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An efficient platform for genetic selection and screening of gene switches in *Escherichia coli*

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ABSTRACT

Engineered gene switches and circuits that can sense various biochemical and physical signals, perform computation, and produce predictable outputs are expected to greatly advance our ability to program complex cellular behaviors. However, rational design of gene switches and circuits that function in living cells is challenging due to the complex intracellular milieu. Consequently, most successful designs of gene switches and circuits have relied, to some extent, on high-throughput screening and/or selection from combinatorial libraries of gene switch and circuit variants. In this study, we describe a generic and efficient platform for selection and screening of gene switches and circuits in *Escherichia coli* from large libraries. The single-gene dual selection marker *tetA* was translationally fused to green fluorescent protein (*gfpuv*) via a flexible peptide linker and used as a dual selection and screening marker for laboratory evolution of gene switches. Single-cycle (sequential positive and negative selections) enrichment efficiencies of >7000 were observed in mock selections of model libraries containing functional riboswitches in liquid culture. The technique was applied to optimize various parameters affecting the selection outcome, and to isolate novel thiamine pyrophosphate riboswitches from a complex library. Artificial riboswitches with excellent characteristics were isolated that exhibit up to 58-fold activation as measured by fluorescent reporter gene assay.

INTRODUCTION

Rewiring of metabolic regulatory pathways and genetic networks for basic as well as applied research requires gene switches and circuits that can respond to various biochemical and physical signals (1,2). Engineering gene switches that can sense desired molecules and control gene expression in a characteristic manner (e.g. activation versus repression) remains a major challenge being addressed by protein and nucleic acid engineers. The underlying mechanisms of gene regulation as well as the design strategies employed in engineered gene switches are highly diverse. However, because all gene switches ultimately turn ON or turn OFF gene expression under defined conditions, it may be possible to develop a generic platform to engineer gene switches independent of how they are implemented.

Due to the inherent complexity of gene switches and the challenges associated with their operation in living cells, most switches have been designed, at least in part, based on combinatorial design strategies. First, critical parts of a gene switch (e.g. amino acid residues contacting a ligand in a protein transcription factor) are randomized to yield libraries of different complexities. Subsequently, variants with desired phenotypes (e.g. altered ligand specificity) are screened or selected. Consequently, a robust and cost-effective selection and/or screening method would be highly useful for engineering gene switches and circuits regardless of their physical implementations (e.g. protein versus RNA, transcription versus translation regulation).

Genetic selection is an old yet very powerful tool for genetic research, especially in bacteria. Numerous selection markers are available for both positive (ON) and negative (OFF) selections in *Escherichia coli* and other bacteria. Selections of gene switches have been performed by alternately employing separate ON and OFF selection markers (3,4). The use of independent selection markers for the ON and OFF selections, however, significantly complicates the selection process by requiring plasmid isolation steps and increases the chances of isolating false positives. To address these problems, our group recently selected engineered riboswitches and logic gates from complex libraries using a single selection marker *tetA* which encodes a tetracycline/H^+ antiporter as both a positive and a negative selection marker (5,6). Expression of TetA confers the cells tetracycline resistance (ON selection) while the overexpression of the membrane-bound protein renders them more sensitive to toxic metal salts such as $NiCl_2$ as well as other compounds (OFF selection) (7–11).

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Use of a single selection marker for ON and OFF selections not only simplifies the selection procedure, but also makes the process more robust against false positives (12). However, genetic selection of gene switches still presents many barriers to implement. For example, selection experiments require handling of hundreds of agar plates in each round of selection, the parameters that affect the success or failure of selection are hard to optimize, and it is difficult to quantitatively monitor the progress of selection without screening and assaying many individual clones after each round.

In this work, we describe a new platform for dual genetic selection and screening of gene switches in *E. coli*. To compensate for the lack of a quantitative readout of the expression level of *tetA*, we designed the TetA-GFPuv translational fusion that enables robust genetic selection and convenient gene expression assay by fluorescence measurement. The platform enables high-density genetic selection in liquid culture, rapid optimization of critical selection parameters and quantitative monitoring of selection progress. The versatility of the gene-switch design strategy was demonstrated by the selection of reengineered riboswitches.

MATERIALS AND METHODS

Plasmid construction

The pLac + thiM#2-tetA-gfpuv (Figure S1A) was constructed from pLac + thiM#2-tetA (5) as described in detail in Supplementary Data. The plasmids pLacthiM-ON-tetA-gfpuv and pLacthiM-OFF-tetA-gfpuv are constitutively ON and OFF (i.e. nonfunctional) variants of the riboswitches that were isolated by screening (Supplementary Methods) and used as controls in the mock selection experiments.

Bacterial strain and culture conditions

Escherichia coli TOP10 cells (Invitrogen) were used throughout this study. Except when indicated otherwise, M9 minimal medium containing 0.8% glycerol as the carbon source supplemented with 100 μg/ml ampicillin (M9-amp) was used to grow TOP10 cells transformed with plasmid in liquid culture. When appropriate, thiamine (Acros Organics), tetracycline (MP Biomedicals) and $NiCl_2$ (Fisher Scientific) were added to M9-amp. Cells were grown at 37°C in 16-mm glass test tubes.

Selective growth conditions

Overnight cultures of TOP10 cells transformed with the appropriate plasmids were diluted to a final OD_{600} of 0.0015 in 1 ml fresh M9-amp with or without thiamine, and cultured for 8 h at 275 r.p.m. (New Brunswick Scientific I2400) to let the expression level of TetA-GFPuv stabilize (OD_{600} = ~0.2). The cells were further diluted to a final OD_{600} of 0.0015 in 2 ml fresh M9-amp with or without thiamine supplemented with an appropriate concentration of a selective agent (tetracycline or $NiCl_2$). After 24 h, cell growth was evaluated by measuring OD_{600}.

GFPuv assay under nonselective growth conditions

Overnight cultures of TOP10 cells transformed with the appropriate plasmids were diluted 100-fold in 1 ml fresh M9-amp with or without thiamine, and cultured for 8 h (4.5 h for reporter genes *gfpuv*, *mcherry* and *luc*) at 275 r.p.m. Cells reached OD_{600} of ~1.2 (8 h) or ~0.8 (4.5 h). Cells were harvested by centrifugation and washed once with 200 μl of phosphate-buffered saline (PBS). The washed cells were suspended in 200 μl of PBS, and GFPuv fluorescence (excitation 395 nm and emission 509 nm) was measured by Safire2 microplate reader (Tecan). Fluorescence intensity of each well was normalized by OD_{600} of the same well. Background fluorescence obtained from wells filled with PBS was subtracted from each fluorescence measurement.

ThiMN$_{15}$ library construction

The pLacthiMN$_{15}$-tetA-gfpuv library was constructed from pLac + thiM#2-tetA-gfpuv as previously described (5). Up to 15 random bases were inserted between the *thiM* TPP aptamer and the SD sequence (Figure 3A) by PCR using primers N15-thiM-SD-f (5′-NNNNN NNNNN NNNNN CAGGA GCAAA CTATG CAAG T CG-3′) and P1-r (5′-GCTTC CCTAC GCTGG CATTA TC-3′), and pLac + thiM#2-tetA-gfpuv as a template. The PCR product was treated with DpnI (Fermentas) and column-purified. The linear DNA was phosphorylated by T4 polynucleotide kinase (New England Biolabs), and column-purified. The DNA was then self-ligated and transformed into TOP10 cells. The transformed cells were plated on LB-ampicillin agar plates, and incubated overnight at 37°C. Approximately 300 000 individual colonies were obtained which approximates the size of the library. The cells were collected by overlaying M9-amp and stored at –80°C in 20% glycerol until use. The constructed library is depicted in Figure 3A. It should be noted that due to errors in oligo DNA synthesis, PCR or ligation, the number of bases in the randomized region of the selected clones were often <15.

Dual genetic selection

The library (mock library or pLacthiMN$_{15}$-tetA-gfpuv library) cells were diluted to a final OD_{600} of 0.0015 in 1 ml M9-amp supplemented with an appropriate concentration of thiamine, and cultured for 8 h at 275 r.p.m. to let the expression level of TetA-GFPuv stabilize. The cells were further diluted to OD_{600} of 0.0015 in 2 ml fresh M9-amp supplemented with an appropriate concentration of tetracycline and cultured for 24 h for positive selection. The selected cells were washed twice with M9-amp and further diluted to a final OD_{600} of 0.0015 in 1 ml M9-amp without thiamine, and cultured for 8 h to stabilize the expression level of TetA-GFPuv. The cells were diluted to a final OD_{600} of 0.0015 in 2 ml of M9-amp supplemented with an appropriate concentration of $NiCl_2$. After 24 h growth under the negative selection condition, the cells were washed twice with M9-amp and stored at –80°C in 20% glycerol. Typical OD_{600} values of the cultures after 24 h (for both tetracycline and

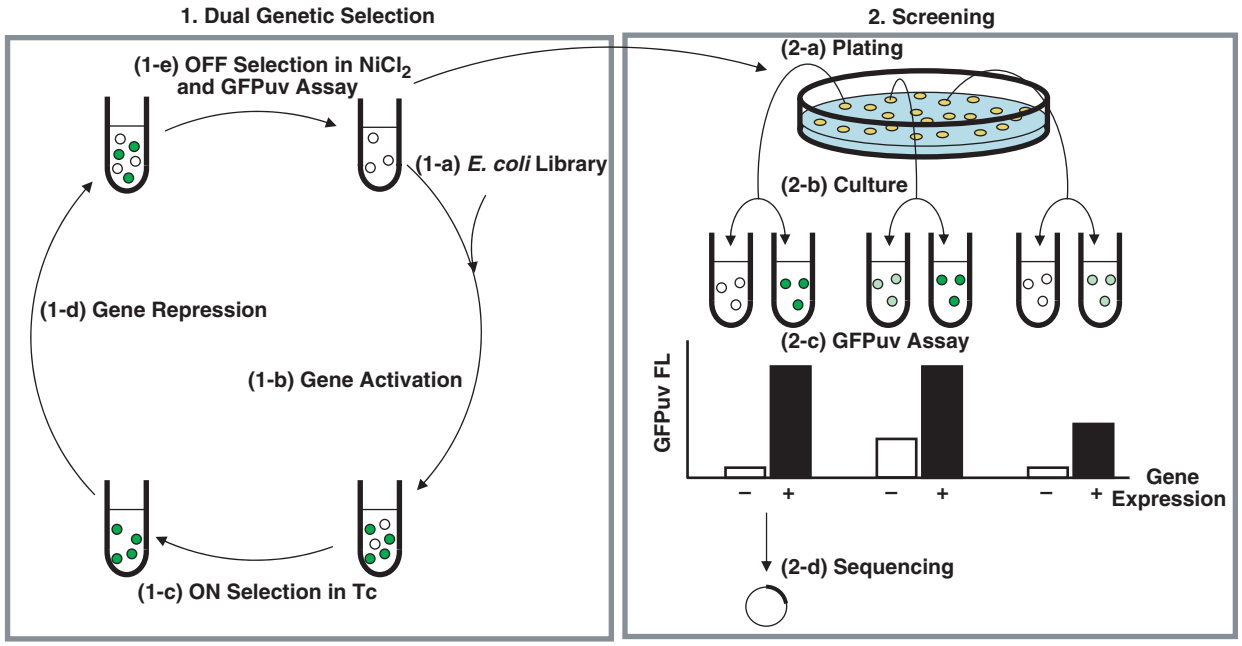

Figure 1. Schematic illustration of the genetic selection and screening process of gene switches. (**1**) Dual genetic selection. (a) A gene switch library is constructed in *E. coli* (b) The library cells are grown for 8 h under nonselective conditions in liquid medium (e.g. with thiamine) to stabilize TetA-GFPuv expression. (c) ON cells are selected by addition of tetracycline and growth for 24 h. d) The selected cells are grown for 8 h under nonselective conditions in liquid medium (e.g. without thiamine) to readjust TetA-GFPuv expression levels. (e) OFF cells are selected by addition of $NiCl_2$ and growth for 24 h. Progress of dual selection is evaluated by culturing the selected cell population under nonselective conditions (e.g. with and without thiamine) and measuring GFPuv fluorescence. (**2**) Screening. (a) Selected cells are plated on LB plates. (b) Individual colonies are transferred into fresh medium and grown under nonselective conditions (e.g. with or without thiamine). (c) Gene switch clones that show desired phenotypes are identified by GFPuv fluorescence measurement for further analysis (e.g. sequencing).

$NiCl_2$ selections) were 0.3–0.6. For evaluations of selected cells as a population, the cells were first diluted in LB-amp and incubated overnight at 37°C. The overnight culture was diluted into fresh M9-amp medium and analyzed as described above under nonselective growth conditions.

Screening and characterization of gene switches

Cell selected by dual genetic selection were plated on an LB-amp agar plate, and incubated overnight at 37°C. Individual colonies were picked and used to inoculate LB-amp liquid medium for overnight cultures that were used to perform GFPuv assays under nonselective conditions as described above. Clones exhibiting the desired riboswitch characteristics were sequenced. Removal of *tetA* was performed by PCR using 5′-phosphorylated primers p-GFP-f (5′-ATTGA GTAAA GGAGA AGAAC T TTTC AC-3′) and p-ORF-r (5′-GGATC CAGCA GGTC G ACTTG CAT-3′), and a *tetA-gfpuv* plasmid as a template. The PCR product was treated with DpnI, column-purified and self-ligated. The ligation product was transformed into TOP10 cells to clone the riboswitch-*gfpuv* constructs.

RESULTS AND DISCUSSION

Overview of the selection process

Outline of the streamlined dual genetic selection process is illustrated in Figure 1. First, a gene switch library is subjected to one or more cycles of ON and OFF selections under appropriate conditions. As opposed to most genetic selection procedures which are plated on solid media, our platform allows the selection to be performed in liquid cultures at densities of 10^6–10^7 cells/ml of growth medium. The gene switches encoded in the plasmid need not be isolated during the selection process. At the conclusion of genetic selection, individual bacterial clones are isolated and quantitatively assayed for the gene switch activity by GFPuv fluorescence. Clones with promising characteristics can be sequenced and further characterized individually. As demonstrated in this study, the selection and screening process up to the fluorescence assays of individual clones can be performed without plasmid manipulations, greatly accelerating gene switch development.

Construction and characterization of TetA–GFPuv fusion

We designed and constructed a translational fusion of TetA and GFPuv linked by a flexible peptide linker. TetA is an inner-membrane protein that consists of 12 transmembrane segments, and its N-terminus and C-terminus are exposed to the cytoplasm (13). Previous studies have shown *tetA* to retain its tetracycline-resistant phenotype when fused to alkaline phosphatase or β-galactosidase at the C-terminus (13,14). GFPuv has also been fused to membrane proteins in *E. coli* to determine their topologies (15). However, TetA-GFPuv fusion protein has not been previously reported in *E. coli*.

The coding region of GFPuv was amplified by PCR and cloned downstream of *tetA* in pLac–thiMwt-tetA and pLac + thiM#2-tetA that contain the TPP-repressed wildtype *thiM* riboswitch (–ThiMwt) (16) and the reengineered TPP-activated riboswitch + ThiM#2 (5), respectively (Figure S1A). A flexible peptide linker sequence encoding $(Gly\text{-}Gly\text{-}Gly\text{-}Ser)_4$ was inserted between TetA and GFPuv to facilitate proper folding of each protein (17). The resulting plasmids pLac–thiMwt-tetA-gfpuv and pLac + thiM#2-tetA-gfpuv were transformed into TOP10 cells and further characterized. Both riboswitches yielded the expected thiamine-dependent gene expression as measured by GFPuv fluorescence (Figure S1B).

To confirm that the function of TetA as a dual selection marker is conserved as a GFPuv fusion, TOP10 cells harboring pLac + thiM#2-tetA-gfpuv were grown in liquid M9 medium under selective conditions (Figure S1C and D). In the presence of tetracycline (20–40 μg/ml), robust cell growth was observed only in the presence of thiamine (100 μM). Conversely, addition of 0.4–0.5 mM $NiCl_2$ resulted in suppression of thiamine-supplemented cell growth, consistent with the riboswitch phenotype (TPP activation of TetA-GFPuv expression). From these results, it was concluded that the TetA–GFPuv fusion is capable of functioning as both a dual selection marker and as a quantitative reporter of gene expression. Additionally, the results shown in Figure S1C and D suggested optimal selection conditions for the ON selection (30 μg/ml tetracycline) and the OFF selection (0.4 mM $NiCl_2$) wherein the growth rate differences between the ON and OFF cells are the greatest.

Mock selections using model libraries

Genetic selections are mostly performed on solid media (agar plates) but rarely in liquid culture. Selection in liquid culture allows high cell density (10^6–10^7 cells/ml) so that multiple selections can be performed in parallel to optimize experimental parameters and increase efficiency. On the other hand, bacterial populations are subject to constant spontaneous mutations some of which could result in false positives in genetic selection experiments. On solid media, such false-positive clones emerge as individually isolated colonies which can be identified by proper screening. However, a false-positive clone that has a shorter doubling time can quickly overtake the population in the exponentially growing liquid culture, making the true positive clones harder to isolate. Emergence of false positives is a more serious challenge in genetic selection of gene switches that utilize independent positive and negative selection markers. For example, any mutation that disrupts the negative selection marker (e.g. frame shift) would result in the host cell being selected in both ON and OFF selection steps. To prevent accumulation of such mutants in the selection marker that yield false positives, Collins and co-workers (4) used physically separate plasmids for the gene switch (LuxR) library, the positive selection marker and the negative selection marker, which required isolation of the plasmid library after each selection step. Isolation of plasmid library and retransformation is not only laborious, but undesirable for the selection of *cis*-regulatory elements such as riboswitches. We anticipated that the robustness of *tetA* as a dual selection marker should allow gene-switch selection in liquid culture without significant complications by false positives. To demonstrate the feasibility and to determine the efficiency of dual genetic selection in liquid culture, we designed and executed mock selections using existing riboswitches and model background clones.

Our model libraries consisted of four TPP riboswitch phenotypes, –ThiMwt (OFF switch), + ThiM#2 (ON switch), ThiM-ON (always ON) and ThiM-OFF (always OFF). Real libraries are expected to contain a range of intermediate phenotypes. However, the model libraries based on the four representative phenotypes allow us to conveniently assess the feasibility and efficiency of liquid culture selection using the new TetA–GFPuv selection marker. The four phenotypes were experimentally confirmed by growth assays in the presence of tetracycline, $NiCl_2$, as well as GFPuv fluorescence measurements (Figure S2).

Model libraries for the selection of –ThiMwt (OFF switch) were prepared by spiking –ThiMwt cells in the background of an equal mixture of cells with the other three phenotypes in ratios ranging from 10^{-1} to 10^{-5}. After performing one round each of positive and negative selections, the selected and the unselected cell populations were cultured in the presence (100 μM) and absence of thiamine under nonselective conditions followed by fluorescence measurement. As shown in Figure 2A, the selected cell populations exhibited thiamine-repressed GFPuv expression in all experiments although the cells selected from the library with 10^{-5} dilution showed a smaller change in gene expression. To validate the selection results further, 96 clones from the 10^{-4} dilution library selection were randomly tested by tetracycline-growth assay. Seventy-two out of 96 clones, or 75%, were found to exhibit the phenotype consistent with –ThiMwt, while the remaining clones were either ThiM-OFF (15 clones) or ThiM-ON (nine clones) (data not shown). This equates to a 7500-fold enrichment in a single cycle of dual genetic selection.

Similarly, selections for + ThiM#2 (ON switch) were performed under the appropriate selection conditions. Again, the selected cell populations exhibited the expected GFPuv activation in the presence of thiamine (Figure 2B). Screening of 96 clones from the 10^{-4} dilution library revealed that 68 out of 96 clones showed the expected phenotype which is equivalent to a single-cycle enrichment efficiency of 7100 (data not shown). These results suggest that GFPuv fluorescence of the selected pools of cells grown under nonselective conditions can be used to evaluate the progress of gene switch selection, eliminating the need to sample and assay a large number of individual clones. Although the selections were performed using model libraries, the observed enrichment efficiencies (>7000) are remarkable and are due, at least in part, to the optimized selection conditions used based on the cell growth data (Figure S1C and D). In a similar characterization of dual selection by Collins and co-workers (4), 490-fold enrichment in a single selection cycle was reported.

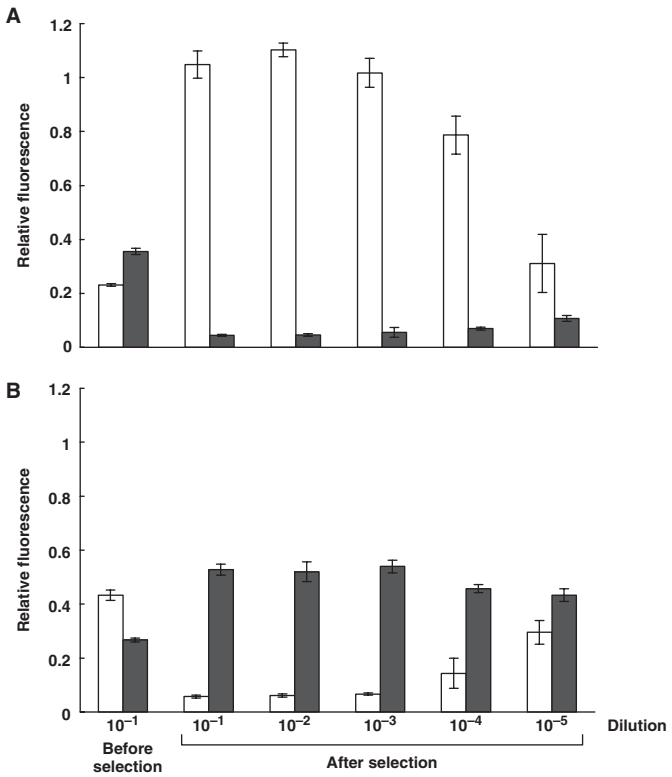

Figure 2. Mock selections of TPP riboswitches from model libraries. GFPuv fluorescence of the cell populations were measured under non-selective conditions in the absence (white bars) and presence (100 μM, dark bars) of thiamine. In (**A**), cells transformed with -ThiMwt riboswitch (fused to tetA-gfpuv) were diluted with the background cells having phenotypes as described in the text at ratios of 10^{-1} to 10^{-5}. The mock library cells were subjected to one round of positive selection in the absence of thiamine followed by one round of negative selection in the presence of thiamine (100 μM). Selected cell populations were evaluated under nonselective conditions. Two independent selections were performed for each dilution and two GFPuv assays were performed using cells from a single selection experiment. The error bars represent SD of the four measurements. In (**B**), analogous selections were performed with model libraries in which cells harboring the +ThiM#2 riboswitch were diluted with the background cells and selected for thiamine-activated gene expression. The fluorescence intensities were normalized to the observed fluorescence of +ThiMwt grown without thiamine (=1.0).

Selection from $ThiMN_{15}$ library

As the next step, we decided to apply the improved selection protocol to select novel TPP riboswitches from a combinatorial library similar to the one used in our recent experiment based on the original *tetA* dual genetic selection method (5). The library $ThiMN_{15}$ which we constructed for this study is depicted in Figure 3A. It contains fewer randomized bases compared to our original library (5), which had up to 30 randomized bases between the *E. coli thiM* TPP aptamer (16) and the Shine–Dalgarno (SD) sequence. Another minor modification made was to conserve the putative P1 stem of the TPP aptamer found in +ThiM#2, as the stem was generally conserved among the selected clones in the previous experiment. The $ThiMN_{15}$ library thus prepared was estimated to contain $\sim 3 \times 10^5$ independent clones.

One of the purposes of introducing the fluorescent reporter fusion was to be able to monitor the progress of genetic selection without screening a large number of individual clones. We expected to optimize the selection conditions by varying critical parameters before committing to investigation of promising individual gene switch clones. Consequently, we first studied the effects of the concentrations of tetracycline and $NiCl_2$ used during dual genetic selection. We performed one round each of positive selection in the presence of 100 μM thiamine and negative selection in the absence of thiamine using the $ThiMN_{15}$ library. Three concentrations of tetracycline (20, 30 and 40 μg/ml) and $NiCl_2$ (0.3, 0.4 and 0.5 mM) were examined, resulting in nine independent selections performed in parallel. The high cell density of liquid culture selection facilitates execution of multiple selections in parallel. At the conclusion of dual genetic selection, the enriched cell populations from each experiment were cultured in the presence (100 μM) and absence of thiamine under nonselective conditions for GFPuv fluorescence assays (Figure 3B–D). The OFF expression levels shown in Figure 3B exhibit lowest levels at 0.4 mM $NiCl_2$. Lower-than-optimal $NiCl_2$ concentrations would be expected to result in growth of cells expressing high levels of TetA, whereas higher-than-optimal concentrations would be expected to minimize the growth advantage of low-TetA expressing cells due to the excessive cellular toxicity. Similarly, 30 μg/ml of tetracycline proved to strike the best balance between the selective power and toxicity of the antibiotic (Figure 3C). As expected, the highest ON/OFF ratio was observed in the selection performed at 30 μg/ml tetracycline and 0.4 mM $NiCl_2$ (Figure 3D). These results are in good agreement with the growth profiles of the +ThiM#2 riboswitch shown in Figure S1C and D, implying that the selection condition can be optimized in the absence of a positive control gene switch.

Next, we examined the effect of the thiamine concentration used in the positive selection procedure. Dual genetic selections were performed as described above (30 μg/ml tetracycline, 0.4 mM $NiCl_2$) using 1, 10 or 100 μM thiamine during the positive selection step. The enriched cells from each selection were evaluated in 0, 1, 10 and 100 μM thiamine for GFPuv fluorescence (Figure 4A). The TPP-activated riboswitches were highly enriched in the selections conducted using 10 or 100 μM thiamine but the 1 μM selection products yielded only a moderate activation by thiamine (Figure 4A). The dissociation constant of the *E. coli thiM* TPP aptamer used in the library has been estimated to be ~600 nM *in vitro* (16), which could be the reason why the 1 μM thiamine selection failed to enrich the desired riboswitches due to the low intracellular TPP concentration.

Parallel attempts to enrich riboswitches that repress gene expression in the presence of thiamine failed to yield the desired phenotypes (data not shown), even though we observed comparable enrichment efficiencies of the ON and OFF switches in our mock selections (Figure 2A and B). We speculate that the design of the library, or the sequence space represented in the library, has a great influence on the potential phenotypes that can be discovered through genetic selection. It is intriguing

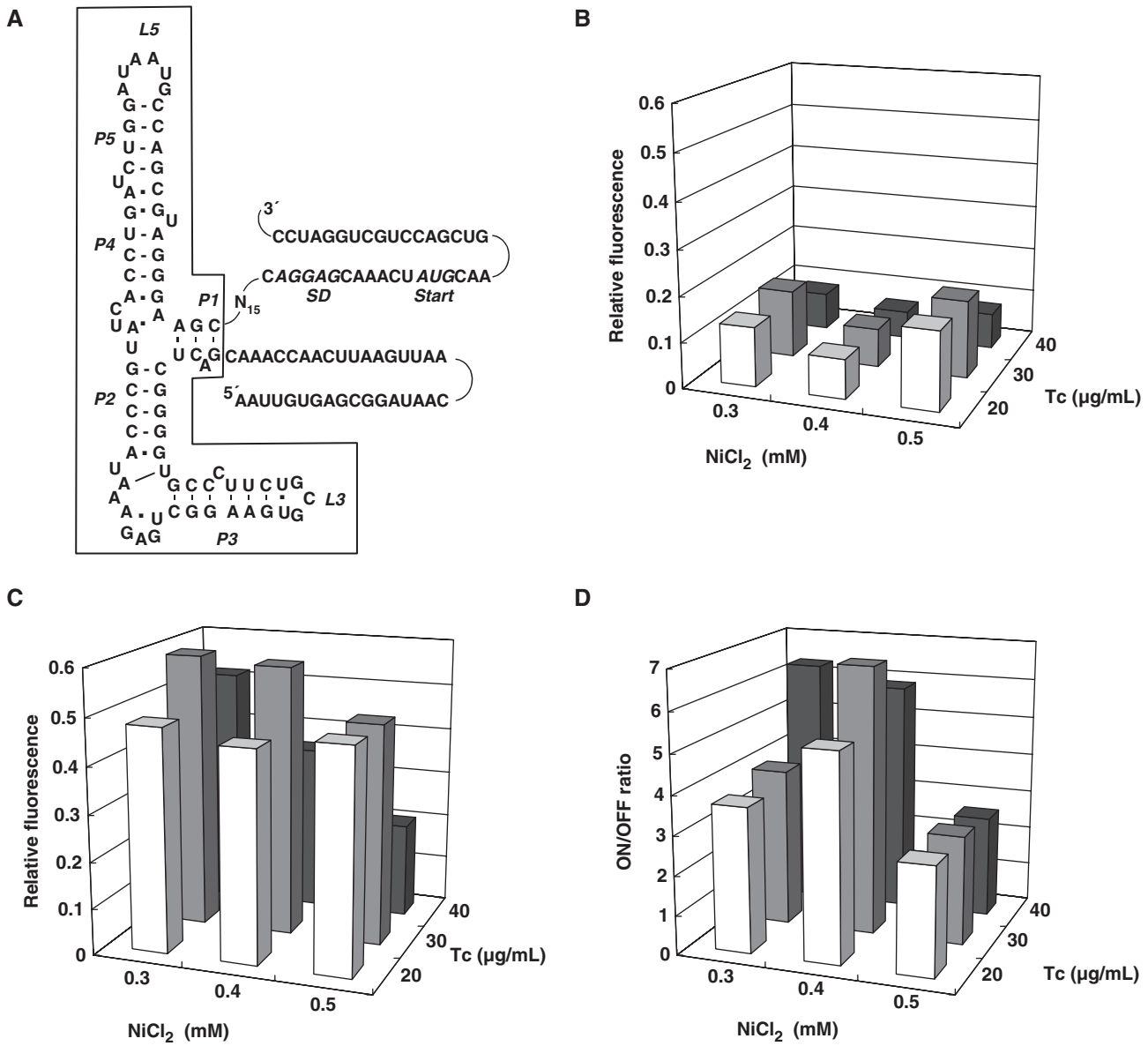

Figure 3. Design of $ThiMN_{15}$ library and optimization of selection conditions. (**A**) The 5′-UTR sequence of $ThiMN_{15}$ TPP riboswitch library. The TPP aptamer derived from the *E. coli thiM* riboswitch (16) is indicated in the box. Fifteen random bases were inserted between the aptamer and the SD sequence by PCR mutagenesis. The library was subjected to sequential positive (20, 30 and 40 μg/ml tetracycline and 100 μM thiamine) and negative (0.3, 0.4 and 0.5 mM $NiCl_2$) selections. Total of nine selections were performed in parallel under the indicated conditions. GFPuv fluorescence of the selected cell populations from each experiment were measured under nonselective conditions without (**B**) and with 100 μM thiamine (**C**). ON/OFF ratios were calculated and plotted in (**D**). The fluorescence data shown are averages of three independent GFPuv assays from a single selection experiment.

that the translationally regulated TPP-repressed riboswitch (*E. coli thiM*) harbors a rather large expression platform compared to that is possible in $ThiMN_{15}$. We anticipate that informed and careful library design is expected to be an important factor in dual genetic selection of synthetic gene switches.

Screening of individual clones

In our previous dual genetic selections of riboswitches and logic gates using *tetA*, the selected clones were first screened based on tetracycline growth assays in the presence and absence of the relevant effectors (5,6). This screening process is not only laborious, but it also does not provide quantitative expression levels at ON and OFF states. Consequently, we often found clones that exhibited apparently excellent switching phenotypes to function suboptimally when studied after subcloning reporter genes such as *gfpuv* and *lacZ*, due to the narrow threshold of the tetracycline growth assays. In contrast, TetA-GFPuv fusion allows screening of the selected clones by quantitative gene expression directly after genetic selection.

Cells selected under the optimized selection conditions (30 μg/ml tetracycline, 0.4 mM $NiCl_2$, 10 μM thiamine)

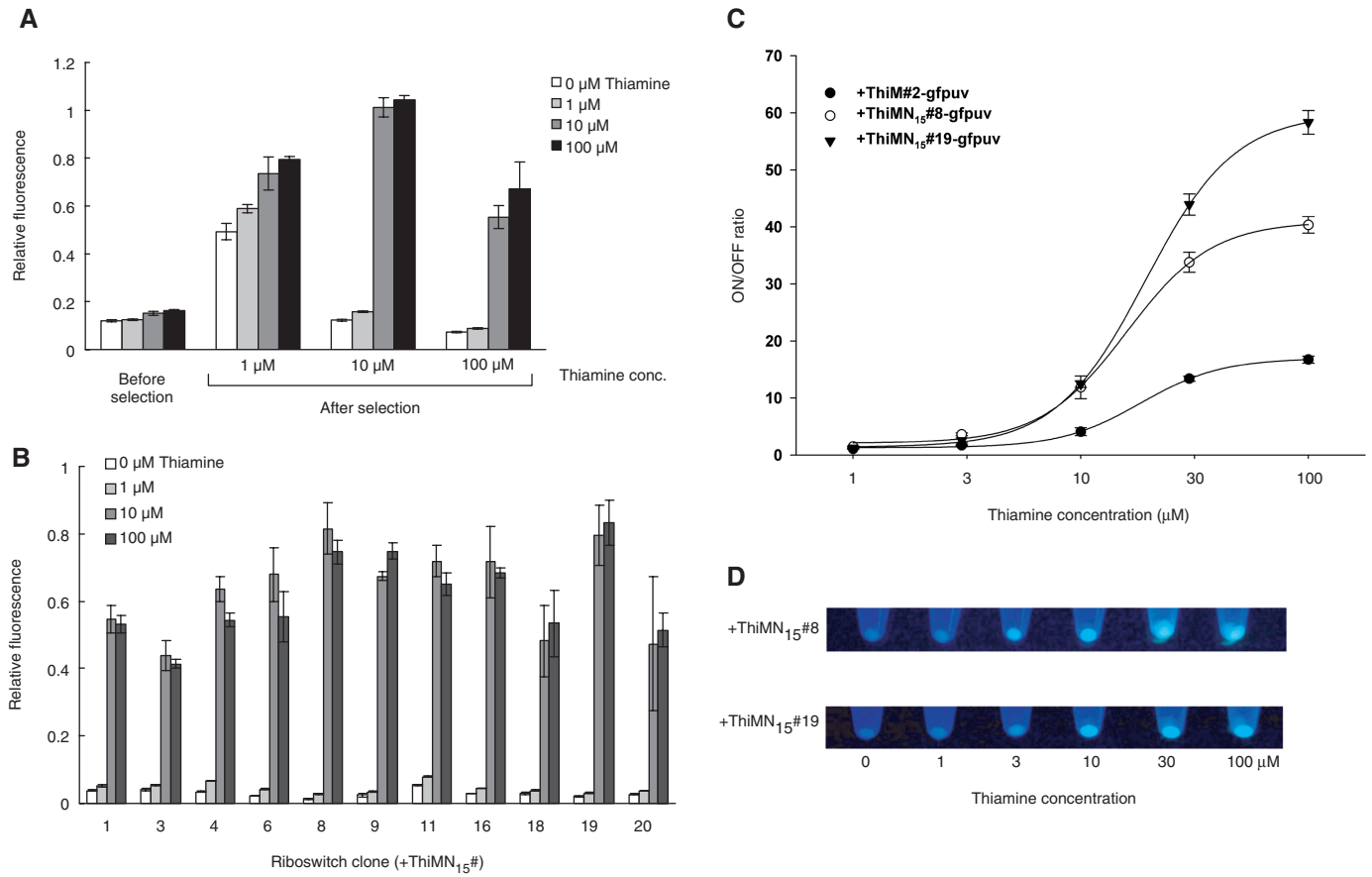

Figure 4. Selection and screening of TPP riboswitches from ThiMN15 library (**A**) Optimization of the thiamine concentration used during the positive selection. ThiMN15 library was subjected to sequential positive (with 1, 10 or 100 μM thiamine) and negative selections (without thiamine). Cell populations from each selection were evaluated under nonselective conditions for thiamine-activated gene expression. (**B**) GFPuv assays of individual riboswitch clones from the 10 μM thiamine selection depicted in (A). (**C**) Thiamine-dependent GFPuv expression of +ThiM#2 (closed circles), +ThiMN$_{15}$#8 (open circles) and +ThiMN$_{15}$#19 (closed triangles) riboswitches as *gfpuv* fusions (after removal of *tetA*). All fluorescence data are averages of three independent measurements and the error bars represent SD. (**D**) Thiamine-activated expression of GFPuv by +ThiMN$_{15}$#8-gfpuv (upper) and +ThiMN$_{15}$#19-gfpuv (lower) visualized in cell pellets. The cells were grown in M9-amp supplemented with the indicated concentrations of thiamine for 8 h, pelleted by centrifugation, and imaged on a 360-nm UV transilluminator.

were plated to isolate and evaluate individual clones. Remarkably, 19 out of 20 randomly chosen clones exhibited TPP-activated GFPuv expression after culturing in the absence and presence (10 μM) of thiamine (data not shown). We analyzed the sequences of the 15 clones that showed lower OFF levels and identified 11 unique sequences (Table 1). Six sequences share the 3′ GAU within the randomized region just upstream of the SD sequence which was also identified in +ThiM#2 (5). It was previously assumed that this 3′ GAU motif in conjunction with the SD sequence plays a critical role in forming a translation-inhibitory stem with a part of the TPP aptamer in the absence of the metabolite. A similar regulatory mechanism seems to have been selected in this experiment, along with possibly new modes of gene switching mechanism involving alternative RNA secondary structures. GFPuv assay of the 11 riboswitch clones (Figure 4B and Table S1) indicates that the selected riboswitches reach their maximum ON level with 10 μM thiamine whereas the previously isolated +ThiM#2 is only partially activated at the same concentration (Figure S1B). Some of the clones yielded exceptional ON/OFF ratios. For instance, riboswitches +ThiMN$_{15}$#8 and +ThiMN$_{15}$#19 were activated 58- and 38-fold, respectively, over the OFF expression levels.

The results thus far demonstrate that the TetA–GFPuv fusion protein is very useful and efficient as a selection and screening marker for gene switch design. Optimization and execution of the dual genetic selection and quantitative gene expression assays of the selected clones does not require plasmid isolation and/or subcloning. However, overexpression and the consequent accumulation of TetA is known to have pleiotropic effects on cell physiology and negative impact on cell growth, which may compromise the performance of the gene switches or complicate their practical applications (18). The design of the selection plasmid allows convenient removal of *tetA* by one-step PCR mutagenesis. To demonstrate this procedure, we deleted *tetA* from the plasmids harboring +ThiMN$_{15}$#8 and +ThiMN$_{15}$#19 linked with *tetA-gfpuv* by PCR deletion mutagenesis using a pair of 5′-phosphorylated universal primers followed by recircularization.

Table 1. Selected expression platform sequences

Clone #	Sequence (5′ to 3′)
Library[a]	**AGC** NNNNNNNNNNNNNNN ***CAGGAG***
+ThiM#2	UAUUACAAGAAGAU
1, 2, 7, 13, 14	CGAU
3	GCGUCGGUGUGAU
4[b]	**G** U
6	UCGGACUCGGAACGU
8	UUCGUAGUGACGCUA
9	GUCAGCAUGUUCAAU
11	UCGAA
16	CAACGGACUCUUGAU
18	GAGGAAUGGUAAGAU
19	UCUUAAGAAUCAGAU
20	GCGGAAGAACGUGAU

[a]The flanking constant sequences are shown in bold. The putative SD sequence is italicized.
[b]Clone #4 had an unintended C-to-G mutation at P1 shown in bold.

The resulting plasmids pLac + thiMN$_{15}$#8-gfpuv and pLac + thiMN$_{15}$#19-gfpuv maintained in TOP10 cells were assayed for thiamine-dependent GFPuv expression (Figure 4C and D). The two riboswitch clones activated GFPuv expression up to 40-fold and 58-fold for + ThiMN$_{15}$#8 and + ThiMN$_{15}$#19, respectively, in the presence of 100 μM thiamine (Figure 4C). In contrast to the corresponding *tetA–gfpuv* fusions (Figure 4B), GFPuv expression was not saturated at 10 μM thiamine, but progressively increased up to 100 μM. Drew and colleagues (19) reported that overexpressed membrane proteins with C-terminal GFP fusion often form inclusion bodies in *E. coli* in which GFP fluorescence is diminished. It is possible that the high expression levels of TetA–GFPuv in some riboswitch clones lead to saturation at 10 μM thiamine due to intracellular aggregation, but not with GFPuv alone.

The riboswitches could also regulate the expression of other reporter gene products such as mCherry (20) and luciferase, consistent with the modular nature of the riboswitch domains (Figure S3A and B). The activation ratios of the selected riboswitches improved considerably compared to the riboswitches discovered in our previous selection (5), and compare favorably with other synthetic bacterial riboswitches discovered by high-throughput screening using the theophylline aptamer (21,22).

While high-throughput screening alone has been shown to identify functional gene switches from carefully designed libraries, costly and labor-intensive screening could become impractical for isolating rarer and more complex phenotypes. For example, we recently discovered two-input logic gates (AND and NAND gates) based on the TPP and theophylline aptamers by *tetA* dual genetic selection (6). Screening of these logic gates would require evaluation of each clone under four different conditions as opposed to two for the simple riboswitches. The TetA–GFPuv dual selection platform described here will enable efficient transition from genetic selection to quantitative screening which will greatly facilitate the discovery of complex gene switches and circuits.

CONCLUSION

The remarkable performance of the isolated riboswitches underscores the power of dual genetic selection as well as the importance of optimizing the selection conditions. We have shown that the fusion of the robust dual selection marker *tetA* and green fluorescent protein (*gfpuv*) facilitates evaluation of the selection progress. Upon successful selection, individual clones can be rapidly screened for the desired characteristics without plasmid isolation or subcloning. The optimization of selection condition is greatly facilitated by performing selection in liquid culture in high cell density compared to agar plates. Moreover, the selection does not require special host phenotypes except for those strains already harboring extrachromosomal elements that confer tetracycline resistance. Other selection methods, such as those based on motility, require deletion of the corresponding host gene (23). The selection method is compatible with both solid and liquid media.

The use of GFPuv also allows the use of both genetic selection and fluorescence-based screening, including the powerful fluorescence-activated cell sorting (FACS) techniques (24–27). Lynch and Gallivan (27) recently identified theophylline-activated riboswitches with impressive ON/OFF ratios using FACS. It can be imagined that the chance of isolating very rare gene switches can be increased by combining the two techniques; first, enriching the gene switches by dual selection, followed by extensive screening based on the fluorescence readout. Finally, dual genetic selection is widely applicable to gene switches other than riboswitches as well as more complex genetic circuits that yield simple ON/OFF gene expression outputs.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online.

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