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Synthetic Chemical Inducers and Genetic Decoupling Enable Orthogonal Control of the *rhaBAD* Promoter

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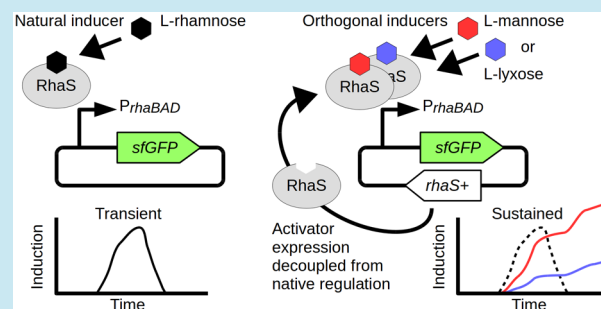
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Supporting Information

ABSTRACT: External control of gene expression is crucial in synthetic biology and biotechnology research and applications, and is commonly achieved using inducible promoter systems. The *E. coli* rhamnose-inducible *rhaBAD* promoter has properties superior to more commonly used inducible expression systems, but is marred by transient expression caused by degradation of the native inducer, L-rhamnose. To address this problem, 35 analogues of L-rhamnose were screened for induction of the *rhaBAD* promoter, but no strong inducers were identified. In the native configuration, an inducer must bind and activate two transcriptional activators, RhaR and RhaS. Therefore, the expression system was reconfigured to decouple the *rhaBAD* promoter from the native *rhaSR* regulatory cascade so that candidate inducers need only activate the terminal transcription factor RhaS. Rescreening the 35 compounds using the modified *rhaBAD* expression system revealed several promising inducers. These were characterized further to determine the strength, kinetics, and concentration-dependence of induction; whether the inducer was used as a carbon source by *E. coli*; and the modality (distribution) of induction among populations of cells. L-Mannose was found to be the most useful orthogonal inducer, providing an even greater range of induction than the native inducer L-rhamnose, and crucially, allowing sustained induction instead of transient induction. These findings address the key limitation of the *rhaBAD* expression system and suggest it may now be the most suitable system for many applications.

KEYWORDS: *rhaBAD*, inducible promoter, expression system, rhamnose, mannose, nonmetabolized inducer



Experimental control of gene expression is a crucial requirement in synthetic biology and biotechnology research and applications and in many studies of natural biological systems. Inducible promoter systems are commonly used to achieve this external control through initiation of transcription. Promoters have been identified that respond to a diverse array of stimuli, including sugars,^{1–3} antibiotics,⁴ metals,^{5,6} quorum sensing signaling molecules,^{7–9} temperature,¹⁰ and many others. The L-rhamnose-inducible promoter of the *rhaBAD* operon (P_{rhaBAD}) of *Escherichia coli* (*E. coli*) has been used extensively to control expression of many heterologous genes in *E. coli* and other bacteria.^{11–20} P_{rhaBAD} is a member of the AraC-XylS family of positively regulated expression systems^{21,22} and has several advantages over other

inducible promoter systems such as the P_{tet} (tetracycline), P_{lac} (lactose) or P_{araBAD} (arabinose) inducible systems and their derivatives. P_{rhaBAD} is capable of very high levels of expression,²³ but unlike P_{lac} , P_{araBAD} , or P_{tet} displays undetectable baseline gene expression in the absence of inducer.^{17,24} This level of control is useful generally, but particularly for the expression of genes encoding toxic products, where “leaky” expression can lead to cellular toxicity, difficulties in generating expression constructs, and selective pressure for mutation of the expressed

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gene.¹⁶ Expression from the *rhaBAD* promoter is linear with respect to inducer concentration, in contrast to the “all or nothing” response of the P_{lac} and P_{araBAD} systems.^{16,23} L-Rhamnose, a naturally occurring deoxy-hexose, is nontoxic to bacterial cells, unlike the inducers of some other systems such as tetracycline, the lactose analogue isopropyl β -D-1-thiogalactopyranoside (IPTG)^{23,25–27} and in certain cases, arabinose.²⁸

In *E. coli* the genes encoding the enzymes required for L-rhamnose metabolism (*rhaBAD*) and the regulatory proteins required for controlled expression of these genes (*rhaSR*) are found in two divergent operons.^{24,29,30} The gene encoding the rhamnose-proton symporter (*rhaT*) is found at another locus. Expression of *rhaBAD* and *rhaT* both result from a regulatory cascade.³¹ Transcription of *rhaS* and *rhaR* is driven by the AraC/XylS family transcriptional activator RhaR, which is constitutively expressed at a low basal level.¹⁷ The binding of L-rhamnose to RhaR results in autoinduction of *rhaSR* expression to high levels.^{24,31,32} In the presence of L-rhamnose, RhaS binds upstream of the RNA polymerase-binding sites of *rhaBAD*²⁴ and *rhaT*,³³ leading to high levels of transcription of the downstream genes.³⁴ Expression of these genes is subject to catabolite repression, with a CRP-binding site located upstream of the RhaS-binding site.³⁴ When bound to L-rhamnose, RhaS also negatively autoregulates its own expression.³⁵

In the absence of a preferred carbon source, L-rhamnose is readily metabolized by *E. coli* in both aerobic and anaerobic conditions.³⁶ The mechanism of L-rhamnose catabolism is well studied and involves uptake of L-rhamnose into the cell by RhaT, an L-rhamnose:proton symporter,³⁷ isomerization by L-rhamnose isomerase (RhaA) to L-rhamnulose,^{29,38} phosphorylation of L-rhamnulose by L-rhamnulose kinase (RhaB),^{39,40} and conversion of the resulting L-rhamnulose-1-phosphate to L-lactaldehyde and dihydroxyacetone phosphate (DHAP) by rhamnulose-1-phosphate aldolase (RhaD).⁴¹ L-Lactaldehyde can be metabolized further by an assimilatory aerobic pathway, to pyruvate *via* lactate, or an anaerobic pathway, to propane-1-2-diol.^{42,43} This efficient metabolism of L-rhamnose gives rise to the one major limitation of *rhaBAD* as an expression system: a decrease in inducer concentration over time, which causes expression to decrease and ultimately to cease.¹⁷

The problem of transient expression caused by inducer degradation has been solved for some other promoter systems by using nonmetabolized inducer analogues, which can be considered effectively orthogonal to native physiology. Commonly used examples include the lactose analogue IPTG^{44,45} and the tetracycline analogue anhydrotetracycline (ATC).⁴⁶ Such inducer analogues have proven widely useful in diverse fields of study and application. To date, no such analogue of L-rhamnose has been described for the *rhaBAD* expression system.

An ideal inducer of *rhaBAD* would have similar useful properties to L-rhamnose (no or low toxicity, readily soluble, and readily taken up by cells) and would cause the *rhaBAD* expression system to respond in a similar way (a linear response to inducer concentration between very low basal expression and high maximal expression) but unlike L-rhamnose, would persist in the cell, resulting in sustained rather than transient induction of the *rhaBAD* promoter. Such a combination of properties might be found in a structural analogue sufficiently similar to L-rhamnose to be recognized by RhaS and RhaR, but which is a poor substrate for native L-rhamnose metabolism. This study aimed to identify such an inducer of *rhaBAD* by screening 35 structural analogues of L-rhamnose. These candidates were

screened, the genetic architecture of the *rhaBAD* expression system was simplified to improve its inducer compatibility, and the induction characteristics of the most promising candidates were studied in detail. Several rare sugars caused substantial induction of *rhaBAD*. L-Mannose in particular was found to be an excellent, metabolically orthogonal inducer of the *rhaBAD* expression system, superior to the natural inducer L-rhamnose.

RESULTS

Construction and Validation of a *rhaBAD* Reporter Construct. To evaluate the ability of candidate compounds to induce the *rhaBAD* promoter, an assay system was required. To this end, we constructed a reporter plasmid (pCK301) containing a transcriptional fusion between the *rhaBAD* promoter of *E. coli* MG1655 and a gene encoding the highly stable and widely used “superfolder” variant of green fluorescent protein, sfGFP.⁴⁷ In this arrangement, fluorescence depends upon transcription from the *rhaBAD* promoter, so the effects of candidate inducers can be readily determined. A similar plasmid lacking the reporter, pCK300, was constructed as a control. *E. coli* MG1655 was used as the host for induction assays, as it has been shown that the levels of RhaS and RhaR expressed from the native *rhaSR* operon in this strain are sufficient for full induction by the native inducer L-rhamnose,⁴⁸ and because this fully sequenced strain contains no mutations in the *rhaSR*, *rhaBAD*, or *rhaT* operons relevant to this study. Before using this assay to evaluate untested candidate compounds, we first validated the reporter plasmid pCK301 using the native inducer L-rhamnose. This allows us to compare the response of the *rhaBAD* promoter of pCK301 to previously reported data. To demonstrate induction by L-rhamnose, and to assess the dependence of the response on L-rhamnose concentration, *E. coli* MG1655 cells containing pCK301 were cultured in complex medium (LB) supplemented with various concentrations of L-rhamnose for 16 h, and then fluorescence (normalized to cell density) was determined as a measure of the sfGFP protein concentration (Figure 1). The fluorescence at the end of induction showed a highly linear relationship with L-rhamnose concentration (Figure 1; R^2 value = 0.97). Fluorescence in the absence of inducer was identical to the negative control plasmid pCK300 (no statistically significant difference; $P > 0.05$), indicating very low basal expression. The linear concentration dependence and very low basal expression are both in excellent agreement with published data,^{16,23} which validates pCK301 as an appropriate system to test candidate inducer compounds.

Screening Analogues of L-Rhamnose as Inducers of the *rhaBAD* Promoter System. Thirty-five sugars structurally related to L-rhamnose (Figure 2) were investigated as potential inducers of the *rhaBAD* promoter system: (i) the four epimers (having one different chiral center from rhamnose) and three other diastereomers of rhamnose (changes of stereochemistry from rhamnose shown in red); (ii) a series of eight sugars with the same stereochemistry at C2, C3, and C4 as that of L-rhamnose (shown in purple); (iii) all eight isomeric pentoses and four pentitols (shown in green); and (iv) nine sugars in which the C2 OH group of L-rhamnose is replaced by other groups (shown in blue). Accordingly these cannot be isomerized by RhaA.

The series of 35 sugar mimics of L-rhamnose were screened for activity as inducers of the *rhaBAD* promoter, using the validated sfGFP reporter plasmid pCK301 and the end point fluorescence assay described above. To maximize sensitivity and

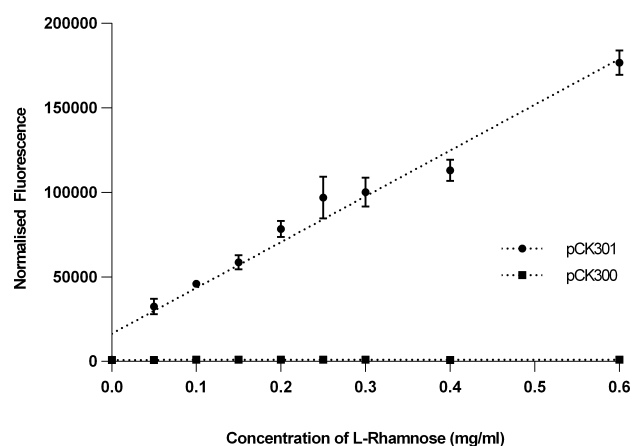


Figure 1. Validation of the P_{rhaBAD} reporter plasmid pCK301 by determining fluorescence response to *L*-rhamnose inducer concentration in complex media. *E. coli* MG1655 cells were transformed with either pCK301 (P_{rhaBAD} -*sfGFP* transcriptional reporter) or the control plasmid pCK300 (lacking P_{rhaBAD} and *sfGFP*), grown in 500 μ L LB media cultures supplemented with different concentrations of *L*-rhamnose. Optical density at 600 nm and GFP fluorescence (excitation 488 nm; emission 530 nm) were measured after 16 h. Lines shown represent a linear regression of each plot of normalized fluorescence (GFP/OD_{600 nm}). A linear dose response to *L*-rhamnose is observed for pCK301 samples (R^2 value: 0.97). Error bars shown are the standard deviation of three independent biological replicates.

allow detection of even weak induction, the compounds were used at a high concentration of 1 mg/mL. Preliminary experiments revealed that most of the compounds were completely inactive as inducers of the *rhaBAD* promoter in this assay system. However, several compounds did induce expression, as shown in Figure 3, which also includes *L*-rhamnose for comparison. None of the analogues resulted in induction to the same level as *L*-rhamnose. However, four sugars showed potentially useful induction of the system after 16 h of growth in complex media: two epimers at C3 and C5 of rhamnose (6-deoxy-*L*-altrose and 6-deoxy-*D*-gulose respectively); and *L*-mannose and *L*-xylose in which the C6 methyl group of rhamnose is replaced by CH_2OH and H, respectively.

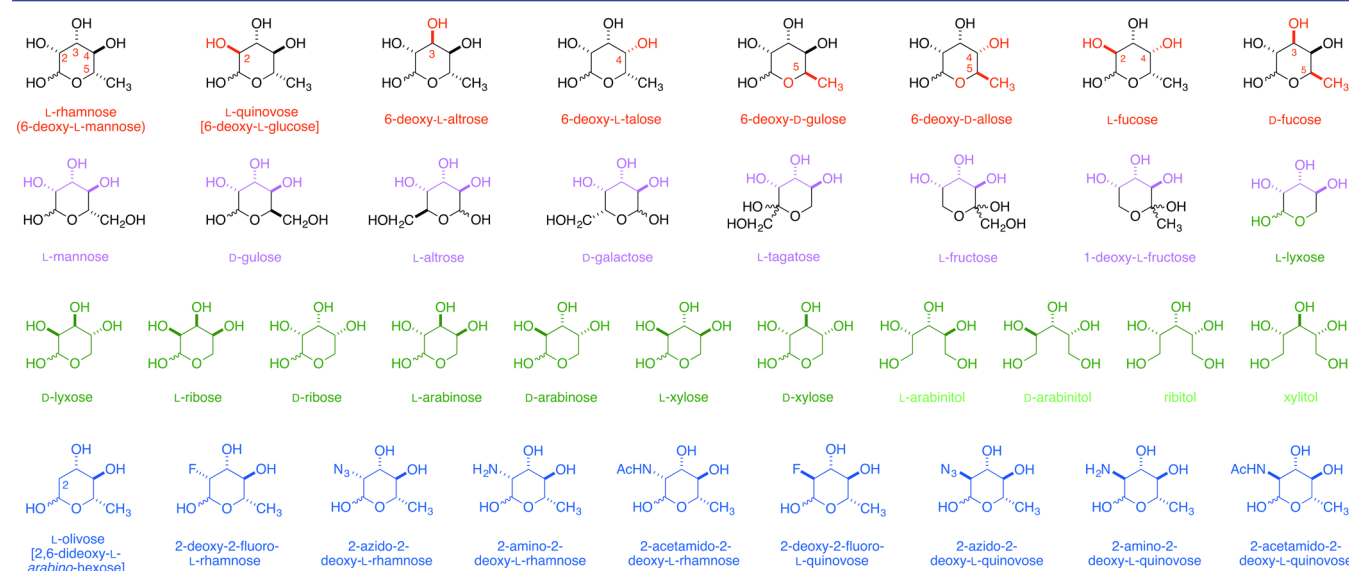


Figure 2. Structural analogues of *L*-rhamnose, as described in the text.

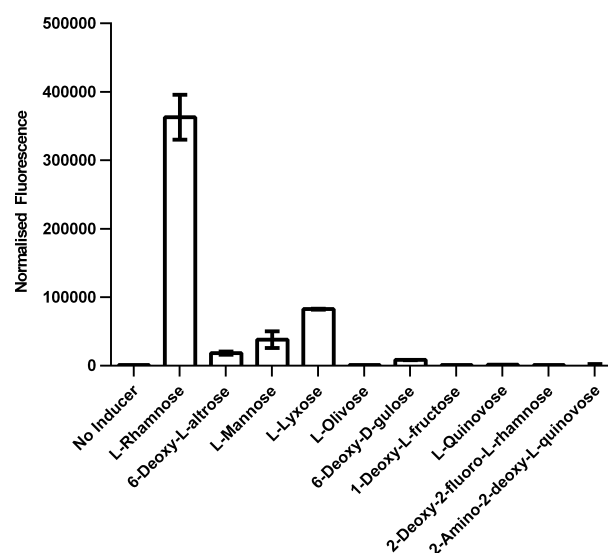


Figure 3. Initial screen of rhamnose analogues as inducers of the *rhaBAD* promoter system. Rhamnose analogues were screened for their ability to induce expression of the fluorescent reporter. *E. coli* MG1655 cells were transformed with pCK301, cultured for 16 h at 37 °C in LB media with each inducer at a final concentration of 1 mg/mL. Optical density (600 nm) and GFP fluorescence was measured at the end of the experiment. Error bars shown are the standard deviation of three independent biological replicates. Statistical significance was determined using a one-way ANOVA, followed by a Tukey's multiple comparison test assuming unequal variance. Analogues which caused no induction (zero fluorescence values) are not shown, except those compared to results later in the study.

Decoupling the *rhaBAD* Promoter from the *rhaSR* Regulatory Cascade Reveals Effective Inducers.

In the native regulatory cascade, *L*-rhamnose binds both to RhaR, inducing expression of the *rhaSR* operon, and to RhaS, inducing expression of the *rhaBAD* operon. To be an effective inducer of the native system, a compound must act *via* both RhaR and RhaS appropriately. These two proteins share only 36% identity and 54% similarity with each other, and *L*-rhamnose binding to the N-terminal domains results in

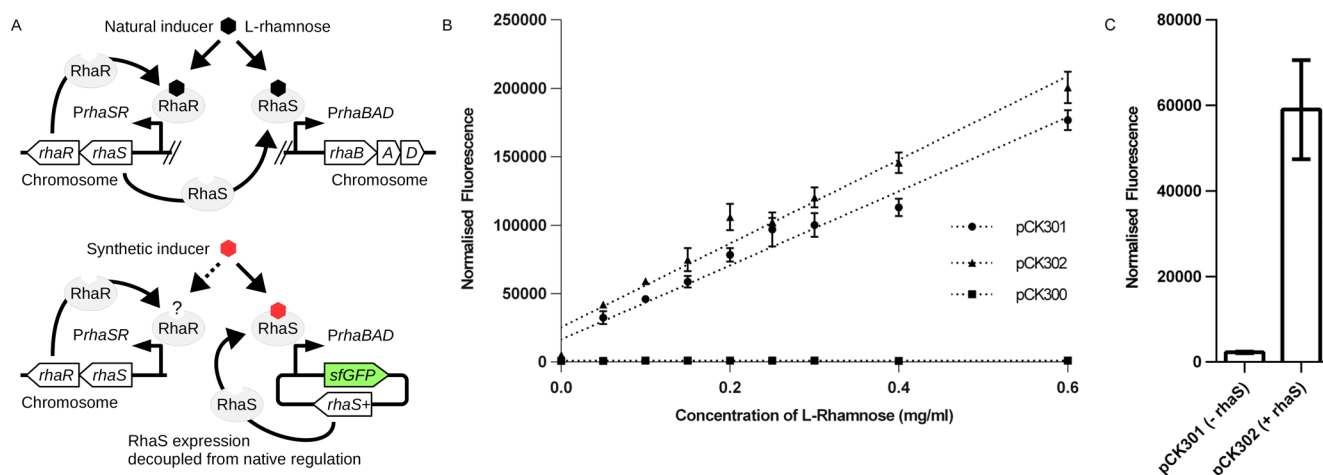


Figure 4. Design and evaluation of improved, *rhaS*-encoding reporter plasmid, pCK302. (a) Design of pCK302. It was hypothesized that weak induction of P_{rhaBAD} resulted from poor *rhaS* expression, which in turn was due to suboptimum interaction of candidate inducers with RhaR. A new reporter plasmid, pCK302, was thus constructed that expresses the MG1655 *rhaS* gene constitutively (*rhaS+*), insulating it from the native *rhaSR* regulatory cascade. (b) Induction response of the *rhaBAD* promoter to L-rhamnose concentration using the *rhaS*-expressing reporter plasmid pCK302. *E. coli* MG1655 cells were transformed with either pCK302 (+*rhaS*), pCK301 (*-rhaS*) or the control plasmid pCK300 (lacking P_{rhaBAD} and *sfGFP*), and transformants cultured for 16 h at 37 °C in LB medium supplemented with different concentrations of L-rhamnose. Optical density (600 nm) and GFP fluorescence were measured at the end of the experiment. Lines shown represent a linear regression of each plot of normalized fluorescence ($GFP/OD_{600\text{ nm}}$). pCK302 samples demonstrate a linear dose–response to L-rhamnose with an R^2 value of 0.97, identical to pCK301 samples. Error bars shown are the standard deviation of three independent biological replicates. (c) Relief of catabolite repression through the constitutive expression of *rhaS* on pCK302. *E. coli* MG1655 cells were transformed with either pCK301 (*-rhaS*) or pCK302 (+*rhaS*) and grown at 37 °C in M9 minimal media supplemented with 0.2% (w/v) glucose and 0.4 mg/mL of L-rhamnose. Fluorescence and optical density and measurements were taken after 16 h. Fluorescence normalized to ($GFP/OD_{600\text{ nm}}$) is shown. The pCK302 samples were 25-fold more fluorescent than pCK301 samples when induced with L-rhamnose ($P < 0.05$; statistical significance was determined using an unpaired *t* test). No fluorescence was observed in the absence of L-rhamnose. Error bars shown are the standard deviation of three independent biological replicates.

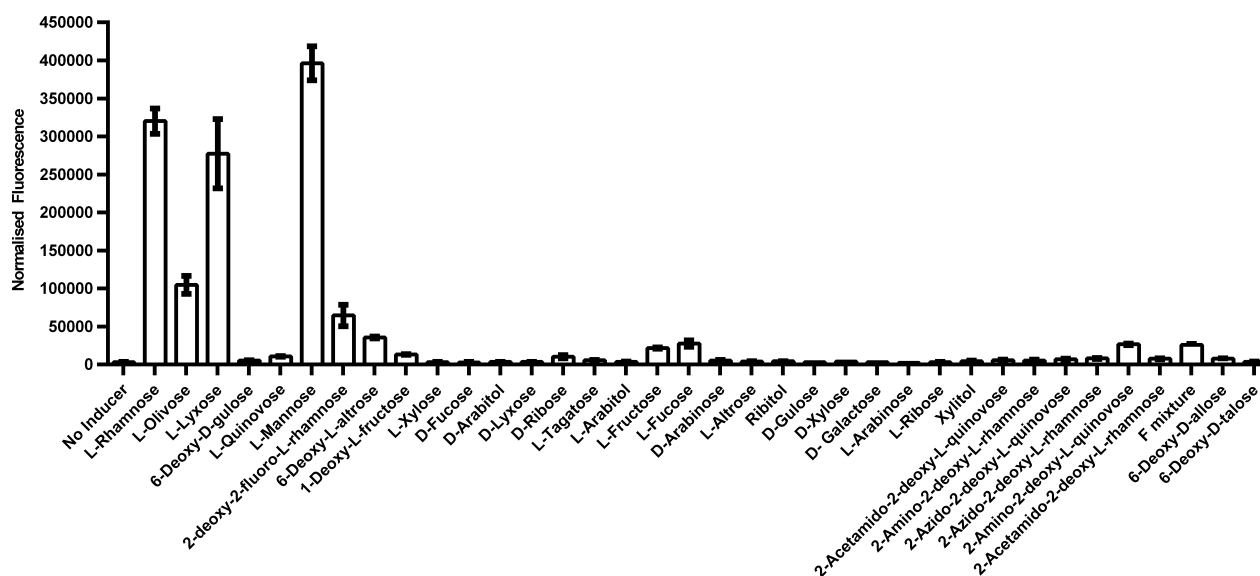


Figure 5. Screen of rhamnose analogues as inducers of the modified *rhaBAD* system expressing *rhaS* constitutively. All previously tested rhamnose analogues were screened again using the new *rhaS*-expressing reporter plasmid pCK302. *E. coli* MG1655 cells were transformed with pCK302 and transformants cultured for 16 h at 37 °C in LB media supplemented with the analogue to be tested at a final concentration of 1 mg/mL. Optical density (600 nm) and fluorescence were measured at the end of experiment. Error bars shown are the standard deviation of three independent biological replicates. Statistical significance was determined using a one-way ANOVA, followed by a Tukey's multiple comparison test assuming unequal variance. L-Rhamnose and five analogues showed statistically significant induction using this improved reporter plasmid.

conformational changes that affect different helix–turn–helix motifs (HTH) in the DNA-binding, C-terminal domains of each protein.⁴⁹ For these reasons, it is quite feasible that a compound might properly bind and induce the correct conformational change in RhaS, but not in RhaR, or *vice versa*. Such compounds would be expected to give weak or

negative results in our initial screen described above, which depended upon the native *rhaSR* regulatory cascade. However, it would be useful to identify compounds that bind well to RhaS, even if they bind poorly to RhaR, as these could be effective inducers of the *rhaBAD* promoter in synthetic systems that do not depend upon the native regulatory cascade for

production of RhaS (Figure 4A).⁵⁰ To investigate this, we implemented such a synthetic system by constructing a modified reporter plasmid pCK302 (Figure 4A) containing a constitutively expressed copy of the *rhaS* gene (referred to herein as *rhaS+*). This effectively decouples induction of the *rhaBAD* promoter from dependence on induction of *rhaSR* by RhaR.³⁴

The new decoupled reporter plasmid pCK302 (including *rhaS+*) was validated using the end-point fluorescence induction assay with various concentrations of L-rhamnose, as before (Figure 4B). *E. coli* MG1655 cells containing pCK302 performed very similarly to those containing pCK301, showing the desired linear relationship between L-rhamnose concentration and fluorescence of sfGFP ($R^2 = 0.97$) and a barely detectable basal level of fluorescence in the absence of inducer (Figure 4B). This confirmed that the reporter system remained a suitable assay following the addition of *rhaS+*, so the decoupled reporter plasmid pCK302 was used for all subsequent reporter experiments. Interestingly, expression of *rhaS+* from pCK302 also substantially relieved catabolite repression of the *rhaBAD* reporter. *E. coli* MG1655 cells containing pCK302 (*rhaS+*) cultured overnight in LB medium supplemented with both 0.4 mg/mL L-rhamnose and 0.2% D-glucose showed 25-fold higher fluorescence than equivalent cultures of cells containing pCK301, which lacks *rhaS* (Figure 4C, fluorescence statistically significantly different, $P < 0.05$). This will allow the *rhaBAD* promoter to be used in combination with glucose, which will be useful particularly in situations in which use of noncatabolite-repressing substrates such as glycerol is complicated or unsuitable, such as in *E. coli* anaerobic fermentation, which is an important metabolic mode for chemical production.

Using the decoupled reporter plasmid pCK302, all 35 analogues of L-rhamnose were screened again for activity as inducers of the *rhaBAD* promoter as before. In this second screen (Figure 5), we observed induction of sfGFP expression by several additional compounds that did not induce expression in the previous screen using pCK301 (Figure 3), including the non-natural sugars L-olivose and 2-deoxy-2-fluoro-L-rhamnose. All of the compounds identified as modest inducers in the previous screen (Figure 3) showed greater induction in this second screen (Figure 5). Among these, L-lyxose induced fluorescence to a level almost equal to that of L-rhamnose, whereas L-mannose actually induced fluorescence to a significantly greater level than the natural inducer L-rhamnose ($P < 0.05$). This suggested that, at least in this assay system, either induction of the *rhaBAD* promoter is more sensitive to L-mannose than the natural inducer L-rhamnose, or that there are differences in the kinetics of induction by these two compounds, or both.

Characterization and Comparison of Promising Inducers. We chose the five strongest analogue inducers from the second end-point fluorescence screen (Figure 5) for further characterization as candidate orthogonal inducers. These promising compounds were L-mannose, L-lyxose, L-olivose, 2-deoxy-2-fluoro-L-rhamnose and 6-deoxy-L-altrose. First, the concentration dependence of induction by each of these analogues was determined using the same range of concentrations tested previously for L-rhamnose (Figure 6A). An additional, lower range of concentrations was also included for L-mannose to determine the concentration dependence of this analogue at the lower fluorescence levels reached with the other analogues (Figure 6A, inset). All analogues showed a

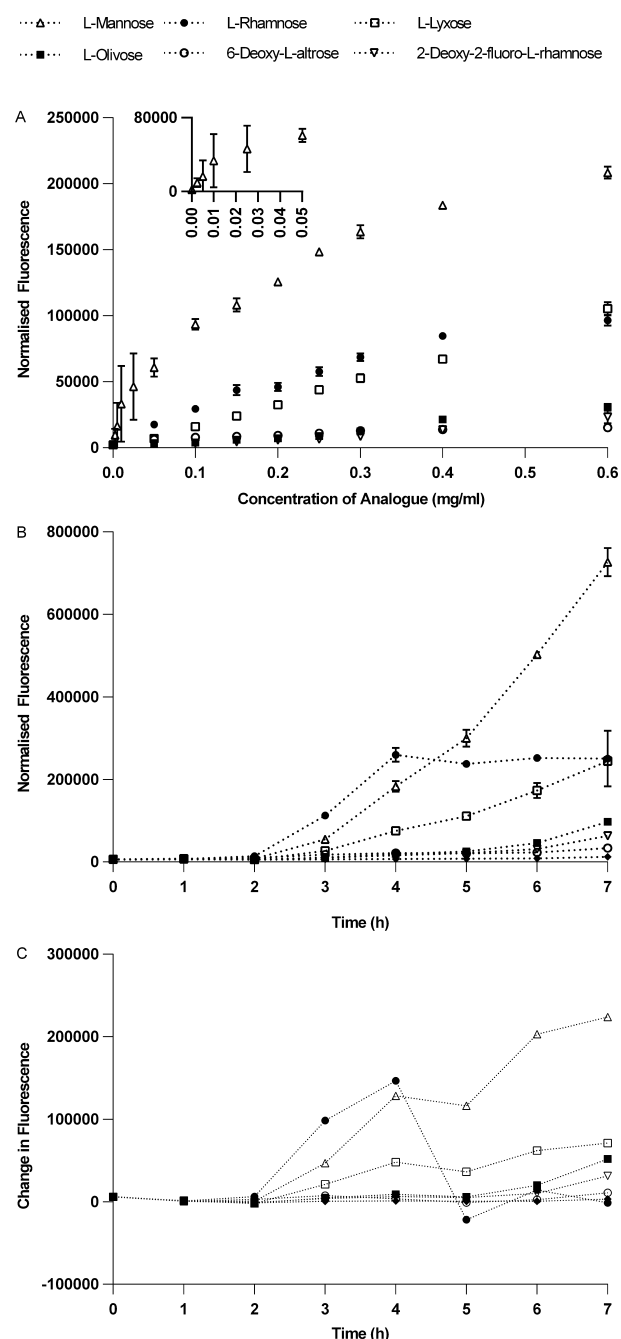


Figure 6. Characterization of the most promising analogue inducers: response to inducer concentration and induction kinetics. (a) Response of the *rhaBAD* promoter to analogue inducer concentrations. The five analogues showing statistically significant induction (L-mannose, L-lyxose, L-olivose, 6-deoxy-L-altrose, and 2-deoxy-2-fluoro-L-rhamnose) were added to 500 μ L cultures of MG1655 containing pCK302 to final concentrations of 0.05–0.6 mg/mL and optical density and fluorescence measured after 16 h at 37 $^{\circ}$ C. The dose response of L-mannose was further characterized at lower concentrations (inset). Error bars shown are standard deviation of three independent biological replicates. (b) Kinetics of *rhaBAD* promoter induction by novel analogue inducers. *E. coli* MG1655 cells transformed with pCK302 were grown in 500 μ L of LB media cultures supplemented with 0.4 mg/mL of the five analogue inducers for 7 h at 37 $^{\circ}$ C and optical density and fluorescence measurements taken every hour. Error bars shown are the standard deviation of three independent biological replicates. (c) Change in mean fluorescence over time as a measure of the rate of sfGFP synthesis.

similar linear response to concentration as L-rhamnose (Figure 6A, R^2 values of linear regression: 0.86–0.99).

Next, the kinetics of induction of each of the five promising analogues was studied by monitoring the fluorescence of cultures (of *E. coli* MG1655 cells containing pCK302 in LB medium, as before) containing 0.4 mg/mL of inducer over the course of 7 h (Figure 6B). Use of sfGFP in this study allows additional interpretation of the kinetic data, as sfGFP is very stable,⁴⁷ so it is a reasonable approximation to treat degradation of the sfGFP protein during the course of this experiment as negligible. Therefore, fluorescence at a given time is a measure of the cumulative total of sfGFP protein produced by that time (Figure 6B), and hence the change in fluorescence per unit time (that is, the first derivative of fluorescence with respect to time) is a measure of the rate of sfGFP production (Figure 6C) due to transcription from the *rhaBAD* promoter of pCK302. The kinetics of induction of the *rhaBAD* promoter by the native inducer L-rhamnose differed qualitatively in two ways from the kinetics of induction of all the other inducers (Figure 6C). First, induction by L-rhamnose occurred slightly earlier than the other analogues, being detectable after 2 h ($P < 0.05$), and already approaching its maximum rate by 3 h. Second and more importantly, induction by L-rhamnose was transient, giving no further increase in fluorescence after 4 h, indicating that transcription from the *rhaBAD* promoter had stopped. In marked contrast, induction by the five promising analogues (especially L-mannose and L-lyxose) was sustained to the end of the experiment. Among the quantitative differences, the most noteworthy is that the relative rate of sfGFP production (increase of fluorescence; Figure 6C) for L-mannose at its maximum (at 7 h) was greater than the rate for the natural inducer L-rhamnose at its maximum (at 4 h). Hence this experiment indicates that differences between L-rhamnose and L-mannose in both the strength and the kinetics of induction contribute to the different end-point fluorescence assay results noted previously (Figure 5).

Sustained induction kinetics by a given inducer are likely to be explained by poor or absent metabolism of that compound. To test if the five promising inducers of the *rhaBAD* promoter were efficiently metabolized by *E. coli* MG1655 containing the reporter plasmid pCK302, growth assays at 37 °C were carried out using M9 minimal medium agar plates, each supplemented with 0.4% (w/v) of one of the analogues as the sole carbon source. Plates supplemented with glycerol and plates lacking a carbon source were used as positive and negative controls, respectively. Cells grew fastest, forming the largest colonies, on the plates containing glycerol. Both L-rhamnose and L-lyxose supported good growth of *E. coli*. Growth on the other analogues including L-mannose was much slower, with very small colonies observed after incubation at 37 °C for 48 h (data not shown). These observations suggest that the most promising strong inducer L-mannose is poorly metabolized. To confirm this, growth assays at 37 °C were carried out using M9 minimal medium liquid cultures, each supplemented with 0.4% (w/v) L-mannose, L-rhamnose, or glycerol as sole carbon source (Figure 7). L-Mannose did not support growth of *E. coli*, whereas L-rhamnose or glycerol did support growth.

Finally, flow cytometry was used to assess whether the most promising analogue, L-mannose, and the natural inducer, L-rhamnose, led to unimodal or multimodal induction of the *rhaBAD* promoter. Induction that leads to subpopulations of cells with different distributions of expression levels is said to be multimodal. Unimodality is typically preferred, as it means that

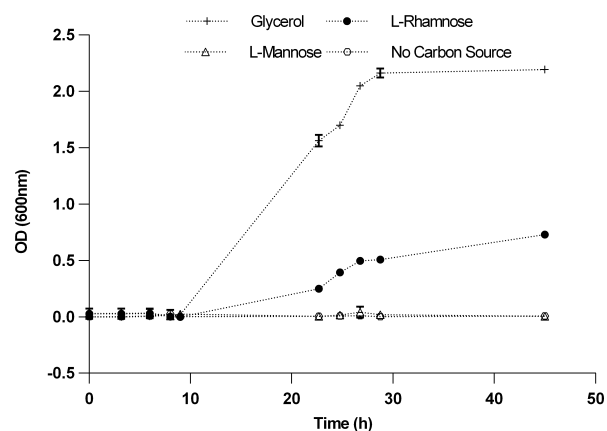


Figure 7. Assessment of growth of *E. coli* with selected inducers as sole carbon source. *E. coli* MG1655 cells transformed with pCK300 were cultured at 37 °C for 45 h in M9 minimal media supplemented with one of the following sole carbon sources: 0.4% (w/v) glycerol, 0.4% (w/v) L-rhamnose, 0.4% (w/v) L-mannose, no carbon source. Samples were taken at various time points, and the optical density at 600 nm was recorded. Error bars shown are the standard deviation of three independent biological replicates.

an inducer can be used to achieve a similar concentration of protein(s) of interest in all cells in a population, rather than different concentrations. *E. coli* MG1655 cells containing pCK302 were grown to the mid (6 h) and late (8 h) exponential growth phase in LB medium supplemented with three concentrations of L-rhamnose or L-mannose (0.2, 0.4, or 0.6 mg/mL), and the fluorescence of cells in samples were measured by flow cytometry (Figure 8). The number of fluorescing and nonfluorescing cells in each sample were counted at each time point for three independent biological replicates. As expected, L-rhamnose induction led to a unimodal distribution of expression for all three inducer concentrations tested and at both time points. Induction with all three concentrations of L-mannose led to a unimodal distribution of expression at the 8 h time point. At the 6 h time point, L-mannose induction led to a unimodal distribution at the medium (0.4 mg/mL) and high (0.6 mg/mL) concentrations, but bimodality was observed at the low (0.2 mg/mL) concentration.

DISCUSSION

This study sought and identified several inducers of the *rhaBAD* promoter of *E. coli* that enable sustained induction from this promoter for much longer durations than the native inducer L-rhamnose. To varying degrees, these compounds can be considered functionally orthogonal to *E. coli* metabolism, as they are evidently not metabolized well enough to cause transient induction, unlike the native inducer L-rhamnose. Overall, L-mannose seems likely to be the most useful of the compounds studied, as it provides the greatest range of expression levels, while retaining very good linearity of response to inducer concentration, and sustained induction. L-Lyxose may also prove useful, particularly at lower expression levels, where it may offer slightly greater control than L-mannose (Figure 6). L-Mannose and L-lyxose are commercially available, unlike most of the other compounds tested, which is an important advantage for their usefulness to most researchers. The only observed disadvantage of L-mannose was bimodality at the lowest inducer concentration tested, at the early time

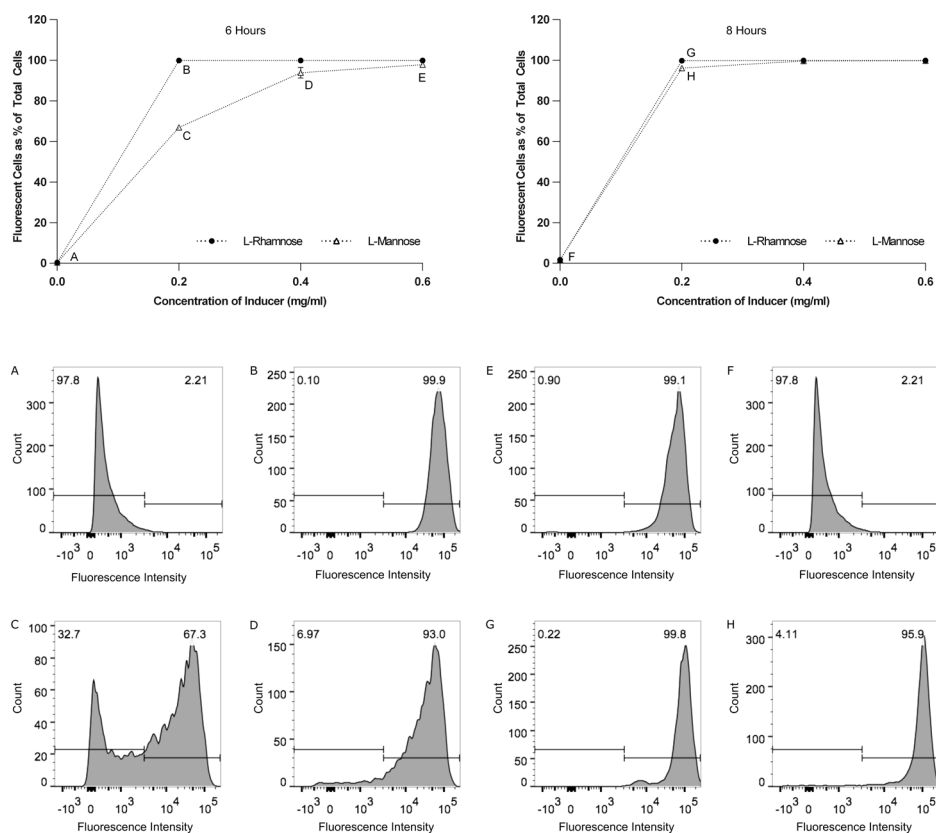


Figure 8. Distribution of expression levels in populations of cells induced by L-rhamnose or L-mannose. *E. coli* MG1655 cells transformed with pCK302 were grown in 300 μ L cultures of LB media supplemented with 0.2, 0.4, or 0.6 mg/mL of L-rhamnose or L-mannose at 37 $^{\circ}$ C. After 6 and 8 h, representing the mid to late exponential phase of growth, cells were analyzed using a LSRFortessa flow cytometer (BD Biosciences). Cells were gated appropriately, and GFP fluorescence was measured at each time point and inducer concentration. Histograms of fluorescence intensity were plotted, and population statistics were extracted. The percentages of events within the fluorescent and nonfluorescent populations are indicated on each histogram. Error bars shown are the standard deviation of three independent biological replicates.

point (6 h). This bimodality may be an issue for some uses. It is known that bimodality occurs in induction of the *lac* promoter by IPTG^{51,52} and induction of the *araBAD* promoter by arabinose⁵³ due to positive feedback between uptake of each inducer and inducible expression of the corresponding uptake system. L-Rhamnose catabolism involves the inducible expression of the uptake system RhaT, so this might explain how *rhaBAD* induction could develop bimodality by this mechanism.

The panel of 35 analogues of L-rhamnose studied here induced expression of the *rhaBAD* promoter to a wide range of levels. It was surprising to observe the apparent greater sensitivity of the *rhaBAD* reporter system pCK302 to L-mannose than to the natural inducer L-rhamnose. As crystal structures of RhaS and RhaR are not currently available, it is difficult to provide meaningful structural interpretation of these observations. However, it is clear that induction of this system, governed by ligand binding to RhaS and/or RhaR, is very sensitive to small changes in the structure of the ligand, including its stereochemistry. If crystal structures of RhaS or RhaR are obtained in future, it would be interesting to reanalyse these data. Differences in rates of uptake of these compounds by RhaT³⁷ and other transporters might also contribute to differences in the induction observed.

It is interesting that decoupling expression of *rhaS* from dependence on RhaR and the *rhaSR* promoter, effectively bypassing the native cascade, did not substantially change the shape, maximum or minimum of the induction response to

inducer concentration (Figure 4B). This suggests that the native architecture of the *rhaSR* cascade is not required for these properties, so presumably exists chiefly for reasons of resource efficiency, as in the uninduced state it minimizes protein expression from *rhaSR* as well as *rhaBAD*.

The results of this study place the *rhaBAD* expression system in the small group of preferred inducible systems for which effective orthogonal inducers are available. The additional control afforded by sustained induction represents a substantial improvement in the usefulness of the *rhaBAD* system for synthetic biology. Due to this, and the other highly desirable induction properties of the *rhaBAD* system, it may now be the best choice of inducible expression system in many cases. It has been shown that the *rhaBAD* system can be used beyond its native host by providing RhaS *in trans*, so the inducers reported here are likely to be useful, and orthogonal to metabolism, in organisms beyond *E. coli*.

This study highlights the synergy between synthetic biology and synthetic chemistry. The combined approach used here could readily be applied to other expression systems to identify useful combinations of orthogonal inducers and compatible genetic configurations. Although this report has focused on identification of the single most effective orthogonal inducer by typical criteria, the same approach could be used to identify a set of inducers with different induction characteristics. L-Rhamnose and L-mannose represent a minimal example of such a set, as L-rhamnose causes transient induction whereas L-mannose causes sustained induction. Such sets of inducers

could be used to interrogate or optimize genetic circuits or networks instead of, or in parallel to, genetic modifications and redesigns. For example, the inducers reported here may have proven useful in optimization of a recently described synthetic system using the *rhaBAD* promoter and RhaS, which required a series of design iterations to achieve the desired functionality.⁵⁰ The use of alternative chemical inducers with differing induction characteristics represents an under-utilized and rarely considered⁵⁴ mechanism for predictable external control of gene expression, which could be better developed and exploited as demonstrated here.

MATERIALS AND METHODS

Rare Sugars. The sugars were prepared either from rhamnose⁵⁵ or by isomerization of other hexoses using enzymatic methods.^{56–58}

Bacterial Strains and Growth Conditions. *E. coli* strain DH5 α was used for all plasmid construction and propagation. Wild-type *E. coli* strain MG1655 was used for all induction assays. Cells were cultured in LB or M9 minimal media at 37 °C. Ampicillin was used at a final concentration of 125 μ g/mL where appropriate.

Plasmid Construction. A list of plasmids and oligonucleotides is provided in Table S1. Sequences of pCK300, pCK301, and pCK302 have been submitted to Genbank, accession numbers are KU555408, KU555409, and KU555410, respectively. The Gibson assembly method was used throughout.⁵⁹ To construct the initial reporter plasmid pCK301, the *E. coli rhaBAD* promoter (P_{rhaBAD}) was amplified from *E. coli* MG1655 genomic DNA using oligonucleotides oligoCK207 and oligoCK208 and inserted in place of the T5 promoter on the medium copy number *E. coli* expression vector pJ404 (DNA2.0), which itself was amplified using oligonucleotides oligoCK97 and oligoCK98. Next, superfolder GFP (*sfGFP*) was amplified from pJT118-sfGFP⁶⁰ using oligonucleotides oligoCK209 and oligoCK210 and inserted immediately downstream of this promoter, following amplification of the vector using oligoCK56 and oligoCK207. An appropriate ribosome-binding site sequence with a translation initiation rate (TIR) of 50 000 was designed using the ribosome binding site calculator,⁶¹ and was incorporated using the forward primer (oligoCK210). pCK302 was constructed through amplification of the native *rhaS* gene of *E. coli* including its native ribosome-binding sequence using oligonucleotides oligoCK173 and oligoCK174, which was inserted immediately downstream of *ampR* (vector amplified using oligonucleotides oligoCK171 and oligoCK172) to ensure constitutive expression from the *ampR* promoter. pCK300 was constructed by removal of the P_{rhaBAD} -*sfGFP* fragment of pCK301 using oligonucleotides oligoCK170 and oligoCK91. All plasmids were verified by nucleotide sequencing.

Assays. To test for induction of the *rhaBAD* promoter by the analogues, MG1655 was transformed with either pCK300, pCK301, or pCK302. Successful transformants were isolated and cultured in 5 mL of LB and ampicillin with shaking at 37 °C for 16 h. These precultures were used to inoculate all subsequent assay cultures throughout this work. A Synergy HT plater reader was used for all absorbance and fluorescence measurements. Optical density (OD) was measured at 600 nm, and fluorescence excitation and emission wavelengths used were 488 and 530 nm, respectively.

End-point induction assays were performed in 500 μ L LB cultures supplemented with ampicillin and various concen-

trations of L-rhamnose or analogues and grown at 37 °C in deep 96-well plates with shaking for 16 h. Cultures were then centrifuged at 4009g for 10 min and resuspended in phosphate buffered saline and diluted 1 in 4; 200 μ L was used to measure optical density and fluorescence. Fluorescence readings were normalized by OD.

Time course experiments of P_{rhaBAD} induction in LB were performed as follows: 300 μ L of LB media supplemented with ampicillin and 0.4 mg/mL of inducer, was inoculated with 1/1000 volume of the preculture and grown at 37 °C in 96-well microplates with shaking for 7 h. Optical density and fluorescence readings were taken every 60 min. Fluorescence readings were normalized to OD.

Catabolite repression was assayed by transforming *E. coli* MG1655 cells with either pCK301 or pCK302, and transformants were used to inoculate 500 μ L of liquid M9 minimal media cultures supplemented with 0.2% glucose and 0.4% of L-rhamnose as inducer and grown at 37 °C in deep 96-well plates with shaking for 16 h; 200 μ L of the culture was used to measure optical density and fluorescence.

To test whether the analogues were capable of supporting growth of *E. coli* cells as the sole carbon source, MG1655 cells were transformed with pCK302 and transformants streaked out on M9 minimal media agar plates supplemented with 0.2% (w/v unless otherwise stated) final concentration of a single carbon source: glycerol (v/v), L-rhamnose, L-lyxose, L-mannose, 6-deoxy-L-altrose, L-olivose, 2-deoxy-2-fluoro-L-rhamnose or no sugar. Plates were incubated at 37 °C for 72 h at which time growth was scored qualitatively. Additionally 3.3 mL of liquid M9 minimal media cultures with either 0.2% glycerol (v/v), L-rhamnose (w/v) or L-mannose (v/v) were incubated for 4 days at 37 °C, and 100 μ L samples were taken at regular time points for optical density measurements.

To test whether L-rhamnose or L-mannose induction led to a bimodal expression profile across a population, MG1655 cells were transformed with pCK302, and transformants were cultured in 300 μ L of LB supplemented with ampicillin and various concentrations of L-rhamnose or L-mannose, at 37 °C in deep 96-well plates with shaking. At the 6 and 8 h time points, cells were diluted 1 in 100 in phosphate-buffered saline and analyzed using a LSRFortessa flow cytometer (BD Biosciences). Cells were gated using forward and side scatter, and GFP fluorescence (excitation and emission wavelengths: 488 and 525 nm [with 20 nm bandwidth] respectively) was measured at each time point and inducer concentration. Histograms of fluorescence intensity were plotted, and population statistics were extracted.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acssynbio.6b00030.

List of plasmids and oligonucleotides (PDF)

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Notes

The authors declare no competing financial interest.

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