Modeling of the genetic switch of bacteriophage TP901-1: A heteromer of CI and MOR ensures robust bistability

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Abstract

The lytic-lysogenic switch of the temperate lactococcal phage TP901-1 is fundamentally different from that of phage lambda. In phage TP901-1, the lytic promoter P\textsubscript{L} is repressed by CI whereas repression of the lysogenic promoter P\textsubscript{R} requires the presence of both of the antagonistic regulator proteins, MOR and CI. We model the central part of the switch and compare the two cases for P\textsubscript{R} repression: the one where the two regulators interact only on the DNA, and the other where the two regulators form a heteromer complex in the cytoplasm prior to DNA binding. The models are analyzed for bistability, and the predicted promoter repression folds are compared to experimental data. We conclude that the experimental data are best reproduced the latter case, where a heteromer complex forms in solution. We further find that CI sequestration by the formation of MOR:CI complexes in cytoplasm makes the genetic switch robust.

Key words: temperate bacteriophage TP901-1, genetic switch, mixed feedback loop, sequestration, protein interaction

1. Introduction

Phenotypic variability under homogeneous conditions can readily be obtained by interlinking multiple gene regulatory pathways. Several well-characterized examples of phenotypic variations are known to be important for different developmental process of bacteria, such as the presence of persister cells in \textit{Staphylococcus aureus} and \textit{E. coli}, development of natural competence and sporulation in \textit{Bacillus subtilis}, and the choice between lytic or lysogenic growth of temperate bacteriophages \cite{1, 2}. Two distinguishable phenotypes may originate from a bistable system, i.e. a system that can toggle between two alternative stable steady-states \cite{3}. Infection of bacteria by temperate bacteriophages provides a classical example of the possibility to choose between two alternative modes of development.

The bacteriophage lambda infecting \textit{Escherichia coli} has been subjected to decades of intensive study, making the lytic-lysogenic switch one of the best understood gene regulatory systems \cite{4, 5, 6}. The bistability of the lambda switch is obtained from a double negative feedback mechanism, where two repressor proteins directly repress transcription of the other repressor gene. This system has a stable state with one promoter on and the other off, and vice versa for the other stable state. Once either state has been established, it would persist indefinitely or until some trigger stimulus forces the system to switch to the other state.

The genetic switch of the temperate lactococcal bacteriophage TP901-1 infecting \textit{Lactococcus lactis} subsp. \textit{cremoris} provides a regulatory system diverse from the lambda genetic switch. A previous study has demonstrated that a DNA fragment obtained from the temperate lactococcal phage TP901-1 shows bistability when introduced into \textit{Lactococcus lactis}. The cloned DNA fragment contains the two divergently oriented promoters, P\textsubscript{R} and P\textsubscript{L}, and the two promoter proximal genes \textit{cI} and \textit{mor} \cite{7}(Fig. 1a). A knockout mutation in the \textit{mor} gene showed that CI ensures tight repression of the P\textsubscript{L} promoter and partially repression of the P\textsubscript{R} promoter whereas a knockout mutation in the \textit{cI} gene results in open states of both P\textsubscript{R} and P\textsubscript{L}, showing that MOR by itself does not exhibit repression of either promoter \cite{8, 9}. Two types of repression has been shown: i) \textit{MOR}-independent repression, which is responsible for repression of P\textsubscript{L}. The P\textsubscript{L} promoter is repressed by cooperative binding of CI to the three operator sites O\textsubscript{R}, O\textsubscript{L} and O\textsubscript{D}, by the formation of a CI-DNA loop structure. ii) \textit{MOR}-dependent repression, which is responsible for repres-
and CI collectively repress transcription from $P$ involved in $P$ where both of the antagonistic repressor proteins are in-

TP901-1 may be described as a mixed feedback loop, hence, the bistability of the genetic switch from phage  

MOR and CI binding at a putative $O$ contains only one of the three CI operator sites, $O_L$, which gives tight repression of $P_L$, thus still sustains the bistable behavior of the construct. In the immune state, the $P_L$ promoter is repressed approximately 1000-fold, but high expression from $P_L$ is allowed due to the absence of $O_S$, which autonomous transcription from $P_L$ in the case of the wild-type switch (Fig. 1b). In the anti-immune state, $P_L$ is repressed approximately 100-fold but high expression from $P_L$ is allowed (Fig. 1b).

Generally speaking, it is not easy to predict the behavior of a bistable switch without quantitative analysis because a bistable switch is a dynamical and highly nonlinear system. In the present case of TP901-1, even the modified version of the switch (Fig. 1b) could involve a number of mechanisms, such as cooperativity binding via homo/hetero-dimerization, sequestration via heterodimerization, intertwined loops of negative and positive feed back via protein interactions, etc. In such a situation, the only way to obtain any reliable results is to perform quantitative analysis on specific models. By confronting numerical results with experimental data, we can restrict possible mechanisms with plausible parameters.

In this paper, we construct mathematical models for this modified bistable system based upon statistical mechanics, examine their behavior in the steady states numerically, and compare the obtained repression folds with experimental observations. We assume that $P_L$ is repressed by the CI dimer binding to the operator $O_S$ since the operator has two inverted repeated sequences. On the other hand, the MOR-dependent
repression of $P_x$ is assumed to be brought about by the MOR:CI:DNA complex formation at the putative $O_x$ operator. Since the amino sequence of MOR shows high similarity to the DNA-binding Helix-turn-Helix domain of the repressor protein encoded by *Escherichia coli* phage 434 [11], MOR is likely to be a DNA binding protein, but the fact that MOR alone does not repress transcription from either $P_r$ or $P_s$ suggests that the DNA-binding affinity of MOR is negligible. CI alone does not repress $P_x$ in this system due to absence of $O_x$ operator. Based upon these observations, we test two different scenarios for $P_x$ repression (Fig. 2): i) repression through direct binding of CI and MOR to $O_x$ (Model A), and ii) repression through binding of MOR:CI complex to $O_x$ with the complex being formed in the cytoplasm prior to the binding to DNA (Model B).

The MOR:CI:DNA complex formation in Model A may be regarded as an extreme case of Model B where the MOR:CI complex formation in cytoplasm is so weak that the complex is stabilized only when it binds to DNA, thus there is no substantial presence of MOR:CI complex in the cytoplasm. However, distinguishing Model A from Model B helps us to recognize two distinct aspects in the $P_x$ repression by the MOR:CI:DNA complex, i.e., co-operativity and sequestration; the latter has been studied in silico as a possible mechanism for a genetic switch [12, 13] and demonstrated to provide strong nonlinearity [14, 15]. In fact, our Model B is a reminiscent of one of the bistable switches obtained by the simulated evolution (Fig. 3A in [12]). We will demonstrate that CI sequestration by the MOR:CI formation in cytoplasm can make a robust bistable system and is actually a plausible switching mechanism for TP901-1.

Theory

The regulatory circuit in the present system consists of the two promoters $P_r$ and $P_s$, which produce MOR and CI, respectively (Fig. 1). The promoter $P_r$ is repressed by CI binding at $O_r$, thus the $P_r$ activity is given by a function of the CI concentration as

$$pL([CI]) = pL_0 \cdot f_{OL}([CI]),$$

where $pL_0$ is the bare activity of the promoter $P_r$. The function $f_{OL}([CI])$ represents the repression factor. In the absence of CI, there is no repression: $f_{OL}(0) = 1$. The $P_s$ activity, on the other hand, depends on the concentrations of both CI and MOR. Accordingly, the $P_s$ activity can be written as

$$pR([MOR], [CI]) = pR_0 \cdot f_{OM}([MOR], [CI]),$$

with $pR_0$ being the bare activity. The function $f_{OM}([MOR], [CI])$ is the repression factor due to the binding of MOR and CI at $O_s$, and satisfies $f_{OM}(0, [CI]) = f_{OM}([MOR], 0) = 1$.

The promoter $P_r$ produces MOR, and $P_s$ produces CI, thus in the modeled feedback system, the total concentration for each protein, $[MOR]_{total}$ and $[CI]_{total}$, is governed by the dynamics equations,

$$\frac{d}{dt}[MOR]_{total} = \frac{1}{\tau_M} \left( pL([CI]) - [MOR]_{total} \right),$$

$$\frac{d}{dt}[CI]_{total} = \frac{1}{\tau_C} \left( pR([MOR], [CI]) - [CI]_{total} \right),$$

where $\tau_M$ and $\tau_C$ are the degradation times for MOR and CI, respectively. To simplify the notation, we have rescaled the promoter activities, Eqs. (1) and (2), by the degradation times, i.e. the promoter activities are now measured in terms of the steady state protein concentrations.

In steady states, the production and the degradation of each protein should balance, therefore, the promoter activities and the concentrations of the expressed proteins in the cytoplasm should satisfy the steady state condition,

$$pL([CI]) = [MOR]_{total},$$

$$pR([MOR], [CI]) = [CI]_{total}.$$  

Not all steady states are stable against small perturbations. A steady state is unstable if a perturbation drives the system out of the state; The stability should be determined by the dynamics equations, Eqs. (3) and (4) (See supplementary material). If there are two stable steady states, the system shows bistability.

We assume that the repression factors, $f_{OL}$ and $f_{OM}$ in Eqs. (1) and (2), are given by the statistical weights at equilibrium that the corresponding operators are not bound by the regulators. This approximation holds when the time that RNA polymerase (RNAP) needs to start elongation after binding to DNA is much shorter than the time scales of binding/unbinding of RNAP and repression factors to the promoter/operator sites [16]. The equilibrium statistical weights depend upon the repressor concentrations, and their dependence is characterized by the Hill coefficient and the affinities of the repressors to the operator sites [12, 18, 19].

For the MOR-independent repression of $P_s$, we suppose that $P_r$ is repressed by CI dimer binding at $O_r$, and
that the dimers are formed in the cytoplasm before binding. Thus, within the above approximation for the repression factor, the \( p_L \) activity is given by

\[
p_L([\text{CI}]) = p_{L_0} \cdot \frac{1}{1 + [\text{CI}]^2/K_{\text{Cl}_2}},
\]

where the affinity \( K_{\text{Cl}_1} \) represents the \( \text{Cl}_2 \) concentration at which \( O_{\text{K}} \) is occupied for 50% of the time. Since the dimer concentration \( [\text{Cl}_2] \) is related to the monomer concentration \( [\text{CI}] \) as

\[
[\text{Cl}_2] = \frac{[\text{CI}]^2}{K_{\text{Cl}_2}} \tag{8}
\]

with the dissociation constant \( K_{\text{Cl}_2} \), Eq.\( (7) \) may be written as

\[
p_L([\text{CI}]) = p_{L_0} \cdot \frac{1}{1 + [\text{CI}]^2/K_{\text{Cl}_2}^2} \tag{9}
\]

with the effective affinity

\[
K_{\text{OL}} \equiv \sqrt{K_{\text{Cl}_1} K_{\text{Cl}_2}} \tag{10}
\]

for \( \text{CI} \) concentration. In Fig.\( 5 \) the activity of \( p_L \) as a function of \( [\text{CI}] \) is plotted by a green line.

As for the MOR-dependent repression of \( P_\text{K} \), we will examine two models. In Model A, monomers of MOR and CI may bind cooperatively at \( O_{\text{K}} \), but we do not assume any MOR:CI complexes formed in cytoplasm before binding to DNA. In Model B, on the other hand, CI and MOR may associate in cytoplasm before they bind at \( O_{\text{K}} \). For both models, \( p_R \) is repressed by the formation of the MOR:CI:DNA complex at \( O_{\text{K}} \). The important point in Model B is that the formation of MOR:CI heteromers competes with CI dimer formation by sequestering CI monomers.

Model A

We first consider a MOR:CI:DNA complex containing one MOR and one CI protein as illustrated in Fig.\( 2 \). Then, we can approximate the total concentration of MOR unit by the MOR monomer concentration,

\[
[MOR]_{\text{total}} = [\text{MOR}]. \tag{11}
\]

The activity of the \( P_\text{K} \) promoter is repressed from the bare activity \( p_{R_0} \) by the statistical weight that the operator \( O_{\text{K}} \) is not occupied by MOR and CI,

\[
p_R([\text{MOR}], [\text{CI}]) = p_{R_0} \cdot \frac{1}{1 + [\text{MOR}][\text{CI}]/(K_{\text{OM}})}. \tag{12}
\]

The affinity \( K_{\text{OM}} \) is the concentration \( \sqrt{[\text{MOR}] \cdot [\text{CI}]} \) where \( O_{\text{K}} \) is occupied by MOR and CI for 50% of the time.

The steady state is determined from the steady state condition Eqs.\( 5 \) and \( 6 \) by eliminating the MOR concentrations. With the help of Eq.\( (11) \), we obtain

\[
p_R\left(p_L([\text{CI}]), [\text{CI}]\right) = [\text{CI}]_{\text{total}}, \tag{13}
\]

which represents the balance between the production and the degradation of CI. This can be solved graphically by plotting the both sides as a function of \( [\text{CI}] \),

\[
p_R\left(p_L([\text{CI}]), [\text{CI}]\right) = p_{R_0} \left[1 + \frac{p_{L_0} \tilde{K}_{\text{OL}}/(K_{\text{OM}})^2}{([\text{CI}]/\tilde{K}_{\text{OL}}) + (\tilde{K}_{\text{OL}}/\tilde{[\text{CI}])}} \right], \tag{14}
\]

\[
[\text{CI}]_{\text{total}} = [\text{CI}] + 2[\text{CI}]^2/K_{\text{Cl}_2}. \tag{15}
\]

Eq.\( (14) \) represents the \( p_R \) activity in the system where MOR is provided by \( p_L \) but [CI] is controlled externally. Note that the relative strength of the bare promoters, \( p_{L_0} \) and \( p_{R_0} \), does not affect the system behaviors, such as bistability or repression folds, because there is no direct interaction between MOR and CI in this model.

Model B

In this model, a MOR CI heterodimer is formed in solution before it binds to the putative \( O_{\text{K}} \) site to repress \( P_\text{K} \) (Fig.\( 2 \)). The activity of the \( P_\text{K} \) promoter is again given by Eq.\( (11) \) but the \( p_R \) activity is

\[
p_R([\text{MOR}], [\text{CI}]) = p_{R_0} \cdot \frac{1}{1 + [\text{MOR}][\text{CI}]/K_{\text{OM}}}. \tag{16}
\]

where \( K_{\text{OM}} \) now represents the concentration of the MOR CI heterodimer at which \( O_{\text{K}} \) is occupied for 50% of the time. The concentration of the MOR CI heterodimer is given as

\[
[MOR \cdot \text{CI}] = \frac{[\text{MOR}] \cdot [\text{CI}]}{K_{\text{MOR} \cdot \text{CI}}}. \tag{17}
\]

with the dissociation constant \( K_{\text{MOR} \cdot \text{CI}} \) for the heterodimer.

The formation of the heterodimers couples the monomer concentrations of CI and MOR through

\[
[\text{CI}]_{\text{total}} = [\text{CI}] + 2[\text{Cl}_2] + [\text{MOR} \cdot \text{CI}], \tag{18}
\]

\[
[MOR]_{\text{total}} = [\text{MOR}] + [\text{MOR} \cdot \text{CI}], \tag{19}
\]

which leads to the competition between the \( \text{Cl}_2 \) formation and the MOR CI formation. Note that \( [\text{Cl}_2] \) is still given by Eq.\( (8) \).

The steady state is determined as in the case of Model A: We consider the \( p_r \) activity as a function of [CI]
when MOR is provided by Pe. From Eqs. (17) and (19), [MOR] is expressed in terms of [CI] and [MOR]total,
\[
[MOR] = \frac{[MOR]_{\text{total}}}{1 + [CI]/K_{\text{MOR:CI}}},
\]
(20)
and then, in the steady state where Eq. (5) holds, [MOR]total is given by the Pe activity with Eq. (7). Then
the steady state condition Eq. (6) for Pe becomes
\[
pr \left( \frac{pl([CI])}{1 + [CI]/K_{\text{MOR:CI}}} \right) = [CI]_{\text{total}},
\]
(21)
which can be solved graphically with the explicit forms for the both sides:
\[
pr \left( \frac{pl([CI])}{1 + [CI]/K_{\text{MOR:CI}}} \right) = pr_0 \left[ 1 + \frac{pl_0 K_{OL} / K_{OM}}{([CI] / K_{OL}) + (K_{OL} / [CI])]^2} \right]^{-1}
\]
(22)
\[
[CI]_{\text{total}} = [CI] + 2 \frac{[CI]^2}{K_{CL}} + pl([CI]) \frac{[CI]/K_{\text{MOR:CI}}}{1 + [CI]/K_{\text{MOR:CI}}},
\]
(23)
where the effective affinities are
\[
\bar{K}_{OM} \equiv \sqrt{K_{\text{MOR:CI}} \cdot K_{OM}} \quad \text{and} \quad \bar{K}_{OL} \equiv \sqrt{K_{CL} \cdot K_{OL}}.
\]
(24)
Eq. (21) represents the balance between the production and degradation of CI in the system where MOR is pro-
vided by Pe. Note that Model B reduces to Model A in the limit of large $K_{\text{MOR:CI}}$ with $\bar{K}_{OM}$ being kept constant
as has been discussed at the end of Introduction.

2. Results

We numerically examine the steady states for the two versions of the models we have constructed (Fig. 2).

Model A

In our first model, we study the possibility for bistability in the system where Pe is repressed by binding a
MOR monomer and a CI monomer to Oa without direct interaction between MOR and CI in the cytoplasm.
The binding affinities for each of the proteins alone at Oa should be negligible because the Pe repression requires
both of the proteins. Hence, the affinity $K_{OM}$ in Eq. (12) may be considered as the effective binding affinity $\bar{K}_{OM}$
for MOR and CI with very weak MOR CI formation, or as the resulting binding affinities from CI:Oa, MOR:Oa,
and the interaction between the bound proteins.

Figure 5 shows a typical example of the Pe activity of Eq. (9) (green line), the Pe activity of Eq. (14) (solid red line), and the total concentration of CI, or degradation
rate, of Eq. (15) (dashed red line) as a function of free [CI] in the logarithmic scale. One can see that Pe (green line)
is fully active and produces a lot of MOR at low [CI], whereas its activity is monotonically decreasing with increasing [CI], due to Pe repression by CI2 binding at Oa; Pe is virtually shut down beyond [CI] ≈ $\bar{K}_{OL} = \sqrt{K_{CL} \cdot K_{OL}} = 6 \cdot 10^{-4}$.

On the other hand, the Pe activity (solid red line) shows a more complicated behavior, i.e., Pe is open both at very low and high [CI] concentrations but re-
pressed at the intermediate concentration. One can understand this behavior by noting that the solid red line,
Eq. (14), represents the Pe activity in the system where MOR is expressed from Pe under CI control; At low
[CI], there is plenty of MOR due to high Pe activity, while at high [CI], no MOR is present. The Pe activity
is repressed only at intermediate [CI] because both MOR and CI are necessary for its repression by means
of the MOR:CI:DNA complex formation.

In the steady state, the production and the degradation of each protein should balance. Since CI is expressed
from Pe and the degradation of CI is assumed to be proportional to the total concentration of CI, the steady
states are identified as $pr(pl([CI]), [CI]) = [CI]_{\text{total}}$, namely, Eq. (13). We thus find the steady states at the
intersection points between the $pr$ and $[CI]_{\text{total}}$ curves represented by Eqs. (14) (solid red line) and (15) (red
dashed line), respectively.

In Fig. 3 only one intersection point is observed be-
tween the two curves, showing that there is only one steady state solution. This uniqueness of steady state holds true for any given value of the parameters, because the activity of \( p_L \) given by Eq. (14) never increases faster than proportional to CI, whereas \([CI]_{\text{total}}\) always increases faster or proportional to [CI]. Therefore, bistability is never realized in Model A with the assumption of binding of one MOR and one CI for repression of \( p_L \).

**Variant of Model A**

Bistability may be obtained in Model A if a larger number of proteins are allowed to form a complex structure at \( O_m \). Suppose \( m \) MOR monomers and \( c \) CI monomers bind at \( O_m \) to form the \( \text{MOR}_m;\text{CI}_c;\text{DNA} \) complex that represses transcription from \( p_L \), then for the expression for the \( p_L \) activity, Eq. (12) should be replaced by

\[
p_{RL}([\text{MOR}], [\text{CI}]) = p_{R0} \frac{1}{1 + [\text{MOR}]^m[\text{CI}]^c/(K_{OM})^{m+c}}, \tag{25}
\]

while all the other equations remain the same. Using this for the left hand side of the steady state equation (13) with \([\text{MOR}] \approx 1/([\text{CI}])^2 \) from Eq. (7), one can see that the largest slope of the \( p_L \) activity as a function of [CI] is \( 2m - c \) in the logarithmic scale. Since the slope of the plot of [CI]_{\text{total}} given by Eq. (15) is between 1 and 2, the steady state equation (15) can have more than two solutions with Eq. (25) when \( 2m - c \geq 2 \). In the case of \( m = 2 \), we could obtain multiple solutions with \( c = 1 \) or 2, i.e., two MOR monomers binding together with one or two CI monomers at \( O_m \). Examples for Model A with \((m,c) = (2,1) \) and \((2,2) \) are shown in Fig. 4. In each example, the intersections between the solid red line (the \( p_L \) activity) and the dashed red line (the total CI concentration) represent steady state solutions. One can see there are three solutions for each case in Fig. 4.

Dynamical analysis shows that the steady state in the middle marked by an open red circle is unstable against small fluctuations, and the states at the ends marked by filled red circles are stable (See supplementary material for detail). Full analysis requires Eqs. (3) and (4), but the stability may be understood in the following way: For the steady state in the middle, if the CI monomer concentration increases by fluctuation from the steady value of [CI], the CI production from \( p_L \) will increase more than the increase in degradation given by [CI]_{\text{total}}, as is seen in Fig 4 where the solid red line of the \( p_L \) activity goes above the dashed red line of [CI]_{\text{total}} upon increasing [CI] from the middle steady state. This means that such a fluctuation causes further increases of [CI], consequently, the state is driven out of the steady state. On the other hand, the steady states at both ends represent stable states. A fluctuation towards larger [CI] leads to insufficient CI production in comparison with the CI degradation, i.e. the solid red line goes under the dashed red line as [CI] increases. This brings the system back to the original state, therefore, they are stable. Thus, the system has two stable steady states, which leads to bistability.

The promoter activities in the two stable states can be determined from the graphic representation in Fig 4. The \( p_L \) activity is read from the ordinates of the intersection (filled red circles) and the \( p_L \) activity is read off from the corresponding [CI] values of the intersection points (green circles), which allows us to estimate the repression folds for \( p_L \) and \( p_R \) between the two stable states. The state at the right represents the immune state with open \( p_R \) and repressed \( p_L \), while the one at the left represents the anti-immune state with open \( p_L \) and repressed \( p_R \).

The relative activities between the two states should be compared with the promoter activities obtained from the in vivo measurements [9]; \( p_L \) is repressed approximately 1,000-fold in the immune state and \( p_R \) approximately 100-fold in the anti-immune state. To reproduce these repression folds in Model A with \((m,c) = (2,1) \) and \((2,2) \), we test the three parameters, \( K_{OM} \), \( K_{CL} \), and \( K_{OL} \), representing the dimerization constant of CI.
the effective binding constant of CI-monomer at $O$, and the binding constant of the MOR:CI complex at $O$, respectively, by setting the criterion that the Ps repression fold should be in the range from 50 to 200 (50 < $pR(\text{open})/pR(\text{closed})$ < 200) and the Ps repression fold should at least be 500 ($pL(\text{open})/pL(\text{closed})$ > 500). Fig.5 shows the distributions of accepted values of parameters out of randomly chosen values in the logarithmic scale. $K_{OL}$ and $K_{OM}$ are narrowly distributed while $K_{CI}$ are much larger than $K_{OL}$. This suggests that, in order for Model A to work, CI must exist as a monomer and act by cooperative binding to form CI$_2$ at $O$. when repressing $P$.

Figure 6 shows the parameters that satisfy the criterion only for the Ps repression fold versus resulting Ps repression fold (left two columns for Model A and right two for Model B). The vertical green lines are drawn at the Ps repression fold 500, thus only the plots on the right side of the lines should be accepted by the repression fold criterion. From the plots for $K_{CI}$ for Model A, one can see that the relatively high values for $K_{CI}$ in this model comes from the requirement for the large Ps repression fold. This can be understood as follows: In order to achieve large repression fold for Ps, the difference in [CI] for the two steady states should be large, which in turn requires smaller slope in the [CI]$_{total}$ curve, namely larger $K_{CI}$ because the slope in [CI]$_{total}$ changes from 1 to 2 around [CI] $\approx K_{CI}$ (Fig.4).

**Model B**

Now, we consider the possibility that $P_s$ is repressed by a MOR:CI complex formed in cytoplasm before binding to DNA. Examples for Model B are shown in Fig.7, where the $P_l$ activity of Eq.(22) (solid red line), and the total density of CI of Eq. (23) (dashed red line) as a function of [CI] in the logarithmic scale; All of them have three steady states.

Striking difference from the case of Model A is that the [CI]$_{total}$ (dashed red line) can be non-monotonic. Therefore, for a given [CI]$_{total}$ within a certain range, there exist three possible states with different [CI]. This suggests that the bistability could be obtained for the system with $P_l$ even if $P_s$ were not regulated, namely, even if $P_s$ would produce CI at a fixed rate within the range. This bistability is due to the MOR:CI heteromer formation in cytoplasm; $P_l$ produces MOR, which sequesters its own repressor, i.e. CI, by forming MOR:CI. For a given [CI]$_{total}$, the state at low [CI] is the state where most of CI’s are incorporated in MOR:CI heterodimers due to the MOR produced by $P_l$, while the state at high [CI] is the state where most of CI is in the dimer with $P_l$ being repressed. The state in the middle is unstable. Such bistability is, of course, not the bistability observed in the experiments, but one can see that this feature of behavior in [CI]$_{total}$ (dashed red line) makes it easier to have three intersections with the $P_l$ activity curve (solid red line) than in the case of Model A.

According to the stability criterion we discussed, the steady state at both ends are stable while the state in the middle is unstable even for the system where both $P_s$ and $P_l$ are regulated. Full analysis, however, shows there are some cases where the states at both ends can be unstable although the stability criterion is correct for most cases (See supplementary material). We analyse the bistability based upon the stability criterion, ignoring the small possibility that the states at both ends could be unstable.
Figure 6: Distribution of possible parameters versus P_L repression fold, \( p_L(open)/p_L(closed) \) for Model A with \((m,c)=(2,1)\) and \((2,2)\), and for Model B with \((m,c)=(1,1)\) and \((2,1)\). The plotted parameters are those that satisfy the P_L repression fold criterion \( 50 < pR(open)/pR(closed) < 200 \) out of randomly chosen parameters from the region \( \tilde{K}_{OL}, \tilde{K}_{OM}, K_{CI}^2 \in [10^{-7}, 1] \) for Model A and \( \tilde{K}_{OL}, \tilde{K}_{OM}, K_{CI}^2, K_{MOR:CI} \in [10^{-7}, 1] \) for Model B. Vertical green lines are drawn at P_L repression fold 500, thus only the parameters that are in the right side of the lines are consistent with the experimentally obtained repression folds. The number of tested data are \( 10^6 \) for Model A with \((m,c)=(2,1)\), \( 5 \times 10^6 \) for Model A with \((m,c)=(2,2)\), and \( 2 \times 10^5 \) for Model B. Note that the effective affinity \( K_{OM} \) for Model B is defined by \( K_{OM} = K_{OM}^{K_{MOR:CI}} \).
We also examine Model B in the case where a larger complex, $\text{MOR}_2\text{Cl}_2$, represses $p_k$. Detailed formalism is given in the appendix.

Figure 8 shows the plots for the extended Model B with $(m,c) = (2,1)$. This version of the model shows sharper transition between the $\text{MOR}_2\text{Cl}_2$ regime and the $\text{Cl}_2$ regime for the form of CI protein as one can see in the lower graphs for CI ratio. As for the form of $\text{MOR}$, substantial fraction of $\text{MOR}_2\text{Cl}_2$ appears only in the intermediate range of $[\text{CI}]$. In contrast to the case with $(m,c) = (1,1)$ in Fig. 7, the heteromer $\text{MOR}_2\text{Cl}_2$ is not formed at high $[\text{CI}]$ because the $p_k$ promoter is closed faster than the $\text{MOR}_2\text{Cl}_2$ is formed. Note that the effective affinity of Ots is $K_{OM} = (K_{OM}, K_{\text{MOR}_2\text{Cl}_2})^{1/3}$.

### 3. Discussion

#### 3.1. Summary

The bacteriophage TP901-1 has provided us with a conceptually new design of a genetic switch, in which the interaction between two antagonistic regulators, CI and MOR, is essential. Bistability between the immune and the anti-immune states has been demonstrated with a genetic switch that consists of the two divergently oriented promoters $p_k$ and $p_\ell$, the two promoter-proximal genes, $cl$ and $mor$, and only one of the three CI operator sites Ots on a low copy number plasmid [8, 9]. The repression folds in the two states have been determined by in vivo measurements as around 1,000-fold for $p_k$ repression in the immune state and around 100-fold for $p_k$ repression in the anti-immune state.

We constructed mathematical models for this cloned bistable system, assuming a putative operator Ots to regulate $p_k$ (Fig. 1). We assumed that $p_k$ is repressed by CI2 bound to Ots whereas $p_\ell$ is repressed by the $\text{MOR}_m\text{Cl}_i$-DNA complex on Ots. We examined two types of models: one where MOR and CI interact only on DNA (Model A), and the other where MOR and CI form $\text{MOR}_m\text{Cl}_i$ complex in cytoplasm first and then the
complex binds to Oₘ (Model B). For each model, we tested bistability and performed parameter scans using the criterion that the repression fold should be consistent with the experiments.

Our results are summarized as follows: For Model A, (i) the system shows bistability only when 2m − c ≥ 2, (ii) the possible values for the operator affinities $K_{OL}$ and $K_{OM}$ are narrowly distributed, (iii) the possible dissociation constant $K_{CI}$ is much larger than the operator affinity $K_{OL}$ due to the large repression fold for Pᵢ, (iv) the possible value for $K_{CI}$ is bounded by the relatively large lower limit. The accepted ranges for the parameters are listed in Table 1. For Model B, (i) the bistability is robust due to the sequestration of CI by the MORₘCI complex formation, (ii) large parameter regions are allowed by the repression fold criterion, (iii) the Pᵢ repression fold is bounded by the lower limit for the parameters that are consistent with the Pᵢ repression fold: $pL(open)/pL(closed) > 5000$ for $(m, c) = (2, 1)$, and > 50 for $(m, c) = (2, 2)$. The accepted ranges for the parameters are listed in Table 2.

### Table 1: Accepted ranges of parameters for Model A. The values are given in the unit of the concentrations that correspond to [CI] and [MOR] at the full activity of the promoter Pₛ and Pᵢ, respectively.

| Parameter | (m, c) | (1,1) | (2,1) |
|-----------|--------|-------|-------|
| $K_{OL}$  | $\lesssim 3 \times 10^{-3}$ | $(3 \times 10^{-6}) - 3 \times 10^{-2}$ | |
| $K_{OM}$  | $\lesssim 5 \times 10^{-3}$ | $(10^{-4}) - 3 \times 10^{-2}$ | |
| $K_{CI}$  | | | |
| $K_{MOR-Cl}$ | $\leq 3 \times 10^{-1}$ | $(10^{-3}) - 1$ | |

### Table 2: Accepted ranges of parameters for Model B. The values are given in the unit of the concentrations that correspond to [CI] and [MOR] at the full activity of the promoter Pₛ and Pᵢ, respectively. The values in the parentheses are the lower limits when the fold criterion for Pᵢ is restricted to 500 < $pL(open)/pL(closed)$ < 5000. The ranges for $K_{CI}$ cannot be set because the accepted values extends over the whole tested range.

| Parameter | (m, c) | (1,1) | (2,1) |
|-----------|--------|-------|-------|
| $K_{OL}$  | $\sim 10^{-2}$ | $10^{-3} - 10^{-2}$ | |
| $K_{OM}$  | $10^{-2} - 10^{-1}$ | $\sim 10^{-3}$ | |
| $K_{CI}$  | $\gtrsim 10^{-2}$ | $\gtrsim 10^{-2}$ | |

### 3.2. Validity of the Models

In order to assess validity of the models, we have to determine our unit for CI concentration first. We employed the unit where [CI] is measured by the concentration in the steady state with the full activity of Pᵢ. This concentration should be compared with [CI] in the immune state of in vivo experiment on the system with around 10 copy-number plasmid containing the modified switch. We estimate this as follows by using the value of 300 nM for the CI concentration in the lysogenic/immune state of the wild lambda phage [20]: First, we assume that this concentration is compatible with that for wild type TP901-1 with a single copy. Then, we multiply this by the following two factors: the factor 10 of the copy-number of plasmid, and the factor 100 of the relative activity of Pᵢ in our modified system in comparison with the wild type switch [3]. With these factors, we estimate that $pR₀$, i.e., [CI] at the full Pᵢ activity in the present system, could be well over $10^5$ nM scale.

**Model A.** With this unit, we might be able to rule out Model A based upon the estimated values of $K_{OL}$ and $K_{CI}$. The possible value of $K_{OL}$ in Model A is between around $10^{-3}$ and $5 \times 10^{-2}$ (Table 1), but this contradicts the in vitro estimate of 28 nM for the CI concentration at which Oᵢ is occupied by CI for 50% of the time [10]. The lower limit of $K_{OL} \sim 10^{-3}$ in Model A should correspond to 100 nM or quite possibly even larger, but it is already well above 28 nM, i.e., the in vivo estimate for repressor-DNA affinity for TP901-1.

We also found that the large repression fold of Pᵢ entails $K_{CI} \gg K_{OL}$ for Model A. This means that CI’s exist as monomers in cytoplasm and form CI₂ when they bind to Oᵢ, but this is in contrast with many phage-encoded repressor proteins, such as those encoded by phage lambda, 434, and 186, which tend to exist as dimers or higher oligomers in solution [21, 22, 23, 24]; Actually, most of the 434 and lambda repressors exist in the dimeric conformation at nanomolar concentrations [23, 21]. Our Model A challenges the presumption that the formation of dimers is a prerequisite for its specific DNA binding.

**Model B.** We found broader distribution of parameter sets that satisfy the repression fold criterion for Model B. In particular, we did not find lower bounds for possible $K_{CI}$ in contrast to the case of Model A.

In the comparison of the two variants of Model B, our results show that the model with the formation of MOR₂CI complex is more favorable than that with
3.3. Experimental Test

One of the distinguishing consequences of Model B is that the system with uncontrolled \( P_R \) can be bistable because of the sequestration of CI by MOR:CI complex formation. Even if \( P_R \) produces CI at a constant rate, there can be the two stable states: one with the repressed \( P_L \) and the other with the derepressed \( P_R \). Such a mechanism of bistability has been proposed by François and Hakim [12, 13] as a theoretical possibility. Our study suggests this mechanism is employed in TP901-1. This may be tested experimentally for the genetic switch of phage TP901-1 by measuring the promoter activity of \( P_R \) in systems containing a functional \( mor \) gene and expressing CI from uncontrolled \( P_R \) promoters at constant but various rates. Plotting the \( P_L \) activity of each system versus the uncontrolled \( P_R \) activity, one should find a characteristic feature for bistability as in Fig.9, where the \( P_L \) activity is doubled-valued for a certain range of the \( P_R \) activity.

3.4. Concluding Remark

The genetic switching mechanism in TP901-1 is remarkably robust; The modified system studied here with only one operator \( O_0 \) contains 100 times more CI molecules in its immune state than the wild type genetic switch with all of the three operators on plasmids, yet still shows bistability. Our model study suggests that the robustness of the genetic switch in TP901-1 is brought about by sequestration of CI through MOR:CI complex formation in cytoplasm.

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Thus, both sides of the steady state condition Eq.(6) are now given by

\[ pR_0[MOR, CI] = pR_0 1 + [MOR]^2/\tilde{K}_{OM} \]  

(31)

\[ [CI]_{total} = [CI] + 2 [CI]_2 + 1/2 ([MOR]_{total} - [MOR]) \]  

(32)

with [MOR] by Eq.(30) and [MOR]_{total} by Eq.(5). The effective affinity \( \tilde{K}_{OM} \) is defined as

\[ \tilde{K}_{OM} \equiv (K_{OM}K_{MOR.CI})^{1/3}. \]  

(33)

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Supplementary material for
“Model Analysis of the Genetic Switch Isolated from the Temperate
Bacteriophage TP901-1:
Repressor Sequestration Effect”

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The stability criterion for the steady state used in the manuscript is examined. Dynamical analysis shows that the criterion is valid as long as the \([\text{CI}]\)\(_{\text{total}}\) curve is a monotonically increasing function. In Model B, however, the \([\text{CI}]\)\(_{\text{total}}\) curve has a part with negative slope for some parameter region, in which case the stability cannot be determined only by comparing the slopes of the curves. The steady state at the left side could be unstable when it is located in the region where \([\text{CI}]\)\(_{\text{total}}\) is decreasing.

1. Steady States

The steady states satisfy the self-consistent conditions

\[
p_L([\text{CI}]) = [\text{MOR}]_{\text{tot}}([\text{MOR}], [\text{CI}])
\]

\[
p_R([\text{MOR}], [\text{CI}]) = [\text{CI}]_{\text{tot}}([\text{CI}], [\text{MOR}]),
\]

with

\[
[\text{CI}]_{\text{tot}}([\text{CI}], [\text{MOR}]) = [\text{CI}] + 2[\text{CI}]_2 + [\text{MOR} \cdot \text{CI}]
\]

\[
[\text{MOR}]_{\text{tot}}([\text{MOR}], [\text{CI}]) = [\text{MOR}] + [\text{MOR} \cdot \text{CI}].
\]

Here, \([\text{CI}]_2\), \([\text{MOR} \cdot \text{CI}]\) are equilibrium concentrations of the protein complexes.

We determined the steady solutions graphically by looking for intersections of the following two curves as a function of \([\text{CI}]\), i.e. the production curve and the \([\text{CI}]_{\text{total}}\) curve that represents degradation rate:

\[
p_R = p_R([\text{MOR}], [\text{CI}])
\]

\[
[\text{CI}]_{\text{tot}} = [\text{CI}]_{\text{tot}}([\text{CI}], [\text{MOR}]),
\]

with \([\text{MOR}]\) being a function of \([\text{MOR}]_{\text{tot}}\) and \([\text{CI}]\) derived from the relation \([\text{MOR}]_{\text{tot}}([\text{MOR}], [\text{CI}]),\) and \([\text{MOR}]_{\text{tot}}\) being given by \(p_L([\text{CI}]),\)

\[
[\text{MOR}] = [\text{MOR}][[\text{MOR}]_{\text{tot}}, [\text{CI}]] = [\text{MOR}](p_L([\text{CI}]), [\text{CI}]).
\]
2. Stability criterion

In the text, the stability of the steady state is determined by the simple criterion. Let the slope of the production curve and the degradation curve be denoted by
\[ \frac{d pR}{d[CI]} \text{ and } \frac{d [CI]_{\text{tot}}}{d[CI]}, \]
respectively. Then the criterion is

the steady state is stable if \[ \frac{d pR}{d[CI]} < \frac{d [CI]_{\text{tot}}}{d[CI]}, \]

the steady state is unstable if \[ \frac{d pR}{d[CI]} > \frac{d [CI]_{\text{tot}}}{d[CI]} \]
at the corresponding intersection.

This criterion is simple and plausible, but based on the single variable picture although the system has at least two dynamical variables: \([CI]\) and \([MOR]\). The full analysis for stability requires dynamical consideration.

3. Dynamical Analysis of Stability

The dynamics for the protein concentrations is given by the set of equations:

\[
\begin{align*}
\frac{d}{dt} [MOR]_{\text{tot}} &= \frac{1}{\tau_M} \left( pL([CI]) - [MOR]_{\text{tot}} \right) \\
\frac{d}{dt} [CI]_{\text{tot}} &= \frac{1}{\tau_C} \left( pR([MOR], [CI]) - [CI]_{\text{tot}} \right).
\end{align*}
\]

The total concentrations \([CI]_{\text{tot}}\) and \([MOR]_{\text{tot}}\) are given by eqs. (3) and (4). Basic assumption for this is that the equilibration among protein complexes in cytoplasm is much faster than the decay rates of CI and MOR: \(1/\tau_C\) and \(1/\tau_M\).

Consider the steady solution with \([CI]^*\) and \([MOR]^*\), which satisfies eqs. (11) and (2). Suppose the steady state is perturbed by small fluctuation as

\[
\begin{align*}
[CI] &= [CI]^* + \delta[CI] \\
[MOR] &= [MOR]^* + \delta[MOR],
\end{align*}
\]

and see if the small deviation will grow or decay in time.

By inserting these into eqs. (9) and (10), we obtain the equations for the time evolution of \(\delta[CI]\) and \(\delta[MOR]\),

\[
\begin{pmatrix}
\left( \frac{\partial M_l}{\partial C} \right)^*, & \left( \frac{\partial M_l}{\partial M} \right)^* \\
\left( \frac{\partial C_l}{\partial C} \right)^*, & \left( \frac{\partial C_l}{\partial M} \right)^*
\end{pmatrix}
\begin{pmatrix}
\delta C \\
\delta M
\end{pmatrix} = \frac{1}{\tau_C} \begin{pmatrix}
\left( \frac{\partial M_l}{\partial C} \right)^*, & \left( \frac{\partial M_l}{\partial M} \right)^* \\
\left( \frac{\partial C_l}{\partial C} \right)^*, & \left( \frac{\partial C_l}{\partial M} \right)^*
\end{pmatrix}
\begin{pmatrix}
\delta C \\
\delta M
\end{pmatrix}.
\]
We further abbreviate the notation as
\[
\frac{1}{\tau_M} \left\{ \left( \frac{\partial L}{\partial C} \right) - \left( \frac{\partial M_i}{\partial C} \right) \right\}, \quad \frac{1}{\tau_C} \left\{ \left( \frac{\partial R}{\partial C} \right) - \left( \frac{\partial C_i}{\partial C} \right) \right\}, \quad \frac{1}{\tau_C} \left\{ \left( \frac{\partial R}{\partial M} \right) - \left( \frac{\partial C_i}{\partial M} \right) \right\}
\]
\[
\begin{pmatrix}
\delta C \\
\delta M
\end{pmatrix}
\]
\[
(13)
\]
where we employ the abbreviated notations:
\[
M_i \equiv [\text{MOR}]_{\text{tot}}, \quad M \equiv [\text{MOR}], \quad C_i \equiv [CI]_{\text{tot}}, \quad C \equiv [CI], \quad L \equiv pL, \quad R \equiv pR.
\]

We further abbreviate the notation as
\[
M_{i,C} \equiv \left( \frac{\partial M_i}{\partial C} \right)^*, \quad L_{C} \equiv \left( \frac{\partial L}{\partial C} \right)^* \equiv \left( \frac{\partial pL([CI])}{\partial [CI]} \right)^*, \quad \text{etc.}
\]
then eq. (13) is expressed as
\[
\begin{pmatrix}
M_{i,C} & M_{i,M} \\
C_{i,C} & C_{i,M}
\end{pmatrix}
\begin{pmatrix}
\delta C \\
\delta M
\end{pmatrix}
= \begin{pmatrix}
\frac{1}{\tau_M} L_{C} - M_{i,C} \quad - \frac{1}{\tau_M} M_{i,M} \\
\frac{1}{\tau_C} R_{C} - C_{i,C} \quad \frac{1}{\tau_C} R_{M} - C_{i,M}
\end{pmatrix}
\begin{pmatrix}
\delta C \\
\delta M
\end{pmatrix}
\]
\[
(14)
\]
\[\text{4. The criterion is always valid for Model A:}\]

In the case of Model A, \([\text{MOR} \cdot CI] = 0\), thus we have
\[
C_{i,M} = 0, \quad M_{i,M} = 1, \quad M_{i,C} = 0,
\]
then eq. (14) becomes
\[
\begin{pmatrix}
0 & 1 \\
C_{i,C} & 0
\end{pmatrix}
\begin{pmatrix}
\delta C \\
\delta M
\end{pmatrix}
= \begin{pmatrix}
\frac{1}{\tau_M} L_{C} \quad - \frac{1}{\tau_M} \\
\frac{1}{\tau_C} R_{C} - C_{i,C} \quad \frac{1}{\tau_C} R_{M}
\end{pmatrix}
\begin{pmatrix}
\delta C \\
\delta M
\end{pmatrix}
\]
\[
(15)
\]
Now we assume the solution as
\[
\delta C, \quad \delta M \propto e^{\omega t}
\]
then we have
\[
\begin{pmatrix}
\frac{1}{\tau_M} L_{C} \quad - \frac{1}{\tau_M} - \omega \\
\frac{1}{\tau_C} (R_{C} - C_{i,C}) - C_{i,C} \omega \quad \frac{1}{\tau_C} R_{M}
\end{pmatrix}
\begin{pmatrix}
\delta C \\
\delta M
\end{pmatrix}
= 0.
\]
\[
(16)
\]
The condition that this equation has non-zero solution gives
\[
-C_{i,C} \omega^2 + \left[ \frac{1}{\tau_C} (R_{C} - C_{i,C}) - \frac{1}{\tau_M} C_{i,C} \right] \omega + \frac{1}{\tau_M \tau_C} [L_{C} R_{M} + R_{C} - C_{i,C}] = 0
\]
\[
(17)
\]
If all the solutions $\omega$ have a negative real part, the state is stable, whereas the state is unstable if there is a solution with a positive real part.

Note that the slopes of the production curve and the degradation curve are given by

$$
\frac{d\ pR}{d\ CI} \equiv \left( \frac{d\ pR([MOR],[CI])}{d[CI]} \right)^* = L_c R_M + R_C
$$

(18)

$$
\frac{d\ CI_{tot}}{d\ CI} \equiv \left( \frac{\partial([CI]_{tot}(\ [CI])}{\partial[CI]} \right)^* = C_{t,C}.
$$

(19)

Using these, eq.(17) becomes

$$
-\frac{d\ CI_{tot}}{d\ CI} \omega^2 + \left[ \frac{1}{\tau_C} \left( \frac{d\ pR}{d\ CI} - \frac{d\ CI_{tot}}{d\ CI} - L_c R_M \right) - \frac{1}{\tau_M} \frac{d\ CI_{tot}}{d\ CI} \right] \omega \\
+ \frac{1}{\tau_C \tau_M} \left[ \frac{d\ pR}{d\ CI} - \frac{d\ CI_{tot}}{d\ CI} \right] = 0
$$

(20)

Note that

$L_c, \ R_c, \ R_M < 0.$

(1) The case of $\tau_M \ll \tau_C$. One solution is of order $1/\tau_M$ and the other is of order $1/\tau_C$.

$$
\omega \approx \begin{cases} 
-\frac{1}{\tau_M} \\
\frac{1}{\tau_C} \left[ \frac{d\ CI_{tot}}{d\ CI} \right]^{-1} \left[ \frac{d\ pR}{d\ CI} - \frac{d\ CI_{tot}}{d\ CI} \right] 
\end{cases}.
$$

This shows the criterion (8) is valid.

(2) The case of $\tau_C \ll \tau_M$. One solution is of order $1/\tau_M$ and the other is of order $1/\tau_C$.

$$
\omega \approx \begin{cases} 
\frac{1}{\tau_C} \left[ \frac{d\ CI_{tot}}{d\ CI} \right]^{-1} \left[ \frac{d\ pR}{d\ CI} - \frac{d\ CI_{tot}}{d\ CI} - L_c R_M \right] \\
-\frac{1}{\tau_M} \left[ \frac{d\ pR}{d\ CI} - \frac{d\ CI_{tot}}{d\ CI} - L_c R_M \right]^{-1} \left[ \frac{d\ pR}{d\ CI} - \frac{d\ CI_{tot}}{d\ CI} \right]
\end{cases}.
$$

The criterion (8) is also valid because

$$
\frac{d\ CI_{tot}}{d\ CI} > 0, \quad L_c R_M > 0.
$$
(3) General case. The criterion (8) can be shown to be valid because eq.(17) always has two real solutions, \( \omega_1 \) and \( \omega_2 \), and the sum of the two solutions is negative when the slope of the production curve is less steep than that of the degradation curve, i.e.

\[
\omega_1 + \omega_2 < 0 \quad \text{when} \quad \frac{d pR}{dCI} - \frac{dCI_{\text{tot}}}{dCI} < 0.
\]

The positivity of the discriminant \( D \) of eq.(17):

\[
D = \left[ \frac{1}{\tau_C} \left( R_C - C_{i,C} \right) - \frac{1}{\tau_M} C_{i,C} \right]^2 + 4C_{i,C} \frac{1}{\tau_M \tau_C} \left[ L_C R_M + R_C - C_{i,C} \right] = \left[ \frac{1}{\tau_C} R_C - \left( \frac{1}{\tau_C} - \frac{1}{\tau_M} \right) C_{i,C} \right]^2 + \frac{4}{\tau_C \tau_M} C_{i,C} R_M L_C > 0,
\]

thus eq.(17) has two real solutions.

5. The criterion is valid for Model B as long as the degradation curve has positive slope, but the state may be unstable otherwise.

In this model, the existence of \( MOR \cdot CI \) makes the expressions for the slopes of the production curve and the degradation curve a bit more complicated:

\[
\frac{d pR}{dCI} \equiv \frac{d}{d[CI]} pR\left([MOR],[M],[CI]\right) = \left( \frac{\partial R}{\partial C} \right)_M + \left( \frac{\partial R}{\partial M} \right)_C \left[ \left( \frac{\partial M}{\partial M}_M \right)_C \left( \frac{\partial L}{\partial C} \right)_M + \left( \frac{\partial M}{\partial C} \right)_M \right] = \left( \frac{\partial R}{\partial C} \right)_M + \left( \frac{\partial R}{\partial M} \right)_C \left( \frac{\partial M}{\partial M}_C \right)^{-1} \left[ \left( \frac{\partial L}{\partial C} \right)_M - \left( \frac{\partial M}{\partial C} \right)_M \right] \right.
\]

\[
= R_C + \frac{R_M}{M_{i,M}} \left( L_C - M_{i,C} \right) \tag{21}
\]

\[
\frac{d CI_{\text{tot}}}{dCI} \equiv \frac{d}{d[CI]} [CI]_{\text{tot}}\left([CI],[MOR],[M],[CI]\right) = \left[ \frac{\partial C_i}{\partial C} \right]_M + \left( \frac{\partial C_i}{\partial M} \right)_C \left[ \left( \frac{\partial M}{\partial M}_M \right)_C \left( \frac{\partial L}{\partial C} \right)_M + \left( \frac{\partial M}{\partial C} \right)_M \right] = \left[ \frac{\partial C_i}{\partial C} \right]_M + \left( \frac{\partial C_i}{\partial M} \right)_C \left( \frac{\partial M}{\partial M}_C \right)^{-1} \left[ \left( \frac{\partial L}{\partial C} \right)_M - \left( \frac{\partial M}{\partial C} \right)_M \right] \right.
\]

\[
= C_{i,C} + \frac{C_{i,M}}{M_{i,M}} \left( L_C - M_{i,C} \right), \tag{22}
\]
where the variables that kept constant upon partial differentiation are explicitly indicated as
\[
\left( \frac{\partial M}{\partial M_t} \right)_C \equiv \frac{\partial}{\partial [MOR]_{\text{tot}}} [MOR] ([MOR]_{\text{tot}}, [CI])
\]
whenever it could be ambiguous. In the derivation, we have used the relations
\[
\left( \frac{\partial M}{\partial M_t} \right)_C = \left( \frac{\partial M}{\partial M_t} \right)^{-1}_C, \quad \left( \frac{\partial M}{\partial C} \right)_M, \quad \left( \frac{\partial M}{\partial M_t} \right)_C \left( \frac{\partial C}{\partial M_t} \right)_M = -1.
\]

5.1. Stability of the system with PL with externally controlled \([CI]_{\text{tot}}\)

First, we will examine the stability of the system without PR, CI being provided externally. The system is shown to be bistable for some parameter region.

Based upon the approximation that the relaxation in the solution is much faster than the protein production rate by PL, we consider the system where \([CI]_{\text{tot}}\) and \([MOR]_{\text{tot}}\) satisfy
\[
[CI]_{\text{tot}} = [CI]_{\text{tot}}([CI], [MOR]),
\]
\[
\frac{d}{dt} [MOR]_{\text{tot}} = \frac{1}{\tau_M} (pL([CI]) - [MOR]_{\text{tot}}),
\]
thus the deviation from the steady state follows
\[
C_t, C_\delta C + C_{t,M} \delta M = 0
\]
\[
M_{t,C} \delta C + M_{t,M} \delta M = \frac{1}{\tau_M} (L_C \delta C - M_{t,C} \delta C - M_{t,M} \delta M),
\]
which results in
\[
\delta \dot{C} = \frac{1}{\tau_M} \frac{(L_C - M_{t,C}) C_{t,M} + M_{t,M} C_{t,C}}{M_{t,C} C_{t,M} - M_{t,M} C_{t,C}} \delta C
\]
\[
= -\frac{1}{\tau_M} \frac{M_{t,M}}{A} \frac{d[CI]_{\text{tot}}}{dCI} \delta C,
\]
with the notation
\[
A \equiv C_{t,C} M_{t,M} - C_{t,M} M_{t,C} > 0,
\]
whose inequality can be shown from the actual expressions of \([CI]_{\text{tot}}\) and \([MOR]_{\text{tot}}\), (3) and (4).

Therefore, we have
\[
\frac{d[CI]_{\text{tot}}}{dCI} < 0 \quad \text{unstable}
\]
\[
\frac{d[CI]_{\text{tot}}}{dCI} > 0 \quad \text{stable.}
\]
5.2. Stability of genetic switch with PL and PR:

The growth rate $\omega$ is determined by the characteristic equation

$$\begin{vmatrix}
\frac{1}{\tau_M}(L_C - M_{i,C}) - M_{i,C} \omega, & -\frac{1}{\tau_M}M_{i,M} - M_{i,M} \omega \\
\frac{1}{\tau_C}(R_C - C_{i,C}) - C_{i,C} \omega, & \frac{1}{\tau_C}(R_M - C_{i,M}) - C_{i,M} \omega
\end{vmatrix} = 0,$$

which can be expanded as

$$\omega^2 \left[M_{i,C}C_{i,M} - C_{i,C}M_{i,M}\right] + \omega \left[\frac{1}{\tau_C} \left(-M_{i,C}(R_M - C_{i,M}) + M_{i,M}(R_C - C_{i,C})\right) - \frac{1}{\tau_M} \left(C_{i,M}(L_C - M_{i,C}) + C_{i,C}M_{i,M}\right)\right]$$

$$+ \frac{1}{\tau_M \tau_C} \left((L_C - M_{i,C})(R_M - C_{i,M}) + (R_C - C_{i,C})M_{i,M}\right) = 0. \quad (29)$$

This can be put in the form

$$-\omega^2 \frac{A}{M_{i,M}} + \omega \left[\left(\frac{1}{\tau_C} \left(-\frac{dR}{dC} - \frac{dCI_{tot}}{dC}\right) - \frac{1}{\tau_M} \frac{dCI_{tot}}{M_{i,M}}\right) - \frac{1}{\tau_C} \left(\frac{L_C}{M_{i,M}}(R_M - C_{i,M})\right)\right]$$

$$+ \frac{1}{\tau_M \tau_C} \left[\frac{dR}{dC} - \frac{dCI_{tot}}{dC}\right] = 0. \quad (30)$$

This is almost the same with the corresponding equation for Model A eq.(20).

(1) The case of $\tau_M \ll \tau_C$. One solution is of order $1/\tau_M$ and the other of order $1/\tau_C$:

$$\omega \approx \left\{\begin{array}{c}
\frac{1}{\tau_M} \frac{M_{i,M}}{A} \left(\frac{dCI_{tot}}{dC}\right) \\
\frac{1}{\tau_C} \left(\frac{dR}{dC} - \frac{dCI_{tot}}{dC}\right) \left(\frac{dCI_{tot}}{dC}\right)^{-1}
\end{array}\right. \quad (31)$$

Therefore, the criterion (8) is valid as long as the slope of degradation curve is positive, i.e. $(dCI_{tot}/dC) > 0$, but the state is always unstable for the negative slope for the degradation curve, i.e. $(dCI_{tot}/dC) < 0$.

(2) The case of $\tau_C \ll \tau_M$.

$$\omega \approx \left\{\begin{array}{c}
\frac{1}{\tau_C} \frac{M_{i,M}}{A} \left[\left(\frac{dR}{dC} - \frac{dCI_{tot}}{dC}\right) - \frac{L_C}{M_{i,M}}(R_M - C_{i,M})\right] \\
-\frac{1}{\tau_M} \left[\left(\frac{dR}{dC} - \frac{dCI_{tot}}{dC}\right) - \frac{L_C}{M_{i,M}}(R_M - C_{i,M})\right]^{-1} \left(\frac{dR}{dC} - \frac{dCI_{tot}}{dC}\right)
\end{array}\right. \quad (32)$$

In this case, the criterion (8) is always valid because

$$\frac{L_C}{M_{i,M}}(R_M - C_{i,M}) > 0, \quad \frac{M_{i,M}}{A} > 0.$$
(3) General case. We can show eq. (30) always has two real solutions, \( \omega_1 \) and \( \omega_2 \), and the steady state stability is determined from the sign of \( \omega_1 + \omega_2 \):

\[
\frac{d\ pR}{d\ CI} - \frac{d\ CI_{tot}}{d\ CI} < 0, \quad \frac{d\ CI_{tot}}{d\ CI} > 0 \quad \text{then stable}
\]

\[
\frac{d\ pR}{d\ CI} - \frac{d\ CI_{tot}}{d\ CI} > 0, \quad \text{then unstable}
\]

but

\[
\frac{d\ pR}{d\ CI} - \frac{d\ CI_{tot}}{d\ CI} < 0, \quad \frac{d\ CI_{tot}}{d\ CI} < 0 \quad \text{stable/unstable.}
\]

This means that the stability cannot be determined only from the slopes of the production and the degradation curves in the case the degradation curve has a negative slope.

Appendix: The positivity of the discriminant \( D \) of eq. (29):

\[
D = \left[ \frac{1}{\tau_C} \left( -M_t C_t(R_c - C_t) + M_{t,M}(R_c - C_t) \right) \right. \\
\quad \left. - \frac{1}{\tau_M} \left( C_t M_t(L_c - M_t) + C_t M_t \right) \right]^2 \\
\quad - \frac{4}{\tau_M \tau_C} \left[ M_t C_t M_{t,M} - C_t M_{t,M} \right] \left( L_c - M_t \right) (R_c - C_t) M_{t,M} \\
\quad = \left[ \frac{1}{\tau_C} \left( A - R_{\ t,M} M_t + R_{\ t,C} M_t \right) - \frac{1}{\tau_M} \left( A + L_c C_{t,M} \right) \right]^2 \\
\quad + \frac{4}{\tau_M \tau_C} A \left[ -A + L_c R_{\ t,M} - L_c C_{t,M} - R_{\ t,M} M_{t,C} + R_{\ t,C} M_{t,M} \right] \\
\quad = \left[ -A \left( \frac{1}{\tau_C} + \frac{1}{\tau_M} \right) + \frac{1}{\tau_C} \left( -R_{\ t,M} M_t + R_{\ t,C} M_t \right) - \frac{1}{\tau_M} L_c C_{t,M} \right]^2 \\
\quad + \frac{4}{\tau_M \tau_C} A \left[ -A + L_c R_{\ t,M} - L_c C_{t,M} - R_{\ t,M} M_{t,C} + R_{\ t,C} M_{t,M} \right] \\
\quad = A^2 \left[ \frac{1}{\tau_C} - \frac{1}{\tau_M} \right]^2 + 2A \left[ \frac{1}{\tau_C} + \frac{1}{\tau_M} \left( \frac{1}{\tau_C} \left( R_{\ t,M} M_t - R_{\ t,C} M_t \right) + \frac{1}{\tau_M} L_c C_{t,M} \right) \right. \\
\quad \left. + \frac{2}{\tau_M \tau_C} \left( L_c R_{\ t,M} - L_c C_{t,M} - R_{\ t,M} M_{t,C} + R_{\ t,C} M_{t,M} \right) \right] \\
\quad + \left( \frac{1}{\tau_C} (-R_{\ t,M} M_t + R_{\ t,C} M_t) - \frac{1}{\tau_M} L_c C_{t,M} \right)^2 \\
\]

The coefficient of 2A is

\[
(R_{\ t,M} M_t - R_{\ t,C} M_t) \left( \frac{1}{\tau_C} - \frac{1}{\tau_M} \right) \frac{1}{\tau_C} + L_c C_{t,M} \left( -\frac{1}{\tau_C} + \frac{1}{\tau_M} \right) \frac{1}{\tau_M} + L_c R_{\ t,M} \frac{2}{\tau_M \tau_C}.
\]
Thus, $D$ is

$$D = A^2 \left( \frac{1}{\tau_C} - \frac{1}{\tau_M} \right)^2 + 2A \left[ \left( \frac{1}{\tau_C} - \frac{1}{\tau_M} \right) \left( \frac{1}{\tau_C} (R_M M_{t,c} - R_C M_{t,M}) - \frac{1}{\tau_M} L_C C_{t,M} \right) + \frac{2}{\tau_M \tau_C} L_C R_M \right]$$

$$+ \left( \frac{1}{\tau_C} (R_M M_{t,c} - R_C M_{t,M}) + \frac{1}{\tau_M} L_C C_{t,M} \right)^2$$

$$= \left[ \left( \frac{1}{\tau_C} - \frac{1}{\tau_M} \right) A + \left( \frac{1}{\tau_C} (R_M M_{t,c} - R_C M_{t,M}) - \frac{1}{\tau_M} L_C C_{t,M} \right) \right]^2$$

$$- \left( \frac{1}{\tau_C} (R_M M_{t,c} - R_C M_{t,M}) - \frac{1}{\tau_M} L_C C_{t,M} \right)^2 + 2A \frac{2}{\tau_M \tau_C} L_C R_M$$

$$+ \left( \frac{1}{\tau_C} (R_M M_{t,c} - R_C M_{t,M}) + \frac{1}{\tau_M} L_C C_{t,M} \right)^2$$

$$= \left[ \left( \frac{1}{\tau_C} - \frac{1}{\tau_M} \right) A + \left( \frac{1}{\tau_C} (R_M M_{t,c} - R_C M_{t,M}) - \frac{1}{\tau_M} L_C C_{t,M} \right) \right]^2$$

$$+ \frac{4}{\tau_C \tau_M} L_C \left( (R_M M_{t,c} - R_C M_{t,M}) C_{t,M} + (C_{t,c} M_{t,M} - C_{t,M} M_{t,c}) R_M \right)$$

The second line of the last expression is shown to be positive as follows:

Note that

$$C_{t,c} = 1 + C_{2,c} + MC_{C}, \quad C_{t,M} = MC_{C}, \quad M_{t,c} = MC_{C}, \quad M_{t,M} = 1 + MC_{C},$$

$$R_C = R' \cdot MC_{C}, \quad R_M = R' \cdot MC_{C},$$

where the abbreviated notations are used,

$$C_{2,c} \equiv \frac{\partial [C_{t,c}]}{\partial [C]}, \quad MC_{C} \equiv \frac{\partial [MOR \cdot C]}{\partial [MOR]}, \quad \text{etc. and} \quad R' \equiv \frac{\partial pR}{\partial [MOR \cdot C]}.$$

Then the second line of $D$ is

$$L_C \left( (R_M M_{t,c} - R_C M_{t,M}) C_{t,M} + (C_{t,c} M_{t,M} - C_{t,M} M_{t,c}) R_M \right)$$

$$= L_C \left( -R_C M_{t,M} C_{t,M} + C_{t,c} M_{t,M} R_M \right)$$

$$= L_C M_{t,M} (-R_C C_{t,M} + R_M C_{t,c})$$

$$= L_C M_{t,M} (-R' \cdot MC_{C} M_{t,M} + R' \cdot MC_{C} (1 + C_{2,c} + MC_{C}))$$

$$= L_C R' \cdot MC_{C} (1 + C_{2,c}) > 0$$
Therefore,

\[ D > 0. \]