circuits differ significantly. For example, repressible circuits tend to involve effector genes that encode biosynthetic enzymes, whereas inducible circuits tend to involve effector genes that encode catabolic enzymes; and of course, repressible and inducible circuits respond oppositely to signals. In a repressible circuit, effector gene expression is downregulated in response to an increase in the level of a signal molecule, whereas in an inducible circuit, effector gene expression is upregulated.

The present work also extends earlier more narrow comparisons\(^3\) of repressible circuits. In this earlier work, two kinds of repressible circuits with special forms of coupling were considered: completely uncoupled circuits, in which the regulator protein has no effect whatsoever on regulator gene expression, and perfectly coupled circuits, in which relative changes in the expression of regulator and effector genes are identical because, for example, regulator and effector genes reside within the same operon. The more general forms of coupling considered here are more common than the completely uncoupled and perfectly coupled patterns of regulator and effector gene expression.

The foundation of the approach that we take to compare circuits is the method of controlled mathematical comparison.\(^10\) This method is implemented, in part, by defining a priori criteria for functional effectiveness\(^11\) and by developing a generalized mathematical model for the different types of systems under consideration. Special cases of the model represent repressible gene circuits with each form of coupling. Systems that are compared are allowed to differ in ways that distinguish alternative circuit designs but are otherwise required to be as much alike as possible. The criteria for functional effectiveness, which are inspired partly by the properties of well-designed man-made controllers, are used to score the results of comparisons. If differences in functional effectiveness are observed between different types of systems, we can hypothesize that these differences explain the evolution of the different circuit designs. On the other hand, if no differences are observed, we can hypothesize that diversity in circuit design is explained by historical accident, which is certainly important in some cases.\(^20\) The method of controlled mathematical comparison is a precise way to discover design principles of biological regulatory networks within the context of evolution and the limitations of tinkering.\(^10\)

The results of our comparisons indicate that there are functional differences associated with the different forms of coupling. For example, for systems in which the regulator protein is a repressor of effector gene expression (i.e. for repressor-controlled systems), we find that direct coupling allows faster responsiveness to signals than uncoupling, which in turn allows faster responsiveness than inverse coupling. These results lead to testable predictions. The predictions, which are similar to those for inducible systems,\(^6\) can be tested to a limited extent by comparing them with data available in the literature and electronic databases. If our predictions are supported by further tests, then we will have identified design principles of repressible gene circuits.
Theory

In this study we wish to understand what gives rise to differences in control of regulator gene expression in repressible gene circuits. Our study is carried out as follows. (1) We develop a generalized mathematical model for repressible gene circuits; a system with any of the three forms of coupling can be described by a special case of this model. (2) We next select systems for comparison. These systems differ in transcriptional control but are otherwise the same. Separate comparisons are made for systems with activator and repressor control of effector gene expression, because this feature of a gene circuit (i.e. the mode of effector gene regulation) appears to be selected on the basis of environmental demand for effector gene expression. (3) In comparisons, we determine the relative functional capabilities of alternative systems using well-defined a priori criteria for functional effectiveness. We also determine how physical constraints on kinetic orders influence the results.

Model

Equations and variables

We will consider systems characterized by Figure 1. A regulator gene encodes a regulator protein, which is a transcription factor, and the effector genes regulated by this protein encode enzymes that participate in biosynthesis of a metabolic end product. The effector genes are coordinated such that relative changes in gene expression are the same for each effector gene. The regulator protein can act as either a repressor or activator; its influence on transcription can be antagonized, unaffected (at the promoter of the regulator gene but not at the promoter of an effector gene), or stimulated by end product binding; and its mode of regulation and sensitivity to end product binding can depend on whether the regulator protein acts at the promoter of a regulator or effector gene. Because we are interested in coupling of regulator and effector gene expression in repressible circuits, we will only consider transcriptional circuitry for which an increase (decrease) in intracellular end-product concentration causes a decrease (increase) in effector gene expression. As illustrated in Figure 1, processes that we will consider include (1) synthesis of effector mRNA, (2) degradation of effector mRNA, (3) synthesis of effector enzymes, (4) dilution of effector enzymes through cell growth, (5) synthesis of end product, (6) consumption of end product, (7) cellular import of end product, (8) synthesis of regulator mRNA, (9) degradation of regulator mRNA, (10) synthesis of regulator protein, and (11) dilution of regulator protein. The metabolic pathway leading to end product is taken to be unbranched, controlled by feedback inhibition, and composed of fast intermediate steps such that we can treat the pathway as a one-step reaction from initial substrate to final end product. Note that the model considered here, which has been tailored to incorporate features of repressible circuits (e.g. end product feedback inhibition), differs from the model used to compare direct coupling, uncoupling, and inverse coupling in inducible circuits.

The state of a system is characterized by the five dependent variables $X_1, ..., X_5$, which represent the intracellular concentrations of effector mRNA ($X_1$), enzyme ($X_5$), end product ($X_6$), regulator mRNA ($X_4$), and regulator protein ($X_3$). The independent variables $X_6, ..., X_9$ are considered to be set by factors external to the system. These independent variables represent the precursor pools for mRNA and protein synthesis ($X_6$ and $X_7$), the amount of substrate available for conversion to end product ($X_9$), and the amount of end product available in the extracellular environment ($X_8$). We treat $X_6$ and $X_7$ as constants, and we consider $X_9$ as a constant.

Figure 1. The model: the numbered arrows represent mass fluxes. Other arrows represent catalytic or regulatory influences. The kinetic orders influencing gene regulation are $g_{15}, g_{13}, g_{45},$ and $g_{45}$. The kinetic order $g_{15}$ characterizes the influence of the end product on effector gene expression, and the kinetic order $g_{45} \neq 0$ characterizes the influence of the regulator protein on effector gene expression. Systems with positive (negative) $g_{15}$ values are under activator (repressor) control. The kinetic order $g_{45}$ characterizes the influence of the end product on regulator gene expression, and the kinetic order $g_{45}$ characterizes the influence of the regulator protein on regulator gene expression. Systems with positive (negative) $g_{45}$ values have positive (negative) autoregulation; systems with $g_5 = 0$ have a constitutively expressed regulator protein with no influence at the promoter of its own gene. We define a repressible system as one for which the steady-state gain $L_{25} = \frac{\log Z_{2}/\log Z_{5}}{g_{45}}$ (equation (2)) is negative. The steady-state gain $L_{53} = \frac{\log Z_{5}/\log Z_{3}}{g_{15}}$ (equation (3)) may be positive (inverse coupling), zero (uncoupling), or negative (direct coupling).
will consider the effects of step changes in the availability of substrate (Xₐ) and exogenous end product (Xₜ).

Based on Figure 1, we derive a system of equations that includes one equation for each dependent variable, with each equation having the form dXᵢ/dt = Vᵢ₊ − Vᵢᵢ. Here, dXᵢ/dt is the rate at which concentration Xᵢ changes with time t. Vᵢ₊ is the net mass-flux directed into the pool characterized by Xᵢ, and Vᵢᵢ is the net mass-flux directed out of the pool characterized by Xᵢ. We use the power-law formalism to specify rate laws for each flux Vᵢ₊ and Vᵢᵢ; thus, we obtain:

\[
\begin{align*}
\frac{dX_1}{dt} &= \alpha_1 X_4^{g_1} X_3^{g_2} X_5^{g_3} - \beta_1 X_i^{h_1} \\
\frac{dX_2}{dt} &= \alpha_2 X_4^{g_2} X_1^{g_3} - \beta_2 X_i^{h_2} \\
\frac{dX_3}{dt} &= \alpha_3 X_4^{g_3} X_3^{g_4} X_5^{g_5} - \beta_3 X_i^{h_3} \\
\frac{dX_4}{dt} &= \alpha_4 X_4^{g_4} X_3^{g_5} X_5^{g_6} - \beta_4 X_i^{h_4} \\
\frac{dX_5}{dt} &= \alpha_5 X_4^{g_5} X_4^{g_6} - \beta_5 X_i^{h_5}
\end{align*}
\]

in which the α and β parameters are rate constants that have strictly positive values, and the g and h parameters are real-valued kinetic orders: a negative value indicates an inhibitory influence, a positive value indicates a stimulatory influence, and a zero value indicates the absence of influence. The kinetic order gᵢ describes the influence of concentration Xᵢ on the flux Vᵢ₊, whereas the kinetic order hᵢ describes the influence of concentration Xᵢ on the flux Vᵢᵢ.

One of the steady states of equation (1) is the steady-state at the threshold of repression, i.e. the steady-state operating point at which effector gene expression is maximal. Note that we consider equation (1) to apply only over a certain range of end product concentration and that we consider effector gene expression to be maximal at the low end of the regulatable range of end product concentration (see Supplementary Material). We denote the steady-state concentrations at the threshold of repression as (Xₐₐ, ..., Xₐ₉) and the steady-state fluxes as (V₁, ..., V₅). Note that influxes and effluxes are equal at steady-state. It is convenient to define the following dimensionless concentrations, which are normalized at the threshold of repression: uᵢ = Xᵢ/Xₐᵢ for i = 1, ..., 9. Likewise, it is convenient to define the following turnover numbers: Fᵢ = Vᵢ/Xₐᵢ for i = 1, ..., 5. See Supplementary Material for further discussion.

Parameters

Repressible circuits

We consider a circuit to be repressible if the steady-state level of effector protein decreases (increases) when the intracellular level of end product increases (decreases). Thus, repressible circuits correspond to systems marked by a negative value of the steady-state logarithmic gain L₂₃, which is defined as

\[
L_{23} = \frac{g_{21}}{h_{13} h_{22}} \left( \frac{g_{13} g_{45} h_{44} h_{55} / g_{54}}{g_{45}} \right)
\]

(2)

The gain L₂₃ characterizes the sensitivity of effector protein levels to changes in the intracellular supply of end product. The expression for L₂₃ in equation (2) is obtained from the steady-state solution of equation (1). Further discussion of L₂₃ is provided in Supplementary Material.

Activator and repressor control

The parameters describing the effect of a change in the concentration of the regulator protein on effector and regulator gene expression are gᵢₜ and gᵢₜ respectively (Figure 1). If gᵢₜ is positive, then the regulator protein is an activator of effector expression, and the system is considered to be positively regulated, i.e. activator controlled. If, on the other hand, gᵢₜ is negative, then the regulator protein is a repressor of effector expression, and the system is considered to be negatively regulated, i.e. repressor controlled. The kinetic order gᵢₜ similarly determines whether the regulator protein is an activator or repressor of regulator gene expression: systems with positive gᵢₜ are positively autoregulated, and systems with negative gᵢₜ are negatively autoregulated.

Form of coupling

The logarithmic gain L₅₃ = d log Xₕ₃/d log X₅₃ characterizes the sensitivity of the level of regulator protein to changes in the concentration of end product:

\[
L_{53} = \frac{g_{43}}{h_{44} h_{55} / g_{54} - g_{45}}
\]

(3)

Like L₂₃, this gain is derived from the steady-state form of equation (1). Note that the signs of L₅₃ and g₄₃ are the same if g₄₅ < h₄₄ h₅₅/g₅₄, which, as we will see, is necessarily true for systems with a stable steady state. A system’s form of coupling is determined by comparing the signs of L₅₃ and L₂₃:

\[†\]

Different formal definitions of a repressible system are possible. The definition that perhaps best corresponds to an experimental test for a repressible system is a system for which the gain L₂₃ is negative. This gain, defined as d log Xₙ₅/d log X₅ₙ, characterizes the steady-state change in the level of enzyme Xₙ caused by a change in the level of exogenous end product Xₗ. The gain L₂₃ is related to the gain L₅₃ as follows: L₂₃ = −g₃ₙ L₅₃/(g₃ₙ g₅₃ + g₃ₙ − h₃ₙ). Thus, systems with the same gain L₂₃ also have the same gain L₅₃ provided these systems have the same values for g₃ₙ, g₅₃, g₅₄, and h₃ₙ, as we will require for internal equivalence. Given the parameter estimates noted in Table 1, L₂₃ < 0 if L₅₃ < 0 or if L₅₃ > (h₃ₙ − g₃ₙ)/g₅₄ > 0. We consider only systems with L₂₃ < 0. Systems with L₂₃ > 0, although they can have L₂₃ < 0, are not considered to be physiologically relevant.
if they have the same sign (i.e. $L_{53} < 0$), the system is directly coupled; if they have opposite sign (i.e. $L_{53} > 0$), the system is inversely coupled; and if $L_{53} = 0$, the system is uncoupled. Recall that $L_{23} < 0$ for systems of interest. See Supplementary Material for further discussion of the gain $L_{53}$.

**Physical limitations on kinetic orders that characterize regulation of gene expression**

We will compare systems that differ in transcriptional control, focusing on differences in the kinetic orders $g_{13}$ and $g_{15}$, which characterize regulation of effector gene expression, and the kinetics orders $g_{43}$ and $g_{45}$, which characterize regulation of regulator gene expression (Figure 1). The magnitudes of these kinetic orders are limited for physical reasons.23 Thus:

\[

g_{13} \leq |g_{13}|_{\text{max}}, \quad g_{15} \leq |g_{15}|_{\text{max}}
\]

\[

g_{43} \leq |g_{43}|_{\text{max}}, \quad g_{45} \leq |g_{45}|_{\text{max}}
\]

where $|g_{13}|_{\text{max}}$, $|g_{15}|_{\text{max}}$, $|g_{43}|_{\text{max}}$, and $|g_{45}|_{\text{max}}$ are small positive integers. We will consider kinetic orders to have magnitudes no greater than 2 or 4.

We compare the different circuit designs (directly coupled, uncoupled, and inversely coupled) in a specific background, i.e. in the context of particular values for the non-regulatory parameters. Our estimates of these non-regulatory parameters are given in Table 1.

### Equivalence conditions for controlled comparisons

We compare systems with the different forms of coupling under conditions of internal and external equivalence.18,19 This procedure is meant to ensure that any functional difference between alternative systems can be attributed to their differences in control of gene expression.

For internal equivalence, we require alternative systems to be identical except in the process of mRNA synthesis. Thus, only $\alpha_{1}, \beta_{2}, \beta_{3}, \gamma_{15}, \alpha_{16}, \alpha_{43}, \beta_{45}$, and $\gamma_{45}$, which characterize transcription and transcriptional control, can have values that differ for two systems.

For external equivalence, we require that alternative systems exhibit the same steady-state response of enzyme level to changes in the level of intracellular end product. This requirement is met if alternative systems have the same derepressed steady-state concentrations $X_{10}, \ldots, X_{50}$ and have the same steady-state logarithmic gain $L_{23}$. We will consider activator and repressor-controlled systems separately because of demand theory.22 As a result, we also require alternative systems to have the same sign for the kinetic order $g_{45}$.

The first step in a comparison is to choose a reference system, the parameter values of which we will denote using primes. This reference system has a particular derepressed steady-state ($X_{10}', \ldots, X_{50}'$), gain $L_{23}'$, and sign of $g_{45}'$. These quantities and the reference system's non-regulatory parameter values (Table 1) are then used to determine the allowable parameter values for

### Table 1. Parameter estimates

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Estimate</th>
<th>Comment</th>
</tr>
</thead>
<tbody>
<tr>
<td>$g_{13}$ or $g_{44}$</td>
<td>1</td>
<td>Rate of protein synthesis is proportional to amount of mRNA</td>
</tr>
<tr>
<td>$g_{52}$</td>
<td>1</td>
<td>Rate of end-product synthesis is proportional to the amount of enzyme that catalyzes the reaction</td>
</tr>
<tr>
<td>$g_{53}$</td>
<td>−1</td>
<td>Biosynthetic pathways commonly involve feedback inhibition, which is indicated in vitro for the trp system in <em>E. coli</em>. A value of −1 can be estimated based on the inhibition of anthranilate synthase activity in vitro</td>
</tr>
<tr>
<td>$h_{51}$ or $h_{54}$</td>
<td>1</td>
<td>Degradation of mRNA is a first-order process</td>
</tr>
<tr>
<td>$h_{52}$ or $h_{55}$</td>
<td>1</td>
<td>Proteins are typically stable in bacteria. Dilution of stable protein through exponential growth is a first-order process</td>
</tr>
<tr>
<td>$h_{53}$</td>
<td>0.5</td>
<td>Assuming Michaelis–Menten kinetics, the value of $h_{53}$ lies between 0 and 1 with a value of 0.5 when the endogenous level of end product equals the $K_m$ for its consumption. Based on analysis of the trp system in <em>E. coli</em>, we estimate that $h_{53} = 0.5$ for this system</td>
</tr>
</tbody>
</table>

Estimates for $g_{43}$, $g_{52}$, $g_{54}$, $h_{51}$, $h_{52}$, $h_{55}$, $F_1$, and $F_2$ are likely to hold for a variety of systems, especially in *E. coli* growing exponentially. Estimates for $g_{53}$, $h_{52}$, $F_1$, and $F_2$ are perhaps less general, being based on data specific for the tryptophan system, is 43 seconds. Because the apparent $K_m$ value for tryptophan in the formation of charged tRNA in *E. coli* (10 µM) is similar to the endogenous level of tryptophan (70 µM), we estimate that $h_{53} = 0.5$ for this system.
Design of Repressible Gene Circuits

alternative systems. Internal equivalence fixes the values of all but eight parameters, which are $\alpha_4$, $g_{1\ell}$, $g_{15}$, $g_{4\ell}$, $a_4$, $g_{45}$, and $g_{44}$. However, because $X_5$ is a constant, the terms $\alpha_4X_6$ and $\alpha_4X_5^6$ in equation (1) can each be treated as a single lumped parameter, which effectively leaves only six free parameters. External equivalence imposes further constraints. Equivalent systems are required to have the same steady-state at the threshold of derepression, from which it follows that $V_1 = V_1'$ and $V_4 = V_4'$ for any system equivalent to the reference system. These two constraints, equation (1), and the parameter values of the reference system determine the values of $\alpha_4X_6$ and $\alpha_4X_6^6$ for equivalent systems. Furthermore, equation (2) and the requirement that $L_{23} = L_{23}'$ fixes one of the four kinetic orders that characterize transcriptional control once the other three kinetic orders are specified. Thus, to examine a family of equivalent systems, it is sufficient to vary only three parameters (e.g., $g_{1\ell}$, $g_{4\ell}$, and $g_{44}$). See Supplementary Material for further discussion.

Functional effectiveness

We compare equivalent systems using five a priori criteria for functional effectiveness: selectivity, stability, robustness, efficiency, and temporal responsiveness. Each criterion is described below.

A system is considered to be selective if $|l_{g53}|$ is less than a threshold value $6,23$ such that the steady-state level of regulator protein is sufficiently insensitive to changes in the level of end product. Limited variation of regulator gene expression avoids three potential problems: non-specific interactions with unrelated systems when the level of regulator protein is high, loss of regulation when the level of regulator protein is low, and possible variation in cellular growth rate caused by large changes in the burden of protein synthesis.

A system is considered to be effective with respect to stability if its derepressed steady state is locally stable and characterized by a large margin of stability. Local stability ensures that the system returns to its steady state following a small disturbance of this state, and a large margin of stability ensures that the system’s steady state remains stable despite small changes in the system’s structure (i.e., the system’s parameter values).

To determine if the derepressed steady state of equation (1) is locally stable, we study the associated linearization,26,27 $dx/dt = Lx$, where $x = (x_1, \ldots, x_5)^T$ and $x_i = u_i - 1$. The elements of $L$ are defined as $l_{ij} = -f_i(h_{ij} - \xi_j)$. If the characteristic polynomial of $L$, $p(\lambda) = \det(L - \lambda I)$, has roots all with negative real parts (i.e. if $p(\lambda)$ is Hurwitz), then the steady state of equation (1) is locally stable.26,28 The characteristic polynomial of $L$ is:

$$p(\lambda) = \lambda^5 + a_1\lambda^4 + a_2\lambda^3 + a_3\lambda^2 + a_4\lambda + a_5$$

$$= [(\lambda + f_1)(\lambda + f_2)(\lambda + f_3) - c_1g_{13}][(\lambda + f_4)(\lambda + f_5)$$

$$+ f_5 + c_2g_{45}] - c_1c_2g_{15}g_{43}$$

(5)

where $c_1 = F_1F_2F_3g_{21}g_{22}$, $c_2 = F_4F_5g_{54}$, $f_1 = F_1h_{11}$, $f_2 = F_2h_{22}$, $f_3 = F_3(h_{33} - g_{33})$, $f_4 = F_4h_{44}$, and $f_5 = F_5h_{55}$. See Supplementary Material for further discussion.

To determine if $p(\lambda)$ is Hurwitz, we use the Liénard–Chipart criterion.26,28 This criterion states that $p(\lambda)$ is Hurwitz if and only if $a_1 > 0$, $a_3 > 0$, $a_5 > 0$, $\Delta_2 > 0$, and $\Delta_4 > 0$, where $a_1$, $a_3$, and $a_5$ are coefficients of $p(\lambda)$ (equation (5)) and $\Delta_2$ and $\Delta_4$ are Hurwitz determinants:26,29

$$\Delta_2 = a_1a_2 - a_3$$

and

$$\Delta_4 = (a_1a_2 - a_3)(a_2a_3 - a_5) - a_1a_5a_3^2.$$ Note that $a_5 > 0$ implies $g_{43} < h_{44}h_{55}/g_{54}$. We will focus on systems that satisfy this necessary condition for stability, which simplifies the analysis of results.

A system’s margin of stability is defined as a distance in parameter space, the distance between the point representing the system and the closest point representing a system with an unstable steady state. The parameter space of interest is the parameter space that are of importance for determining the margin of stability: the divergence curve, along

Figure 2. Representation of systems with stable and unstable steady states in the parameter space of $g_{13}$ and $g_{45}$. The domain that represents systems with a stable steady state is bordered by two boundaries of instability: the line along which $\Delta_4 = 0$ and the algebraic curve of third degree along which $\Delta_1 = 0$. Systems with an unstable steady state lie in the shaded region; systems represented elsewhere in this plot have a stable steady state.
which \( a_5 = 0 \), and the unstable oscillation curve, along which \( \Delta t = 0 \). Systems with stable steady states are bounded by these two curves (Figure 2). The divergence curve, which is so called because points on this curve correspond to a zero eigenvalue, is the straight line \( g_{45} = h_{45} \). The unstable oscillation curve, which corresponds to points with a pair of conjugate pure imaginary eigenvalues, is an algebraic curve of order 3, which follows from the expression given above for \( \Delta t \). Thus, the margin of stability can be calculated as the shortest distance between a (stable) system of interest and either of these curves. We will use the Manhattan metric to measure distance. See Supplementary Material for further discussion.

A system is considered to be robust if its steady states are characterized by parameter sensitivities with small magnitudes. \(^{34,35}\) Parameter sensitivities with small magnitudes indicate that a system’s steady state is insensitive to disturbances that affect the system’s structure (i.e. the system’s parameter values). \(^{32}\)

Sensitivities are defined as follows. The sensitivity of the steady-state concentration \( X_i \) to a change in a parameter \( p \) is defined as:

\[
S(X_i, p) = \frac{\partial \log X_i}{\partial \log p}
\]

where \( p \) can be a rate constant or kinetic order. Likewise, the sensitivity of the steady-state flux \( V_i \) to a change in a parameter \( p \) is defined as \( S(V_i, p) = \frac{\partial \log V_i}{\partial \log p} \). Expressions for \( X_i \) and \( V_i \) are found from the steady-state form of equation (1), which can be written as \( A y = b + i \). Here, \( i \) is a vector involving the independent concentrations \( \{X_r, X_7, X_6, \ldots, X_0\} \) and their kinetic orders, \( b \) is a vector defined as \( \{b_1, \ldots, b_5\} \), in which each \( b_1 = \log \beta_i - \log \alpha_i, \) \( y \) is a vector defined as \( \{y_1, \ldots, y_5\} \), in which each \( y_i = \log X_i \), and \( A \) is a \( 5 \times 5 \) matrix with elements \( a_{ij} = y_j - y_i \). All parameter sensitivities are proportional to sensitivities of the form \( S(X_i, \beta_i) \). \(^{33}\) which compose the elements of \( A^{-1} \). See Supplementary Material for further discussion.

As explained elsewhere, \(^{14}\) a system is considered to be efficient if it produces appropriate steady-state input–output behavior. We are concerned with the steady-state responses of effector protein \( \{X_3\} \) and intracellular end-product \( \{X_3\} \) to three types of changes: (1) changes in the level of flux \( V_i \); (2) changes in the level of exogenous end product \( \{X_3\} \); and (3) changes in the rate of end product consumption \( \{\beta_i\} \). The relevant quantities that characterize responses to these changes are the following gains and sensitivities: \( L_{28}, L_{38}, \) \( L_{29}, L_{39}, S(X_2, \beta_3) \) and \( S(X_3, \beta_3) \). Each of these gains and sensitivities can be related to the gain \( L_{23} \) as follows:

\[
\begin{align*}
L_{28} &= L_{29} \quad \text{for } g_{38} = g_{39} = -S(X_2, \beta_3) = \frac{L_{23}}{h_{33} - g_{33} - g_{32}L_{23}} \\
L_{38} &= L_{39} \quad \text{for } g_{38} = g_{39} = -S(X_3, \beta_3) = \frac{1}{h_{33} - g_{33} - g_{32}L_{23}}
\end{align*}
\]

A system is considered to be temporally responsive if it has a small settling time following a step decrease in the level of substrate \( \{X_3\} \) or exogenous end product \( \{X_3\} \). Settling time is defined as the time required for the level of enzyme to approach and remain within 5% of its steady-state value. Time-courses of derepression are found by using standard methods \(^{34}\) to numerically solve the following initial value problem, which is derived from equation (1):

\[
\begin{align*}
\epsilon \frac{du_1}{dt} &= u_1^{\infty} u_1^{\infty} - u_1, \\
\mu \epsilon \frac{du_2}{dt} &= f_t u_2^{\infty} u_3^{\infty} - u_3^{\infty} \\
\epsilon \frac{du_4}{dt} &= u_3^{\infty} u_5^{\infty} - u_4, \\
\frac{du_5}{dt} &= u_4^{\infty} - u_5
\end{align*}
\]

where:

\[
f_t(\tau) = u_6^{\infty} u_9^{\infty} \begin{cases} f_{\tau, max} & \text{if } \tau < 0 \\
f_{\tau, min} & \text{if } \tau \geq 0 \end{cases}
\]

The initial condition is given by:

\[
u(0) = f_{\tau, max}^{E_2} u_3^{\infty} u_5^{\infty} \quad \text{and} \quad u_5(0) = f_{\tau, max}^{E_3}
\]

Recall that the variables \( u_i \) are normalized concentrations \( X_i / X_0 \) and that the parameters \( F_r \) are turnover numbers \( V_i / X_0 \). Several new quantities are introduced in equations (7)–(9). Equation (7) is obtained from equation (1) by setting \( X_6 = X_6^n, X_7 = X_7^n, \) \( g_{21} = 1, \) \( h_{11} = 1, h_{32} = 1, h_{44} = 1, h_{55} = 1, F_4 = F_1, \) and \( F_5 = F_2 \) (Table 1) and by introducing the dimensionless variables \( \epsilon = F_2 / F_1, \mu = F_1 / F_3, \) and \( \tau = F_3 \). The dimensionless repression factor \( f_r \), which is related to \( X_4 \) and \( X_5 \) is defined in equation (8). The parameters in the exponents of equation (9) are defined as follows: \( E_1 = g_{43} / (1 - g_{45}), E_2 = g_{43} + g_{45} E_1, \) and \( E_3 = h_{33} - g_{33} - g_{32} E_2 \). As indicated in equation (8), we follow the dynamics of derepression initiated by a step decrease in the repression factor \( f_r \) from \( f_{\tau, max} \) to \( f_{\tau, min} \). The final derepressed steady-state corresponds to \( f_r = f_{\tau, min} \), which we set equal to 1 without loss of generality. As indicated in equation (9), the initial repressed steady-state is determined by the value of \( f_{\tau, max} \), which is 100 for all cases considered (Table 1).

We compare the responsiveness of systems with a particular gain \( L_{23} < 0 \) and a particular sign for \( g_{35} \) by considering families of systems in the \( (g_{15} g_{45} g_{60}) \) parameter space. For each of several planes of constant \( g_{15} \) in this space, we find three settling times: the settling time of uncoupled circuits \( f_{\tau, max}, g_{45} = 0 \), the minimal settling time for directly coupled circuits \( f_{\tau, max}, g_{45} < 0 \), and the minimal settling time for inversely coupled circuits \( f_{\tau, max}, g_{45} > 0 \). To find the minimal settling times \( f_{\tau, min}^{D} \) and \( f_{\tau, min}^{I} \) we sample the regions of parameter space where directly and inversely coupled circuits are represented by (pseudo) randomly selecting values for \( g_{43} \) and \( g_{45} \).
within the ranges allowed by equation (4). Note that uncoupled systems have settling times that are independent of \( g_{53} \). Results are obtained for a variety of values of \( L_23 \) and \( g_{53} \).

To determine how comparisons depend on estimates of turnover numbers, for each family of systems described above, we obtained and compared the settling times \( t^{(L)}_1 \), \( t^{(D)}_1 \), and \( t^{(D)}_2 \) for systems with (pseudo) randomly generated values for the turnover numbers \( F_1 \) (\( = F_3 \), \( F_2 \) (\( = F_3 \)), and \( F_3 \). Sampling of each turnover number was centered on the estimated value given in Table 1 and log uniform in the range of 10% to 1000% of the estimated value.

**Results**

Below, we compare directly coupled, uncoupled, and inversely coupled circuits that are described by the model illustrated in Figure 1. We begin by considering the constraints that limit the magnitudes of kinetic orders (equation (4)). We then report detailed results for comparisons of circuits based on selectivity, stability, robustness, efficiency, and temporal responsiveness. The comparisons depend in part on an assumption of specific parameter estimates (Table 1).

**Physical limitations on the form of coupling**

We find that a constraint on the magnitude of the kinetic order \( g_{15} \) (equation (4)) can limit the form of coupling in a system. The limitation follows from equations (2)–(4) and our requirements that \( g_{15} \neq 0 \) and \( L_{23} < 0 \), and it depends on the relationship between \( L_{23} \) and a critical gain \( L_{23}^{*} \), which is defined as \(-L_{max}/(h_1 h_2 / g_{21})\):

\[
L_{23} < L_{23}^{*} \leftrightarrow g_{15} L_{53} < -g_{13} - |g_{13}|_{max}
\]

\[
L_{23} = L_{23}^{*} \leftrightarrow g_{15} L_{53} = -g_{13} - |g_{13}|_{max}
\]

\[
L_{23} > L_{23}^{*} \leftrightarrow g_{15} L_{53} > -g_{13} - |g_{13}|_{max}
\]

Equation (10) simply indicates that \( g_{15} L_{53} \) is negative when \( |L_{23}| > |L_{23}^{*}| \), negative or zero when \( L_{23} = L_{23}^{*} \), and negative, zero, or positive when \( |L_{23}| < |L_{23}^{*}| \). Thus, for systems with high gain (i.e. \( |L_{23}| > |L_{23}^{*}| \)), only inverse coupling (\( L_{53} > 0 \)) is possible with repressor control of effector gene expression (\( g_{15} < 0 \)), and only direct coupling (\( L_{53} < 0 \)) is possible with activator control of effector gene expression (\( g_{15} > 0 \)). For systems with intermediate gain (i.e. \( |L_{23}| = |L_{23}^{*}| \)), uncoupling (\( L_{53} = 0 \)) is also possible, and for systems with low gain (i.e. \( |L_{23}| < |L_{23}^{*}| \)), each form of coupling is possible. The above results are closely related to those obtained in an earlier study for inducible circuits.\(^\dagger\) For further discussion, see Supplementary Material.

**Selectivity**

Systems that have \( |L_{23}| \) below a certain threshold are selective. To estimate an upper bound on \( |L_{23}| \), we make a number of assumptions. We assume that more than one molecule per cell is required to maintain regulation, and fewer than 1000 molecules of regulator protein per cell are required to avoid dysfunctional cross-talk or an excessive protein burden on the cell. We also assume that regulation takes place over an approximate 30-fold range of end product concentration. From these assumptions, we find that the upper bound on the magnitude of \( L_{53} \) is 2, which implies that a directly coupled or inversely coupled system has the potential to be selective if \( |L_{23}| < 2 \) but not otherwise. If \( |L_{23}| > 2 \), the level of regulator protein can fall below one molecule per cell, which abolishes selectivity, or rise above 1000 molecules per cell, which also abolishes selectivity. Thus, if \( |L_{23}| < 2 \), each form of coupling allows for selectivity. Note that systems with strong negative auto-regulation of the regulator gene will tend to be selective, because decreasing \( g_{53} \) decreases the magnitude of \( L_{53} \) (equation (3)).

**Stability**

We find that negative autoregulation of the regulator gene favors stability and that the form of coupling has little impact on stability, especially when negative autoregulation of the regulator gene is strong. In Figure 2, equivalent systems with the parameter values of Table 1 can be compared graphically on the basis of stability. Systems with a stable steady-state are represented in a region of \((g_{13}, g_{53})\)-parameter space bounded by the divergence (\( \Delta g = 0 \)) and unstable oscillation (\( \Delta g = 0 \)) curves; systems with an unstable steady state are represented in the shaded region. As can be seen, most systems lie closer to the divergence curve than the unstable oscillation curve. We use the Manhattan metric to measure distance, but the same qualitative result is obtained using other metrics. Among the systems closer to the divergence curve, which include systems with each form of coupling, systems with identical \( g_{53} \) values have the same margin of stability, because they are equidistant from the divergence curve, the nearest boundary of instability. It can be further seen that the margin of stability increases as \( g_{53} \) decreases, which also holds for systems that are closer to the unstable oscillation curve than the divergence curve. Thus, it seems that the three forms of coupling each allow the same margin of stability, and any difference in margin of stability

\(^\dagger\) To consider the temporal responsiveness of different families of equivalent systems, we specified repressor and activator-controlled reference systems with a range of values for the gain \( L_{23} \) (\( -1/4, -1/2, -3/4, -1, -5/4, -3/2, -7/4 \)); for each family, we considered a range of values for \( g_{15} \) (\( 1/4, 1/2, 3/4, 1, 5/4, 3/2, 7/4, 2 \)).
betwee the two systems can be reduced by decreasing $g_{45}$. We conclude that the different forms of coupling are indistinguishable on the basis of our stability criterion and that stability is promoted by strong negative autoregulation (i.e., negative values of $g_{45}$ of large magnitude), at least for our estimates of non-regulatory parameters.

**Robustness**

Our analysis shows that robustness is enhanced by negative autoregulation of regulator gene expression and that the different forms of coupling allow similar robustness. The robustness of a system’s steady state is measured by parameter sensitivities, which were calculated numerically using the parameter estimates of Table 1. Results for sensitivities of the form $S(X_i, \beta_j)$ are shown in Figure 3 for repressor-controlled systems. Recall that other sensitivities are proportional to these sensitivities. As illustrated in Figure 3(a), equivalent systems with direct coupling, uncoupling, and inverse coupling can be found that have similar sensitivities. Moreover, sensitivities involving $X_2$ (enzyme) and $X_3$ (end product), which are likely to have greater physiological importance than sensitivities involving $X_1$, $X_2$, or $X_3$, are the same for each system. As can be seen by comparing Figure 3(b) and (c), differences between equivalent systems with the different forms of coupling are reduced overall when the value of $g_{45}$ shared by these systems is decreased, which indicates that negative autoregulation promotes robustness. Note that the systems considered in Figure 3 have robust steady states: a 1% change in any of the rate constants $\beta_i$ leads to less than a 1% change in any of the concentrations $X_i$. Similar results are obtained for activator-controlled systems (results not shown). A more detailed analytical comparison supports the numerical results and confirms that negative autoregulation is associated with increased robustness (see Supplementary Material). It seems that there is no necessary distinction among the different forms of coupling on the basis of our robustness criterion.

**Efficiency**

The efficiency of a system is determined by the logarithmic gains and parameter sensitivities in
Because equivalent systems have the same non-regulatory parameter values and the same gain $L_{23}$, the gains and sensitivities of equation (6) are identical for equivalent systems, and directly coupled, uncoupled and inversely coupled systems are equal with respect to our efficiency criterion.

**Temporal responsiveness**

We find significant differences in temporal responsiveness for systems with different types of coupling. Temporal responsiveness requires that the level of effector enzyme adjust quickly to changes in the level of substrate or exogenous end product. Time-courses triggered by such a change are shown in Figure 4 for systems with (a) repressor and (b) activator control of effector gene expression.

Figure 4. Time-courses of derepression. The parameter values in Table 1, in addition to $L_{23} = -1$ and $g_{45} = -2$, hold for all curves in both panels. For (a), $g_{15} = -2$, and for (b), $g_{15} = 2$. Values of $g_{45}$ and $g_{13}$ are as follows for curves (1)–(10): (1) $g_{45} = -1, g_{13} = -0.5, g_{15} = -1.33$; (2) $g_{45} = -0.5, g_{13} = -0.667$; (5) $g_{45} = 1, g_{13} = -0.333$; (6) $g_{45} = -1, g_{13} = -0.333$; (7) $g_{45} = 0.5, g_{13} = -0.667$; (8) $g_{45} = 0, g_{13} = -1$; (9) $g_{45} = 0.5, g_{13} = -1.33$; and (10) $g_{45} = 1, g_{13} = -1.67$. Time-courses are calculated by solving equation (7). As indicated in equation (8), derepression is stimulated by a step change in the repression factor $r$ from 100 to 1 at $\tau = F_2 t = 0$. The initial condition is given by equation (9).

Design of Repressive Gene Circuits

Figure 5. Dimensionless settling time as a function of $g_{43}$ and $g_{45}$. (a) Repressor-controlled systems with $g_{15} = -2$. (b) Activator-controlled systems with $g_{15} = 2$. The parameter estimates in Table 1, in addition to $L_{23} = -1$, apply to both panels. Settling time is defined as the dimensionless time $\tau$ required for enzyme to settle within 5% of its final steady-state value after derepression is initiated. Contours are equally spaced at 0.5 unit intervals. The dotted lines mark discontinuities. Settling times greater than 5.0 are not shown in (a) and settling times greater than 3.0 are not shown in (b). Settling times are calculated for the region of parameter space bounded by $|g_{43}|_{\text{max}} = |g_{45}|_{\text{max}} = |g_{13}|_{\text{max}} = 2$ and where $g_{45} < h_0 h_f / g_{44}$. The same qualitative results are obtained if the region of parameter space considered is instead bounded by $|g_{43}|_{\text{max}} = |g_{45}|_{\text{max}} = |g_{13}|_{\text{max}} = 4$ (not shown).
coupled systems with $L_{03} = 1/6$ and $1/3$, respectively. These results suggest that, for a repressor-controlled system, direct coupling ($L_{03} < 0$) allows faster responsiveness than uncoupling ($L_{03} = 0$), which in turn allows faster responsiveness than inverse coupling ($L_{03} > 0$). This ordering of the three forms of coupling with respect to responsiveness is supported by exhaustive calculations of settling times for equivalent systems within each of various families of equivalent systems. Representative results are shown in Figure 5(a). A number of genes in E. coli, and other bacteria, are regulated in response to changes in the availability of small-molecule metabolites. Genes that are turned off in the presence of a metabolite are said to have a repressible pattern of expression, whereas genes that are turned on are said to have an inducible pattern of expression. Here, we considered regulator gene expression in systems where effector gene expression is repressible.

Let us now consider activator-controlled systems. The values of $L_{03}$ that produce time-courses (i) shown in Figure 4(a) and (i + 5) in Figure 4(b) are the same for $i = 1,...,5$. Inspection of time-courses (7)–(9), which correspond to systems with direct coupling (7), uncoupling (8), and inverse coupling (9), suggest that the trend shown in Figure 4(a) is perhaps reversed. In other words, for these cases, settling time increases, rather than decreases, as the gain $L_{03}$ decreases: $\tau_6 ( = 0.53) < \tau_5 ( = 1.45) < \tau_7 ( = 1.48)$. However, time-courses (6) and (10) are inconsistent with a simple reversal in trend. The settling time of time-course (6), $\tau_6 = 1.19$, is smaller, not larger, than that of time-course (7), and because of overshoot, the settling time of time-course (10), $\tau_{10} = 2.18$, is larger, not smaller, than that of time-course (9). Greater insight is obtained by exhaustive calculations of settling time for equivalent systems within each of various families of equivalent systems. Typical results are shown in Figure 5(b). As for repressor-controlled systems, activator-controlled systems with large negative $g_{03}$ values (i.e. strong negative autoregulation) have faster settling times. It can be seen that the minimal settling time in this plot corresponds to a system with $g_{03} > 0$ (inverse coupling). It can also be seen that some systems with $g_{03} < 0$ (direct coupling) have a settling time less than that of a system with $g_{03} = 0$ (uncoupling). These and other results (not shown) suggest the following conclusions. For an activator-controlled system, inverse coupling allows faster responsiveness than direct coupling, which in turn allows faster responsiveness than uncoupling.

### Discussion

A number of genes in E. coli, and other bacteria, are regulated in response to changes in the availability of small-molecule metabolites. Genes that are turned off in the presence of a metabolite are said to have a repressible pattern of expression, whereas genes that are turned on are said to have an inducible pattern of expression. Here, we considered regulator gene expression in systems where effector gene expression is repressible. Using the method of controlled mathematical comparison, we have attempted to determine whether the regulator gene should be expected to have a repressible (direct coupling), inducible (inverse coupling), or constitutive (uncoupling) pattern of expression. We have also considered whether the regulator should positively or negatively regulate its own gene, or alternatively have no effect at all.

Our results indicate that the regulator protein should generally be expected to negatively regulate its own gene, i.e. to act as a repressor at the promoter of its own gene. Negative autoregulation is associated with selectivity, stability, robustness, and temporal responsiveness in repressible gene circuits. Consistent with the importance of negative autoregulation suggested here and in earlier studies, many of the regulator proteins in E. coli are negatively autoregulated. Also, consistent with the results reported here and in earlier reports, experimental studies of synthetic gene circuits have indicated that negative autoregulation is associated with stability, and temporal responsiveness.

The expected type of coupling depends on whether the effector gene is under activator or repressor control. The functional consequences of changing the type of coupling are seen in analyzing the temporal responsiveness of alternative circuit designs. For repressor-controlled systems, we obtained results that are qualitatively the same as those obtained earlier for inducible systems with repressor control: direct coupling allows faster responsiveness than uncoupling, which in turn allows faster responsiveness than inverse coupling. In contrast, for activator-controlled
Design of Repressible Gene Circuits

Figure 6. Comparisons of systems on the basis of temporal responsiveness are insensitive to uncertainty in estimates of turnover numbers. Results are shown for activator-controlled systems. Parameter values are the same as for Figure 5(b), except each point corresponds to a set of equivalent systems with randomly selected values for \( F_1 (= F_0) \), \( F_2 (= F_0) \), and \( F_3 \). In (a), sets of equivalent systems with inverse coupling or direct coupling are considered; in (b), sets of equivalent systems with inverse coupling or uncoupling are considered; and in (c), sets of equivalent systems with direct coupling or uncoupling are considered. On the x-axis of each panel, each point indicates a minimal settling time, the fastest settling time found for a system of the type indicated by the x-axis label among equivalent systems that share the point-specific turnover numbers. On the y-axis of each panel, each point indicates the ratio of minimal settling times found for the two types of systems being compared. Thus, in (a), \( x = t_{min}^{(0)} / t_{min}^{(D)} \), in (b), \( x = t_{min}^{(0)} / t_{min}^{(U)} \), and in (c), \( x = t_{min}^{(0)} / t_{min}^{(U)} \), where \( t_{min}^{(0)} \) is the minimal settling time among systems, we obtained results that are similar to but qualitatively different from those obtained earlier for inducible systems with activator control. Inverse coupling allows faster responsiveness than direct coupling, which in turn allows faster responsiveness than uncoupling. For both repressible and inducible systems, inverse coupling is optimal, but direct coupling is superior to uncoupling in a repressible system. These results are robust to changes in turnover numbers (Figure 6).

Because qualitative results are the same for repressor-controlled systems regardless of whether effector genes are inducible or repressible but not for activator-controlled systems, we carefully examined the results for repressible systems under activator control. Although direct coupling allows a faster settling time than uncoupling, we find that the ability to establish a steady-state level of enzyme faster comes at some cost: (1) there is an initial delay in enzyme response at early times, which is less for an uncoupled system; and (2) there is transient expression of the activator protein above its steady-state level. Activator overexpression can continue for several doubling times, which could be a disadvantage in rapidly changing environments. Thus, the relative effectiveness of directly coupled and uncoupled activator-controlled systems with respect to dynamics of response is more ambiguous than indicated by comparisons of setting times alone.

As predicted for inducible systems and in an earlier more limited study of repressible systems, not all types of coupling are generally physically realizable because of limits on the magnitudes of kinetic orders (equations (4) and (10)). With repressor control, systems that have high gain \((|L_{23}| > |L_{23}|)\) are limited to inverse coupling, and systems that have intermediate gain \((|L_{23}| = |L_{23}|)\) are limited to inverse coupling or uncoupling. With activator control, systems that have high gain are limited to direct coupling, and systems that have intermediate gain are limited to direct coupling or uncoupling. Only systems that have low gain \((|L_{23}| < |L_{23}|)\) are unconstrained with respect to the form of coupling.

The results of our comparisons allow us to formulate predictions for gene circuits with repressible effector genes. In repressible systems, we generally expect the regulator protein to negatively regulate its own expression, ensuring stability, robustness, selectivity and temporal responsiveness. In addition, the optimal form of coupling is a set of equivalent inversely coupled systems, \( t_{min}^{(0)} \) is the minimal settling time among a set of equivalent directly coupled systems, and \( t^{(0)} \) is the settling time of all equivalent uncoupled systems. A ratio less than 1 indicates that \( t_{min}^{(0)} < t_{min}^{(D)} \) in (a), \( t_{min}^{(0)} < t^{(U)} \) in (b), and \( t_{min}^{(0)} < t^{(U)} \) in (c). The sampling of ratios is interpreted statistically by calculating a moving median of ratios, indicated by the curve in each panel. The moving median is based on a window size of 50 points.
the most responsive one among the forms of coupling that are physically realizable, which is
determined by the magnitude of the gain $L_{23}$. For repressor-controlled systems, we expect direct
coupling if the gain is low, uncoupling if the gain is intermediate, and inverse coupling if the gain is
high. For activator-controlled systems, we expect inverse coupling if the gain is low and direct
coupling if the gain is intermediate or high. These predictions are similar to those for inducible
systems. The only difference is that for repressible systems we never expect uncoupling with activator
control, whereas we do for inducible systems with intermediate gain.

How well do these predictions compare with the historical results of evolution? There are over 400
promoter regions for which regulatory interactions have been documented in RegulonDB. To test
our predictions, we must first identify those systems, more or less consistent with Figure 1, for
which the mode of regulator action has been determined at the promoters of effector and regulator
genes (i.e. systems for which the signs of $z_{15}$ and $z_{45}$ are known) and for which the signal molecule
has been identified. Ideally, we would also have available for each system a direct measurement of the
gain $L_{23}$. This gain is usually unavailable, but as discussed elsewhere in the context of inducible
systems, we can expect the gain in effector gene expression to correlate with a more readily avail-
able measurement, the ratio of maximal to minimal level of effector gene expression, which has been
called the capacity of effector gene expression. Although RegulonDB and a derivative contain
information about whether a regulator is an activator or repressor of a promoter, they do not contain
detailed information about the influence of signal molecules. Thus, to test predictions, we must rely
mostly on a careful reading of the primary literature. Our reading of the literature has uncovered
about ten repressible systems in bacteria that can serve as test cases for our predictions.

Let us first consider the examples of repressible activator-controlled genes. Regulator–effector
gene pairs in this class include $asnC$-$asnA$ in E. coli, $cysB$-$cysP$ in E. coli and Salmonella, $fadR$-$fabA$
in E. coli, and $frr$-$ppsA$ in E. coli and Salmonella. Two of the four regulator genes in these systems are autoregulated: $asnC$ and $cysB$, both of which are negatively auto-
regulated. In each case, expression of the regulator gene is apparently unresponsive to the signal for
repression/derepression of the effector gene. A constitutively expressed activator gene is inconsis-
tent with our predictions, but an inversely coupled system may appear to be uncoupled unless experiments are designed to detect subtle changes in regulator gene expression. As can be
seen in Figure 5(b), responsive activator-controlled systems with inverse coupling tend to have a
value for $z_{45}$ that is small in magnitude and a value for $z_{45}$ that is large in magnitude. Thus, from equation (3), we expect only modest changes
in regulator gene expression. For inducible systems with inverse coupling, we have estimated that regulator gene expression may vary over less than a
twofold range. This estimate applies for repressible systems as well. Of course, the criteria for func-
tional effectiveness considered here may not be the most relevant for the particular systems under consideration, or the predictions may not apply, because the systems are inadequately represented by the generic model shown in Figure 1 and/or the parameter estimates in Table 1. The latter explanation might be relevant in the case of $asnC$ and $asnA$. Recent data suggest that expression of these genes is perhaps inversely coupled through a mechanism more complicated than that con-
sidered in Figure 1. In this system, $asnA$ encodes an asparagine synthetase, the expression of which is controlled through AsnC in response to the level of asparagine. Expression of $asnC$, which is negatively autoregulated, appears to be unaffected by asparagine but is repressed by the nitrogen assimilation control (Nac) protein when ammonia is limiting. Because ammonia is the nitrogen donor in AsnA-catalyzed asparagine biosynthesis, upregulation of $asnA$ could poten-
tially lead to Nac-mediated downregulation of $asnC$. In any case, because few examples of activator-controlled repressible genes are known, further tests of our predictions, as more data become available, seem necessary to reach conclu-
sions about the relevance of the predictions.

Let us now consider the examples of repressible repressor-controlled genes. Regulator–effector
gene pairs in this class include $traP$-$trpLEDCBA$ in E. coli, $alaS$ (AlaS has both regulator and effector functions) in E. coli, $met$-$metBL$ in E. coli and Salmonella, $fur$-$iucABCD$ in E. coli, $purR$-$purB$ in E. coli, $argR$-$argF$ in E. coli, the pyr operon (the regulator gene $pyrR$ is the first gene in this operon) in Bacillus subtilis and Enterococcus faecalis, $tyrR$-$aroF$ in E. coli, and $nadR$-$nadB$ in Salmonella. Eight of these systems exhibit negative autoregulation: only $nadB$ is expressed constitutively. With the exception of the last two cases, which are characterized by an uncoupled pattern of expression, the experimental evidence indicates direct coupling. In each case, the capacity for effector gene expression is 50-fold or less, which suggests a low gain of $L_{23}$ for each system. Thus, seven of the nine examples seem consistent with our prediction of direct coupling in the case of low gain.

The survey of regulator gene expression in repressible systems summarized above and a similar survey for inducible systems are consistent empirically. In systems, inducible or repressible, controlled by a repressor of effector gene expression, expression of the regulator protein tends to follow that of effector gene products (i.e. there is a pattern of direct coupling), whereas in systems controlled by an activator of effector gene expression, expression of the regulator protein tends to remain more constant during changes in
effector gene expression (i.e. there is a real or apparent pattern of uncoupling). These surveys, which reveal that activator and repressor-controlled systems exhibit distinct preferential forms of coupling, and that negative autoregulation is common, suggest that there are indeed rules that govern the pattern of regulator and effector gene expression. They also suggest that the system properties considered here and in earlier studies, particularly temporal responsiveness, can explain the rules, at least in part, particularly for repressor-controlled systems.

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