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Dual selection of a genetic switch by a single selection marker

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Abstract

Forward engineering of synthetic genetic circuits in living cells is expected to deliver various applications in biotechnology and medicine and to provide valuable insights into the design principles of natural gene networks. However, lack of biochemical data and complexity of biological environment complicate rational design of such circuits based on quantitative simulation. Previously, we have shown that directed evolution can complement our weakness in designing genetic circuits by screening or selecting functional circuits from a large pool of nonfunctional ones. Here we describe a dual selection strategy that allows selection of both ON and OFF states of genetic circuits using *tetA* as a single selection marker. We also describe a successful demonstration of a genetic switch selection from a 2000-fold excess background of nonfunctional switches in three rounds of iterative selection. The dual selection system is more robust than the previously reported selection system employing three genes, with no observed false positive mutants during the simulated selections.

Keywords: Genetic circuit; Genetic switch; Directed evolution; Genetic selection

1. Introduction

While significant progress has been made recently in computational modeling of biological systems at the molecular and genetic level, the daunting complexity of real biological systems and environment in which they operate often preclude accurate quantitative simulation of even the smallest synthetic genetic circuits. Simulations provide crucial information and insights into genetic circuit design but significant experimental efforts are still required to implement such circuits. We have demonstrated that well planned and executed screening or selection of functional genetic circuits from a large pool of nonfunctional variants can be an efficient strategy for implementing or optimizing genetic circuits (Yokobayashi and Arnold, 2005; Yokobayashi et al., 2003, 2002). This approach is based on the directed evolution technologies that have been successfully used to improve various protein functions (Otten and Quax, 2005).

Screening may be well suited for directed evolution of genetic circuits because many circuits provide simple ON/OFF output that can be coupled to autofluorescent protein (e.g., GFP) expression which can be measured in high-throughput. Yokobayashi et al. (2002) demonstrated the screening of genetic inverters in *Escherichia coli*. Guet et al. (2002) generated a small library of randomly connected gene networks and screened for various Boolean logic functions by fluorescence screening. However, screening often requires significant cost and labor as the library size becomes larger because each mutant in a library must be physically isolated and individually evaluated.

Selection of genetic circuits by coupling the circuit output with the survival of the host cells would allow sorting of larger libraries which may be necessary

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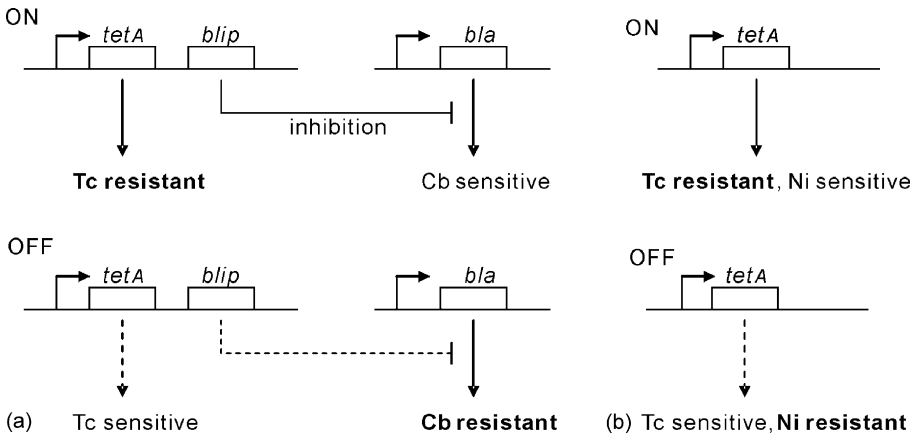

Fig. 1. Schematic representations of dual selection systems. (a) Three gene selection module based on *bli–tetA* coexpression by Yokobayashi and Arnold. (b) Single gene selection marker based on *tetA* described in this article.

for evolving more complex circuits. Towards this goal Yokobayashi and Arnold (2005) designed a dual selection system that allows the circuit designer to select for circuits that are ON or OFF under appropriate selective agents. The selection was achieved by coupling the circuit output with the expression of two genes *bli* and *tetA*, encoding β-lactamase inhibitory protein (BLIP) and tetracycline/H^+ antiporter, respectively, and constitutive expression of another gene *bla* that encodes β-lactamase (Fig. 1a). When the reporter genes (*bli* and *tetA*) are ON, the host cell becomes resistant to tetracycline by expression of *tetA*, but becomes sensitive to carbenicillin (an ampicillin analog) due to the inhibition of β-lactamase by BLIP. Conversely, attenuation of *bli* and *tetA* expression results in a tetracycline sensitive and carbenicillin resistant phenotype. The selection module was used to enrich a genetic inverter from a 200-fold excess of nonfunctional circuits in two selection cycles with a single round enrichment factor of up to 155-fold.

However, the authors also noticed the emergence of false positive mutants that are resistant to both tetracycline and carbenicillin. Although the exact nature of the mutations was not identified, many of the mutants contained larger than expected plasmids suggesting recombination as one of the potential processes leading to false positives. It is also expected that any mutation that disrupts BLIP function or expression, such as a frame shift of *bli* coding gene or damaged ribosome binding site, could render the cell harboring the mutant selection module to be resistant to both antibiotics regardless of the associated circuit output. Unless these false positive mutants are eliminated by DNA purification, iteratively selected pool would be dominated by such mutants.

This work describes an alternative dual selection strategy based on a single reporter gene *tetA* which encodes the tetracycline/H^+ antiporter (Fig. 1b). It is widely known that *tetA* expression confers resistance to the antibiotic tetracycline (Griffith et al., 1994). Moreover, accumulation of TetA in the cell membrane renders the host cell sensitive to various chemicals such as cadmium (Griffith et al., 1982) and nickel (Podolsky et al., 1996) cations, fusaric acid (Bochner et al., 1980; Maloy and Nunn, 1981), and aminoglycosides (Merlin et al., 1989). Consequently, *tetA* has the unique characteristic of being able to function as both positive and negative selection markers. The mutations that retain *tetA*'s antibiotic resistance and lose sensitivity to chemicals such as $NiCl_2$ simultaneously are expected to be rare because the two phenotypes, chemical sensitivity and tetracycline resistance, have not been attributed to independent subsequences of TetA (Griffith et al., 1994). Therefore, we anticipated that the use of *tetA* as the single selection marker for genetic circuits could result in a robust ON/OFF dual selection module.

2. Materials and methods

2.1. Plasmids

Three plasmids were used for the demonstration of *tetA* dual selection strategy (Fig. 2). pLacT1 is the model genetic switch which encodes *tetA* under the control of the modified *lac* promoter $p_{lac}{}^*$ (refer to Section 3.1 below). This plasmid expresses *tetA* in the presence of isopropyl-β-D-thiogalactopyranoside (IPTG), thus functioning as a genetic switch in *lacI*^{q} host cells. As nonfunctional switches, pΔLacT1 and pCIG were constructed. pΔLacT1 was derived from pLacT1 by partial deletion of the *lac* operator which resulted in constitutive expression of *tetA* (always ON). pCIG is a GFP expression vector that represents a nonfunctional switch that is always OFF. All three plasmids contain ColE1 backbone and encode