6424–6434 *Nucleic Acids Research, 2012, Vol. 40, No. 14* *Published online 11 April 2012*
doi:10.1093/nar/gks297

Conditional cooperativity in toxin–antitoxin regulation prevents random toxin activation and promotes fast translational recovery

Ilaria Cataudella[1,*], Ala Trusina[1], Kim Sneppen[1], Kenn Gerdes[2] and Namiko Mitarai[1,*]

[1]Center for Models of Life, Niels Bohr Institute, University of Copenhagen, Blegdamsvej 17, DK-2100 Copenhagen, Denmark and [2]Centre for Bacterial Cell Biology, Institute for Cell and Molecular Biosciences, Newcastle University, Newcastle NE2 4AX, UK

Received November 7, 2011; Revised March 19, 2012; Accepted March 20, 2012

ABSTRACT

Many toxin–antitoxin (TA) loci are known to strongly repress their own transcription. This auto-inhibition is often called 'conditional cooperativity' as it relies on cooperative binding of TA complexes to operator DNA that occurs only when toxins are in a proper stoichiometric relationship with antitoxins. There has recently been an explosion of interest in TA systems due to their role in bacterial persistence, however the role of conditional cooperativity is still unclear. We reveal the biological function of conditional cooperativity by constructing a mathematical model of the well studied TA system, *relBE* of *Escherichia coli*. We show that the model with the *in vivo* and *in vitro* established parameters reproduces experimentally observed response to nutritional stress. We further demonstrate that conditional cooperativity stabilizes the level of antitoxin in rapidly growing cells such that random induction of *relBE* is minimized. At the same time it enables quick removal of free toxin when the starvation is terminated.

INTRODUCTION

Toxin–antitoxin (TA) loci are present in many bacteria and archaea (1). Toxin normally inhibits cell growth, whereas antitoxin neutralizes the activity of toxin by forming a tight TA complex.

One of the known functions of TA loci is to respond to nutritional stress, namely, toxins are activated upon nutritional starvation and slow down the rate of translation (2). Another significant feature of TA loci is that they contribute to persister cell formation in growing bacterial cultures (3–5). Persisters are cells that have entered a slow-growing or dormant state in which the cells are tolerant to environmental insults such as antibiotics; thus persisters are multidrug tolerant and therefore pose a medical problem. Especially, the recent experiments by Maisonneuve *et al.* (5) demonstrated that successive deletion of 10 mRNase-encoding TA loci of *Escherichia coli* progressively reduced the level of persisters. TA loci have multiple complex levels of regulation involving both positive and negative feedbacks and sequestration through binding. The importance and role of these regulations is still an open question.

The *relBE* locus of *E. coli* is one of the best studied TA model systems. The *relBE* locus encodes for antitoxin RelB and toxin RelE. RelE is an mRNase that cleaves mRNA positioned in the ribosomal A site (6), including its own mRNA, while RelB inactivates RelE by forming a tight complex with it (7). RelB is a metabolically unstable protein whereas RelE is stable (2). However, RelB is translated at a higher rate than RelE, and in exponentially growing cells the abundant RelB molecules {[RelB] $\approx$ 10[RelE] (8)} will quench RelE activity completely.

It has been shown that RelB and the RelB-RelE complex autoregulate *relBE* transcription in a complex way (9) (Figure 1): if only RelB is present then a RelB dimer ($RelB_2$) will repress *relBE* transcription. When RelE is present at a concentration such that $[RelB_2] > [RelE]$ then a $RelB_2RelE$ complex binds strongly and cooperatively to the *relBE* promoter and represses transcription (9). In contrast if RelE increases such that $[RelE] > [RelB_2]$, then the excess RelE molecules will destabilize the $RelB_2RelE$-operator complex and thereby induce strong transcription from the *relBE* promoter (8). This sensitivity to the proper ratio between RelBE proteins is called *conditional cooperativity* (9, 10).

*To whom correspondence should be addressed. Tel: +45 353 25273. Email: cataudel@nbi.dk
Correspondence may also be addressed to Namiko Mitarai. Tel: +45 353 25402. Email: mitarai@nbi.dk

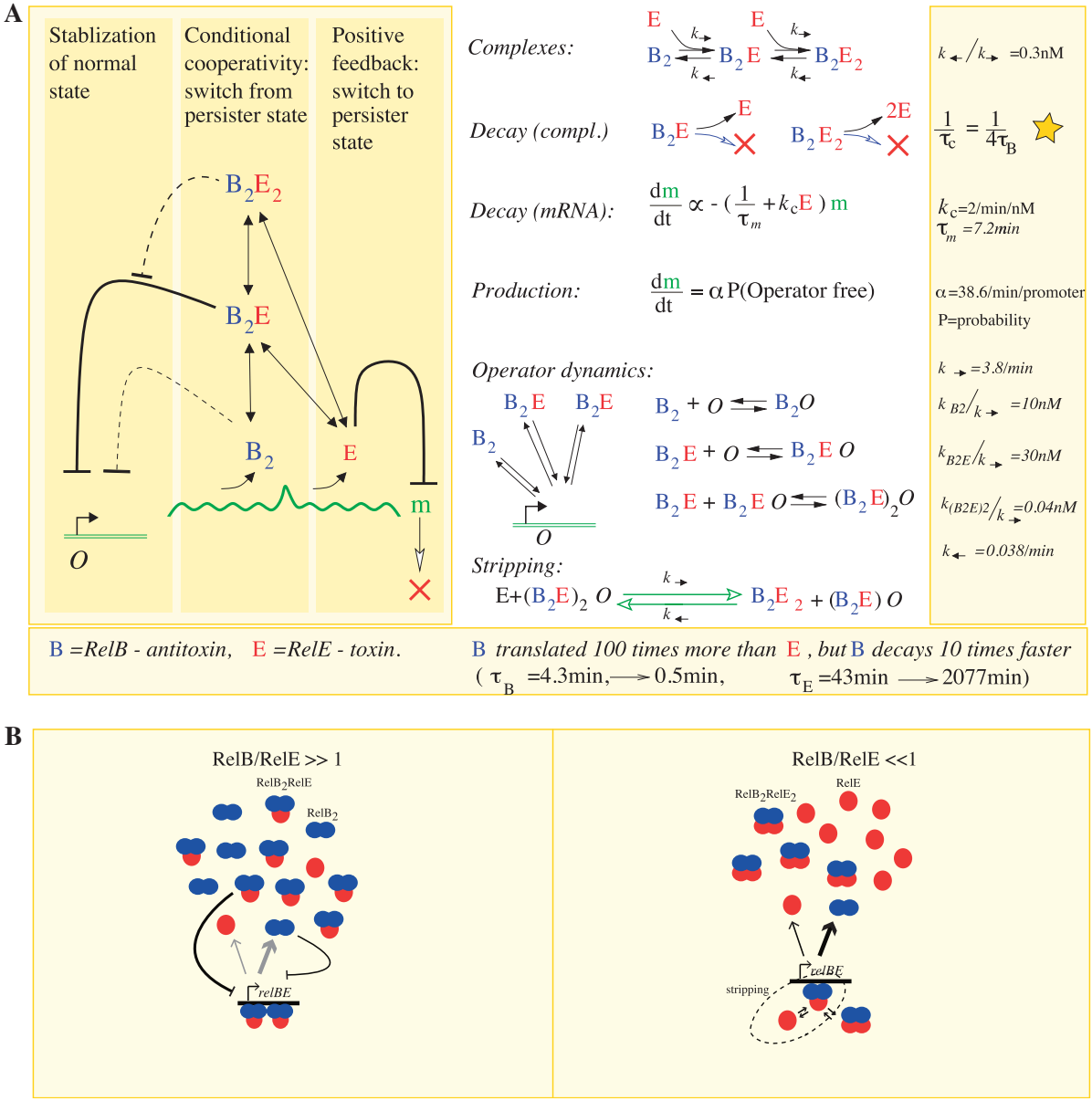

Figure 1. (**A**) Model description: antitoxin RelB (B) and toxin RelE (E) are encoded on the same mRNA (*m*), with only 1/100 of the ribosome that translate *relB* continues to translate *relE*. RelE cleaves mRNAs when it is free. RelB forms dimer $RelB_2$ when it is free, and $RelB_2$ and RelE can form two kinds of complexes: $RelB_2RelE$ and $RelB_2RelE_2$. For simplicity, we use $RelB_2$ as a unit for RelB and do not consider RelB monomers explicitly. Translation rates for $RelB_2$ is $trans_B$ = 15/min, and for RelE is $trans_E$ = 0.3/min. In our simulation there will be 44 nM RelE and 200 nM of $RelB_2$ in total in the non-starved condition. A list of parameters and references used are given in the 'Materials and Methods' section. (**B**) Visualization of conditional cooperativity due to the formation of $RelB_2RelE_2$, that does not repress the promoter.

Conditional cooperativity has been found in all plasmids and chromosome-encoded TA loci investigated so far, including *relBE* of *E. coli* (8), *vapBC* of *Salmonella enterica* (11), *phd*/*doc* of plasmid P1 (12) and *ccdA*/*ccdB* of plasmid F (13). These systems belong to evolutionary independent families (14) and function by different molecular mechanisms. Thus conditional cooperativity must have an essential role in the biology of the TA genes. This role is difficult to analyse because of the complex interweaving of the components that control TA operon transcription.

The *relBE* locus is also one of the first systems whose response to nutritional stress has been studied in detail (2). It has been found that exponentially growing wild type *E. coli* cells that are starved for amino acids reduce their global translation rate to a new steady state level of ~5% of that of the non-starved level. This dramatic change occurs within 20 min after starvation. Interestingly, deletion of *relBE* instead results in a post-starvation level of translation of ~10%. This 2-fold increase in translation is consistent with RelE being a global inhibitor of translation.

In an attempt to understand the biological role of conditional cooperativity and to analyse the TA operon transcription in general we present a mathematical model of *relBE* operon activity that takes into account the known features. We subsequently investigate the role of conditional cooperativity, and show that it provides a mechanism to stabilize the level of antitoxin in rapidly growing cells such that random induction of *relBE* is minimized. Another important prediction is that conditional cooperativity enables quick recovery from the RelE-mediated reduction of translation when the starvation is terminated.

MATERIALS AND METHODS

In our mathematical model of the *relBE* system, the transcription of the *relBE* operon (production of mRNA) and translation of *relBE* mRNA (proteins production) are taken into account as two separate processes. RelB form tight dimers, hence we can assume that RelB is always present as a dimer. In addition, $RelB_2$ and RelE can form complexes in the two stoichiometric forms $RelB_2RelE$ and $RelB_2RelE_2$ (32).

A key goal of our model is to investigate the complex autoregulation feedback of *relBE* promoter activity. $RelB_2$ represses transcription. With moderate amount of RelE added, this repression become stronger because the RelB2RelE has larger affinity to the operator. When RelE is further increased, however, the promoter is de-repressed because $RelB_2RelE_2$ does not bind to the operator.

RelE works as an mRNase only in free form, by degrading the *relBE* mRNA as well as all other mRNAs in the cell. In our modeling, only the cleavage action of RelE on *relBE* mRNA is directly taken into account.

Free $RelB_2$ has a very short half-life since it is actively degraded by Lon protease. RelB half-life was measured to be ~3 min (9), whereas RelE is stable and its half-life is equal to the average *E. coli* doubling time (~30 min in normal growing condition). We do not model the cell division explicitly, it only enters implicitly into the RelE half-life.

Based on the fact that roughly 10 times more RelB monomers than RelE monomers are present during steady state cell growth in spite of the 10-fold difference in the half-life, we assume that the translation rate of RelB (monomers) is 100-fold higher than that of RelE.

$RelB_2$ in complex with RelE is known to be relatively stable (8). However, for RelE to become active, RelB in complex with RelE must be degraded at some rate. We assume $RelB_2$ in $RelB_2RelE$ and $RelB_2$ $RelB_2$ complexes has a half-life roughly 3-4 to fold longer than the free $RelB_2$ (~12 min).

The half-life of *relBE* mRNA is not known, and in our model it is set to 5 min. This is on the long side of typical *E. coli* mRNA half-life (29), which helps to keep the maximal promoter activity and the translation rate within biologically plausible values while having enough proteins.

The behavior of the system is investigated throughout three different phases.

A first phase, from time 0 to time 200 min in the plots, is what we call the 'non-starved state', where all the parameters used in the simulation refer to the exponential growth phase of the cell.

At time 200 min we switch to the nutritional stress phase (amino acid starvation). Within the framework of our model this means a sudden decrease in translation rate for both RelB and RelE by 10-fold, based on the observation that a $relBE^-$ deletion strain shows a reduction of translation to a post-starvation level of 10%. Because the dilution by cell division does not happen in this phase, the half-life of τ_E is changed to 24 h, which is much longer than the examined amino acid starvation duration (5 h). We investigate whether enough free RelE can be released upon nutritional stress, since this circumstance could explain the 2-fold difference of the translation rate in starvation between wild type and $relBE^-$ strain (2). In addition, starvation is known to significantly increase Lon activity (17), thus during starvation the half-life of the $RelB_2$ is reduced by a factor of 8, both in the free form and in complex.

At time 500 min, we then switch-back to the non-starved set of parameters corresponding to refeeding of the cells with amino acids and monitor the recovery of the system during the switch-back to exponential growth.

Note that the change of the parameters at the switching of the states has been done instantaneously. We discuss the effect of a time delay in the parameter change later.

We used the Gillespie algorithm (15) and performed stochastic simulations (simulation procedures given below). *relBE* mRNA, $RelB_2$, RelE, $RelB_2RelE$ and $RelB_2RelE_2$ are the molecular players in the simulations. The concentrations are converted to number of molecules so that 1 nM corresponds to 1 molecule in a cell, which is a typical estimate based on the size of *E. coli*. Each chemical reaction event happens at a random time and it is chosen according to the reaction rates in Table 1. The possible reaction events are listed in Table 2.

In the results in the main text, we consider the presence of four chromosomes in each cell, that means four *relBE* promoters, which is an average number of chromosomes for exponentially growing *E. coli* cells. We also tried the one chromosome case, which exhibit increased noise, but the average trajectories remains similar. Each chromosome has two operators: each of them can be bound either by a $RelB_2RelE$ complex or RelB. Since cooperativity in the interaction between $RelB_2$ and the *relBE* operator is not proven (9) we assume that only one $RelB_2$ can bind to the promoter at a given time, while either one or two $RelB_2RelE$ can be bound to the operator (9). When the promoter is free, it shows maximal promoter activity, and $\alpha_0/4$ *relBE* mRNA {per promoter} per minute are produced. When either one $RelB_2$ is bound to the promoter or two $RelB_2RelE$ bind cooperatively, the promoter is repressed and no *relBE* mRNAs are produced. In the present simulation, we did not consider the repression by one $RelB_2RelE$ because experimentally the Hill coefficient close to 2 in repression is observed in the wide range of $RelB_2RelE$ concentration (9).

Table 1. Set of parameters

Symbol and meaning Description Value	Units	Reference
α_0 total promoter activity 154.665	nM min^{-1}	See text
$[B_2]_{ss}$ steady state total concentration of RelB dimers 200	nM	See text c.f. (8)
$[E]_{ss}$ steady state total concentration of RelE 44	nM	See text c.f. (8)
τ_m *mRNA* half-life 7.2	min	See text c.f. (29)
τ_B RelB half-life 4.3	min	See text c.f. (8)
τ_E RelE half-life 43	min	See text
τ_c $RelB_2$ half-life in complexes 17	min	See text c.f. (8)
n_H Hill's coefficient 2.3		(8)
$trans_B$ RelB translation rate 15	min^{-1}	See text
$trans_E$ RelE translation rate $trans_B/50$ 0.3	min^{-1}	See text
k_{bind} binding on-rate $4\pi\ Da/V_{cell}$ 3.8	min^{-1}	(28)
K_dB2E dissociation constant for B2E complexes formation $\frac{[B2][E]}{[B2E]} = K_dB2E$ 0.3	nM	(9)
K_dB2E2 dissociation constant for B2E2 complexes formation $\frac{[B2E][E]}{[B2E2]} = K_dB2E2$ 0.3	nM	
k_uB2E dissociation rate for B2E $k_uB2E = k_{bind} \times K_dB2E$ 1.14	nM	
k_uB2E2 dissociation rate for B2E2 $k_uB2E2 = k_{bind} \times K_dB2E2$ 1.14	nM	
K_{D1} dissociation constant for B binding to DNA 10	nM	See text c.f. (16)
K_{D2} dissociation constant for second B2E bound to DNA 0.04	nM	See text c.f. (16)
K_{D3} dissociation constant for first B2E binding to DNA 30	nM	See text c.f. (16)
k_c cleavage rate 2.0	$nM^{-1}\ min^{-1}$	See text c.f. (6)

Table 2. Events and rates in the simulation

Event	Rate
mRNA transcription	$\frac{\alpha_0}{4} *$ no. of operators with two free binding sites
RelB dimers translation	$[mRNA]$ * $trans_B$
RelE translation	[mRNA] * $trans_E$
relBE mRNA degradation	$\frac{[mRNA]}{\tau_m}$
RelB dimer degradation by Lon	$\frac{[RelB_2]}{\tau_B}$
RelE degradation due to cell dilution	$\frac{[RelE]}{\tau_E}$
$RelB_2RelE$ formation	k_{bind} * $[RelB_2]$ * $[RelE]$
$RelB_2RelE_2$ formation	k_{bind} * $[RelB_2RelE]$ * $[RelE]$
$RelB_2RelE$ dissociation	k_uB2E * $[RelB_2\ RelE]$
$RelB_2RelE_2$ dissociation	k_uB2E2 * $[RelB_2RelE_2]$
degradation of $RelB_2RelE$ due to cell dilution	$\frac{[RelB_2RelE]}{\tau_E}$
degradation of $RelB_2RelE_2$ due to cell dilution	$\frac{[RelB_2RelE_2]}{\tau_E}$
degradation of $RelB_2$ in $RelB_2RelE$ complex	$\frac{[RelB_2RelE]}{\tau_c}$
degradation of $RelB_2$ in $RelB_2RelE_2$ complex	$\frac{[RelB_2RelE_2]}{\tau_C}$
binding of $RelB_2$ to operator	k_{bind} * $[RelB_2]$ * no. of operators with two free binding sites
binding of $RelB_2RelE$ to operator	k_{bind} * $[RelB_2]$ * no. of operators with at least one free binding site
unbinding of $RelB_2$ from operator	(K_{D1} * k_{bind}) * no. of operators with 1 $RelB_2$ bound
unbinding of one out of two $RelB_2RelE$ from operator	(K_{D2} * k_{bind}) * $[RelB_2]$ * no. of operators with two $RelB_2RelE$ bound
unbinding of single $RelB_2RelE$ from operator	(K_{D3} * k_{bind}) * $[RelB_2]$ * no. of operators with a single $RelB_2RelE$ bound
cleavage of *relBE mRNA*	k_c * $[RelE]$
stripping of $RelB_2RelE$ complex bound to operator from it by RelE	k_{bind} * $[RelE]$ * no. of operators with at least on $RelB_2$RelE

Algorithm of the Gillespie simulation

We use the Gillespie algorithm in our simulation, where the chemical reactions are treated as discrete stochastic events that happen at given rates with time interval between events drawn from the exponential distribution (15).

The state of the system at each time step is defined by the concentrations of the five molecular players, i.e. *RelBE* mRNA (m), free RelE (E_f), free $RelB_2$ (B_f), $RelB_2RelE$ (B_2E) and $RelB_2RelE_2$ (B_2E_2). The total copy number of $RelB_2$ (B_T) and RelE (E_T) are given by

$$B_T = B_f + B_2E + B_2E_2,$$
$$E_T = E_f + B_2E + 2B_2E_2.$$

The chemical reaction with a rate specified by the parameters showed in Table 1 results in a change in the number of molecules as follows.

$$m \xrightarrow{\alpha_0/4 \cdot \text{no. of unrepressed promoters}} m+1$$
$$m \xrightarrow{k_c \cdot m \cdot E_f} m-1$$
$$B_f \xrightarrow{\text{trans}_B \cdot m + B_2E \cdot K_{DB2E} \cdot k_b} B_f+1$$
$$B_f \xrightarrow{B_f/\tau_B} B_f+1$$
$$E_f \xrightarrow{B2E2/\tau_c} B_f+2$$
$$E_f \xrightarrow{\text{trans}_E \cdot m + B_2E \cdot K_{DB2E} \cdot k_b + B_2E_2 \cdot K_{DB2E2} \cdot k_b + B_2E/\tau_c} E_f+1$$
$$E_f \xrightarrow{E_f/\tau_E} E_f-1$$
$$B_2E \xrightarrow{B_f \cdot E_f \cdot k_b + B_2E_2 \cdot K_{DB2E2} \cdot k_b} B_2E+1$$
$$B_2E \xrightarrow{B2E \cdot K_{DB2E} \cdot K_b + B_2E/\tau_c} B_2E-1$$
$$B_2E_2 \xrightarrow{B_2E \cdot E_f \cdot k_b} B_2E_2+1$$
$$B_2E_2 \xrightarrow{B_2E_2 \cdot K_{DB2E2} \cdot k_b + B_2E_2/\tau_c} B_2E_2-1$$

In addition, $RelB_2$ and $RelB_2RelE$ can bind to the four operator sites. The binding happens to each operators independently, and the binding rates are given as follows (O expresses the operator):

$$O + B_f \xrightarrow{k_b \cdot B_f \cdot O} O \cdot B_f$$
$$O \cdot B_f \xrightarrow{K_{D1} \cdot k_b \cdot (O \cdot B_f)} O + B_f$$
$$O + B_2E \xrightarrow{k_b \cdot O \cdot B_2E} O \cdot B_2E$$
$$O \cdot B_2E \xrightarrow{K_{D3} \cdot k_b \cdot (O \cdot B_2E)} O + B_2E$$
$$O \cdot B_2E + B_2E \xrightarrow{k_b \cdot B_2E \cdot (O \cdot B_2E)} O \cdot (B_2E)_2 \quad (1)$$
$$O \cdot (B_2E)_2 \xrightarrow{K_{D2} \cdot k_b \cdot (O \cdot (B_2E)_2)} O \cdot B_2E + B_2E$$
$$O \cdot B_2E + E_f \xrightarrow{k_b \cdot E_f \cdot (O \cdot B_2E)} O + B_2E_2$$
$$O + B_2E_2 \xrightarrow{k_b \cdot (K_{DB2E2}/K_{D3}) \cdot O \cdot B_2E_2} O \cdot B_2E + E_f$$
$$O \cdot (B_2E)_2 + E_f \xrightarrow{k_b \cdot E_f \cdot (O \cdot (B_2E)_2)} O \cdot B_2E + B_2E_2$$
$$O \cdot B_2E + B_2E_2 \xrightarrow{k_b \cdot (K_{DB2E2}/K_{D2}) \cdot O \cdot B_2E_2} O \cdot (B_2E)_2 + E_f$$

The last four reactions are what we call 'stripping' (18,19), where RelE forming complex with $RelB_2RelE$ bound on the operator and removing it, and the reverse reaction of the stripping.

Each run is from time 0 to time 600. At time $t = 200$ min the values of τ_B, τ_c, τ_E, trans_B and trans_E are changed from values that mimic fast growth conditions to values typical of amino acid starvation. At time $t = 500$ the same parameters are changed back to the fast growth value.

All the presented results refer to the averages of the concentrations over a sample of 1000 simulations, unless otherwise noted.

RESULTS

Outline of the model

The overall regulations and feedbacks in our model are summarized in Figure 1A. The mechanism of the conditional cooperatively is illustrated in Figure 1B. RelB and RelE can form two types of complexes, namely $RelB_2$·RelE (B_2E) and $RelB_2$·$RelE_2$ (B_2E_2). B_2 and B_2E repress the promoter activity of *relBE* operon, while B_2E_2 does not. This is a scenario proposed to reproduce the observed conditional cooperativity (9). Both $RelB_2RelE$ complex (9, 32) and $RelB_2RelE_2$ complex (as heterotetramer); (32) have been observed *in vitro*.

All the molecules are exposed to either degradation by proteases or dilution by cell division, where the details are described in the next subsection. The mRNA m can be also actively degraded by the free toxin E.

Model parameters

The parameters of the model are constrained by (i) Stoichiometric data showing that when [total amount of RelE]:[total amount of RelB monomer] is in 1:2 ratio, $RelB_2RelE$ and operator O complex, $(RelB_2RelE)_2 \cdot O$, is formed, while increasing the ratio of RelE further destabilizes the complex (9). This can be reproduced when the binding of RelE to $RelB_2$ and binding of RelE to $RelB_2RelE$ occur with similar dissociation constants, hence when the total amount of RelE exceeds the total amount of $RelB_2$, $RelB_2RelE$ are converted to $RelB_2RelE_2$ (See Supplementary Material A for detail).

(ii) That under normal growth in rich medium the *in vivo* concentration of RelE is about one-tenth of that of RelB (8). The actual concentration level was estimated to be 550 – 1100 nM for RelB while 50–100 nM for RelE (8). Here we choose parameters so that total RelB is ~500 nM and the total RelE is ~50 nM in non-starved exponential growth condition.

The *in vivo* lifetime of RelB is ~4.3 min, whereas RelE is metabolically stable but diluted by a rate set by cell division, giving it a characteristic lifetime of ~43 min in the exponential phase. This 10-fold shorter lifetime for RelB than RelE, with the 10-fold higher concentration of RelB in the exponential growing condition mentioned above gives us a translation rate of RelE ~1% of that of RelB. [Since the estimate of the RelB and RelE levels *in vivo* was difficult due to the low cellular amounts of the

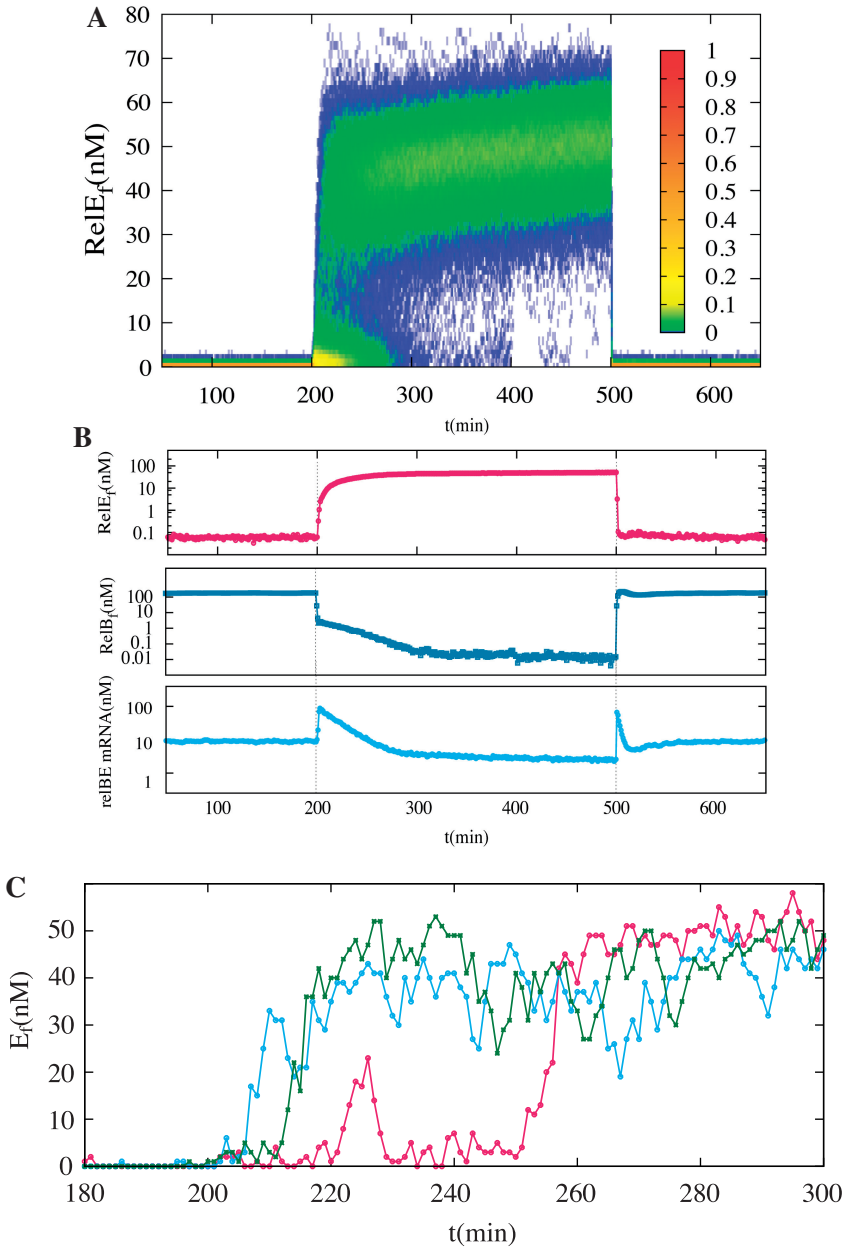

Figure 2. Response to amino acid starvation and later recovery. The system is starved for amino acids from 200 min to 500 min. (**A**) Probability distribution $P([E_f], t)$ of a cell having a certain concentration $[E_f]$ nM of free RelE at a given moment t. (**B**) Time courses averaged over 1000 cells, for free RelE, free RelB and *relBE mRNA*, illustrating how the system switches between a state of high antitoxin to a state of high free toxin. (**C**) The dynamics at entrance to the starvation at the single cell level. Three examples are shown, and the total amount of free RelE is plotted as function of time, from time 180 to time 300.

proteins (8), the 10-fold difference in concentration can be smaller in reality, which would indicate higher translation rate ratio (up to 10%).] (iii) That $RelB_2$ represses RelBE promoter by a factor 16, whereas $(RelB_2RelE)_2$ represses the same promoter by a factor 800 under exponential growth in normal medium. These repression folds are about one-tenth of what was observed using *lacZ* fusion on low copy number (~10) plasmids (16). We adapted these repression folds mainly because they are close to the upper limit of the repression folds which can give biologically plausible promoter strength for the *relBE* promoter and translation rate with still being able to reproduce the observed concentrations of RelB and RelE in exponential growth, where promoters are fully repressed.

In the next subsections, we demonstrate that the model with these evaluated parameter values show expected response to amino acid starvation and recovery when the starvation is over. We also investigate the robustness of the behavior against the parameter changes.

Amino acid starvation drives the switch to toxin activation

The model is examined when switching from rich medium to amino acids starvation, and subsequently exposed to refeeding after 5 h. There are three main processes taking place during starvation: (i) The overall translation rate is reduced, because of the lack of amino acids, here simulated by a 10-fold reduction. (ii) Cells stop dividing, thus dilution of RelE decreases, allowing for accumulation of this long lived protein. (iii) Lon activity is believed to be increased under starvation (17), increasing the degradation rate of RelB. We chose to make the degradation 8-fold stronger during amino acid starvation. The result presented here holds as long as the degradation of RelB in the $RelB_2RelE$ complex is large enough during the starvation, and the 8-fold is close to the minimum fold needed.

Figure 2 shows switching from the antitoxin dominated state to the toxin dominated state elicited by amino acid starvation. Importantly, in order to make this response work we had to assume that RelB in complex with RelE must be actively degraded, at least during starvation. If such degradation was not included, then the transition to the high-RelE state cannot be achieved. Here, we assume that RelB is degraded 4-fold less effectively when in complex with RelE than when it is free (*in vitro* data supports that RelB is partly protected from degradation when it is in complex with RelE (8)). To demonstrate the necessity of this process, we compare the model with and without active degradation of RelB in the complex in the Supplementary Material B.

From Figure 2B, we also see that there is a some time-lag to enter the toxin dominated state, while the time to exit this state is very short. This time lag is due to the combined effect of 'stripping' and conditional cooperativity. By 'stripping' we mean the process where a free toxin molecules 'invades' the $RelB_2RelE$ complex bound to the promoter inducing the complex to be released from operator *DNA*. This has been shown to occur both *in vitro* and *in vivo* (8). Note that it is possible to have conditional cooperativity without stripping, in which case excess RelE will form $RelB_2RelE_2$ complex in the bulk and thus sequester $RelB_2RelE$, but does not remove bound $RelB_2RelE$ actively from the operator site.

The conditional cooperativity with stripping opens for an active and relatively long battle between degradation of RelB and a de-repression of the promoter with an associated rise of *relBE* mRNA (2,8) and hence increase in production of RelB. [It should be noted that in the experiment that *relBE* mRNA level was observed to rise ~30-fold just after the amino acid starvation (2,8), while in the present model we observe only ~2-fold rise (Figure 2). The height of this peak depends strongly on the cleavage rate of mRNA by free RelE, k_c, but just lowering this value delays to reach the high free toxin state at the starvation (details in Supplementary Material C). This disagreement can be in principle improved if we take into account the fact that free RelE will interact with all the mRNAs in the cell, thus it is quite likely that free RelE will be sequestered and will not cleave much of *relBE* mRNA when its concentration is very low, which should give bigger rise for *relBE* mRNA. In the present model, however, we do not take this effect into account because of large ambiguity in detailed interactions between all mRNAs the free RelEs.] Without stripping, this rise becomes much smaller than this, and hence the delay becomes less (details in Supplementary Material D). Central in this 'battle' is cleavage of mRNA by free RelE, since it reduces the *relBE* mRNA for both RelE and RelB, and thereby favor the long-lived toxin RelE. The cleavage rate k_c of *relBE* mRNA by toxin is not known *in vivo*, and we use $k_c = 2$/min/nM to obtain reasonably fast rise of the toxin upon starvation. For comparison, the *in vitro* cleavage activity per codon with empty ribosome A-site was estimated to be between 0.000042 to 2.4 /min/nM depending on the codon (6). Considering there are 79 codons for RelB, one expect 2–3 ribosomes at any time to translate the mRNA, and thus an effective cleavage rate that should not exceed 5/min/nM. Thus our assumed value is in the high end, but using for example a 10 times smaller value of k_c would delay the transition into toxin dominated state by hours, as shown in Supplementary Material E.

Single cell activation of RelE is binary

The behavior of single cells are summarized in Fig. 2A as the probability distribution $P([E_f], t)$ of a cell having a certain concentration $[E_f]$ nM of free toxin at a given moment t. We can see that the response is binary. There is a high peak at low toxin at the start of starvation (time 200), but another peak for high free toxin state (around $[E_f] \approx 45$ nM) appears already at 10 min after starvation. The low probability to take the value in between ($10\,\text{nM} < [E_f] < 30\,\text{nM}$) suggest that each cell switches from low (<10 nM) to high free toxin (>30 nM) quickly. On cell population level (Figure 2B) in contrast it takes almost 40 min for free RelE to reach 30 nM. This contrast reflects the big variation in switching time between different cells.

Figure 2C shows three representative trajectories of single cells entering starvation. At the beginning, free RelE is almost zero because RelE are bound by RelB's, but after some time the balance switches and the concentration of free RelE rises quickly to high level. The examples show variation in switching timing over 60 min but none of them spend much time at the intermediate free toxin level. Overall this shows the significance of stochastic modeling of this type of system as well as the need for single cell measurement of the TA systems.

Amino acid starvation is terminated at $t = 500$ min in Figure 2. Compared with the entry to the starved state, the recovery from the starved state is found to be extremely quick both on average and at the single cell level. As demonstrated below the fast recovery depends on conditional cooperativity.

Conditional cooperativity primes fast exit from the toxin dominated state

To clarify the role of conditional cooperativity, we compare the system with conditional cooperativity (the same system as in Figure 2: referred as 'cc') and the

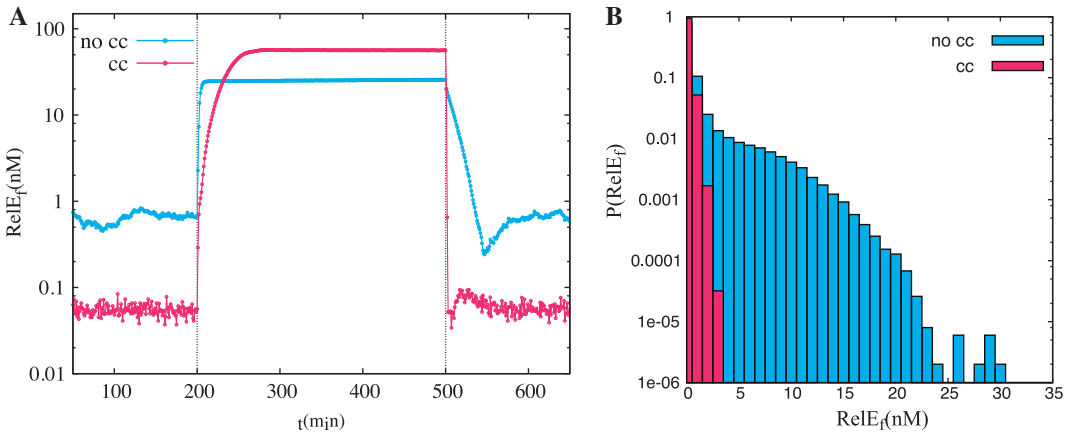

Figure 3. Role of conditional cooperativity. (**A**) The time evolution of free RelE level for the system with (red) and without (blue) conditional cooperativity. The system is starved for amino acid from 200 to 500 min. (**B**) Probability distribution of free RelE in the non-starved state without conditional cooperativity (blue) and with conditional cooperativity (red). Free RelE takes higher value without conditional cooperativity.

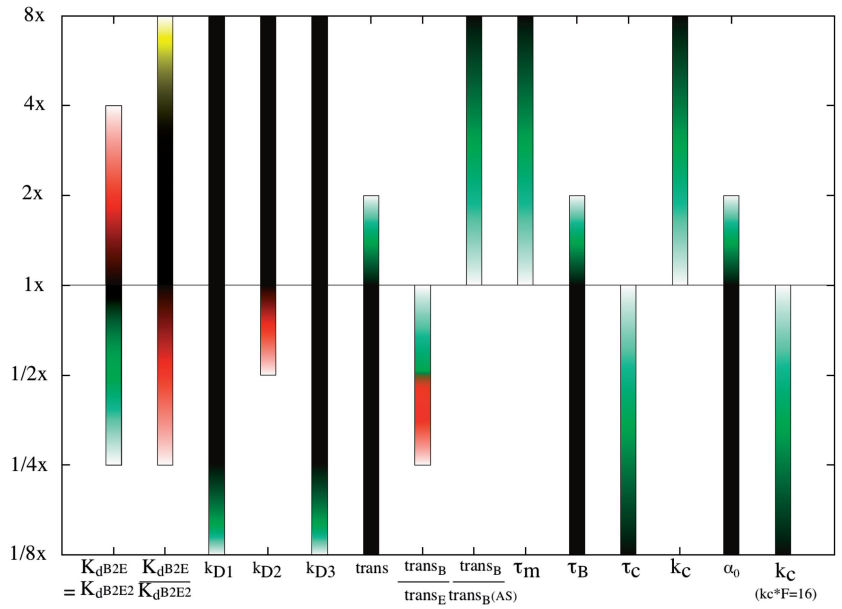

Figure 4. Summary of the model behavior against parameter changes. For each parameters (horizontal axis), fold change of the values from our reference values are tried one by one. The color gradients indicate how the model deviates from the reference behavior: yellow indicates too many free toxins in the healthy states, green indicates too slow rise of free RelE at amino acid starvation and red indicates too slow drop of toxins after the removal of amino acid starvation. In the first entry, $K_dB2E = K_dB2E2$, the ratio of the dissociation constants K_dB_2E and K_dB2E2 are kept to be one, but the value itself is changed. In the second entry, the ratio K_dB_2E/K_dB2E2 is changed, while keeping smaller dissociation constant to be the reference value 0.3 nM. For the entry *trans*, the translation rates for RelB and RelE are changed by the given folds, while $trans_B/trans_E$ and $trans_B/trans_B(AS)$ [$trans_B(AS)$ is the translation rate of RelB during the amino acid starvation] are kept to the reference values. For the entry $trans_B/trans_E$ and $trans_B/trans_B(AS)$ the given ratio is changed with keeping the value of the translation for RelB $trans_B$ to be 15/min. For the entry τ_B (τ_C), the lifetime of the $RelB_2$ (RelB's in the complexes) are changed with keeping the 1/8-fold reduction of the lifetime during the amino acid starvation. For the entry k_c (the 12th entry), the value of the cleavage rate is changed, while for the entry $k_c(k_c \times F = 16)$ (the last entry), the value of k_c and the fold-change of the RelB degradation rate F are changed, so that $k_c \times F$ is kept to the reference value 16.

system without conditional cooperativity (referred as 'no cc'): We removed the conditional cooperativity by preventing $RelB_2RelE_2$ complex formation completely, while keeping the other parts of the system unchanged. Therefore, regardless of the relative concentrations of total RelE and total RelB, the only possible complex they can form is $RelB_2RelE$. Figure 3A illustrates that the conditional cooperativity is required for the fast recovery from the toxin dominant state under starvation to the non-starved state. Mechanistically this reflects that formation of $RelB_2RelE_2$ derepresses the promoter in the high-*RelE* state and thereby primes the system for recovery already during starvation.

Figure 3B shows that conditional cooperativity also reduces the probability to have high free toxin in the non-starved state. In the non-starved state the excess of RelB buffers for an increase in free RelE (by the sequestration into the complex $RelB_2RelE$). If RelB by

fluctuations becomes low, the conditional cooperativity provides a negative feedback that secures additional RelB. This reduces free RelE by complex formation and thus the concentration of free toxin is kept low.

Robustness of the observed behaviors against the parameter changes

Finally, we study the robustness of the observed behaviors against parameter changes. This is not only to see how general our conclusion is regarding the choice of the parameter values, but also to indirectly test how the feedbacks that are not modeled in the present framework would affect the behavior. Especially, growth-rate dependences (24–27) are observed in cell physiology through for example the partitioning of the ribosomes (which affects the translation rate) or the RNAP availability (which affects the transcription rate). Such dependences will affect parameter values upon amino acid starvation. The robustness test gives idea about how significant such a feedback can be in the studied behavior.

In Figure 4, we summarize the robustness of the observed behaviors against parameter changes. We change parameters (or ratio of parameters) one by one by 2^n-fold, with $-3 \leq n \leq 3$, and we check whether the model is working with that parameter value based on the following criteria: (i) The free RelE level in the healthy state is <1 nM. (ii) The free RelE level reaches >10 nM within 20 min after the start of the amino acid starvation. (iii) The free RelE level drops <1 nM within 5 min after starvation is stopped.

The robustness analyses shows that the condition (ii) is hardest to satisfy. Most parameters are split in two subregions, i.e. the model is very sensitive to the change in one direction (increase or decrease) but insensitive to change in another direction. This is because our reference parameter is at borderline, i.e. just fast enough, to satisfy the criterion (ii). Even at this borderline we had to introduce the faster degradation of RelB's in complexes during starvation, to achieve fast increase in free RelE. This again support the necessity of the fast degradation of the antitoxins at amino acid starvation.

The only case where condition (i) tends to be violated is when the dissociation constant K_dB2E for $RelB_2RelE$ formation is very large, hence RelE are not tightly sequestered in the complex. Increasing both K_dB_2E and K_dB2E2 has similar effect, but it affects stronger on the recovery from the free high-toxin state (iii), by freeing up toxin easier at the transient state.

In addition, the third criterion (iii) is violated when the unstable antitoxin RelB is not produced high enough, which happens when the ratio between the translation rate of RelB and RelE ($trans_B/trans_E$) is too small or the repression by $RelB_2RelE$ (characterized by K_{D2}) is too tight. When K_dB_2E/K_dB2E2 is too small (1/4-fold or less), $RelB_2RelE_2$ is not formed as much and the conditional cooperativity becomes ineffective, which also makes the recovery slow.

The robustness of the transcription rate α_0 and the translation rate (trans) is of particular interest in the context of the growth-rate dependent feedback. We see that the model behavior is robust 1/8- to 2-fold change of these parameters. Namely, even if there is a feedback from the growth rate to these parameters, the model behavior will not be altered as long as the change is within this fold. Especially, the transcription rate α_0 is expected to decrease upon slower growth (24), which is the direction where the model behavior is robust.

We also investigated how the time scale of the parameter changes (when the conditions are shifted to or from starvation) affect the kinetics of the transitions between two states. The relevant parameters changed are the RelB lifetime and the translation rates. Overall, we find that the time scale of parameter changes is rate-limiting for the entry into the starved state, i.e. the kinetics of the transitions is fully determined by how rapidly we change these parameters (Supplementary Material E). This is again because our reference parameters are at the borderline to reproduce the fast entry.

The recovery phase is, however, less sensitive to the time scale of the parameter changes. The translation rate has the biggest effect, but as soon as the translation rate increases by some amount, the RelB accumulate enough and free RelEs are repressed (Supplementary Material E). Furthermore, the main result regarding the conditional cooperative is robust: the recovery is always much faster for the model with conditional cooperativity than without conditional cooperativity (Supplementary Material F).

DISCUSSION

We constructed a mathematical model of how *relBE* is regulated, with the main focus centered around the conditional cooperativity in the autoregulation of *relBE* operon. With our model we inteded to capture the available experimental data, test the known and estimate the values of unknown parameters and investigate the systems dynamics when the cells are shifted between non-starved and starved states.

Our current modeling framework highlights several interesting features:

(1) A fast entry to the high-toxin state can only be realized if antitoxin is degraded both when it is free and in complex with toxin during amino acid starvation.
(2) The transition from the antitoxin dominated state to the toxin dominated state upon sudden amino acid starvation is not graded but binary at the level of single cells.
(3) When amino acid starvation is terminated conditional cooperativity mediates fast recovery from the toxin dominated to the growing state.
(4) Conditional cooperativity also reduces the occasional occurrence of high free toxin state in the non-starved condition.

In the following we discuss these four features. Active degradation of antitoxin in the TA complex during the starvation [Feature (1) above] should be closely coupled to Lon protease activation during amino acid starvation (17). More detailed understanding of Lon activity during

the starvation as well as fluctuations of Lon activity in growing cell will provide more insights about toxin activation through this pathway, which can be included in the future development of the model.

The switch-like activation of the toxin at the single cell level [Feature (2) above] comes from the positive feedback; when free toxin starts to increase, the cleavage-rate of TA mRNA increases, that in turn reduces the amount of antitoxin and thereby forces more free toxin to accumulate (see also Figure 1). Positive feedback facilitated switches have been seen in many other systems that require decision making, for example, the lysis/lysogeny switch in temperate phage (20), sugar utilization in bacteria (21) and cell differentiation (22). In the present case, the decision that a cell needs to make is whether it should translate or shut down translation; we believe that it makes biological sense that the cells do not waste time between these two states.

Conditional cooperativity [Feature (3)] facilitates the switch to work in a robust manner, favoring the antitoxin dominated state by making the switching back dynamics fast and by reducing the probability to randomly switch to toxin dominated state without stress [Feature (4)].

Even though the present modeling relies on parameters measured for *relBE* systems, we believe that our model is relevant for the mRNase TA systems in general, where the basic framework of the regulations is believed to be similar to the one in RelBE. Especially, the observed switching—between low- and high-toxin states upon starvation and recovery—is interesting in relation to persisters. A current view on the persister mechanism suggest that by toxin fluctuations some cells happen to end in a toxin dominated state [cf. (4,23,24)], while the present study shows that if the TA system has the conditional cooperativity such fluctuations will be strongly suppressed (Figure 3B). The extent to which the system is subject to fluctuations, i.e., how often a cell can be in high-toxin state by chance without amino acid starvation, is an important quantity to study in the future in relation to the persister formation. It is in general an interesting theoretical and experimental problem to understand the role of TA system in persister cells formation in the light conditional cooperativity.

In order to generalize the present model, especially to the persister system, it should be noted that the several known feedbacks are not taken into account in the present model. As mentioned earlier, a number of parameters are growth-rate dependent (24–27) due to e.g. RNAP availability, stringent response via ppGpp, DksA regulation, etc. In the present level of modeling, these factors would mainly affect the transcription rate. Within the interest of the present work, the conclusions remain qualitatively the same for changing the transcription rate by 1/8- to 2-fold (Figure 4). However, the feedback where high-toxin state imposes slow growth and slow transcription which in turn favors high-toxin state opens for an interesting direction as it can have a strong effect on the stability of the persister state (24).

Finally, we propose several possible experiments based on the present results.

The direct test of the predictions of the model about the conditional cooperatively would be to construct a mutant that does not form $RelB_2RelE_2$ and yet keep other properties of RelB and RelE, and then compare the dynamics with the wild type scenario. This, however, requires the detailed structural knowledge of the proteins.

The observation of the dynamics of the entrance to and recovery from the starved state at the single cell level will give a lot of information about the system. Especially, the binary response [Step (2)] should be confirmed experimentally, by for example visualizing the RelE level or *relBE* mRNA level in each cell.

The low copy number of RelE (~50 nM) or *relBE* mRNA (~a few molecules) makes it challenging to monitor these molecules using usual fluorescent microscopy, to overcome this challenge superresolution microscopy (e.g STORM) has been successfully used to monitor the single cell/single molecule dynamics in bacterial cells (31). The dynamics of the recovery is also interesting to observe, especially the expected short recovery time due to the conditional cooperativity.

Another interesting experiment is to study the dependence of recovery dynamics on the duration of the amino acid starvation. The duration of typical experiments is ~5 h, and the toxin RelE is expected to be stable on that time scale. This is consistent with (7), where a pulse of RelE found to sufficient to prevent cell division for similar time scale. The limit of the stability of RelE in non-dividing cells, though, is not known. This factor, according to our model, can have a strong effect on the recovery behavior from high-toxin state after very long lasting amino acid starvation.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online: Supplementary Data A–F and Supplementary References [33,34].

ACKNOWLEDGEMENTS

K.G. thanks Dagmar Iber for discussions in the initial phase of the project.

FUNDING

Center for Models of Life, Danish National Research Foundation; Steno Fellowship, The Danish Council for Independent Research. Funding for open access charge: Danish National Research Foundation.

Conflict of interest statement. None declared.

REFERENCES

1. Pandey,D. and Gerdes,K. (2005) Toxin-antitoxin loci are highly abundant in free-living but from host-associated prokaryotes. *Nucleic Acids Res.*, **33**, 966–976.
2. Christensen,S.K., Mikkelsen,M., Pedersen,K. and Gerdes,K. (2001) RelE, a global inhibitor of translation, is activated during nutritional stress. *Proc. Natl Acad. Sci. USA*, **98**, 14328–14333.
3. Gefen,O. and Balaban,N.Q. (2009) The importance of being persistent: heterogeneity of bacterial populations under antibiotic stress. *FEMS Microbiol. Rev.*, **33**, 704–717.

4. Rotem,E., Loinger,A., Ronin,I., Levin-Reisman,I., Gabay,C., Shoresh,N., Biham,O. and Balaban,N.Q. (2010) Regulation of phenotypic variability by a threshold-based mechanism underlies bacterial persistence. *Proc. Natl Acad. Sci. USA*, **107**, 12541–12546.
5. Maisonneuve,E., Shakespeare,L.J., Jørgensen,M. and Gerdes,K. (2011) Bacterial persistence by RNA endonucleases. *Proc. Natl Acad. Sci. USA*, **108**, 13206–13211.
6. Pedersen,K., Zavialov,A.V., Pavlov,M.Y., Elf,J., Gerdes,K. and Ehrenberg,M. (2003) The bacterial toxin RelE displays codon-specific cleavage of mRNAs in the ribosomal A site. *Cell*, **112**, 131–140.
7. Pedersen,K., Christensen,S.K. and Gerdes,K. (2002) Rapid induction and reversal of a bacteriostatic condition by controlled expression of toxins and antitoxins. *Mol. Microbiol.*, **45**, 501–510.
8. Overgaard,M., Borch,J. and Gerdes,K. (2009) RelB and RelE of Escherichia coli form a tight complex that represses transcription via the ribbon-helix-helix motif in RelB. *J. Mol. Biol.*, **394**, 183–196.
9. Overgaard,M., Borch,J., Jørgensen,M.G. and Gerdes,K. (2008) Messenger RNA interferase RelE controls relBE transcription by conditional cooperativity. *Mol. Microbiol.*, **69**, 841–857.
10. Garcia-Pino,A., Balasubramanian,S., Wyns,L., Gazit,E., Greve,H.D., Magnuson,R., Charlier,D., van Nuland,N. and Loris,R. (2010) Allostery and intrinsic disorder mediate transcription regulation by conditional cooperativity. *Cell*, **142**, 101–111.
11. Winther,K.S. and Gerdes,K. (2012) Regulation of enteric vapBC transcription: induction by VapC toxin dimer-breaking. *Nucleic Acid Res.*, **40**, 4347–4357.
12. Magnuson,R. and Yarmolinsky,M.B. (1998) Corepression of the p1 addiction operon by phd and doc. *J. Bacteriol.*, **180**, 6342–6351.
13. Feyter,R., Wallace,C. and Lane,D. (1989) Autoregulation of the ccd operon in the F plasmid. *Mol. Gen. Genet.*, **218**, 81–86.
14. Gerdes,K., Christensen,S.K. and Lobner-Olesen,A. (2005) Prokaryotic toxin-antitoxin stress response loci. *Nat. Rev. Microbiol.*, **3**, 371–382.
15. Gillespie,D.T. (1977) Exact stochastic simulation of coupled chemical reactions. *J. Phys. Chem.*, **81**, 2340–2361.
16. Gotfredsen,M. and Gerdes,K. (1998) Escherichia coli relBE genes belong to a new toxin-antitoxin gene family. *Mol. Microbiol.*, **29**, 1065–1076.
17. Kuroda,A., Nomura,K., Ohtomo,R., Kato,J., Ikeda,T., Takiguchi,N., Ohtake,H. and Kornberg,A. (2001) Role of inorganic polyphosphate in promoting ribosomal protein degradation by the Lon protease in E. coli. *Science*, **293**, 705–708.
18. Bergqvist,S., Alverdi,V., Mengel,M., Hoffmann,A., Ghosh,G. and Komives,E.A. (2009) Kinetic enhansement of NF-κB · dna dissociation by IκBα. *Proc. Natl Acad. Sci. USA*, **106**, 19328–19333.
19. Zabel,U. and Baeuerle,P.A. (1990) Purified human IκB can rapidly dissociate the complex of the NF-κB transcription factor with its cognate DNA. *Cell*, **2**, 255–265.
20. Ptashne,M. (1986) Gene regulation by proteins acting nearby and at a distance. *Nature*, **322**, 697–701.
21. Ozbudak,E., Thattai,M., Lim,H., Shraiman,B. and Oudenaarden,A.V. (2004) Multistability in the lactose utilization network of Escherichia coli. *Nature*, **427**, 737–740.
22. Becskei,A., Seraphin,B. and Serrano,L. (2001) Positive feedback in eukaryotic gene networks: cell differentiation by graded to binary response conversion. *EMBO J.*, **20**, 2528–2535.
23. Lou,C., Li,Z. and Ouyang,Q. (2008) A molecular model for persister in E. coli. *J. Theor. Biol.*, **255**, 205–209.
24. Klumpp,S., Zhang,Z. and Hwa,T. (2009) Growth rate-dependent global effects on gene expression in bacteria. *Cell*, **139**, 1366–1375.
25. Klumpp,S. and Hwa,T. (2008) Growth-rate-dependent partitioning of RNA polymerases in bacteria. *Proc. Natl Acad. Sci. USA*, **105**, 20245–20250.
26. Scott,M., Gunderson,C.W., Mateescu,E.M., Zhang,A. and Hwa,T. (2010) Inter-dependence of cell growth and gene expression: origins and consequences. *Science*, **330**, 1099–1102.
27. Zaslaver,A., Kaplan,S., Bren,A., Jinich,A., Mayo,A., Dekel,E. and Uri,A. (2009) S. Itzkovitz Invariant distribution of promoter activities in Escherichia coli. *PLoS Comp. Biol.*, **5**, e1000545.
28. Sneppen,K. and Zocchi,G. (2005) *Physics in Molecular Biology*. Cambridge University Press, Cambridge, UK.
29. Pedersen,S. and Reeh,S. (1978) Functional mRNA half lives in *E. coli*. *Mol. Gen. Genet.*, **166**, 329–336.
30. Takagi,H., Kakuta,Y., Okada,T., Yao,M., Tanaka,I. and Kimura,M. (2005) Crystal structure of archaeal toxin antitoxin RelERelB complex with implications for toxin activity and antitoxin effects. *Nat. Struct. Mol. Biol.*, **12**, 327–331.
31. Elf,J., Li,G.W. and Xie,X.S. (2007) Probing transcription factor dynamics at the single molecule level a living cell. *Science*, **316**, 1191–1194.
32. Li,G.-Y., Zhang,Y., Inouye,M. and Ikura,M. (2008) Structural mechanism of transcriptional autorepression of the Escherichia coli RelB/RelE antitoxin/toxin module. *J. Mol. Biol.*, **380**, 107–119.
33. Slutsky,M., Kardar,M. and Mirny,L.A. (2004) Diffusion in correlated random potentials with applications to DNA. *Phys. Rev.*, **69**, 061903.
34. Elf,J., Li,G.W. and Xie,X.S. (2007) Probing transcription factor dynamics at the single molecular level in a living cell. *Science*, **316**, 1191–1194.