Comparative analysis of inducible promoters in cyanobacteria

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Abstract

Research in the field of synthetic biology highly depends on efficient, well-characterized promoters. While great progress has been made with other model organisms such as *Escherichia coli*, photosynthetic cyanobacteria still lag behind. Commonly used promoters that have been tested in cyanobacteria show weaker dynamic range or no regulation at all. Alternatives such as native metal-inducible promoters pose the problem of inducer toxicity.

Here, we evaluate four different inducible promoters, both previously published and new, using the modular plasmid pSHDY, in the model cyanobacterium *Synechocystis* sp. PCC 6803 - namely the vanillate-inducible promoter P_{vanCC} , the rhamnose-inducible P_{rha} , and the aTc-inducible P_{L03} , and the Co^{2+} -inducible P_{coaT} . We estimate individual advantages and disadvantages, as well as dynamic range and strength of each promoter in comparison with well-established constitutive systems. We observed a delicate balance between transcription factor toxicity and sufficient expression to obtain a dose-dependent response to the inducer. In summary, we expand the current understanding and employability of inducible promoters in order to facilitate the construction of more complex regulatory synthetic networks, as well as more complicated biotechnological pathways for cyanobacteria.

Keywords: *Synechocystis*, Inducible promoter, synthetic biology, cyanobacteria, pSHDY

Abbreviations

aTc	anhydrotetracycline
<i>Escherichia coli</i>	<i>E. coli</i>
LAHG	light-activated heterotrophic growth
RBS	ribosome binding site
<i>Synechocystis</i>	<i>Synechocystis</i> sp. PCC 6803
WT	wild type
OD ₇₅₀	Optical density at 750 nm
TSS	Transcription start site

Introduction

Cyanobacteria are versatile photoautotrophic organisms that are becoming more and more interesting for various research applications. Due to their ability to fix carbon photosynthetically, they are emerging as promising candidates for the biotechnological production of different compounds, including biofuels¹ and complex secondary metabolites².

Their ancestral relation to today's plant chloroplasts makes them important model organisms for foundational research in the field of photosynthesis³. In addition to this, many cyanobacteria are naturally competent and possess the ability to incorporate free DNA into their genomes as well as receive conjugative plasmids, making them attractive from a genetic engineering perspective⁴.

In recent years, more and more tools have been developed and characterized for diverse cyanobacterial species⁵. This includes well-studied model organisms such as *Synechocystis* sp. PCC 6803⁶, *Synechococcus elongatus* PCC 7942^{7,8} and *Anabaena* sp. PCC 7120⁹, but also some more recently discovered, fast-growing strains, such as *Synechococcus elongatus* UTEX 2973¹⁰ or *Synechococcus* sp. PCC 7002¹¹. One challenging aspect when applying promoters previously established in *Escherichia coli* is the difference in RNA polymerase architecture, which results in different binding affinities and overall responses to promoter and operator regions¹².

Nevertheless, a range of promoters, both constitutive^{6,10} and inducible¹³, has been engineered and successfully implemented in *Synechocystis* sp. PCC 6803 (referred to as *Synechocystis* hereafter). These publications tend to either focus on a single promoter construct with detailed work on sequence variations, or have a different core angle such as metabolic engineering. For the purpose of engineering more extensive synthetic regulatory networks, the availability of multiple differentially regulated promoters or regulatory building blocks is essential; for comparability, they should be characterized in a way that one can easily choose from depending on the application. In order to efficiently fine-tune and optimize more complex systems which combine transcriptional and translational output, fundamental evaluation of precise expression dynamics and strength in the context of a range of constitutive promoters is highly desirable.

One of the most standard inducible promoter systems, which is based on the *lac*-operon from *E. coli* and is inducible by the lactose analog IPTG, has been tested and implemented in some cyanobacterial species. The P_{trc} promoter, for example, performs well in *S. elongatus* and is commonly used in many applications¹⁴. However, efforts to implement similar constructs in *Synechocystis* have mostly failed, resulting in either extremely leaky expression under non-induced conditions or little to no regulation at all¹⁵. For example, Camsund *et al.* 2014 investigated sequence-specific repression patterns in *Synechocystis*¹⁶. They reported a 2.3-fold induction ratio for the original P_{trc} promoter, stating that this was likely due to insufficient levels of the repressor *lacI* in the cell, and furthermore that previous success in *S. elongatus* was likely a result of higher expression of *lacI*. Albers *et al.* 2015 investigated different IPTG-inducible constructs by modification of the gap between the sigma factor binding sites¹⁷. They placed *lacI* under the control of P_{sigA}, which promotes expression of the housekeeping sigma factor *sigA* and therefore assures stable, strong expression of the repressor. For their promoter construct P_{sca6-2}, they were able to show approximately 10-fold induction ratios.

Another well-characterized promoter in *Synechocystis* is the aTc (anhydrotetracycline)-inducible, *tetR*-regulated system. Huang *et al.* 2013 constructed a library by altering the region downstream of the -10 promoter region¹⁸. They reported induction ratios of up to 239 for their best performing promoter, P_{L03}, under LAHG (light-activated heterotrophic growth) conditions. This promoter suite was also successfully implemented by Yao *et al.* 2016 for dCas9-mediated gene repression, although they reported better success with the

weaker, more tightly repressed P_{L22} due to leaky expression of dCas9 from P_{L03} ¹⁹. Unfortunately, an issue with aTc in general is the fact that it is light-degradable, making its performance difficult to predict and also preventing it from stable expression under photoautotrophic growth conditions, as are common for most studies focusing on cyanobacteria.

A third system which was reported for *Synechocystis* by Kelly *et al.* in 2018 is the L-rhamnose-inducible promoter P_{rha} , which is regulated by the transcriptional activator $rhaS$ ²⁰. They thoroughly investigated this promoter under different light and nutrient conditions and found it to be tightly repressed under non-induced conditions, with a linear response upon induction and a good dynamic range, in addition to the inducer, L-rhamnose, being non-toxic to and non-metabolizable by the cells. To date, this is the best working promoter system in *Synechocystis* in terms of both performance and inducer characteristics.

A general issue when selecting promoters for different applications is the data reproducibility. Depending on factors like the choice of measurement methods, (reporter) genes, RBS / 5'UTR or growth conditions, effects on mRNA stability, fold activation or promoter strength may strongly differ between publications²¹⁻²³. While it remains true that each lab should reproduce measurements under their own conditions to ensure reproducibility, an evaluation of constructs in a side-by-side manner using comparable genetic elements and culturing conditions can be helpful in choosing a suitable promoter to begin with.

In contrast to cyanobacteria, there has been ongoing, successful work published for more accessible model organisms such as *E. coli*. For example, Ruegg *et al.* reported the optimization of a promoter system in *E. coli*, previously identified in *Enterobacter lignolyticus*²⁴, which responds to a variety of cationic dyes at very low, non-toxic concentrations, including the cheap inducer compound crystal violet, for which they report a dynamic range of four orders of magnitude.

Another recent publication focused on optimization of parameters such as binding of the transcription factor to the operator, full repression under non-induced conditions, and elimination of cross-talk using a two-phase directed evolution approach²⁵. Here, a positive selection process involving expression of DNA polymerase was combined with a negative selection involving the toxic expression of a mutant aminoacyl tRNA-synthetase. This

yielded 12 highly optimized promoter/sensor pairs, including a vanillate-inducible system originating from *Caulobacter crescentus*.

In this work, we constructed and investigated a comprehensive, comparative library of available different inducible promoters by evaluating them in the same genetic architecture, using the modular plasmid pSHDY. Alongside established aTc-, L-rhamnose-, and Co²⁺-inducible systems, we also present the newly tested vanillate-inducible promoter system.

Finally, we estimated individual promoter performance in a controlled setting for various downstream applications.

Results and Discussion

Design framework of all promoter constructs tested in *Synechocystis*.

In order to assay each promoter while ensuring comparability/reproducibility, a suitable reporter system was required. We considered a vector with two spatially separated cloning sites, in which the reporter construct comprising promoter, RBS and reporter gene could be located distantly from the repressor/activator and could easily be switched out. For this reason, we constructed pSHDY, a conjugative shuttle vector based on pVZ321²⁶, but much more suitable for cloning due to multiple restriction sites flanking the antibiotic resistance cassettes. In addition, pSHDY also contains the *mobAY25F* point mutation investigated by Taton *et al.*, which leads to an increase in supercoiled plasmid and therefore more efficient downstream cloning applications such as restriction digest²⁷.

The basic pSHDY cloning vector contains a total of three antibiotic resistance cassettes, chloramphenicol and kanamycin, which are flanked by two independent cloning sites termed the BioBrick and the NeoBrick site, respectively, and a spectinomycin resistance separating the two (*Fig. 1A*). For the purpose of comparability, we cloned each promoter/reporter construct into the BioBrick site, while keeping the corresponding repressor constructs in the NeoBrick site (*Fig. 1B*).

Fig. 1: Genetic composition of the different promoter and sensor constructs measured in this work.

A: Detailed overview of the two modular cloning sites, the NeoBrick (NB) shown in green and the BioBrick (BB) sites shown in yellow. Restriction site abbreviations: K: *KpnI*; Sa: *SalI*; Xh: *XhoI*; H: *HindIII*; E: *EcoRI*; Xb: *XbaI*; Sp: *SpeI*; P: *PstI*

B: Overview of the plasmid composition of reporter constructs used in this work.

P_{const.}: Constitutive promoter. Sp^R: Spectinomycin resistance. P_{ind.}: inducible promoter. ASV: *ssrA*-based ASV-degradation tag.

The promoter/reporter devices were constructed in a comparable manner. For the reporter, we chose mVenus, an eYFP variant with enhanced brightness²⁸. The gene was further codon-optimized for *Synechocystis* and a C-terminal *ssrA*-based ASV degradation tag for moderate protein turnover²⁹ was added to prevent measuring stable protein instead of inducer-dependent expression. For the RBS, we used the established synthetic RBS³⁰, which was shown to perform well in *Synechocystis* on multiple occasions, except in the native promoter constructs, P_{coaT}, as well as P_{cpc560}, which has been reported to require its native RBS for maximum strength¹⁰. In the case of P_{vanCC}, the *riboJ* insulator was kept in the 5'UTR as was constructed in the original publication²⁵.

Table 1 contains a basic description of all promoters tested in this work, while detailed descriptions and sequences of each promoter construct can be found in the Supplementary information (Table S1).

Table 1: Overview of promoter constructs tested in this work.

Inducible promoters are shown above, constitutive promoters below the double line.

Name	Full genotype of construct	inducer	regulator	5'UTR	Source
P _{vanCC}	NB_P _{J23100::vanR} _NB BB_P _{vanCC::riboJ} - RBS*::mVenus_BB	vanillic acid	<i>vanR</i>	riboJ + RBS*	²⁵
P _{rha} (111) P _{rha} (119)	NB_P _{J23111::rhaS} _NB NB_P _{J23119::rhaS} _NB BB_P _{rha::RBS*} ::mVenus_BB	L-rhamnose	<i>rhaS</i>	RBS*	²⁰
P _{L03(tetR-mut5)} P _{L03(tetR-mut8)}	NB_P _{J23119-mut5::rhaS} _NB NB_P _{J23119-mut8::rhaS} _NB BB_P _{L03::RBS*} ::mVenus_BB	aTc	<i>tetR</i>	RBS*	¹⁸
P _{coaT}	BB_ <i>coaR</i> _P _{coaT::mVenus} _BB	CoCl ₂	<i>coaR</i>	native	<i>Synechocystis</i>
P _{mpB}	BB_P _{mpB::RBS*} ::mVenus_BB	-	-	RBS*	<i>Synechocystis</i>
P _{cpc560}	BB_P _{cpc560::mVenus} _BB	-	-	native	<i>Synechocystis</i> ³¹
P _{J23119}	BB_P _{J23119::RBS*} ::mVenus_BB	-	-	RBS*	BioBrick
P _{J23100}	BB_P _{J23100::RBS*} ::mVenus_BB	-	-	RBS*	BioBrick
P _{rbcl}	BB_P _{rbcl::RBS*} ::mVenus_BB	-	-	131 bp native +RBS*	<i>Synechocystis</i>

Another consideration was encoding the repressor constructs on the genome and the promoter constructs on a plasmid, but we decided against it for two reasons.

Firstly, the copy number of the *Synechocystis* genome can fluctuate depending on different conditions such as growth phase, light intensity or nutrient availability, potentially resulting in different repressor copy numbers and subsequent strength of gene repression. In contrast, plasmid copy number is more stringently regulated within the cell, leading to more consistent results³². This also relates to the fact that expression may vary depending on the genomic context. Since different working groups have been using different genomic integration sites, data may not be directly reproducible.

Secondly, it takes longer to generate fully segregated genomic mutants, extending the amount of time between conceiving a project and measuring the data, further complicating rapid genetic screens.

Therefore, we determined a plasmid-encoded reporter system to be the best option.

Introducing the vanillate-inducible promoter P_{vanCC} in *Synechocystis*

While there have been publications on vanillate inducible systems, mainly in α -proteobacteria³³, but also in *E. coli*²⁵, to the best of our knowledge, this is the first detailed, dose-dependent promoter study in *Synechocystis*. One publication focusing on NOT-

gates in *S. elongatus* PCC 7942 used the promoter/repressor pair *vanR*/*P_{vanA}* from *Corynebacter glutamicum*³⁴. There, the regulation of the promoter-sensor pair was successfully implemented independently of the inducer vanillate. However, vanillate-dependent induction was not further investigated.

We ultimately chose to evaluate *vanR*/*P_{vanCC}* from the recent publication by Meyer *et al.*, 2018²⁵, in *Synechocystis*. Here, the promoter/repressor pair *vanR*/*P_{vanCC}* from *Caulobacter crescentus* was rationally designed and then further optimized via directed evolution for *E. coli*, yielding a vanillate sensor with both improved dynamic range, as well as lower cross-reactivity.

We chose the weak constitutive promoter *P_{J23100}* from the Anderson library and the published *van3* RBS to control *vanR*, shown schematically in Fig. 2A. The *van3-vanR* fusion was amplified from sAJM.1504, the Marionette-Clo strain (addgene ID 108251). For *P_{vanCC}*, we amplified the original promoter construct, including the *riboJ* insulator, from pAJM.714 (addgene ID 108515), but replaced the RBS with the synthetic RBS*. Detailed descriptions and sequences are provided in the supplementary information (Table S1).

The conjugative plasmid containing *P_{J23100}::vanR* and *P_{vanCC}::mVenus* were transferred into *Synechocystis* via conjugation. Transconjugants were validated, cultured, induced and mVenus fluorescence, as well as the optical density at 750 nm, was measured in a microplate reader. An empty vector control (EVC) was included for each concentration. 24h post-induction, we observed a linear dose-response to vanillate, which saturated at 1 mM (Fig. 2B). Furthermore, under non-induced conditions, the promoter remained tightly repressed, reaching the same autofluorescence levels observed in the empty vector control.

Fig. 2 Dose-dependent response of the vanillate-inducible promoter in *Synechocystis*.

A: Schematic overview of genetic construct used. Top: Genetic composition of regulator. Bottom: Genetic composition of regulated promoter. -10, -35 and +1 are highlighted in grey; RBS is shown in bold. Operator regions are underlined.

B: Dose-response of the vanillate-inducible promoter P_{vanCC} to different concentrations of vanillate after 24h

C: Dose-response of the vanillate-inducible promoter P_{vanCC} to different concentrations of vanillate after 48h

D: Response of $P_{vanCC}::mVenus$ to 500 μ M vanillate over time.

E: Growth of WT *Synechocystis* in different vanillate concentrations.

Three biological replicates were cultured in BG11 + vanillate and fluorescence and optical density was measured in a microplate reader.

¹van3 RBS ²⁵; ²RBS* from ³⁰

After 48h, a decrease in fluorescence to a fraction of that after 24h could be observed at lower concentrations (100-500 μ M), while fluorescence increased or remained at a similar level at saturating concentrations of 1-2 mM (Fig. 2C). While there is no evidence of light-mediated degradation of vanillate, it is an intermediate in the biochemical degradation of lignin³⁵, so we hypothesized that vanillate might be degraded by *Synechocystis* after longer periods of time. We therefore chose to investigate mVenus fluorescence over time. To account for possible inducer degradation, we chose a vanillate concentration of 500 μ M, which was below saturation of expression and at which concentration a decrease in fluorescence was observable.

After induction, cultures were measured every 6h. To account for effects caused by cell density, an aliquot of each culture was sampled and cell density was adjusted to the start OD₇₅₀ of 0,25 prior to each measurement.

P_{vanCC} rapidly responded to vanillate induction, reaching a fluorescence maximum approximately 24h post-induction. This level was maintained until 48h post-induction, after which fluorescence gradually decreased in a linear fashion, reaching autofluorescence levels 90h post-induction (Fig. 2D).

Since vanillate appears to be completely degraded by *Synechocystis* after 90h, we chose to investigate whether it had any effect on its growth. WT *Synechocystis* cells were treated with different concentrations of vanillate, and OD₇₅₀ was monitored over 7 days (Fig. 2E). Interestingly, vanillate had no positive or negative influence on growth of *Synechocystis*. We therefore hypothesize that degradation of vanillate occurs in an unspecific manner, not significantly contributing as a nutrient.

Overall, P_{vanCC} performs well in *Synechocystis* in a dose-dependent manner, showing no toxicity, tight repression and good dynamic range, with a maximum fold-induction of 16x (2 mM vanillate, 48h post-induction).

The strong rhamnose-inducible promoter can be fine-tuned via activator expression

While the degradation of vanillate shown for P_{vanCC} can be positive for certain applications, it can also be a drawback when a more long-term expression is desired.

Since the P_{rha} promoter published by Kelly *et al.*²⁰ showed such promising results both in dynamic range and stability over time, we aimed to reproduce the data. In accordance with our design framework, which allows for modular exchange of parts, we chose to investigate whether L-rhamnose response could be further tuned by fusing two different minimal constitutive promoters upstream of the activator gene *rhaS* - P_{J23119} , containing the *E. coli* consensus core elements and reportedly the strongest of the Anderson promoter library, and P_{J23111} , which was shown to be approximately half as strong as P_{J23119} in *Synechocystis*¹⁰ (Fig. 3A). Plasmids containing these fusions, as well as $P_{\text{rha}}::\text{mVenus}$, were transferred into *Synechocystis* via conjugation. Transconjugants were validated, cultured, induced and mVenus fluorescence, as well as the OD₇₅₀, was measured in the plate reader. An empty vector control (EVC) was included for each concentration. The strains were termed $P_{\text{rha}}::\text{mVenus}(119)$ or $P_{\text{rha}}::\text{mVenus}(111)$, with the number in parentheses corresponding to the respective Anderson promoter number.

A typical dose-dependent response can be observed in both reporter constructs 24 h post induction, saturating at approximately 10 mM L-rhamnose (Fig. 3B). A maximum fold induction of 55x and 39x is achieved for $P_{\text{rha}}::\text{mVenus}(119)$ and $P_{\text{rha}}::\text{mVenus}(111)$ at this concentration, respectively. While Kelly *et al.* don't specify fold change, a 15x increase under similar conditions can be roughly estimated from their data, indicating an overall improvement of promoter function. We hypothesize that this improvement can be attributed to both an increase of activator levels inside the cell and the use of the well-established RBS* instead of the native *E. coli* RBS, which may increase the maximal expression achievable with P_{rha} .

Fig. 3 Dose-dependent response of the rhamnose-inducible promoter *P_{rha}* in *Synechocystis*.

A: Schematic overview of genetic constructs used. Top: Genetic composition of regulator. Bottom: Genetic composition of regulated promoter -10, -35 and +1 are highlighted in grey; RBS is shown in bold. Operator regions are underlined.

B: Dose-response of the rhamnose-inducible promoter *P_{rha}* to different concentrations of aTc after 24h

C: Dose-response of the rhamnose-inducible promoter *P_{rha}* to different concentrations of aTc after 48h

D: Response of *P_{rha}::mVenus*(119) to 5 mM rhamnose over time. OD₇₅₀ of each sample was adjusted to 0,25 prior to fluorescence measurement.

E: Growth of WT *Synechocystis* in different rhamnose concentrations.

Three biological replicates were cultured in BG11 + inducer and fluorescence and optical density was measured in a microplate reader.

¹ RBS* from ³⁰

When growing induced cultures for a longer amount of time, the general dose-dependent pattern remained the same for both strains. However, both overall fluorescence intensity, as well as fold induction at 10 mM appeared to further increase over time, up to 165x and 143x after 76 h for $P_{rha}::mVenus(119)$ and $P_{rha}::mVenus(111)$, respectively (Fig. 3C). Therefore, we decided to also evaluate the short- and long-term temporal expression dynamics. To account for possible inducer degradation and reliably assert expression dynamics, we chose a rhamnose concentration of 5 mM, which was below saturation of expression.

After induction, cultures were measured every 6h. To account for effects caused by cell density, an aliquot of each culture was sampled and cell density was adjusted to the start OD_{750} of 0,25 prior to each measurement.

Fluorescence rapidly increased directly after induction. 18h post-induction, this increase became linear. Fluorescence continued to increase linearly until 136h post-induction, after which fluorescence levels remained stable for three more days (Fig. 3D). To investigate whether this was reversible, we performed a step-down by washing the cells twice with BG11 to remove all L-rhamnose from the media. OD_{750} was adjusted to 1.0. Fluorescence rapidly decreased after step-down, reaching pre-induction autofluorescence levels after 3 days.

Finally, we chose to investigate whether L-rhamnose had any effects on *Synechocystis* growth, since some of the concentrations used were higher than previously tested by Kelly *et al.* WT *Synechocystis* cells were treated with different concentrations of L-rhamnose, and OD_{750} was monitored over 7 days (Fig. 3E). Consistent with previous results, L-rhamnose had neither a positive nor a negative effect on *Synechocystis* growth. Moreover, the fluorescence time-course results further support the hypothesis that *Synechocystis* is unable to use L-rhamnose as a carbon source.

As already stated by Kelly *et al.*, P_{rha} performs exceptionally well as an inducible promoter, with a high dynamic range, tight repression, stable expression over at least 7 days, and no toxic effects or metabolization of the inducer.

The aTc-inducible promoter P_{L03} shows improved function by increasing the protein levels of the *tetR* repressor

Next, we chose to evaluate the P_{L03} promoter published by Huang and Lindblad¹⁸. Despite their promising results of 300x fold induction this promoter was also reported to lead to leaky expression of dCas9 by Yao *et al.*¹⁹. For these reasons, we chose multiple different design strategies to possibly reduce leakiness. Firstly, we removed the *ssrA*-based LVA degradation tag to overcome rapid degradation of the repressor protein. Secondly, we chose the strong promoter P_{J23119} in place of P_{J23101} to further increase intracellular TetR. Finally, we applied the same plasmid-based design strategy used for the other promoters to be able to compare the results later on.

Interestingly, we were unable to obtain clones with the expected regulatory sequences upstream of *tetR* planned *in silico*. Instead, each sequenced clone showed point mutations either in the promoter or RBS sequence, suggesting toxicity resulting from excessive expression of *tetR*. Since we preselected clones that showed no fluorescence in *E. coli* for sequencing, indicating tight repression of P_{L03} in *E. coli*, we decided to investigate two of them despite the point mutations. We termed them *tetR*-mut5 and *tetR*-mut8. Fig. 4A highlights the genetic composition of the two mutants compared to the desired construct. Cultures containing the plasmid constructs were treated identically to the ones containing the P_{vanCC} and P_{rha} promoter constructs. For the purpose of employing this promoter in broad, standard applications, we limited our experimental setup to photoautotrophic growth conditions (see the Method section for details), despite Huang *et al.* reporting better results for cultures grown in red light and LAHG.

Fig. 4B shows the dose response of the two mutant constructs 24h post-induction. Interestingly, the dose response assay shows the expected linear aTc-dependent increase of relative fluorescence. The fold change at 1000 nM aTc was lower than for rhamnose with 16-fold and 11-fold for *tetR*-mut5 and *tetR*-mut8, respectively. While the *tetR*-mut8 strain outperforms *tetR*-mut5 both in dynamic range and maximum strength of the promoter in terms of relative fluorescence achieved, it also shows minimally higher leaky expression under uninduced conditions (Fig. 4 B).

Fig. 4 Dose-dependent response of the aTc-inducible promoter P_{L03} in *Synechocystis*.

A: Schematic overview of the mutant variants with the intended construct ($P_{J23119}::tetR$) as a reference. Top: Genetic composition of regulator. Bottom: Genetic composition of the regulated promoter P_{L03} . -10, -35 and +1 are highlighted in grey; RBS is shown in bold. Point mutations / deletions are shown in blue.

B: Dose-response of the aTc inducible promoter P_{L03} to different concentrations of aTc after 24h

C: Dose-response of the aTc inducible promoter P_{L03} to different concentrations of aTc after 48h

D: Growth of *Synechocystis* WT supplemented with different concentrations of aTc. Three biological replicates each were cultured in BG11 and measured in the spectrophotometer.

E: Response of P_{L03} (*tetR*-mut5) to 500 nM aTc over time. OD_{750} of each sample was adjusted to 0,25 prior to fluorescence measurement.

Two and three biological replicates for *tetR*-mut5 and *tetR*-mut8, respectively, were cultured in BG11 + inducer and fluorescence and optical density was measured in a microplate reader.

¹ RBS* from ³⁰

The dynamic range of mVenus expression decreased over time; by 48h post-induction, fluorescence had significantly decreased to a fraction of what was measured before (Fig. 4C). We therefore decided to also evaluate the short- and long-term temporal expression dynamics.

To account for possible inducer degradation, we chose an aTc concentration of 500 nM, which was below saturation of expression.

After induction, cultures were measured every 6h. To account for effects caused by cell density, an aliquot of each culture was sampled and cell density was adjusted to the start OD₇₅₀ of 0,25 prior to each measurement.

Consistent with the results observed for P_{vanCC} and P_{rha}, fluorescence rapidly increased, reaching a maximum after 18h (Fig. 4D). However, in contrast to P_{vanCC}, fluorescence decreased again just as rapidly, reaching autofluorescence levels after 66h. In accordance with published literature, the rapid decrease in fluorescence is most likely a result of light-mediated degradation of aTc. As reported in Huang *et al.*, this promoter likely performs much better under LAHG in darkness or red light. However, for reasons stated earlier, we chose not to further investigate P_{L03} under these conditions.

Since aTc is a derivative of the antibiotic tetracycline, there have been reports on its toxicity in *E. coli* at high concentrations³⁶. Thus, we were interested in its effects on the growth of *Synechocystis* WT at the relevant concentrations used for induction of P_{L03}.

Interestingly, aTc-treated cells show minimally improved growth compared to untreated cells (Fig. 4E). We attribute this effect to hormesis, a positive effect on growth often observed in bacteria as a result of a global stress response to sublethal concentrations of antibiotics³⁷. At concentrations relevant for the induction of P_{L03}, aTc appears to have no growth-inhibiting effect on *Synechocystis*.

As previously shown by Huang *et al.*, P_{L03} performs well as an inducible promoter. Providing a suitable intracellular amount of TetR, it shows minimal leakiness and good dynamic range. Especially during the first 24h, it shows rapid, strong induction, making it a suitable tool for applications within this time-frame. Due to the light-sensitive properties of aTc, this promoter may be better suited under red light or darkness for longer term induction experiments. It also may be beneficial for the half-life of aTc to adjust the culture conditions to a higher cell density, thereby preserving the aTc due to shading.

Evaluating the native Co²⁺-responsive promoter, P_{coaT}, as an inducible promoter

Next, we decided to investigate a commonly used metal-inducible promoter. Since the most efficient and commonly used Ni²⁺-responsive promoter, P_{nrsB}, has already been investigated in detail elsewhere¹³, we chose P_{coaT}. This promoter was successfully used towards small-scale biotechnological production of plant terpenes³⁸ and ethylene³⁹ in *Synechocystis*, as well as for mimicking a null mutant in the filamentous cyanobacterium *Anabaena* sp. by selectively removing Co²⁺ and Zn²⁺ from the media⁴⁰.

Since the TSS of P_{coaT} has, to the best of our knowledge, not been mapped previously, we performed 5'RACE (rapid amplification of cDNA ends) to determine the TSS of P_{coaT}. Our results indicate at least 3 putative TSS for P_{coaT} (Fig. 5A, Fig. S1). We therefore decided to maintain the native promoter+5'UTR architecture, and fused the entire 1195 bp upstream of *coaT*, including the *coaR* repressor, upstream of mVenus.

Upon induction with different concentrations of CoCl₂, a linear response could be observed up to a concentration of 10 μM (Fig 5B). For higher concentrations, the values measured became erratic for both P_{coaT}::mVenus and EVC. This is likely due to toxic effects of Co²⁺ ions.

Upon investigating effects of relevant CoCl₂ concentrations on growth of WT *Synechocystis*, a slight defect in growth was observed at 10 μM (Fig. 5C). This effect was even stronger in P_{coaT}::mVenus (data not shown), which is consistent with previous observations, where it was reported that deletion of *coaT* led to higher cobalt sensitivity⁴¹. Increasing the amount of CoaR repressor in the cell, as done in this work by expression of an additional copy from a plasmid, likely has the same effect. Moreover, the maximum working concentration of Co²⁺ reported throughout the literature for the P_{coaT} promoter is 6 μM⁴² indicating toxic effects at higher concentrations.

Fig. 5 Dose-dependent response of *Synechocystis* to Cobalt.

A: Schematic overview of genetic construct used. -10, -35 and putative +1 are highlighted in grey.

B: Dose-response of *P_{coaT}::mVenus* to different concentrations of CoCl₂ after 48h

C: Cobalt-dependent growth behavior of WT *Synechocystis* over time. Optical density

Three biological replicates were cultured in BG11 + inducer and fluorescence and optical density was measured in a microplate reader.

More importantly, for complete repression of *P_{coaT}*, it is necessary to culture strains in Co²⁺-depleted BG11. Since Co²⁺ ions are required for the synthesis of coenzyme B₁₂ in diverse cyanobacteria⁴³, this means that complete repression of the promoter may require a defect in growth as a result of nutrient limitation.

When looking into temporal expression dynamics, Englund *et al.* could show a decrease of fluorescence for *P_{nrsB}*, due to Ni²⁺ actively being pumped out of the cells¹³. We hypothesize that this is also the case for Co²⁺, since *coaT* also encodes an efflux pump. *P_{coaT}* specifically, as well as metal-inducible promoters in general, are unsuitable as inducible promoters in synthetic biology. They lack orthogonality, require laborious alteration of standard culture media, show inducer toxicity at higher concentrations and are outperformed by all three inducible systems shown in this work, both in terms of dynamic range and maximum strength.

Different inducible promoters cover a wide range of expression levels

Finally, we measured the performance of each promoter alongside each other, either uninduced or induced. In order to categorize each promoter within a broader range, we included the native promoter constructs P_{cpc560} , P_{rnpB} and P_{rbcL} , as well as the minimal constitutive promoters P_{J23100} and P_{J23119} .

All strains were cultured in accordance with the dose-response assays shown previously. Transconjugants were validated, cultured, and mVenus fluorescence, as well as the optical density at 750 nm, was measured in a microplate reader after 24h. For all four inducible promoters, cultures both uninduced and induced with 10 mM L-rhamnose, 1 μ M aTc, 1 mM vanillate or 10 μ M $CoCl_2$, were grown and measured.

Consistent with the previous results, the fluorescence of P_{rha} is the strongest of the inducible promoters, closely followed by P_{L03} and P_{vanCC} (Fig. 6 A).

While these three show promise both in terms of dynamic range and strength, P_{coaT} is by far the weakest of the four. The uninduced control, which was cultured in regular BG11 instead of BG11 lacking $CoCl_2$, shows leaky induction, leading of a fold change of only 2x for P_{coaT} .

The strongest inducible promoter, P_{rha} , is still weaker than P_{cpc560} , the “super-strong” promoter published by Zhou *et al*³¹. This promoter enabled expression of heterologous proteins leading up to 15 % of total soluble protein. However, the data shown in Fig. 6A was measured 24h post-induction, and P_{rha} shows a steady and strong increase in fluorescence over 7 days (Fig. 3D). It could be assumed that P_{rha} is able to reach levels similar to P_{cpc560} after a sufficient induction time.

Fig. 6 Comparison of established constitutive promoter and inducible promoters.

A: Comparison of inducible with constitutive promoters. EVC: Empty vector control. P_{rha}: P_{rha}::mVenus(119). P_{L03}: P_{L03}::mVenus (*tetR*-mut5).

B: Evaluation of inducer specificity.

Three biological replicates each were cultured in BG11 + inducer (10 mM rhamnose, 1 mM vanillate or 1 μM aTc or combinations thereof, marked by a + when present or a – when absent) and fluorescence and optical density was measured in a microplate reader after 24 h.

In order to investigate inducer specificity, each single promoter construct was also induced with all possible combinations of inducers. Cultures were induced with 10 mM L-rhamnose, 1 μ M aTc or 1 mM vanillate, or a combination thereof. If left uninduced, the corresponding volume of solvent (H₂O or ethanol) was added.

All promoters show specific induction only in the presence of the respective inducer molecule (Fig. 6B). The level of fluorescence appears to be the same regardless of the presence or absence of the other inducers for each promoter. In terms of inducer specificity, the promoter constructs are therefore compatible with one another. It remains to be investigated whether they are truly orthogonal to each other in terms of transcription factor binding specificity, i.e., whether the transcriptional regulators are able to bind to unspecific operator sequences and activate or repress gene expression.

Conclusions & Outlook

In this work, we constructed and evaluated a library of different inducible promoters in a way that enables a useful comparison for later selection of a suitable promoter in *Synechocystis* sp. PCC 6803. Using the pSHDY plasmid allowed efficient exchange of parts to build this library, as well as comparable conditions. We observed a delicate balance between transcription factor toxicity and sufficient expression to obtain a dose-dependent response to the inducer. This observation should be kept in mind for future works, as it might significantly improve the performance of other promoters. Next to the established aTc- and rhamnose-inducible promoters P_{L03} and P_{rha}, we report the vanillate inducible promoter P_{vanCC} as a new tool for *Synechocystis*. All three promoters show a linear induction over a range of inducer concentrations, as well as little to no leakiness in the absence of the inducer. Interestingly, they show different strengths of expression, as well as different temporal expression patterns, with the potential for a wide range of biological applications. Thus, our promoter library allows moving away from metal-inducible promoters and towards well-characterized, defined and orthogonal parts, a key requirement of synthetic biology.

The next step in applying the three inducible promoters for future works would be evaluating their performance in a strain genomically encoding the transcriptional regulators. Ultimately, encoding the regulators on the genome using a markerless

genomic manipulation strategy would facilitate working with cyanobacteria, since it would free available space on the plasmid, as well as antibiotic resistances. This strategy has proven successful in the past in *E. coli*, resulting in many expression strains for different applications.

Finally, all three promoters should be combined with different reporter genes each and encoded in one strain to evaluate whether they are truly orthogonal and whether they can be used in combination to control multiple genes or operons, enabling the possibility for larger synthetic networks or metabolic engineering by optimization of metabolic pathways.

Material & Methods

Plasmid and strain construction.

A detailed list of all relevant genetic modules and information regarding their origin, as well as plasmids constructed from them, is provided in the Supplemental Information (Table S1).

All parts were amplified and fused using overlap extension PCR ([dx.doi.org/10.17504/protocols.io.psndnde](https://doi.org/10.17504/protocols.io.psndnde)) and integrated into the pSHDY backbone via Gibson assembly ([dx.doi.org/10.17504/protocols.io.n9xdh7n](https://doi.org/10.17504/protocols.io.n9xdh7n)).

Plasmids were transferred to *Synechocystis* sp. PCC 6803 wild type using triparental mating ([dx.doi.org/10.17504/protocols.io.psndnde](https://doi.org/10.17504/protocols.io.psndnde)). Clones were verified via colony PCR ([dx.doi.org/10.17504/protocols.io.mk5c4y6](https://doi.org/10.17504/protocols.io.mk5c4y6)).

Culture conditions.

All strains were maintained on BG11 plates containing 40 µg/mL spectinomycin.

Prior to each assay, BG11 + 20 µg/mL spectinomycin were inoculated with the strain of interest, grown for 5 days, diluted to an OD₇₅₀ of 0.2, grown for 3 more days, and diluted again to the desired OD₇₅₀ (specified in each assay) prior to starting the experiment.

Liquid cultures were grown in constant white light (80 µmol·m⁻²·s⁻¹, 16% intensity setting in the Infors HT multitron) at 30 °C and 75% humidity with constant agitation at 150 rpm without added CO₂.

Detailed protocols for each assay can be found on protocols.io:

Dose response assay → [dx.doi.org/10.17504/protocols.io.55wg87e](https://doi.org/10.17504/protocols.io.55wg87e)

Toxicity assay → [dx.doi.org/10.17504/protocols.io.6tghejw](https://doi.org/10.17504/protocols.io.6tghejw)

Fluorescence time course assay → [dx.doi.org/10.17504/protocols.io.6tkhekw](https://doi.org/10.17504/protocols.io.6tkhekw)

Measurements & settings.

To determine cell density, absorbance of cells was measured in a Specord 200 Plus spectrophotometer (Analytik Jena) at 750 nm.

Fluorescence measurements were performed using a BMG Clariostar. Absorbance at 750 nm, as well as fluorescence at $\lambda^{\text{ex}}/\lambda^{\text{em}}$ 511/552, was measured every time. Prior to each measurement, the plate was shaken at 500 rpm for 30 seconds.

The exact protocol for the BMG can be found in Supplementary File 1.

Data analysis & -treatment.

For dose response assays, fluorescence values were divided by OD₇₅₀.

For fluorescence time course assays, fluorescence values were divided by OD₇₅₀. Then, the mean of the values measured for the uninduced control culture was subtracted from each individual value measured for the induced culture.

For the fluorescence time courses, all raw fluorescence values were normalized to OD₇₅₀, then, the mean fluorescence of the uninduced control was subtracted from each value of the induced culture.

The plasmid pAJM.714, as well as the strain sAJM.1504 were a gift from Christopher Voigt (Addgene plasmid # 108515; Bacterial strain # 108251).

Author Contributions:

AB and IMA designed and conceived the study. AB and PS performed the experiments and analyzed the data. AB wrote the manuscript with input from PS and IMA.

Conflict of Interest Disclosure:

The authors declare no conflict of interest.

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