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Fine-Tuning of Photoautotrophic Protein Production by Combining Promoters and Neutral Sites in the Cyanobacterium *Synechocystis* sp. Strain PCC 6803

Andrew H. Ng,[a] Bertram M. Berla,[b] Himadri B. Pakrasi[a,b]

Department of Biology[a] and Department of Energy, Environmental, and Chemical Engineering,[b] Washington University, St. Louis, Missouri, USA

Cyanobacteria are photosynthetic cell factories that use solar energy to convert CO_2 into useful products. Despite this attractive feature, the development of tools for engineering cyanobacterial chassis has lagged behind that for heterotrophs such as *Escherichia coli* or *Saccharomyces cerevisiae*. Heterologous genes in cyanobacteria are often integrated at presumptively "neutral" chromosomal sites, with unknown effects. We used transcriptome sequencing (RNA-seq) data for the model cyanobacterium *Synechocystis* sp. strain PCC 6803 to identify neutral sites from which no transcripts are expressed. We characterized the two largest such sites on the chromosome, a site on an endogenous plasmid, and a shuttle vector by integrating an enhanced yellow fluorescent protein (EYFP) expression cassette expressed from either the P_{cpc560} or the P_{trc1O} promoter into each locus. Expression from the endogenous plasmid was as much as 14-fold higher than that from the chromosome, with intermediate expression from the shuttle vector. The expression characteristics of each locus correlated predictably with the promoters used. These findings provide novel, characterized tools for synthetic biology and metabolic engineering in cyanobacteria.

Cyanobacteria are oxygenic photosynthetic prokaryotes that use solar energy to fix CO_2, converting it into biomass and valuable products. Since these organisms do not require fixed carbon feedstocks, they have great potential for synthetic biology and metabolic engineering applications (1–4). Several strains of cyanobacteria have been engineered to act as microbial cellular factories for the production of fuels and chemicals, such as isobutanol, 2,3-butanediol, free fatty acids, and D-lactate (5–7). Despite these advances, none of these engineered strains has been able to achieve industrially relevant levels of productivity. A lack of effective and well-characterized tools for synthetic biology in cyanobacteria has limited progress relative to that with other established microbial chassis, such as *Escherichia coli* or *Saccharomyces cerevisiae* (1). *Synechocystis* sp. strain PCC 6803 is a naturally transformable cyanobacterial chassis for synthetic biology. This strain carries several endogenous plasmids in addition to its single chromosome (8–11). Since there are a limited number of self-replicating exogenous plasmids for the expression of heterologous genes in *Synechocystis* PCC 6803 (12) and other cyanobacteria, these endogenous plasmids may prove to be attractive parts for synthetic biology (13). Additionally, these plasmids increase their copy numbers during the transition from the exponential-growth phase to the stationary phase (14). Thus, we have hypothesized that the expression of heterologous genes from sites in these plasmids could be autoinduced by such a growth transition. This induction could be advantageous in a production system that first yields a dense culture of light-harvesting cyanobacteria and later uses those microbial cell factories to churn out a product of interest.

One strategy for advancing synthetic biology applications in cyanobacteria has been to transfer tools such as promoters and ribosome binding sites from *E. coli* to model cyanobacterial strains and characterize the tools in those strains. Unfortunately, such tools often do not function as expected in cyanobacteria (15, 16). This is due to differences in the transcription (17) and translation (18) machinery, cyanobacterial traits such as polyploidy (19), an efficient recombination system (20), and a circadian rhythm (21, 22), and other features that distinguish cyanobacteria from their heterotrophic cousins. Repositories of biological parts, such as the Registry of Standard Biological Parts, have only recently begun to include information and parts for cyanobacteria. An alternative strategy is to pull parts from within the cyanobacterial phylum. While these parts may perform more predictably, there is also the possibility of increased cross talk with native regulatory systems.

Control over heterologous protein expression is a major need for cyanobacterial synthetic biologists. Protein expression can be controlled by modulating gene dosage, transcription, translation, or RNA and protein turnover (23). In *E. coli*, extensive knowledge of methods for manipulating protein expression has led to the creation of complex synthetic systems such as analog computational circuits (24), logic gates (25), and oscillators (26). To facilitate advanced synthetic biology applications in cyanobacteria, broad-host-range vectors (16), promoter libraries (27, 28), and ribosome binding sites (15) have been characterized.

In heterotrophic systems, gene dosage can be controlled by using a number of off-the-shelf expression vectors. These systems can achieve recombinant protein expression at levels as high as

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Address correspondence to Himadri B. Pakrasi, Pakrasi@wustl.edu.

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50% of total cellular protein (29). However, such systems are limited for cyanobacteria, and most systems described integrate heterologous genes into so-called "neutral sites" in the host genome. Neutral sites are genomic locations where a modification causes no noticeable phenotypic change under the relevant growth conditions. In the naturally transformable cyanobacterium *Synechocystis* PCC 6803, previously used neutral sites include *psbA1* (30), a gene that encodes one of several isoforms of the photosystem II reaction center protein D1, and *slr0646* (31), a putative carboxypeptidase (32). Although mutations engineered at such sites could segregate, disruption of coding DNA sequences may decrease the robustness of the organisms, since evolution has likely selected for the maintenance of the entirety of a genome on the basis of some advantage under some conditions. Other investigators have turned to intergenic regions for inserting genes of interest (33). However, transcriptome-sequencing (RNA-seq) analysis of the genome of *Synechocystis* PCC 6803 has revealed that many regions of DNA previously thought to be noncoding are actually transcribed with unknown or antisense function (34). This suggests the need for a rigorous analysis of the genome of *Synechocystis* PCC 6803 in order to identify neutral sites for heterologous gene expression. Moreover, the small endogenous plasmids (pCA2.4, pCB2.4, pCC5.2) of *Synechocystis* PCC 6803 have been shown to increase their copy numbers at stationary phase to ca. 3 to 8 copies per chromosome (14). This could make these genomic loci attractive as insertion sites for the expression of heterologous genes during stationary phase. In metabolic engineering, this is an advantage because it may allow the decoupling of growth and production, leading to higher product yields.

Another element that limits synthetic biology studies in cyanobacteria is the availability of well-characterized, strong promoters. In *Synechocystis* PCC 6803, strong promoters, such as P_{trc1O}, a broad-host-range promoter, and P_{cpc560}, a cyanobacterial promoter that drives the expression of the β subunit of phycocyanin, a pigment-binding protein that is one of the most abundant proteins in cyanobacteria, have been used to enhance the transcription of genes of interest (35, 36). Although both of these promoters are regarded as strong, they have not been compared directly in terms of either strength or composability (how their characteristics measured in isolation compare to their characteristics when they are combined with other genetic parts used in cyanobacteria). Since P_{cpc560} is a native promoter from *Synechocystis* PCC 6803 that controls the transcription of highly regulated endogenous genes (37, 38), possible issues related to cross talk need to be explored.

In this study, we identified a set of novel neutral sites in the chromosome of *Synechocystis* PCC 6803 and an endogenous plasmid. Strong promoters were characterized at each locus of expression using a fluorescent reporter protein, enhanced yellow fluorescent protein (EYFP). Expression from the endogenous plasmid and expression from P_{cpc560} were found to depend on the growth phase of the organism. Together, a site on the endogenous plasmid and P_{cpc560} resulted in the highest level of EYFP expression, during exponential growth. The combination of that insertion site with P_{trc1O} produced nearly as strong protein expression as the culture reached stationary phase. We expect that these well-characterized neutral sites and strong promoters will enable further synthetic biology and metabolic engineering applications in *Synechocystis* PCC 6803.

MATERIALS AND METHODS

Strains and culture conditions. All cloning was performed in *Escherichia coli* strain XL1-Blue grown in LB medium in culture tubes or on agar plates at 37°C, supplemented with 50 μg/ml kanamycin as needed. *Synechocystis* PCC 6803 cells were grown in BG11 medium (39) supplemented with kanamycin (10 to 100 μg/ml as needed) under continuous white light at 30 μmol m^{-2} s^{-1} at 30°C. Cultures were grown in 125-ml glass Erlenmeyer flasks, in TPP tissue culture treated 12-well plates (Sigma-Aldrich), or on agar plates.

Construction of vectors for neutral-site targeting and promoter analysis. Upstream and downstream flanking regions of 600 bp for each neutral site were amplified from *Synechocystis* PCC 6803 genomic DNA via PCR. A cassette consisting of a kanamycin resistance gene and the P_{trc1O} promoter driving EYFP expression was obtained from a derivative of the pPMQAK1 broad-host-range vector (16). Suicide vectors for cloning into *Synechocystis* PCC 6803 were constructed using the pUC118 backbone. Upstream and downstream flanking regions, the EYFP-Km^r cassette, and the pUC118 backbone were assembled using circular polymerase extension cloning (CPEC) (40). pPMQAK1, pSL2264 (P_{trc1O}-*eyfp* in NSC1), and pSL2309 (P_{trc1O}-*eyfp* in NSP1) were modified to replace P_{trc1O} with P_{cpc560}. P_{cpc560} was amplified from *Synechocystis* PCC 6803 genomic DNA as a 560-bp fragment upstream (35) of the translation start site of the *cpcB* gene. To replace the promoters in pSL2264 and pSL2309, PCR was used to amplify the entire plasmid excluding P_{trc1O}, and the resulting product was assembled with each promoter using CPEC. To replace the promoter in pSL2183, PCR was used to amplify the entire plasmid excluding P_{trc1O} and the ampicillin resistance gene, and the resulting products were assembled with each promoter by using CPEC. All the plasmids used in this study are listed in Table S1 in the supplemental material.

All amplifications and assemblies were performed using Phusion High-Fidelity DNA polymerase (Thermo Scientific). Plasmids were purified using the GeneJET plasmid miniprep kit (Thermo Scientific), and PCR purifications were performed using the GeneJET PCR purification kit (Thermo Scientific). All oligonucleotides were designed in SnapGene (GSL Biotech LLC) and were synthesized by IDT (Coralville, IA). The oligonucleotides used in this study are listed in Table S2 in the supplemental material.

Transformation of *Synechocystis* PCC 6803. Wild-type (WT) *Synechocystis* PCC 6803 was transformed with 1 μg plasmid DNA via natural transformation, and inserts were integrated into the genome via homologous recombination. Transformants were isolated on BG11 agar plates containing 10 μg/ml of kanamycin. Colonies were first restreaked onto BG11 agar plates containing 20 μg/ml of kanamycin and then restreaked again, onto BG11 agar plates containing 40 μg/ml of kanamycin. Mutations were confirmed using colony PCR. Conjugal transfer of pPMQAK1 derivative vectors to *Synechocystis* PCC 6803 cells was performed using a helper strain of *Escherichia coli* containing the pRL443 and pRL623 plasmids (41). Transformants were isolated on BG11 agar plates containing 20 μg/ml of kanamycin.

Fluorescence measurements. Seed cultures for each mutant strain of interest were grown in 30 ml of BG11 medium supplemented with 20 μg/ml kanamycin in 125-ml Erlenmeyer flasks for 3 days. Cultures were adjusted to an optical density at 730 nm (OD_{730}) of 0.02 (corresponding to $\sim 2 \times 10^7$ cells/ml) at the start of the experiment. Three independent replicates of each culture were then transferred to 12-well plates for growth measurements. The excitation and emission of EYFP, as well as the optical densities of the cultures in 96-well black-walled clear-bottom plates (Corning), were determined every 24 h for the next 8 days on a BioTek Synergy Mx plate reader (BioTek, Winooski, VT). The excitation and emission wavelengths were set to 485 nm and 528 nm, respectively, for EYFP. Culture density was also measured as the absorbance at 730 nm. All fluorescence measurements were normalized by culture density.

Total-protein extraction, SDS-PAGE, and Coomassie staining. Fifty milliliters of an exponential-phase *Synechocystis* PCC 6803 culture was

TABLE 1 List of putative chromosomal neutral sites identified by analysis of a transcriptomic data set[a] and suggested neutral sites on small endogenous plasmids

Neutral site	Replicon	Locus[b] (bases deleted)	Neutral site length (bp)
NSC1	Chromosome	1599539–1600658 (1600073–1600150)	1,119
NSC2	Chromosome	3027658–3028500 (3028002–3028117)	842
NSC3	Chromosome	3449111–3449927	816
NSC4	Chromosome	3546659–3547441	782
NSC5	Chromosome	2950392–2951098	706
NSC6	Chromosome	1639248–1639943	695
NSC7	Chromosome	1606646–1607303	657
NSC8	Chromosome	2080121–2080718	597
NSC9	Chromosome	1923473–1924065	592
NSC10	Chromosome	2972205–2972781	576
NSC11	Chromosome	1679804–1680334	530
NSC12	Chromosome	3324552–3325053	501
NSC13	Chromosome	2553476–2553961	485
NSC14	Chromosome	3359043–3359457	414
NSC15	Chromosome	2790247–2790655	408
NS-pCA-1	pCA2.4	2138–647 (390–523)	887
NS-pCA-2	pCA2.4	1659–1835 (1747–1819)	177
NS-pCB-1	pCB2.4	430–849 (560–660)	420
NS-pCB-2	pCB2.4	1497–1595 (1501–1590)	99
NS-pCC-1	pCC5.2	1046–1599 (1293–1382)	554
NS-pCC-2 (NSP1)	pCC5.2	5137–2 (5151–5199)	80

[a] See reference 33.
[b] Given as base pair positions in GenBank accession no. BA000022.2 (chromosomal loci), L13739.1 (pCA2.4 loci), L25424.1 (pCB2.4 loci), and CP003272.1 (pCC5.2 loci).

collected and was resuspended in 20 mM potassium phosphate buffer (90.8% K_2HPO_4, 9.2% KH_2PO_4), pH 7.8. A 0.5-ml volume of sterile, acid-washed glass beads was added to the cells, and the mixture was disrupted using a bead beater (BioSpec Products). The resultant mixture was centrifuged for 3 min at 2,500 × *g*, and the supernatant was removed and was transferred to a new tube. The protein concentration was quantified using a bicinchoninic acid (BCA) protein assay (Thermo Scientific). A 20-μg total-protein extract from each sample was separated on an SDS (0.1%, wt/vol)-polyacrylamide (12.5%, wt/vol) gel by electrophoresis and was stained with Coomassie blue. The gel was then destained in a solution of acetic acid (7%, wt/vol) and methanol (25%, wt/vol) and was imaged.

RESULTS AND DISCUSSION

Identification of potential neutral sites in *Synechocystis* PCC 6803. We identified putative neutral sites within the *Synechocystis* PCC 6803 chromosome as loci from which no transcripts were observed in a recent RNA-seq transcriptome-profiling study (34). We used Artemis genome-browsing software (42) to find large regions of intergenic DNA within the annotated genome (GenBank accession no. BA000022.2) and then manually examined these for any corresponding transcripts, including rRNA, tRNA, ncRNA (noncoding RNA), and asRNA (antisense RNA). We found 15 potential neutral sites with ~400 to 1,100 bp of nonexpressed DNA on the chromosome (Table 1). Since these regions are untranscribed, we expect that their interruption will not have any phenotypic effect.

Additionally, the high copy numbers of several small endogenous plasmids in *Synechocystis* PCC 6803 made them attractive as locations in which to identify additional neutral sites. Since no RNA-seq data were available for these plasmids (pCA2.4, pCB2.4, and pCC5.2), we retrieved their annotated sequences from GenBank and identified two regions on each plasmid without annotated transcripts or repeated and/or inverted regions of DNA, which have previously been suggested to have significant functions (8–11) (Table 1).

Construction of neutral-site-targeting plasmids and analysis of neutral sites. We constructed suicide vectors to insert a P_{trc1O}-*eyfp* expression cassette at the two largest putative chromosomal neutral sites (NSC1 and NSC2) and all six putative neutral sites in the small plasmids (NSP1 to -6) via double homologous recombination (Fig. 1a). Each construct replaced the central ~100 bp of the targeted site and included a selectable marker (kanamycin resistance). After natural transformation, the resulting colonies were patched several times with increasing antibiotic concentrations to induce segregation. Both NSC1 and NSC2 produced successful transformants, whereas among the six plasmid sites, only NSP1 on pCC5.2 produced a transformant. All transformations were performed with 1 μg of plasmid DNA, and all successful transformations produced approximately 100 colonies. The expected insertions were confirmed using colony PCR (Fig. 1b). Segregation was indicated by the disappearance of a band corresponding to the WT genotype. We found that while mutations at NSC1 and NSC2 segregated completely, the mutation at NSP1 did not segregate fully, even after repeated patchings over several months. This could be because the high copy numbers of these small plasmids make it difficult to isolate individuals without any wild-type allele from a merodiploid population, or because the insertion at NSP1 interrupts an essential sequence. A recent report indicates that *Synechocystis* PCC 6803 may harbor as many as 200 copies of its chromosome per cell (19), with many more copies of its small plasmids.

After finding that we could not segregate a mutant in NSP1, we tested the stability of the engineered mutations. After growing mutant strains in liquid BG11 medium without any antibiotic in shake flasks for 30 days with weekly subcultures, we extracted genomic DNA and repeated the previous PCR to examine relative allele frequencies (Fig. 1c). Mutants with mutations at NSC1 and NSC2 maintained nearly the same allele frequencies. The mutation in NSP1 was maintained, but at a reduced frequency. These results suggest that mutations in NSC1, NSC2, and NSP1 can all be maintained without the need for antibiotic selection. However, segregation of mutations in NSP1 may pose a concern for stability of expression in practical applications.

The choice of neutral site influences heterologous protein expression levels. We analyzed the relationship between growth phase and heterologous protein expression from various neutral sites by measuring EYFP fluorescence daily for 8 days (Fig. 2). As a control, we also included an RSF1010-based derivative of plasmid pPMQAK1 (16) in this study. Across the growth phases, we found that EYFP expression was nearly identical in NSC1 and NSC2, while expression from pPMQAK1 was approximately 3 times higher. The NSP1 mutant expressed 8 to 14 times more EYFP than NSC1 and NSC2, and that ratio increased from the exponential to the stationary phase. This agrees with our earlier observations of plasmid copy number (14) and shows that expression of heterologous genes from pCC5.2 can result in a highly favorable expression profile for the use of *Synechocystis* PCC 6803 as a microbial cell factory that first produces cellular biomass and later produces products of interest in stationary phase. As also shown in Fig. 2, expression of EYFP from all four of these loci produced no differ-

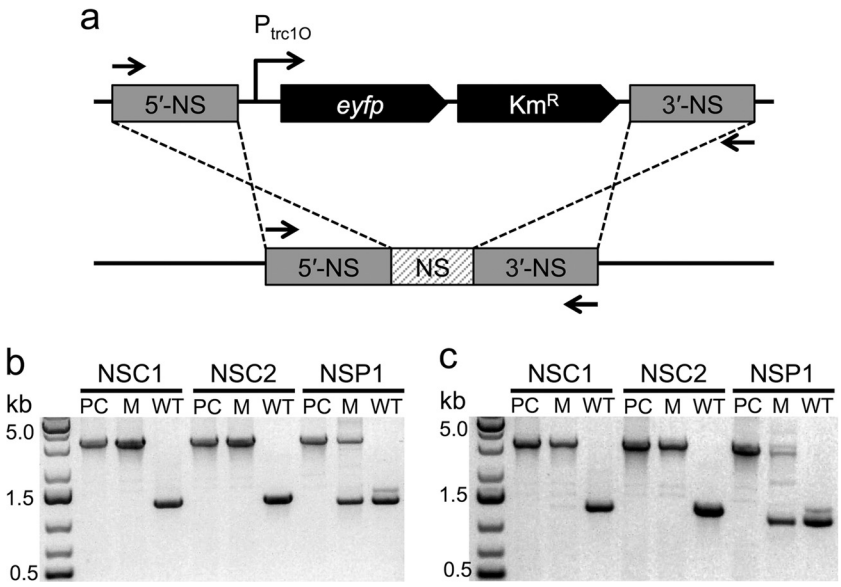

FIG 1 Neutral sites in the genome of *Synechocystis* PCC 6803. (a) Insertion of reporter cassette into neutral sites (NS). Double homologous recombination was used to integrate a cassette containing *eyfp* and a Km^r marker into each neutral site, replacing approximately 100 bp in the middle of each site. (b and c) Genomic DNA was extracted from mutants grown with (b) and then for 30 days without (c) antibiotic selective pressure, and PCR using flanking primers in the 5′ and 3′ neutral site flanking regions was performed to test for the presence of the desired mutations and the relative frequency of mutant alleles. Lanes: PC, positive-control PCR performed on the suicide vector used to transform *Synechocystis* PCC 6803; M, colony PCR performed on the *Synechocystis* PCC 6803 mutant strain; WT, colony PCR performed on wild-type *Synechocystis* PCC 6803.

ences in growth between recombinant strains. Thus, gene dosage can be influenced by the choice of a neutral site and can, in turn, influence protein expression levels in cyanobacterial synthetic biology applications.

Promoter characterization in neutral sites. In addition to testing loci that could influence gene dosage and expression, we compared expression from P_{trc1O}, a strong promoter derived from heterotrophic bacteria, with that from P_{cpc560}, a promoter that drives the production of one of the most abundant proteins in *Synechocystis* PCC 6803. We found that both of these promoters combined in a modular fashion with the different neutral sites tested and that both gave rise to strong expression levels. To test such modularity, we replaced P_{trc1O} with P_{cpc560} in variants of the NSC1 and NSP1 targeting vectors outlined above, as well as in pPMQAK1 (see Table S1 in the supplemental material). Mutants were confirmed using colony PCR, and EYFP expression was measured as a function of growth phase (Fig. 3a to c). Across all three loci tested, P_{cpc560} produced approximately 2 to 4 times more EYFP than P_{trc1O} during early growth phases. As the culture density increased, the amount of EYFP produced by P_{cpc560} decreased, while the amount of EYFP produced by P_{trc1O} remained steady or increased slightly (Fig. 3d) on a per-cell basis. We also observed a similar trend in *cpcB* expression with growth phase in an earlier microarray-based study (38). Thus, while the use of a native cyanobacterial promoter led to the strongest EYFP expression measured, cross talk from native regulatory systems appears to have reduced the expression level during stationary phase. Future promoter-engineering efforts could remove such cross talk or even

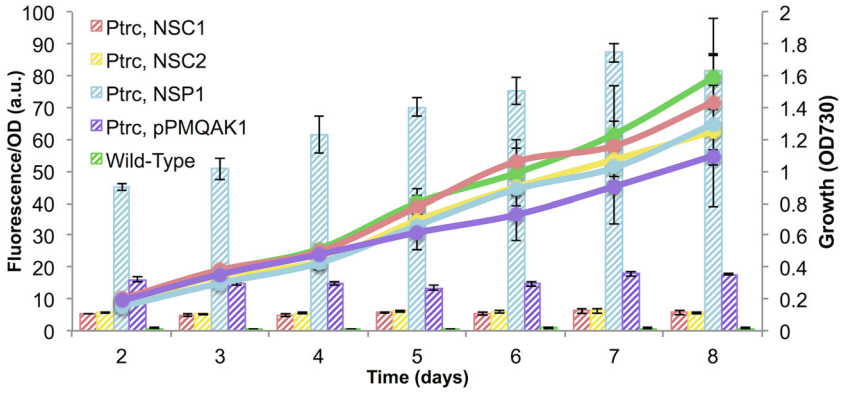

FIG 2 The genetic location of P_{trc1O}-*eyfp* influences gene expression. Shown are normalized fluorescence intensities ± standard deviations and growth curves over a time course of 8 days for mutants expressing P_{trc}-*eyfp* in NSC1, NSC2, NSP1, and a broad-host-range vector containing the RSF1010 replicon. Cells were grown in BG11 medium (39) supplemented with kanamycin (10 μg/ml) as needed under continuous white light at 30 μmol m^{-2} s^{-1} at 30°C. Cultures were grown in TPP tissue culture-treated 12-well plates (Sigma-Aldrich).

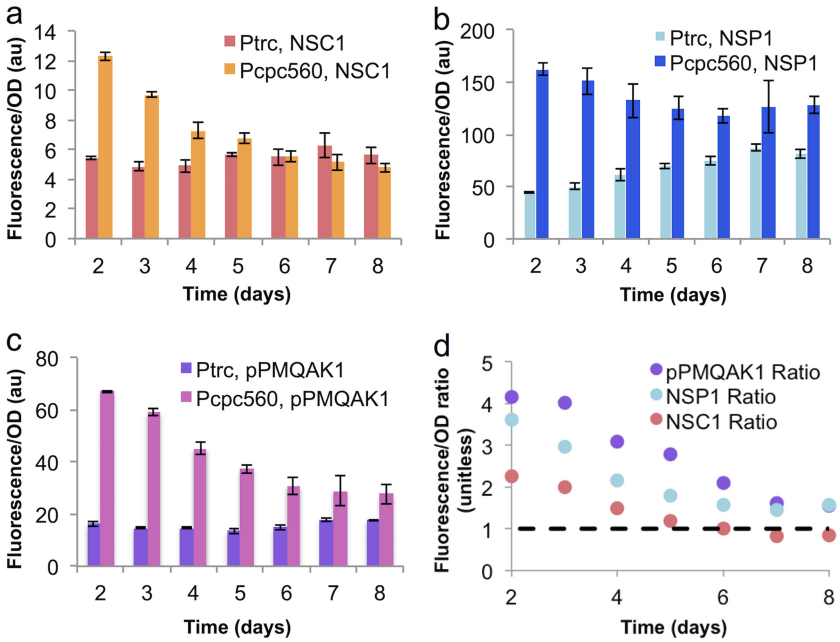

FIG 3 Composability of promoters in different expression loci. (a to c) Normalized fluorescence intensities ± standard deviations over a time course of 8 days in NSC1, NSP1, and pPMQAK1, respectively. au, arbitrary units. (d) Ratio of P_{cpc560}-*eyfp* to P_{trc}-*eyfp* expression at each expression locus over a time course of 8 days.

reverse its effect by using promoters that result in higher expression at stationary phase.

To assess the abundances of EYFP in these mutants relative to those of other cellular proteins, we extracted total protein from each mutant strain and analyzed it using SDS-PAGE (Fig. 4). In most strains, it was difficult to identify EYFP due to the presence of another band of similar molecular mass (27.2 kDa). A distinct band for EYFP was, however, clearly visualized in the constructs at NSP1 with either P_{cpc560} or P_{trc1O}. In fact with P_{cpc560}, EYFP was clearly the most abundant cellular protein. Such high levels of expression and modular behavior should make these neutral sites and promoters very useful parts for future synthetic biology applications. In addition, overexpression of EYFP from these sites with either of these strong promoters caused no detectable growth defect in our experiments (see Fig. S1 in the supplemental material). A recently published report on the overproduction of ethylene in *Synechocystis* PCC 6803 (43) estimated that ~10% of fixed carbon was diverted into ethylene with no negative effect on photosynthetic growth rates. If we assume a biomass yield of 2.4 g biomass/g C and a cellular protein content of 36% (g/g), and we also assume that 20% of total cellular protein is EYFP in the strain with P_{cpc560} in NSP1 (from Fig. 4), then ~8% of total cellular carbon is contained within that heterologous product. Thus, this strain compares well with the highest photosynthetic partitioning that has been shown to date.

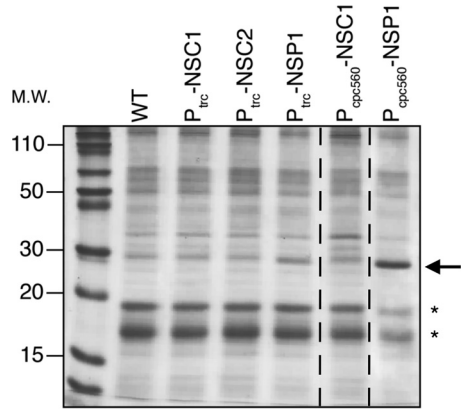

FIG 4 Coomassie blue-stained SDS-PAGE gel of total proteins extracted from cultures of *Synechocystis* PCC 6803 mutants expressing EYFP from different locations using the promoters shown above the gel. Lanes were loaded on an equal-total-protein basis. The band for EYFP is indicated by the arrow, and phycobiliproteins are indicated by asterisks. M.W., molecular weight (in thousands). The gel shown here was spliced along the dashed lines to remove two lanes containing samples not discussed in this work.

In this communication, we present the selection and characterization of novel neutral sites in the genome of *Synechocystis* PCC 6803. The sites that were identified in this work will contribute to the growing toolbox for genetic manipulation of cyanobacteria. A methodical approach was used to identify the largest nontranscribed regions in the *Synechocystis* PCC 6803 genome, using RNA-seq data that revealed previously unknown RNA coding regions. Our approach has the potential to be used to identify neutral sites in other organisms as well. Of six putative neutral sites identified in the small endogenous plasmids without the benefit of existing RNA-seq data, only one (NSP1) yielded any transformant, suggesting that the transcriptomic data set is a valuable tool for neutral-site identification. Although targeting of NSP1 for the insertion of our expression cassette produced verifiable transformants, we were unable to obtain a fully segregated mutant at this location, even after numerous passages through antibiotic-containing plates. This contrasted with NSC1 and NSC2, both of which yielded fully segregated mutants after only two such passages. Thus, although NSP1 may not be a true "neutral site," it is a

very useful part for its ability to express large amounts of heterologous protein. Finally, we found that the various loci tested for heterologous expression could be combined with different promoters in a modular fashion, giving consistent patterns of relative expression at different sites. The appropriate combination of promoters and neutral sites can result in strong expression with a profile that is favorable for the industrial production of fuels and chemicals.

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