THE PENNSYLVANIA STATE UNIVERSITY
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DEPARTMENT OF BIOCHEMISTRY AND MOLECULAR BIOLOGY

DIFFERENTIAL AND INDUCIBLE EXPRESSION OF YELLOW FLUORESCENT PROTEIN IN THE MARINE CYANOBACTERIUM SYNECHOCOCCUS SP. PCC 7002

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i. Abstract

Synechococcus sp. PCC 7002 serves as a model system for studying the molecular biology of photosynthetic microorganisms. Its fast growth rate and natural transformability also make *Synechococcus* sp. PCC 7002 amenable to bioengineering. Recently, a system for expressing non-native genes based in an endogenous plasmid (pAQ1) was developed in this organism. This study explored the capability of the pAQ1 expression system to serve as a platform for differential and inducible expression of yellow fluorescent protein (YFP). It was found that varying the promoter region of the pAQ1 plasmid lead to differential expression when transformed into cyanobacteria. Furthermore, inducible expression was achieved using the promoter from a sodium-dependent bicarbonate transporter gene (*sbtA*) derived from *Synechocystis* sp. PCC 6803. This initial foray into the development of the pAQ1 expression system demonstrates both its aptitude for studying the molecular biology of *Synechococcus* sp. PCC 7002 and its potential for use as a tool for bioengineering.

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Introduction

Synechococcus sp. PCC 7002 is a marine, unicellular, euryhaline cyanobacterium. As a fast growing, naturally transformable, high light tolerant photoheterotroph, *Synechococcus* sp. PCC 7002 (formerly called *Agmenellum quadruplicatum* PR-6) has been shown to be a suitable model system for basic photosynthetic research (1,2,3,4). Furthermore, its genome was mapped in 1993 (5) and its sequence was recently deposited in the National Center for Biotechnology Information Database (http://www.ncbi.nlm.nih.gov/), making *Synechococcus* sp. 7002 an ideal candidate for bioengineering.

In addition to its chromosome, *Synechococcus* sp. PCC 7002 houses six endogenous plasmids, called pAQ1 (4809 bp), pAQ3 (16,103 bp), pAQ4 (31,972 bp), pAQ5 (38,515 bp), pAQ6 (124,030 bp), and pAQ7 (186,459 bp) (Ref. 6). The smallest of these plasmids, pAQ1, is thought to be present in the highest copy number and has served as a template for the construction of an overexpression platform in *Synechococcus* sp. PCC 7002. The pAQ1 plasmid has been modified so that it can be passed through *E. coli* before its final incorporation into the cyanobacterial genome (7). The plasmid comprises an exogenous promoter, multiple restriction sites, a deca-histidine (His_{10}-) affinity tag, a transcription terminator, and an antibiotic resistance cassette (*Figure 1*, Results and Discussion). Flanking DNA sequences derived from the cyanobacterial-pAQ1 direct the overexpression fragment to specific sites in pAQ1, thereby incorporating the engineered expression system into the *Synechococcus* sp. PCC 7002 genome (7).

The prospect of its possible use for basic molecular biological research and bioengineering warrants exploration of the capabilities of the pAQ1 expression platform. An immediate choice for investigating the potential to express non-native protein at varying levels

would be to vary the promoter region of the pAQ1 plasmid. A variety of promoters, each operating according to its own mechanism of molecular regulation, should produce yellow fluorescent protein at differential levels of expression. For the purpose of this study, *differential expression* shall refer exclusively to differences in the levels of expression of YFP (as reported by fluorescence), unless otherwise specified.

While the ability to constitutively express protein may prove useful, it would be equally useful to be able to control protein expression levels by altering some trivially adjustable experimental condition (e.g. light intensity, temperature, presence of a particular reagent). Toward that end, the 5' untranslated region of a sodium-dependent bicarbonate transporter gene (*sbtA*) derived from *Synechocystis* sp. PCC 6803 was selected to pioneer the investigation inducible expression in the pAQ1 system.

SbtA is located in the plasma membrane in *Synechocystis* sp. PCC 6803 and is thought to act as a Na^+/HCO_3^- symporter (8, 9), though this has yet to be confirmed. It has been shown, however, that Na^+ is required for HCO_3^- transport and that higher levels of HCO_3^- influx attributable to *sbtA* are found in inorganic carbon (C_i) limiting conditions (10,11). Consistent with these observations is the finding that *Synechocystis* sp. PCC 6803 grown under C_i limiting conditions showed an increase in *sbtA* transcripts relative to cultures that had been grown under high C_i conditions (12). This evidence strongly suggests that *sbtA* is an inducible, Na^+ dependent bicarbonate transporter.

SbtA functions as part of a larger network of genes that serve to effectively increase the availability of carbon dioxide near the carboxysome, collectively termed "carbon concentrating mechanisms", or CCMs. Cyanobacteria have developed these mechanisms for scavenging inorganic carbon from their environment and localizing it near the carboxysome (13, 14, 15).

Relative to the extracellular concentration, up to 1000-fold increases levels of intracellular Ci have been observed, helping to compensate for the low rate of CO_2 fixation by ribulose 1,5-bisphosphate carboxylase/oxygenase (RubisCO, k_{cat} = 1000-2000 mol CO_2 •mol^{-1} RubisCO• min^{-1}) (Ref. 16). Carboxysomal carbonic anhydrases (CA) oxidize HCO_3^- that has been accumulated in the cytosol, allowing for CO_2 fixation. Alkalophilic cyanobacteria in particular have evolved mechanisms for importing C_i given the low availability of bicarbonate under basic conditions.

There are four main mechanisms by which cyanobacteria scavenge for extracellular inorganic carbon. The first two are constitutively activated systems associated with NADP-H dehydrogenase complexes (8, 14). The other two mechanisms use inducible bicarbonate transporters to pump Ci from the extracellular environment into the cytosol. These two inducible pumps are activated when Ci becomes limiting in solution. *SbtA* codes for one of two inducible cyanobacterial bicarbonate transporters. The other, BCT 1, is an ATP binding cassette (ABC)-type transporter. BCT 1 is an inducible, high affinity bicarbonate transporter (14, 17) encoded by the *cmp* operon. The *cmp* operon is present in *Synechococcus elongatus* sp. PCC 7942 and *Synechcocystis* sp. PCC 6803, however no *cmp* homologue is known in *Syenchococcus* sp. PCC 7002 (http://www.ncbi.nlm.nih.gov/).

Since *Synechococcus* is only known to possess an s*btA* homologue, this was the promoter that was chosen as a candidate. Expression of *sbtA* in *Synechococcus* sp. PCC 6803 is thought to be regulated by an Abr-type transcription factor, *sll0359*. A recent study reported that *sbtA* (along with *cmp*) that is normally expressed only under Ci-limiting conditions is expressed equally in Δ*sll0359* mutants when grown under both high-Ci and Ci-limiting conditions. The *sll0359* gene product was also shown (*in vitro*) to bind the untranslated region of *sbtA* (19).

These results suggest that *sll0359* may act as a transcriptional repressor, binding to the promoter region of *sbtA* in high-Ci conditions and releasing when Ci is limiting.

Sequences for the 5' untranslated region of *sbtA* are similar between *Synechococcus* sp. PCC 7002 and *Synechocystis* sp. PCC 6803. Additionally, *Synechococcus* harbors an Abr-type transcriptional regulator with a 69% sequence similarity to the *Synechocystis* sp. PCC 6803 *sll0359* gene (http://www.ncbi.nlm.nih.gov/). If the mechanisms for regulating expression of *sbtA* are similar enough between *Synechococcus* sp. PCC 7002 and *Synechocystis* sp. PCC 6803, then expression of YFP on pAQ1 should mimic expression of native *sbtA* located in the *Synechococcus* chromosome. This work explores the possibility of inducible expression of genes in the pAQ1 expression system in *Synechococcus* sp. PCC 7002.

Materials and Methods

Media:

For making chemically competent E. coli *cells*

Ψ Broth (Total volume = 1L)

Yeast Extract	5 g
Tryptone	20 g
$MgSO_4$	4 g
KCl	.74 g

The pH was adjusted to 7.6 with KOH.
The solution was autoclaved and cooled at room temperature.

TfB I (Total volume = 1 L)

KOAc	2.94 g
$MnCl_2$	9.90 g
RbCl	12.10 g
$CaCl_2$	1.48 g
Glycerol	150 mL

The pH was adjusted to 5.8 with acetic acid.
The solution was filter sterilized and stored at 4 ^{o}C

TfB II (Total volume = 1L)

MOPS	2.10 g
$CaCl_2$	11.0 g
RbCl	1.2 g
Glycerol	150 mL

The pH was adjusted to 7.0.
The solution was filter sterilized and stored at 4 ^{o}C.

Tris-acetate-EDTA (TAE) (Total Volume = 1L)

Tris Base	242 g
Glacial acetic acid	57.1 mL
0.5 M EDTA	100 mL

Distilled, deionized (dd) H_2O was added to a final volume of 1L

SOC Medium (Total volume = 1L)

Bacto-Tryptone	10 g
Yeast Exctract	5 g
5M NaCl	3 mL
1M KCl	2.5 mL
1M $MgCl_2$	10 mL
1M $MgSO_4$	10 mL
1M glucose	10 mL

The solution was autoclaved and cooled at room temperature.

LB (Luria-Bertani) (Total volume = 1L)

Bacto-tryptone	10 g
Yeast extract	5 g
NaCl	10 g

The pH was adjusted to 7.0 with NaOH.
The solution was autoclaved and cool at room temperature.

For maintaining cyanobacterial cultures

P1 Metals (1000X)

H_3BO_3	34.26 g
$MnCl_2 \cdot 4H_2O$	4.32 g
ZnCl	0.315 g
MoO_3 (85%)	0.03 g
$CuSO_4 \cdot 5H_2O$	0.003 g
$CoCl_2 \cdot 6H_2O$	0.01215 g

ddH_2O was added to a final volume of 1 L

BG-11 Stock Solution (100X)

$NaNO_3$	149.6 g
$MgSO_4 \cdot 7H_2O$	7.49g
$CaCl_2 \cdot 2H_2O$	3.6 g
Sodium citrate	0.89 g
NaEDTA (pH 8.0, 0.25 M)	1.12 mL
Trace Minerals	100 mL

ddH_2O was added to a final volume of 1 L.

BG-11 (Total volume = 1L)

100X BG-11 Stock Solution	10 mL
1 M TES/NaOH, pH 8.2	5 mL
1000X Ferric ammonium citrate (6 mg/mL)	1 mL
1000X Na_2CO_3 (50 mg/mL)	1 mL
1000X KH_2PO_4 (30 mg/mL)	1 mL

ddH_2O was added to a final volume of 1L
The solution was autoclaved and cooled at room temperature.

*To make BG-11 solid agar plates, Na-thiosulfate (3g) and Bactoagar (15g) were added per 1L of solution. The solution was autoclaved for 45 minutes and cooled to 50-55°C before pouring.

Liquid A+ Medium (Total Volume = 1L)

NaCl	18 g
KCl	0.6 g
$NaNO_3$	1.0 g
$MgSO_4 \cdot 7H_2O$	5.0 g
KH_2PO_4 (50 g/L)	1 mL
$CaCl_2$ (37 g/L)	7.2 mL
NaEDTA tetra (3 g/L)	10 mL
$FeCl_3$ (3.89g/ 1L 0.1 M HCl)	1 mL
Tris (100 g/L, pH 8.2) ‡	10 mL‡
P1 Metals (1000X)	1 mL

ddH_2O was added to a final volume of 1L.
Solution was autoclaved and cooled to room temperature, to which 4 μg of Vitamin B12 was added.

*To make A+ agar plates, 15 g Bactoagar was added per liter of solution, the solution was autoclaved for 45 minutes, then Vitamin B12 was added. After cooling to 50-55°C, apspectinomycin was added to a final concentration of 50 $\mu g \cdot mL^{-1}$.

‡ *Tris* was used to grow cultures at a pH of 8.2. For experiments in which a culture buffered to a different pH value was desired, appropriate buffer was substituted for *Tris*. The molarities for each buffer are indicated where appropriate in the Results section.

Conditions for growing liquid cultures of Synechocystis *sp. PCC 6803*

Sterilized glass bubbling tubes containing liquid BG-11 medium were inoculated with wild-type *Synechocystis* sp. PCC 6803 that had been growing on solid BG-11 plates. The inoculated bubbling tubes were transferred to a 32 ^oC water bath and then connected to a sterile CO_2 bubbling system (2% CO_2, 98% N_2). Cultures were continuously illuminated using fluorescent lamps at a photon flux of 250 $\mu E \cdot m^{-2} \cdot s^{-1}$.

Conditions for growing liquid cultures of Synechococcus *sp. PCC 7002*

Sterilized glass bubbling tubes containing liquid A+ medium were inoculated with *Synechocystis* sp. PCC 7002 that had been growing on solid A+ plates. Appropriate antibiotics were added. The inoculated bubbling tubes were first transferred to a 37 ^{o}C water bath and then connected to a sterile CO_2 bubbling system (2% CO_2, 98% N_2). Cultures were continuously illuminated using fluorescent lamps at a photon flux of 250 $\mu E \cdot m^{-2} \cdot s^{-1}$. For CO_2 starved cultures, bubbling with 2% CO_2 was replaced by bubbling with air using a commercial air pump.

Isolation of cyanobacterial DNA

Cyanobacterial cultures were grown photoautotrophically as described above to an OD_{730} of 0.8-1.2. Cells were spun down by centrifugation (5000 X *g*) and the cell pellets were resuspended in 400 µL TES (5 mM Tris, pH 8.5; 50 mM NaCl; 5 mM EDTA, pH 8.0), then transferred into a 1.5 mL microcentrifuge tube. To the cell suspension was added 100 µL of lysozyme (50 mg/mL), at which point the cells were incubated for 15 min. at 37 ºC with occasional mixing. After incubation, 50 µL of 10% sarkosyl was added to the tube, followed by 600 µL phenol. The tube was vortexed for several minutes, then centrifuged for 5 min. at 10,000 x *g*. The supernatant was transferred to a new microcentrifuge tube, to which 5 µL RNase (10 mg/mL) was added and incubated for 15 min. at 37 ºC. After incubation, 100 µL, 100 µL, and 600 mL of 5M NaCl, CTAB-NaCl solution (700 mM NaCl, 10% CTAB w/v), and chloroform were added, respectively, and the tube was vortexed for several minutes. The solution was centrifuged for 5 min. at 10,000 X *g* and the upper layer was transferred to a new microcentrifuge tube. An equal volume of isopropanol was added and the tube was centrifuged a

final time for 10 min. at 10,000 x *g* to pellet the DNA. The DNA pellet was washed with 70% ethanol and dried at 37 ^{o}C. The pellet was resuspended in 30 µL of ddH_2O and stored at -20 ^{o}C.

PCR Amplification and Purification of Synechocystis *sp. PCC 6803* promotors

Cultures of *Synechocystis* sp. PCC 6803 were grown photoautotrophically in BG-11 medium (see above) to an OD_{730} of 0.8-1.2. Genomic DNA was isolated as described above and diluted to a concentration of 100 ng·mL^{-1}.

Consistent with the design of the pAQ1 expression plasmid, primers were designed such that *Synechocystis* promoters were included in the "Promoter X" position (**Figure 1**). The forward PCR primers were designed to include an EcoRI restriction site. Reverse primers were designed to include an NdeI restriction site. Sequences for primers used in this work can be found in Xu *et. al.* (7). The primers used to amplify the *sbtA* promoter region from *Synechocystis 6803* are found in **Table 1**, below.

PCR was performed using Phusion high fidelity polymerase (New England Bioloabs). PCR reactions using genomic DNA from *Synechocystis* sp. PCC 6803 as template contained: 10 µL 5x Phusion HF (High Fidelity) buffer, 1 µL of 10 mM dNTP, 3 µL of each 10 µM primer, 100 ng genomic DNA, 0.5 µL Phusion™ DNA polymerase, and sterile water to produce a final volume of 50 µL. The reaction conditions were: One cycle of initial denaturation at 98 °C for 30 s, 35 cycles of denaturation at 98 °C for 10 s, 20 s of annealing at a temperature +3 °C of the lower T_m primer, 20 s per kb of extension at 72 °C. One cycle of final extension was included at 72 °C for 10 min. The PCR product was purified using the *MoBio* Lab DNA purification kit (Carlsbad, CA, Catalog Number 12400-250) according to the protocol suggested by the manufacturer.

Table 1: *DNA oligonucleotides sequences (engineered restriction sites are underlined)**

Primer name	Sequence (5' to 3')
pAQ1flankAF	AAGGCAGTCGACCTTTCTCTTATGCACAGATGGG
pAQ1flankBR	TCACGGTCTAGAGCGAATTCGCCTCCTGAATAAATCTATTTATAC
cpcBproF	AGGAATTCGTTATAAAATAAACTTAACAAATCTAT
cpcBproR	CGTCGACCATGGAATTAATCTCCTACTTGACTTTATG
PkanF	GAACACGTAGAATTCCAGTCCGCAGAAAC
PkanR	CATCGAGCTCAACCATGGGAAACGATCC
eyfpF	GAATTCTCGCATATGGTGAGCAAGGGCGAGGA
eyfpR	GTCGCGGGATCCTTACTTGTACAGCTCGTCCATG
fdcpecpR	CAAATGCCATGGATTGTTTCTCCTGTTAACGA
fdcpecpF	CACTAAGAATTCCGGTGACACAGCATTATACTTA
6psbAF	GTTTACGAATTCAAAAAACGACAATTACAAGAAAGTA
6psbAR	TCGTGGCCATGGGGTTATAATTCCTTATGTAT
6803 sbtA F	CAACATGGGAGAATTCGAAGAACTGGGCAAGAAGGCA
6803 sbtA R	ACAAAAAATTCATGATGTAAATTGTCTCCTTGGTTG

*Table adapted from Xu *et. al*., 2009 (Ref. 7).

Making Chemically Competent E. coli DH5α

A single colony of *E. coli* DH5α was grown overnight at 37 ^{o}C in 5 mL Luria Bertani (LB) medium. The overnight culture was transferred to 100 mL of pre-warmed ΨB and grown for 90 minutes with vigorous shaking until the OD_{550} was 0.485. The cells were transferred into

two 50 mL Falcon tubes, chilled on ice (~4 ^{o}C) and centrifuged for 5 minutes. The supernatant was decanted and the cells were re-suspended in 30 mL cold TfB I by vortexing. The cells were then incubated on ice for two hours, then centrifuged for another 5 minutes. The cell pellet was re-suspended in 4 mL of cold TfB II and aliquoted (100 µL each) into pre-chilled 0.5 mL microcentrifuge tubes. The aliquots were then frozen in a liquid nitrogen bath and stored at -80 ^{o}C until further use.

Transformations of Chemically Competent E. coli *DH5α Cells*

The procedure for transformation of *Synechococcus* sp. PCC 7002 has been described in detail previously by Frigaard et. al (14). The chemically competent DH5α cells were removed from -80 ^{o}C and thawed on ice (~4^{o}C), to which 10 µL of DNA was added. The contents were mixed gently by pipetting and incubated on ice for 20 minutes. After the incubation period, the cells were heat shocked in a water bath at 42^{o}C for 30 seconds. The cells were allowed to recover in 1 mL SOC at 37^{o}C for one hour with vigorous shaking. After incubation the cells were centrifuged and the pellet was re-suspended in 100 µL SOC. The re-suspended pellet was plated on LB with ampicillin (100 µg·mL^{-1}), spectinomycin (100 µg·mL^{-1}), and streptomycin (50 µg·mL^{-1}). Plates were incubated overnight (at least 12 hours) at 37 ^{o}C.

Plasmid Miniprep of E. coli *Transformants*

Cultures of transformed colonies were prepared in 5 mL LB and incubated at 37 ^{o}C with vigorous shaking overnight (for at least 12 hours). Appropriate antibiotics were added. Plasmids were extracted from the transformant cultures using the Zyppy Plasmid Miniprep Kit (Zymo Research Corp., Organge, CA, Catalog Number D4020). In a 1.5 mL microcentrifuge tube, 150

μL of 7x blue lysis buffer was added to 600 μL of overnight culture and mixed by inverting. Cold neutralization buffer (350 μL) was added and the tube was mixed by vortexing. After 3-5 minutes of centrifugation (10,000 x *g*), approximately 900 μL of supernatant was removed by pipetting and added to a filter column. The column was centrifuged for 30 seconds (10,000 x *g*), 400 μL of Zyppy wash buffer was added and the column as centrifuged for an additional 30 seconds to remove residual ethanol. The filter column was then transferred to a clean 1.5 mL microcentrifuge tube, to which 30 μL of distilled, deionized water was added. The column was allowed to stand at room temperature for 1-2 minutes to allow water to adequately permeate the membrane. The column was then centrifuged a final time for 30 seconds (10,000 x *g*) to elute the plasmid. Plasmid solutions that were not immediately used were divided into 10 μL aliquots and stored at -20 ^{o}C until further use.

Transformations of Synechococcus *sp. PCC 7002*

Liquid A+ medium (25 mL) was inoculated with wild-type *Synechococcus* sp. PCC 7002 from A+ plates and grown to OD_{730} of 0.5-1.0. To 2 mL of this culture was added 10 μL of purified pAQ1 plasmid. The culture was grown overnight with CO_2 bubbling (no antibiotics were added). The overnight culture was centrifuged (4,000 x *g*) for 2 minutes and the loose pellet was re-suspended in 200 μL of liquid A+ medium. The re-suspended pellet was plated on A+ with spectinomycin (50 $\mu g \cdot mL^{-1}$). Transformant colonies typically took 7-10 days to appear, at which point 6 were selected and transferred to new A+ plates. Colonies were PCR-checked for transformation using the forward and reverse primers from the initial *Synechocystis* sp. PCC 6803 amplification step. If no successful transformants were found, then new transformant colonies were selected and the process was repeated until viable transformants were found.

Analysis of YFP content of Synechococcus *sp. PCC 7002 transformants*

Yellow fluorescent protein was isolated from *Synechococcus* sp. PCC 7002 transformants as described by Xu et al. (7). A 1-L culture of *Synechococcus* sp. PCC 7002 transformed with pAQ1Ex-P_X::YFP was grown photoautotrophically to late exponential phase ($0.8 < OD_{730} < 1.2$) in A^+ medium with spectinomycin (50 $\mu g \cdot mL^{-1}$). Cells were harvested by centrifugation (5,000 × *g*, Sorvall SLC-4000 rotor). The pelleted cells were then re-suspended in 40 mL lysis/binding buffer (50 mM Na_2HPO_4, 300 mM NaCl, 10 mM imidazole) and transferred to a 50-mL Falcon tube. The suspension was centrifuged again (5,000 × *g*, Piramon Technologies F13-14x50cy rotor) and the resulting pellet was re-suspended in 20 mL lysis/binding buffer. *Synechococcus* cells were then disrupted by three passages through a chilled French pressure cell operated at approximately 138 MPa. The cell extract was clarified by ultracentrifugation (90,000 × *g* for 35 min, Beckman 70Ti rotor). A 1.0-mL aliquot of Ni-NTA resin (Qiagen) that had been washed and re-suspended in lysis/binding buffer was then added to the cleared lysate and incubated with constant mixing for 1 h at room temperature. The resulting slurry was applied to a column and YFO was eluted using treatments with lysis/binding buffer containing imidazole at 30, 50, 100, 250, and 500 mM concentrations, using wash volumes of 4 mL, 0.5 mL, 0.75 mL and 0.5 mL, respectively. The YFP content of each sample was then examined visually using UV excitation light. The YFP content was further analyzed by sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE) on a 10% (w/v) polyacrylamide gel in the presence of sodium dodecylsulfate.

Spectroscopic Assay of YFP content

Starting cultures of *Synechococcus* sp. PCC 7002 transformed with pAQ1Ex-P_X::YFP (where the subscript '*X*' represents a given promoter) were grown photoautotrophically as described above to an OD_{730} of 0.8-1.2. This starting culture was used to inoculate experimental cultures (initial OD_{730} = 0.2), which were grown until the desired OD_{730} was reached. Spectinomycin (50 $\mu g \cdot mL^{-1}$) was added to starting and experimental cultures. Spectra were taken on an Olis® DM 45 Spectrofluorimeter.

Single-cell fluorescence measurements using flow cytometry

Starting and experimental cultures were grown as for the spectroscopic assay (see above section). Single cell fluorescence measurements were taken on a Coulter XL-MCL cytometer (15 mW Ar^+ ion laser, λ_{exc} = 488 nm).

DNA sequencing and oligonucleotides synthesis

Oligonucleotides were synthesized on a MerMade 12 DNA synthesizer. Both oligonucleotides synthesis and DNA sequencing were performed by the Genomics Core Facility at the Huck Institutes for Life Sciences, University Park, PA.

Results and Discussion

This section of the report is divided into three portions: 1) explanation how transformants were constructed, 2) examination of differential expression using constitutive promoters, and 3) examination of inducible expression using an inducible promoter.

Construction of Synechococcus *sp. PCC 7002 transformants*

Five promoters were selected for this study (*Table 2*): four were selected to examine the capability for differential expression on pAQ1 (*psbA2*, *km pro*, *cpe*, and *cpc*) and one was selected to examine the potential to induce expression by changing growth conditions (*sbtA*). For the *differential expression* study, promoters were taken from a variety of source organisms in an attempt to obtain a range of expression levels. The *cpcBA* promoter (phycocyanin promoter) is from *Synechocystis* sp. PCC 6803. Using the primers *fdcpecpF* and *fdcpecpR* (*Table 1*), the *cpeC* promoter was derived from *Fremyella diplosiphon* for the *cpe* transformant strain. The promoter for the *aph2A* gene in the kanamycin resistance cassette of pRL161 was derived using the primers $P_{kan}F$ and $P_{kan}R$ for the *km pro* transformant (Ref. 18). The *psbA2* promoter was derived from *Synechocystis* sp. PCC 6803 using primers *6psbAF* and *6psbAR* for the *psbA2* transformant. Sequences for each primer can be found in *Table 1* of the Materials and Methods section.

Table 2. Promoters selected to evaluate expression capabilities of pAQ1

Promoter *(abbreviation)*	Source of promoter	Explanation
cpcBA *(cpc)*	*Synechocystsis* sp. PCC 6803	phycocyanin promoter
cpeC *(cpe)*	*Fymmyella diplosiphon*	phycoerythrin promoter
aph2A *(km pro)*	pRL161 plasmid	promoter for the *aph2A* gene in the kanamycin resistance cassette of pRL161
psbA2 *(kmpro)*	*Synechocystsis* sp. PCC 6803	promoter for photosystem I reaction center protein operon
sbtA *(sbtA)*	*Synechocystsis* sp. PCC 6803	promoter for sodium dependent bicarbonate transporter; low Ci inducible

Candidate promoters were amplified, digested with appropriate restriction endonucleases, and cloned into the "Promoter X" region of the pAQ1 plasmid (as represented below in *Figure 1A*). Primers used to amplify promoters from host organisms are found in *Table 1* in the Methods section. Linearized fragments (*Figure 1B*) that had been amplified using the appropriate pAQ1 flanking promoters (*pAQ1 flankAF and pAQ1 flankBR*) were used to transform *Synechococcus* sp. PCC 7002 as described in the Materials and Methods section.

Figure 1: *Model of the pAQ1Ex-P$_X$-YFP expression vector and linearized fragment*

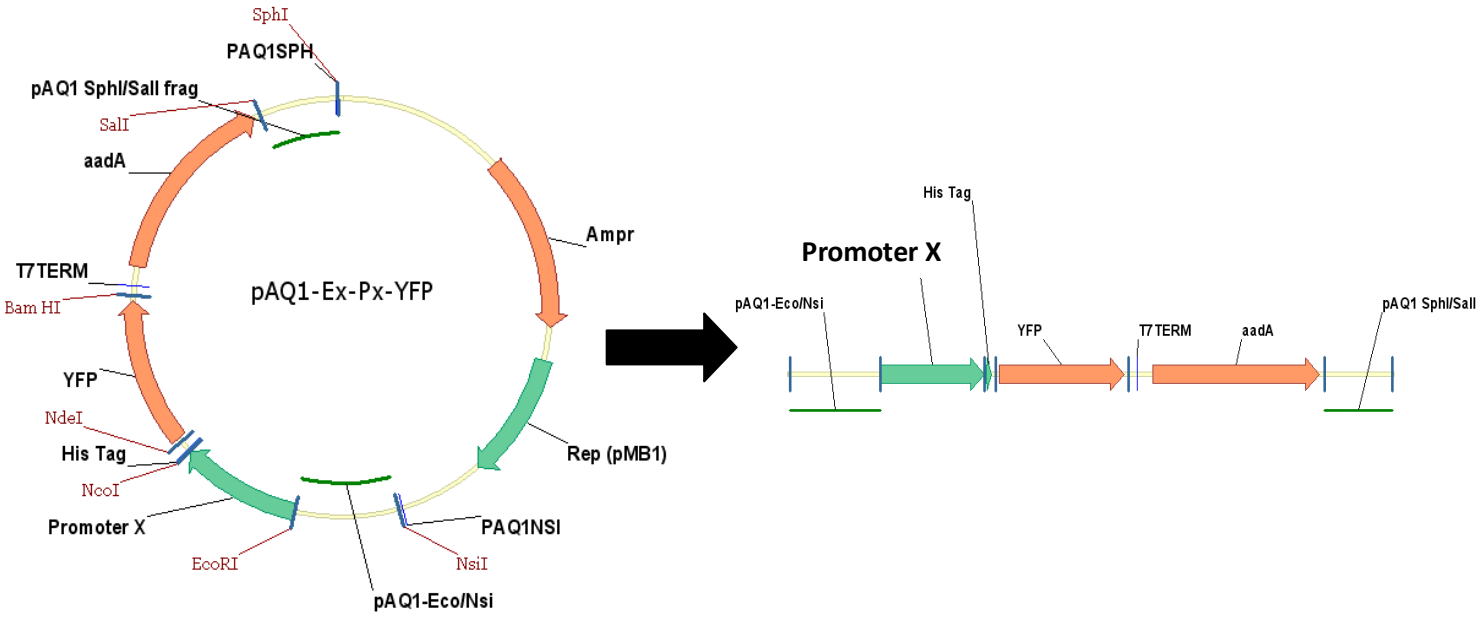


Figure 1A-B. A schematic of linearization of a section of pAQ1-Ex-P$_x$-YFP for transformation into *Synechococcus* sp. PCC 7002. Restriction sites are shown in red. Promoters are shown as green arrows, genes are shown in orange. **A)** Circular pAQ1-Ex-P$_x$-YFP harboring a promoter site, a deca-His Tag, antibiotic resistance cassettes (*aadA* and *Ampr*) and restriction sites. **B)** PCR amplification using the *pAQ1 FlankAF* and *pAQ1 flank BR* primers and the circular plasmid in part 1A as a template yielded a linear DNA fragment. This fragment was then used to transform wild type *Synechococcus* sp. PCC 7002

After the 7-10 day incubation period, colonies were PCR-screened to determine if viable transformants had been obtained. Either isolated cyanobacterial DNA or whole cells were used

as a template. To screen any given transformant, two promoters were used: 1) *eyfpR* and 2) the forward primer used to amplify the particular promoter. The choice of these two primers ("*Forward-Primer*$_{Promoter\ X}$" anneals to the 5'-end of the promoter region, *eyfpR* anneals to the 3'-end of YFP) ensured that *Promoter X* and YFP were oriented correctly.

Figure 2, below, illustrates one example of a successful PCR screen (for the *sbtA*-transformant), however analogous PCR-screens were performed for each other transformant used in this study (*psbA2*, *km pro*, *cpe*, and *cpc*). For the *sbtA*-transformant, the PCR amplification product was of appropriate length (~1100 base pairs) for the *sbtA/YFP* region of the cyanobacterial pAQ1. DNA that had been amplified from the PCR reaction was isolated and sequenced to confirm that the promoter and YFP had been incorporated into *Synechococcus*. Sequences obtained from transformants were compared to known DNA sequence data for YFP and *sbtA* (taking into account experimentally introduced restriction sites) to ensure the fidelity of the transformation.

Figure 2, below, illustrates an example of a successful PCR screening of seven *Synechococcus* colonies transformed with *pAQ1Ex-P*$_{sbtA}$*-YFP*. Again, though only the data for the *sbtA* transformation are shown here, the same experimental scheme was followed for constructing each *Synechococcus* sp. PCC 7002 *pAQ1Ex-P*$_X$*-YFP* transformants (PCR amplification of cyanobacterial DNA using analogous primers, followed by sequencing).

Figure 2: PCR confirmation of promoter and YFP in 7002

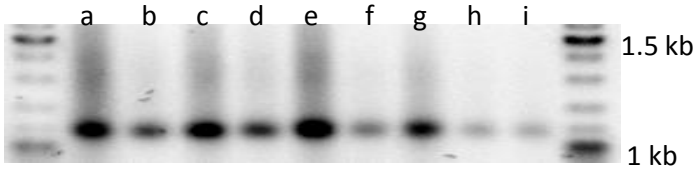


Figure 2. PCR was used to screen for successful transformant colonies. *Lanes a-h* are the result of PCR reactions using whole *Synechococcus* transformant cells. *Lane i* is the positive control, using *pAQ1Ex-P*$_{sbtA}$*-YFP* plasmid (isolated from *E. coli*) as a template.

Analysis of Differential Expression of YFP in pAQ1

The process of integrating promoters into the cyanobacterial pAQ1 plasmid was repeated until the desired *Synechococcus* sp. PCC 7002 pAQ1Ex-P_X-YFP transformants had been constructed. Relative total population fluorescence was measured for each transformant using a bench top spectrofluorometer (λ_{exc} = 493 nm). The results of the fluorescence assay are shown below in *Figure 3*. Values for "$Fluor_{527}$ per OD_{730}" are represented on the y-axis. The y-values reported represent the peak value for the fluorescence spectrum at 527 nm. The fluorescence is given per absorbance unit (OD_{730}) to account for any differences in fluorescence that might have occurred due to different cell densities.

Figure 3: Spectroscopic analysis of *Synechococcus* 7002 pAQ1Ex-P_X-YFP transformants

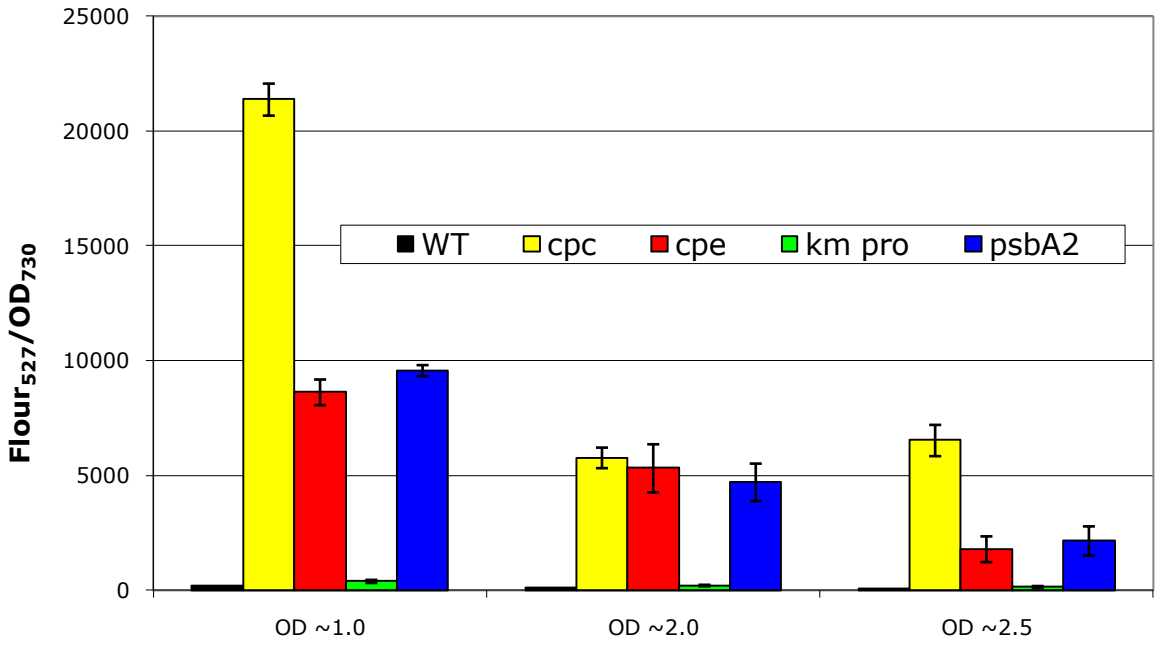


Figure 3. . In the figure above, "cpc" corresponds to *Synechococcus* sp. PCC 7002 harboring pAQ1Ex-P_{cpcBA}-YFP. Abbreviations for promoters are those used in *Table 2*. Here, 'WT' refers to wild-type *Synechococcus* sp. PCC 7002.

In general, voltages as a result of YFP fluorescence (per absorbance unit) were highest in exponential phase (OD_{730} = 1.0) and decreased gradually as the cultures grew into stationary and late-stationary phase for each transformant. Transformants harboring the *cpcBA* promoter exhibited the highest levels of fluorescence initially, after which the fluorescence per absorbance unit decreased nearly four-fold between logarithmic and stationary phase (*Figure 3*). Fluorescence values for the *cpc* transformant did not change (within experimental error) between stationary and late stationary phases.

YFP fluorescence levels were comparable for the two *cpe* and *psbA2* transformants throughout the duration of the experiment (*Figure 3*). Initial fluorescence levels were about half the maximum value for the *cpc* transformant, after which fluorescence decreased by a factor of two between each stage of the experiment. Except for the measurement at log phase, differences in fluorescence values between the two transformants remained within experimental error. Flourescence values for the *kmpro* transformant remained consistently low throughout the experiment (25 times less than the maximum value for the *cpc* transformant in exponential phase). Though the fluorescence was low in the *kmpro* transformants (*Figure 3*), a distinguishable peak, albeit not very pronounced, was visible at 527 nm during each stage of the experiment (spectra not shown).

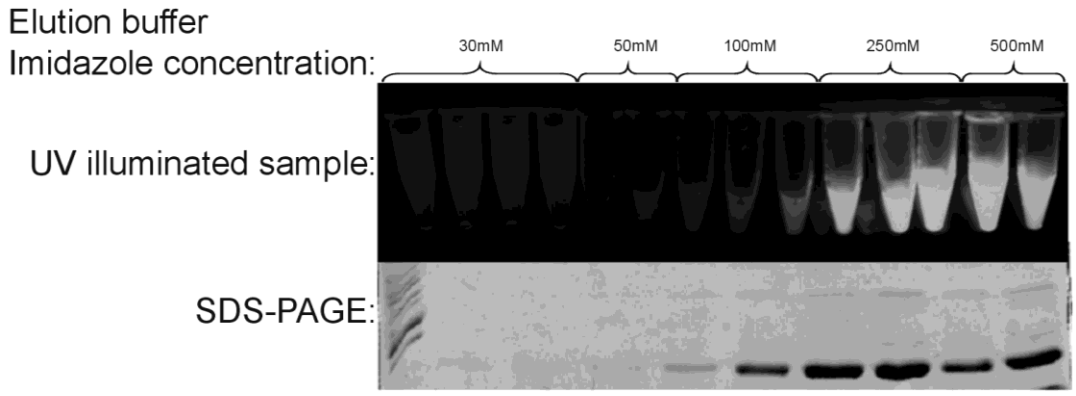
Spectrofluorimetric analysis of these four transformants showed that differential expression of YFP can be achieved in *Synechococcus* using the pAQ1 expression system. It should be noted that, while the fluorescence per absorbance unit decreased with time, the total population fluorescence in each culture increased. Presumably, expression of the *Synechococcus* homologues of *psbA2* and *cpe*C decreased regularly as the cell density in each culture increased.

Interestingly, fluorescence per absorbance unit in the *cpcBA* transformant was high initially, then decreased by half and remained constant for the remainder of the experiment.

No conclusions as to the levels of YFP transcripts can be made from the data shown here, especially since the quantity measured (YFP fluorescence) depended explicitly on whole protein content and not directly on transcript level. Nevertheless, it would be worthwhile to conduct comprehensive expression analysis of YFP and the homologous gene in *Synechococcus* for each PromoterX-transformant strain (e.g. collect transcripts for YFP and psbA2 in the 7002 pAQ1Ex-P_{psbA2}-YFP transformant). Although the promoter regions on the chromosome and pAQ1 are different (pAQ1 promoters are derived from other organisms to prevent homologous recombination on the chromosome), the transcription analyses would allow for a comparison of transcription on pAQ1 versus transcription on the cyanobacterial chromosome. Such analyses might be used as a criterion for selecting candidate promoters for future pAQ1 transformants.

Levels of yellow fluorescent protein in each transformant were also characterized qualitatively by protein gel electrophoresis as described in the Materials and Methods section. *Figure 4*, below, shows the results of Nickel-NTA column isolation and SDS-PAGE blot of His-tagged yellow fluorescent protein from *Syenchococcus* cell cultures. In contrast to the spectrophotometric assay, this analysis allows for isolation of whole protein.

Figure 4: Visual and immunoblot characterization of *Synechococcus* 7002 YFP content



The intrinsic fluorescence of YFP and the deca-His tag that was engineered into pAQ1 allowed for straightforward isolation and identification of YFP. For the purpose of this study, Spectrofluorimetric data were sufficient to characterize levels of YFP. Though these types of analyses were not performed here, SDS-PAGE can be used to quantitatively analyze whole yellow fluorescent protein content. This figure merely demonstrates the feasibility of such an analysis for a future study.

Analysis of Inducible Expression of YFP in pAQ1

To examine inducible expression of YFP using the pAQ1 system, *Synechococcus* sp. PCC 7002 harboring the pAQ1Ex-P_{sbtA}-YFP plasmid (hereafter, *sbtA* transformant) were subjected to a Spectrofluorimetric assay as a function of time. *SbtA* transformants were grown photoautotrophically under bubbling with 2% CO_2 until the cultures reached an OD_{730} of about 0.5, at which time 1 mL of the starting culture was used to inoculate new, experimental cultures. Experimental cultures were grown under two sets of conditions: 1) bubbling with 2% CO_2 or

2) bubbling with air. Henceforth, "*bubbling with 2% CO_2*" shall be referred to as either "high CO_2" or "high Ci" conditions, and "bubbling with air" shall be referred to as "low CO_2" or "C_i limiting" conditions. All other variables were kept constant (light, temperature, medium). Fluorescence spectra were taken at intervals of t= 3, 6, and 22 hours. Experiments were performed in triplicate and the fluorescence values were averaged to obtain the result in *Figure 5*.

Figure 5 shows that the bicarbonate-sensitive nature of the *sbtA* promoter resulted in induction of YFP expression for cells that were grown under Ci limiting conditions (bubbling with "low CO_2"). Total population fluorescence in the *Syenchococcus* cell cultures increased as a function of time between t=0 and t=22 hours. Fluorescence values for the sb*tA* transformant were similar after 3 hours, however after 6 hours total population fluorescence increased by a factor of four in cells grown under low CO_2. After 22 hours, differences in fluorescence between cells grown in low CO_2 and high CO_2 ranged from five- to eight-fold. Importantly, fluorescence did not increase dramatically for cultures grown under high-CO_2, reflecting tight control over induction.

It should be noted that although *Synechococcus* transformants grown to an OD_{730} of about 0.5 were used as starting cultures for these experiments, starting cultures having OD_{730} values ranging from zero to 2.5 could be used to achieve the same result. That is to say, induction of YFP expression (after switching to bubbling with air) was achieved regardless of the growth time of the starting culture (bubbled with 2% CO_2).

Figure 5: Time-evolved spectroscopic analysis of YFP Fluorescence in *Synechococcus* 7002 pAQ1Ex-P_{sbtA}-YFP

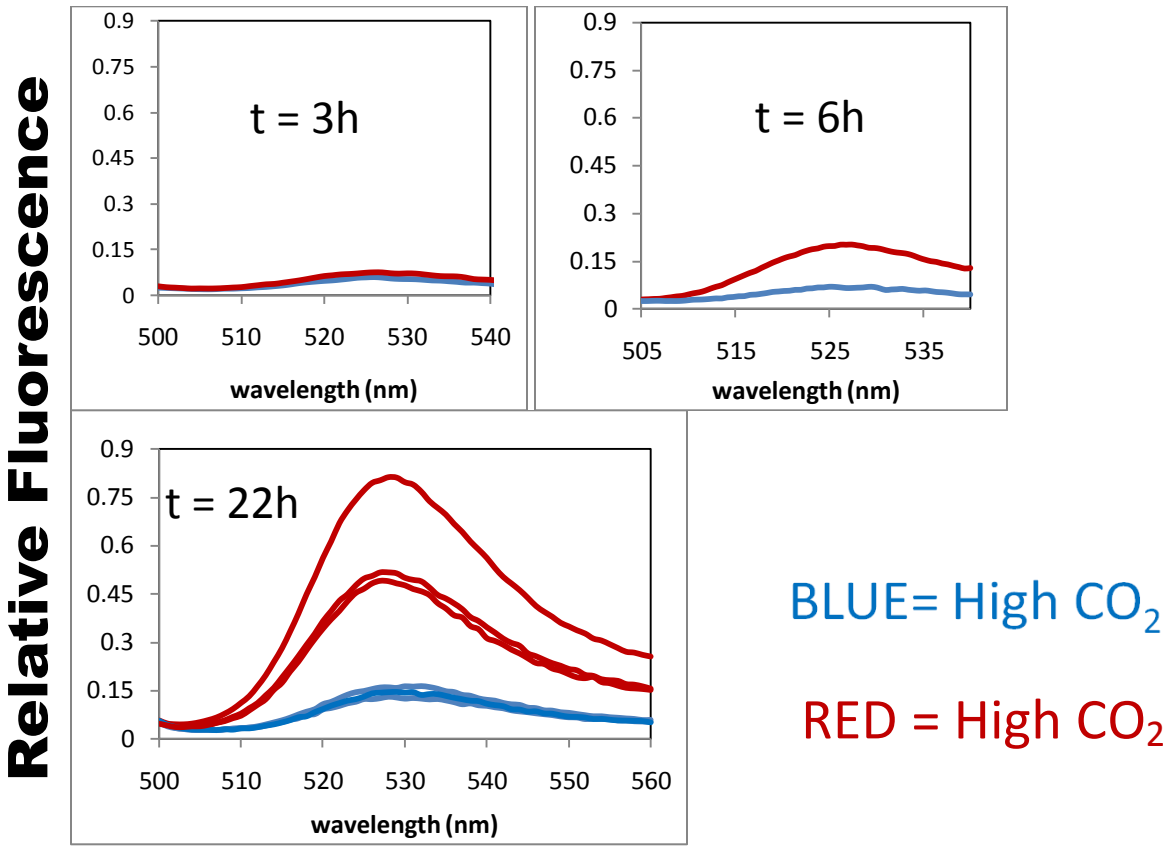


Figure 5. The *y*-axis in each graph shows relative fluorescence (peak at 527 nm). The red lines represent cultures grown under high CO_2, the blue lines represent growth under C_i limiting conditions. The results for *t= 3h* and *t= 6h* are an average of three separate cultures (all values agreed to within 5%). Because of the unusually high value for one of the low-C_i-grown cultures at *t= 22h*, the three cultures were reported separately.

To control for phenotypic variability within the monoclonal cell culture, the same experiment was run using a flow cytometer (Coulter XL-MCL). As was the case on the spectrofluorimeter, cells grown under high CO_2 bubbling showed little to no increase in fluorescence, whereas the average fluorescence at 527 nm per cell increased from zero to 22 hours. Wild type *Synechococcus* sp. PCC 7002 was also grown for a period of 22 hours.

Fluorescence values for wild-type were consistently low (*relative fluorescence* < 1, results not shown) in accordance with the results in *Figure 3*.

Figure 6: Time-evolved flow-cytometric assay of YFP content in *Synechococcus* 7002 pAQ1Ex-P_{sbtA}-YFP cultures

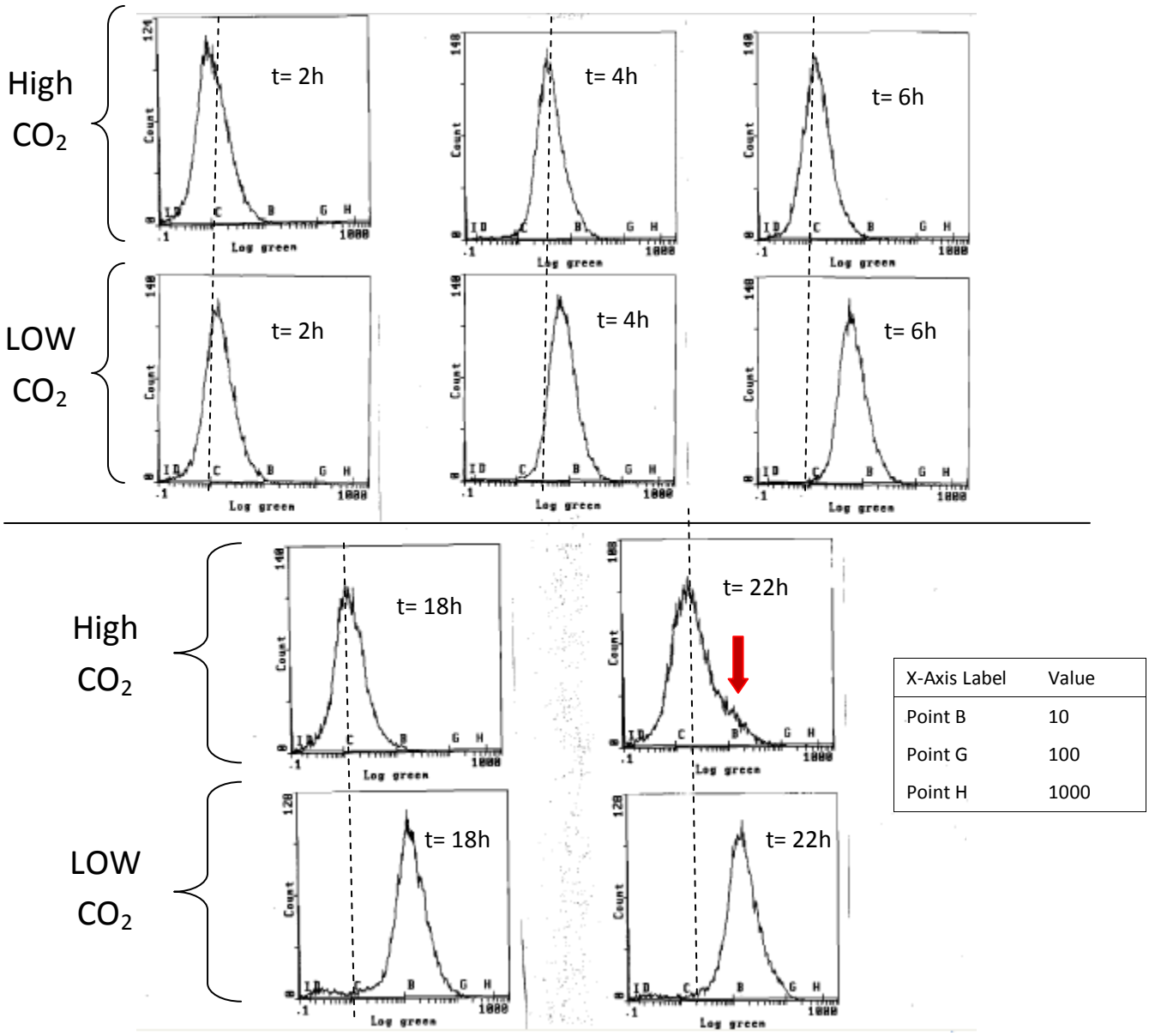


Figure 6. The *x*-axis of each histogram gives the $\log_{10}$(fluorescence). Labels on the *x*-axes are shown in the legend above. The *y*-axis (*"count"*) gives the number of cells counted at each fluorescence value. The total number of cells counted for each run was at least 20,000. The dotted lines are used to compare corresponding cultures of *sbtA*-transformants at each time point during the experiment. The red arrow points to a possible sub-population of high CO_2-grown *sbtA* transformants that appear to be fluorescing.

While the distribution of fluorescence values appears to be Gaussian for cells grown under Ci limiting conditions, a shoulder appears at 18 hours for cells grown under high CO_2, which then becomes more pronounced at 22 hours. This indicates that, even under "high CO_2" conditions, there is a portion of the culture population that is a population that is fluorescing as the cell density of the culture increases (*Figure 6*, High CO_2, t= 22h, red arrow). This observation suggests that as the cell density increases, competition for inorganic carbon increases amongst individuals in the culture population. As Ci becomes limiting, cells could activate their Ci scavenging pathway, thereby activating expression of YFP on pAQ1 in turn.

Figure 7. Fluorescence measurements for *sbtA* transformant in late-stationary phase

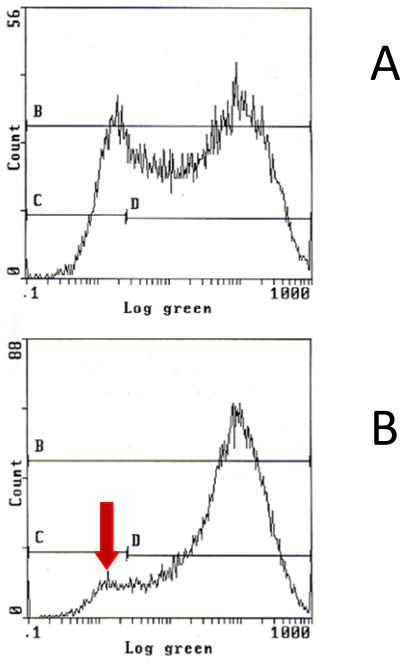


Figure 7. High CO_2-grown *sbtA* transformants grown to late stationary phase exhibited two distinct populations, one fluorescing and one non-fluorescing.

When cells are allowed to grow into stationary phase (OD_{730} of about 2.5), dual populations are seen for cultures grown under high CO_2 (*Fig. 7A*) and low CO_2 (*Fig. 7B*). The small shoulder that appeared during exponential phase (*Figure 6*, red arrow) is now a pronounced peak (*Fig. 7A*), implying that Ci does indeed become limiting when the cell density

becomes high enough. It may be possible that a high copy number of pAQ1 is to blame, especially considering the proposed mechanism for regulation of *sbtA*-expression in *Synechocystis* sp. PCC 6803. If expression of genes located downstream of the *sbtA* promoter are in fact regulated by a transcriptional repressor located on the chromosome, then perhaps the number of repressors encoded by the chromosomal gene is insufficient to repress expression of *YFP* on pAQ1. This possibility would need to be investigated further, since the pAQ1 copy number has only been characterized in exponential phase and is thought to vary with experimental growth conditions (7). It is unknown whether the high copy number persists into stationary phase.

It is unclear why a second, low-fluorescing population appeared in *Figure 7B* (red arrow). Cells that are grown continuously in Ci limiting conditions should presumably activate their carbon scavenging mechanisms throughout the experiment. However this result implies that is not the case. From these data, it is unclear as to why a portion of the cells would not be actively working to scavenge inorganic carbon in Ci-limiting conditions. It is possible that this low-fluorescing population comprises transformants that have lost their plasmid, though this would need to be confirmed. A cell sorting experiment might shed light on this mystery, as the high-fluorescing population could be separated from the low-fluorescing one. PCR screening of the separate populations would confirm whether or not the low-fluorescing population still harbored the pAQ1 plasmid. Furthermore, the experiment in *Figures 6-7* could be replicated to see if the fluorescent and low-fluorescent populations exhibited different responses to Ci-limiting stress.

Generally, the result that expression of YFP in the *sbtA*-transformant was inducible under Ci limiting conditions implies that the mechanisms for regulation of expression of *sbtA* are

indeed similar between *Synechococcus* sp. PCC 7002 and *Synechocystis* sp. PCC 6803. Of course, this study only suggests the possibility that the mechanisms are similar, though this hypothesis could easily be tested by conducting an experiment analogous to that of Lieman-Hurwiz and coworkers (19), in which expression of *sbtA* was studied in *Synechocystis* sp. PCC 6803 with *sll0359* (the putative *sbtA* transcriptional repressor) knocked out. A strain of *Synechococcus* sp. PCC 7002 pAQ1Ex-P_{sbtA}-YFP with the *sll0359* homologue knocked out could be tested to see if the *sll0359* homologue plays a role in the regulation of *sbtA* expression.

These possibilities give way to further considerations on pAQ1and its possible uses. It is not unreasonable to imagine, for example, that the *sbtA* inducible expression system (or some other inducible system employing some other inducible promoter) could be employed to study the gene function in *Synechococcus* sp. PCC 7002. Genes that have been knocked out on the chromosome could be re-introduced into the organism's genome via an *sbtA*-inducible pAQ1, allowing the experimenter to control when their gene of interest could be expressed. Furthermore, the inducible nature of the pAQ1 expression system could be employed for future bioengineering projects. For instance, expression of a particular gene set could be controlled as expression of YFP was controlled in this study.

The possibility that pAQ1 is present in high copy number in stationary phase has yet to be rectified, and the problem of homologous recombination of native genes remains as a barrier to studying native genes in pAQ1. If high copy number does in fact persist into stationary phase, Xu and coworkers demonstrated that a similar expression system can be employed using the pAQ3 plasmid, leaving open the possibility that a plasmid of lower copy-number might be employed (7).

Additionally, the *sbtA*-pAQ1 plasmid represents only one example of inducible expression. Other cyanobacterial genes are known that are differentially expressed under different growth conditions. For example, expression of the *psbA2* gene, whose promoter was employed in this study, is known to be up-regulated under high-light conditions (20). Further exploration of known inducible promoters and their subsequent integration into pAQ1 should result in a library of clones harboring inducible expression systems tied to a variety of controllable experimental conditions.

Conclusions

The pAQ1 expression platform developed by Xu and coworkers (7) has been investigated. It has been demonstrated that differential expression of YFP can be achieved by varying the promoter region in front of YFP. A wide range of expression levels was observed using four promoters. Furthermore, the capability to experimentally dictate the time of YFP induction was demonstrated using the promoter from the *sbtA* gene in *Synechocystis*, a gene whose expression was known to be affected by changes in inorganic carbon in solution. These initial findings have demonstrated the relative ease with which desired levels of YFP can be produced. In the future, incorporation of inducible promoter candidates should add another degree of versatility to the pAQ1 expression system.

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Publications

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