

# Engineering a portable riboswitch-LacP hybrid device for two-way gene regulation

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## ABSTRACT

**Riboswitches are RNA-based regulatory devices that mediate ligand-dependent control of gene expression. However, there has been limited success in rationally designing riboswitches. Moreover, most previous riboswitches are confined to a particular gene and only perform one-way regulation. Here, we used a library screening strategy for efficient creation of ON and OFF riboswitches of *lacI* on the chromosome of *Escherichia coli*. We then engineered a riboswitch-LacP hybrid device to achieve portable gene control in response to theophylline and IPTG. Moreover, this device regulated target expression in a 'two-way' manner: the default state of target expression was ON; the expression was switched off by adding theophylline and restored to the ON state by adding IPTG without changing growth medium. We showcased the portability and two-way regulation of this device by applying it to the small RNA CsrB and the RpoS protein. Finally, the use of the hybrid device uncovered an inhibitory role of RpoS in acetate assimilation, a function which is otherwise neglected using conventional genetic approaches. Overall, this work establishes a portable riboswitch-LacP device that achieves sequential OFF-and-ON gene regulation. The two-way control of gene expression has various potential scientific and biotechnological applications and helps reveal novel gene functions.**

## INTRODUCTION

Inducible gene regulation is required for diverse applications such as gene function investigations, gene therapies and industrial uses. So far, inducible promoters are the major tools for specifically controlling target gene expression. However, there are only limited numbers of inducible promoters which, on some occasions, are insufficient for

independent control of multiple genes by the addition of distinct inducers. It is, therefore, highly desirable to develop alternative approaches to enrich the repertoire of inducible systems. A promising candidate is riboswitch that is a RNA-based, ligand-dependent genetic control element located in non-coding regions of messenger RNAs. Riboswitches control biological processes at various regulatory levels. Lysine riboswitches prematurely halt RNA transcription by modulating the formation of a Rho-independent terminator (1–3). Translationally controlling riboswitches in the 5'-untranslated region modulate the ribosome access to the ribosome-binding site (RBS) (4–6). In addition, some riboswitches undergo self-cleavage upon ligand binding, resulting in rapid RNA degradation (7,8). Unlike inducible promoters, these RNA-based control elements are potentially numerous as a result of high-throughput screen for ligand-specific aptamers (the sensor domain of a riboswitch) (9–13).

In recent years, advancements have been made in the ability to convert ligand-specific aptamers to functionally active riboswitches by various random screening approaches (14–16) or rational design (4,5,8). However, library screening is labor intensive and time consuming, and successful rational design is not guaranteed even though a long list of design principles are followed, due to incomplete understanding of RNA-based gene regulation mechanisms. In addition, a common disadvantage of riboswitches as well as inducible promoters is that they only allow for one-way gene regulation. Once activated, a target gene cannot be turned off unless promoter inducers or riboswitch ligands are removed. The one-way regulation mode makes the existing regulation systems unsuitable for quick gene control in live animals or in vitro studies where intact cell cultures are required. Thus, there is a great need for a two-way regulation system, in which target gene can be switched off and on in response to distinct effector molecules.

Here, we set out to develop a portable and two-way regulatory device by integrating an ON theophylline-responsive riboswitch with the *lac*-inducible promoter system (abbreviated as LacP), in which the LacI repressor

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binds as a homotetramer to two LacI-binding sites positioned immediately downstream of the *lac* promoter. First, we used a library screening strategy to create ON and OFF theophylline-responsive riboswitches of the *lacI* gene that encodes the LacI transcriptional regulator. Then, combining an ON-riboswitch of LacI synthesized in the first part of this study with two LacI-binding sites (LacIbs), we established a portable hybrid device that acted as a versatile key for controlling gene expression without the need for further rational design or library screening. We demonstrated the portability of this device by applying it to *rpoS* (encoding RpoS, a master regulator of acid resistance) and *csrB* (encoding a small non-coding RNA CsrB). We showed that this portable device regulates target genes in a two-way manner, switching off the targets in response to theophylline and restoring the target expression in response to isopropyl  $\beta$ -D-1-thiogalactopyranoside (IPTG). The sequential two-way control of RpoS and CsrB by the riboswitch-LacP hybrid device reversibly fine-tuned virulence-associated cellular behaviors including acid resistance, intercellular autoaggregation and biofilm formation. Finally, we used this device to explore unidentified functions of RpoS and revealed for the first time that removing RpoS promotes acetate assimilation after the acetate switch is flipped. This finding is opposite to the previous ones made by using conventional *rpoS* mutants, which might have accumulated secondary mutations. Since the portable two-way device does not silence target genes until theophylline is added, its host cells are less likely to generate secondary mutations and therefore able to provide more reliable information.

## MATERIALS AND METHODS

### Bacterial strains, plasmids and growth conditions

*Escherichia coli* strain MG1655 was used in this study. All bacterial mutants were grown at 37°C, with shaking at 220 rpm, in Luria–Bertani (LB) medium supplemented with 2 mM magnesium sulfate. The antibiotics ampicillin (50  $\mu$ g/ml), kanamycin (50  $\mu$ g/ml) and chloramphenicol (12.5  $\mu$ g/ml) were used for selection when appropriate.

### Mutagenesis

Gene deletion was performed using the recombineering system (17). *Escherichia coli*-K12 MG1655 was transformed with plasmid pSim6 (a gift from Dr Donald Court) from which the expression of the  $\lambda$  recombination proteins is induced at 42°C. PCR fragments encompassing a loxP-*cat*-loxP with homology (45 nt) to the regions immediately flanking each deletion locus were transformed via electroporation into MG1655 cells harboring pSim6. After induction of  $\lambda$ red functions, recombinants were selected for chloramphenicol resistance (encoded by the *cat* gene) and were further verified by colony PCR.

### Construction of randomized riboswitch libraries

For construction of theophylline-responsive riboswitch libraries on the chromosome, the loxP-*cat*-loxP fragment was linked to a theophylline-responsive aptamer followed

by a 5-nt-random sequence by ligation PCR. The resulting PCR products with homology (45 nt) to the regions surrounding the ribosome-binding site of each target gene were then integrated into the chromosome using the above-described recombineering technique. In theory, each thus established library contains 4<sup>5</sup> riboswitch candidates.

### Construction of chromosomal *lacZ* translational fusions and $\beta$ -galactosidase assays

The loxP-cm-loxP selectable cassette was inserted immediately after the stop codon of the *lacZ* gene on the MG1655 chromosome using recombineering (as described above). Next, the *lacZ*-loxP-cm-loxP cassette was PCR amplified and inserted (in frame) immediately prior to the stop codon of the target gene on the chromosome. The inserted *lacZ* fragment started from the eighth codon of *lacZ* gene and was co-transcribed and translated with the fused genes. The expression of gene-*lacZ* fusions was quantified using a  $\beta$ -galactosidase assay as described previously (4,18). Levels of  $\beta$ -galactosidase were calculated using the following formula:

$$\text{Units (U)} = \text{OD}_{420} \times 1000 / (\text{OD}_{600} \times \text{hydrolysis time} \times \text{volume of lysate}).$$

### Acid resistance assay

Overnight cultures were treated with acid (pH 2) for 2 h and then serially diluted in neutral medium. After overnight culture, colony forming units (CFU) of acid-treated and untreated cells were determined. Acid survival (%) was calculated with the following formula:

$$\text{Acid survival (\%)} = 100 \times (\text{CFU of treated} / \text{CFU of untreated})$$

### Autoaggregation

Overnight cultures were diluted 1:500 and incubated at 37°C, with shaking at 220 rpm, in liquid LB medium. When cells aggregated (visualized macroscopically by the clumping or ‘fluffing’ of cells in liquid cultures), 100  $\mu$ l of each cell suspension was transferred to flat-bottom 96-well plates (Iwaki, Tokyo, Japan) and the images of the cell aggregates were captured by scanning. To better visualize the cell aggregates, 1  $\mu$ l of crystal violet (0.1%) was added to each cell suspension immediately prior to image capture. Cellular autoaggregation was also examined microscopically. Cell suspensions were spread on a microscope slide, heat-fixed, stained with DAPI (4′6-diamidino-2-phenylindol) and imaged using a fluorescence stereomicroscope SZX12 (Olympus, Tokyo, Japan) with DAPI filter sets.

### Biofilm formation

Biofilms were formed on polystyrene, flat-bottom 96-well microtiter plates (Iwaki, Tokyo, Japan). Two hundred microliters of each cell suspension (10<sup>5</sup> cells/ml) was transferred into each well of a microtiter plate and incubated

for 24 h at 37°C in a shaker at 75 rpm. Resulting biofilms were washed thrice with PBS and air dried. Then, biofilms were stained with 100 µl of 0.4% aqueous crystal violet solution for 15 min. Afterward, biofilms were washed thrice with sterile distilled water and immediately destained with 200 µl of 95% ethanol. After 30 min of destaining, 100 µl of destaining solution was transferred to a new well and measured with a microtiter plate reader (SpectraMAX 340 Tunable Microplate Reader; Molecular Devices Ltd) at 595 nm.

### Semi-quantitative RT-PCR

Total RNA was isolated from overnight cultures in LB medium. Subsequently, 2 µg of RNA was reverse transcribed in a total reaction volume of 20 µl using the ThermoScript RT-PCR system (Invitrogen). Each reaction was incubated at 55°C for 50 min followed by 15 min at 70°C. Two microliters of the resulting reverse transcript products (cDNA) were then used for 18, 20, 22 and 24 rounds of PCR (30 s each at 94°C, 55°C, and 72°C) with Ex Taq DNA polymerase (Takara Bio, Inc.) and primers complementary to *csrB* (RT-CsrB-F: 5'-GTCAGACAAC GAAGTGAACATCAGG-3' and RT-CsrB-R: 5'-GGAG CACTGTATTCACAGCGCT-3') and the 16S rRNA gene (RT-16S-F: 5'- CTCCTACGGGAGGCAGCAG-3' and RT-16S-R: 5'-CTCCGTATTACCGCGGCTG-3') to co-amplify the gene of interest and the internal control. PCR products were separated in 1.5% agarose gel.

### Acetate determination

To quantify extracellular acetate, cell suspensions were centrifuged at 10 000 rpm for 5 min at 4°C. The resulting supernatants were then subjected to acetate determination using an acetate detection kit (Megazyme, Bray, Ireland) according to the manufacturer's instructions.

### Statistical analysis

Paired *t*-tests were used to compare the two means obtained from β-galactosidase assays; one-way ANOVA was used for the comparison of multiple means from the biofilm formation assay.  $P < 0.05$  was considered statistically significant.

## RESULTS

### Establishment of a random library for engineering functionally active riboswitches on the chromosome

To construct a riboswitch-LacP hybrid device, our first task was engineering ligand-responsive riboswitches for the *lacI* gene, which encodes the LacI transcriptional regulator in the Lac system. Previously, we have successfully engineered a theophylline-responsive ON riboswitch residing upstream of the *csrA* gene in *E. coli* and managed to modulate the accessibility to ribosome binding and to flexibly control the *csrA* expression (4). However, the riboswitch did not work when grafted to *lacI* (data not shown), indicating that riboswitches besides the RBS are sensitive to changes in flanking regions and have to be specifically designed and constructed for each target gene.

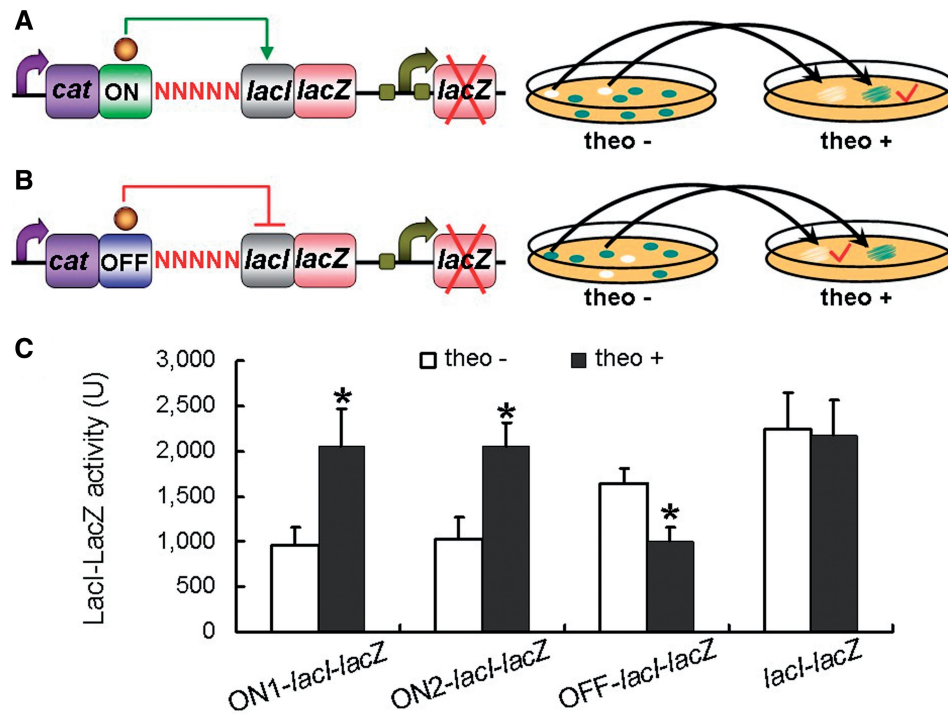
However, rational design is laborious and does not guarantee successful engineering of functionally active riboswitches. We, therefore, sought out to explore the possibility of establishing a chromosomal riboswitch library, with the aid of the recombineering technique, to screen for active riboswitches.

We used the theophylline-responsive aptamer as the sensor domain of our riboswitches. The riboswitches were proposed to adopt multiple conformations at equilibrium, some of which permitted downstream target expression whereas some of which did not. If theophylline binding to the aptamer favored the formation of conformations that permitted target expression, then the riboswitch was an ON switch. Conversely, if theophylline binding shifted the equilibrium distribution to conformations that repressed the target expression, the riboswitch was an OFF switch.

To engineer a theophylline-responsive riboswitch for *lacI*, we first constructed a riboswitch library and then screened for clones that could effectively control the target expression in response to theophylline (Figure 1A and B). We used the genomic DNA isolated from the previously reported switch-*csrA* strain (4) to PCR amplify the theophylline-specific aptamer fragment. Then, the aptamer was linked to a chloromphenical resistance gene (*cat*) by ligation PCR, generating a riboswitch cassette library containing a *cat*-aptamer-linker-random fragment on the chromosome. The *cat* gene facilitates recombineering of the cassette into the genome, and the 5-nt-random sequence allows construction of a riboswitch library. The cassette was integrated, using the recombineering technique, into the genome of the *E. coli* MG1655 strain right upstream of the RBS of target genes, resulting in numerous mutant strains each of which carried a riboswitch candidate (functional or nonfunctional) on the chromosome. In theory, the mutant library contained 4<sup>5</sup> riboswitch candidates.

### Effective ON and OFF riboswitches of the *lacI* gene are constructed using the efficient library screening approach

LacI is a well-known repressor of *lacZ* and therefore the expression of *lacI* can be detected on X-gal plates. However, we did not use this strategy but instead constructed a *lacI-lacZ* translational fusion on the chromosome of a MG1655 *lacZ* null mutant, to illustrate that the library screening on X-gal plates allows for construction of riboswitches for any target genes fused with *lacZ*. To engineer an ON riboswitch of *lacI*, the riboswitch cassette *cat*-aptamer-linker-random sequence was integrated upstream of the RBS of the *lacI-lacZ* fusion on the chromosome by recombineering (Figure 1A). Then, the resulting mutant collection was placed on agar with 0.06 mg/ml X-gal and 2 mM caffeine (used as a negative control whose chemical structure differs from theophylline only by the additional presence of one methyl group) but without theophylline. Approximately 2000 colonies were checked for color, and two clones were whiter than others. Both clones turned into blue when re-streak on X-gal plates plus 2 mM theophylline. The riboswitches in the two clones were sequenced and the random sequence was TGTAT



**Figure 1.** Creation of ON and OFF riboswitches for the *lacI* gene using a library screening-based method. (A) A random cassette mixture containing a theophylline-responsive aptamer and five random bases was ‘recombined’ into the chromosome of a MG1655 mutant that was defective in native *lacZ* but carried the *lacI-lacZ* translational fusion, generating a riboswitch library for *lacI*. Cells were placed on X-gal plates without theophylline but with 2 mM caffeine. Relatively whiter colonies were searched for and transferred to X-gal plates with 2 mM theophylline. Those that turned into blue were considered to carry ON riboswitches of *lacI*. (B) Cells were placed on X-gal plates without theophylline but with 2 mM caffeine. Blue colonies were searched for and transferred to X-gal plates with 2 mM theophylline. Those that turned into relatively white were considered to carry OFF riboswitches of *lacI*. (C) Evaluation of the ON and OFF riboswitches of *lacI* using  $\beta$ -galactosidase assay. The mutant strain (named *lacI-lacZ*) that carries the *lacI-lacZ* translational fusion without the ON riboswitch was used as a control.

and CGTAT, respectively. The riboswitch carrying TGTAT was named ON1-*lacI*, and the one with CGTAT was named ON2-*lacI*. The effectiveness of the two ON riboswitches of *lacI* was confirmed by  $\beta$ -galactosidase assay comparing *lacI-lacZ* fusion levels in the presence of either caffeine or theophylline (Figure 1C).

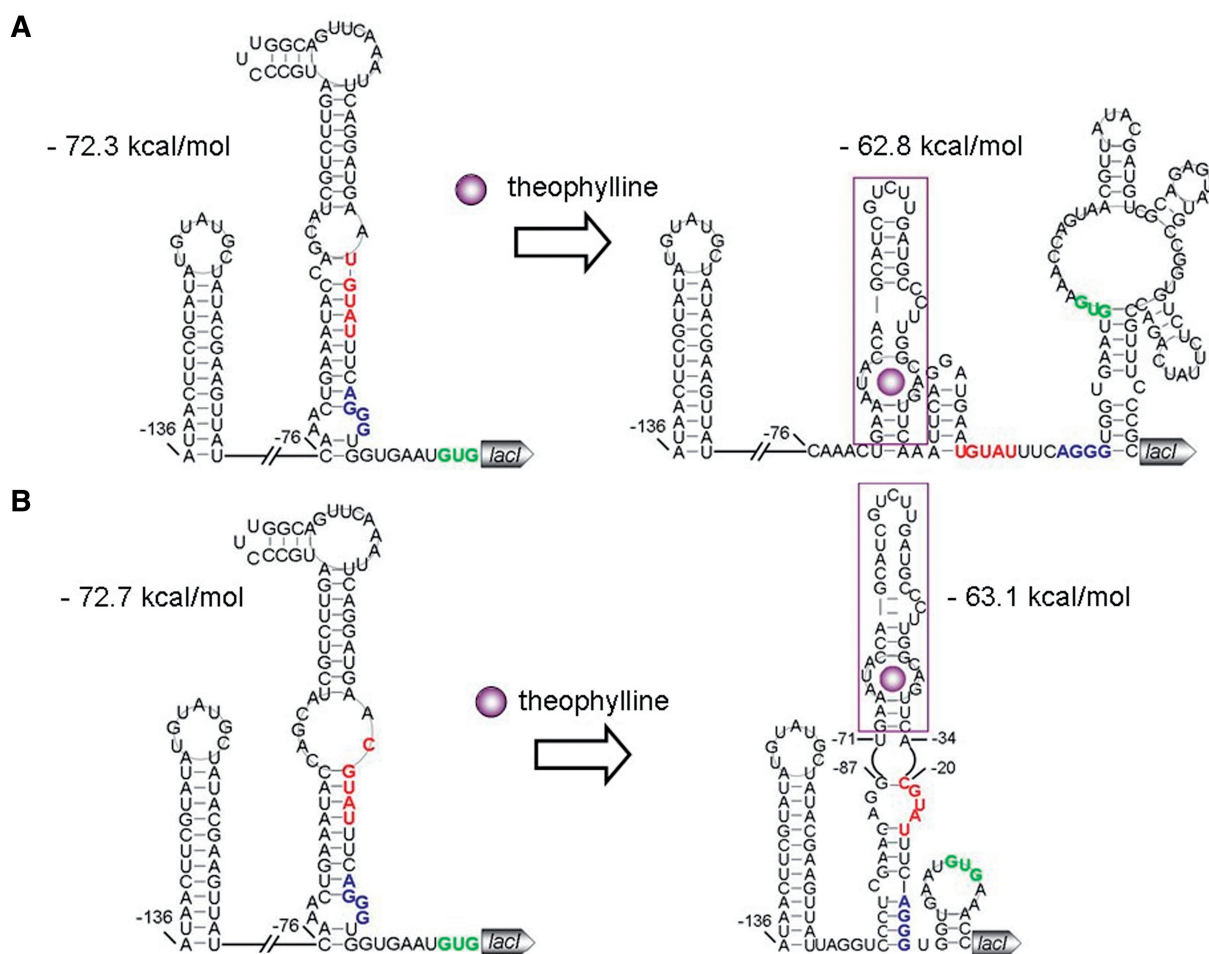
To screen for effective OFF riboswitches of *lacI*, approximately 3000 blue colonies on the caffeine-supplemented X-gal plates were re-streak on plates plus 2 mM theophylline (Figure 1B). After 6 h culture, one clone gave whiter color relative to others. Its responsiveness to theophylline was verified by  $\beta$ -galactosidase assay (Figure 1C), indicating that the clone carries an OFF riboswitch of *lacI*. We named it OFF-*lacI*. Sequencing analysis revealed that the 5-nt random sequence in OFF-*lacI* is CTGGT.

#### The artificial riboswitches control the *lacI* expression via a long stem structure adjacent to the ribosome-binding site

Secondary structures of the ON and OFF riboswitches of *lacI* were then predicted using RNAstructure 5.1 (19,20). The secondary structure prediction of all the riboswitches was performed for a 237-nt long fragment starting with a 33-nt stem-loop structure (–136 nt relative to the translational start site) and ending at the 100th nucleotide of *lacI*. For each riboswitch, the optimal structure with the

minimum free energy was chosen for the structure analysis. To predict riboswitch structures in the ligand-bound form, we utilized the ‘force pair’ function of RNAstructure 5.1 to force the theophylline binding pocket to form.

The structure analysis revealed that ON1-*lacI* in the non-ligand-bound state forms a 9-bp stem immediately adjacent to the RBS (Figure 2A). This long stem exists in not only the optimal structure but also alternative structures with higher free energy (data not shown). Except for the first nucleotide, the random sequence contributes to the formation of the stem. When theophylline binds to the riboswitch, the conformation changes and the long stem adjacent to the RBS does not form (Figure 2A). In ON2-*lacI*, the same was found (Figure 2B). Interestingly, the 5-nt random sequences of the two ON riboswitches share the latter 4 nt, which is unlikely to be coincident. We proposed that the latter four nucleotides GUAU is critical for the action of ON1-*lacI* and ON2-*lacI* by being part of the long stem immediately upstream of the RBS. If this is true, then the riboswitch OFF-*lacI* in the absence of theophylline would not contain a long stem next to the RBS, allowing for the gene translation; but would form the stem structure when bound by the ligand, repressing the expression. As shown in Figure 3A, the predicted structures of OFF-*lacI* in the presence and in the absence of theophylline are totally in agreement with this hypothesis.



**Figure 2.** Predicted structures of the riboswitches ON1-*lacI* (A) and ON2-*lacI* (B). In the non-ligand-bound state, there is a 9-bp stem structure immediately upstream of the ribosome binding site and is likely to block the gene translation. In the theophylline-bound state, the long stem does not form and consequently permits the gene translation. The random sequence is shown in red; the ribosome binding site in blue; and the start codon in green.

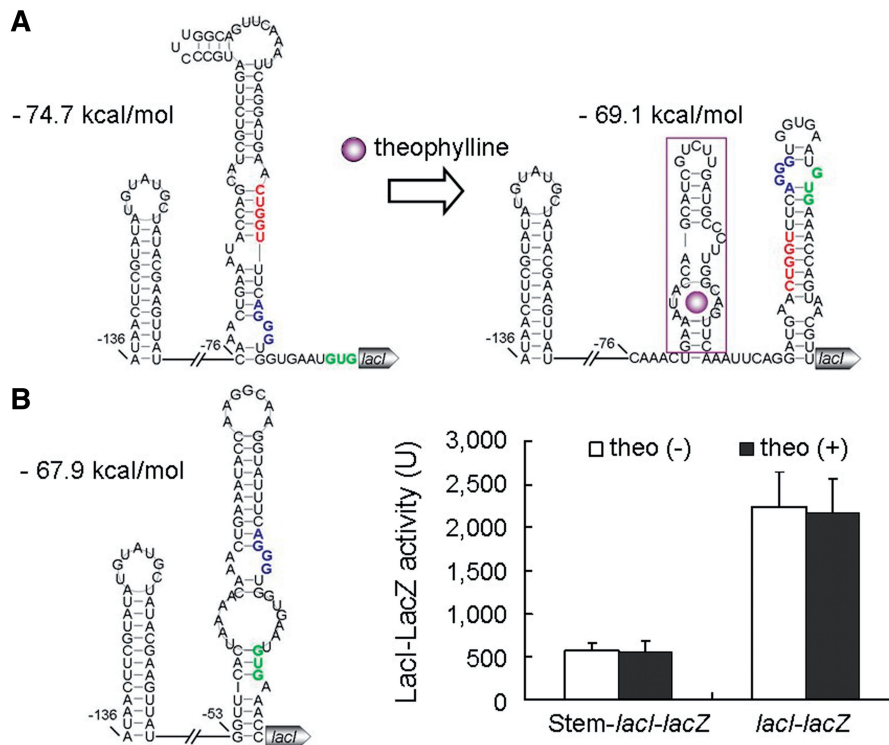
According to the prediction of free energy using RNA structure 5.1, all the structures in the non-bound state have lower free energy than the structures in the ligand-bound state (Figures 2 and 3), and therefore are proposed to predominate in the absence of theophylline. Only when theophylline binds to the riboswitches would the equilibrium distribution be shifted to the formation of structures with a theophylline-binding pocket, switching ON or OFF the target. To confirm the role of a long stem adjacent to RBS in the riboswitch-mediated gene control, we integrated a long stem (10 nt)-loop structure immediately upstream of the RBS of *lacI* fused with *lacZ* (Figure 3B). In the resulting mutant named Stem-*lacI-lacZ*, the expression levels of the *lacI-lacZ* fusion were reduced by 74% as a result of the insertion of the long stem (Figure 3B). Taken together, the synthetic riboswitches regulate *lacI* expression by forming or disrupting a long stem (9–10 nt) immediately upstream of the RBS in response to theophylline.

#### ON-*lacI*-LacIbs acts as a magnified, tunable and two-way riboswitch device

Although the above-described riboswitches managed to fine-tune their target genes in response to theophylline

and modulate corresponding biological processes, they had following limitations. First, they did not restore wild-type levels in the ON state (i.e. low ligand-saturating target levels) and failed to completely shut off the target genes in the OFF-state (i.e. high target leakage) (Figure 1C). Second, these riboswitches performed either ON or OFF function and thus were ‘one-way’ switches. It would be desirable to develop a ‘two-way’ device that not only switches off but also switches on gene expression without changing growth medium. Here, we utilized a ligand-responsive riboswitch in combination with the LacI protein and LacIbs to solve these problems. Given that the two ON riboswitches are equally effective in controlling *lacI* and both have a wider dynamic range than OFF-*lacI* (Figure 1C), we chose ON1-*lacI* for the construction of the riboswitch-LacP hybrid device.

LacI is a transcriptional repressor and inhibits *lacZ* transcription by binding to two LacIbs upstream of *lacZ*. Although the cells carrying ON1-*lacI* displayed high basal levels of LacI in the ligand-free form and had only 1-fold increase in LacI levels in the ligand-bound form (Figure 1C), the narrow dynamic range of the ON1-*lacI* riboswitch would be magnified when the LacI repressor in



**Figure 3.** Predicted structures of the riboswitch OFF-*lacI* and the long stem besides the ribosome binding site in the mutant Stem-*lacI*. (A) In OFF-*lacI*, a 9-bp stem structure forms immediately upstream of the ribosome binding site in the absence of theophylline but does not exist in the presence of the ligand. The random sequence is shown in red; the ribosome binding site in blue; and the start codon in green. (B) In Stem-*lacI*, there is a 10-bp stem structure located immediately upstream of the ribosome binding site. LacZ activity of the strain Stem-*lacI*-*lacZ* was determined using the  $\beta$ -galactosidase assay. The ribosome binding site is shown in blue, and the start codon in green.

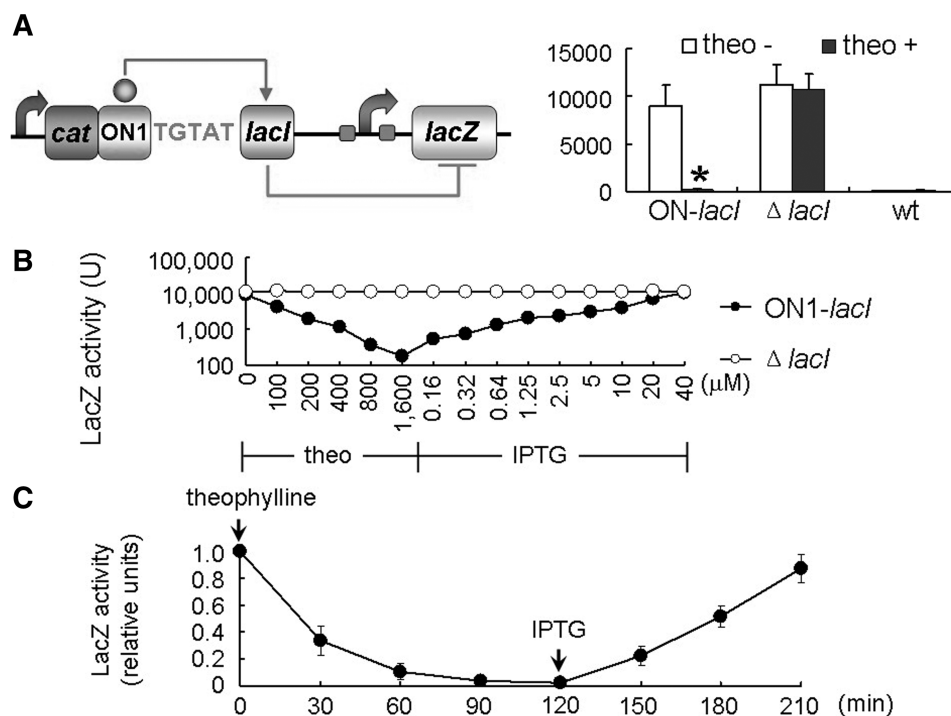
turns controlled its downstream target. To test this possibility, we integrated the ON riboswitch upstream of the intact *lacI* gene (not fused with *lacZ*). The LacI protein represses expression of *lacZ* so that the ON riboswitch indirectly switches off *lacZ* in response to theophylline. In support of the above magnification hypothesis, ON1-*lacI* when equipped with two LacIbs (upstream of *lacZ*) displayed little leaky repression on *lacZ* in the presence of caffeine but in the absence of theophylline, and almost completely switched off *lacZ* when theophylline was added to the cells (Figure 4A). Thus, LacI as an intermediary modulator can greatly increase the dynamic range and improve the performance of the ON riboswitch. ON1-*lacI* equipped with LacI and two LacIbs was hereinafter referred to as ON-*lacI*-LacIbs.

Using LacI in the riboswitch device also makes possible sequential two-way gene regulation as the LacI protein can be inactivated by isopropyl  $\beta$ -D-1-thiogalactopyranoside (IPTG). Thus, adding theophylline should switch on *lacI* and repress target genes of LacI, whereas adding IPTG should inactivate LacI and derepress the targets. The sequential control was verified by determining  $\beta$ -galactosidase levels of the cells carrying the ON1-*lacI* switch and intact *lacZ*. Theophylline and IPTG regulated *lacZ* in opposite directions in a concentration-dependent manner (Figure 4B). The effective concentration range of theophylline was 100–1600  $\mu$ M. Theophylline concentrations <100  $\mu$ M gave little regulation and those higher

than this range failed to cause additional repression. When IPTG was added to the medium containing 1600  $\mu$ M theophylline, *lacZ* was derepressed. The effective range of IPTG was 0.16–40  $\mu$ M. In addition, we performed time-course experiments to further characterize the response of ON-*lacI*-LacIbs to theophylline and IPTG. All the data were normalized to those of the same strain grown in LB plus 1600  $\mu$ M caffeine. As shown in Figure 4C, the hybrid device switched off ~66% of the LacZ activity within 30 min after the addition of 1600  $\mu$ M theophylline. Maximum silencing (~98% of the LacZ activity was switched off) was observed at 120 min. Forty micrometer IPTG was then added to the culture and switched on the *lacZ* expression rapidly. Within 90 min after the IPTG addition, ~85% of the LacZ activity was restored.

#### Application of ON-*lacI*-LacIbs to portable, tunable and two-way regulation of sigmaS (RpoS) subunit of RNA polymerase

Inducible gene control built on binding of LacI to LacIbs has been widely used for various non-*lacZ* genes in prokaryotic and eukaryotic cells (21–25), showing that the inducibility of the LacI-responsive promoters are less likely to be affected by downstream targets than that of riboswitches. Thus, the ON-*lacI*-LacIbs hybrid device should be capable of regulating any target gene when integrated

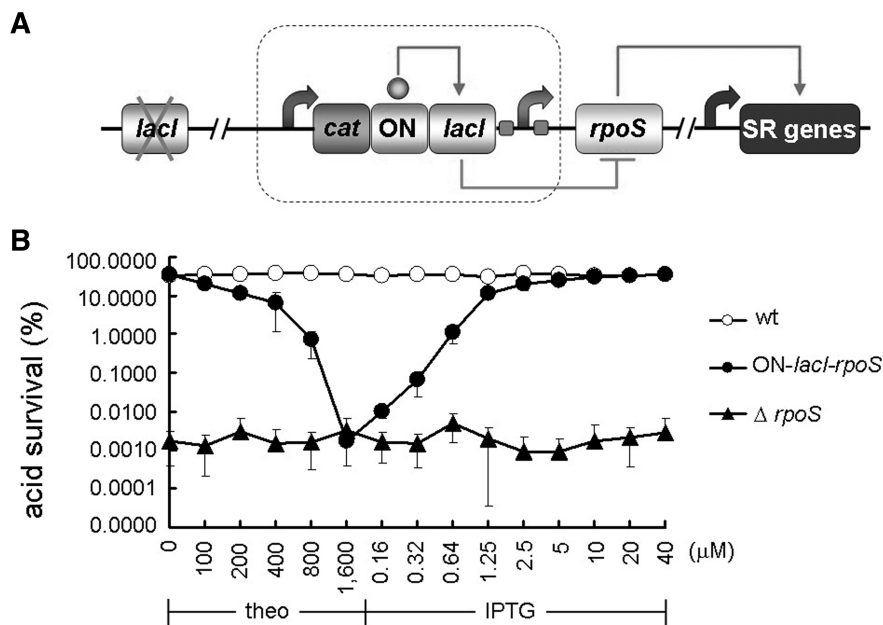


**Figure 4.** Magnified, tunable, two-way gene control mediated by the hybrid device ON-*lacI*-LacIbs. (A) Using the LacI repressor as a modulator, the ON-*lacI* riboswitch indirectly controlled *lacZ* expression and its regulatory effects on *lacZ* were magnified compared to its effects on *lacI*. (B) Tunable and two-way control of target gene mediated by the combinatorial use of the ON-*lacI* riboswitch and LacI-binding sites. Using LacI as a modulator, the ON-*lacI* riboswitches finely tuned *lacZ* expression in response to theophylline (0–1600  $\mu$ M) and reversed *lacZ* expression by adding increasing concentrations of IPTG (0.16–40  $\mu$ M) without removal of medium. Error bars represent standard deviation (\* $P < 0.05$ ). (C) Kinetics of the LacZ activity in the strain carrying ON-*lacI*-*lacZ*. 1600  $\mu$ M theophylline was added to exponentially growing culture at 0 min, and 40  $\mu$ M IPTG was added to the culture at 120 min. Samples were collected for measurement of LacZ activity at the indicated time points. All data were normalized to the LacZ activity of the strain grown in LB medium plus 1600  $\mu$ M caffeine.

upstream of the RBS. We next showcased the portability of this device by applying it to the *rpoS* gene.

RpoS is an alternative sigma factor of RNA polymerase, governing expression of over 200 genes (26,27) and playing a critical role in survival of a diverse number of stresses such as acid shock (28,29), osmotic stress (30), heat shock (31), oxidative damage (29,32) and starvation (29,33). RpoS negatively regulates *Salmonella* virulence (29) and has, therefore, been mutated to engineer live attenuated vaccine strains (34,35). Given the significance of RpoS as a global response regulator and its potential medical use, it is desired that the *rpoS* gene could be placed under the control of the ON-*lacI*-LacIbs hybrid device for tunable and two-way expression control. To do this, we PCR amplified the ON-*lacI*-LacIbs cassette (2500 bp) from the genome of the strain carrying the ON riboswitch of *lacI*. The cassette is composed of the *cat* gene, the ON-*lacI* riboswitch, the *lacI* gene and the *lacI*-*lacZ* intergenic region containing the *lac* promoter (LacP) with two LacIbs. The *cat* gene facilitates integration of this cassette immediately upstream of any target gene via recombineering. Next, we simply integrated the riboswitch cassette upstream of the RBS of the *rpoS* gene, without adjusting the sequence of the device. Then, the native *lacI* gene was deleted from this strain, generating a new strain named ON-*lacI*-*rpoS* (Figure 5A). This strain was used to test if *rpoS* could be controlled by the

theophylline-responsive and LacI-repressible regulation cassette. Since *rpoS* is required for acid resistance, we assayed survival of the strain under acidic conditions. We observed that the *rpoS* expression was on in the presence of caffeine and the absence of theophylline as revealed by normal survival percentage compared to the wild-type strain after 2-h acid treatment (pH 2) (Figure 5B). This was because the switch turned off *lacI* and therefore *rpoS* was in the ON state. When theophylline was added, the cells became sensitive to acid (Figure 5B), indicating that the addition of theophylline turned on the expression of *lacI* and the latter repressed the expression of *rpoS*. Acid survival in the absence of theophylline was close to the wild-type levels and decreased with increasing concentrations of theophylline (Figure 5B). Acid survival reached the minimum that was comparable to that of an *rpoS* null mutant when theophylline was increased to 1600  $\mu$ M. When increasing concentrations of IPTG were added to the medium containing 1600  $\mu$ M theophylline, the acid survival increased and was restored to the wild-type levels in the presence of 40  $\mu$ M IPTG, consistent with the response curve of the ON1-*lacI* strain. These *rpoS* data not only confirmed the portability, tunability and two-way control features of the ON-*lacI*-LacIbs device, but also suggested the potential utility of this device in flexibly manipulating virulence and immunogenicity of live vaccines and bacteria for other medical purposes.



**Figure 5.** Gene control mediated by the ON-*lacI*-LacIbs hybrid device. (A) The ON-*lacI*-LacIbs hybrid device. The boxed is a portable riboswitch cassette ON-*lacI*-LacIbs (LacI-binding sites) integrated immediately upstream of ribosome binding site of the *rpoS* gene that is a master regulator of stress resistance (SR). (B) Tunable and two-way control of *rpoS* via the ON-*lacI*-LacIbs device, as revealed by acid survival. The strain ON-*lacI*-*rpoS* that carried the ON-*lacI*-LacIbs device upstream of the ribosome binding site became increasingly sensitive to acid with theophylline (0–1600 μM). When increasing concentrations of IPTG were added to cells grown in media with 1600 μM theophylline, acid survival was increased and restored to wild-type levels in the presence of 40 μM IPTG. Error bars represent standard deviation.

#### ON-*lacI*-LacIbs coupled with a small non-coding RNA CsrB for *trans*-acting regulation of autoaggregation and biofilm formation

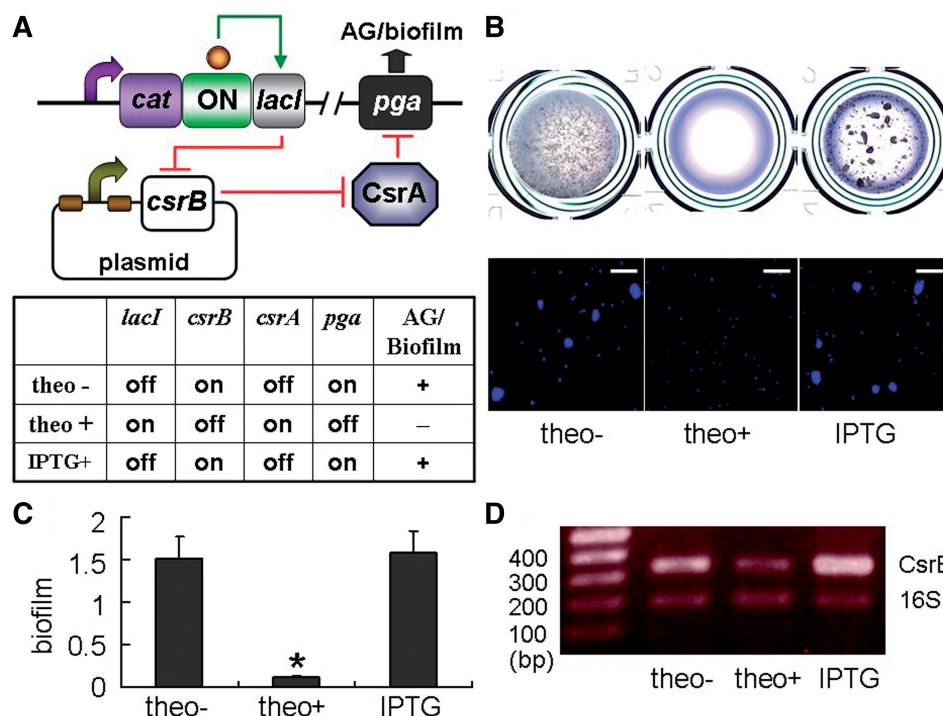
Next, we asked if we could accomplish *trans*-acting regulation of target expression using the ON-*lacI*-LacIbs device so that there is no need for chromosome engineering. We illustrated this using a naturally existing small non-coding RNA CsrB which is well known as an antagonist of the CsrA protein (36). It has previously been demonstrated that CsrB promotes cellular autoaggregation (4) and biofilm formation by indirectly activating the expression of the *pgaABCD* operon via CsrA (37). Considering these obvious phenotypes of CsrB-overproducing cells, we decided to couple CsrB with the ON-*lacI*-LacIbs device to control the *pga* operon (Figure 6A). We cloned *csrB* in a multi-copy vector downstream of the *lac* promoter to place the CsrB expression under the control of the ON1-*lacI* switch. Since one LacIb is after the transcriptional start site, the resulting CsrB slightly differs from the native one by the extra 21-nt sequence (i.e. LacIb). We induced the CsrB expression by IPTG and observed that autoaggregation (which serves as an indicator of CsrB expression) occurred (data not shown), indicating that this CsrB is functional. We introduced this CsrB-carrying vector into the ON1-*lacI* strain and examined autoaggregation and biofilm formation. In the presence of caffeine and the absence of theophylline, LacI levels were low and CsrB expression should be on (Figure 6A). As predicted, the cells autoaggregated and formed high levels of biofilms, showing that the *pga* expression was induced due to the overproduction of CsrB

(Figure 6B and C). In the presence of theophylline, *lacI* expression is turned on and CsrB production should be repressed by LacI. The repression of CsrB is expected to result in upregulation of CsrA, which inhibits autoaggregation and biofilm formation (Figure 6A). In agreement with this, the cells were negative with autoaggregation and formed low levels of biofilms (Figure 6B and C). When IPTG was added together with theophylline, the activity of LacI was inhibited, and CsrB should be at high levels (Figure 6A). In support of this, autoaggregation and biofilm formation were restored in the presence of IPTG and theophylline (Figure 6B and C). The changes in CsrB expression in response to theophylline and IPTG were also confirmed by semi-quantitative RT-PCR (Figure 6D). These results demonstrate that the hybrid device can be used in combination with a small non-coding RNA to exert *trans*-acting control of target expression, alleviating the need for modification of the chromosome and expanding the applications of this riboswitch system.

#### Use of ON-*lacI*-LacIbs to uncover a role of RpoS in acetate assimilation

Next, we applied ON-*lacI*-LacIbs to identify new gene functions that may otherwise be neglected using traditional methods. It is well established that bacterial cells switch from rapid growth that produces and excretes acetate (dissimilation) in the presence of abundant nutrients to slower growth supported by the import and utilization of the excreted acetate (assimilation) when glucose is exhausted [for a review, see reference (38)]. This physiological event





**Figure 6.** Trans-acting gene control through combinatorial use of the ON-*lacI*-LacIbs hybrid device and a small non-coding RNA CsrB. (A) Trans-acting system and predicted autoaggregation (AG) and biofilm phenotypic changes of the ON-*lacI* strain carrying pLacIb-CsrB in response to theophylline and IPTG. A *csrB*-carrying plasmid (pLacIb-CsrB) was transformed into the ON-*lacI* strain. The *csrB* gene was preceded by two LacI-binding sites and thus under the control of the ON-*lacI* riboswitch. (B) Scanned images of cell suspensions (stained with crystal violet) of the ON-*lacI* strain harboring pLacIb-CsrB in microtiter plates and fluorescence microscopic images of the corresponding cell suspensions (stained with DAPI) in the absence of theophylline, in the presence of 2 mM theophylline or in the presence of both 2 mM theophylline and 1 mM IPTG. (C) Biofilm formation of the ON-*lacI* strain harboring pLacIb-CsrB in the absence of theophylline, in the presence of 2 mM theophylline or in the presence of both 2 mM theophylline and 1 mM IPTG. (D) Detection of intracellular CsrB small non-coding RNA levels by semi-quantitative reverse transcription-PCR. Total RNA was isolated from overnight cultures of the ON-*lacI* strain carrying pLacIb-CsrB grown in the absence of 2 mM theophylline, in the presence of 2 mM theophylline or in the presence of both 2 mM theophylline and 1 mM IPTG. cDNA was amplified for 18, 20, 22 and 24 cycles using gene-specific primers. 16S rRNA was used as an internal control. PCR products formed after 18 cycles are included in the agarose gel image shown in the figure, along with a DNA ladder (100-, 200-, 300-, and 400-bp bands indicated). Densitometry indicated that for both the CsrB and 16S rRNA products, cycle 18 was within the linear range for the PCR amplification (data not shown). Expected product size for CsrB and 16S rRNA is 347 and 200 bp, respectively. Error bars represent standard deviation (\* $P < 0.05$ ).

is defined as acetate switch, a survival response allowing cells to compete successfully during carbon starvation. It was previously reported that RpoS positively regulated consumption of acetate by activating the expression of acetyl-coenzyme A synthetase (Acs) (39,40). However, we obtained opposite results with ON-*lacI*-*rpoS*. ON-*lacI*-*rpoS* in the ON state in the absence of theophylline and IPTG exhibited acetate assimilation similar to that of the wild-type strain (Figure 7A). When grown in the presence of theophylline (2 mM) and absence of IPTG, the *rpoS* expression was shut off (detailed above) and accordingly, the acetate assimilation was accelerated. When IPTG (1 mM) was added to the medium to free up the *rpoS* expression, the acetate assimilation was restored to the wild-type levels (Figure 7A). Neither theophylline nor IPTG had effect on acetate assimilation of the wild-type strain (data not shown), excluding the possible non-specific effects of the two chemicals. The enhanced acetate assimilation was also observed with the newly constructed *rpoS* null mutant (Figure 7B). All the cell suspensions were adjusted to an OD of 0.08 prior to the acetate

assays, and the cells with varying RpoS activities showed comparable cell growth rate (data not shown), ruling out the possible effects of growth rate on acetate assimilation. Taken together, RpoS represses acetate assimilation.

Next, we explored the underlying mechanism by which RpoS regulates the ability to scavenge for extracellular acetate. Acetate assimilation depends on the Acs and the phosphotransacetylase (Pta)-acetate kinase (AckA) pathway (38,41) (Figure 7C). We therefore proposed that RpoS inhibited the Acs and/or the Pta-AckA pathway and that deleting *rpoS* should increase their expression levels. To test this hypothesis, we incubated the cells for 6 h when acetate switch had been flipped and then examined the possible effects of RpoS on the levels of Acs and AckA-Pta. Our  $\beta$ -galactosidase data showed that the removal of *rpoS* slightly reduced the expression of an *acs-lacZ* translational fusion on the chromosome, excluding the first possibility. However, deleting *rpoS* resulted in a 5-fold increase in the expression levels of an *ackA-lacZ* translational fusion (Figure 7D). *Pta* and *ackA* are in the same operon. The *ackA-lacZ* data



riboswitches. Unlike previously reported riboswitch libraries that are all carried on plasmids (14–16), our libraries are established on the chromosome with the aid of recombineering. Therefore, our riboswitches are more genetically stable than the previous plasmid-harbored ones. We built up a riboswitch random library for the *lacI* gene that was fused in frame with *lacZ* whose expression could be easily examined on X-gal plates. By checking the color of colonies grown on X-gal plates with or without theophylline, both ON and OFF riboswitches of *lacI* were created. Structure predictions revealed that the above riboswitches regulate the *lacI* expression by adopting or disrupting a long stem structure immediately upstream of the RBS in response to theophylline. The long stem forms in the OFF state and fails to exist in the ON state. Thus, the long stem besides the RBS is responsible for the gene silencing mediated by the riboswitches, consistent with previous reports (5).

Building on the synthetic riboswitches created in the first part of the work, we constructed a portable and two-way regulation device that act as a versatile key to any target without rational design or library screening. We achieved this by adopting the LacI repressor, which inhibits transcription by binding to LacIbs, as a modulator that links the ligand-responsive riboswitch to ultimate targets. As described above, effective ON riboswitches had been created for *lacI*. Upstream of the *lacI* gene in the strain ON-*lacI* is a riboswitch cassette that carries all necessary regulation elements including the  $\text{Cm}^R$  gene (*cat*), a constitutive promoter, the theophylline-responsive ON riboswitch of *lacI*, the *lacI* gene and two LacIbs. Without any sequence adjustment, we simply inserted this 2500-bp cassette immediately upstream of the RBS of the *rpoS* gene and showed that the ON-*lacI* riboswitch fine-tuned *rpoS* expression (as revealed by acid survival). When applied to the *csrB* gene, this device controlled the expression of the small non-coding RNA CsrB and consequently modulated biofilm formation and cellular autoaggregation. The success of this device in regulating the expression of *lacZ*, *rpoS* and *csrB* demonstrates its portability.

The combinatorial use of the ON riboswitch of *lacI* and LacIbs has other advantages in addition to portability. LacI can magnify the regulatory effects of the ON-*lacI*-LacIbs device and maximize its dynamic range that is defined as the ratio of the highest and lowest target levels. Previous regulation systems mediate control of gene expression in a one-way manner: target expression is turned on in response to a specific inducer and turned off when the inducer is removed by replacement of medium. However, this maneuver is infeasible in animal experiments and may cause problems in some demanding bacterial experiments where cultures have to be kept intact. The ON-*lacI*-LacIbs system overcomes this problem as its target gene is in the ON state in the absence of theophylline, switched off in response to the ligand, and switched back to the ON state when IPTG is added without removal of theophylline.

Finally, strains armed with this device are more genetically stable and therefore provide more reliable information compared to those constructed using traditional approaches. It has been demonstrated that deleterious

mutations and those that cause reduced fitness can bring about secondary mutations that compensate for undesired changes (45–50). The compensatory mutations can give misleading results in gene function studies and destabilize geno- and phenotypes of genetically engineered bacteria for medical and industrial purposes. One solution to minimize the compensatory mutations is to engineer a strain in which the gene to be controlled is normally expressed and not shut off unless an inducer is applied. Moreover, it would be ideal if the wild-type levels of gene expression could be restored in response to a second effector molecule, to ensure that the observed mutation phenotype is not caused by pleiotropic effects of the inducer. The ON-*lacI*-LacIbs riboswitch device meets all these requirements. For example, the strain ON-*lacI*-*rpoS* exhibits normal *rpoS* expression in the absence of theophylline and IPTG and therefore has no fitness burden. Therefore, compared to an *rpoS* null mutant, ON-*lacI*-*rpoS* is less likely to accumulate compensatory mutations and thus more reliable. Only in the presence of theophylline, can ON-*lacI*-*rpoS* behavior as an *rpoS* mutant, allowing for *rpoS* function investigation. Phenotypes in the two ON states (in the absence of any inducer and in the presence of both theophylline and IPTG) should be identical, excluding the non-specific effects of theophylline. Here, the use of the ON-*lacI*-LacIbs device helped uncover a novel function of RpoS, which may otherwise be neglected or misunderstood by using traditional approaches. That is, RpoS inhibits acetate assimilation. Acetate assimilation is a critical process allowing the cell to efficiently utilize nutrient resources when favorable carbon sources are exhausted, and turning the waste product acetate into a carbon and energy source. Acetate assimilation is also responsible for managing levels of acetyl-coA and acetyl phosphate, both of which are important central metabolites [for a review, see reference (38)]. Interestingly, previous studies reported the opposite results that RpoS appeared to positively regulate acetate assimilation (39,40). One explanation of the discrepancy is the above-mentioned occurrence of compensatory mutations. Indeed, it has been demonstrated that secondary mutations within the *hns* gene frequently occur in strains carrying *rpoS* mutations to compensate for the loss of RpoS function (42). The action of H-NS is generally opposite to that of RpoS (51), and H-NS downregulates both *rpoS* mRNA translation and RpoS stability (52). This, together with our observation that deleting *hns* represses acetate assimilation makes it reasonable to speculate that compensatory mutations within *hns* may have occurred in the *rpoS* mutant strains used in the previous studies so that they observed opposite phenotypes with their *rpoS* mutants.

In summary, we have developed a portable device that performs sequential OFF-and-ON gene regulation in response to theophylline and IPTG, respectively. This study also indicates that the number of gene control elements available to us can be greatly increased by combining ligand-dependent riboswitches and inducer-responsive promoters. Suppose the number of riboswitch ligands is  $n$  and the number of inducers of inducible promoters is  $m$ , then  $n \times m$  combinatorial control elements can be created,

greatly enriching the repertoire of gene regulatory elements. Although this work focuses on developing a riboswitch-LacP hybrid device in *E. coli*, similar strategy (i.e. a ligand-responsive riboswitch integrated with an inducible promoter system) may be extended to eukaryotic cells to achieve portable, two-way expression regulation.

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*Conflict of interest statement.* J.-D.H. and Y.J. declare competing financial interests in the form of a patent application whose value may be affected by the publication of this manuscript.

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