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# Synthetic Biology Toolbox for Controlling Gene Expression in the Cyanobacterium Synechococcus sp. strain PCC 7002

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S *Supporting Information*

ABSTRACT: The application of synthetic biology requires characterized tools to precisely control gene expression. This toolbox of genetic parts previously did not exist for the industrially promising cyanobacterium, *Synechococcus* sp. strain PCC 7002. To address this gap, two orthogonal constitutive promoter libraries, one based on a cyanobacterial promoter and the other ported from *Escherichia coli*, were built and tested in PCC 7002. The libraries demonstrated 3 and 2.5 log dynamic ranges, respectively, but correlated poorly with *E. coli* expression levels. These promoter libraries were then combined to create and optimize a series of IPTG inducible cassettes. The resultant induction system had a 48-fold dynamic range and was shown to out-perform P$_{trc}$ constructs. Finally, a RBS library was designed and tested in PCC 7002. The presented synthetic biology toolbox will enable accelerated engineering of PCC 7002.
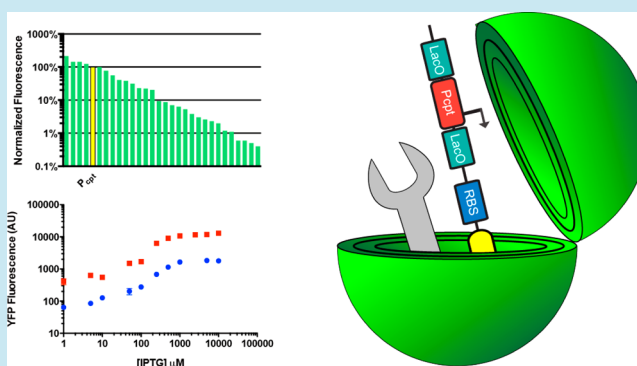
At the most basic level, synthetic biology relies on the ability to integrate a set of well-defined DNA sequences to obtain a desired phenotype. Protein levels are commonly controlled by the modulation of transcription and translation initiation rates. For example, transcription rates are changed by fusing a gene to promoters of varied strength or modulating the degree of repression by titrating inducers (e.g., the lac promoter family is modulated by IPTG addition)[1] while translation rates are controlled by changing the strength and accessibility of ribosome binding sites.[2] Libraries of constitutive promoters have been developed via mutagenesis of base promoters for several model organisms including *Escherichia coli* and *Saccharomyces cerevisiae*.[3−6] Inducible promoter systems, such as T7/lac[7] and P$_{trc}$,[8] have been created by fusing repressor sequences to promoter sites. Alternatively, biophysical models have been developed to predict and guide engineering of translation initiation signals in bacteria, thereby providing an additional point of control.[2,9,10] While these approaches have allowed for the development of complex genetic circuits in *E. coli*, many of these tools have not been developed or validated for use in nonmodel organisms.

Cyanobacteria are an attractive host for metabolic engineering due to their innate ability to convert CO_2 and sunlight directly into a chemical product of interest, thereby bypassing the need for expensive feedstocks. Model strains of cyanobacteria have been modified to overproduce a wide variety of products,[11] including sucrose,[12] 2,3-butanediol,[13] and ethylene;[14] however, none of these photosynthetically produced products have been at a rate that is economically viable. Attempts to increase productivity and titer have been hampered by a limited set of genetic tools available for metabolic engineering in cyanobacteria. Few promoters have been developed for applied projects in cyanobacteria; well-characterized endogenous promoters such as P$_{psbA}$ or P$_{cpcB}$ are typically used in synthetic biology strategies. These promoters control transcription of photosystem genes making them often too strong and undesirably sensitive to light.[15] Endogenous metal responsive promoters have been developed for use as induction systems with better than 100 fold dynamic range; however, the low sensitivity and complex off target regulatory effects make these systems difficult to use in practice.[16] Finally, several orthogonal genetic tools originally developed in *E. coli*, such as P$_{trc}$, P$_{lacI}$, and P$_{tet}$, have been tested in cyanobacteria with poor or inconsistent results across model cyanobacterial systems.[17]

Recently, improvements have been made to small molecule induction systems in model cyanobacteria. In particular, new IPTG induction systems have been described for *Synechocystis* sp. strain PCC 6803 (PCC 6803) and *Synechococcus* sp. strain PCC 7942 (PCC 7942).[18,19] While these works are improvements of previously available systems, the dynamic ranges were limited: 13-fold for PCC 6803 and 18-fold for PCC 7942. As an alternative to IPTG induction, an anhydrotetracyline induction

system was developed for PCC 6803.[20] This system demonstrated a 239-fold dynamic range under dark heterotrophic growth conditions but only 49-fold dynamic range under photoautotrophic growth conditions when induced with an exceptionally high concentration of anhydrotetracycline (10 ug/mL).[20] While there has been some development of synthetic biology tools for PCC 6803 and PCC 7942, other cyanobacteria used in metabolic engineering remain limited to a few native promoters. In particular, *Synechococcus* sp. strain PCC 7002 (PCC 7002), an attractive strain for metabolic engineering due to its rapid growth rate, high CO_2 fixation rate, and halotolerance, has very few promoters of known strength and only one reported inducible promoter.[16,21]

The objective of this work was to develop a set of defined synthetic biology tools that would enable precise control of gene expression in PCC 7002. Herein, we present two constitutive promoter libraries in PCC 7002 containing promoters with a range of strengths spanning 3 orders of magnitude. These promoter libraries were used to construct an IPTG induction system based on a promoter of cyanobacterial origin. Iterative modifications to this system resulted in stronger repression and higher intrinsic expression levels. The resulting IPTG induction system was capable of a 48 ± 7 fold increase of YFP expression. This induction ratio represents over an order of magnitude improvement over the next best small molecule inducible system in PCC 7002[16] but is an order of magnitude lower than P_{trc} in *E. coli*.[22] Finally, a cyanobacterial ribosome binding site (RBS) library was designed with *in silico* modeling tools and validated in PCC 7002. The models were sufficient to predict gross changes in expression, but were not capable of predicting subtle differences. We predict that these tools will enable construction of more complex genetic circuits in PCC 7002 and increase the number of successful metabolic engineering projects in this promising cyanobacterium.

■ RESULTS AND DISCUSSION

Promoter Library Based on the PCC 6803 *cpcB* Promoter. Until recently, the *cpcB* promoter from PCC 6803 ($P_{PCC6803cpcB}$) was the best characterized promoter for use in PCC 7002. It was initially designed to drive gene overexpression as part of the high-copy plasmid pAQ1 homologous recombination system.[21] Recently, a chromosomal recombination system based on acrylic acid counter selection was developed that also used $P_{PCC6803cpcB}$ for gene expression.[23] In preliminary studies, we found that a single strong constitutive promoter was not applicable for most metabolic engineering applications. Therefore, we mutagenized $P_{PCC6803cpcB}$ to create a library of constitutive promoters capable of generating more modest levels of gene expression. To simplify library construction, $P_{PCC6803cpcB}$ derived from plasmid pAQ1_Exp[21] was truncated from its original 500 bp length to a more easily modifiable 89 bp core promoter region. This region was chosen based on homology of *cpcB* promoters in closely related cyanobacteria. The resulting promoter was called P_{cpt} for cyanobacterial phycocyanin truncated promoter. $P_{PCC6803cpcB}$ strength was compared to P_{cpt} using a homozygous chromosomal YFP system in PCC 7002. The truncation reduced YFP fluorescence by 50%; however, P_{cpt} expression was not significantly affected by high light intensity; in contrast, a ~30% reduction was seen in $P_{PCC6803cpcB}$ controlled YFP expression under high light (Figure 1). Previous transcription profiling in PCC 7002 showed a similar decrease in full length P_{cpcB} expression under high light conditions.[24] These data
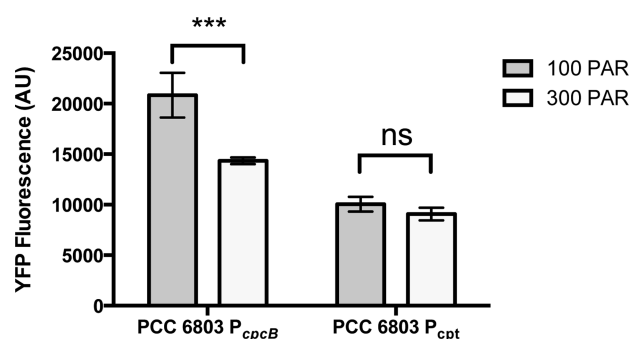

Figure 1. Expression of full length and truncated PCC 6803 *cpcB* promoters under high and low light conditions. Light intensity is shown as PAR (photosynthetically available radiation) as measured in $\mu E/m^2 \cdot s$. The full length $P_{PCC6803cpcB}$ exhibited reduced expression under high light (p-value <0.005). No significant difference was observed between high and low light conditions for the truncated promoter. Error bars represent standard deviations of biological replicates ($n = 3$).

suggest that additional transcription factors beyond core RNA polymerase may be involved in generating maximum transcription rates from the P_{cpcB} core promoter.

To generate a promoter library, P_{cpt} was mutagenized with error prone PCR and fused with a YFP expression cassette. The promoter sequences from 107 strains were determined and aligned based on YFP expression. Several of these promoters had duplications or mutations outside of the core promoter region and were eliminated from further analysis, leaving 29 unique promoters (see Supporting Information Table 4 for sequences). YFP expression levels were screened in these segregated strains of PCC 7002 (Figure 2A). Of these promoters, 11 representative samples were chosen for further testing, covering the full range of YFP expression levels (Figure 2B). Expression from individual members of the P_{cpt} library spanned 3 orders of magnitude. Several strains displayed higher fluorescence than the basal P_{cpt} construct. One such strain, c223, displayed two times the YFP fluorescence as P_{cpt}, approaching the levels observed with the full length $P_{PCC6803cpcB}$ promoter. The sequence of c223 contained only two point mutations relative to the wild type promoter sequence (Figure 2C). These two mutations, A30T and A67T, were tested individually. The latter mutation was sufficient to double YFP expression by itself. Interestingly, this mutation changed the P_{cpt} -10 σ^{70} recognition site from TATAAA to TATAAT, the *E. coli* consensus sequence.[25]

Development of a Synthetic Promoter Library in PCC 7002. The latter finding raised the question of whether promoter libraries would be consistent between *E. coli* and PCC 7002. We therefore cloned and tested a series of well-characterized *E. coli* promoters to determine if they could serve as a second library in PCC 7002. A subset of the BioBrick promoters derived from part BBa_J23119, a σ^{70} consensus promoter, were combined with a synthetic 5′ UTR containing a RBS designed using a ribosome binding site calculator[2] and YFP (Figure 3A).

The expression level of these constructs were quantified under standard growth conditions and compared to constructs using $P_{PCC6803cpcB}$.[21] The expression cassette containing the promoter BBa_J23119, the strongest *E. coli* promoter, generated $35 \pm 3\%$ of the fluorescence of the strain using promoter $P_{PCC6803cpcB}$, one of the strongest promoters in PCC 7002. The closely related PCC 7002 homolog of $P_{PCC6803cpcB}$,
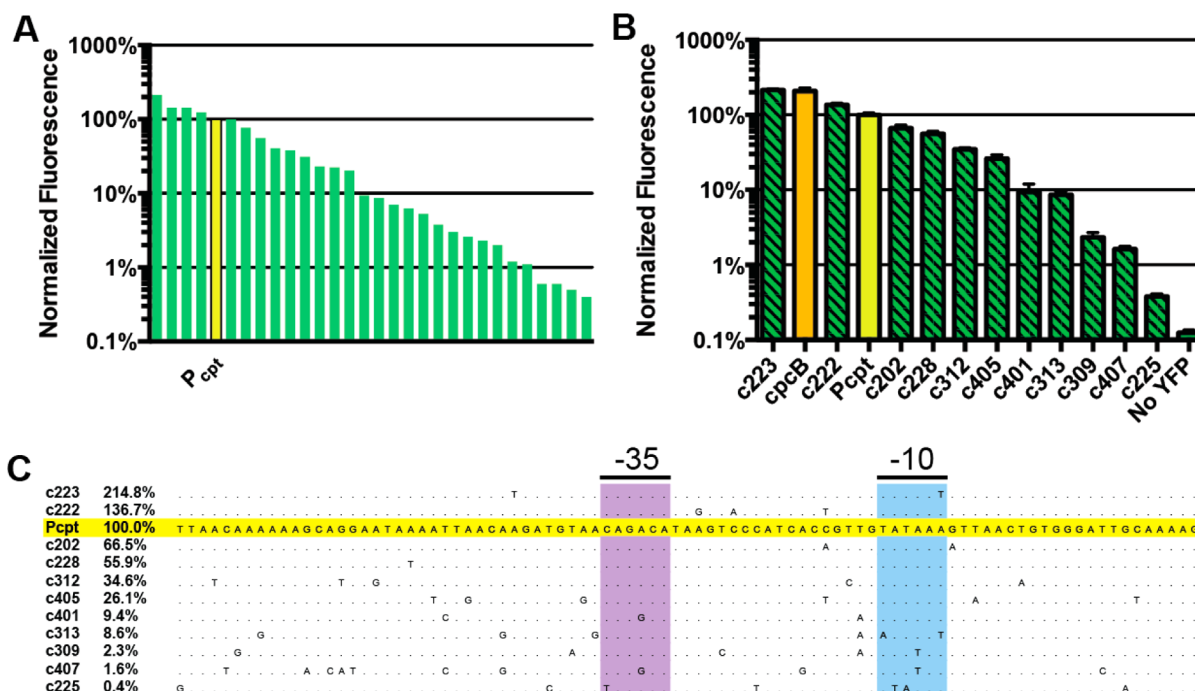
Figure 2. Normalized expression levels and sequences of members of the P_cpt promoter library. (A) Fluorescence values of library members normalized to expression level of P_cpt (yellow). (B) Fluorescence values of a representative sample of library members normalized to P_cpt (yellow). P_PCC6803cpcB is shown in orange and wild type PCC 7002 without a YFP expression construct is shown in solid green. Error bars represent the standard deviation of biological replicates ($n = 3$). (C) Sequence alignment of the representative library members. The predicted −35 and −10 regions are highlighted in purple and blue, respectively.

P_PCC7002cpcB, drives the expression of SYNPCC7002_A2209, the third most abundant transcript in PCC 7002.[24] Fluorescence from the other library members were all lower than BBa_J23119 spanning 2.5 orders of magnitude (Figure 3B). To increase the range of expression levels, additional permutations of BBa_J23119 were made, resulting in promoters pMB1, pMB2, and pMB3 (Figure 3A). When the relative fluorescence generated by these promoters in PCC 7002 were compared with the corresponding E. coli strengths published on the BioBrick Web site (http://parts.igem.org/Part:BBa_J23119), a weak correlation was observed ($R^2 = 0.434$) (Figure 4A). The mechanism behind this weak correlation is unclear but could be due to differences in the transcription machinery of these two organisms. Interestingly, the σ^{70} transcription factors of E. coli and PCC 7002 share strong homology, particularly at regions involved in DNA binding. In response to this weak interspecies correlation, the P_cpt promoter library was evaluated in E. coli. YFP expression cassettes from the 11 representative library members were inserted into an E. coli plasmid in order to test relative expression. As was seen with the synthetic promoter library, there was only weak correlation between the relative expression of the P_cpt library in PCC 7002 and E. coli ($R^2 = 0.366$) (Figure 4B).

Redesigning the P_cpt Promoter for IPTG Induction. While the constitutive promoter libraries offered a range of expression levels, many metabolic engineering projects require promoters that can be controlled in response to environmental signals. To make P_cpt responsive to the common laboratory inducer IPTG, the core promoter sequence was modified by replacing native sequence with lac operators to allow for binding of the lac repressor, LacI. Specifically, two lac operator sequence variants Oid and O1[7,17] were individually inserted 42

base pairs upstream of the predicted −35 promoter region and directly downstream of the predicted −10 promoter region of P_cpt, respectively, resulting in a promoter called P_cptOO (Figure 5A). Insertion of these two operators caused a $76 \pm 5\%$ decrease in its intrinsic promoter strength relative to P_cpt (Figure 5B). Next, the lacI gene under the E. coli P_lacI^Q promoter was inserted downstream of the YFP terminator. Addition of the lacI cassette resulted in decreased fluorescence relative to P_cptOO. Fluorescence was restored upon addition of 1 mM IPTG (Figure 5B). The resulting construct demonstrated only 4-fold induction but provided a starting point for promoter engineering.

Since this construct was fully inducible, an increased dynamic range would require stronger repression in the absence of the inducer and/or stronger expression in the absence of LacI. Poor repression could be attributed to suboptimal spacing of the operators or poor LacI expression. Attempts to move the operators closer together resulted in dramatic decreases in the intrinsic expression level of the promoter, resulting in constructs with limited utility and little increase in repression (data not shown). As an alternative, the promoter driving lacI, P_lacI was replaced with members of the synthetic promoter library (Figure 5C). Several of these promoters, in particular P_MB2, resulted in reduced induction system leakiness relative to the E. coli lacI^Q promoter. Replacing the P_lacI^Q promoter in the original induction system with P_MB2 resulted in a strain with 12-fold induction. This system was further improved by making a single amino acid substitution, W220F, in lacI which had previously been shown to strengthen LacI dimerization and provide tighter repression than wild type LacI.[22] This construct, cLac94, with P_MB2 driving the expression of a modified lacI, resulted in a system with 38 ± 6 fold induction using 5 mM IPTG (Figure 6A). A higher IPTG concentration was needed

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Figure 3. Schematic drawing and expression levels of the synthetic promoter library. (A) Drawing of synthetic promoter and 5′ UTR. Sequences for promoters J23119, pMB1, pMB2, and pMB3 are presented. The labeled transcription start site was determined for J23119 and pMB2. The promoter region is outlined in light green with the −35 and −10 regions in purple and light blue, respectively. The 5′ UTR is shown in blue with the RBS in red. (B) Expression levels of the synthetic promoter library relative to J23119. Error bars represent standard deviations of biological replicates ($n = 3$).

Figure 4. Comparison of promoter libraries in PCC 7002 and *E. coli*. (A) Comparison of BBa_J23119 derived promoters in *E. coli* and PCC 7002 relative to the expression level of J23100. $R^2 = 0.434$. (B) Comparison of the *cpcB* derived promoter library in *E. coli* and PCC 7002 relative to the truncated *cpcB* promoter. $R^2 = 0.366$. Error bars represent the standard deviation of biological replicates of biological replicates ($n = 3$).

to achieve maximum induction, possibly due to the tighter DNA binding exhibited by *lacI* W220F.

Increasing the Intrinsic Strength of P*cpcB*-Based Induction System. Optimizing *lacI* expression resulted in tight repression and a larger dynamic range upon induction of the P$_{cptOO}$ promoter. However, with these modifications, the maximum expression reached only $15 \pm 2\%$ relative to P$_{cpt}$ (Figure 6A). To address this limitation, the P$_{cptOO}$ region of cLac94 was mutated to incorporate advantageous base-pair

changes discovered during the construction of the P$_{cpt}$ promoter library (Figure 6B). The first P$_{cptOO}$ mutation tested was A67T, which was identified by testing the mutations present in the strong P$_{cpt}$ library member c223. The A67T mutation in the P$_{cptOO}$ construct, cLac109 increased YFP expression by $255 \pm 21\%$ upon induction with IPTG; however, it also significantly increased the leakiness of the promoter (defined as expression in the absence of IPTG), resulting in only a 11 ± 1 fold induction range (Figure 6B). Deletion of
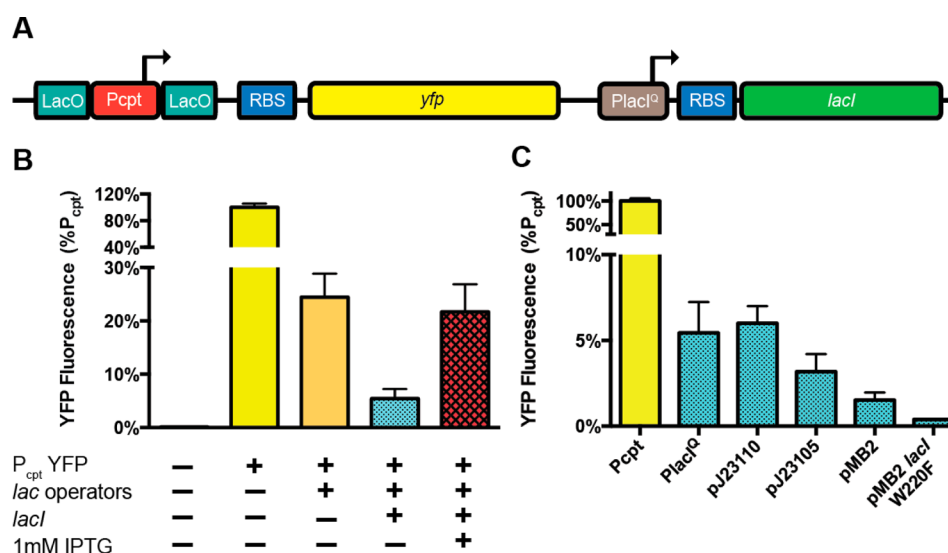
Figure 5. Initial development of an IPTG induction system based on the P_{cpt} promoter. (A) Fluorescence of PCC 7002 strains harboring promoters constructs normalized to P_{cpt} expression. Insertion of *lac* operators resulted in a ~80% decrease in expression relative to P_{cpt}. Introduction of *lacI* resulted in repression that was removed by the addition of IPTG. (B) Expression levels of uninduced *lac* induction strains with various *lacI* promoters relative to expression of P_{cpt}. (C) Optimization of *lacI* expression and activity using different promoters driving *lacI* and the W220F mutation improving lacI binding. Error bars represent the standard deviation of biological replicates ($n = 3$).
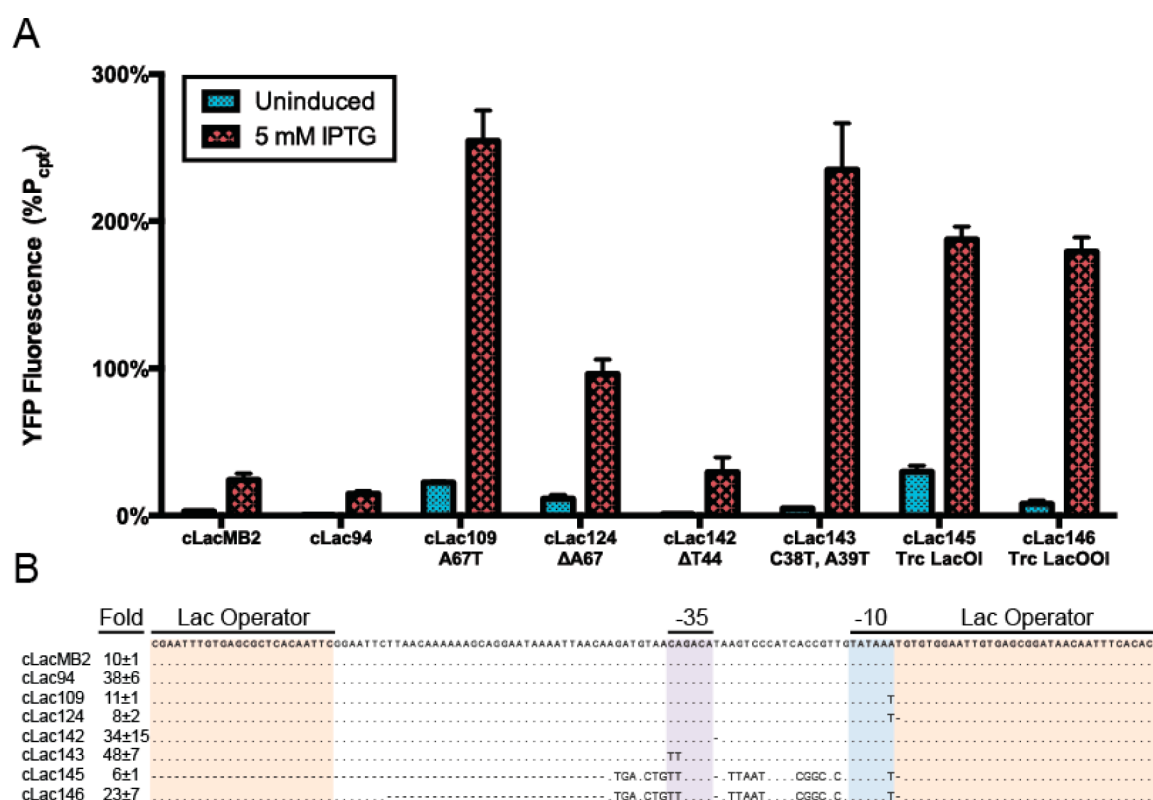
Figure 6. Diagram and induction levels for IPTG inducible promoters. (A) Expression of induced and uninduced IPTG inducible promoters relative to P_{cpt}. With the exception of the P_{cpt} control, all strains contain *lacI* expressed by P_{MB2}. All cLac### strains have the additional W220F mutation in *lacI* while cLacMB2 contains wildtype *lacI*. Error bars represent standard deviation of biological replicates ($n = 3$) (B) List of IPTG inducible promoters with fold induction potential (fold change between 0 and 5 mM IPTG) and sequence comparison. The *lac* operators are highlighted in yellow, the −35 sequence is highlighted in purple, and the −10 sequence is highlighted in green.

A67 (cLac124) provides a shorter overlap between the −10 and the downstream operator, analogous to the strong P_{trc} *E. coli* promoter. This deletion resulted in 96 ± 10% derepressed expression but only an 8-fold dynamic range. Finally, the spacing between the P_{cptOO} −35 and −10 binding site was reduced from 18 to the more common 17 base pairs, resulting in construct cLac142 (See Figure 6B). This resulted in a maximum derepressed expression of 30 ± 10% and a 34-fold induction. See Supporting Information Table 5 for all *lac* induction promoter sequences.

The increased expression seen when mutating the −10 promoter sequence to the *E. coli* σ[70] consensus sequence led us to try mutating the −35 P_{cptOO} from CAGACA to the *E. coli* consensus sequence TTGACA, creating the construct cLac143. This mutation increased the derepressed promoter strength of P_{cptOO} to 235 ± 32% relative to constitutive P_{cpt}. In the presence of LacI and absence of IPTG, YFP was expressed at 4.9 ± 0.2% of P_{cpt} resulting in a potential 48 ± 7 fold range of expression upon addition of IPTG (Figure 6A). Titration of IPTG resulted in qualitatively similar fluorescence curves (basal expression up to 10 μM and maximum expression at 1 mM) for the constructs with the highest dynamic range, cLac94 and cLac143 (Figure 7).
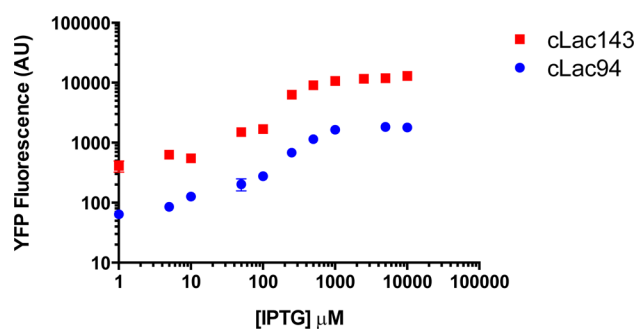
Figure 7. Titration of inducible promoter constructs cLac94 and cLac143 with varying concentrations of IPTG. Promoter cLac94 is shown in blue and promoter cLac143 is shown in red. Both strains contain *lacI* W220F expressed with pMB2. Error bars represent standard deviation of biological replicates ($n = 3$).

Comparison with trc Promoters. The performance of the P_{cptOO} induction system increased as mutations moved its sequence toward the *E. coli* consensus. This observation led us to reconsider P_{trc} as a viable inducible promoter in PCC 7002. Using our optimized *lacI* repression cassette, we replaced the P_{cpt} core promoter with P_{trc} using either one or two *lac* operators. The maximum derepressed YFP expression was similar for the one and two operator constructs, 188 ± 9% and 179 ± 10% relative to constitutive P_{cpt}, respectively. However, the noninduced P_{trc} promoter expression with one operator was significantly more leaky than the version with two operators resulting in a 6 (cLac145) and 23 fold induction range (cLac146), respectively (Figure 6A). The cLac146 induction system has the advantage of using a shorter promoter region; however, it has a lower dynamic range than both cLac94 and cLac143 making it less useful for genetic control in PCC 7002. P_{trcOO} could prove be a useful compliment to PcptOO, avoiding unwanted chromosomal recombination events due to regions of strong homology in strains with multiple gene insertions.

Design and Construction of a PCC 7002 RBS Library. To test whether an RBS library could be predictably constructed for PCC 7002, the Shine−Delgarno region of the previously described pACSA_J23119_YFP (AGGGAT) was modified using the RBS Library Calculator[26] to create an 8-member library (degenerate base sequence ASGMGR) specific to PCC 7002 with a predicted 143 fold range of translation initiation rates.[2] Two additional Shine−Delgarno sequences that were predicted to induce strong translation initiation rates were added (AGGGGG, AGGGAG), which increased the predicted library range to 213-fold. YFP fluorescence data in PCC 7002 showed that, in practice, this library provided a 30-fold range of protein expression with a weak correlation to their predicted strengths (Figure 8A). qPCR analysis of these strains
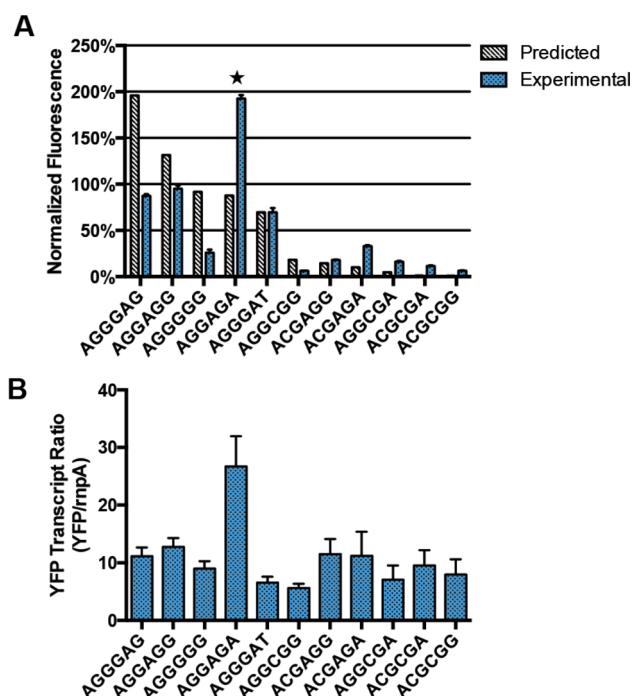

Figure 8. Evaluation of the RBS library versus predicted transcription initiation rates. (A) RBS Calculator v1.1 transcription initiation rate data was compared with YFP fluorescence by anchoring both data sets to AGGGAT, which is the RBS sequence of the original pJ23119_YFP construct. YFP fluorescence is represented as a percentage of constitutive P_{cpt} YFP fluorescence. Error bars on experimental data represent standard deviation of biological replicates ($n = 3$). Starred library member AGGAGA had significantly higher YFP transcript than every other library member (Figure 8B), which likely explains its observed deviation from the prediction. (B) Relative YFP transcript levels quantified by qPCR normalized to *rnpA*. Transcript levels for AGGAGA was significantly higher (95% CI) than every other strain in the RBS library. All other library members showed no significant difference between them (95% CI).

showed that one library member, AGGAGA, had a significantly higher YFP transcript level than any of the other library members (Figure 8B). The YFP expression cassettes were sequenced and no mutations were present (data not shown). Excluding AGGAGA removed an outlier and improved the fit of the model from $R^2 = 0.3760$ to $R^2 = 0.7443$ (Figure 9A and B). The RBS library sequences were also back calculated with two alternative translation rate prediction programs: the UTR Designer[9] and the RBSDesigner.[10] In this case, the correlation of the experimental data to the predicted expression level provided by the UTR Designer (Figure 9C) was similar to the RBS Calculator prediction; however, the analogous correlation to the RBSDesigner prediction was weaker (Figure 9D). These data suggest that the RBS Calculator is useful for designing RBS parts in PCC 7002; however, as shown in a recent paper by Li et al.,[27] fine control of RBS strength may not be possible with this prediction program.

Conclusions. PCC 7002 has the potential to become an important photosynthetic chemical production platform. To date, few products have been successfully made with this strain. This may, in part, be due to the dearth of genetic tools available for the strain. In this study, we developed two orthogonal
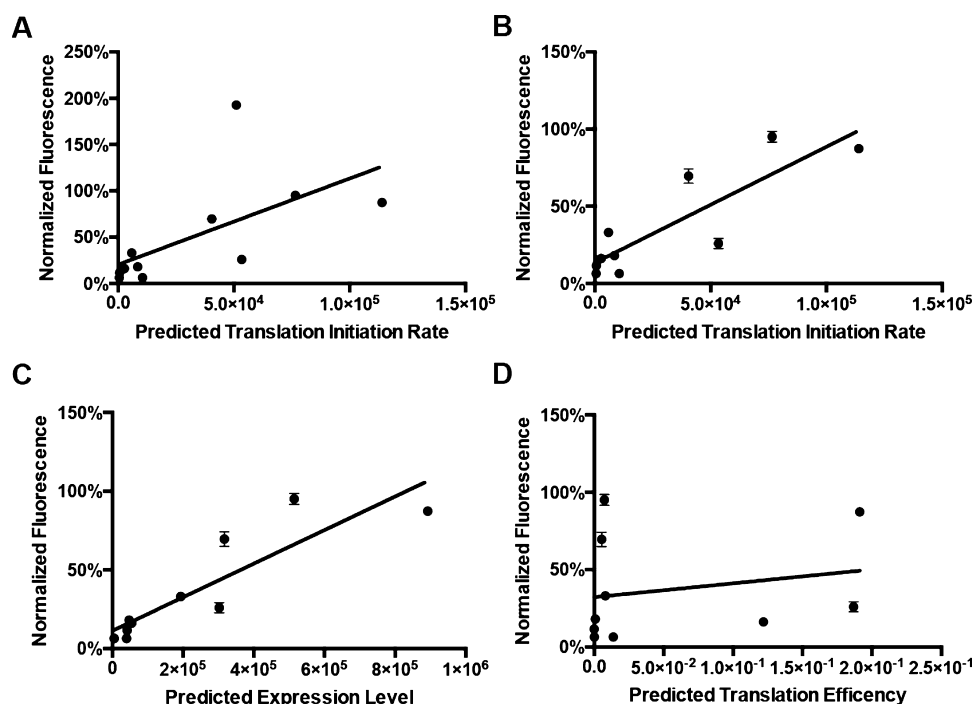
Figure 9. Correlation of the predicted translation initiation rates from the RBS Calculator to experimentally determined YFP fluorescence. (A) Correlation with AGGAGA included ($R^2 = 0.3760$). (B) Correlation with AGGAGA removed ($R^2 = 0.7443$). (C) Correlation of the experimental data set, excluding AGGAGA, to the UTR Designer ($R^2 = 0.7843$). (D) Correlation of the experimental data set, excluding AGGAGA, to the RBS Designer ($R^2 = 0.04448$). Error bars on experimental data represent standard deviation of biological replicates ($n = 3$).

constitutive promoter libraries with a 3 and 2.5-fold dynamic range respectively and used these libraries to optimize the most tightly controlled IPTG induction system available for any cyanobacterial species. Finally, we performed the first evaluation of the RBS Calculator in PCC 7002. The resultant RBS library showed a moderate correlation to the predicted values suggesting that the RBS library calculator can be used in PCC 7002 to predict gross differences in expression. A representative subset of the promoter library, RBS library, and IPTG inducible strains were also tested for relative expression at high cell densities ($OD_{730} \sim 5$) with high correlation to the data collected during log phase (OD_{730} 0.5, data not shown). This suggests that strains produced using these tools can be reliably used in higher density chemical production environments. The gene expression tools presented here, along with our previously published acrylic acid counter selection system[23] enable rapid construction of finely controlled genetic circuits within PCC 7002 and should accelerate the field of synthetic biology within cyanobacteria.

■ METHODS

Chemicals, Reagents, and Media. Acrylic acid was obtained from Fisher Scientific (AC16425). Restriction enzymes, Phusion DNA polymerase, and T4 DNA ligase were purchased from New England Biolabs (Ipswich, MA). All other chemicals and reagents were purchased from either Fisher Scientific or Sigma-Aldrich. Unless otherwise noted, PCC 7002 was grown in Media A+ and maintained on 1.5% agar plates.[28] Unless otherwise noted, cultures were grown in 10 mL volumes bubbled with air at 35 °C with a light intensity of 200 $\mu E/m^2 \cdot s$. E. coli were grown in lysogeny broth (LB).

Strain Construction. All strains used in this study are listed in Supporting Information Table 1. E. coli DH5α was used for

cloning of plasmids. Wild Type PCC 7002 was obtained from the Pasteur Culture Collection. Unless otherwise indicated, all genetic tools (i.e., promoters, ribosome binding sites) were constructed as fusions to the EYFP reporter gene[29] and cloned into pACSA_pcpcB_YFP.[23] The resulting plasmids contained the YFP expression cassette flanked with DNA sequences that would enable homologous recombination at the acsA loci of the PCC 7002 chromosome. Plasmids were amplified and stored in E. coli DH5α prior to transformation into PCC 7002 according to published protocols.[21] Homozygous recombinants were isolated using an acrylic acid counter selection as previously described.[23] Segregation was confirmed by colony PCR. See Supporting Information Table 3 for the oligonucleotide sequences used in this study. The standard deviations of the correlated values were propagated whenever average YFP fluorescence levels were compared.

Measurement of Fluorescence. All experiments began with isolation of single colonies from freezer stocks of segregated mutants. Cultures of PCC 7002 expressing YFP were grown in triplicate to an OD_{730} 0.5−1. Cultures were normalized to 1.5−3 $OD_{730} \cdot mL$ and centrifuged at 3000g for 12 min. The resulting pellets were aspirated, resuspended in 300 μL BugBuster Protein Extraction Reagent (Novagen), rocked at room temperature for 30 min, and centrifuged at 16 000g for 25 min at 4 °C. The florescence of the resulting supernatant was measured (excitation 514 nm, emission 527 nm) using a Tecan M1000 plate reader.

YFP expression in E. coli was similarly tested by growing DH5α containing pACSA plasmids with promoter or RBS library members in 5 mL of LB overnight at 37 °C with shaking. After 16 h the cultures were normalized to OD_{600} and split into triplicate 5 mL tubes and grown overnight again. The cultures were normalized to OD_{600} and 200 μL of each culture was analyzed for YFP fluorescence by plate reader.

Construction of a Promoter Library Based on the PCC 6803 P_{cpcB} Promoter. P_{cpt}, a truncated version of the $P_{PCC6803cpcB}$ promoter, was constructed by annealing two complementary, phosphorylated oligonucleotides. The resulting double stranded DNA cassette was cloned by restriction digestion and ligation to yield pACSA_P_{cpt}_YFP. The library of P_{cpt} mutants was created using the GeneMorph II Random Mutagenesis Kit (Agilent). Error prone PCR was conduced using the kit's instructions with primers cpt error prone F and cpt error prone R (Supporting Information Table 3). pACSA_P_{cpt}_YFP plasmid DNA (0.1 ng) was used as a template to maximize mutation frequency. The resulting PCR products were serially used as a template for additional rounds (four total) of error-prone PCR, each time using 0.1 ng template per reaction. Once amplified (using Phusion polymerase), the promoter libraries were cloned into pACSA_P_{cpt}_YFP cassette using *EcoR* I/*Sac* I restriction sites. The resulting plasmids were replicated in DH5α, pooled, and transformed into PCC 7002 for double homologous recombination into the *acsA* locus as previously described.[23,30] Approximately 100 mutants were segregated and confirmed to be homozygous. The truncated P_{cpt} promoter region was amplified from each isolate using GoTaq DNA polymerase (Promega, Madison WI) and sequenced (Functional Biosciences, Madison WI). Promoters from 11 of the library members were moved back into the pACSA_P_{cpt}_YFP construct using *in vitro* recombination assembly with overlapping primers (Gibson 2011) using the primer pairs indicated in Supporting Information Table 3. The resulting assemblies were transformed into *E. coli* DH5α and confirmed by sequencing.

Construction of a Synthetic Promoter Library. The J23119 promoter sequence was obtained from the BioBrick part BBa_J23119 (iGEM Registry of Standard Biological Parts, www.partsregistry.org). The RBS was designed to have 40 000 arbitrary translation initiation units as predicted by the RBS calculator.[2] These two parts were combined with the plasmid pACSA_P_{cpt}_YFP[23] to create pACSA_J23119_YFP in a two part Gibson Assembly using primer pairs indicated in Supporting Information Table 3. Derivatives of this construct were made by replacing promoter Bba_J23119 with Bba_J23100, Bba_J23101, Bba_J23105, Bba_J23108, Bba_J23109, Bba_J23110, Bba_J23114, and Bba_J23117, along with three novel promoter sequences pMB1, pMB2, and pMB3. These constructs were made using a two piece Gibson Assembly using primer pairs indicated in Supporting Information Table 3. Transcription start sites were mapped using the 5′ RACE System for Rapid Amplification of cDNA Ends, Version 2.0 (Invitrogen).

Construction of the IPTG Inducible PCC 7002 Strains. pACSA_P_{cpt}_YFP was modified to be IPTG inducible by sequentially inserting two operators and the $P_{lacI}{}^{Q}$_*lacI* repressor cassette proximal to YFP. The downstream operator was added by Quikchange site directed mutagenesis (Agilent) and then the upstream operator was inserted by Gibson assembly to produce the construct, pACSA_P_{cptOO}_YFP. The $P_{lacI}{}^{Q}$_*lacI* −expression cassette was added to pACSA_P_{cptOO}_YFP using the *Xba* I restriction site. Primers used to produce these constructs are described in Supporting Information Table 3. LacI mediated repression was improved by replacing the $P_{lacI}{}^{Q}$ promoter in pACSA_P_{cptOO}_YFP_$P_{lacI}{}^{Q}$_*lacI* with members of the synthetic promoter library using Gibson Assembly (see Supporting Information Table 3 for primers used). LacI repression was

further improved by making a W220F point mutation via Quikchange site directed mutagenesis. Additional mutations within P_{cptOO} were created using Quikchange (see Supporting Information Table 3 for primers used). P_{TrcO}_YFP_P_{MB2}_*lacI*^{WF} and P_{TrcOO}_YFP_P_{MB2}_*lacI*^{WF} were constructed using two part Gibson Assembly using the optimized pACSA_P_{cptOO}_YFP_P_{MB2}_*lacI*^{WF} plasmid as a template and primers identified in Supporting Information Table 3. All *lac* constructs were stably inserted into the *AcsA* locus of PCC 7002 as previously described. Segregation was confirmed by colony PCR.

Design and Construction of the RBS Library. The RBS Library Calculator[26] was used to design an RBS library based on the untranslated region and YFP coding sequence of pACSA_J23119_YFP. The library was constructed using Quikchange mutagenesis with primers shown in Supporting Information Table 3. A degenerate oligonucleotide pair was used to construct the majority of the library. All library members were stably inserted into the *AcsA* locus of PCC 7002 as previously described. Segregation was confirmed by colony PCR.

qPCR Analysis of the RBS Library in PCC 7002. RNA extraction methods and DNase treatment were adapted from prior studies[31] by adding an additional glass bead lysis step during the lysozyme treatment. Samples were vortexed every 2 min during the 6 min incubation at 64 °C. cDNA was synthesized using random hexamers and 500 ng total RNA by the iScript cDNA Synthesis Kit (Bio-Rad) according to the given instructions. RT-qPCR was performed on 10 μL reactions using iQ SYBR Green Supermix on a CFX Connect Real-Time PCR Detection System (Bio-Rad). Then, 1 μL of the cDNA reaction was added to each reaction, and purified PCR products of genes of interest were used to create standard curves for each primer pair. Denaturation at 95 °C for 3 min was followed by 36 cycles of 95 °C for 15 s, 54 °C for 30 s, and 72 °C for 1 min. A melt curve with was generated with increments of 0.5 °C every 5 s. The statistical significance of transcript levels differences between the library members were calculated using Tukey's multiple comparisons test.

■ ASSOCIATED CONTENT

ⓢ Supporting Information

Tables 1−5. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes
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