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Forward engineering of synthetic bio-logical AND gates

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ABSTRACT

The field of synthetic biology has produced genetic circuits capable of emulating functional paradigms seen in digital electronic circuits. Examples are bistable switches, oscillators, and logic gates. The present work combines detailed mechanistic-kinetic models and stochastic simulation techniques as well as the techniques of *in vivo* molecular biology to study the potential of a synthetic, single promoter AND gate. This device is composed of elements of the *tet*, *lac*, and λ-phage promoters and is responsive to the commonly used inducers IPTG and aTc, producing GFP as an output signal. The quantitative behavior of the AND gate phenotype is studied both *in numero* and *in vivo* as a function of promoter topology. The model is constructed from kinetic data obtained from the literature and yields clearly defined ON/OFF logical behavior at realistic inducer concentrations. These behaviors are matched with observed *in vivo* data obtained through fluorescence-activated cell sorting. The effect of incomplete repression by weaker LacI repressor is also investigated and quantified. The simulation results, coupled with *in vivo* data, not only identify important design degrees of freedom, but also provide parameters that can be used to guide future synthetic designs using these common regulatory elements.

1. Introduction

A plethora of synthetic gene regulatory networks has been created by arranging naturally occurring regulatory proteins to produce novel biological phenotypic functions [1–4]. To date, the network paradigms that have been most thoroughly studied are the bistable switch [5–8], the oscillator [7,9], and various logic gates [10–17].

In this work we combine multiscale models with synthetic bioengineering experiments to design, build, and characterize a high-fidelity logical AND gate in bacteria. A reductionist modeling approach is pursued. We adopt the molecular biology dogma that there are universal molecular mechanisms underlying the emergence of phenotypes. Consequently, we represent all gene expression molecular level events with reactions, including all the biomolecular interactions involved in transcription, translation, regulation and induction. This way, we relate each model reaction to a real *in vivo* reaction event and any biological system can be modeled by a network of reactions.

Because biological interactions regularly occur away from the thermodynamic limit, the simulations of reaction networks we conduct are stochastic, i.e., they generate probability distributions of molecular concentration. These distributions are directly comparable to experimentally observed variation. Detailed models then provide the opportunity to gain molecular level insight [18–22].

Experimentally, we constructed an *in vivo* synthetic-hybrid system consisting of multiple operators within a single promoter. The operator sequences employed are derived from three unrelated natural regulatory elements: the tetracycline (*tet*), lactose (*lac*) and λ-phage operons arranged logically within a single transcriptional unit. Specifically, we built six single promoter regulatory motifs by shuffling *tet* and *lac* operator sites (T and L, respectively) in and around the P_L (λ-phage): LLT, LTL, TLL, TLT, TTL and LTT (Fig. 1; detailed sequence information is available in supplement). The promoters drive expression of green fluorescent protein (GFP). The regulatory architecture is designed such that each operator's position efficiently interferes with RNA polymerase (RNAp) promoter binding while causing the least perturbance to promoter function [23]. To study the logical gate fidelity among the designed variants, the synthetic promoter sequences were incorporated in the reporter vector pGLOW to direct the transcription of GFP. We engineer the system in an *Escherichia Coli* strain that constitutively expresses lactose repressor (LacI) and tetracycline repressor (TetR) proteins.

The output fluorescence is then dependent on the input of two small molecule inducers, anhydrotetracycline (aTc) and isopropyl-β-thiogalactoside (IPTG). IPTG and aTc interact with LacI and TetR, respectively, which then free the operator sites for RNAp to bind and initiate transcription. The simplicity of these designs is

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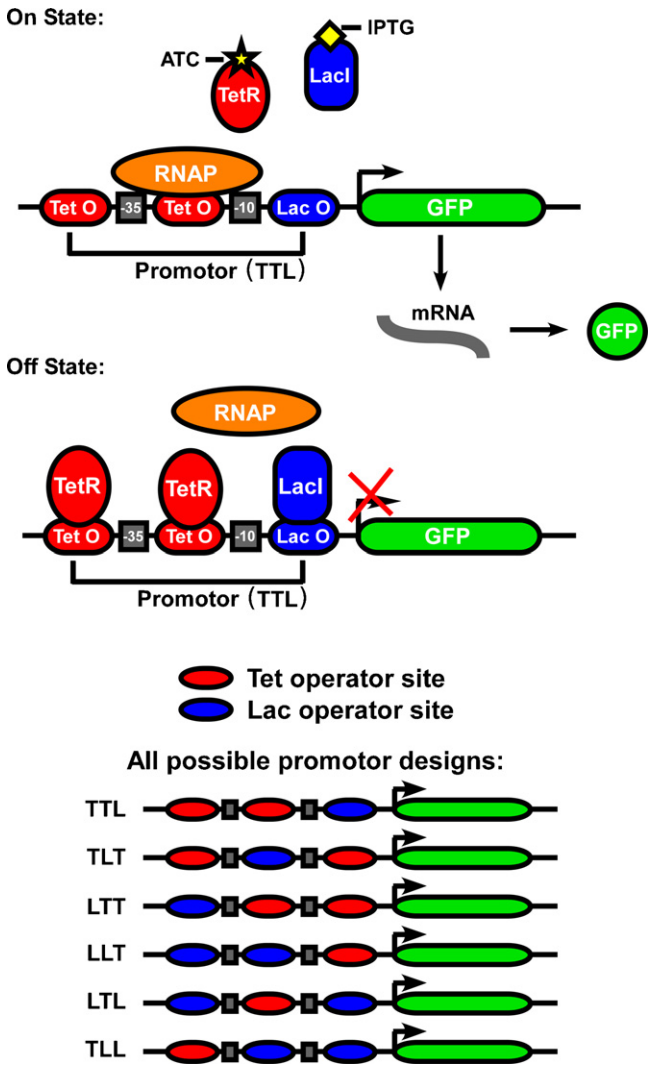

Fig. 1. Schematic representation of the synthetic bio-logical AND gates. Promoter sequence data is available in supplement.

highlighted by the minimal number of regulatory components required to achieve high-fidelity, robust AND gate functionality.

Prior studies served as a prelude to the construction of "TLT" first [24], in which a single *lacO* is inserted between −35 and −10 hexamers, reportedly the most effective operator position. More recently, Cox et al. [25] presented a powerful, combinatorial method for quickly generating single promoter motifs with a wide variety of logical gate phenotypic behavior. The methods presented herein represent a different design philosophy, though some of the same promoter designs are ultimately reached. Rather than construct and screen a large library of putative promoters, we are guided by models to rationally construct a small number of promoters in a targeted fashion. Importantly, the modeling approach is general enough that it is in principle applicable to any gene regulatory network, and this work serves to validate the approach.

2. Methods and models

2.1. Promoter and plasmid construction, strains

Functional synthetic modules of six designed promoters were constructed using standard molecular biology techniques. They were designed using naturally existing, well-characterized genetic elements from Tet, Lac and λ-phage operons differing in relative positions within the transcriptional unit (Fig. 1; details in Supplementary material). The architecture was based on the modular system of Lutz and Bujard [24,26] however differing in the individual elements used (sequences of *lac* and *tet* operators and λ-phage promoter). All promoter/operator sequences, transcriptional start site and the ribosome binding sites are obtained from published sequences [24]. Two overlapping synthetic oligonucleotides (~110 bp each) corresponding to the sequence of each synthetic-hybrid promoter were assembled using polymerase chain reaction (PCR) amplification with outside primers corresponding to the terminal 20 bp of each larger oligonucleotide. After gel purification, each promoter variant was introduced in the pGLOW-TOPO plasmid (Invitrogen, Carlsbad, CA) upstream of Cycle 3 GFP for use in *in vivo* promoter activity assays. Integrity of the promoter sequences was confirmed by DNA sequencing and visual verification of constitutive GFP expression in Top 10 cells (*lacI*$^-$, *tetR*$^-$). Subsequently, plasmids were transformed into DH5αPro (*lacI*$^+$, *tetR*$^+$) to assess promoter function in the presence of repressors.

2.2. In vivo promoter activity studies

E. coli strain DH5αPro was used for all *in vivo* promoter activity assays in the presence of IPTG and aTc inducers. A promoter-less pGLOW variant (containing a non-functional DNA fragment) served as a negative control. Overnight cultures were inoculated 1:100 into fresh LB medium containing 200 μg/mL ampicillin and 50 μg/mL spectinomycin. Inducer concentrations varied from 0 to 2 mM of IPTG and 0 to 200 ng/mL aTc, resulting in a matrix of 36 different inducer pair combinations, each of which was monitored for GFP output over a 24-h period. Specifically, cultures were maintained at 37 °C with shaking (250 rpm). At 3, 6 and 9 h time points samples were taken and the cells diluted 1:10 to restrict growth to logarithmic phase and retain constant cellular parameters (e.g., σ^{70} levels). Samples were monitored for growth state by OD_{600}.

2.3. GFP quantification using flow cytometry

In vivo GFP fluorescence was measured using a Becton Dickinson FACS Calibur flow cytometer equipped with a 488 nm argon laser and a 515–545 nm emission filter (FL1) at low flow rate. Samples were fixed with paraformaldehyde (PFA) to halt GFP production and degradation after harvesting at each time point. One milliliter of cell culture was centrifuged to collect the cells, fixed for 15–30 min in 4% paraformaldehyde (PFA) and re-suspended in phosphate buffered saline (PBS). For each sample, 100,000 gated events were collected and analyzed using Cellquest software (BD Biosciences). GFP fluorescence detected by the FL1 channel was represented as the mean fluorescence versus the normalized population distribution after subtraction of background fluorescence. Background fluorescence was determined using two sets of controls. First, from non-induced cells harboring functional pGLOW plasmids and second, from induced cells maintaining a pGLOW variant with a non-functional promoter sequence. Each of the six synthetic-hybrid promoter variants was characterized under identical experimental conditions for comparison of promoter activity and AND logic gate behavior.

2.4. Kinetic models and parameters

The set of reactions modeling the LTT logical AND gate synthetic circuit is presented as an example in Table 2. The novelty of the approach lays with the detailed incorporation of all biomolecular interactions describing all of the known interaction events in the transcription, translation, repression, and induction processes. This reductionist approach results in large, complex reaction networks that are difficult to simulate. However, although the resulting