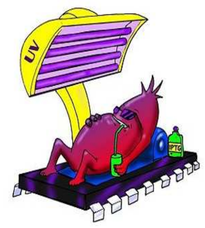
E.coli PROM: an Erasable and Programmable Genetic Memory in *E. coli*

Alma Mater Studiorum University of Bologna

Laboratory of Cellular and Molecular Engineering - II Faculty of Engineering - Campus of Cesena

Students: M. V. Amaduzzi, B. Angelici, F. Avanzi, I. Cenci, G. Mariotta, S. Menozzi, R. Morini, C. Nicoletti, L. Onofri, G. Selvaggio, M. Tramonti;

Instructors: S. Cavalcanti, F. Ceroni, S. Furini; *Advisors:* M. Caprini, E. Giordano, M. Tartagni



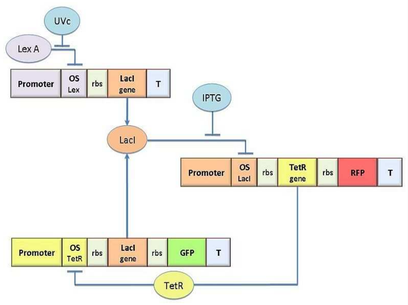
1. The E.coli PROM project

The project aims to design a bacterial reprogrammable memory (EPROM) with genetically engineered E.coli. To engineer bacteria we designed a genetic Flip-Flop formed by an epigenetic binary memory sensitive to IPTG -for the memory reset- and an UV-sensitive trigger to set the memory ON. We designed the Flip-Flop circuit by model-based analysis and computer simulation. The core elements are two mutually regulated promoters. Each of them is composed of a constitutive promoter and an independent operator sequence. Thus, transcriptional strength and repressor binding affinity can be independently tuned. Since operator sites are still lacking in the Registry as standard parts, we cloned operator sequences for LacI, TetR, Lambda and LexA repressors and established an experimental procedure to characterize them. These parts allow the rational design of regulated promoters and we expect them to be a benefit in many Synthetic Biology applications.

2. The genetic Flip-Flop

The molecular circuit

The molecular circuit can switch between two different stable states (LacI-ON and TetR-ON), in response to two external stimuli (UVc and IPTG). LacI-ON represents the stable state in which LacI gene is active and represses TetR gene expression. On the contrary, TetR-ON represents the state with TetR gene active and LacI silenced (LacI-OFF). Thanks to the coexistence of two stable states (bistability) this circuit may work as a binary memory. We named it genetic Flip-Flop since it operates as a SR Latch: LacI state is the input and TetR state is the output. UVc is the set signal and IPTG is the reset signal.



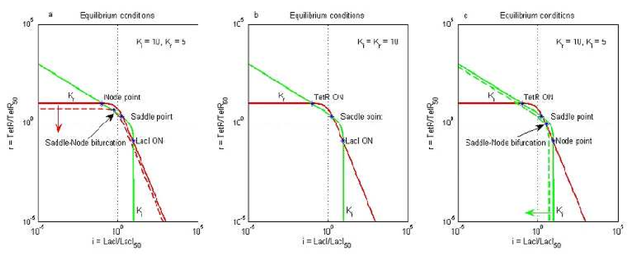
Flip-Flop model equations

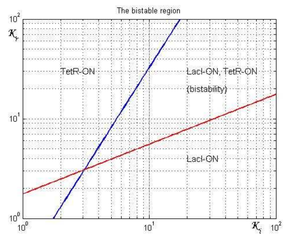
$$\begin{cases} \dfrac{dI}{dt} = \dfrac{\alpha_I}{1+\left(\dfrac{R}{R_{50}}\right)^{\mu_R}} + \dfrac{\alpha_A}{1+\left(\dfrac{A}{A_{50}}\right)^{\mu_A}} \cdot e^{\beta \cdot UVc} - \delta \cdot I \\ \dfrac{dR}{dt} = \dfrac{\alpha_R}{1+\left(\dfrac{I^F}{I_{50}}\right)^{\mu_I}} - \delta \cdot R \\ I^F = \dfrac{I}{1+\left(\dfrac{IPTG}{IPTG_{50}}\right)^2} \end{cases}$$

Symbol	Definition
$I,\ R,\ A$	LacI, TetR and LexA repressors
I^F	Active quote of LacI repressor
$I_{50},\ A_{50},\ R_{50}$	Affinity constant of the repressor-operator binding
$\alpha_I,\ \alpha_R,\ \alpha_A$	Maximum velocity of repressor synthesis
$\mu_I,\ \mu_R,\ \mu_A$	Cooperativity coefficients of repressor-operator binding
δ	Protein degradation/dilution rate
$IPTG$	Isopropylbeta-D-thiogalactopyranoside
$IPTG_{50}$	IPTG concentration blocking half of induction
UVc	UV Radiation absorbed by the sample (253.7 nm)

Equilibrium conditions

Equilibrium conditions depend on Ki and Kr assignment. Changing the value of these parameters a saddle-node bifurcation can occur.





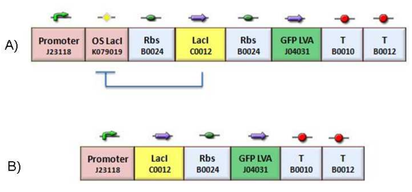
Circuit is bistable for a limited range of Ki and Kr indexes. Bistability (LacI-ON and TerR-ON) is guaranteed in the region of the Ki-Kr plane where:

$$\begin{cases} K_r < \dfrac{3\sqrt{3}}{4^2} \cdot {K_i}^2 \\ K_r > \dfrac{4}{\sqrt{3\sqrt{3}}} \cdot \sqrt{K_i} \end{cases}$$

Procedure for Ki-index identification

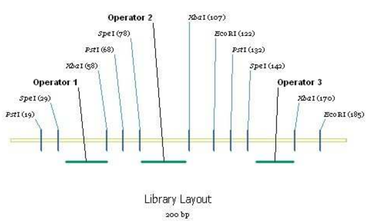
For a regulated promoter (constitutive promoter flanking an operator sequence), the value of Ki parameter can be identified after measuring the GFP expressed in closed- (A) and open-loop (B) configurations by the relationship :

$$K_i = \frac{GFP_0}{GFP_C} \cdot \sqrt{\frac{GFP_0 - GFP_C}{GFP_C}}$$



3. Operator sites as BioBricks

We introduced operator site parts in order to build regulated promoter with independent transcriptional strenght and repression sensitivity. We first synthesized four libraries of operator sequences, respectively for LacI, TetR, cI and LexA repressor proteins. The libraries were synthesized by Geneart in their standard pGA18 and pMA plasmids, with both ColE1 high copy number origin of replication and Ampicillin resistance. In each library there are three sequences, each with a different binding affinity to the repressor protein. We isolated each operator with the intention to clone them into BioBrick standard assembly plasmids.



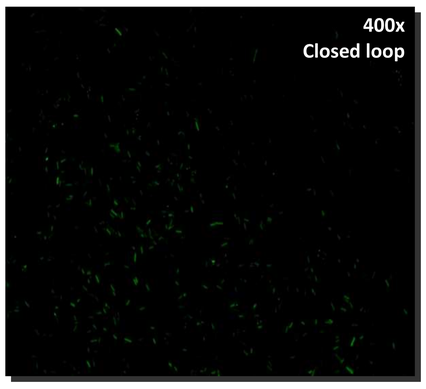
LacI			Lambda		
Name	Sequence	Affinity	Name	Sequence	Affinity
Lac SymL	aattgtgagcgctcacaatt	Very Strong	Lambda OR1	tatcaccgccagaggta	Strong/Intermediate cI/Cro
Lac O1	aattgtgagcggataac aatt	Intermediate	Lambda OR2	taacaccgtgcgtgttg	Intermediate/Weak cI/Cro
Lac O2	aaatgtgagcgagtaacaacc	Weak	Lambda OR3	tatcaccgcaagggata	Intermediate/Intermediate cI/Cro

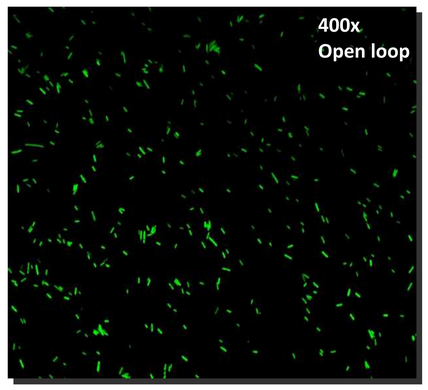
Tet			Lex		
Name	Sequence	Affinity	Name	Sequence	Affinity
TeT O	cctatcagtgatagag	Strong	LexA 1	ctgtatatatatacag	Very Strong
TetO-4C	cctgtcagtgacaga	Intermediate	LexA 2	ctgtatgagcatacag	Quite Weak
TetO-wt/4C5G	cctatcagtgacgga	Weak			

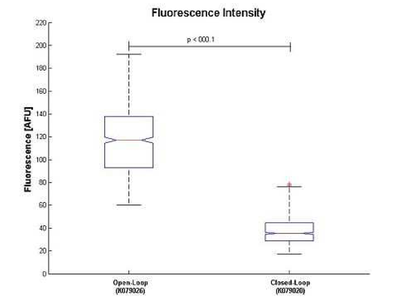
4. Construct Characterization

LacI Operator

LacI O2 operator (K079019) was tested by the circuits shown in section *Procedure for Ki-index identification*. In the closed-loop configuration , GFP expression is auto-regulated by the synthesis of LacI repressor protein (left panel). The open-loop configuration expresses the maximum fluorescence, lacking the operator site (right panel). The comparison of bacteria fluorescence confirmed the functionality of Lac operator site.







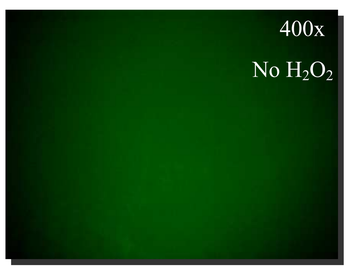
	Bacteria Number	Mean	Standard Deviation	Median
BBa_K079020 (Closed- Loop)	683	116	29	117
BBa_K079026 (Open-Loop)	683	38	12	35

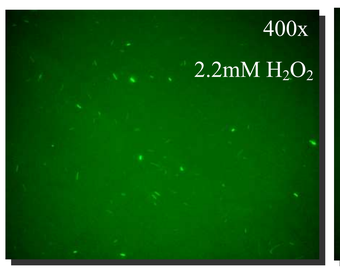
The Ki-index, was 4.43, thus, the Kr - range for the bi-stability is from 4 to 6.

LexA2 Operator

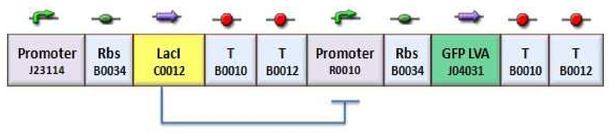
To test the functionality of the LexA operator, we assembled a circuit were GFP is under the control of the BBa_J23100 constitutive promoter and the LexA operator site 2 (K079050, Figure below).

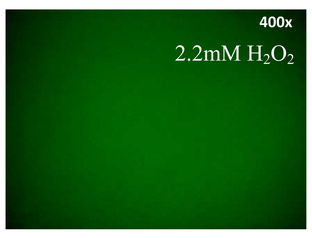
UV signals failed to induce GFP expression, likely because of unevenly irradiation of the sample. Therefore we used an LB medium with 2.2mM H_2O_2, to stimulate the SOS system [Imlay and Linn, 1987]. IPTG was proved to not interact with the SOS circuit.

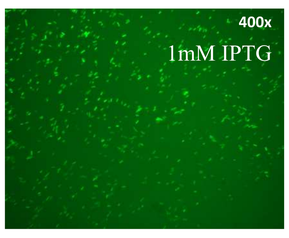




The circuit below was used to asses that H2O2 does not affect the LacI functioning. This result allows the contemporary usage of the two stimuli fn the genetic flip-flop.







7. Discussion

Our results on the use of operator sequences, as independent parts in the assembling of regulated promoters, are still preliminary. However, we are confident that these parts can give a fine-tuning of promoter sensitivity to the repressor allowing the rational design of regulation promoters for Synthetic Biology.

Medal Requirements. The team worked according to the following iGEM2008 medal requirements: - Characterization and improvement of an existing BioBrick (BBa_K079015; - all our experiments and results are entered on the Registry; - we got a collaboration with the Pavia team.

Acknowledgements