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# A Rhamnose-Inducible System for Precise and Temporal Control of Gene Expression in Cyanobacteria

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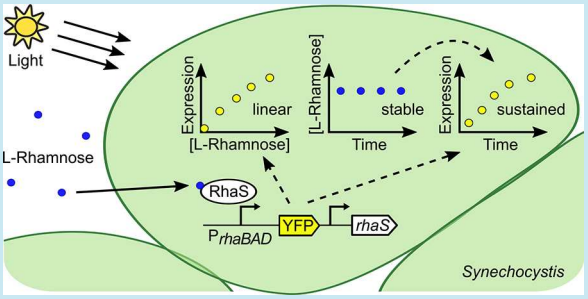
Supporting Information

**ABSTRACT:** Cyanobacteria are important for fundamental studies of photosynthesis and have great biotechnological potential. In order to better study and fully exploit these organisms, the limited repertoire of genetic tools and parts must be expanded. A small number of inducible promoters have been used in cyanobacteria, allowing dynamic external control of gene expression through the addition of specific inducer molecules. However, the inducible promoters used to date suffer from various drawbacks including toxicity of inducers, leaky expression in the absence of inducer and inducer photolability, the latter being particularly relevant to cyanobacteria, which, as photoautotrophs, are grown under light. Here we introduce the rhamnose-inducible *rhaBAD* promoter of *Escherichia coli* into the model freshwater cyanobacterium *Synechocystis* sp. PCC 6803 and demonstrate it has superior properties to previously reported cyanobacterial inducible promoter systems, such as a non-toxic, photostable, non-metabolizable inducer, a linear response to inducer concentration and crucially no basal transcription in the absence of inducer.




**KEYWORDS:** *Synechocystis, cyanobacteria, rhamnose, inducible promoter, gene expression, synthetic biology*



Photoautotrophic microorganisms have great potential for the sustainable production of chemicals from carbon dioxide using energy absorbed from light. Cyanobacteria including *Synechocystis* sp. PCC 6803 (abbreviated to "*Synechocystis*" in this report) and *Synechococcus* sp. PCC 7002 have been successfully engineered to produce 2,3-butanediol,[1,2] lactate,[3] isobutanol,[4] plant terpenoids[5] and ethanol,[6−9] and to allow the utilization of xylose.[10] Cyanobacteria, particularly *Synechocystis*, are also used as model organisms for fundamental studies of important processes such as photosynthesis,[11−16] circadian rhythms[17−19] and carbon-concentrating mechanisms.[20−23] Due to specific challenges, genetic modification of cyanobacteria is more difficult than genetic modification of model heterotrophic microorganisms such as *Escherichia coli* and *Saccharomyces cerevisiae*. These challenges include polyploidy,[24,25] which makes the isolation of segregated recombinant strains slow and laborious, genetic instability of heterologous genes,[26] and limited synthetic biology tools and parts such as promoters and expression systems. Improved synthetic biology capabilities for cyanobacteria would be useful for both fundamental and applied studies.

Inducible promoters are important tools which allow flexible control over gene expression. Unlike the limited number of constitutive promoters which have been shown to function well in cyanobacteria,[6,10,27−32] inducible promoters provide access to a wide, continuous range of gene expression levels using a single genetic construct, simply by varying inducer concentrations.[33] Furthermore, inducible promoters also allow control over the timing of expression of a gene of interest. An ideal inducible promoter system would have the following properties: Firstly, the promoter should not "leak", that is, there should be no basal transcription in the absence of inducer, allowing very low expression levels to be used, and avoiding premature expression during strain construction and segregation, which can be associated with toxicity and mutation.[26,34,35] Secondly, the inducer molecule should be non-toxic, non-metabolizable, readily available and stable under experimental conditions (including under light in the case of photoautotrophic organisms), allowing sustained expression with no impact on growth caused as an artefact of the expression system itself. Thirdly, expression should demonstrate a linear response to inducer concentration allowing fine-tuning of induction over a wide range. Finally, expression should have a consistent unimodal distribution across a population of cells.

Several inducible promoter systems have been described in *Synechocystis* spp. and *Synechococcus* spp., but none are ideal. Metal ion-inducible promoters have been described which respond to nickel, copper, cadmium, arsenic and zinc.[36−40] Unfortunately these systems have disadvantages including the presence of many of the metals in standard growth media,[41] a narrow range of useful concentrations because the concentrations required for detectable and unimodal induction are

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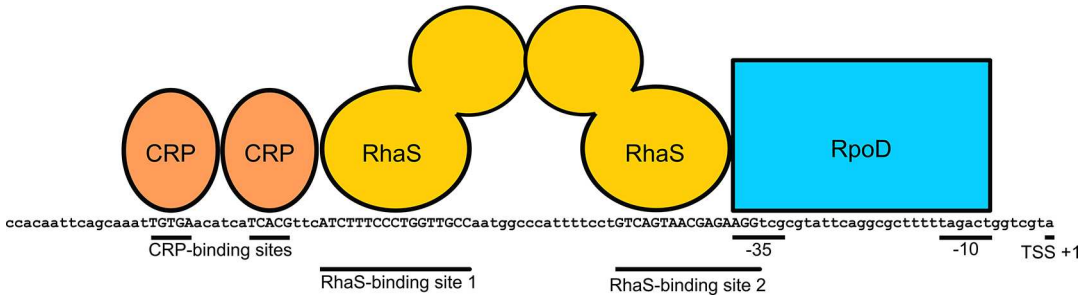


**Figure 1.** The *rhaBAD* promoter of *E. coli* showing transcription factors and binding sites. Binding site sequences are in uppercase letters and labeled. The −35 and −10 operators[53,77] are the binding sites for RpoD, the sigma 70 factor of RNA polymerase. TSS + 1 is the transcriptional start site.

close to toxic levels,[37] and some are "leaky" in the absence of inducer. The use of metals as inducers also has the potential to disrupt metal homeostasis, resulting in the sequestration of metals required as essential cofactors of many enzymes involved in photosynthesis and related metabolic pathways.[11,42−44] Synthetic inducible promoters have also been constructed and used in cyanobacteria. Two promoter systems using the tetracycline-responsive repressor TetR and its cognate operator sites have been engineered for use in cyanobacteria. The first example for use in *Synechocystis* resulted in a well-characterized, anhydrotetracyline (aTc)-responsive promoter with low leakiness and a good dynamic range.[45] Unfortunately the inducer aTc is extremely sensitive to light and therefore induction from this promoter was transient and required high concentrations of aTc. The second example, in *Synechococcus* sp. PCC 7002, suffered the same issues with photolability of the inducer and low expression in comparison to a commonly used strong constitutive promoter.[46] Therefore, aTc-based induction is unsuitable for photoautotrophic growth conditions. The non-metabolisable analogue of lactose, isopropyl *β*-D-1-thiogalacto-pyranoside (IPTG) has also been tested for use as an external inducer of *lac*-based promoters in a variety of cyanobacterial strains,[1,27,47−51] with mixed performance in terms of dynamic range and leakiness in absence of inducer. Finally, use of a green-light inducible promoter in *Synechocystis* sp. PCC 6803 has been reported,[52] but isolating the specific wavelengths required for induction from natural or white light used for growth is difficult, leading to unwanted induction.

To-date, heterologous promoters associated with the AraC/XylS family of positive transcriptional regulators have not been used in cyanobacteria. One promising candidate is the L-rhamnose-inducible *rhaBAD* promoter system of *E. coli*, which naturally has almost all of the ideal properties described above.[53−55] Recently this system was optimized in *E. coli* by constitutive expression of the activating transcription factor RhaS, obviating the need to also induce the native regulatory *rhaSR* operon; and identification of L-mannose as a non-metabolisable inducer, allowing sustained expression rather than transient expression.[56]

Here we introduce the *rhaBAD* promoter of *E. coli* into *Synechocystis* sp. PCC 6803, characterize its behavior, assess inducer stability and investigate the effects of modifying various promoter sequence elements and of varying expression of the transcription factor RhaS. The result is an inducible expression system with several important advantages over expression systems previously characterized in cyanobacteria including precise control of the strength and timing of induction as well as sustained gene expression in the presence of light. This system is likely to be very useful and widely applicable in *Synechocystis* and other cyanobacteria.

## RESULTS AND DISCUSSION

**Analysis of the *E. coli rhaBAD* Promoter in a Heterologous *Synechocystis* Context.** The *E. coli rhaBAD* promoter might be expected to perform differently in the heterologous context of a *Synechocystis* cell than in the native host. To assess this, we considered the known functional features of the *rhaBAD* promoter and whether the relevant transcription factors in *Synechocystis* were present, and if present, investigated the conservation of their functionally-important amino acid residues.

The *rhaBAD* promoter (Figure 1) contains three types of operator sequences for the binding of three distinct transcription factors: the cAMP (cyclic adenosine monophosphate) receptor protein (CRP); RhaS, which in *E. coli* mediates transcriptional activation of the *rhaBAD* operon in response to L-rhamnose; and RpoD, the primary vegetative sigma 70 factor of *E. coli* RNA polymerase (RNAP).

The genome of *Synechocystis* encodes SYCRP1, a homolog of *E. coli* CRP[57,58] with 27% identity and 49% similarity to the *E. coli* protein. In *E. coli*, CRP binds to promoters containing specific binding sites when the concentration of cAMP is high, for example when glucose is scarce and other carbon sources must be metabolized for growth. The CRP-binding site in the *rhaBAD* promoter has been shown to be essential for this promoter to function fully in *E. coli*.[53,59] In *Synechocystis*, SYCRP1 has been shown to positively and negatively regulate a number of promoters in response to changing cAMP concentrations.[58,60] The sequence of the CRP-binding site in *Synechocystis* (tgtgaNNNNNNtcaca) differs by only one nucleotide to the CRP-binding site sequence found in the *E. coli rhaBAD* promoter (tgtgaNNNNNNtcacg), which suggests SYCRP1 might bind to this heterologous promoter sequence.[61,62]

To the best of our knowledge, positively-regulated AraC/XylS-type expression systems like those in *E. coli* have not been reported in *Synechocystis* or in other cyanobacteria. In *E. coli*, the positive transcriptional regulator RhaS is essential for transcription from the *rhaBAD* promoter. We used BLASTP[63] to search the genome of *Synechocystis* for a homolog of *E. coli* RhaS. No protein with significant similarity to *E. coli* RhaS was identified, suggesting that heterologous expression of the *rhaS* gene of *E. coli* which encodes this protein would be required for the heterologous *rhaBAD* promoter from *E. coli* to function in *Synechocystis*.

It has been hypothesized that differences in the RNA polymerase components between cyanobacteria and *E. coli* are


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                                                    1
E. coli RpoD          427 FSTYATWWIRQAITRSIADQARTIRIPVHMIETINKLNRISRQMLQEMGR
                          |||||||||||||||:||||:||||:|||:.|||:::.:.:::.:.|||.|
Synechocystis SigA    241 FSTYATWWIRQAITRAIADQSRTIRLPVHLYETISRIKKTTKLLSQEMRR

E. coli RpoD          477 EPTPEELAERMLMPEDKIRKVLKIAKEPISMETPIGDDEDSHLGDFIEDT
                          :||.||:|||:|.|..:|:|.:.|.|:.|||:|||||.:|||.|||||| .
Synechocystis SigA    291 KPTEEEIAEKMEMTIEKLRFIAKSAQLPISLETPIGKEEDSRLGDFIE-A

E. coli RpoD          527 TLELPLDSATTESLRAATHDVLAGLTAREAKVLRMRFGIDMNTDYTLEEV
                          ..|.|.|..:...||....:||..|:.||..|||:|:|:|.....||||:
Synechocystis SigA    340 DGETPEDEVSKNLLREDLENVLDTLSPRERDVLRLRYGLDDGRMKTLEEI
                                 2
E. coli RpoD          577 GKQFDVTRERIRQIEAKALRKLRHPSRSEVLRSFLDD
                          |:.|:|||||||||||||||||||:|:.:|:.::.
Synechocystis SigA    390 GQIFNVTRERIRQIEAKALRKLRHPNRNSILKEYIR-
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**Figure 2.** Sequence alignment of RNA polymerase sigma 70 factors from *E. coli* and *Synechocystis*. RpoD (NP_417539.1) of the *E. coli* K12 strain MG1655 was aligned pairwise with SigA (ALJ69094.1) of *Synechocystis* sp. PCC 6803 using EMBOSS Needle[78] accessed at https://www.ebi.ac.uk/Tools/psa/. Only the C-terminal portion of the alignment is shown, where the key features of interest are found (see Figure S1 for full alignment). Box 1. Residues involved in binding to the −10 promoter region (region 2). Box 2. Residues involved in binding to the −35 promoter region (region 4.2). Underlined are the two residues in the *E. coli* sigma 70 factor RpoD, K593 and R599, required for interaction with two residues of RhaS (D250 and D241 respectively) and the conserved residues found in the *Synechocystis* ortholog (R412 and K406 respectively).

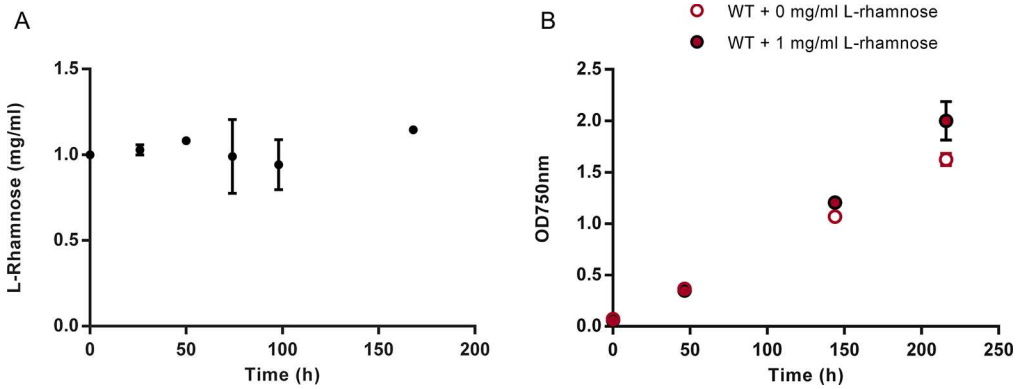


**Figure 3.** L-Rhamnose stability and impact on growth in photoautotrophic cultures of *Synechocystis*. (A) Concentration of L-rhamnose over time in the supernatant of photoautotrophic cultures of wild-type *Synechocystis*, as measured by HPLC-RID. (B) Growth of wild-type *Synechocystis* in photoautotrophic conditions and constant light; with and without 1 mg/mL L-rhamnose. Error bars represent the standard deviation of three independent biological replicates.

one reason for *E. coli* promoters failing to function as expected when used in cyanobacteria.[64] With this in mind, the RpoD sigma factor of *E. coli* and the SigA sigma factor of *Synechocystis* were compared by alignment of their amino acid sequences (Figure 2). RpoD (accession number: NP_417539.1) is the *E. coli* primary vegetative sigma 70 factor, which binds to the −35 and −10 regions of the *rhaBAD* promoter in *E. coli*, and SigA (accession number: ALJ69094.1) is the *Synechocystis* primary sigma factor, and has been shown to be the most abundant sigma factor under standard growth conditions.[65] The two orthologs share 59% identity and 78% similarity but as the *Synechocystis* protein is much smaller than the *E. coli* ortholog (425 and 613 amino acids, respectively), the overall coverage is only 46%, with the N-termini sharing little similarity in contrast to the good alignment at the C-termini, which is the most conserved region across the sigma 70 family of transcription factors.[66−68] The C-termini of sigma 70 factors contain the DNA-binding domains, with conserved and well-defined functional regions.[69] Region 2 is responsible for interaction with the −10 element of the promoter and region 4.2 is responsible for interaction with the −35 element.[70,71] The sequence of the −10 element-binding domain of the *Synechocystis* sigma 70 factor, RTIRLPVH differs only in one amino acid from the *E. coli* sequence RTIRIPVH (Figure 2), which suggests this protein is likely to bind to the −10 element of the *rhaBAD* promoter. Even more encouragingly, the amino acid sequence of the −35 element-binding domain, VTRERI-RQIEAKALRKLRHP, is perfectly conserved between both *Synechocystis* and *E. coli* proteins (Figure 2). Finally, it is known that two residues of the *E. coli* RNAP sigma 70 factor protein, RpoD are essential for interaction with two residues of RhaS when both proteins are bound to the DNA.[72] These interactions are formed between R599 of the sigma 70 factor and D241 of RhaS, as well as K593 of the sigma 70 factor and D250 of RhaS. Both of these residues are found in the *Synechocystis* sigma 70 factor protein, corresponding to R412 and K406, respectively (Figure 2). The above analysis suggested that the *E. coli rhaBAD* promoter is likely to be functional in *Synechocystis*, and will probably require RhaS to be provided.

**L-Rhamnose Is Not Metabolized by nor Toxic to *Synechocystis*.** Before testing whether the *E. coli rhaBAD* promoter is functional in *Synechocystis*, we first wanted to check if the natural sugar inducer L-rhamnose was metabolized by the cyanobacterium or if the use of a non-metabolisable analog of rhamnose would be required, as previously found in *E. coli*.[56]


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Wild-type *Synechocystis* cells were cultivated in BG11 medium under constant light, with L-rhamnose added to the culture to a final concentration of 1 mg/mL L-rhamnose or omitted in the control. The concentration of L-rhamnose in the culture supernatant was monitored over time using HPLC-RID (Figure 3A). The concentration of L-rhamnose does not change over the course of the experiment, indicating that it is not metabolized by *Synechocystis* in photoautotrophic conditions, nor degraded by exposure to light. Next the effect of L-rhamnose on growth was investigated by monitoring the optical density (OD) at 750 nm of cultures over time, with or without L-rhamnose (Figure 3B). No statistically-significant effect of L-rhamnose on growth was observed indicating that L-rhamnose is not inhibitory to *Synechocystis* growth.

**Characterization of *rhaBAD* Promoter System in *Synechocystis*.** To facilitate the testing of the *rhaBAD* promoter from *E. coli* in *Synechocystis*, an *E. coli-Synechocystis* shuttle reporter plasmid pCK306 (Figure 4) containing the

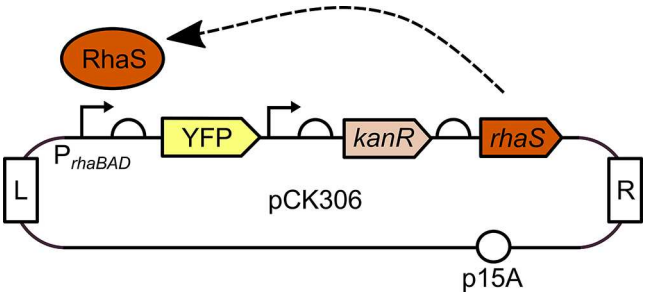


**Figure 4.** *E. coli*−*Synechocystis* shuttle plasmid pCK306 containing a YFP reporter of the *rhaBAD* promoter. L and R denote left and right homology arms respectively for integration into the *Synechocystis* sp. PCC 6803 genome at a previously used insertion site within the ssl0410 ORF adjacent to the *ndhB* locus.[74] $P_{rhaBAD}$ is the *rhaBAD* promoter sequence from *E. coli*. YFP encodes yellow fluorescent protein. *kanR* encodes an aminoglycoside phosphotransferase that confers resistance to kanamycin in both *Synechocystis* and *E. coli*. *rhaS* encodes the transcriptional activator of *rhaBAD* promoter, RhaS. p15A is the medium-copy origin of replication that allows replication of the shuttle plasmid in *E. coli*.

*rhaBAD* promoter sequence and the *rhaS* gene encoding its transcriptional activator was constructed (see Plasmid Construction section of Supporting Information for details). This plasmid contains homology arms for integration into the genome of *Synechocystis* at the ssl0410 locus, the p15A origin of replication for *E. coli*, the promoter of the *rhaBAD* operon from *E. coli* ($P_{rhaBAD}$), a reporter gene encoding yellow fluorescent protein (YFP), a kanamycin-resistance gene functional in both *Synechocystis* and *E. coli*, and *rhaS* from *E. coli*, which encodes the transcriptional activator of the *rhaBAD* promoter, RhaS. In this genetic context, the native *E. coli* RBS of *rhaS* was predicted to have a translation initiation rate (TIR) of just 72.[73] To determine whether it is necessary to supply *rhaS* heterologously, a control reporter plasmid, pCK305, identical to pCK306 but lacking *rhaS*, was also constructed.

To test for L-rhamnose induction of the *rhaBAD* promoter in *Synechocystis*, wild-type cells were transformed with either pCK305 or pCK306 and kanamycin-resistant transformants were passaged until complete segregation was confirmed by PCR. These transformants were then cultured under constant light in BG11 media supplemented with kanamycin, with or without glucose. The experiments lacking glucose represent photoautotrophic growth conditions. Characterization under mixotrophic growth conditions (supplemented with glucose) is also of interest as *Synechocystis* is often used to study photosynthesis mutants with growth impairments under photoautotrophic conditions. Cultures were adjusted to a starting optical density (measured at 750 nm) of 0.1, grown for 24 h and then L-rhamnose was added to a range of final concentrations. To determine the response of the promoter to the concentration of the inducer L-rhamnose, the fluorescence intensity of each cell was measured using flow cytometry after 116 h of growth for both photoautotrophic and mixotrophic cultures (Figure 5A,C). Cell density was monitored during growth by measuring optical density of cultures at 750 nm (Figure S5). At the time of sampling, cultures were in the midlinear phase of growth, with little difference in optical density between cultures containing glucose and those without glucose (0.8−1.1 $OD_{750\ nm}$). Fluorescence intensity of individual cells (10 000 cells per sample) was measured by flow cytometry, avoiding the need to normalize the fluorescence intensity of culture volumes by optical density, which can be problematic as highly pigmented cyanobacterial cells can partially quench fluorescence. Cells containing the reporter plasmid pCK305, lacking *rhaS*, were unresponsive to any concentration of L-rhamnose added, whereas cells containing the plasmid constitutively expressing *rhaS*, pCK306, show a linear response in YFP fluorescence to increasing concentrations of L-rhamnose in both photoautotrophic and mixotrophic conditions. Saturation of induction occurs at lower concentrations in mixotrophic conditions (0.6 mg/mL) than photoautotrophic conditions (no saturation at 1 mg/mL). To determine the kinetics of YFP expression from the *rhaBAD* promoter in *Synechocystis*, the fluorescence intensity of cells sampled from in the same transformant cultures was monitored over a longer period (Figure 5B,D). Fluorescence was observed in cells containing pCK306 after only 24 h of induction and showed sustained induction in both photoautotrophic and mixotrophic growth conditions, with no decrease in fluorescence observed after >250 h of growth. Finally, as levels of gene expression can differ among cells in a population of either natural or engineered strains, flow cytometry was used to investigate the modality (distribution) of fluorescence across *Synechocystis* cells containing pCK306. Induction of the *rhaBAD* promoter in *Synechocystis* containing pCK306 in photoautotrophic conditions was unimodal at all time points measured (Figure S2A). In mixotrophic conditions at the early stages of induction (120 h), a small amount of bimodality was observed (Figure S2B), with 3−6% of cells failing to be induced at this time point, however when induction was complete at a later time point (215 h) the induction was unimodal once again (Figure S2C). These data demonstrate that the *rhaBAD* promoter from *E. coli* is functional in *Synechocystis*, allows the strength of expression of a gene of interest to be precisely controlled in both phototrophic and mixotrophic growth conditions and that the transcriptional activator RhaS from *E. coli* is required for function in *Synechocystis*.

Having confirmed that the *rhaBAD* promoter was functional in *Synechocystis* and demonstrated many of the desired properties of an ideal inducible promoter system, we next investigated if modifications to the promoter sequence itself or varying the concentration of RhaS in the cell affected the behavior of the system. As the role of CRP is still poorly understood in *Synechocystis* and as the CRP-binding site is


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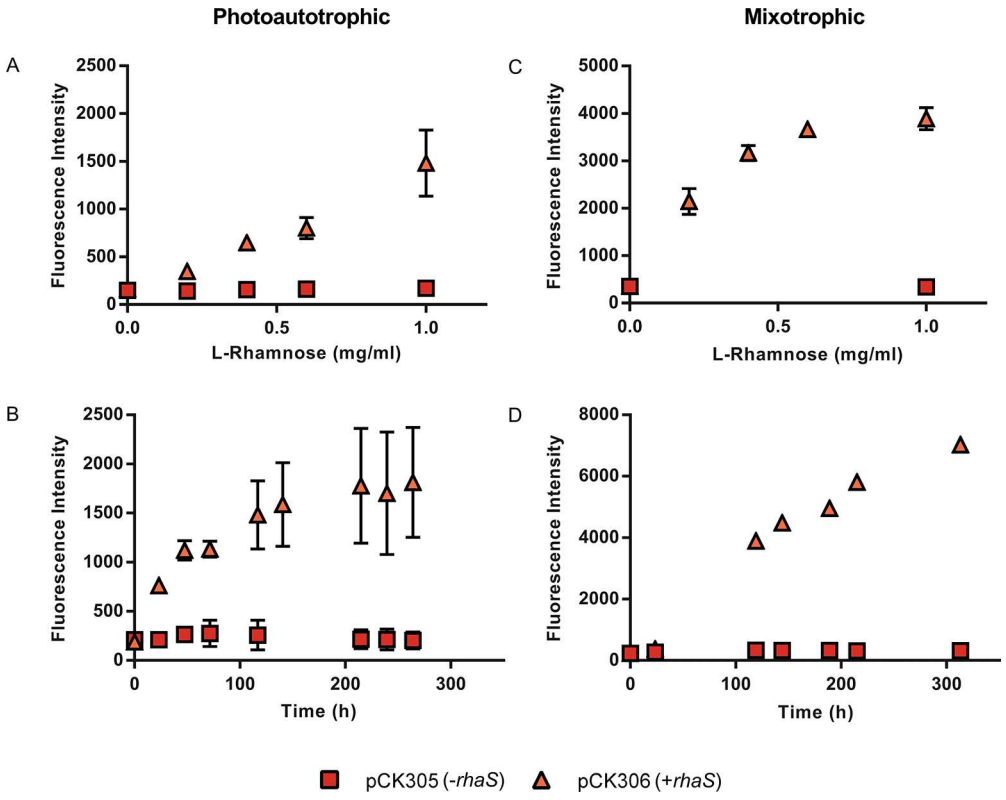


**Figure 5.** Response to concentration of inducer L-rhamnose and timecourse of induction of *rhaBAD* promoter in *Synechocystis*. (A) *Synechocystis* cells containing either pCK305 (*rhaBAD* promoter and YFP only) or pCK306 (*rhaBAD* promoter, YFP and *rhaS*) were cultured in BG11 media supplemented with specified concentrations of L-rhamnose in photoautotrophic conditions and constant light; and the fluorescence intensity of 10 000 cells measured after 116 h using flow cytometry. (B) The same strains of *Synechocystis* were cultured in BG11 media supplemented with L-rhamnose to a final concentration of 1 mg/mL in photoautotrophic conditions and constant light; and the fluorescence intensity of 10 000 cells measured at specified time points using flow cytometry. (C) Equivalent experiment to A but strains cultured in BG11 supplemented with 5 mM D-glucose (mixotrophic growth). (D) Equivalent experiment to B but strains cultured in BG11 supplemented with 5 mM D-glucose (mixotrophic growth). Error bars shown are the standard deviation of the mean for three independent biological replicates.

required for *rhaBAD* function in *E. coli*, we investigated what effect deletion of this operator sequence from the promoter would have on its performance. The reporter plasmids pCK305 and pCK306 were both modified through deletion of the CRP-binding operator sites, resulting in pCK313 and pCK314 respectively. Wild-type *Synechocystis* cells were transformed with either plasmid and integration and segregation confirmed as before. Transformants were then cultured in both photoautotrophic and mixotrophic growth conditions and the timecourse experiments repeated (Figure 6). A minimal inducer response experiment was performed using three concentrations of L-rhamnose (0, 0.2, and 1 mg/mL) in order to determine whether the constructs with the CRP-binding site deleted still showed strong expression at high inducer concentration and low expression at low inducer concentration. Cell density was monitored during growth by measuring optical density of cultures at 750 nm (Figure S6). At the time of sampling, cultures were in the midlinear phase of growth. There was little difference in optical density between cultures containing glucose (1−1.2 $OD_{750\ nm}$) and those without glucose (0.8−1.1 $OD_{750\ nm}$). Results were very similar to those observed with pCK305 and pCK314, indicating that the CRP-binding site is not required for the *rhaBAD* promoter to function in *Synechocystis*.

Next we investigated whether increasing the cellular concentration of the transcriptional activator RhaS would change the response to inducer concentration, dynamic range or kinetics of *rhaBAD* promoter induction. The original *rhaS* RBS was predicted to have a low TIR of just 72, which might result in poor translation of RhaS, and in turn limit expression from the *rhaBAD* promoter. Therefore, in order to improve RhaS expression, two synthetic RBSs were designed using the RBS Calculator[73] with much higher predicted TIR values of 5000 and 18 000, and these new RBS sequences were inserted in place of the *rhaS* RBS used in pCK306, resulting in pCK320 and pCK321 respectively. These constructs were introduced into *Synechocystis*, integration and complete segregation was confirmed as before, then these transformants were used for inducer-response and timecourse experiments as before (Figure S3). As before cell density was monitored during growth by measuring optical density of cultures at 750 nm (Figure S7). At the time of sampling, cultures were in the midlinear phase of growth. There was little difference in optical density between cultures containing glucose (0.8−1.1 $OD_{750\ nm}$) and those without glucose (1−1.2 $OD_{750\ nm}$). The *Synechocystis* strains transformed with the new *rhaS* RBS variant plasmids pCK320 or pCK321 showed similar fluorescence responses to inducer concentration and timecourses to cells transformed with pCK306 (Figure S3), with no statistically-significant difference between cells transformed with pCK320 (*rhaS* RBS with TIR of 5000) or pCK321 (*rhaS* RBS with TIR of 18 000) in both experiments and in both growth conditions. This implies that translation from the original *rhaS* RBS found on pCK306 was sufficient.


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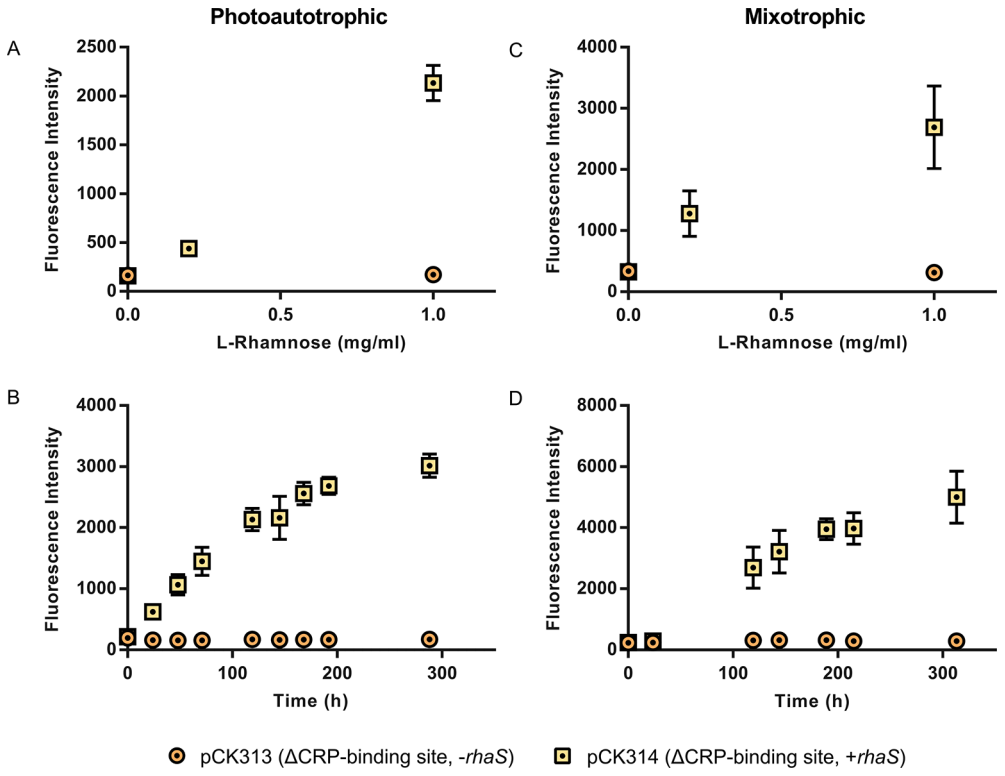


**Figure 6.** Response to concentration of inducer L-rhamnose and timecourse of induction of a variant of the *rhaBAD* promoter with CRP-binding site deletion. (A) *Synechocystis* cells containing either pCK313 (*rhaBAD* promoter minus CRP-binding site and YFP only) or pCK314 (*rhaBAD* promoter minus CRP-binding site, YFP and *rhaS*) were cultured in BG11 media supplemented with specified concentrations of L-rhamnose in photoautotrophic conditions and constant light, and fluorescence intensity of 10 000 cells measured after 116 h using flow cytometry. (B) The same strains of *Synechocystis* were cultured in BG11 media supplemented with L-rhamnose to a final concentration of 1 mg/mL in photoautotrophic conditions and constant light; and the fluorescence intensity of 10 000 cells measured at specified time points using flow cytometry. (C) Equivalent experiment to A but strains cultured in BG11 supplemented with 5 mM D-glucose (mixotrophic growth). (D) Equivalent experiment to B but strains cultured in BG11 supplemented with 5 mM D-glucose (mixotrophic growth). Error bars shown are the standard deviation of the mean for three independent biological replicates.

Next we sought to directly compare all of the functional *rhaBAD* expression system variants, as subtle differences had been observed between cells containing different constructs in different culturing conditions. Absolute levels of fluorescence measured using flow cytometry cannot always be directly compared between different days and experiments due to experimental variation. This is sometimes overcome in reporter studies by normalizing to a reference promoter included in each separate experiment, allowing relative comparisons. Here, as we had a defined set of constructs to compare, we compared these directly in a single experiment. *Synechocystis* cells containing each of the *rhaBAD*-promoter reporter plasmids were cultured, both photoautotrophically and mixotrophically, in BG11 media supplemented with 1 mg/mL L-rhamnose, and the fluorescence intensity measured by flow cytometry after 191 h (Figure S4 and S9). No statistically-significant difference was observed between cells containing constructs pCK306 (+*rhaS*), pCK314 (+*rhaS*, ΔCRP-binding site), pCK320 (+*rhaS*, TIR of RBS of *rhaS* = 5000) or pCK321 (+*rhaS*, TIR of RBS of *rhaS* = 18 000).

As natural light follows diurnal light-dark cycles, it is important to understand how synthetic biology tools for photoautotrophic microorganisms perform under these real-world conditions. Furthermore, it is known that levels of cAMP increase in cyanobacteria at night,[60] so the effect of the CRP-binding site in the *rhaBAD* promoter may be different under diurnal light-dark cycles than under continuous light. Therefore, cells containing a promoter construct including the CRP-binding site (pCK306) or lacking the CRP-binding site (pCK314) were cultured both photoautotrophically and mixotrophically in diurnal cycles of 12 h light and 12 h dark and the fluorescence intensity and optical density of the cultures monitored over time (Figure 7 and Figure S8 respectively). The induction response to different concentrations of inducer was assessed after 72 h, at midlinear stage of growth (1.0−1.5 $OD_{750\ nm}$). The results show that the *rhaBAD* promoter system, with or without a CRP-binding site, behaves similarly when cultured in light-dark photoautotrophic or light-dark mixotrophic conditions as in the previous experiments with continuous light, including the crucial characteristics of strong expression at high inducer concentration, low expression at low inducer concentration, and a near-linear response in between. When cells transformed with pCK306 or pCK314 were grown under light-dark mixotrophic conditions, the rate of increase of fluorescence over time was greater, and saturation was reached earlier, than the same strains grown under light-dark photoautotrophic conditions. Differences were not observed between cells transformed with pCK306 or pCK314, indicating that the presence of the CRP-binding site had no impact.

The inducible reporter constructs described above show nonzero levels of fluorescence in *Synechocystis* even in the complete absence of inducer, which could suggest that the promoter is "leaky". However, it was noted that even cells


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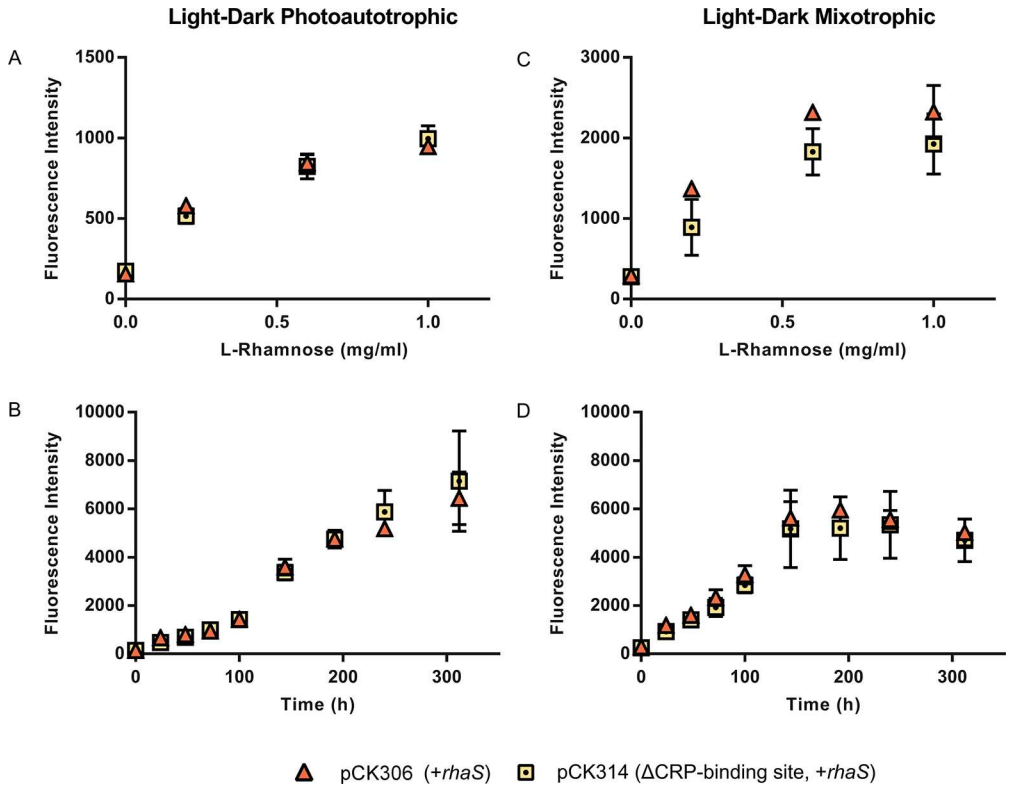


**Figure 7.** Response to concentration of inducer L-rhamnose and timecourse of induction of variants of the *rhaBAD* promoter with and without the CRP-binding site in diurnal cycles of light and darkness. (A) *Synechocystis* cells containing either pCK306 (*rhaBAD* promoter, YFP and *rhaS*) or pCK314 (*rhaBAD* promoter minus CRP-binding site, YFP and *rhaS*) were cultured in BG11 media supplemented with specified concentrations of L-rhamnose in photoautotrophic conditions and diurnal cycles of 12 h light/12 h dark; and fluorescence intensity of 10 000 cells measured after 72 h using flow cytometry. (B) The same strains of *Synechocystis* were cultured in BG11 media supplemented with L-rhamnose to a final concentration of 1 mg/mL in photoautotrophic conditions and diurnal cycles of 12 h light/12 h dark; and the fluorescence intensity of 10 000 cells was measured at specified time points using flow cytometry. (C) Equivalent experiment to A but strains cultured in BG11 supplemented with 5 mM D-glucose (mixotrophic growth). (D) Equivalent experiment to B but strains cultured in BG11 supplemented with 5 mM D-glucose (mixotrophic growth). Error bars shown are the standard deviation of the mean for three independent biological replicates.

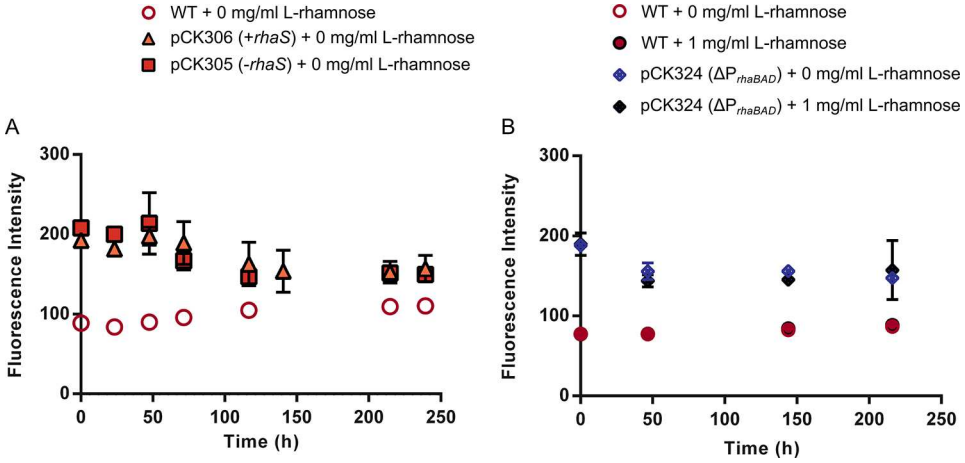


**Figure 8.** Measurement of transcriptional read-through at the site of chromosomal integration using a deletion of the *rhaBAD* promoter. (A) Wild-type *Synechocystis* (WT) or *Synechocystis* containing pCK305 (*rhaBAD* promoter and YFP only) or pCK306 (*rhaBAD* promoter, YFP and *rhaS*) were cultured in BG11 media without L-rhamnose and the fluorescence intensity of 10 000 cells measured by flow cytometry. (B) Wild-type *Synechocystis* (WT) or *Synechocystis* containing pCK324 (a control vector lacking the *rhaBAD* promoter) were cultured with or without 1 mg/mL L-rhamnose and the fluorescence intensity of 10 000 cells measured by flow cytometry. Error bars shown are the standard deviation of the mean for three independent biological replicates.

containing the non-functional promoter reporter constructs (such as pCK305) were slightly more fluorescent than wild type cells lacking any reporter plasmid (Figure 8A). As these constructs are integrated into the *Synechocystis* genome, it was hypothesized that this basal fluorescence resulted from transcriptional read-through from the chromosome rather



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than leaky expression from the *rhaBAD* promoter itself. To test this hypothesis, the *rhaBAD* promoter of pCK321 (one of the above-described derivatives of pCK306 that performs identically) was deleted resulting in the promoterless plasmid pCK324. This construct was integrated into the same site on the *Synechocystis* genome as all other reporter plasmids, fully segregated and the timecourse experiments in mixotrophic and photoautotrophic growth conditions performed as before. Cells containing pCK324, lacking the *rhaBAD* promoter, had the same level of basal YFP fluorescence whether L-rhamnose was added to the media or not and the level of fluorescence in both cases was the same as cells containing pCK305 or pCK306 without inducer (Figure 8B). This confirmed that chromosomal read-through was the cause of basal YFP fluorescence and the *rhaBAD* promoter itself was not leaky in the absence of inducer.

## CONCLUSIONS

This study showed that the *E. coli rhaBAD* promoter performs excellently as an inducible promoter in the cyanobacterium *Synechocystis* sp. PCC 6803, with a linear response to inducer concentration, good dynamic range, sustained induction in light over long periods and crucially no basal expression in the absence of inducer. In many cases, the use of this promoter should allow more precise control of the timing and strength of expression in *Synechocystis* than alternative cyanobacterial inducible promoters. Heterologous expression of *rhaS* was required for promoter function in *Synechocystis*, which is consistent with the apparent absence of an ortholog in the *Synechocystis* genome. This lack of complementation of the *rhaBAD* promoter system by any native *Synechocystis* protein suggests that the heterologously-supplied transcriptional activator RhaS is unlikely to interact with other *Synechocystis* promoters, providing a useful level of independence (or orthogonality). Deletion of the CRP-binding sites from the *rhaBAD* promoter had no substantial effect on promoter function in *Synechocystis* in the experimental conditions tested, including photoautotrophic and mixotrophic growth under constant light or diurnal light-dark cycles. This was unexpected for two reasons: firstly the function of the *rhaBAD* promoter in *E. coli* requires binding of CRP and the presence of glucose leads to reduced cellular cAMP levels preventing CRP binding to the *rhaBAD* promoter, and secondly in *Synechocystis* it is known that cellular cAMP levels increase at night,[60] thus promoting CRP binding. This characterization is important for researchers interested in using this inducible promoter in fundamental studies of the circadian clock or photosynthesis, or in applications where cyanobacteria are grown in light and dark cycles.

The only observed flaw with this implementation of the *rhaBAD* promoter in *Synechocystis* was a low level of basal expression, which we found was independent of the *rhaBAD* promoter. The *sll0410* insertion site adjacent to *ndhB* has been used previously, but seems to result in transcriptional read-through of inserts, presumably from the promoter found inside the *ndhB* ORF.[74] For most inducible expression studies, this observation will be unimportant and expression constructs reported here will be ideal, because in many cases the ability to specify extremely low expression levels is not required. Where extremely low or zero basal and induced expression is required, alternative integration sites or extrachromosomal plasmids may prove more suitable.[75]

We found that the *rhaBAD* promoter of *E. coli* was functional and inducible in *Synechocystis* without any modification of the promoter sequence itself. This was not obvious in advance given reports of difficulties in using *E. coli* promoters in cyanobacteria. In this case our analysis of the relevant transcription factor machinery and interacting residues successfully predicted function of this promoter in *Synechocystis*. Although the primary *Synechocystis* sigma factor, SigA is almost certainly responsible for transcription from the *rhaBAD* promoter under typical laboratory growth conditions, the alternative sigma factors SigC, SigD and SigE, which are used in alternative growth conditions such as salt stress, nitrogen limitation or when photosystem II function is impaired,[65] also contain most of the residues required for both *rhaBAD* promoter and RhaS binding. This suggests that these alternative sigma factors may be able to initiate transcription of the *rhaBAD* promoter under the corresponding growth conditions. It is also interesting to consider whether this promoter might function in other cyanobacteria such as *Synechococcus* sp. PCC 7002 or *Arthrospira* species. For example, one of the sigma 70 factor residues important for interaction with RhaS, K593, is not found in the *Synechococcus* sp. PCC 7002 ortholog but is found in the *Arthrospira plantensis* ortholog. The residue found in the *Synechococcus* ortholog is an arginine, a similar basic amino acid, so may still interact appropriately with RhaS for function.

This study represents an important step toward addressing the shortage of reliable synthetic biology tools for the manipulation of cyanobacteria, both for fundamental and applied studies. The characteristics of the rhamnose-inducible expression system shown in this work will allow greater control of gene expression in cyanobacteria than previously possible. Despite this progress, much work remains in the development and characterization of other synthetic biology tools to address the unique challenge of engineering these important photoautotrophic organisms and realizing their applied potential.

## MATERIALS AND METHODS

**Bacterial Strains and Growth Conditions.** *E. coli* strain DH5$\alpha$ was used for all plasmid construction and propagation. *Synechocystis* sp. PCC 6803 (the glucose-tolerant derivative of the wild type, obtained from the Nixon lab at Imperial College London) was used for all cyanobacterial experiments. *E. coli* were routinely cultured in LB at 37 °C with shaking at 240 rpm and *Synechocystis* cultured in TES-buffered (pH 8.2) BG11 media[41] with 5 mM glucose (mixotrophic growth) or without glucose (photoautotrophic growth) at 30 °C with agitation at 150 rpm, supplemented with 30 $\mu$g mL$^{-1}$ kanamycin where required. *Synechocystis* were either grown in constant white light at 50 $\mu$mol m$^{-2}$ s$^{-1}$ or in cycles of 12 h of white light and 12 h of darkness as indicated in the text.

**Plasmid Construction.** A table of all plasmids and oligonucleotides (Table S1) is provided in the Supporting Information. All plasmid construction was carried out using standard molecular cloning methods. Full details are provided in the Supporting Information.

**Strain Construction.** Wild-type *Synechocystis* cells were cultured in BG11 supplemented with 5 mM D-glucose to an optical density (measured at 750 nm) of 0.5 and 4 mL harvested by centrifugation at 3200*g* for 15 min. Pellets were resuspended in 100 $\mu$L BG11, 100 ng of plasmid DNA was added and the mixture was incubated at 100 $\mu$mol m$^{-2}$ s$^{-1}$ white light for 60 min. Cells were spotted onto BG11 glucose plates and incubated at 100 $\mu$mol m$^{-2}$ s$^{-1}$ white light for 24 h at 30 °C. Cells were collected and transferred onto BG11 glucose


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plates supplemented with 30 $\mu$g mL$^{-1}$ kanamycin. When single colonies appeared after approximately 7 days, transformants were segregated through passaging on selective plates approximately weekly, and full segregation after approximately 3 weeks was confirmed by PCR.

**Assays.** After confirmation by PCR that *Synechocystis* transformants were fully segregated, cells were cultured to midlinear phase before subculture to a final optical density (measured at 750 nm) of 0.1. Cultures were grown for 24 h and then L-rhamnose added to a variety of final concentrations. The optical density of cultures was monitored at 750 nm, and high-resolution fluorescence intensity measurements of each cell was performed using flow cytometry using an Attune NxT Flow Cytometer (ThermoFisher). 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. Histograms of fluorescence intensity were plotted, and mean statistics extracted. The inducer response curves and timecourses were analyzed using the "compareGrowthCurves" function from the statistical modeling package, statmod,[76] to determine whether differences between groups were statistically significant.

## ■ ASSOCIATED CONTENT

### Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acssynbio.7b00435.

Supporting methods, tables, and figures (PDF)

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### Author Contributions

CK and JH designed the study; CK and GT performed experiments; CK, AH and ATM performed plasmid construction; CK and JH prepared the manuscript with input from GT, AH and ATM.

### Notes

The authors declare no competing financial interest.

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