Riboswitch Signal Amplification by Controlling Plasmid Copy Number

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Riboswitch Signal Amplification by Controlling Plasmid Copy Number

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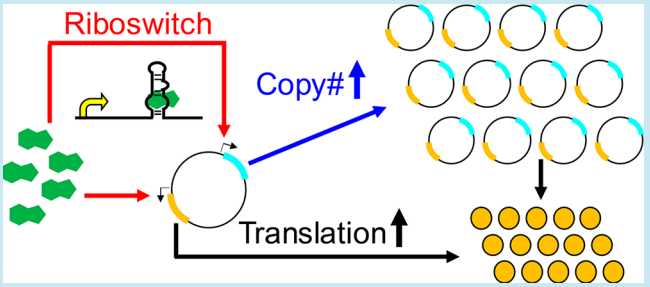
Supporting Information

ABSTRACT: Riboswitches are *cis*-acting RNA devices in mRNAs that control gene expression in response to chemical inputs. As RNA aptamers that recognize diverse classes of molecules can be isolated by *in vitro* selection, synthetic riboswitches hold promise for various applications in synthetic biology. One of the major drawbacks of riboswitches, however, is their limited dynamic range. A high level of gene expression in the OFF state (leakage) is also a common problem. To address these challenges, we designed and constructed a dual-riboswitch plasmid in which two genes are controlled by theophylline-activated riboswitches. One riboswitch controls the gene of interest, and another riboswitch controls RepL, a phage-derived replication protein that regulates the plasmid copy number. This single-plasmid system afforded an ON/OFF ratio as high as 3900. Furthermore, we used the system to control CRISPR interference (CRISPRi) targeting endogenous genes, and successfully observed expected phenotypic changes in *Escherichia coli*.



KEYWORDS: *signal amplification, riboswitch, theophylline, plasmid copy number, CRISPRi*

Riboswitches are *cis*-acting small RNA gene regulatory elements found in the 5′ untranslated regions (UTRs) of mRNAs which operate at the transcriptional or the translational level.[1−3] A typical riboswitch is composed of two parts; an aptamer that recognizes a specific ligand, and an expression platform that mediates the change in secondary structure in response to aptamer−ligand interaction.[4,5] This change in the secondary structure leads to either upregulation or downregulation of gene expression (ON or OFF switch). For example, an ON switch that functions at the translation level can adopt a secondary structure that sequesters the ribosome binding site (RBS) in the absence of the ligand, but the structural change upon aptamer−ligand interaction can make the RBS more accessible to the ribosomes.

Compared to the major mechanism of gene regulation in response to chemical signals in bacteria that involves protein transcription factors, riboswitches have several advantages. Riboswitches are genetically compact sequences (up to few hundred bases) that can be inserted upstream of the gene of interest. Furthermore, riboswitches are modular in nature[6−8] and the demonstrated flexibility of *in vitro* selected RNA aptamers to recognize a wide variety of small molecules and proteins broadens the potential applications of riboswitches. Consequently, we and others have engineered synthetic riboswitches in bacteria by genetic selection, high-throughput screening, and rational design.[9−12] However, one of the common challenges with synthetic riboswitches is their limited dynamic range (ON/OFF ratio), typically less than 10-fold to ∼ 100-fold in few cases.[9,10] A riboswitch with an ON/OFF ratio as high as 383 was reported by the Salis group who developed and used a biophysical model to rationally design riboswitches.[12] The low ON/OFF ratios are often due to relatively high OFF levels, in other words, the leakage of gene expression in the OFF state. These device characteristics sometimes limit riboswitch applications.

To address these problems, several groups designed cascading systems in which the riboswitch ligand controls the expression of a transcription factor or an RNA polymerase (e.g., T7 or SP6 RNA polymerase) which in turn controls the expression of the gene of interest. For example, Ogawa et al.[13] placed an aptazyme-based riboswitch upstream of the gene coding for SP6 RNA polymerase, and another one controlling the reporter gene transcribed from the SP6 promoter. This cascading system resulted in an ON/OFF ratio of 13 in response to theophylline, an approximately 4-fold improvement over the original riboswitch.[13] Another cascading strategy for riboswitch signal amplification based on quorum sensing components was reported by Goodson et al.[14] In that study, the riboswitch ligand (2-aminopurine) induced expression of an acyl homoserine lactone (AHL) synthase (RhlI) that produces the quorum sensing signal molecule *N*-butyryl-L-homoserine lactone (C4-HSL). C4-HSL then diffuses to a receiver cell where it binds to its cognate transcription factor

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RhlR and activates expression of the reporter green fluorescent protein (GFPa1). The amplification circuit increased reporter gene expression (ON level) as well as the ON/OFF ratio to 11, whereas the ON/OFF ratio was only 1.3 when the riboswitch directly controlled the reporter gene expression.

In the current study, we investigated an alternative strategy to amplify the riboswitch signal to improve the dynamic range and minimize leakage. Our strategy is to introduce two ligand-activated riboswitches that respond to the same ligand on a single plasmid. One riboswitch controls the gene of interest and the other controls plasmid replication. Ligand addition results in upregulation of the gene of interest as well as an increase in the plasmid copy number. Together, the riboswitches exhibit dramatic improvement in the dynamic range of gene expression in *Escherichia coli* (Figure 1).

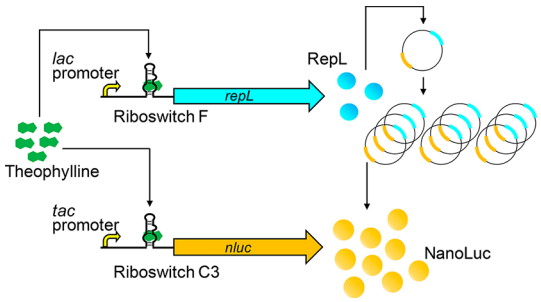

Figure 1. Schematic illustration of the dual-riboswitch signal amplification system. Two different theophylline-responsive riboswitches control *repL* and *nluc* genes. The addition of theophylline activates the first riboswitch which upregulates RepL expression and increases the plasmid copy number. Theophylline also acts on the other riboswitch encoded in the same plasmid that activates the NanoLuc luciferase (*nluc*) expression.

RESULTS AND DISCUSSION

Using the previously published pTrig plasmid,[15] we first investigated the possibility of controlling plasmid replication by a synthetic riboswitch. The pTrig plasmid has two origins of replication; the mini-F origin for stable plasmid maintenance, and the P1 phage-derived origin *oriL* which is located within the coding region of the replication protein RepL.[16] Expression of RepL is driven by the *lac* promoter. Induction of the RepL expression, therefore, initiates plasmid replication from the *oriL* origin which in turn increases pTrig copy number. Sheth et al.[15] observed a 635-fold increase in the plasmid copy number upon induction of RepL expression by isopropyl *β*-D-1-thiogalactopyranoside (IPTG).

On the basis of these observations, we inserted theophylline-responsive Riboswitch F previously described by Topp et al.[9] (denoted as Theo-E* in the original report) upstream of the *repL* gene (F-*repL*). This riboswitch was inserted immediately downstream of the *lac* promoter replacing the *lac* operator sequence. The reporter gene encoding the engineered NanoLuc luciferase (*nluc*) was inserted downstream of the *tac* promoter and a moderate strength RBS sequence suggested by the RBS Calculator[17] (Table S1). Addition of theophylline (1 mM) resulted in a 550-fold increase in luciferase expression (Figure 2A). When Riboswitch F was inserted upstream of the *nluc* gene (F-*nluc*) instead of the *repL*, however, NanoLuc expression was upregulated by only 13-fold. It is likely that the discrepancy in the ON/OFF ratios of gene expression by the same riboswitch reflects the nonlinear relationship between the RepL expression level and the plasmid copy number due to positive feedback.

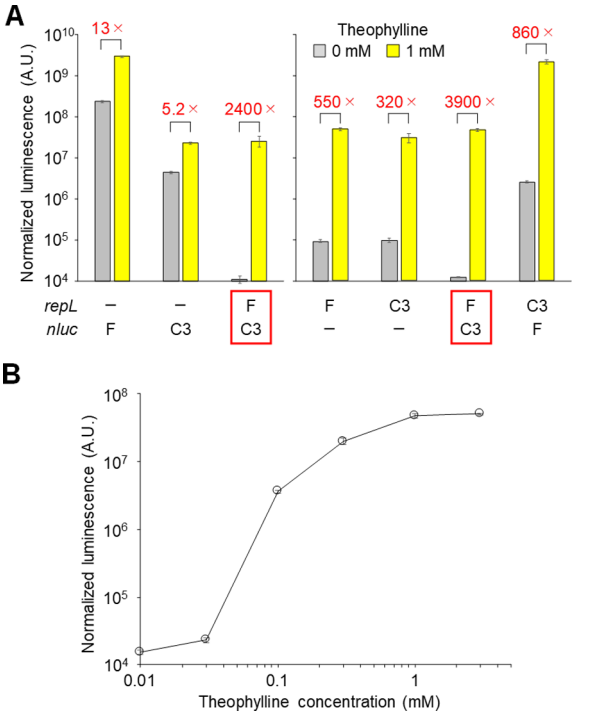

Figure 2. Characterization of riboswitch constructs. (A) NanoLuc expression levels in the presence and absence of theophylline (1 mM). ON/OFF ratios are indicated in numerical values in red. The left and right graphs are based on experiments performed on different days. F-*repL*/C3-*nluc* (red box) was characterized in both experiments as a measure of reproducibility. The luminescence levels are generally reproducible in different experiments. High ON/OFF ratios, however, vary significantly due to the sensitivity of the expression levels to experimental conditions. (B) Dose-dependence of the dual-riboswitch construct F-*repL*/C3-*nluc*. Luminescence values were normalized by the cell density (OD_{600}). The values are averages of at least three replicate cultures with the error bars indicating the standard deviation.

We then explored the possibility of controlling both the plasmid replication and the reporter gene expression by riboswitches to further improve the ON/OFF ratio. We first attempted to insert Riboswitch F upstream of both *repL* and *nluc* within the same plasmid. Unfortunately, this was not possible as the plasmid was unstable due to spontaneous recombination between the two homologous riboswitch sequences. We then tried to insert a different riboswitch construct (Riboswitch C)[9] from Topp et al.[9] upstream of the *nluc* gene which reduces the homologous sequence down to 38 bp. This construct still proved to be too unstable (data not shown). We introduced two additional mutations in the nonessential positions of the theophylline aptamer (Riboswitch C3) which finally allowed us to isolate the dual-riboswitch plasmid. Riboswitch C3 (Table S1) showed an ON/OFF ratio of 5.2 when placed upstream of the *nluc* gene alone (C3-*nluc*), albeit with much lower expression levels for both OFF and ON states compared to Riboswitch F (53-fold lower for OFF, 130-fold lower for ON) (Figure 2A). Despite the significant difference in the relative gene expression levels when the two riboswitches control *nluc*, the expression levels are comparable when the riboswitches control *repL* (Figure 2A) with Riboswitch F (F-*repL*) showing only 1.6-fold higher ON

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level compared to that of Riboswitch C3 (C3-*repL*), implying a threshold-like response of plasmid copy number in response to RepL. The plasmid copy number after 24 h of culturing was 157-fold or 522-fold higher for theophylline-induced cultures when *repL* was controlled by Riboswitch F or Riboswitch C3, respectively.

The relative translation efficiencies of the uninduced riboswitch constructs fused to *repL* and *nluc* were estimated by the RBS Calculator[17] which predicts that the OFF level of Riboswitch F is substantially higher (19- and 75-fold) than that of Riboswitch C3 (Table 1). This is reflected in the

Table 1. Translational Efficiency Predicted by RBS Calculator

gene	Riboswitch F	Riboswitch C3
repL	3.3	0.17
nluc	98	1.3

significantly lower expression level of C3-*nluc* compared to that of F-*nluc* in the absence of theophylline (Figure 2A). However, the difference is minimal between C3-*repL* and F-*repL* even though the predicted (uninduced) expression level of F-*repL* is 19-fold higher than that of C3-*repL*. Again, this could be attributed to the nonlinear effects of the RepL expression level, or due to the substantially lower baseline expression levels compared to *nluc* fusions as predicted by the RBS Calculator (Table 1).

We also observed an approximately 2-fold decrease in the cell density (OD_{600}) when RepL expression was induced by Riboswitch F compared to Riboswitch C3 (Table S2). We speculate that the higher ON level of Riboswitch F (higher RepL expression) and the resulting stronger and earlier induction of luciferase expression may impose a heavier metabolic burden on the host cell.

The dual-riboswitch construct that harbors Riboswitch C3-*nluc* and Riboswitch F-*repL* (C3-*nluc*/F-*repL*) (Figure S1) yielded an ON/OFF ratio of 2400 or 3900 indicative of an additive effect of the two riboswitches (Figure 2A). It should be noted that the apparent ON/OFF ratio of this system varies between 2000 and 4000 due to the sensitivity of the expression level to experimental conditions such as incubation time and growth stage of the cells. To the best of our knowledge, however, this is the highest ON/OFF ratio ever reported by a riboswitch-based circuit. Notably, the dual-riboswitch system showed a marked reduction in the OFF-expression level compared to those of C3-*nluc* and F-*repL*. The dose-dependence curve of C3-*nluc*/F-*repL* dual-riboswitch construct exhibited a sigmoidal response with a half-maximal response at ~0.3 mM theophylline (Figure 2B). In contrast, F-*nluc*/C3-*repL* dual-riboswitch construct resulted in higher ON and OFF levels and a reduced but still high ON/OFF ratio (860). Qualitatively similar results were observed when the *nluc* gene was replaced with a fluorescence reporter gene (*mCherry*) (Figure S2).

We next applied the C3-*nluc*/F-*repL* platform to control endogenous gene expression by harnessing the CRISPR interference (CRISPRi) technology.[18] The *nluc* gene was replaced with the *dCas9* gene (a mutant of the Cas9 nuclease that lacks the endonuclease activity but retains the RNA-guided DNA binding activity), and an expression cassette for a small guide RNA (sgRNA) was inserted into the plasmid (Figure 3).[18,19] Expression of sgRNA was driven by a relatively

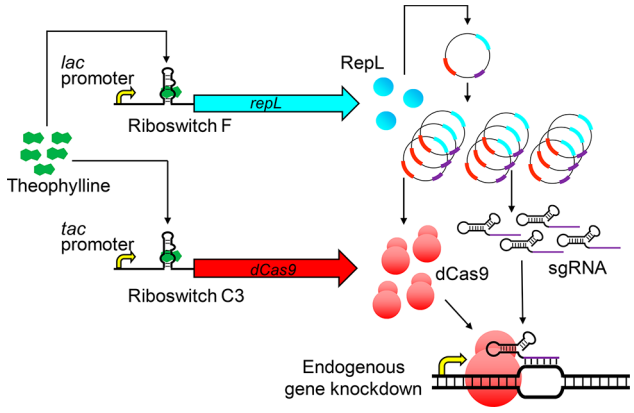

Figure 3. Schematic illustration of the theophylline-regulated CRISPRi system. The theophylline-responsive Riboswitch F and Riboswitch C3 were inserted upstream of *repL* and *dCas9* genes, respectively. Note that the *lac* promoter controlling *repL* expression lacks the *lac* operator sequence, and therefore is not repressed by LacI. The plasmid also contains an expression cassette that transcribes sgRNA driven by a constitutive promoter (pJ23110). The sgRNA targets dCas9 to a specific endogenous gene on the chromosome. Addition of theophylline upregulates RepL resulting in the plasmid copy number amplification, and therefore, increased transcription of the mRNAs encoding dCas9 and sgRNA. At the same time, the theophylline riboswitch activates dCas9 translation. The dCas9-sgRNA complex then targets and downregulates the endogenous gene.

strong synthetic constitutive promoter pJ23110 (Biobricks, http://parts.igem.org/Part:BBa_J23110). First, we targeted the gene coding for the cell division protein FtsZ. FtsZ is necessary for developing the division ring in *E. coli*.[20] In fact, a previous study showed that mutations which reduce the concentration of this protein in *E. coli* render the cells defective in cell division resulting in elongated cells up to hundreds of micrometers.[21] Consequently, we expected to observe elongated *E. coli* cells upon induction of the sgRNA targeting *ftsZ* and *dCas9* by theophylline. The sgRNA sequence was designed to target the early coding region of the *ftsZ* gene. As expected, *E. coli* cells transformed with the plasmid pTrig-F-repL-C3-dCas9-sgRNA(*ftsZ*) did not show altered cellular morphology. However, the addition of theophylline (0.1 mM) resulted in a dramatic elongation of the *E. coli* cells when observed under a microscope with some cells reaching lengths of around 100 μm (Figure 4A, Figure S2). As a control, an analogous plasmid containing the sgRNA which lacks the N_{20} region complementary to the target sequence (pTrig-F-repL-C3-dCas9-Trimmed sgRNA) was constructed. Cells containing this plasmid did not show any change in morphology upon theophylline addition confirming that the effect observed was due to the riboswitch-controlled CRISPRi effect targeting *ftsZ*.

We also targeted the *fliC* gene which codes for the flagellin, the main component of the flagellum. Therefore, silencing FliC expression was expected to diminish the flagellar motility of *E. coli* cells as previously demonstrated.[22] As shown in Figure 4B, the addition of theophylline (0.1 mM) resulted in a moderate reduction in the motility of *E. coli* MG1655 cells harboring the control plasmid (pTrig-F-repL-C3-dCas9-Trimmed sgRNA) which can be attributed to theophylline toxicity or the metabolic load caused by the induced plasmid replication. However, the cells harboring the plasmid pTrig-F-repL-C3-dCas9-sgRNA(*fliC*) that express the *fliC*-targeting sgRNA

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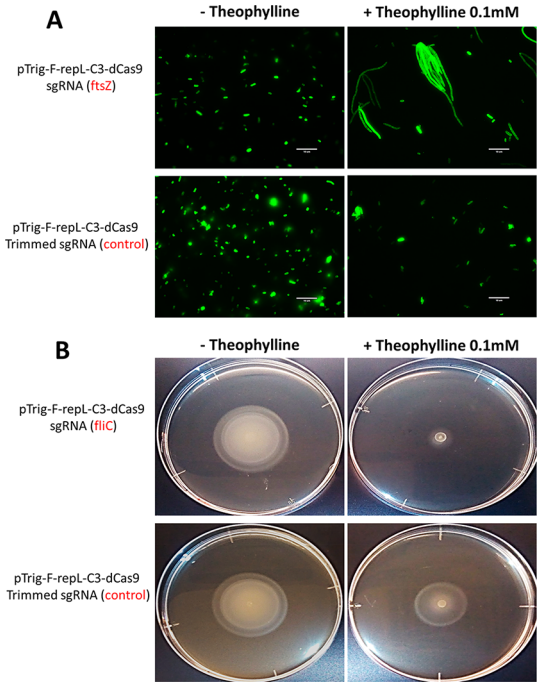

Figure 4. Controlling *E. coli* phenotype by conditional downregulation of an endogenous gene using CRISPRi. (A) Theophylline-triggered downregulation of *ftsZ* gene expression in *E. coli* TOP10 cells. The cells were imaged after 24 h of culturing in the presence or absence of theophylline (0.1 mM). The cells were fluorescently stained with Alexa Fluor 488 conjugated to wheat germ agglutinin which binds to the cell wall. Scale bar: 10 μm. (B) Theophylline-triggered downregulation of *fliC* gene in *E. coli* MG1655. *E. coli* cells were cultured for 24 h in liquid medium in the presence or absence of theophylline (0.1 mM). The cells were then spotted on LB motility agar plates supplemented with theophylline at the same concentration. The plates were imaged after 8 h of further incubation at 37 °C.

showed a clearly stronger reduction in cell motility (Figure 4B). To support these results, the flagella were isolated from *E. coli* MG1655 cells harboring pTrig-F-repL-C3-dCas9-sgRNA-(*fliC*) or pTrig-F-repL-C3-dCas9-Trimmed sgRNA grown in liquid cultures for 24 h with or without theophylline (0.1 mM). The extracted flagellin was subsequently analyzed by SDS-PAGE (Figure S4). While theophylline lowers the amount of isolated flagellin, the reduction observed in cells that express *fliC*-targeted sgRNA is more pronounced than in cells that express the trimmed sgRNA.

Finally, genetic stability of a dual riboswitch construct (pTrig-F-repL-C3-mCherry) was evaluated by colony PCR. Eighteen colonies randomly picked from liquid cultures in the absence of theophylline were used as templates in colony PCR using primers that amplify a 3.3 kbp fragment that spans most of the key genetic elements of the plasmid including the two riboswitch regions. All colonies yielded a PCR product of the expected size (Figure S5A). Similarly, 16 out of 18 colonies yielded the expected PCR product in the theophylline-induced (1 mM) cultures while two colonies yielded a ~4 kbp product (Figure S5B), suggesting minor plasmid instability under prolonged high-copy induction conditions.

In conclusion, we developed a new strategy for amplifying synthetic riboswitch output by simultaneously controlling the translation of the gene of interest and the plasmid copy number in bacteria (via RepL), achieving an ON/OFF ratio as high as 3900 with minimal leakage. An ON/OFF ratio greater than 500 was observed with a riboswitch controlling RepL expression alone which may be sufficient for many applications. This may be more convenient because riboswitch optimization for individual genes of interest would not be necessary. The generality of the dual-riboswitch system was demonstrated by targeting two endogenous genes (*ftsZ* and *fliC*) in *E. coli* by CRISPRi and observing expected phenotypes. By significantly improving the dynamic range and the basal gene expression level, the dual-riboswitch signal amplification system should broaden the potential applications of riboswitches.

METHODS

Strains and Plasmids Used. MG1655 *E. coli* cells were used for cell motility experiments, and TOP10 cells (Invitrogen) were used in all other experiments involving *E. coli*. The original pTrig vector described by Sheth et al.[15] was obtained from Addgene. The *dCas9* gene was amplified by PCR from the plasmid pdCas9-bacteria described by Qi et al.[18] and obtained from Addgene. The NanoLuc luciferase gene was PCR amplified from pNL1.3 (Promega). The mCherry gene was cloned from the plasmid pSUP-p1753-TheoF-mCherry.[23] The Ribowitch F sequence controlling *nluc* and *dCas9* was PCR amplified from plasmid pSUP-p1753-TheoF-mCherry along with an extra coding sequence of mCherry to ensure that the riboswitch function was preserved (Table S1, Figure S1). This extra sequence, however, was not included when Riboswitch F was placed upstream of *repL*. Other shorter sequences were constructed from synthetic oligonucleotides. Sequences of the constructed plasmids were verified by Sanger sequencing.

Luminescence and Fluorescence Measurements. A 200 mM stock solution of theophylline was prepared in 0.1 N NaOH and stored at −20 °C. All *E. coli* strains transformed with an appropriate plasmid were streaked from −80 °C glycerol stocks onto LB agar plates. Three separate fresh colonies per strain were picked and cultured in LB medium for 24 h. Aliquots from these cultures were then diluted in fresh LB medium either at 1% (for all strains with F-*repL* or C3-*repL* constructs) or at 2% (for all other strains in which the *repL* is not controlled by a riboswitch). This was done to compensate for the difference in the growth rate between these two groups. The LB medium and agar plates were supplemented with kanamycin (50 μg/mL). The freshly inoculated LB cultures were then incubated in a shaking incubator (250 rpm) at 37 °C. After 2.5 h (at $OD_{600} \approx 0.4$), aliquots were taken for OD_{600} measurement, and the remaining cultures were divided into different tubes to which theophylline was added to the appropriate final concentrations. An equivalent volume of 0.1 N NaOH was added for control cultures. All cultures were then grown for additional 24 h. From each tube, a 200 μL aliquot was transferred to a well in a transparent 96-well plate (Nunc), and OD_{600} was measured using a microplate reader (Tecan Infinite M1000 PRO). NanoLuc luminescence was measured after appropriate dilution of the cultures in phosphate buffered saline (PBS). Ten microliters of the original or the diluted samples were then mixed with 10 μL of Nano-Glo luciferase assay reagent (Promega) according to the manufacturer's instructions. Luminescence was measured in a 384-well white low-volume plate (Corning) and normalized by OD_{600}. The cells were prepared similarly for mCherry measurement except that the cellular fluorescence was measured at an excitation wavelength of 587 nm and an

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emission wavelength of 610 nm with 200 μL of each culture in 96-well plates. Fluorescence values were normalized by OD_{600}.

CRISPRi Targeting *ftsZ* in *E. coli* TOP10. The plasmid pTrig-F-repL-C3-dCas9-sgRNA (*ftsZ*) was constructed by replacing the *nluc* gene in pTrig-F-repL-C3-Nluc plasmid with the *dCas9* gene and inserting the sgRNA expression cassette targeting *ftsZ* within the same plasmid. The sgRNA expression was driven by the constitutive promoter pJ23110 (Biobricks, http://parts.igem.org/Part:BBa_J23110). As a control, another plasmid (pTrig-F-repL-C3-dCas9-Trimmed-sgRNA) was constructed in which the sgRNA lacked the N_{20} targeting region, and therefore, cannot target dCas9 to the *E. coli* chromosome. Plasmids pTrig-F-repL-C3-dCas9-sgRNA (*ftsZ*) and pTrig-F-repL-C3-dCas9-Trimmed-sgRNA were separately transformed into *E. coli* TOP10 cells. The colonies were picked and cultured in LB medium as described above and theophylline (0.1 mM) was added to appropriate cultures when OD_{600} reached approximately 0.4. After 24 h, *E. coli* cells in each culture were stained green using Alexa Fluor 488-conjugated wheat germ agglutinin (Invitrogen) which binds to the cell wall. The cells were centrifuged and washed to remove excess dye, then resuspended in PBS and examined by fluorescence microscopy.

CRISPRi Targeting *fliC* in *E. coli* MG1655. pTrig-F-repL-C3-dCas9-sgRNA (*fliC*) in which the sgRNA guides dCas9 toward the *fliC* gene in *E. coli* MG1655, and pTrig-F-repL-C3-dCas9-Trimmed-sgRNA were transformed into *E. coli* MG1655. The cells were grown in LB medium, and theophylline (0.1 mM) was added to appropriate cultures when OD_{600} reached approximately 0.4. The cells were further incubated for 24 h as described above. Two microliters of cells from each culture were spotted on an LB motility plate with or without theophylline (0.1 mM). The motility plates were prepared fresh with 0.3% agar. The plates were then left to dry for 1 h at room temperature, transferred to a 37 °C incubator, and imaged after 7 h.

Flagellin Extraction and Observation on SDS-PAGE. *E. coli* MG1655 cells harboring pTrig-F-repL-C3-dCas9-sgRNA (*fliC*) or pTrig-F-repL-C3-dCas9-Trimmed-sgRNA were cultured, and appropriate cultures were treated with theophylline (0.1 mM) at $OD_{600} \approx 0.4$. The cells were incubated for 24 h, diluted 1000-fold, and grown for an additional 24 h with or without theophylline (0.1 mM). Aliquots were then taken to measure OD_{600}, and the same amount of cells (based on OD_{600} values) from each culture were centrifuged and washed. The flagella were then extracted by resuspending the pellets in normal saline adjusted to pH 2.0 as described previously.[24,25] The mixtures were shaken for 1 h at room temperature, centrifuged, and the supernatants were separated. Equal volumes of the supernatants were then mixed with the protein loading dye and analyzed on 12.5% SDS–polyacrylamide gel. The gel was then stained using Takara CBB Protein Safe Stain (Takara) and imaged on Lumino-Graph II (ATTO).

Plasmid Copy Number Measurement. *E. coli* TOP10 cells harboring pTrig-F-repL-Nluc or pTrig-C3-repL-Nluc were grown and treated with theophylline (1 mM) at $OD_{600} \approx 0.4$ as described above. The cells were cultured for additional 24 h, and 500 μL from each culture was centrifuged and resuspended in deionized water. The cells were lysed by heating at 95 °C for 10 min[26] and frozen until analysis. The gene copy number of *repL* relative to that of the 16S rRNA gene was measured by quantitative PCR (qPCR). Primers were designed using the online tool provided by GenScript (https://www.genscript.com/tools/real-time-pcr-tagman-primer-design-tool). Additional sequence flap (5′-AATAAAT-CATAA) was added to the 5′ end of the primers as it was found to improve the reliability of qPCR.[27] Primers used for qPCR are shown in Table S3. The qPCR samples were analyzed by StepOnePlus Real-Time PCR System (Thermo Fisher) using Power SYBR Green PCR Master Mix (Thermo Fisher). The reaction volume was 20 μL. The thermal cycling was programmed as follows: 95 °C for 15 min, 50 cycles of 95 °C for 15 s, 54 °C for 15 s, and 60 °C for 20 s.

Evaluation of Genetic Stability of the Dual Riboswitch Construct. All LB liquid medium and agar plates were supplemented with kanamycin at 50 μg/mL. Three colonies of *E. coli* TOP10 cells harboring the plasmid pTrig-F-repL-C3-mCherry were picked and cultured in LB medium in a shaking incubator (250 rpm) at 37 °C for 24 h. The cultures were diluted 100-fold in fresh LB medium and cultured for additional 24 h. The cells were plated on LB agar plates. Six colonies were randomly picked from each plate (18 total) which were used as templates in colony PCR. The PCR was performed using primers F1 (5′-ATGATTTCTGGCCTCGCT-3′) and R1 (5′-ACCTTTGTTTACAGCTTCACGG-3′) designed to amplify a ∼3.3 kbp fragment which spans the entire *mCherry*, *repE*, oriS, and a part of *repL*. The fragment also includes the two riboswitches, therefore, should detect any homologous recombination occurring at these regions. The PCR products were analyzed by agarose gel electrophoresis (Figure S5A) along with a positive control (PCR using the purified plasmid as a template) and a negative control (PCR without template). Similarly, the cells were cultured in the presence of theophylline (1 mM) after dilution for 48 h and plated. Eighteen colonies from the theophylline induced culture were also analyzed as described above (Figure S5B).

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acssynbio.8b00454.

Sequence information, cell density data, plasmid map, mCherry expression data, flagellin expression data, and fluorescence micrographs (PDF)

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Notes

The authors declare no competing financial interest.

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