

Metabolic regulation
**Using Evolution to understand genome
structure and transcription regulation**

Experimental and Modeling strategies

Experiments: use 'controlled conditions'

Mini-models: can study parameter space and 'choose' parameters based on outcome (fitting experiments)

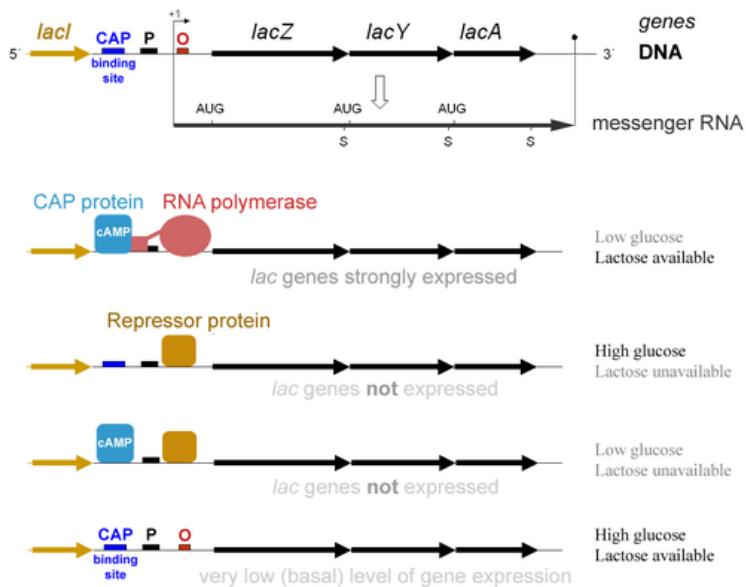
Detailed models: use (MANY) measured / estimated ('reasonable') parameters

minimal evolutionary optimization models
('what is it good for?') (bet-hedging)

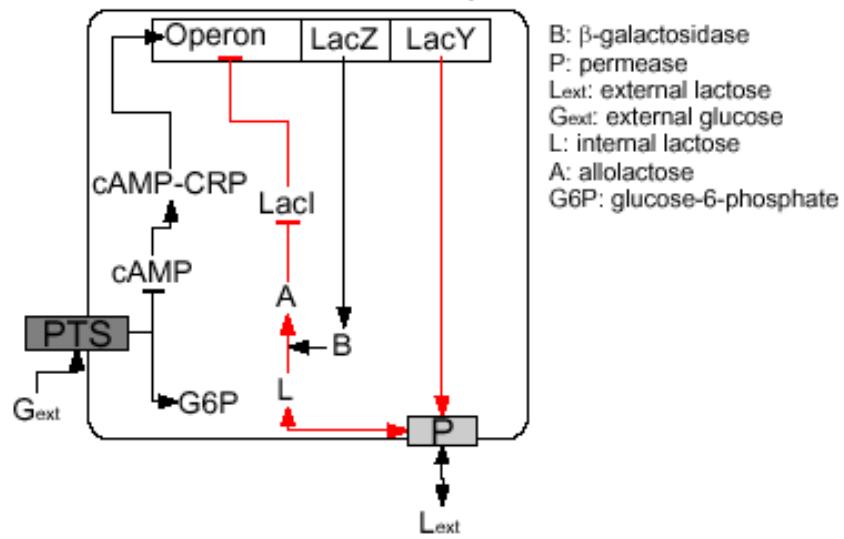
*Here use multilevel (evolutionary) modeling
to generate parameters and debug the above*

Prototype gene regulation: Lac operon

The *lac* Operon and its Control Elements



An overview of the *lac* operon



genome structure

regulatory network

B: β-galactosidase
P: permease
Lext: external lactose
Gext: external glucose
L: internal lactose
A: allolactose
G6P: glucose-6-phosphate

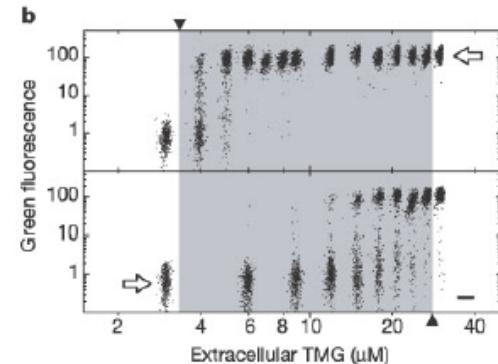
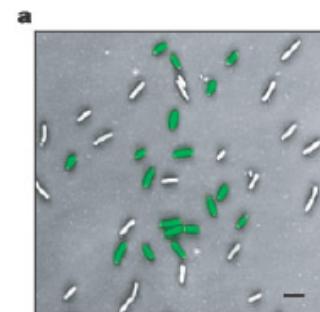
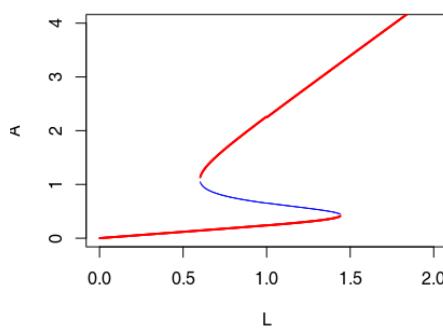
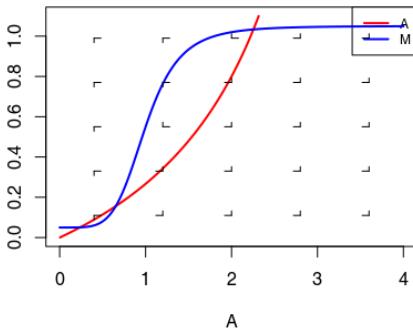
Lac Operon: Prototype bi-stability in gene regulation: classical mini-model, experiments

$$R = 1/(1 + A^n)$$

$$dM/dt = c_0 + c(1 - R) - dM$$

$$dA/dt = ML - \delta A - vMA$$

$$L = 1.0; c = 1.0; c_0 = .05; \delta = .2; v = .25; n = 5)$$



bi-stability

experimentally “verified”

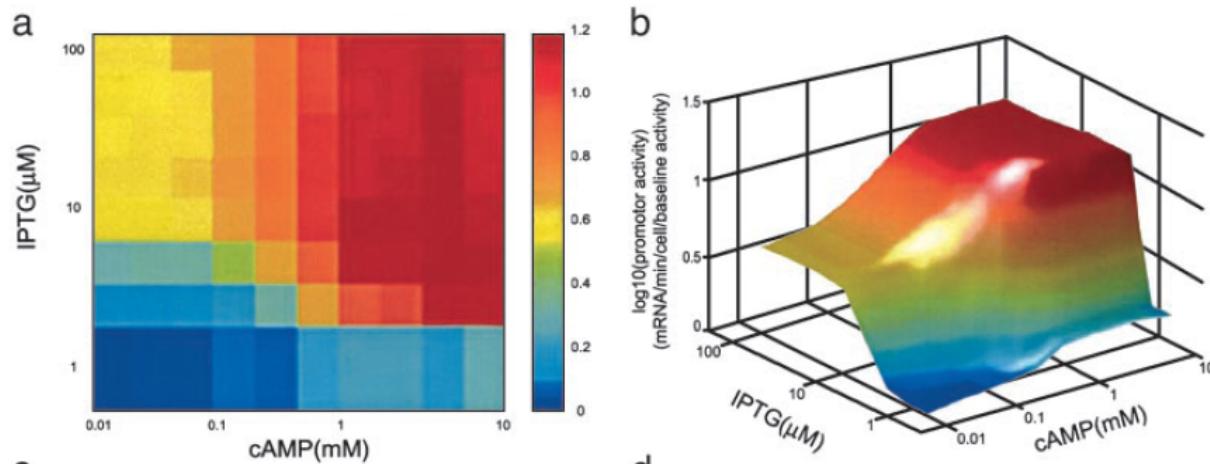
cf Novick and Weiner 1957, Griffith 1968, Ozbudak et al 2004

Metabolic regulation in E.coli

Using Evolution to understand transcription regulation

experimental measurement of promotor function

Setty...Alon 2003



Not a simple AND function

“the wild-type region is selected to perform an elaborate computation in setting the transcription rate.”

measurements fitted to model of promoter function

$$PA(A, C) = V_1 \frac{1 + V_2 \mathcal{A} + V_3 \mathcal{R}}{1 + V_4 \mathcal{A} + V_5 \mathcal{R}}, \quad (1)$$

where A stands for the allolactose concentration and C for the cAMP concentration and \mathcal{A} and \mathcal{R} are the fraction of active CRP and repressed LacI, respectively.

$$\mathcal{A} = \frac{(C/k_C)^n}{1 + (C/k_C)^n} \quad (2)$$

$$\mathcal{R} = \frac{1}{1 + (A/k_A)^m}, \quad (3)$$

where n and m are the Hill-coefficients of cAMP binding to CRP and allolactose binding to LacI. k_C and k_A are the dissociation constants for these reactions. Furthermore we have defined

$$\begin{aligned} V_1 &= (a\alpha + \gamma)/(1 + a) \\ V_2 &= d(b\beta + \gamma)/(a\alpha + \gamma) \\ V_3 &= \gamma c/(a\alpha + \gamma) \\ V_4 &= d(b + 1)/(a + 1) \\ V_5 &= c/(a + 1) \end{aligned} \quad (4)$$

$V_1 \dots V_5$ depend on 7 affinity parameters

$a = RNAP/k_{RNAP}$, RNA-polymerase in units of its dissociation constant for binding to a free site.

$b = RNAP/k_{RNACP}$, RNA-polymerase in units of its dissociation constant for binding to a site with bound CRP.

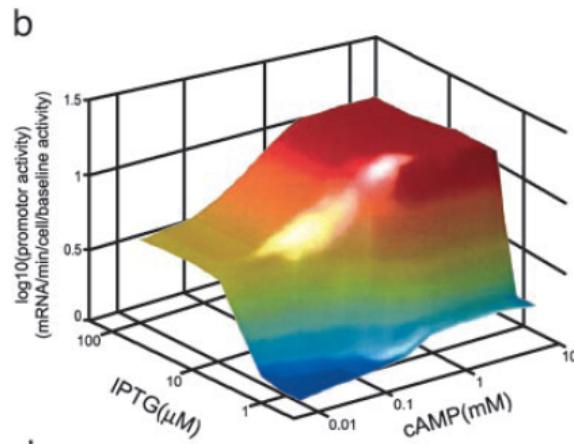
$c = LACI_T/k_{LACI}$, the total LacI concentration in units of its dissociation constant for binding to its site.

$d = CRP_T/k_{CRP}$, the total CRP concentration in units of its dissociation constant for binding to its site.

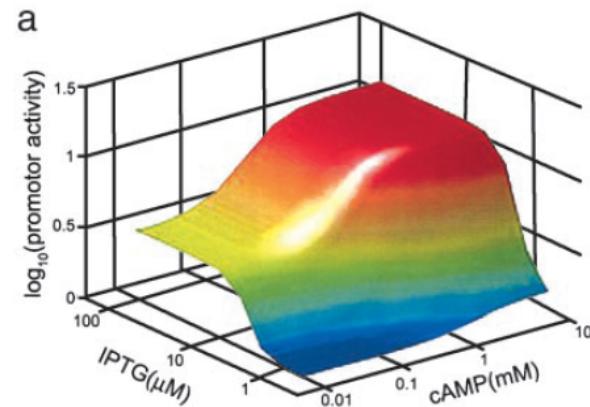
α , the transcription rate when RNA Polymerase is bound to the DNA, but CRP and LacI are not.

β , the transcription rate when both RNA Polymerase and CRP are bound, but LacI is not bound to the DNA.

γ , the “leakiness”, the transcription rate when RNA Polymerase is not bound to the DNA.

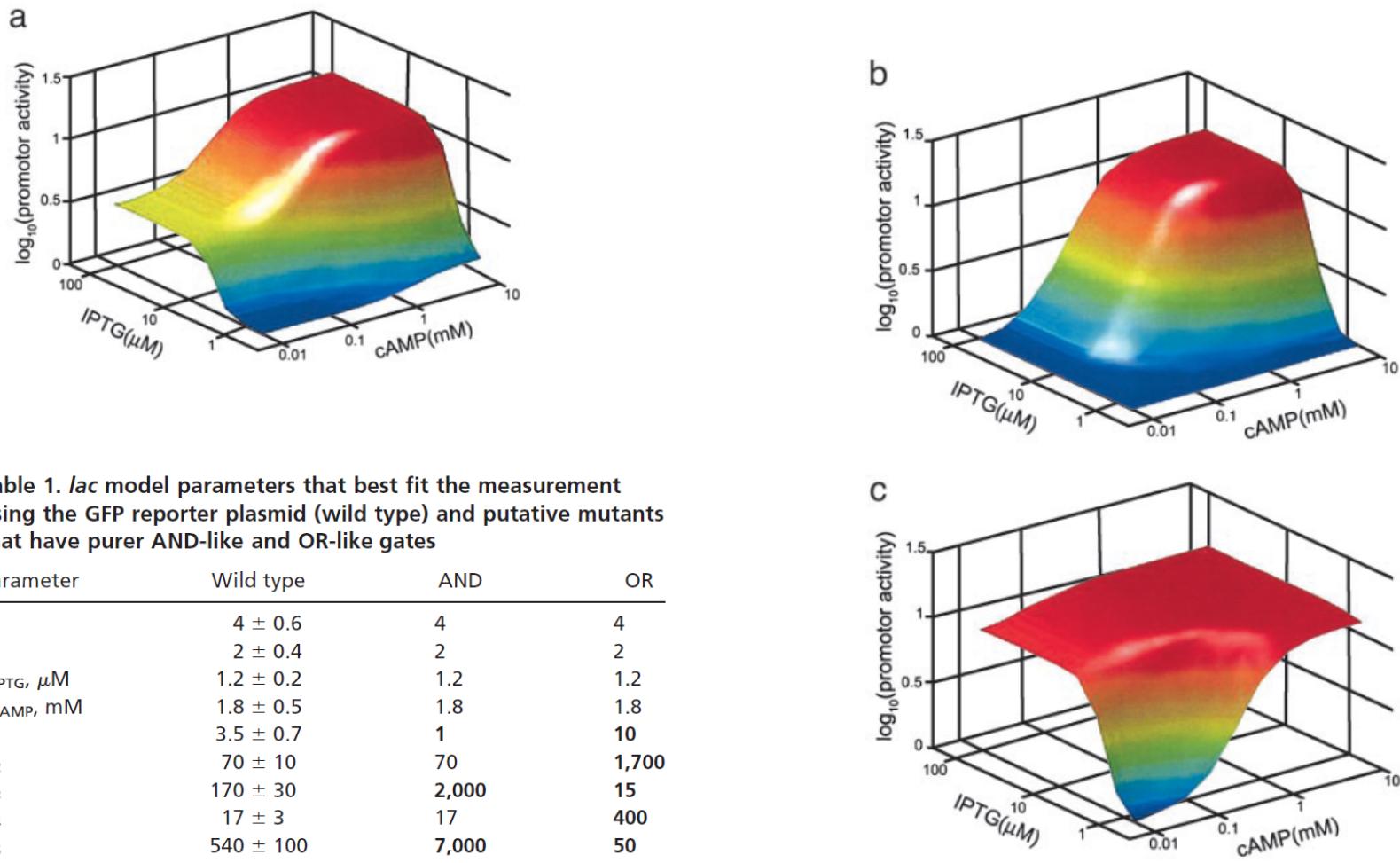


data



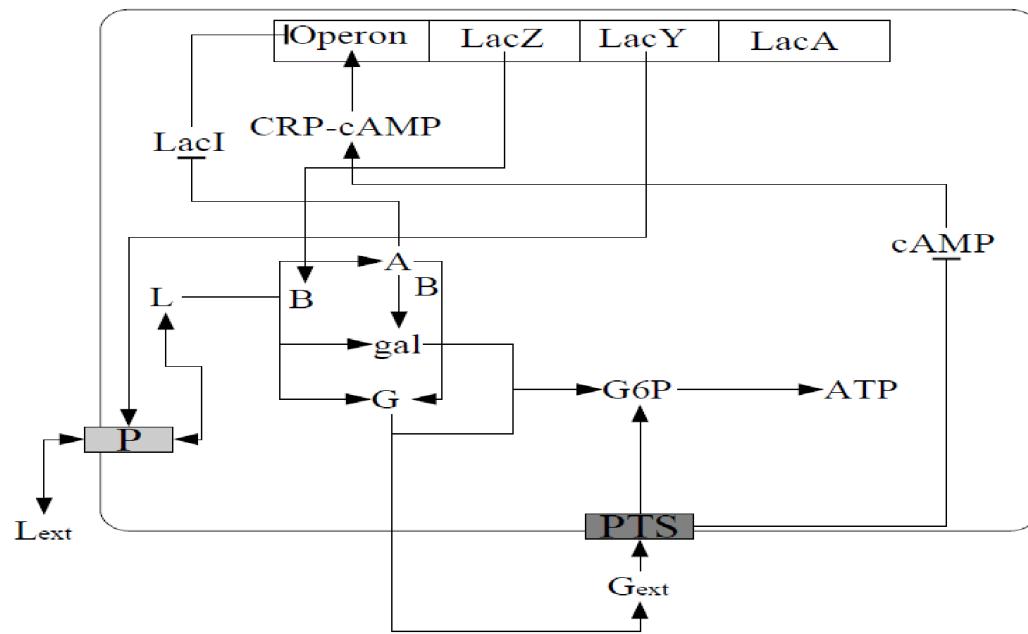
bestfit

Parameter sensitivity / parameter curse (1)



more complex model of the lac operon

Wong et al 1997 , adapted by van Hoek & Hogeweg 2006



eqs determining operon activity

$$\begin{aligned} PA(A, C) &= V_1 \frac{1 + V_2 \frac{(C/k_C)^n}{1+(C/k_C)^n} + \frac{V_3}{1+(A/k_A)^m}}{1 + V_4 \frac{(C/k_C)^n}{1+(C/k_C)^n} + \frac{V_5}{1+(A/k_A)^m}} \\ \frac{dM}{dt} &= PA(A, C) - (\gamma_M + \mu)M \\ \frac{dB}{dt} &= k_B M - (\gamma_B + \mu)B \\ \frac{dP}{dt} &= k_P M - (\gamma_P + \mu)P \\ \frac{dL}{dt} &= P \frac{k_{L,i} L_{ext}}{K_{L,i} + L_{ext}} - P \frac{k_{L,o} L}{K_{L,o} + L} \\ &\quad - B \frac{(k_{c,L} + k_{L-A})L}{L + K_{m,L}} - (\gamma_L + \mu)L \\ \frac{dA}{dt} &= B \frac{k_{L-A} L}{L + K_{m,L}} - B \frac{k_{c,A} A}{A + K_{m,A}} - (\gamma_A + \mu)A \end{aligned}$$

eqs determining further metabolism and cell growth(X)

(cell division if cell size = 2*basic size)

$$\begin{aligned}
 \frac{dG}{dt} &= \frac{k_{c,L}B * L}{L + K_{m,L}} + \frac{k_{c,A}B * A}{A + K_{m,A}} - \frac{k_{c,G}G}{G + K_{m,G}} - k_{G,o}(G - G_{ext}) - \mu G \\
 \frac{dG6P}{dt} &= \frac{k_{t,G}G_{ext}}{G_{ext} + K_{t,G}} + \frac{k_{c,G}G}{G + K_{m,G}} + \frac{k_{c,L}B * L}{L + K_{m,L}} + \frac{k_{c,A}B * A}{A + K_{m,A}} - \\
 &\quad \frac{k_{G6P,R}G6P}{G6P + K_{G6P,R}} - \frac{k_{G6P,F}G6P^8}{K_{G6P,F}^8 + G6P^8} - \mu G6P \\
 \frac{dC}{dt} &= \frac{k_{s,C}K_{s,C}}{\frac{k_{t,G}G_{ext}}{G_{ext} + K_{t,G}} + K_{s,C}} - (\gamma_C + \mu)C \\
 \frac{dATP}{dt} &= \frac{Y_R * k_{G6P,R} * G6P}{G6P + K_{G6P,R}} + \frac{2k_{G6P,F} * G6P^8}{K_{G6P,F}^8 + G6P^8} - BMC - \\
 &\quad \frac{\mu_{max} * GC * ATP^4}{ATP^4 + K_{ATP}^4} - PC * PA - \frac{k_{c,L}B * L}{L + K_{m,L}} - \frac{k_{c,A}B * A}{A + K_{m,A}} \\
 \frac{dX}{dt} &= \mu_{max} \frac{ATP^4}{ATP^4 + K_{ATP}^4} X
 \end{aligned}$$

Wong concluded : bistable switch

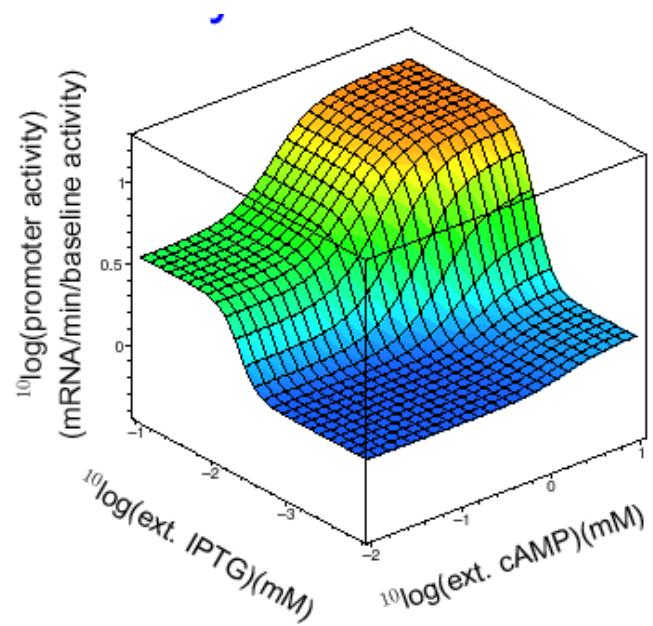
Table 1: All model parameters with their values.

parameter	equation	value	comments
k_C	Eq. 2	evolvable, mM	initial value: 1.0×10^{-3} mM
n	Eq. 2	evolvable	initial value: 4.0
E_A	Eq. 3	evolvable, mM	initial value: 5.5×10^{-4} mM
m	Eq. 3	evolvable	initial value: 8.0
a	Eq. 4	evolvable	initial value: 1.0
b	Eq. 4	evolvable	initial value: 1.0
c	Eq. 4	evolvable	initial value: 1.0×10^6
d	Eq. 4	evolvable	initial value: 50
α	Eq. 4	evolvable, mM/min	initial value: 1.1×10^{-7} mM/min
β	Eq. 4	evolvable, mM/min	initial value: 2.2×10^{-5} mM/min
γ	Eq. 4	evolvable, mM/min	initial value 1.1×10^{-9} mM/min
γ_M	Eq. 5	0.693/min	Wong et al. (2)
k_B	Eq. 6	9.4 mM enzyme/(mM mRNA min)	Wong et al. (2)
γ_B	Eq. 6	0.01/min	Wong et al. (2)
k_P	Eq. 7	18.8 mM enzyme/(mM mRNA min)	Wong et al. (2)
γ_P	Eq. 7	0.01/min	Wong et al. (2)
$k_{Lac,in}$	Eq. 8	2148 mmol lac-tose/(mmol permease min)	Wong et al. (2)
$K_{Lac,in}$	Eq. 8	0.26 mM	Wong et al. (2)
$k_{Lac,out}$	Eq. 8	2148 mmol lac-tose/(mmol permease min)	Wong et al. (2)
$K_{Lac,out}$	Eq. 8	0.26 mM	unlike Wong et al. (2), intracellular concentrations are in mM
$k_{Lac-Allo}$	Eq. 9	8460/min	Wong et al. (2)
$K_{m,Lac}$	Eq. 9, Eq. 10	1.4 mM	Martinez-Bilbao et al. (13), referred to by Wong et al. (2)
$k_{cat,Lac}$	Eq. 10	9540/min	Wong et al. (2)
γ_L	Eq. 11	0.15/min	assumed, to get a significant bistable region, compare Yildirim and Mackey (4)
$k_{cat,Allo}$	Eq. 12	18000/min	Wong et al. (2)
$K_{m,Allo}$	Eq. 12	0.28 mM	Wong et al. (2)
γ_A	Eq. 13	0.15/min	assumed, to get a significant bistable region, compare Yildirim and Mackey (4)
$k_{cat,Glu}$	Eq. 14	11.5 mM/min	fitted with data of Hogema et al. (6)
$K_{m,Glu}$	Eq. 14	0.45 mM	fitted with data of Hogema et al. (6)
$k_{Glu,out}$	Eq. 15	0.093/min	fitted with data of Hogema et al. (6)
$k_{t,Glu}$	Eq. 15	45 mM/min	Wong et al. (2), Carlson and Srienc (11)
$K_{t,Glu}$	Eq. 15	0.015 mM	Wong et al. (2)

parameter	equation	value	comments
$k_{G6P,Rsp}$	Eq. 18	34 mM/min	assumed, saturated respiratory flux assumed for maximal glucose influx. Andersen and Von Meyenburg (10)
$K_{G6P,Rsp}$	Eq. 18	0.5 mM	idem. Andersen and Von Meyenburg (10)
$k_{G6P,Frm}$	Eq. 19	200 mM/min	assumed, maximal fermentative flux is much larger than maximal respiratory flux. Andersen and Von Meyenburg (10)
$K_{G6P,Frm}$	Eq. 19	20 mM	assumed, fermentation saturates much slower than respiration. Andersen and Von Meyenburg (10)
$k_{syn,cAMP}$	Eq. 21	0.001 mM/min	Wong et al. (2)
$K_{syn,cAMP}$	Eq. 21	1.0 mM/min	assumed, to have a large range of possible cAMP concentrations.
γ_{cAMP}	Eq. 21	2.1/min	Wong et al. (2)
Y_{Rsp}	Eq. 22	32 mM ATP/mM glucose-6-phosphate	assumed equal to the ATP-yield of aerobic respiration.
BMC	Eq. 22	23.5 mM/min	Carlson and Srienc (11)
GC	Eq. 22	7.28×10^5 mM	estimated with data of Carlson and Srienc (11)
PC	Eq. 22	2.36×10^6 mM ATP/mM mRNA	calculated assuming 3% growth cost at maximal activity, Koch (14). (for high cost a value ten times higher is used)
μ_{max}	Eq. 23	0.0233/min	Wong et al. (2)
Q	Eq. 25, Eq. 26	0.00035	assumed
D		$0.0020(\text{gridsize})^2/\text{min}$	assumed, scalable
Δ_a		0.075	assumed
Δ_b		0.075	assumed
Δ_c		0.15	assumed
Δ_d		0.15	assumed
Δ_α		0.075	assumed
Δ_β		0.075	assumed
Δ_γ		0.075	assumed
Δ_{k_A}		0.15	assumed
Δ_{k_C}		0.05	assumed
Δ_n		0.5	assumed
Δ_m		0.5	assumed
$V_{mRNA,max}$		2.2×10^{-5} mM/min	assumed, to have a realistic maximal lactose uptake rate.

Functionality of Lac-operon Bistability?

- Most studied regulatory system
- often considered as AND gate
ON if lactose and not glucose; otherwise OFF
- recent direct promoter measurements: more graded response
- Bistability? exp 'seen' and expected from minimodels and 'verified' in more extensive parametrized models
- many (all) parameters measured
HOWEVER
may be orders of magnitude different
parameter curse (2)!

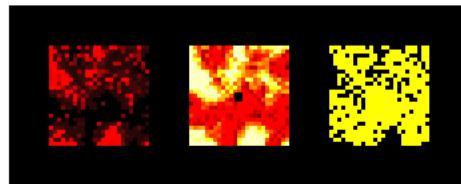


Setty Alon 2003

**Does such a promotor function evolve
DOES BISTABILITY EVOLVE?; alleviate parameter uncertainty**

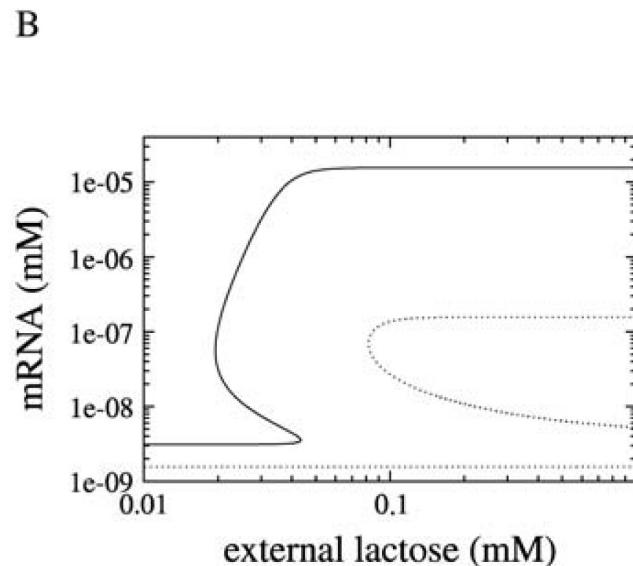
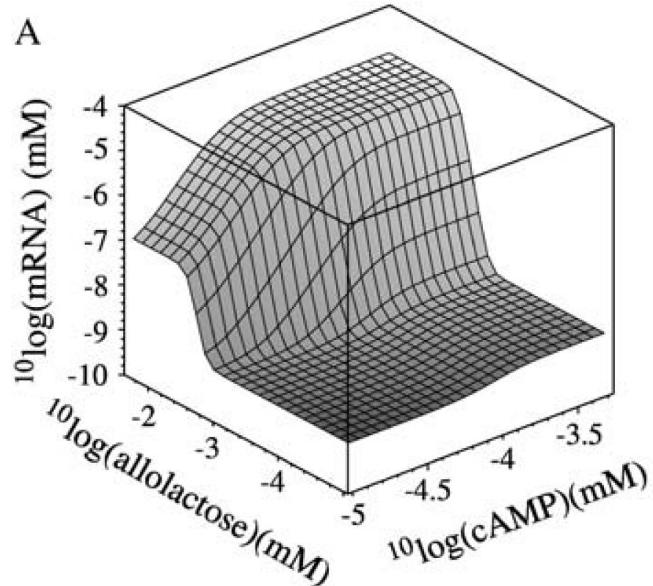
**“experimental setup” evolution of the Lac operon:
timescales: metabolism, cell growth/division, prot. stab,
environmental switches, evolution**

- Adapt existing detailed quantitative model of lac operon dynamics (Wong et al 1997)
- use measured parameters EXCEPT for lac operon parameters
- evolve 11 lac-operon parameters
DO NOT use dimension reduction!
otherwise evolutionary lock-in
- Design environment! (“cover all possibilities”)
- global/aperiodic influx of lactose and glucose in medium, diffusion, scaling
- growth (dependent on ATP), division (2*size), decay (density dep; no ATP)
- encountered environments depend on dynamics! dynamics!



evolution as trick to cope with parameter uncertainty

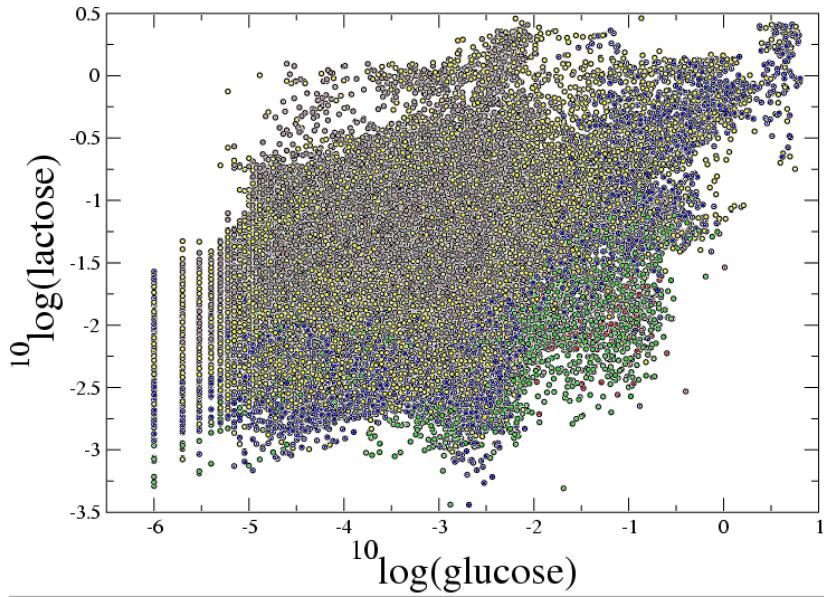
**initialize as a bistable switch
(because no bistable switch evolved...)**



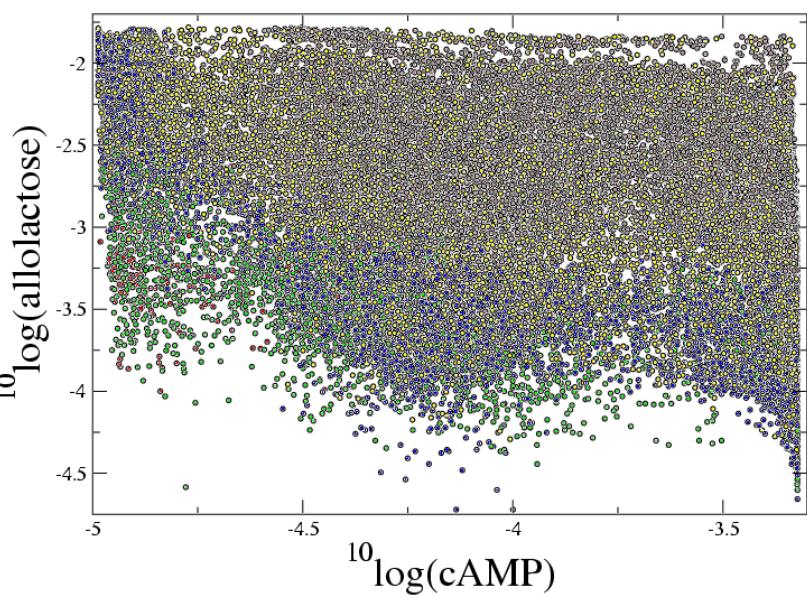
solid low glucose; dotted high glucose

Designing external environment coverage of environmental statespace, while response to environments

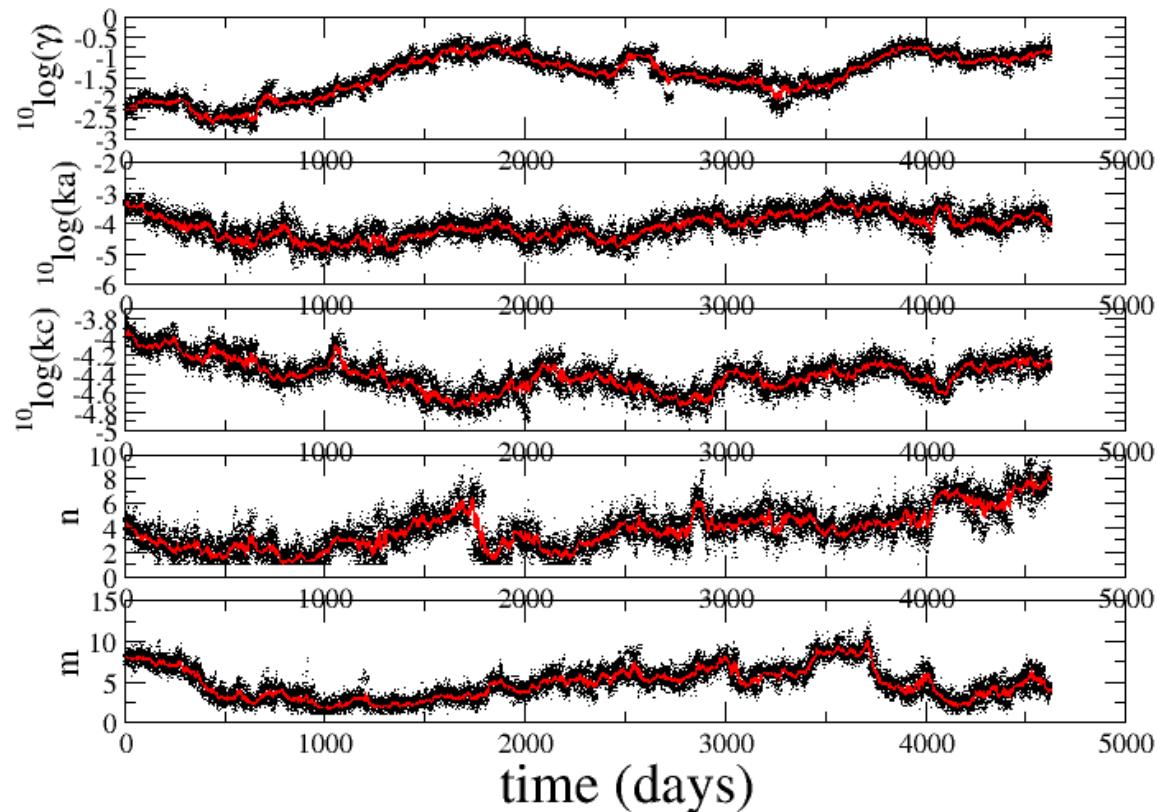
Glucose vs. lactose, at end of evolution



cAMP vs. allolactose, at end of evolution

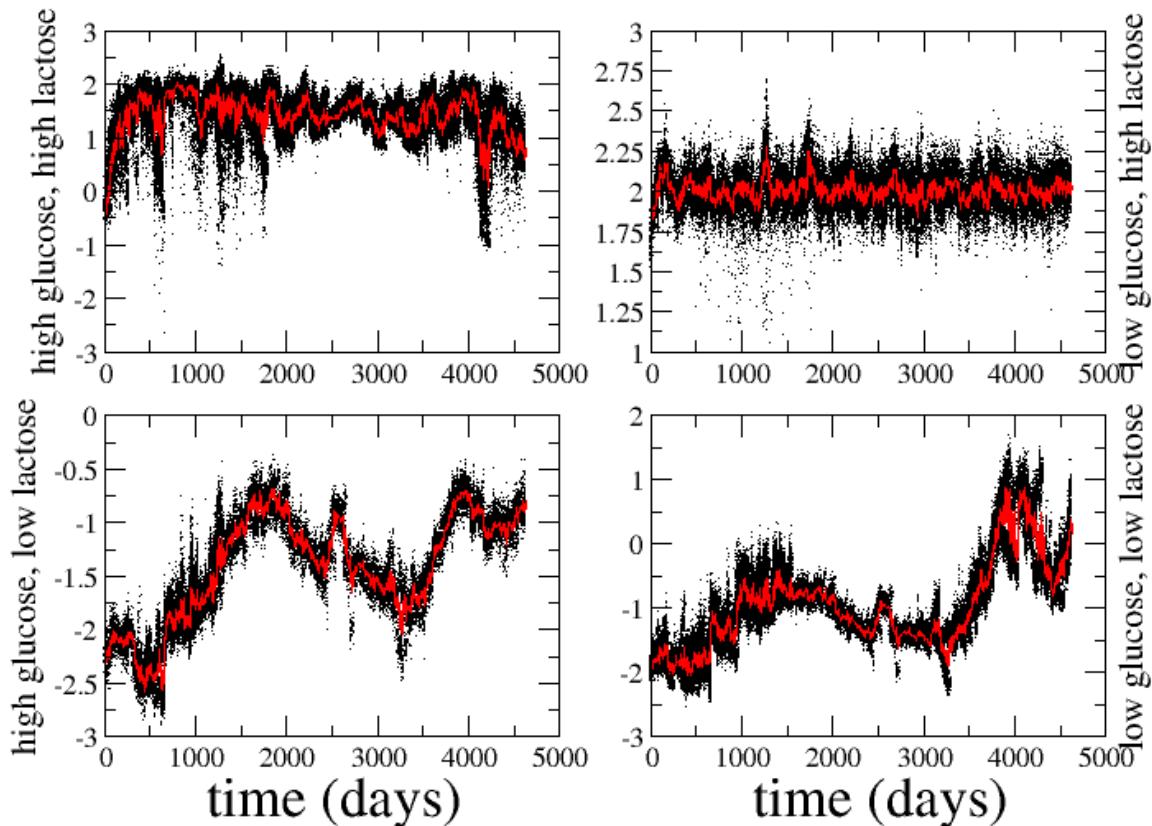


Evolution: how to observe parameter of individuals in pop. in time



Evolution: how to observe phenotypic features in time (4 extremes)

Evolution of 4 corners



Evolution: how to observe comparison of evolutionary outcomes

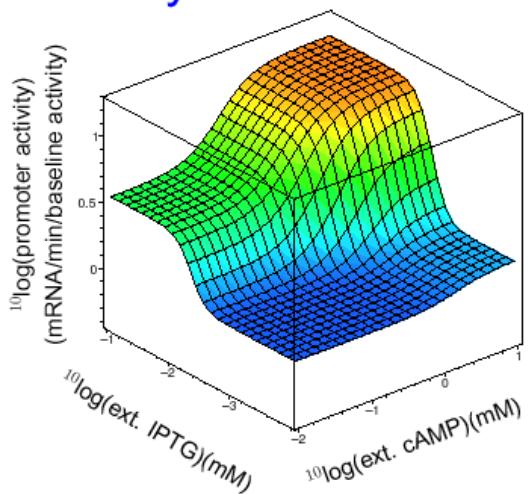
Ancestor trace!

Compete last common ancestors (n*)

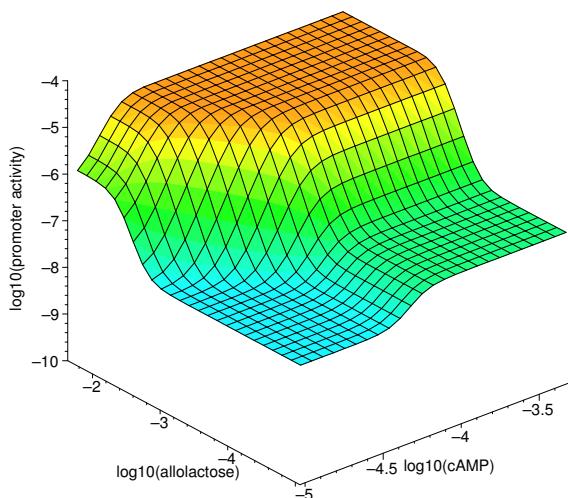
Compete last populations (n*)

-- > “BEST” evolved promoter function

**'Best' evolved last common ancestor
deterministic intracellular dynamics; 11hr average influx regime
spatial pattern formation**

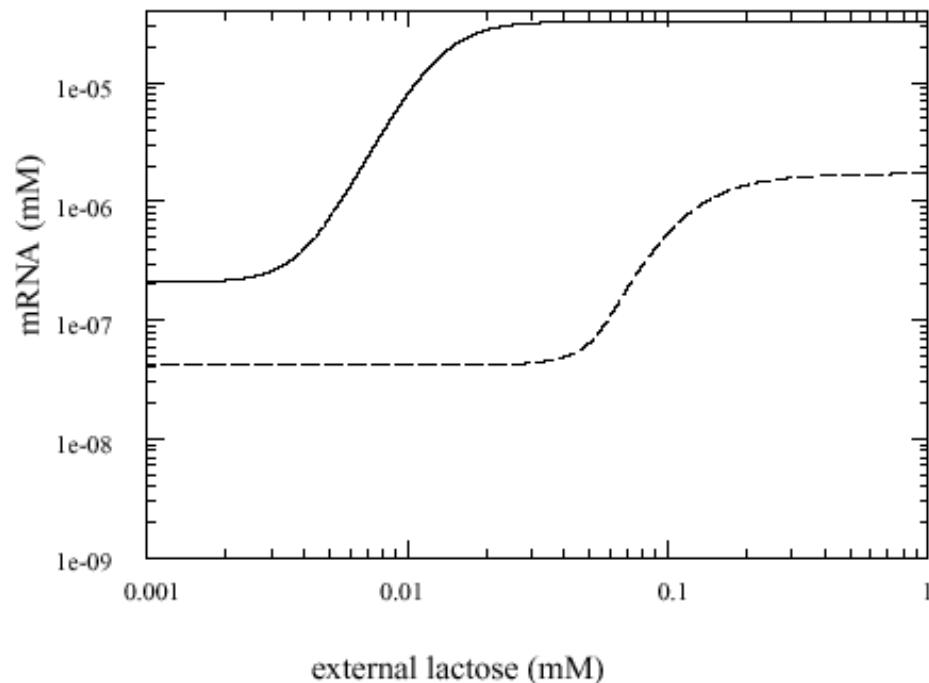


OBSERVED (Setty 2003)



BEST EVOLVED LCA

**Similar to measured promoter function
However NO bistability**



What about experiments / prior modeling?

Conditions for bistability for artificial inducer VERY different from those for lactose.

$$\lambda(C) \equiv \frac{PA(0, C)}{(m-1)^2} \left(\frac{(m+1)^2}{PA(\infty, C)} + 4m\zeta \right) < 1.$$

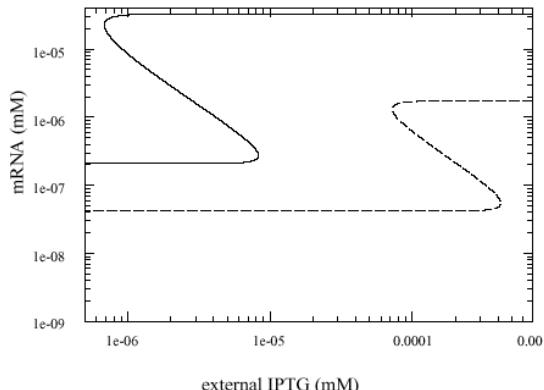
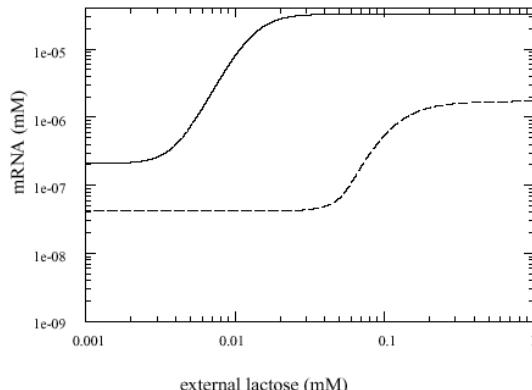
for lactose:

$$\frac{PA(0, C)4m\zeta}{(m-1)^2} < 1.$$

for artificial inducer

$$\frac{PA(0, C)}{(m-1)^2} \frac{(m+1)^2}{PA(\infty, C)} < 1.$$

Evolved promoter function bistable for artificial inducer!

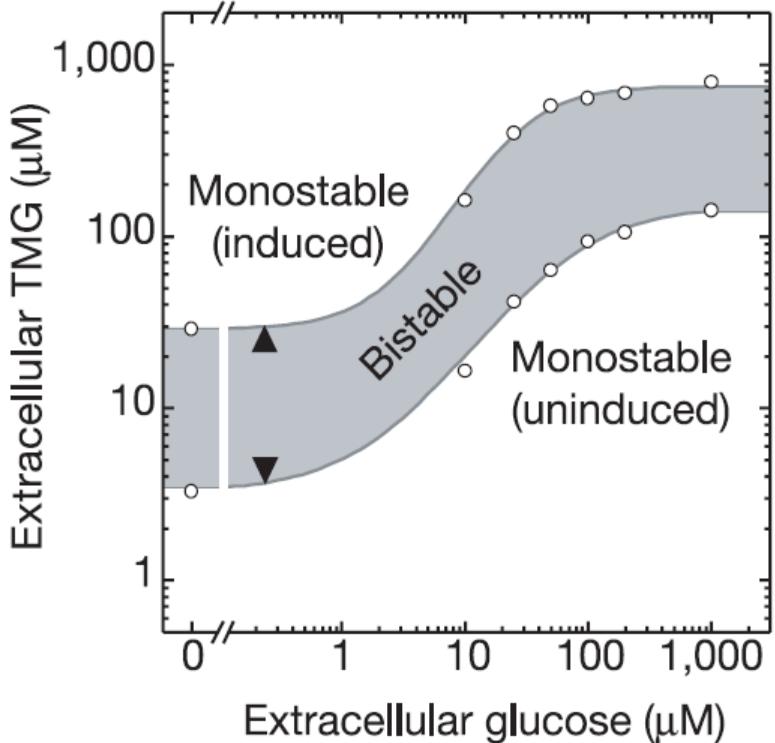


LACTOSE

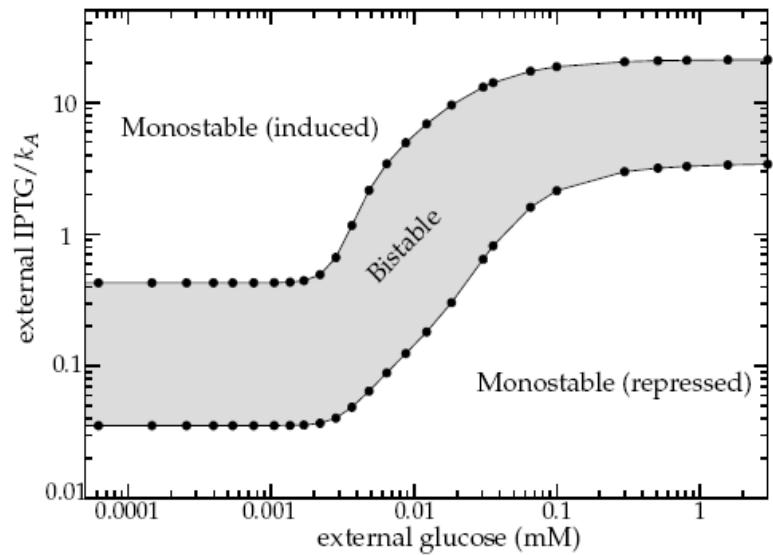
ART INDUCER

evolved vs measured bistability for artificial inducer

c

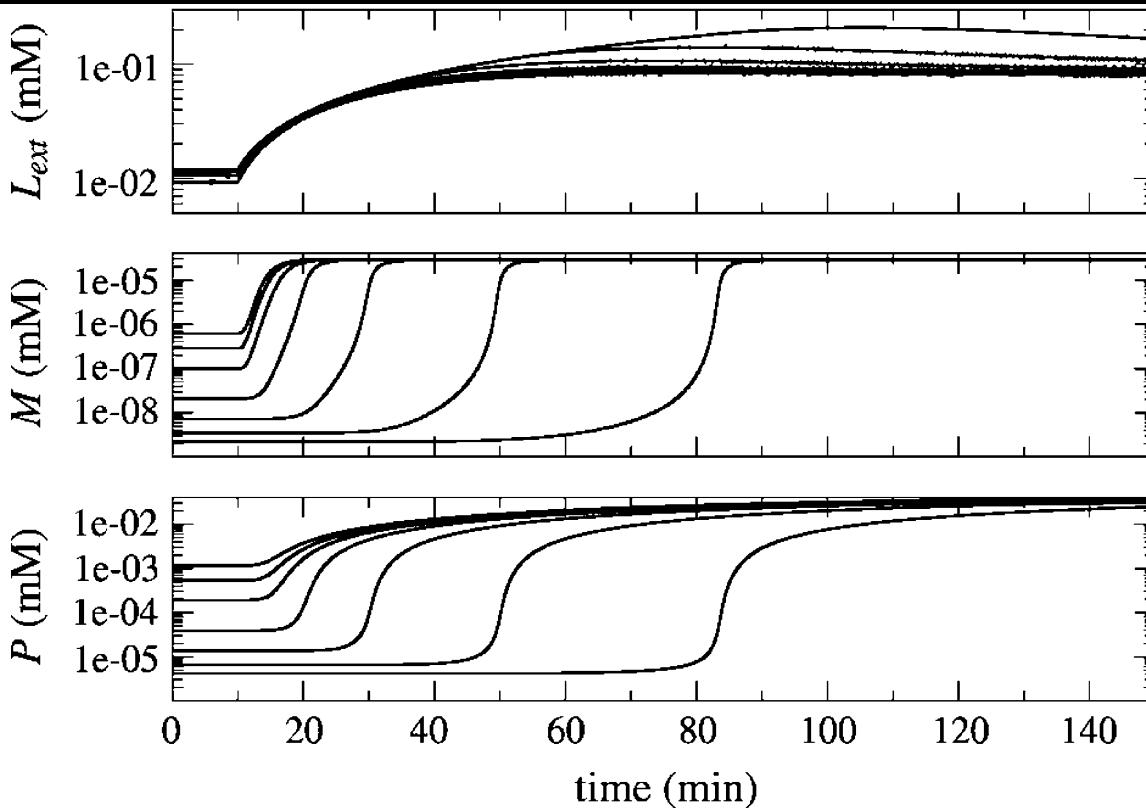


measured (van Oudenaarden)



evolve (van Hoek)

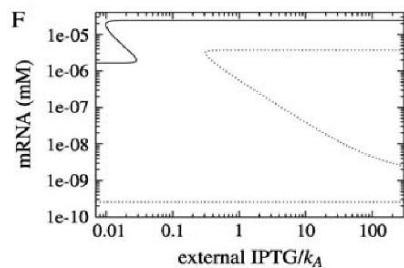
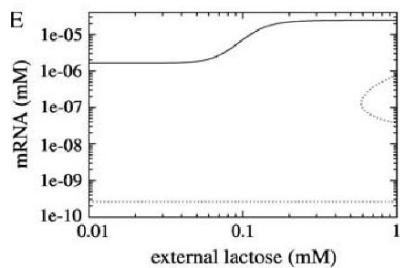
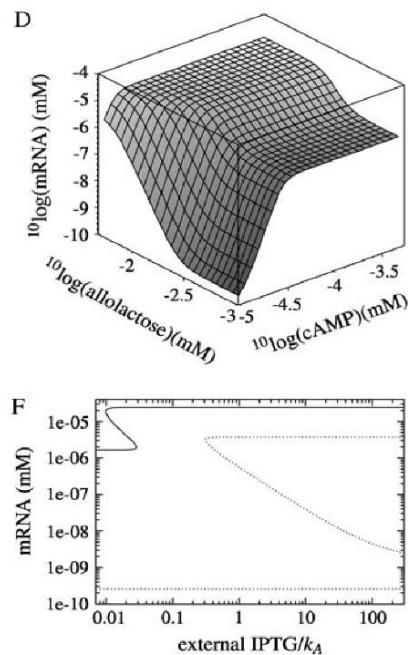
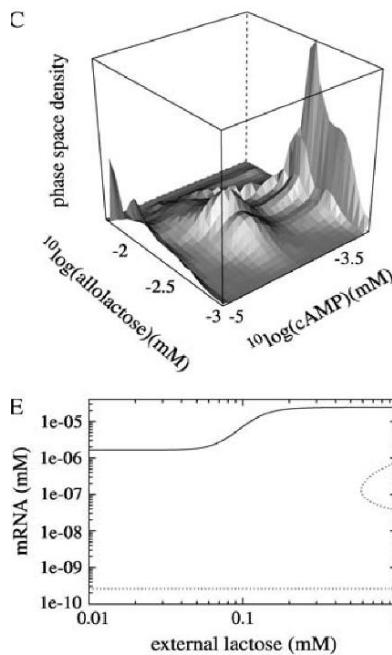
Why avoid bistability
why 'waste' expression when no (low) Lactose available
Non-equilibrium: delays!



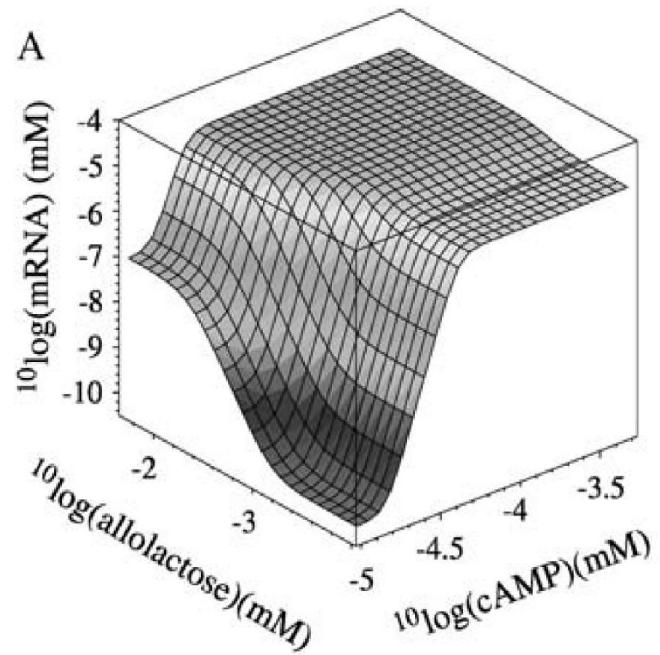
lines for different γ values ($P(0, C)$)

(E.coli division time ca 1hr)

sensitivity to experimental design cost of expression and frequency of environmental switching



high cost bistab at rare high glucose



fast switch : loss of regulation

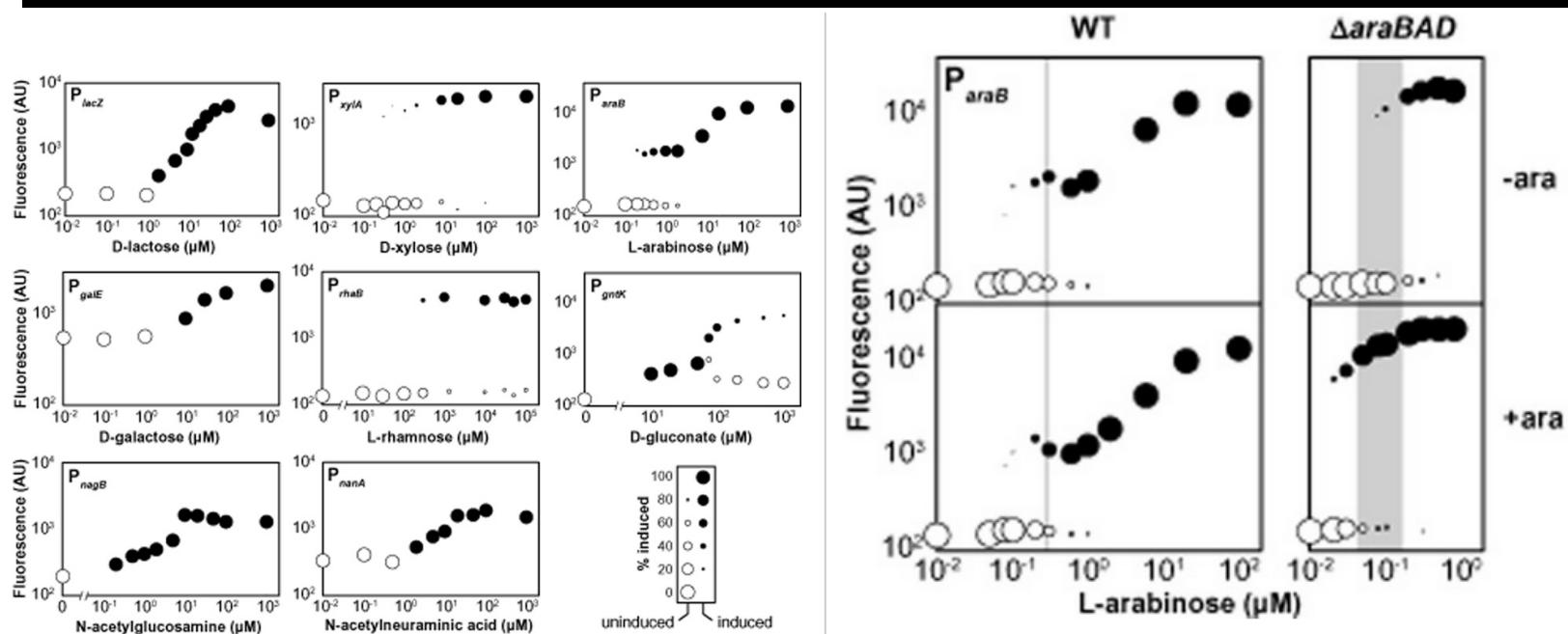
Experimental support for evolutionary model

E.M. Ozbudak, M. Thattai, H.N. Lim, B.I. Shraiman, A. Van Oudenaarden Multistability in the lactose utilization network of *Escherichia coli*. *Nature*, 427 (2004), pp. 737

in supplementary material

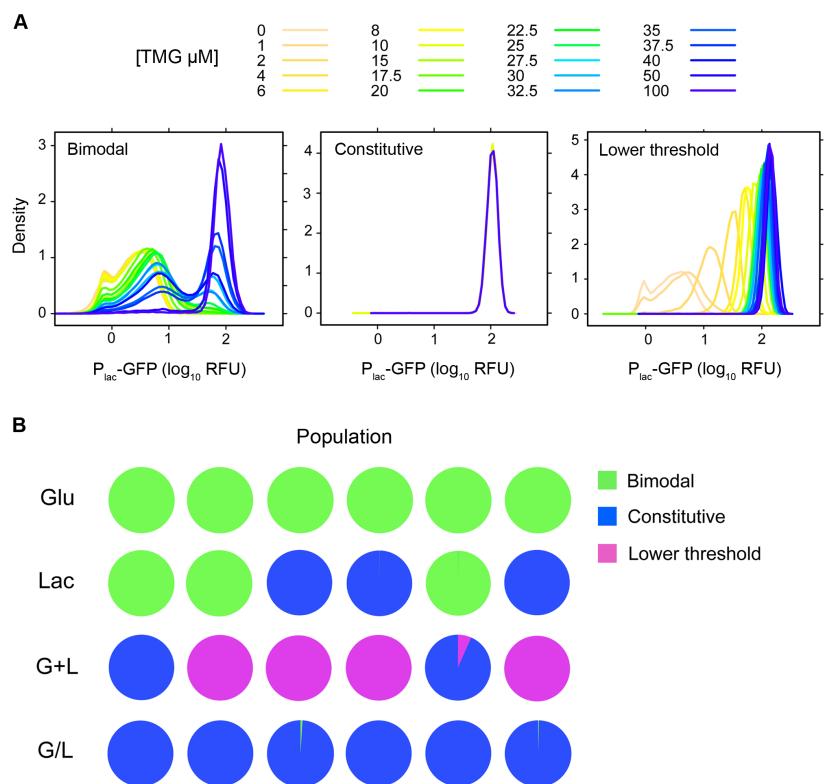
**During induction with lactose, as opposed to IPTG, TMG.....
the steady state distribution after 4 hours of growth is
always uni-modal, and we never observe hysteresis.**

various responses for different sugars suppressing catabolism enhances hysteresis



experimental support of fast evolutionary change, avoidance of bistability even relative to TMG

cf Adaptive Evolution of the
Lactose Utilization Network
in Experimentally Evolved
Populations of
Escherichia coli
Quan et al 2012



Bistability and Nonmonotonic Induction of the *lac* Operon in the Natural Lactose Uptake System

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¹Max-Planck-Institute for Dynamics of Complex Technical Systems, Magdeburg, Germany

ABSTRACT The *Escherichia coli lac* operon is regulated by a positive feedback loop whose potential to generate an all-or-none response in single cells has been a paradigm for bistable gene expression. However, so far bistable *lac* induction has only been observed using gratuitous inducers, raising the question about the biological relevance of bistable *lac* induction in the natural setting with lactose as the inducer. In fact, the existing experimental evidence points to a graded rather than an all-or-none response in the natural lactose uptake system. In contrast, predictions based on computational models of the lactose uptake pathway remain controversial. Although some argue in favor of bistability, others argue against it. Here, we reinvestigate *lac* operon expression in single cells using a combined experimental/modeling approach. To this end, we parameterize a well-supported mathematical model using transient measurements of LacZ activity upon induction with different amounts of lactose. The resulting model predicts a monostable induction curve for the wild-type system, but indicates that overexpression of the LacI repressor would drive the system into the bistable regime. Both predictions were confirmed experimentally supporting the view that the wild-type *lac* induction circuit generates a graded response rather than bistability. More interestingly, we find that the *lac* induction curve exhibits a pronounced maximum at intermediate lactose concentrations. Supported by our data, a model-based analysis suggests that the nonmonotonic response results from saturation of the LacI repressor at low inducer concentrations and dilution of Lac enzymes due to an increased growth rate beyond the saturation point. We speculate that the observed maximum in the *lac* expression level helps to save cellular resources by limiting Lac enzyme expression at high inducer concentrations.

model and parameters used (literature + measured) ((slightly) different to previous model)

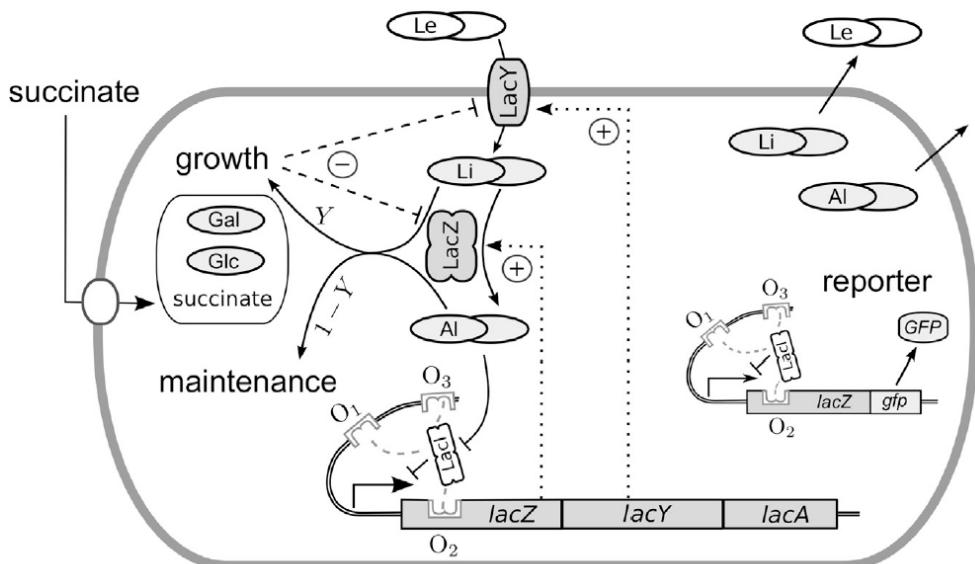
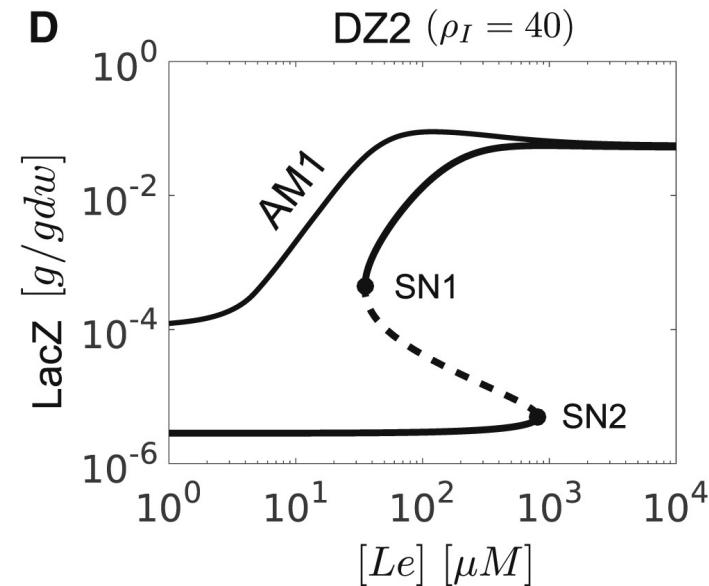
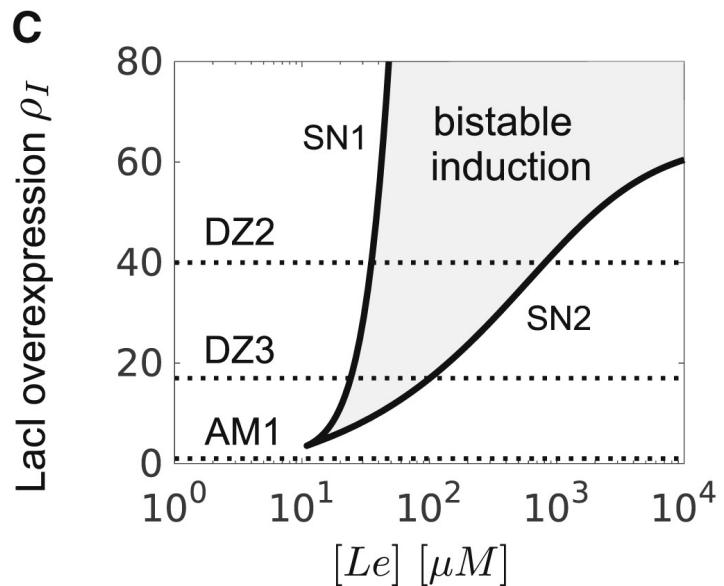
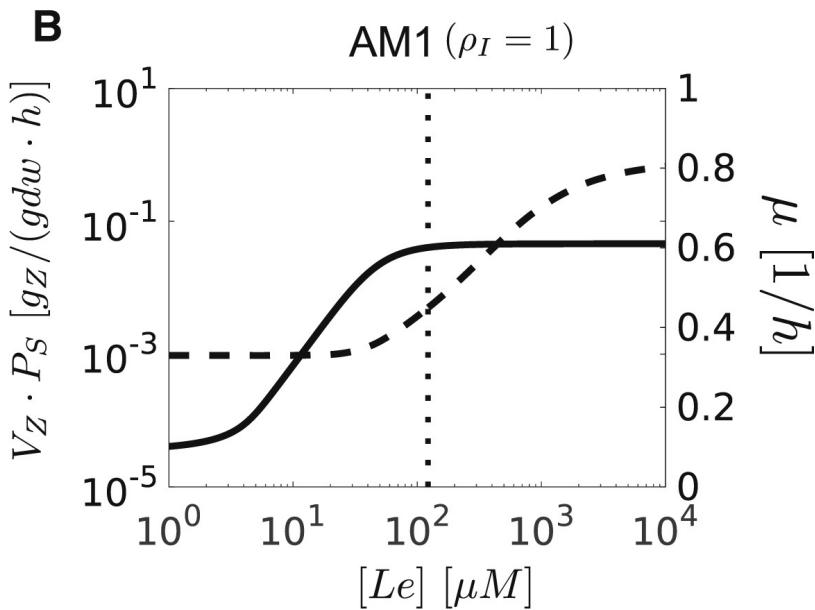
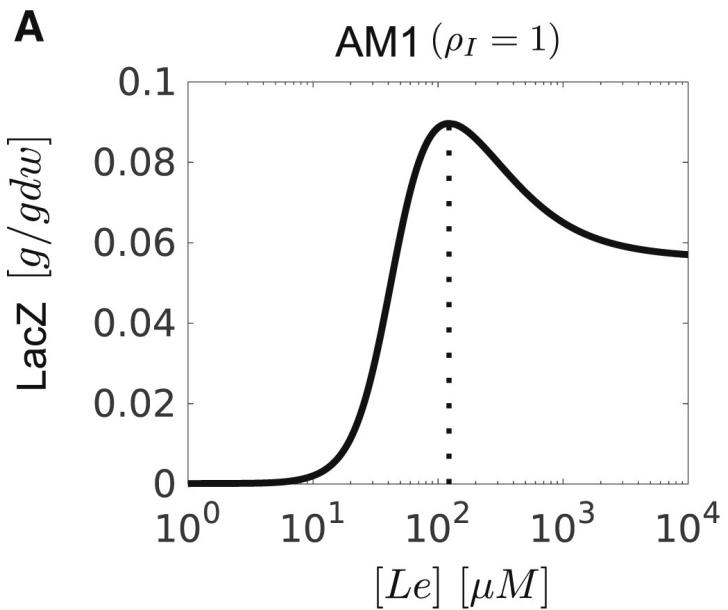


TABLE 2 Model Parameters

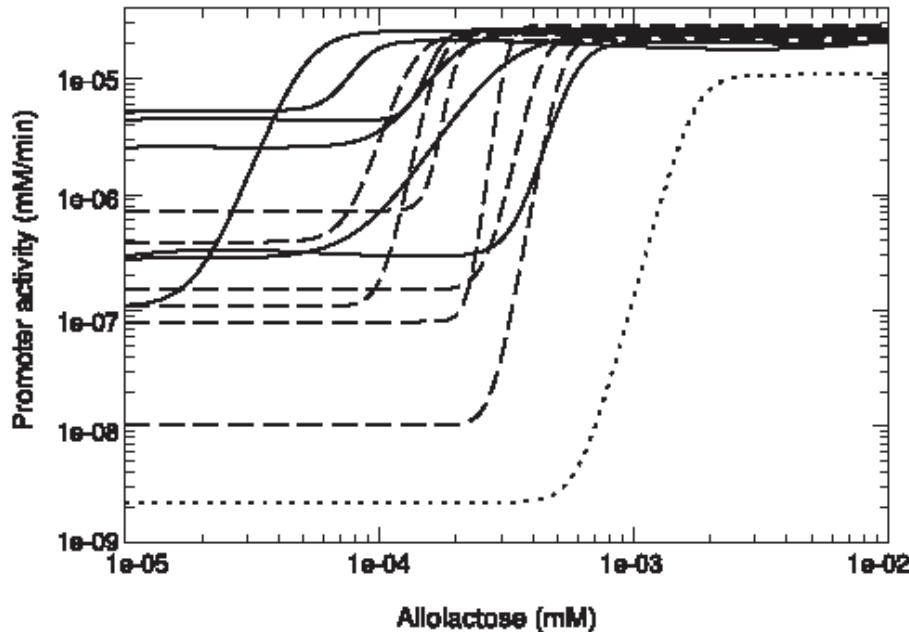
Name	Value	Reference	Name	Value	Reference
V_L	$1271 \frac{g_L/gdw}{g_Y/gdw} \frac{1}{h}$	(20)	κ_2	0.38	(34)
K_L	$0.68 \frac{g_L/L}{(\approx 2 mM)}$	estimated	α_1	31	(34)
K_i	$0.00013 \frac{g_L}{gdw}$	estimated	$\hat{\alpha}_1$	1420	(34)
V_{lac}	$670 \frac{g_L/gdw}{g_Z/gdw} \frac{1}{h}$	(30)	α_2	19	(34)
K_{lac}	$0.0029 \frac{g_L}{gdw}$	(30)	$\hat{\alpha}_2$	322	(34)
V_{al}	$1019 \frac{g_A/gdw}{g_Z/gdw} \frac{1}{h}$	(30)	α_3	3	(34)
K_{al}	$0.0014 \frac{g_A}{gdw}$	(30)	Y	$0.092 \frac{g_L}{gdw}$	estimated
V_Z	$0.046 \frac{g_Z/gdw}{h}$	(28)	k_e^-	$60 \frac{1}{h}$	(21)
K_a	$1.5 \times 10^6 \frac{1}{g_A/gdw}$	(27)	μ_b	$0.33 \frac{1}{h}$	measured

g_X/gdw denotes gram of species $X = L, A, Y, Z$ per gram dry weight (see Table 1).

No bistability for measured parameters, but can be induced by over-expression of LacI. LacZ expression saturates, and by dilution LacZ concentration peaks at intermediate Le, because growth rate increases. Zander et al 2017



Indeed in the model evolution of lac operon avoids bistability by increasing repressed expression level
(and even more so in stochastic version)



dotted: start (bistable); solid evolved stoch.; dashed evolved det.

conclusions

Evolutionary modeling to 'test' regular systems biology models/experiments

Evolutionary perspective helped to debug long held misconceptions

which were prior “verified” theoretically AND experimentally

Evolutionary modeling powerful tool for alleviating parameter uncertainty

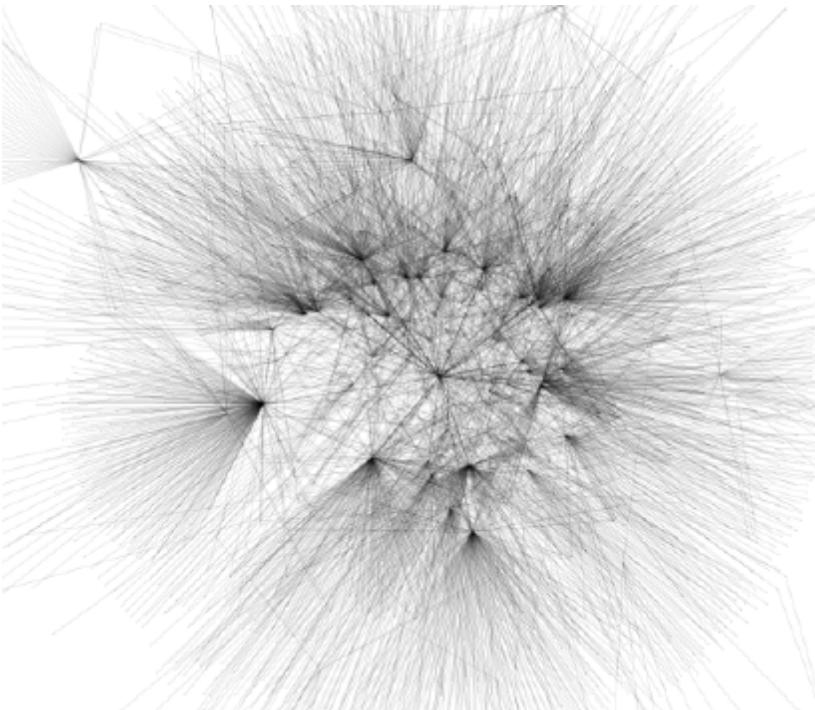
Evolutionary change in parameters very uninformative

Parameter uncertainty inherent in evolutionary context

(parameter redundancy; condition dependent parameter
change (“TRUE” parameters do not exist))

*Non-supervised modeling 'fits' better then fitted supervised
models*

Modeling gene regulation/signal transduction Monster of Loch Ness syndrome



"quod erat demonstrandum"
evolution as trick to cope with parameter uncertainty

HOWEVER: “function” of bistability is often assumed increased population variability, and therewith rapid adaptation, GIVEN stochastic gene expression

Above results artifact of deterministic modeling?

study: bistability and stochasticity
in the lac operon

cf Thattai & van Oudenaarden (2004):
noise + bistability can be ‘good’ because it allows rapid switching due to population heterogeneity.

However: minimization of expression noise in essential genes (Fraser et al 2004)

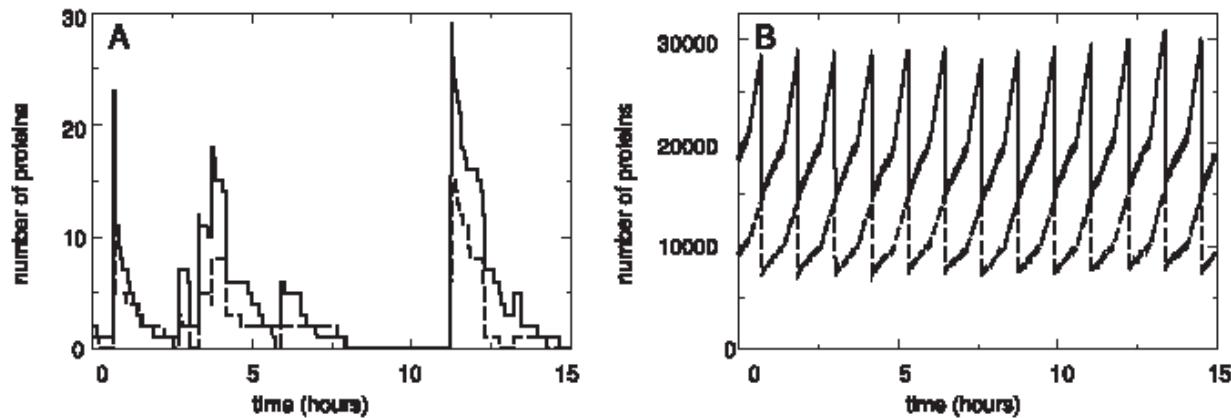
But: Excessive stochasticity of promoter function measured in E. coli (Wolf & van Nimwegen 2016)

from deterministic to stochastic model of lac operon only one (measured) parameter added

protein translation occurs in bursts:
geometrically distribution, average size 5 proteins
(Cai et al 2007)

model chance of burst proportional to # mRNA

at cell division distribute proteins binomially over the cells

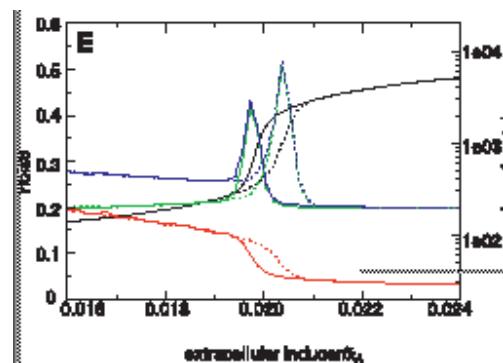
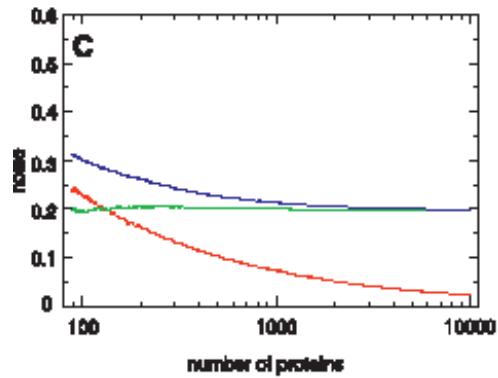
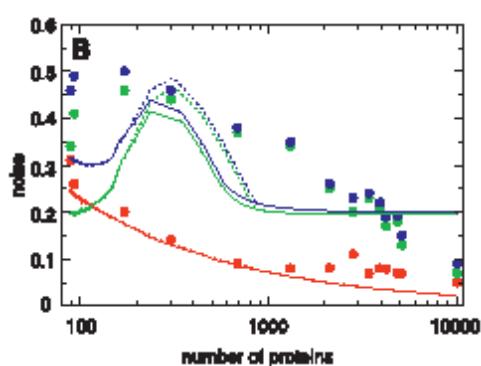


intrinsic vs extrinsic noise: experiments and model

extrinsic noise: cell cycle + intracellular inducer concentration (green)

intrinsic noise: difference in expression of 2 identical promoters in a single cell (red)

$$N_{tot} = N_{ext} + N_{int} = std/mean \text{ in population (blue)}$$



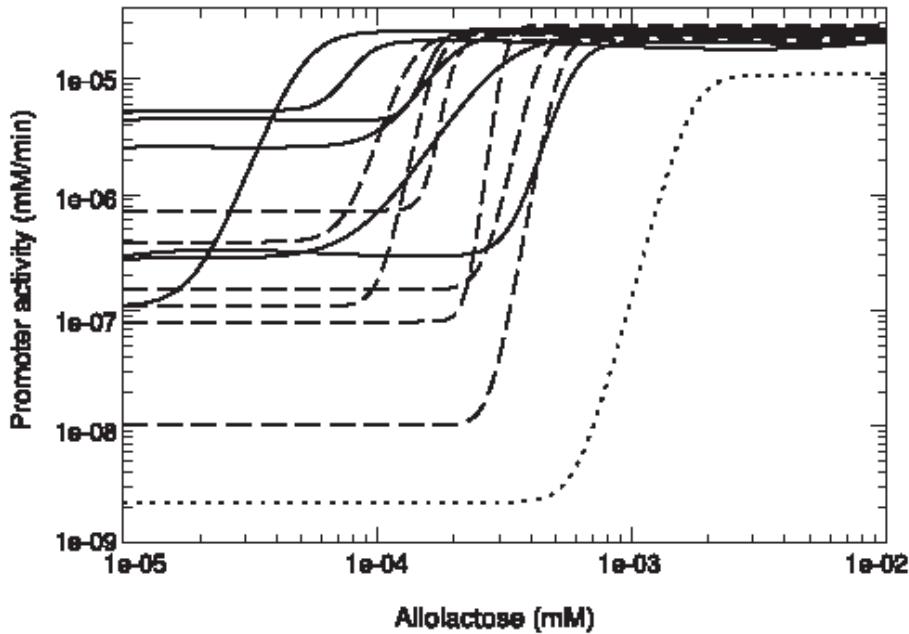
IPTG as inducer

Lac as inducer

IPTG as inducer

noise relative to internal protein numbers relative to external IPTG
black: internal protein number

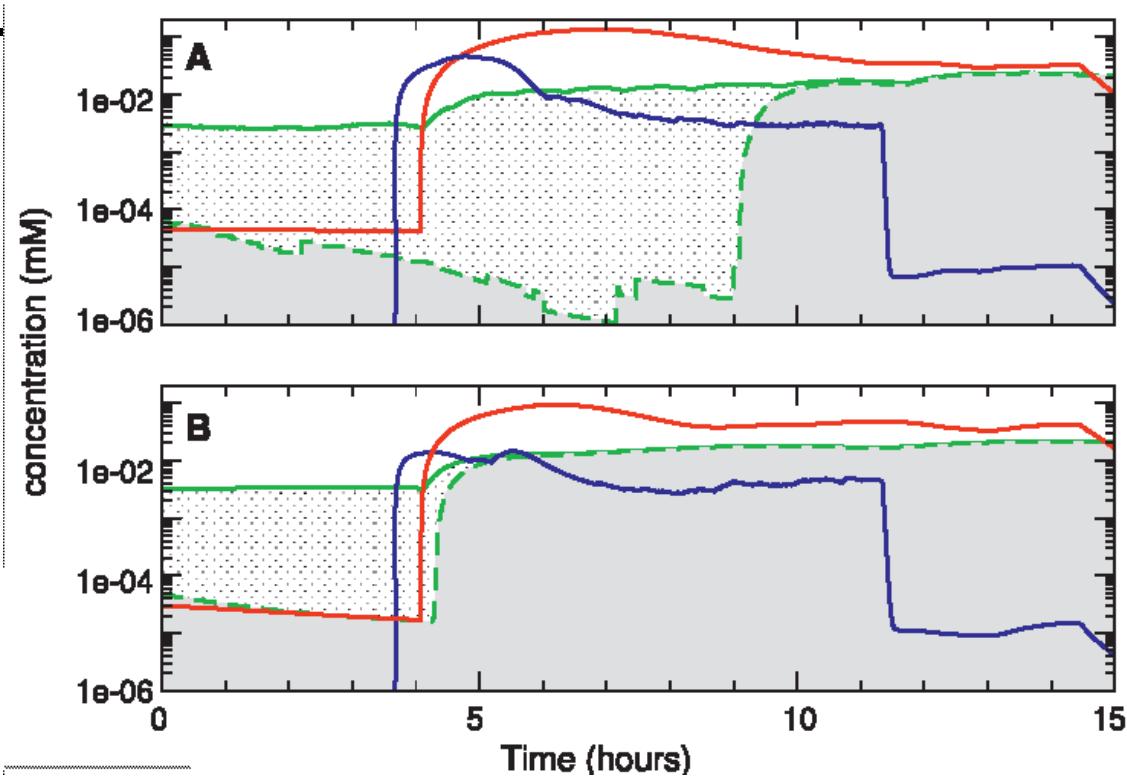
evolution of lac operon with stoch. prot. expression avoids bistability even more



dotted: start (bistable); solid evolved stoch.; dashed evolved det.

WHY?

**long delay in induction in stoch model
when in bistable regime (i.e. low repressed expression)**



red ext. lac; blue ext. gluc; green β Galactosidase (high/low)

Growth rates of true promotor functions in deterministic and stochastic models

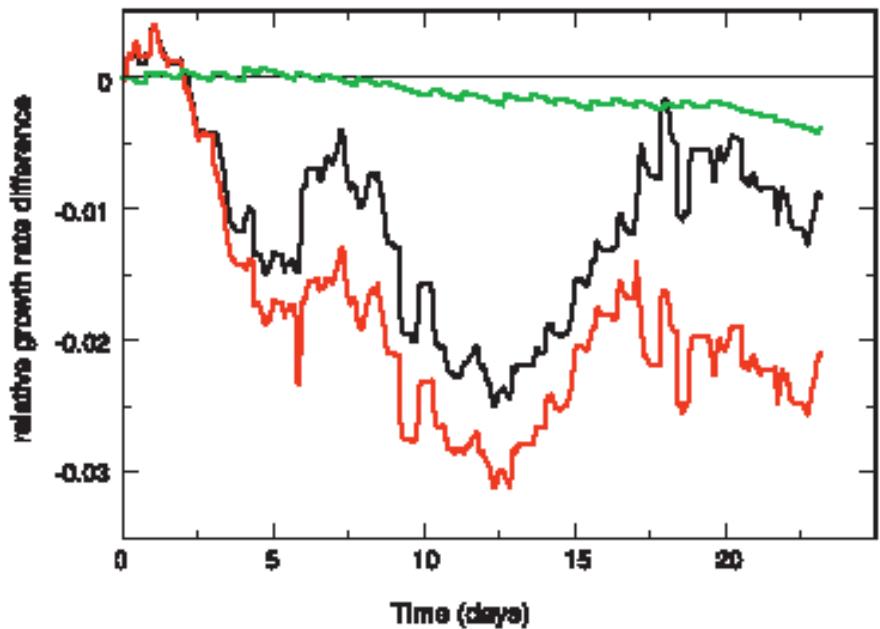
all times resource pulses are different

*low repressed expression 'better' when no lactose and vv
therefor compare growth-rates over time relative to
deterministic, low repres*

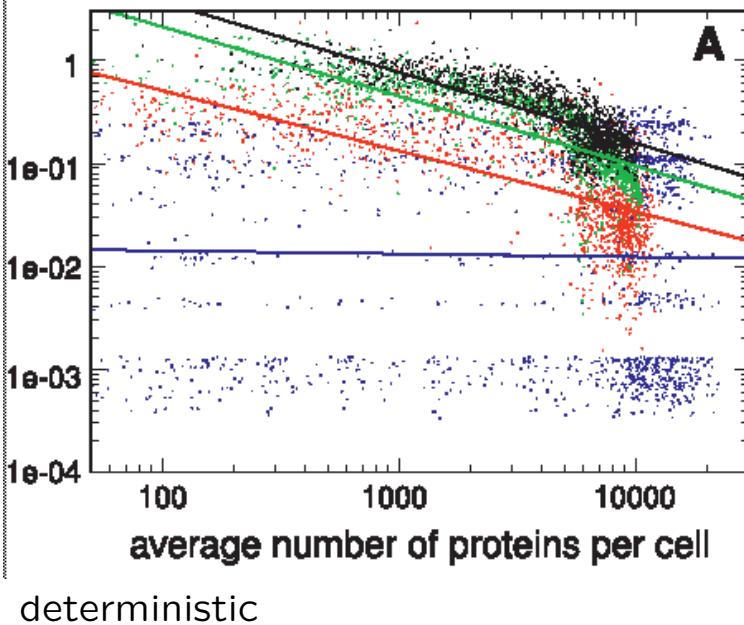
green: deterministic, low repressed

black stoch. : high repressed rates

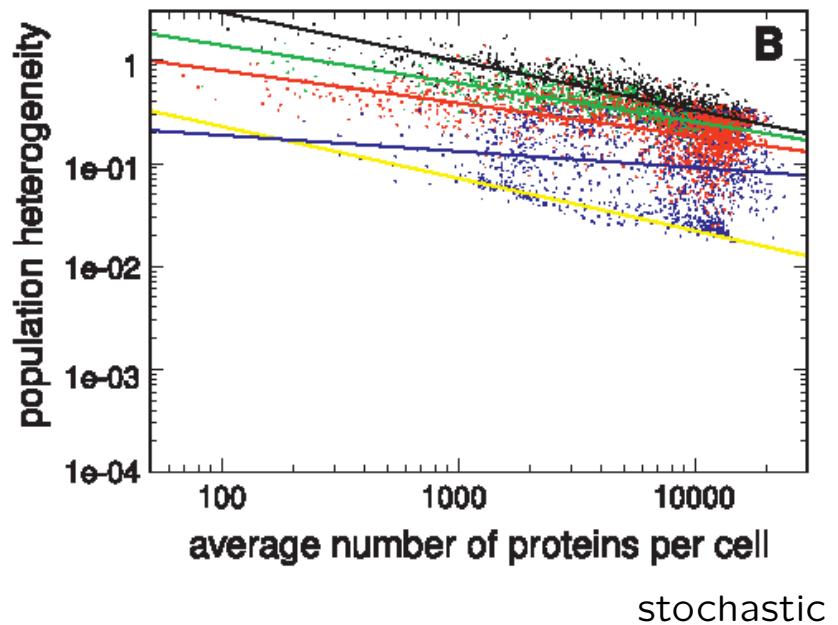
red stoch: low repressed rates



population heterogeneity in various model variants:
deterministic vs stochastic; genetic vs one clone, spatial vs well mixed



deterministic



stochastic

black: full model; red: well mixed; green 1 clone full model; blue 1 clone well mixed;
note partial synchronization; yellow intrinsic noise

conclusions

Bistability even more detrimental when stochasticity is taken into account
on induction: long waiting for large bursts.

role of stochasticity overestimated by considering genetically identical cells in a homogeneous environment in equilibrium

non-equilibrium conditions can reduce population heterogeneity

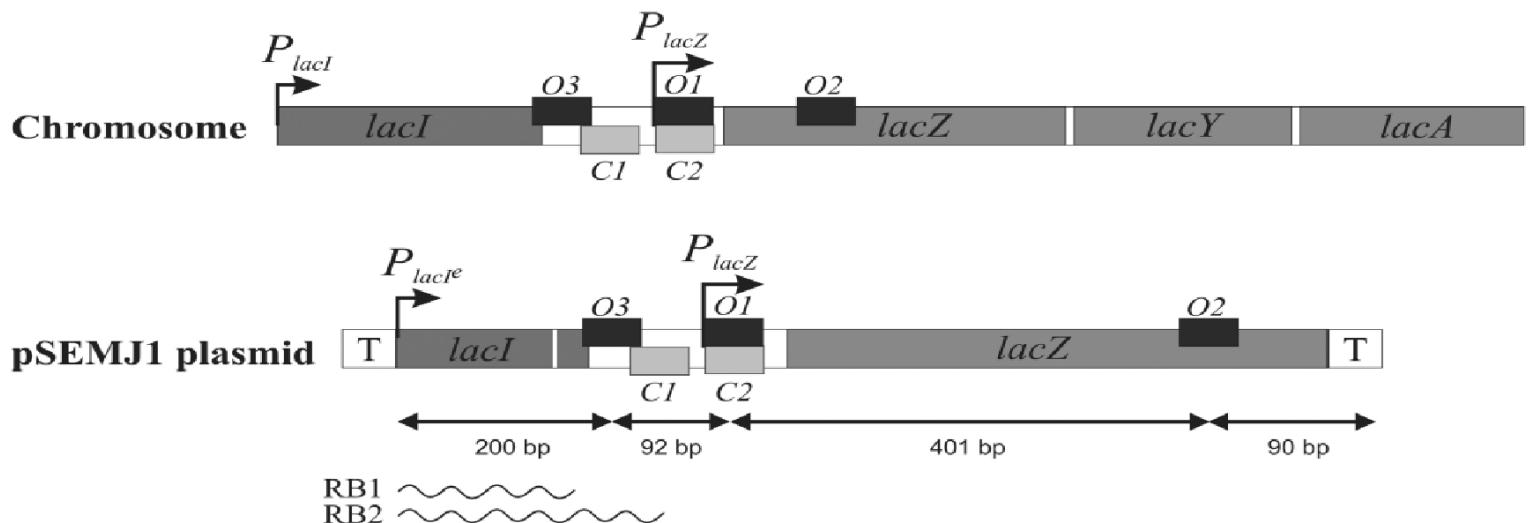
large genetic heterogeneity in natural populations: fast adaptation to environmental condition

interlocking of evolutionary and regulatory timescales!

Parameter uncertainty inherent in evolution

MOREOVER: LACI autoregulator!

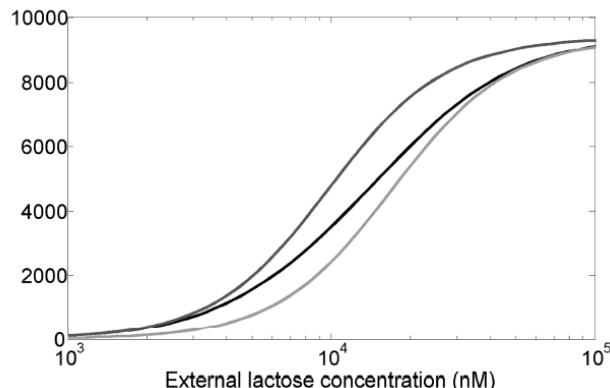
Semsey et al 2013



Binding state	Relative probability	LacI production	LacZYA production
unbound	1	1	1
<i>O1</i> bound	$\varepsilon_1 I^*$	1	0
<i>O1-O2</i> loop	$\varepsilon_2 I^*$	1	0
<i>O1-O3</i> loop	$\varepsilon_3 I^*$	0	0

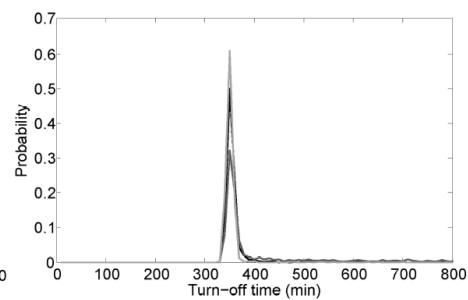
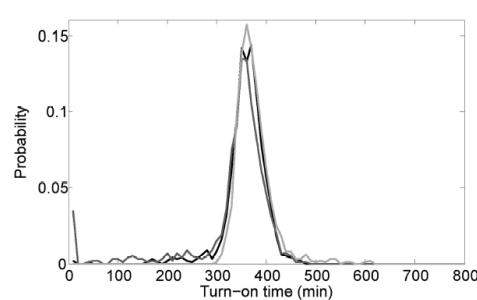
LacI autoregulation leads to smoother activation, and less variation in delays relative to constitutive expression.

LacY expression



deterministic model

Delay in expression after switch



stochastic model

Table 2. Mean \pm standard deviations of turn-on and turn-off times obtained in the simulations shown in Figure 6

LacI range: 30–90 molecules			LacI range: 10–30 molecules		
	WT (autoregulated)	Constant low	WT (autoregulated)	Constant low	Constant high
Turn-on time (minutes)	354.2 ± 41.7	330.5 ± 85.4	368.8 ± 36.0	349.3 ± 44.2	308.7 ± 103.7
Turn-off time (minutes)	363.7 ± 74.4	468.9 ± 246.0	348.4 ± 33.0	394.0 ± 128.6	602.0 ± 392.5