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A Synthetic Anhydrotetracycline-Controllable Gene Expression System in *Ralstonia eutropha* H16

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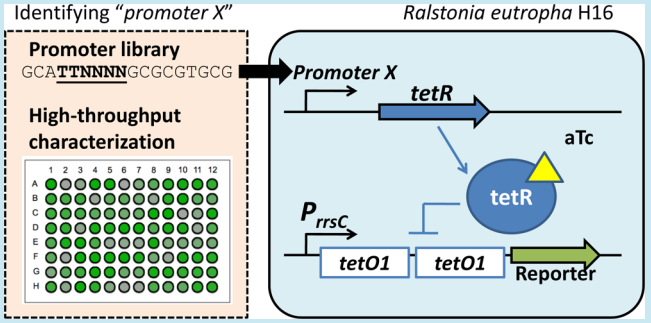
Supporting Information

ABSTRACT: Controllable gene expression systems that are orthogonal to the host's native gene regulation network are invaluable tools for synthetic biology. In *Ralstonia eutropha* H16, such systems are extremely limited despite the importance of this organism in microbiological research and biotechnological application. Here we developed an anhydrotetracycline (aTc)-inducible gene expression system, which is composed of a synthetic promoter containing the operator *tetO*, the repressor TetR, and the inducer aTc. Using a reporter-activity based promoter library screen, we first identified the active hybrids between the *tetO* operators and the *R. eutropha* native *rrsC* promoter (P_{rrsC}). Next, we showed that the hybrid promoters are repressable by TetR. To optimize the dynamic range of the system, a high-throughput screening of 300 mutants of *R. eutropha phaC1* promoter was conducted to identify suitable promoters to tune the *tetR* expression level. The final controllable expression system contains the modified P_{rrsC} with two copies of the *tetO1* operator integrated and the *tetR* driven by the mutated P_{phaC1}. The system has decreased basal expression level and can be tuned by different aTc concentrations with greater than 10-fold dynamic range. The system was used to alleviate cellular toxicity caused by AlsS overexpression, which impeded our metabolic engineering work on isobutanol and 3-methyl-1-butanol production in *R. eutropha* H16.



KEYWORDS: *Ralstonia eutropha, tetracycline regulatory system, controllable promoter, metabolic engineering, synthetic biology*

Ralstonia eutropha H16 is a Gram-negative, facultative lithoautotrophic bacterium of scientific and biotechnological importance. For example, it is one of the best studied microorganisms for biosynthesis of polyhydroxyalkanoates (PHAs),[1] which could be used as biodegradable materials. Besides a broad range of organic compounds, it can also utilize CO_2 as the carbon source through the Calvin−Benson−Bassham (CBB) cycle,[2] which can be powered by the energy derived from H_2 or formate.[3] Recently, metabolic engineering work in *R. eutropha* H16 has demonstrated the production of biofuels from sugars, CO_2 and H_2, formic acid, or electricity and CO_2 in an integrated process.[4−6] However, advanced metabolic engineering work requires various synthetic biology tools especially controllable gene expression systems, which are still limited in this organism.

Many native genes in *R. eutropha* H16 have been found to be expressed in an inducible manner. For example, the expresssion of the *phaP1* gene, which encodes a PHA-granule associated phasin protein, is regulated by phosphate level in the medium[7] and highly coupled to PHA accumulation.[8,9] However, metabolic engineering and synthetic biology work requires non-native gene expression systems, which can be controlled independently of the host's metabolic state. To this end, some heterologous controllable gene expression systems have also been tested in *R. eutropha* H16. The Isopropyl *β*-D-1-thiogalactopyranoside (IPTG) controllable P_{lac} system from *Escherichia coli* could not be effectively induced.[9] And the L-arabinose controllable P_{BAD} system from *E. coli* requires more than 1 g/L of inducer to achieve good induction at certain conditions and may also affect the host's metabolism as indicated by the inhibited growth upon addition of the inducer.[9] These observations suggested that the widely used carbon-catabolite-repression based systems may not be suitable for implementing in *R. eutropha* H16, possibly because of the fact that the sugar uptaking and metabolism in *R. eutropha* H16 is very different from that in *E. coli*.[10] As such, we sought to develop the anhydrotetracycline (aTc)-inducible gene expression system in *R. eutropha* H16.[11] The inducer aTc is freely diffusible through the cell membrane and does not require specific transporters. Furthermore, the system has not been shown have crosstalk with the host's gene regulation network.

Although similar system naturally exists in many organisms such as *E. coli*, creating it in a new species is often still a challenge in synthetic biology. Each part of the naturally existing controllable gene expression system is optimized for

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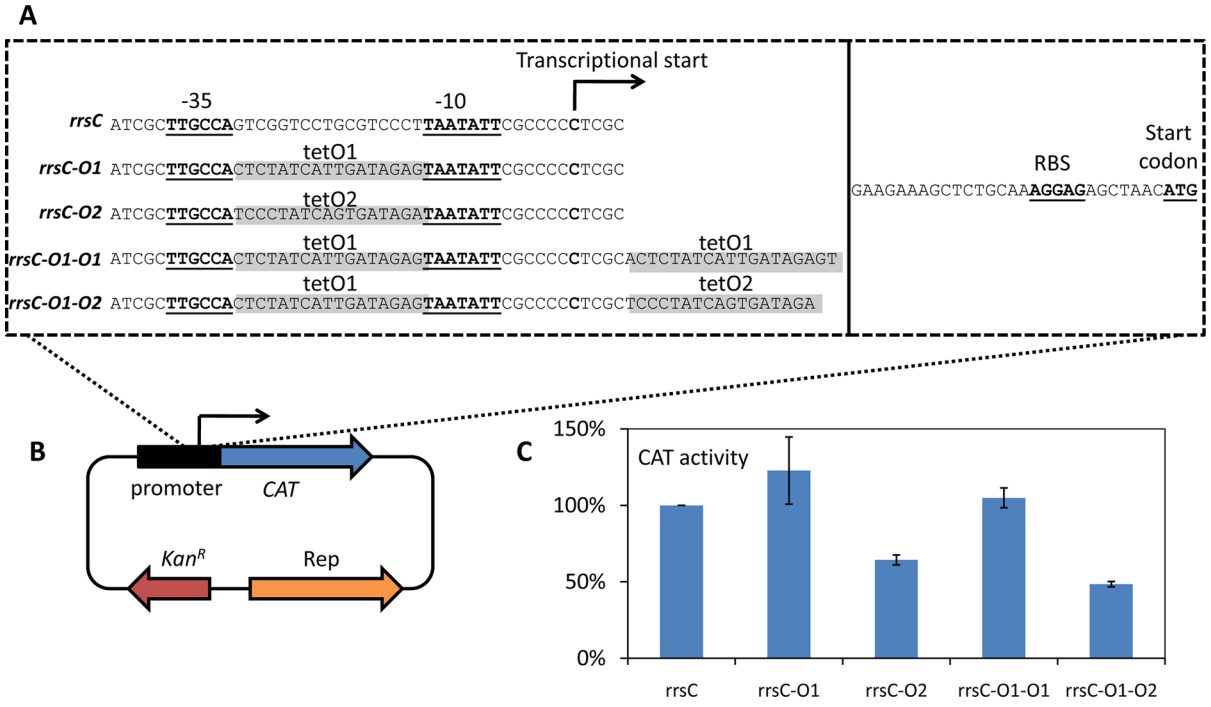

Figure 1. Characterization of the P_{rrsC} *tetO* hybrid promoters. (A) Sequence of the cloned wild type P_{rrsC} promoter from *R. eutropha* H16 and 4 hybrid promoters with *tetO1* or *tetO2* operators placed at different positions of P_{rrsC}. (B) Plasmid map showing the chloramphenicol acetyltransferase (CAT) reporter cassette for promoter characterization. (C) Activity of different promoters as measured by CAT activity level. Error bars stand for the standard deviation of 3 independent repeats. (n = 3).

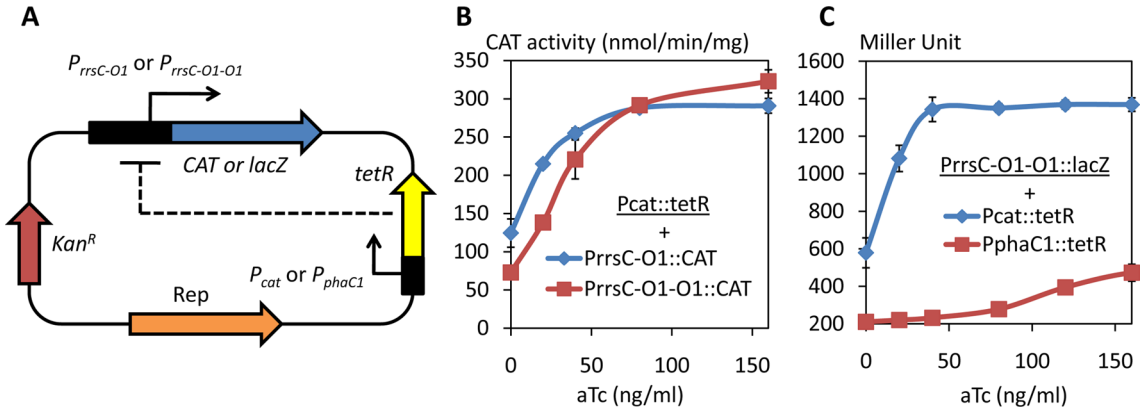

Figure 2. Repression of the P_{rrsC} *tetO* hybrid promoters by tetR. (A) Plasmid map showing the *CAT* or *lacZ* reporter cassette for promoter characterization and the *tetR* expression cassette driven by P_{cat} or P_{phaC1}. The two cassettes were placed in opposite direction to avoid interference. (B) Induction profile of the $P_{rrsC\text{-}O1}$ and $P_{rrsC\text{-}O1\text{-}O1}$ promoters in combination with P_{cat}::*tetR* with different concentration of the inducer aTc. (C) Induction profile of the $P_{rrsC\text{-}O1\text{-}O1}$ promoter in combination with P_{cat}::*tetR* or P_{phaC1}::*tetR* with different concentration of the inducer aTc. Error bars stand for the standard deviation of 3 independent repeats. (n = 3).

function in natural condition in the unique genetic context of its native host. Therefore, it is often difficult to directly transfer the entire system from one species to another. Instead, using synthetic biology approaches, controllable gene expression system can be built from scratch using elements derived from a variety of species following similar design principles of the naturally existing systems. However, such synthetically built systems usually require substantial optimization since different parts often do not match. The anhydrotetracycline (aTc)-inducible gene expression system is composed of a repressor protein TetR and a controllable promoter containing the TetR binding sequence (*tetO* operators). Upon binding of the inducer aTc to TetR, the latter disassociates from *tetO*, allowing expression of the target gene. The level of TetR, the position and number of *tetO*, and the transcription activity of the core promoter region of the controllable promoter all have to be tuned. Here we report the development of the anhydrotetracycline (aTc)-inducible gene expression system in *R. eutropha* H16 by stepwise optimization of the individual parts of the synthetic system. Especially, a mutagenesis method followed by a high throughput screening was used to optimize the level of TetR. Using this system, we alleviated the cellular toxicity caused by overexpression of acetolactate synthase (AlsS), a key enzyme in the isobutanol and 3-methyl-1-butanol production pathway.[4] In addition, the promoter libraries constructed and characterized in this work during the development of the controllable gene expression system also represented significant effort for expanding the synthetic biology tool kits in *R.*

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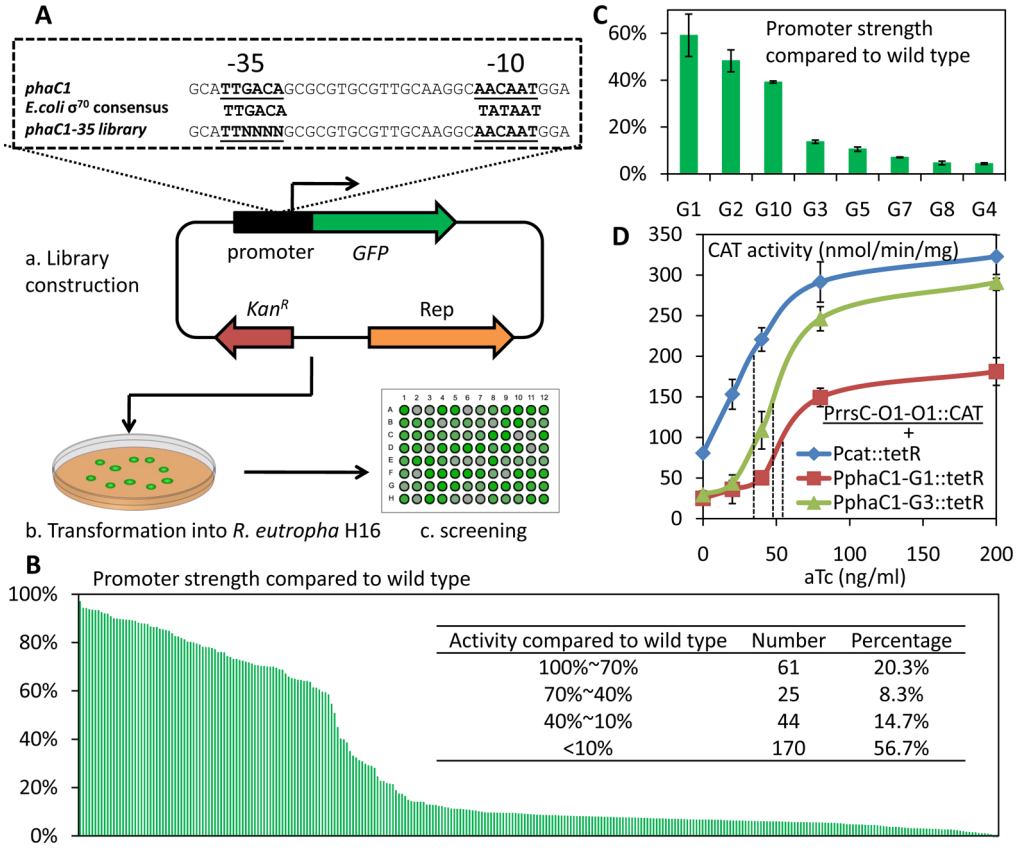

Figure 3. Identification of promoters of different strength to drive *tetR* expression using high-throughput promoter library screening. (A) Illustration of the high-throughput promoter library screening method. (a) To construct the library, the last 4 nucleotides of the −35 region of P_{phaC1} were randamized and inserted in front of the gfp reporter gene. (b) The library was transformed into *R. eutropha* H16. (c) Single colonies were picked and cultured in 96-well plates. Fluorescence of each sample was then measured using 96-well plate reader. (B) Distribution of the promoter activities of 300 candidates measured compared to the wild type P_{phaC1} promoter. Each vertical line represents one candidate. (C) Characterization of 8 promoter variants from the screen with stepwise decreased activities as measured by GFP level. (D) Induction profile of the $P_{rrsC\text{-}O1\text{-}O1}$ promoter in combination with P_{cat}::*tetR*, $P_{phaC1\text{-}G1}$::*tetR*, or $P_{phaC1\text{-}G3}$::*tetR* with different concentration of the inducer aTc. The dash lines indicate the Ind50 (concentration of aTc required to achieve 50% induction). Error bars stand for the standard deviation of 3 independent repeats. (n = 3).

eutropha. The stepwise optimization process to build the final controllable gene expression system also provided insight into the design principle and construction methodology of such systems in general.

Controllable hybrid promoters can be created by placing the operator sequences in a constitutive parent promoter.[12] In *R. eutropha* H16, we chose the promoter of the *rrsC* gene as the parent promoter (Figure 1A), which drives the transcription of an operon containing 16s, 23s, and 5s rRNAs and other translation-related genes. Using the bioinformatic tool (BPROM, Softberry), we identified the putative −10 and −35 elements of the P_{rrsC} and the transcriptional start site (Figure 1A). There are two different *tetO* operators (*tetO1* and *tetO2*) that can both be recognized by the tetR repressor.[13] We took a two-step approach to survey their compatibility with the parent promoter. First, when placed between the −35 and −10 regions of P_{rrsC}, the *tetO2* lowered the promoter's strength as measured by chloramphenicol acetyl transferase (CAT) reporter assays in *R. eutropha* H16 (Figure 1A,B,C). Thus, *tetO1* was chosen in this position, resulting in the hybrid promoter $P_{rrsC\text{-}O1}$ (Figure 1A). Next, a second operator was inserted downstream of transcriptional start site, which can potentially confer more stringent repression[14,15] (Figure 1A). The insertion of *tetO2*, but not *tetO1*, at this position decreased the activity of $P_{rrsC\text{-}O1}$ (Figure 1A,C). Therefore, the hybrid promoter the $P_{rrsC\text{-}O1}$ and $P_{rrsC\text{-}O1\text{-}O1}$ (Figure 1A) were chosen for subsequent development, which maintained the strength of the wild type P_{rrsC} (Figure 1C).

Next, we introduced the repressor protein TetR to the system by overexpressing it on the same plasmid that also contains the reporter gene cassette driven by $P_{rrsC\text{-}O1}$ or $P_{rrsC\text{-}O1\text{-}O1}$ (Figure 2A). We first employed the promoter P_{cat}, which drives the expression of the CAT antibiotics marker in the widely used broad host-range plasmids pBBR122 and pBHR1 (MoBiTec, Göttingen, Germany)[16] and has been used for heterologous gene expression in *R. eutropha* H16.[17] When P_{cat}::*tetR* cassette was added (Figure 2A), the $P_{rrsC\text{-}O1}$ and $P_{rrsC\text{-}O1\text{-}O1}$ promoters can be repressed (Figure 2B). The gene expression level can be regulated with different concentration of the inducer as measured by the CAT reporter assays (Figure 2B). Noticeably, the $P_{rrsC\text{-}O1\text{-}O1}$ promoter had lower basal level expression (the expression when no aTc was added) compared to $P_{rrsC\text{-}O1}$ (Figure 2B), which is possibly attributed to its extra TetR binding site (Figure 1A). Thus, further development was focused on the $P_{rrsC\text{-}O1\text{-}O1}$ promoter.

To further improve the stringency of the gene expression system, we tried to increase the expression level of the TetR repressor. We switched the promoter of the *tetR* expression cassette to P_{phaC1} (Figure 2A), which has been shown to be a relatively strong and constitutive promoter in *R. eutropha*

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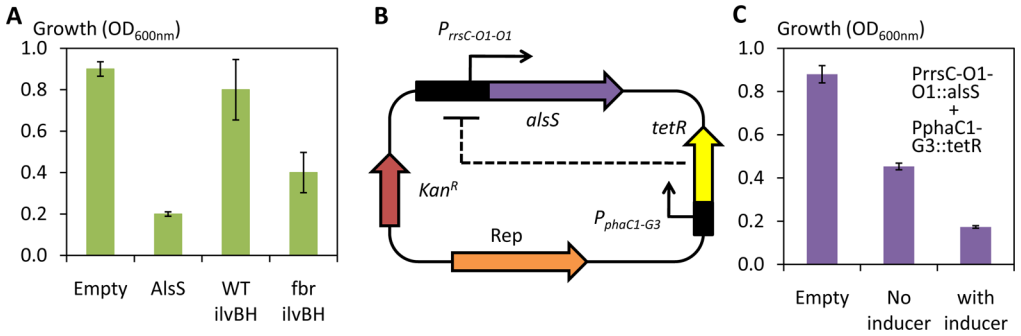

Figure 4. Application of the developed aTc-controllable gene expression system in alleviating the AlsS toxicity. (A) Overexpression of the *alsS* from *Bacillus subtilis* or the feedback resistant *ilvBH* (*fbr ilvBH*) from *R. eutropha* H16 on multiple-copy plasmid caused growth retardation, while the wild type *ilvBH* has no toxicity effect. (B) Plasmid map showing the *alsS* under the control of $P_{rrsC\text{-}O1\text{-}O1}$ promoter in combination $P_{phaC1\text{-}G3}$*::tetR*. (C) With no inducer added, the aTc-controllable gene expression system was repressed, and the AlsS toxicity was alleviated. Error bars stand for the standard deviation of 3 independent repeats. (n = 3) The "empty" data set was obtained using *R. eutropha* H16 transformed with a plasmid that only contains $P_{phaC1\text{-}G3}$*::tetR* cassette.

H16.[9,18] Using β-galactosidase reporter assays, we showed that P_{phaC1}*::tetR* cassette indeed lowered the basal level expression activity of the $P_{rrsC\text{-}O1\text{-}O1}$ promoter (Figure 2C). However, the strength of the resulting system plateaued at a very low level even with high concentration of aTc (160 ng/mL) (Figure 2C), which suggested an overly tight repression caused by the high *tetR* level.

As shown above, the P_{cat} and P_{phaC1} promoters delivered too low or too high TetR level to match with $P_{rrsC\text{-}O1\text{-}O1}$ promoter, respectively. Therefore, it might be helpful to fine tone the TetR level with promoters of intermediate strength.[19] The *R. eutropha* H16 P_{phaC1} promoter has a −35 region sequence that is identical to the −35 sequence of the *E. coli* consensus σ70 promoters (5′-TTGACA-3′) (Figure 3A). Previous studies have shown that *R. eutropha* H16 promoters with higher similarity to *E. coli* σ70 promoter consensus sequence tend to have higher activities.[20] Thus, mutations in the −35 region of P_{phaC1} were hypothesized to generate promoters of lower activity. We constructed a promoter library by mutating the last four nucleotides of the −35 region in P_{phaC1} promoter (Figure 3A). The promoter library was used to drive the green fluorescent protein (GFP) reporter gene, which enabled the high-throughput screening of promoter activity *in vivo* (Figure 3A). A total of 300 candidates from the library were screened (Figure 3B). More than 50% of the candidates showed severely reduced activity (<10% compared to the wild type promoter) as measured by GFP level. ∼20% of the candidates have slightly reduced activity (100−70%). And the candidates with intermediate activities (70−10%) were relatively hard to obtain. This all-or-none distribution of the promoter activities suggested that promoter activity is very sensitive to changes in the −35 region. Eight P_{phaC1} mutants were characterized, which have stepwise reduced activities compared to the wild type promoter (Figure 3C). Promoters $P_{phaC1\text{-}G1}$ (with −35 sequence of 5′-TTGACT-3′) and $P_{phaC1\text{-}G3}$ (with the −35 sequence of 5′-TTCGGC-3′) have ∼60 and ∼15% activity compared to the wild type P_{phaC1}, respectively. These two promoters have strength between that of P_{cat} and P_{phaC1} and were tested to drive the expression of *tetR*.

The induction profiles of the gene expression system containing $P_{phaC1\text{-}G1}$*::tetR* or $P_{phaC1\text{-}G3}$*::tetR* in combination with $P_{rrsC\text{-}O1\text{-}O1}$*::CAT* are shown in Figure 3D. These two systems indeed had significantly lower leaky expression compared to the system with P_{cat}*::tetR*. The Ind50 (inducer concentration needed to yield 50% induction) of the three systems were Ind50(P_{cat}) < Ind50($P_{phaC1\text{-}G3}$) < Ind50($P_{phaC1\text{-}G1}$) (Figure 3D, indicated by dash lines), which corresponds to the levels of *tetR* expression in these systems. The system with the best dynamic profile contains $P_{phaC1\text{-}G3}$*::tetR* in combination with the $P_{rrsC\text{-}O1\text{-}O1}$, which has relatively low leaky expression and an 11-fold induction. The dynamic range is comparable with the L-arabinose controllable P_{BAD} system (∼12 fold induction)[9] in *R. eutropha* H16, which has been the only non-native controllable gene expression system available in this organism.

We next sought to demonstrate the application of the synthetic aTc-controllable gene expression system. In our previous work, production of biofuels isobutanol and 3-methyl-1-butanol has been achieved autotrophically in engineered *R. eutropha* H16.[4] However, one of the key enzymes in the biofuel production pathway, the acetolactate synthase (encoded by *alsS* from *Bacillus subtilis*), caused toxicity when overexpressed in *R. eutropha* H16 especially in minimal medium (Figure 4A). As a result, the biofuel production strain reported previously can only have one copy of *alsS* integrated in the chromosome,[4] since strains with high level *alsS* overexpression on a multiple-copy plasmid grow extremely poorly and are not viable in autotrophic condition. AlsS catalyzes the formation of acetolactate from pyruvate, which can also be catalyzed by the acetohydroxy acid synthase (AHAS) encoded by *ilvBH* in *R. eutropha* H16. Although ilvBH did not cause significant growth retardation (Figure 4A), much less biofuel was produced when ilvBH was used in place of AlsS (Supporting Information, Figure S1). Thus, a dilemma exists that on one hand, AlsS cannot be replaced by ilvBH and needs to be highly overexpressed to achieve high biofuel production, and on the other hand, AlsS's toxicity effect needs to be overcome.

It is well-known that AHAS enzymes such as ilvBH catalyze the first comitted step of the branched-chain amino acid biosynthesis pathways and are subjected to feedback inhibition *in vivo* by downstream metabolites such as valine and leucine. On the other hand, the AlsS in *B. subtilis* functions in the acetoin fermentation pathway and is not feedback regulated. Therefore, one hypothesis is that the unregulated activity of AlsS caused imbalance of the metabolic pathways *in vivo*. To test this hypothesis, feedback-resistant *R. eutropha* H16 ilvBH (fbr ilvBH) was constructed by mutating the allosteric regulator binding site in the ilvH protein (Supporting Information,

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Figure S2). Consistent with the hypothesis, the fbr ilvBH also caused growth retardation (Figure 4A). These results indicate that the activity of AlsS needs to be delivered in a controllable manner. In fact, the expression of the *alsS* operon is tightly controlled in its native host *B. subtilis* on transcriptional level by an inducible promoter.[21]

We placed the *B. subtilis alsS* under the control of the $P_{rrsC\text{-}O1\text{-}O1}$ promoter on a multiple-copy plasmid, which also contains the $P_{phaC1\text{-}G3}$*::tetR* repressor cassette (Figure 4B). When the strain is cultivated in minimal medium with the inducer aTc, growth retardation was observed (Figure 4C), suggesting that *alsS* was expressed with relatively high level. When inducer was not added, the cell reached higher OD compared to the induced condition (Figure 4C), suggesting that the expression level was repressed and the toxicity effect was alleviated.

The dynamic range of the aTc-controllable system developed in this study is not optimal compared to similar systems in *E. coli*[12] or *Synechocystis sp.*,[22] which is largely due to the relatively high leaky expression. As the "empty" data set in Figure 4C was obtained using *R. eutropha* H16 transformed with a plasmid that only contains $P_{phaC1\text{-}G3}$*::tetR* cassette, the growth difference between "empty" and "no inducer" was not likely because of the potential stress caused by tetR overexpression. Instead, the leaky expression may be responsible for the incomplete rescue of AlsS toxicity (Figure 4C). In this system, the relatively strong promoter P_{rrsC} was chosen as the backbone with the rationale that it may deliver high expression when fully induced, which is especially useful for metabolic engineering applications. The idea is that a controllable gene expression system will repress the expression of the toxic genes such as *alsS* during lag phase and early log phase and thus enable the accumulation of cell mass to certain level. Subsequently, with a strong promoter as the backbone of the controllable promoter, the toxic enzymes can be expressed with relatively high level upon induction, which can lead to product formation by the synthetic metabolic pathway with a high rate during late log and stationary phase. However, strong promoters may also be difficult to repress completely. In this study, we improved the stringency of the system by introducing two *tetO* operators to P_{rrsC} and tuning the *tetR* expression level. Previous studies have shown that small changes in the promoters involving only a few nucleotides can cause critical changes in the regulation profile of the system.[22] Further optimizations are possible by systematically mutagenizing other regions in both the working promoter and the promoter driving *tetR*.

Controllable gene expression systems, especially the non-native ones, are extremely useful in metabolic engineering studies. In the case of the AlsS toxicity issue, high level isobutanol production has been achieved with the inducible P_{LlacO1} system in recombinant *E. coli*,[23] while in recombinant *Clostridium cellulolyticum*, constitutive *alsS* expression inhibited growth and hampered biofuel production.[24] In this study, AlsS toxicity was alleviated using the developed aTc-controllable system. Further studies are needed to test the biofuel production performance using this system in *R. eutropha* H16.

In conclusion, we developed an aTc-controllable gene expression system in *R. eutropha* H16 that can be gradually regulated with different aTc concentration with an ~11 fold dynamic range. A TetR repressable promoter was first constructed by hybridizing the *tetO* operator with the *R. eutropha* H16 P_{rrsC} promoter. The regulation profile of the system was then improved by fine-tuning the expression level of the repressor TetR using suitable mutant promoters of P_{phaC1}, which were identified from a high-throughput promoter library screening. The AlsS toxicity issue, which impeded our metabolic engineering work on isobutanol production, was alleviated using the developed system. This aTc-controllable gene expression system is a useful synthetic biology tool for future scientific research and metabolic engineering in *R. eutropha* H16.

METHODS

Chemicals and Reagents. All chemicals were purchased from Sigma-Aldrich (St. Louis, MO) or Fisher Scientifics (Pittsburgh, PA). Restriction enzymes were purchased from New England BioLabs (Ipswich, MA). Oligonucleotides were purchased from IDT (San Diego, CA). The Rapid DNA ligation kit was from Roche (Mannheim, Germany). KOD DNA polymerase was from EMD Chemicals (San Diego, CA).

Strains and Culture Condition. *Ralstonia eutropha* H16 strain was purchased from American Type Culture Collection (ATCC). *R. eutropha* strains were regularly cultured in rich medium (16g/L of nutrient broth, 10g/L of yeast extract, 5g/L of $(NH_4)_2SO_4$) at 30 °C. If the strains contain plasmids, 200 mg/L of kanamycin was added. All promoter testing was performed using the wild type strain. Transformation of the plasmids to *R. eutropha* H16 was done using the previously described method.[4]

All cloning and plasmid preparation were done using *E. coli* XL1-blue cells (Stratagene, La Jolla, CA). *E. coli* cells with plasmids were cultured in LB medium containing 50 mg/L of kanamycin.

Characterization of the Induction Profile of the Controllable Gene Expression System. The plasmid containing the controllable gene expression system was transformed into *R. eutropha* H16. Single colonies were picked from transformation and inoculated into rich medium with 200 mg/L of kanamycin and cultured overnight. The overnight culture was reinoculated into 40 mL of rich medium with 200 mg/L of kanamycin in shake flask to mid log phase. Then the culture was aliquoted into 4 mL each in test tubes and induced with different concentration of aTc. After ~6 h, the cells were harvested and assayed for reporter gene activity. The CAT reporter assay was performed as follows: cell lysate was prepared from each sample using Qiagen Tissuelyser II in 100 mM Tris-HCl pH = 8.0. The CAT assay system contains 100 mM Tris-HCl pH = 8.0, 0.1 mM Acetyl-CoA, 1 mM 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB), 0.25 mM Chloramphenicol, and appropriate amount of cell lysate. The absorbance at 412 nm was monitored. Reactions with no Chloramphenicol served as blank. The β-galactosidase was performed using previously described method.[4]

High-Throughput Screening of the Promoter Library. The DNA containing promoter library (pLH309) was transformed into *R. eutropha* H16. Single colonies were picked and inoculated in 96-well culture plates, which contained 300 μL of rich medium with 200 mg/L of kanamycin in each well. The plates were sealed with porous paper covers and incubated for 24 h. The culture was then diluted by 5 fold before being assayed for fluorescence level in 96-well plate (excitation: 485 nm, emission: 510 nm, cutoff: 495 nm). The fluorescence was normalized by cell density as measured by OD600 nm.

AlsS Toxicity Test. The strains harboring *alsS* or *ilvBH* were cultivated in rich medium overnight. The cells were harvested and then resuspended in German minimal medium[25]

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containing 4g/L of fructose and 200 mg/L of kanamycin with the initial OD600 nm of ~0.01. After ~24 h, the OD600 nm was measured. To test the performance of the developed controllable gene expression system for alleviating AlsS toxicity, a similar method was used, except the 200 ng/mL of aTc was used with the "with inducer" samples.

■ ASSOCIATED CONTENT

Supporting Information

The plasmids and primers used in this study (Tables S1 and S2). Comparison of *R. eutropha* H16 *ilvBH* and *B. subtilis alsS* for biofuel production (Figure S1). Construction and characterization of the feedback resistant ilvBH (Figure S2). This material is available free of charge *via* the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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