Stability Puzzles in Phage λ

Erik Aurell¹, Stanley Brown², Johan Johanson³ and Kim Sneppen⁴

February 1, 2008

¹ Dept. of Mathematics, Stockholm University, SE-106 91 Stockholm, Sweden
² Dept. of Molecular Cell Biology, University of Copenhagen,
   Øster Farimagsgade 2A, DK-1353 Copenhagen K, Denmark
³ Dept. of Microelectronics and Nanoscience, CTH, SE-412 96 Göteborg, Sweden
⁴ NORDITA, Blegdamsvej 17, DK-2100 Copenhagen, Denmark

Keywords: Gene regulation, stability, robustness, phage lambda, lysogeny

Corresponding author K. Sneppen, NORDITA
Blegdamsvej 17, DK-2100 Copenhagen, Denmark
phone +45 3532 5365, fax +45 3538 9157
e-mail sneppen@nbi.dk
Abstract

In the absence of RecA-mediated cleavage of the repressor, the λ prophage is exceptionally stable. In fact, the repressed state is then more stable than the gene encoding the repressor. We develop a mathematical treatment that predicts the stability of such epigenetic states from affinities of the molecular components. We apply the model to the behavior of recently published mutants of $O_R$ and find that their observed stability indicates that the current view of the $O_R$ switch is incomplete. The approach described here should be generally applicable to the stability of expressed states.

1 Introduction

The lysogeny maintenance switch in phage λ can be viewed as one of the simplest examples on the molecular level of computation, command and control in a living system. If, following infection of the bacterium *Escherichia coli*, the virus enters the lysogenic pathway, it represses its developmental functions, in response to a small set of sensory inputs, and integrates its DNA into the host chromosome. In this state the prophage may be passively replicated for many generations of *E. coli*.

Established lysogeny is maintained by the protein CI which blocks operators $O_R$ and $O_L$ on the λ DNA, and thereby prevents transcription of all lytic genes including *cro* (Ptashne, 1992). In lysogeny the CI concentration functions as a sensor of the state of the bacterium: if DNA is damaged the protease activity of RecA is activated, leading to degradation of CI. A low CI concentration allows for transcription of the lytic genes, starting with *cro*, the product of which is the protein Cro.

According to the picture so beautifully conveyed by Ptashne (Ptashne, 1992), the λ lysogeny maintenance switch is centered around operator $O_R$, and consists of three binding sites $O_R1$, $O_R2$ and $O_R3$, each of which can be occupied by either a Cro dimer or a CI dimer. As illustrated in Fig. 1 these three binding sites control the activity of two promoters $P_{RM}$ and $P_R$ for respectively *cI* and *cro* transcription. Transcription of *cro* starts at $P_R$, which partly overlaps $O_R1$ and $O_R2$. Transcription of *cI* starts at $P_{RM}$, which partly overlaps
OR2 and OR3. The affinity of RNA polymerase for the two promoters, and subsequent production of the two proteins, depends on the pattern of Cro and CI bound to the three operator sites and thereby establishes lysogeny with about 200 CI molecules per bacterium. If, however, CI concentration becomes sufficiently low, the increased production of Cro throws the switch to lysis.

There does not seem to be a quantitative theoretical understanding of how stable the lysogenic state is to spontaneous switching. We will demonstrate that the large stability observed experimentally puts constraints on the mechanism of the switch, constraints which we explore in a quantitative model. We will show that in order to span the timescales from single molecule on–off binding times of order about 10 sec, to the stability of order 10^{12} sec, a simple model, where lysis occurs every time cro is transcribed, is inadequate. We elaborate a dynamical model that takes into account all known mechanisms of OR, including in particular nonspecific binding between Cro dimers and E. coli DNA. We can then reproduce the observed high stability. However, we will see that the recently reported robustness of the switch against mutational changes of the operators (Little et al., 1999) suggest that an additional mechanism of stability must be present.

Bistable switches are expected to be important functional units in many systems of control of gene expression (Monot & Jacob, 1961, Glass & Kauffman, 1973). One general consequence of the mathematical model presented here is, that in living systems such switches can often only be of finite stability, on account of the small number of molecules involved. The model is hence also relevant to the stability of data storage in synthetic gene-regulatory networks with similar characteristics, in particular if intended for use in biotechnology or gene therapy (Gardner et al, 2000).

2 Experiments on lysogen stability

Repression of bacteriophage λ can be eliminated either by cleavage of the repressor by the RecA protease (reviewed by Roberts & Devoret, 1983) or by the absence of repressor at the operators. The frequency of spontaneous induction in strains deleted for the recA gene has recently been reported.

3
Rozanov et al., (1998) found the frequency of infectious centers to be $1.1 \cdot 10^{-7}$. Little et al., (1999) measured the ratio of released phage per lysogen and the burst size. They estimate the frequency of one cell to switch to the lytic state to be $4 \cdot 10^{-7}$ per generation, assuming the switch occurred one hour before lysis. Little et al. verified the lysogens contained only a single prophage by PCR. Rozanov et al., (1998) do not report testing the number of prophages in the lysogens they used. We repeated these measurements using single lysogens (Powell et al., 1994) of MG1655 (Jensen, 1993) containing the del(srl-recA)306::Tn10 allele (Csonka et al. 1979). We measured the frequency of switching to the lytic state by measuring the appearance of turbid plaques in a limiting dilution experiment at 37°C in YT broth (Miller, 1972). We find that lysed cells to appear with frequency between 1.0 and $1.8 \cdot 10^{-8}$, which, if we include the same correction factor as (Little et al., 1999) to account for the increase in cell numbers during the period of phage development, implies a rate in the range of $3.5 \cdot 10^{-8}$ and $6.0 \cdot 10^{-8}$ per generation and cell. Thus, all three sets of measurements fall within a factor of 10, in spite of the use of different strain backgrounds.

Recent results of J. Little (Univ. of Arizona, personal communication) show that at 37°C about 99% of the turbid plaques are not wild type, but instead a marginally stable PRM240 mutant. As a consequence, Little estimates that wild type $\lambda$ phages inside E. coli cells grown on nutrient rich medium have a frequency of spontaneous lysis that does not exceed $2 \cdot 10^{-9}$ per generation and cell.

### 3 Elements of a model

The considerations in this section are in part closely similar to the model of (Shea & Ackers, 1985). The main difference is that our interest is in longer time scales, and in the stability of the switch against spontaneous stochastic fluctuations. We will therefore begin with a discussion of characteristic time scales, then discuss chemistry, production and decay. The stochastic element is included in a definite model, see next section.

**Time scales:** The measured half lives of CI and Cro dissociation from $O_R1$ at 22°C are respectively 34 sec for CI (Nelson & Sauer, 1985), and varies
from 1200 sec for Cro (Jana et al., 1998) dissociation from $O_R$ alone down to 20 sec (Kim et al. (1987)) from OR flanked by 1kb of $\lambda$–DNA. At equilibrium dissociation balances association, and thus the time it takes a CI or Cro dimer to bind to the operator is

$$\tau_1 = \tau_{\text{off}} V [1M] \exp(\Delta G/RT) \quad (1)$$

where $\Delta G$ is the free energies of binding, which for CI and Cro are tabulated in Table 1, and $RT = 0.617 \text{kcal/mol}$. Assuming a bacterial volume of $V = 2 \cdot 10^{-15} l$ (Bremmer et al., 1996) we have $V[1M] = 12 \cdot 10^8$, and can then estimate the association rates of single molecules to be of order tens of seconds, for both CI and Cro. This is compatible with diffusion-limited association into a region of size $L = 1$ nm, at a rate determined by the Smoluchowski equation (Berg et al., 1982) $\tau_1 = V/(4\pi DL)$, using a diffusion constant $D = 10^{-11} m^2/s$, similar to that of other proteins in bacterial cytoplasm (Elowitz et al., 1999). The association rate from $N$ molecules is proportional to $N$, and thus for a bacterium with 10-200 CI and Cro molecules association events will occur at a frequency of fractions of a second, for both CI and Cro.

The shortest time scale in which we are actually interested is that which it takes for a switching event to take place. This cannot be much less than those of significant changes in CI and Cro concentrations, which are of order one bacterial generation, 34 minutes in the strains used in (Little et al., 1999). Hence, all binding-unbinding events of Cro and CI can be assumed to take place in homeostatic equilibrium. In addition, we need to characterize RNA polymerase binding and initiation of transcription from promotor sites $P_{RM}$ and $P_R$. As discussed below (see Production) these events also have to occur on time scales much shorter than one bacterial generation, and can therefore be described by their overall rates. We note that a more detailed description of RNA polymerase action may be necessary to resolve the shorter timescales associated to the lysis-lysogeny entry decision, as discussed in modelling by McAdams, Arkin and Ross (1997,1998).

**Chemistry:** We assume that CI and Cro molecules in the cell are in homeostatic equilibrium. This does not mean that there is always the same number of CI dimers bound to the operators at any particular time. These numbers
are fluctuating, and the equilibrium assumption gives the size of these fluctuations. The key inputs are CI and Cro dimerization constants and the Gibbs free energies for their bindings to the three operator sites OR1, OR2 and OR3 (Koblan & Ackers, 1992; Takeda et al., 1989, Kim et al. (1987), Jana et al. (1997,1998)), see Table 1.

We remark that these free energies are taken from in vitro studies, and that the in vivo conditions could be different, e.g. the measured protein-DNA affinities could depend sensitively on the ions present in the buffer solutions. On the other hand, in vivo the effects of such changes should be compensated for, as e.g. changed KCl concentrations are by putrescine (Capp et al. (1996)) and other ions and crowding effects (Record et al. (1998)) The data quoted in Table 1 was obtained at KCl concentration of 200mM, which most closely resembles in vivo conditions. Parameter sensitivity is analysed separately below.

Following Ackers et al. (1982), we encode a state s of CI and/or Cro bound to OR by three numbers (i, j, k) referring to respectively OR3 OR2 and OR1. The coding is 0 if the corresponding site is free, 1 if the site is occupied by a CI dimer, and 2 if the site is occupied by a Cro dimer. The probability of a state s with i CI dimers and j Cro dimers bound to OR is in the grand canonical approach of Shea and Ackers (Shea & Ackers, 1985)

\[
P_R(s) = N^{-1}[\text{CI }]^i[\text{Cro }]^j e^{-G(s)/T}
\]  

where \(N\) is the total number of molecules of this type, \(N_\lambda\) and \(N_{\text{chr}}\) the average number of copies present of the \(\lambda\) genome and of the full E. coli chromosome, \(n_U\) the number of dimers bound nonspecifically to one chromosome DNA and \(n_M\) the number of free monomers. We assume free dimers to be in equilibrium with free monomers and with dimers bound nonspecifically
to DNA, such that \( n_M \) and \( n_U \) can be expressed in terms of \( n_D \), volume, and dimerization and association constants. In this paper we take \( N_\lambda \) and \( N_{\text{chr}} \) both equal to 3 \([5]\). In our analysis we have mostly assumed that CI does not bind nonspecifically to DNA, while Cro does so with considerable affinity. The effect of a putative nonspecific CI binding to DNA is analysed separately, see Table 1 and \([5]\). Note that we also include occupancy of \( O_L \) in order to account for finite depletion of CI, which has some significance for the fractional binding at \( O_R \) when the total number of CI molecules is small.

Parameters for \( O_L \) are listed in caption to Table 1.

In Table \([4]\) we have calculated \( \mathcal{P}(s) = \mathcal{P}(s \mid N_{\text{CI}} = 200, N_{\text{Cro}} = 0) \). Of particular interest for the stability are the states in which cro can be transcribed, i.e. \((000), (100) \) and \((200) \). The numbers in the table show that cro is always transcribed at some rate in lysogeny, which if every cro transcription induced lysis, would destabilize the lysogenic state within a few generations. In Fig. 2a we show \( P(0,0,0) + P(1,0,0) + P(2,0,0) \) as function of \( N_{\text{CI}} \) at \( N_{\text{Cro}} = 0 \). We remark that Fig. 2a corresponds to the scaling of \( P_R \) promoter strength in the in vitro experiment of Hawley & McClure (1983). The small gap in CI number before full activation of \( P_{\text{RM}} \) at low CI in Fig. 2a originates in part from finite system filling of \( O_R \) and \( O_L \).

**Production:** CI and Cro are produced from mRNA transcripts of \( cI \) and \( cro \), which are initiated from promotor sites \( P_{\text{RM}} \) and \( P_R \). The rate of initiation of transcription from \( P_{\text{RM}} \) when stimulated by CI bound to \( O_R \) is denoted \( R_{\text{RM}} \), and when not stimulated \( R_{\text{RM}}^u \). The number of CI molecules produced per transcript is \( S_{cI} \), and overall expected rate of production of CI is

\[
\text{Overall CI rate} = R_{\text{RM}} S_{cI} (P(010) + P(011) + P(012)) + R_{\text{RM}}^u S_{cI} (P(000) + P(001) + P(002) + P(020) + P(021) + P(022))
\]

For the relative values of the coefficients, Hawley & McClure (1982) report \( R_{\text{RM}}^u \) to be equal to \( R_{\text{RM}} / 11 \). The absolute values are not known, and are likely to at least be proportional to the concentration of RNA polymerase in the cell. We discuss how we determine \( R_{\text{RM}} \) below. According to (Shean & Gottesman, 1992) the number of CI molecules produced from one transcript
of CI is a factor 20 – 70 smaller compared to lacZ. Thus $S_{CI}$ is small, between one and five, and we take $S_{CI}$ to be one.

As there are about 200 CI in a lysogenic cell, and as each transcription only results in a small number of CI molecules, transcription has to be initiated from $P_{RM}$ many times per generation in lysogeny. We can therefore safely assume that the time scales for RNA polymerase to find the operator sites is also much less than one generation. This justifies our description of production by the overall rates, without distinguishing RNA polymerase binding, the rate of formation of open complexes, and possible temporary stalling of RNA polymerase at promoter sites. Fig. 2b shows average CI production according to (4) as function of CI number in the cell, volume held constant. We note that the overall behaviour resembles the in vitro measurements of Hawley & McClure (1983).

The rate of initiation of transcription from promoter $P_R$ is denoted $R_R$ and the number of Cro molecules per transcript $S_{cro}$. From Ringquist et al., 1992 one can estimate $S_{cro}$ to be 51% of ideal lacZ and (Kennel & Riezman, 1977) thus allow a lower estimate of $S_{cro}$ to be 20. Thus, in contrast to the many small production events of CI , Cro production is intermittent. The ratio of $R_R$ to $R_{RM}$ has been reported to be between 1.3 and 20, and depends strongly on RNA polymerase concentration in the cell (Hawley & McClure, 1982). In practice we determine $R_{RM}$ by balancing CI production and decay, as discussed below, and use $R_R$ as a free parameter. We end this part by stressing that $R_R$, $R_{RM}$, and $R^{u}_{RM}$ parametrize the total production rates from all copies of λ DNA in the cell. The real rates of transcription per $O_R$ complex will generally be smaller, e.g. about a factor $N_\lambda \sim 3$ smaller at high growth rates.

**Decay:** Concentrations decay due to dilution and degradation. For CI we only take into account dilution from cell growth and division, while for Cro we include in addition an *in vivo* half life time $t_{cro}$ of about one hour at 37°C (Pakula et al., 1986), which is significant compared to *e.g.* generation time of 34 minutes in the experiments of Little et al., 1999.

**The steady state:** Balance between production and decay of CI over one
generation, where production is given by \( \frac{N_{CI}}{t_{lye}} \) and decay by \( \frac{N_{CI}}{t_{lye}} \), gives one equation for the parameter \( R_{RM} \), if the total numbers of CI and Cro in lysogeny are assumed known. Fig. 2b displays the steady state as the intersection between the linear dilution and the \( P_{RM} \) activity curve, with 200 CI and no Cro molecules in the system.

The average number of Cro molecules in lysogens has not been measured, but in the models considered here this number is not zero. Equation (4) below gives the average rate of Cro production. If we assume that \( R_{R} \) is not very different from \( R_{RM} \), as Shea & Ackers (1985), table III fixes the rate of cro transcription in lysogeny to be once in five to ten generations, and if we assume \( R_{R} \) to be an order of magnitude larger than \( R_{RM} \), then cro is transcribed on average once per generation. In both cases, this production is balanced by Cro decay to give an average number of Cro. Hence, the balance of only CI production and decay does not completely determine \( R_{RM} \), since the probabilities depend parametrically on the Cro concentrations, which in turn depend on \( R_{R} \). However, the implied dependence of \( R_{RM} \) on \( R_{R} \) is not very pronounced. In the model presented below we fit \( R_{RM} \) and \( R_{R} \) to data, where the value of 0.115 for \( R_{RM} \) is not very sensitive to changes in model assumptions.

4 The model

The basic time scale in our model is one bacterial generation, where we resolve shorter times around cro transcription events when necessary. A simulation goes as follows. Each bacterial generation starts with \( N_{CI} \) total number of CI, and \( N_{Cro} \) total number of Cro. From these one computes by equation (4) above the occupation probabilities \( P_{s} \) of the states \( s \) of CI and Cro dimers bound to \( O_{R} \), and from these the average CI and Cro production rates \( f_{CI} \) and \( f_{Cro} \). The first is computed by equation (4) above, and the second by

\[
\text{Overall Cro rate} = S_{cro} R_{R} (P_{000} + P_{100} + P_{200})
\]

CI production is assumed continuous, since \( S_{CI} \) is about one. Cro production is on the other hand treated as discrete events. In each generation the bacterial volume \( V \) is assumed to start at \( V_{start} = \frac{2}{3} V_{ave} \), and then to grow linearly until it has doubled. \( V_{ave} = 2 \cdot 10^{-15} l \) is the average volume over
The growth of bacterial DNA is treated similarly, with an average of 3 DNA chromosomes (∼ $15 \cdot 10^6$ base pairs per cell).

1) The rates $f_{CI}$ and $f_{Cro}$ are computed with the current values of $N_{CI}$, $N_{Cro}$ and $V$. cro transcription events are assumed Poisson distributed with mean waiting time $S_{cro}/f_{Cro}$. A random time $t_r$ is drawn from this distribution. If this time is less than the remaining time of the current bacterial generation, one cro transcription will occur, and we continue the simulation under point 2b) below. Else, no more cro transcription will take place in this generation, and we continue under 2a).

2a) The time since beginning of the generation, or since last cro transcription in the generation, is $t$. We update CI numbers by the expected number produced in the interval $t$, $f_{CI} \cdot t$, plus $\xi$, a random number drawn from a Gaussian distribution with zero mean and variance $f_{CI} \cdot t$. We update Cro number by removing each Cro molecule with a probability $p = p(t) = 2^{-t/t_{Cro}}$, and then proceed to cell division under point 3) below.

2b) We update CI number and degrade Cro number as above, 2a), but under time $t_r$. The number of Cro molecules produced from this cro transcription is a random variable drawn from a Poisson distribution of mean $S_{cro}$, and is added to the remaining Cro. We now return back to point 1) above for the remaining time of the current generation.

3) This is the stage of cell division. The volume of a daughter cell is restarted at $V_{start}$, and will contain each CI and Cro molecule of the mother cell with probability one half. If CI number has shrunk below a threshold, a switch to the lytic pathway has occurred, and we leave the loop. Otherwise we return to 1) with the new values of CI and Cro.

The expected number of generations before lysis is calculated by an average over at least 10 independent trajectories. The value of the threshold in CI number is unimportant, provided it is chosen sufficiently low. In the model presented here lysis practically always follows if $N_{CI}$ goes below 20.
5 Results

Standard parameter behaviour: The two parameters $R_{RM}$ and $R_R$ have been adjusted such that the average number of CI molecules in lysogeny is 200, and the mean rate of spontaneous lysis is $\sim 2 \cdot 10^{-9}$ generations. The other parameters values are discussed above in section 3. The values thus obtained are $R_{RM} = 0.115 \text{sec}^{-1}$ and $R_R = 0.30 \text{sec}^{-1}$. The ratio between these two rates is hence about three, well within the limits set by Hawley & McClure (1982) but larger than the ratio 1.3 used in the model by (Shea & Ackers, 1985).

For these parameters we show in Fig. 3 the last few hundred generations before lysis in a typical simulation. The numbers of CI and Cro fluctuate for a long time around the metastable lysogenic state. A particularly large fluctuation (of both CI and Cro) then builds up over a few generations, leading to lysis. This kind of switching event is similar to the escape over an activation barrier as in the well-known diffusion model of chemical reactions. Thus, spontaneous lysis is the result of a number of unlikely events happening in a given short time interval. If each event has probability $p < 1$, and lysis depends on $n$ events, the resulting frequency of lysis is $\sim p^n$. This frequency is therefore very sensitive to $p$, that means to parameters in the model.

In Fig. 4 we explore the activation barrier picture in a more detailed way by computing the probability that the system over time visits various values of $N_{CI}$ and $N_{Cro}$. The distribution has a maximum around the lysogenic state, as it should. From there to the origin, along the $N_{CI}$ axis, the probability becomes almost vanishingly small, which reflects the fact that a fluctuation of CI alone, without Cro, cannot induce lysis. The switch instead happens along a ridge in the $(N_{CI}, N_{Cro})$ plane, where probability does not go down so quickly. In Fig. 4b we examine the tail of the distribution in Fig. 4a. One observes a saddle for high Cro number and $N_{CI}$ about 20 – 25. For the parameter values used here, lysis follows with 50% probability, as soon as $N_{CI}$ goes below 23. It is natural to assume that lysogeny is separated from lysis by a basin boundary (a separatrix), which runs close to parallel to the $N_{Cro}$ axis around the saddle point. The value of Cro at the saddle can be varied from about a hundred to well over a thousand in the model, and depends sensitively on the numerical values of Cro affinities to specific and nonspecific
DNA.

**Parameter sensitivity:** A main objective to build a model for stability is to address which key quantities that determines the stability. As we have already discussed, stability is governed by near simultaneous occurrences of many rare events. Consequently, the stability will be very sensitive to the probability of these events, which in turn depend sensitively on parameters, in particular of the parameters describing Cro production in the cell. We now examine parameter sensitivity, i.e. lysis frequency as function of production rates and binding affinities. The least uncertain combination is the product $S_{cl} \cdot R_{RM}$, which is to good accuracy determined by the number of CI in lysogeny, known to be between 180 and 350 for growth in rich medium. The individual terms in this product are less well known, but only influence the dynamics through the relative amplitude of noise in CI production, which will be proportional to the square root of $S_{cl}$, all else equal. This uncertainty is much less than what stems from Cro, as we describe next, and will therefore be ignored.

In Table 2 the top row refers to a reference set-up, and the three following rows show the effects of varying, subsequently, Cro degradation rate $t_{Cro}^{-1}$, $R_{R}$, and finally $R_{R}$ and $S_{cro}$, keeping the product $R_{R} \cdot S_{cro}$ fixed. Thus, with given binding affinities the mean rate of switching increases with any of $S_{cro}$, $R_{R}$ and $t_{Cro}$, but by varying two of these parameters in opposite directions, one may also reproduce the measured stability. Varying different binding affinities we find that stability primarily is determined by the free energy difference between Cro binding to $O_{R3}$ and Cro binding to nonspecific DNA. In Fig. 5 we show the dependence of stability with this difference, for three different values of $R_{R}$. At low lysis frequency, a four-fold change in $R_{R}$ corresponds to a little less than a $RT \ln 4$ change in binding energy difference (approximately equal to 0.9kcal/mol) Changes in Cro affinities to $O_{R1}$ and $O_{R2}$ do not influence stability significantly.

CI influences the stability of a lysogen by binding to $O_{R1}$ and $O_{R2}$, which determines the fraction of time $P_{R}$ is open (see Fig. 1.). Increasing for instance binding to these sites by 0.5kcal/mol means that the probability that $P_{R}$ is open is reduced from 0.000071 to 0.000013, a five-fold weaker activation of $P_{R}$. CI also indirectly influences the stability of a lysogen through the bind-
ing to $O_R3$, which determines the fraction of time $P_{RM}$ is open. To balance decay through dilution at given total number of CI, such a change must be compensated by an increase in the product $R_{RM}S_{ef}$. An increase in this must then be matched by a change in $R_R$ to maintain the measured stability. Thus any CI affinity increase can be compensated by a corresponding increase in $R_R$.

The conclusion so far is that with present parameter information the model is compatible with observed rate of spontaneous lysis in recA– E. coli strains, but we have to look elsewhere for a more stringent test, given the present lack of knowledge of especially parameters related to Cro.

**The Little mutants:** The last section of the Table 2 shows the mean rate of switching in variants of the model corresponding to the recently reported lysogenic state in $\lambda$ mutants, where either operator $O_R1$ is replaced by a copy of $O_R3$, or vice versa (Little et al., 1999). Thus while wild type (321) has an $O_R$ site made up of $O_R3O_R2O_R1$, the mutant labelled mutant 121 has $O_R1O_R2O_R1$ at $O_R$, and mutant 323 $O_R3O_R2O_R3$. Varying parameters related to Cro and promoter strengths we find it impossible to reproduce simultaneously the measured stability of wild type recA– lysogens and the existence of stable lysogens in these mutants. For 323 the cause of decreased stability is demonstrated in Fig. 2a, which shows that $P_R$ is activated much more than in wild type. For 121, on the other hand, the lack of stability is reflected in a much lower CI level in lysogens, as demonstrated in Fig 2b. Thus the two mutants demonstrate two mechanisms of destabilization, one through enhanced Cro, the other through depleted CI.

When adjusting $R_R$ down by a factor 10 we can obtain stable lysogens for 121, and if by a factor 100, then also for 323. In both cases the stability of wild-type increases enormously, and does not match the supposed lysis frequency of $2 \cdot 10^{-9}$ per cell and generation. These parameter changes allow us however to analyse what lysogens in the mutants would look like, as in the last six lines of Table 2. We see that the number of CI for 121 is 15-20% of wild-type, while for 323 the number is 50-60%. Both of these numbers are similar to the CI levels reported by Little et al. (1999). These CI levels can also be found by balancing production and decay of CI only, as in Fig. 2b. In further qualitative agreement with experiment we observe that in spite of
having more CI, the 121 mutant is markedly more stable than 323. This is mainly caused by the much larger probability for open $P_R$ in 323, as seen from Fig. 2a.

Finally we report that the above conclusions remain valid also if CI binds significantly to nonspecific DNA, see appendix: In that case we may simultaneously fit stability of wild-type and 121, but not wild-type and 323.

6 Discussion

The switch to lysis in our model is essentially a first exit time problem, in a system influenced by a combination of deterministic and stochastic forces. A well-known analogy is thermal escape of a particle from a potential (Kramers, (1940); Hänggi et al., 1990), a model of chemical reactions with activation energies. There the rate of escape depends exponentially on the height of the potential barrier, and is therefore very sensitive to small changes in the potential. Our case is not exactly in Kramers’ form, but the analogy is nevertheless illuminating. We also find that the rate of spontaneous switching depends very sensitively on model parameters. In fact, the negative of the logarithm of the probability from Fig. 4 can be identified with the Wentzel-Freidlin quasi-potential (Freidlin & Wentzell, 1984), (Maier & Stein, 1997), which plays the same role in this more general exit time problem as the potential in Kramers’ problem. An analogy to temperature is on the other hand the noises associated to typical fluctuations of CI and Cro numbers in a lysogen. We intend to return to these questions in a future publication.

The large stability of the lysogenic state of the $\lambda$ puts constraints on working mechanisms of the switch. First, we have shown that a simple model, where every transcription of cro leads to lysis, only provides stability for a few generations. A mechanism must therefore exist which stabilizes the switch against most transcriptions of cro. We have explored a straight-forward model, where lysogens are stabilized by a spontaneous lysis that require a number of cro translations within each of 3-5 subsequent bacteria generations. Thereby the small stability arising from a single cro translation is raised to a high power, implying a very rare spontaneous breakdown of lysogeny. The need for multiple cro translations for breakdown of lysogen stability is associated to the
following mechanisms: (i) Cro bound to operators is in homeostatic equilibrium, and CI production is therefore possible with Cro present; (ii) Cro is degraded and diluted over time; (iii) Cro binds also to nonspecific DNA, with significant affinity. Measured lysogenic stability of wild-type recA− E. coli can then be reproduced in the model.

The actual stability depends sensitively on the parameters in the model, and we thus predict a stability which changes dramatically with changes in e.g. growth conditions. The model allows for quantitative examination of mutants, by changing parameters in the model, and we have in this sense investigated the $O_R$ mutants reported by Little et al., 1999. Although the model can reproduce the ratios of CI numbers in mutant and wild-type lysogens, and of the stabilities of each one of wild-type, 121 and 323 mutants, it fails for all parameter values to reproduce simultaneously all of the stabilities. The discrepancy is largest between wild type and 323. This suggests that some additional mechanism outside the $O_R$ complex contributes to the stability. Origins of such a mechanism could be either an increased repression of Cro production by CI relative to our model, or, conversely, a decreased repression of CI production by Cro. Examples of such mechanisms are: i) additional cI transcription directed from $P_{RE}$; ii) an unknown interaction between Cro and CI that allows Cro to stabilize lysogens; iii) CI mediated binding between $O_L$ and $O_R$ if that would significantly repress $P_R$; iv) that Cro is unable to block $P_{RM}$ completely, even when bound to $O_R3$.

The first mechanism i) would give a second role to $P_{RE}$ in repressor maintenance, while mechanism ii) was suggested already by (Eisen et al., 1982) based on a study of the Hyp phenotype. An argument for mechanism iii) is the recent report by Révet et al (1999), which shows a fourfold increase in repression of $P_R$ in a plasmid construction involving two operator complexes, both of which can bind CI dimers, and one of which overlaps with $P_R$ as in wild-type $\lambda O_R$. These mechanisms are of course not exclusive.

In summary, we have developed a quantitative model for the stability of lysogens. The model builds a direct connection between processes, their affinities and the resulting stability. The model allows for quantitative numerical tests of genetic feed-back mechanisms on the molecular level, and points to possible shortcomings in the standard model of $\lambda$ lysogeny maintenance switch.
Finally, we wish to point out again that the model developed here speaks directly to the stability of data storage in biocomputing (Gardner, et al. 2000).

Appendix: Consequences of nonspecific CI binding

(Koblan et al, 1992) report that binding per base pair between CI dimers and nonspecific DNA is at least 9kcal/mol weaker than binding between CI and Or3. This sets a limit on nonspecific binding to be at most \(-3.5\text{kcal/(mol·bp)}\). Here we consider the effects of a \(-3.0\text{kcal/(mol·bp)}\) nonspecific binding between CI dimers and DNA, see also Table 1. The \(2 \cdot 10^{-9}\) stability of lysogens with \(N_{CI} = 200\) is then obtained with \(R_{RM} = 0.085/sec\) and \(R_{R} = 0.02/sec\). In this state \(O_{R3}\) is 20% occupied in lysogeny, in agreement with (Maurer et al., 1980). When we also apply these parameters to the 121 and 323 mutants we find that 121 can form stable lysogens (with \(N_{CI}(121) \approx 40\) and lysis frequency in the range \(10^{-3}\) to \(10^{-5}\)), whereas 323 fails to do so. This lack of stability of 323 in the model fails to reproduce the stability observed by (Little et al., 1999).

Acknowledgements

We thank D. Court, I. Dodd, B. Egan, H. Eisen, J. Little, M. Mossing and P. Muratore-Ginanneschi for discussions and valuable comments. We thank I. Dodd abd B. Egan for suggesting repression by Cro may be incomplete. We thank the Swedish Natural Research Council for support under grant M-AA/FU/MA 01778-333 (E.A), and the Danish research council for financial support (S.B.). We also thank K.F. Jensen and B.E. Uhlin for gifts of MG1655 and the recA allele, respectively.

References
[1] Ackers, G., Johnson, A. & Shea, M. (1982). Quantitative model for gene regulation by λ phage repressor. *Proc. Natl. Acad. Sci. USA* **79**, 1129-1133.

[2] McAdams H.H. & Arkin A. (1997). Stochastic mechanisms in gene expression. *Proc. Natl. Acad. Sci. U S A* **94**, 814-819.

[3] Arkin A., Ross J. & McAdams H. H. (1998) Stochastic kinetic analysis of developmental pathway bifurcation in phage lambda-infected Escherichia coli cells. *Genetics* **149**, 1633-1648.

[4] Berg, O. G., Winter, R.B. & von Hippel, P. H. (1982). How do genome-regulatory proteins locate their DNA target sites? *Trends Biochem. sci* **7** 52-55.

[5] Bremmer, H. & Dennis P. P. (1996). Modulation of chemical composition and other parameters of the cell by growth rate, Escherichia coli and Salmonella, pp 1553-1569, edd. F.C. Neidhardt, ASM press. At doubling time of 30 minutes one *E. coli* cell has dry weight $6.4 \times 10^{-13} \text{g}$ and consists of $\sim 70\%$ water (Neidhardt and Umbarger, *ibid* page 13-16), giving a volume of $2.1 \times 10^{-15} \text{l}$. At same conditions *E. coli* DNA is on average present in three copies, the part belonging to λ in 2.4 copies.

[6] Capp M. W., Cayley D. S., Zhang W., Guttmann H. J., Melcher S. E., Saecker R. M., Anderson C. F. and Record M. T. Jr. Compensating Effects of Opposing Changes in Putrescine (2+) and $K^+$ Concentrations on lac Repressor-lac Operator Binding: in Vitro Thermodynamic Analysis and in Vivo Relevance. (1996), *J. Mol. Biol.* **258** 25-36.

[7] Csonka, L. N. & Clark A. J. (1979). Deletions generated by the transposon Tn10 in the srl recA region of the Escherichia coli K-12 chromosome. *Genetics* **93**, 321-343.

[8] Eisen, H., Barrand, P., Speigelman, W. Reichardt, L. F., Heinemann, S. & Georgopoulos, C. (1982). Mutants in the $y$ region of bacteriophage λ constitutive for repressor synthesis: Their isolation and the characterization of the Hyp phenotype. *Gene* **20**, 71-81; 83-90.
[9] Elowitz, M. B., Surette, M. G., Wolf, P.-E., Stock, J. B. & Leibler, S. (1999). Protein mobility in the cytoplasm of Echerichia Coli. *J. of Bacteriol.* **181**, 197-203.

[10] Fong, R. S.-C., Woody, S. & Gussin, G. N. (1993). Modulation of PRM activity by the lambda $P_R$ promoter in both the presence and absence of repressor. *J. Mol. Biol.* **232**, 792-804.

[11] Fong, R. S.-C., Woody, S. & Gussin, G. N. (1993). Direct and Indirect Effects of Mutations in $\lambda P_{RM}$ on open Complex Formation at the Divergent $P_R$ promoter. *J. Mol. Biol.* **240**, 119-126.

[12] Freidlin, M. & Wentzell, A. (1984) *Random perturbations of Dynamical Systems*, Springer-Verlag, New York/Berlin.

[13] Gardner, T. S., Cantor, C. R. & Collins, J. J. (2000). Construction of a genetic toggle switch in Escherichia coli. *Nature* **403** (6767), 339-342.

[14] Glass, L. & Kauffman, S. A.. (1973). The logical analysis of continuous, non-linear biochemical control networks. *J. Theor. Biology* **39**, 103-129.

[15] Hänggi, P., Talkner, P. & Borkevic, M. (1990). Reaction-rate theory: Fifty years after Kramers. *Rev. Mod. Phys.* **62**, 251-341.

[16] Hawley, D. K. & McClure, W. R. (1982). Mechanism of activation of transcription initiation from the lambda PRM promoter. *J. Mol. Biol.* **157**, 493-525.

[17] Hawley, D. K. & McClure, W. R. (1983). The Effect of a Lambda Repressor Mutation on the Activation of Transcription Initiation from the Lambda $P_{RM}$ Promoter. *Cell* **32**, 327-333.

[18] Jana R., Hazbun, T. R, Mollah, A. K. M. M. & Mossing, M. C. (1997). A folded monomeric intermediate in the formation of Lambda Cro dimer-DNA complexes. *J. Mol. Biol.* **273**, 402-416.

[19] Jana, R., Hazbun, T. R., Fields, J. D. & Mossing, M. C. (1998). Single-chain lambda Cro repressors confirm high intrinsic dimer-DNA affinity. *Biochemistry* **37**, 6446-6455.
[20] Jensen, K. F. (1993). The Escherichia coli K-12 “wild-types W3110 and MG1655 have an rph frameshift mutation that leads to pyrimidine starvation due to low pyrE expression levels. J. Bacteriol. 175, 3401-3407.

[21] Johnson, A. D., Poteete, A. R., Lauer, G., Sauer, R. T., Ackers, G. K. & Ptashne, M. (1981). Lambda repressor and cro–components of an efficient molecular switch. Nature 294, 217-223.

[22] Johnson, A. D., Meyer, B. J. & Ptashne, M. (1979). Interactions between DNA-bound repressors govern regulation by the lambda phage repressor. Proc. Natl. Acad. Sci. U S A 76, 5061-5065.

[23] Kennell, D. & Riezman, H. (1977). Transcription and translation initiation frequencies of the Escherichia coli lac operon. J. Mol. B 114, 1-21.

[24] Kim, J. G., Takeda Y., Matthews B. W. & Anderson W. F. (1987). Kinetic Studies on Cro Repressor-Operator DNA Interaction. J. Mol. B 196, 149-158.

[25] Koblan S. K. & Ackers, G. K. (1991). Energetics of Subunit Dimerization in bacteriophage lambda cI Repressor: Linkage to Protons, Temperature and KCL Biochemistry 30, 7817-7821.

[26] Koblan S. K. & Ackers, G. K. (1992). Site-Specific Enthalpic Regulation of DNA Transcription at Bacteriophage λ O R. Biochemistry 31, 57-65.

[27] Kramers, H. A. (1940). Brownian motion in a field of force and the diffusion model of chemical reactions. Physica 7, 284-304.

[28] Little, J. W., Shepley, D. P. & Wert, D. W. (1999). Robustness of a gene regulatory circuit. EMBO J. 18, 4299-4307.

[29] Maurer R., Meyer, B. J. & Ptashne M. (1980). Gene Regulation at the Right Operator ( O R ) of Bacteriophage λ. J. Mol. Biol. 139, 147-161.

[30] Maier R.S. & Stein D.S. (1997). Limiting exit location distribution in the stochastic exit problem, SIAM J. Appl. Math. 57, 752-790.

[31] Miller, J. H. (1972) Experiments in molecular genetics, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York.
[32] Monot, J. & Jacob F. (1961). General conclusions: teleonomic mechanisms in cellular metabolism, growth and differentiation. Cold Spring Harbour Symp. Quant. Biology 26, 389-401.

[33] Nelson, H. M. & Sauer, R. T. (1985). Lambda repressor mutations that increase the affinity and specificity of operator binding. Cell 42, 549-558.

[34] Pakula, A. A., Young, V. B. & Sauer R. T. (1986). Bacteriophage λ cro mutations: Effects on activity and intracellular degradation. Proc. Natl. Acad. Sci. USA 83, 8829-8833.

[35] Powell, B. S., Court, D. L., Nakamura, Y., Rivas, M. P. & Turnbough, C. L. (1994). Rapid confirmation of single copy lambda prophage integration by PCR. Nucl. Acids Res. 22, 5765-5766.

[36] Pray, T. R., Burz, D. S. & Ackers, G. K. (1999). Cooperative non-specific DAN binding by octamerizing λ CI repressors: A site-specific thermodynamic analysis. J. Mol. Biol. 282, 947-958.

[37] Ptashne, M. (1992). A Genetic Switch; Phage λ and Higher Organisms, Blackwell Scientific Publications & Cell Press.

[38] Record M. T., Courtenay E. S., Cayley D. S. & Guttman H. G. (1998). Biophysical compensation mechanisms buffering E.coli protein-nucleic acid interactions against changing environments. Trends Biochem. Sci. 23 (5) 190-194.

[39] Révet B., von Wilcken-Bergmann B., Bessert H., Barker A. & Müller-Hill B., Four dimers of λ repressor bound to two suitably spaced pairs of λ operators form octamers and DNA loops over large distances. Current Biology 9, 151-154.

[40] Ringquist, S., Shinedling, S., Barrick, D., Green, L., Binkley, J, Stormo, G.D. & Gold, L. (1992). Translation initiation in Escherichia coli: sequences within the ribosome-binding site. Mol. Microbiol. 6, 1219-1229.

[41] Roberts, J. W. & Devoret, R. (1983). Lysogenic induction. Lambda II, Hendrix, R.W., Roberts, J.W., Stahl, F.W. & Weisberg, R.A. eds., (Cold Spring Harbor, New York: Cold Spring Harbor Laboratory), pp. 123-144.
[42] Rozanov, D. V., D’Ari R. & Sineoky, S. P. (1998). RecA-independent pathways of lambdoid prophage induction in Escherichia coli. *J. Bacteriol.* **180**, 6306-6315.

[43] Shea, M. A. & Ackers, G. K. (1985). The $O_R$ control system of bacteriophage lambda – A physical-chemical model for gene regulation. *J. Mol. Biol.* **181**, 211-230.

[44] Shean, C. S. & Gottesman, M. E. (1992). Translation of the prophage lambda cI transcript. *Cell* **70**, 513-522.

[45] Takeda, Y., Sarai, A. & Rivera, V. M. (1989). Analysis of the sequence-specific interactions between Cro repressor and operator DNA by systematic base substitution experiments. *Proc. Natl. Acad. Sci. USA* **86**, 439-443.

[46] Takeda, Y., Ross, P. D. & Mudd, C. P. (1992). Thermodynamics of Cro protein-DNA interactions. *Proc. Natl. Acad. Sci. USA* **89**, 8180-8184.

Figure 1: Right operator complex, $O_R$, consisting of the three operators $O_{R1}$, $O_{R2}$ and $O_{R3}$. cI is transcribed when $O_{R3}$ is free and $O_{R2}$ is occupied by CI. cro is transcribed when both $O_{R2}$ and $O_{R1}$ is free. CI dimers bind cooperatively to $O_{R1}$ and $O_{R2}$.

Figure 2: a) Probability of open cro promoter, $P(00) = P(000) + P(100) + P(200)$, as function of CI number in *E.coli* cell of volume $2 \cdot \mu m^3$. The dashed lines marked 121 and 323 show repression for the two $O_R$ mutants investigated by J. Little (Little *et al*, 1999). b) Activity of $P_{RM}$ in units of activity when $O_{R3}$ is free and $O_{R2}$ is occupied by CI. Same notation as in a). For relative activity of nonstimulated $P_{RM}$ promoter we use the data of (Hawley & McClure, 1982).
Figure 3: Time course of last few hundreds of generations before lysis in a typical simulation with the standard set of parameters. The average switching rate is $1.4 \cdot 10^{-9}$ per cell and generation. The real level of Cro in a lysogenic cell is unknown. In our model, almost all of the Cro present is bound nonspecifically to DNA in lysogeny, the total level could be changed by at least a factor 10 by changing the (uncertain) difference in Cro affinity to $O_{R3}$ and to nonspecific DNA.

Figure 4: a) Probability to be at various values of CI and Cro number in the cell during lysogeny. Due to finite number of samples, one cannot see the rare fluctuations leading to lysis. b) As in a), but limited to the rare events where $N_{Cro} > 50$.

Figure 5: Frequency of spontaneous lysis ($f$) as function of the affinity ratio $r = \exp(\Delta\Delta G/RT)$ between specific and nonspecific Cro binding. The full line is for standard setup (e.g. $R_R = 0.30/s$), and one observe a large relative change in $f$ with $r$, implying that the adjustment in bindings that leads from high to very high stability is small. The +) show effect of increased nonspecific Cro – DNA binding by 1kcal/(mol-bp), and its similarity to the full line marks that no free Cro are present. The dashed lines show lysis frequency with respectively $R_R = 1.2/s$ and $R_R = 0.075/s$. 
\[ \Delta G_{\text{CI-DNA}} = 0.0 \text{kcal/(mol·bp)} \]

\[ \Delta G_{\text{CI-DNA}} = -3.0 \text{kcal/(mol·bp)} \]

| State s | \( \Delta G \) [kcal/mol] | \( P(s,N=200) \) | \( P(s,N=200) \) |
|---------|----------------|----------------|----------------|
| (000)   | 0.0            | 0.00005        | 0.00063        |
| (001)   | -12.5          | 0.00301        | 0.01269        |
| (010)   | -10.5          | 0.00012        | 0.00050        |
| (100)   | -9.5           | 0.00002        | 0.00010        |
| (011)   | -25.7          | 0.59291        | 0.80189        |
| (101)   | -22.0          | 0.00147        | 0.00199        |
| (110)   | -22.9          | 0.00632        | 0.00855        |
| (111)   | -35.4          | 0.39606        | 0.17366        |
| CI dimer| -11.1          |                |                |
| (002)   | -14.4          |                |                |
| (020)   | -13.1          |                |                |
| (200)   | -15.5          |                |                |
| Cro dimer| -7.0          |                |                |

Table 1: CI and Cro dimer affinities to \( O_R1 \), \( O_R2 \) and \( O_R3 \) after, respectively, (Koblan & Ackers, 1992), and (Takeda et al., 1989, 1992), (Jana et al. 1997, 1998) and (Kim et al. 1987). A major source of uncertainty of in vivo Cro bindings is that all in vitro experiments for Cro have been performed at or below room temperature. The CI dimerization is from (Koblan & Ackers 1991), Cro dimerization from (Jana et al. 1997), and nonspecific Cro dimer binding to DNA from (Kim et al., 1987). The third and fourth columns display the probabilities of different states of binding of CI to the sites in \( O_R \), with different assumed nonspecific affinity of CI to DNA. For binding of single CI dimers to left operators the relative affinities compared to \( O_R1 \) (\( \Delta G_R(0,0,1) \)) are \( \Delta \Delta G_L(s) = \{1.0, 0.8, -0.2\} \) in units of kcal/mol, where \( s \) are the states \((0,0,1), (0,1,0)\) and \((1,0,0)\), respectively. The relative affinities of binding of single Cro dimers are similarly \( \Delta \Delta G_L(s) = \{-0.1, 0.5, 1.0\} \), where the reference energy is \( \Delta G_R(0,0,2) \) (Takeda et al.).
Table 2: Switching stabilities and CI and Cro numbers as function of model parameters. for wild type and the Little mutants. Top row is the standard configuration, as described in the main text. Second through fourth row explores the changes in lysis frequency and average number of Cro present in wild type by varying $R_R$, $S_{cro}$ and $t_{Cro}$. Fifth to twelfth row show the changes in Little 121 and 323 mutants upon changing $R_R$. The rows marked 121* and 323* explore an additional change of $R_u^{aRM}$, the rate of unstimulated transcription from $P_{RM}$ to $R_u^{aRM} = 0.30R_{RM}$. The standard parameter value (without *) is $R_u^{aRM} = R_{RM}/11$. We observe that the 323 mutants fail to stabilize unless $R_R$ is decreased a hundredfold. The stability of 121 is increased a million times (compare row 5 and 11), as is also wild-type.

| $O_R$ type | $R_R$ [s$^{-1}$] | $S_{cro}$ number | $t_{Cro}$ [s] | CI number | Cro number | lysis frequency [generations$^{-1}$] |
|------------|------------------|------------------|--------------|-----------|------------|-------------------------------------|
| 321        | 0.30             | 20               | 3600         | 200       | 0.8        | $1.4 \times 10^{-9}$               |
| 321        | 0.30             | 20               | 7200         | 200       | 0.8        | $1.7 \times 10^{-8}$               |
| 321        | 0.60             | 20               | 3600         | 200       | 1.4        | $3 \times 10^{-7}$                 |
| 321        | 0.30             | 40               | 3600         | 200       | 1.6        | $2 \times 10^{-5}$                 |
| 121        | 0.30             | 20               | 3600         |           |            | $> 0.1$                            |
| 323        | 0.30             | 20               | 3600         |           |            | $> 0.1$                            |
| 323        | 0.030            | 20               | 3600         | 32        | 8          | $1.3 \times 10^{-6}$               |
| 121        | 0.030            | 20               | 3600         | 32        | 8          | $4 \times 10^{-6}$                 |
| 323*       | 0.030            | 20               | 3600         | 85        | 34         | 0.04                                |
| 121        | 0.0030           | 20               | 3600         | 33        | 1          | $5 \times 10^{-7}$                 |
| 323        | 0.0030           | 20               | 3600         | 122       | 2          | $1.5 \times 10^{-6}$               |
PRM activity vs. N(Cl) for different dilutions.
frequency of lysis

$\exp \left[ \frac{G(\text{Cro-DNA})}{T} - \frac{G(2,0,0)}{T} \right]$