

Upregulation of Plasmid Genes during Stationary Phase in *Synechocystis* sp. Strain PCC 6803, a Cyanobacterium

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We analyzed DNA microarrays to identify highly expressed genes during stationary-phase growth of *Synechocystis* sp. PCC 6803. Many identified genes are on endogenous plasmids, with copy numbers between 0.4 and 7 per chromosome. The promoters of such genes will be useful for synthetic biology applications with this phototrophic host.

Bacterial cultures enter stationary phase when either nutrient limitation or a buildup of growth by-products ceases cell division. However, this does not necessarily imply that cells become metabolically inactive. Artificial “leaves” have been constructed from *Rhodospseudomonas palustris* cells embedded in latex that can produce H₂ photoheterotrophically for over 5 months without cell growth (5). *Pfic* in *Escherichia coli* was recently used to produce a high titer of a bacteriotoxin at stationary phase without any inducer and during exponential phase without detectable growth-limiting toxin during exponential phase (1).

Cultures and microarray analysis. We used DNA microarray analysis to identify genes and promoters active during stationary phase in *Synechocystis* sp. PCC 6803. This naturally competent cyanobacterium is a widely used host organism for autotrophic synthetic biology (3, 4, 13, 18). Synthetic biology is an emerging field in which genes, promoters, and other units of genetic code either taken from across the diversity of life or created entirely from scratch are mixed and matched in a host organism (chassis) to improve existing cellular functions or create entirely new ones. Such studies demand the use of a wide variety of promoters that are active under different conditions in the chassis organism.

We grew replicate cultures of *Synechocystis* PCC 6803 in BG11 medium bubbled with air plus 5% CO₂ (autotrophic) or with air plus 5 mM glucose (mixotrophic). The temperature was maintained at 30°C, and light intensity was 100 μE m⁻² s⁻¹ from cool white fluo-

rescent lamps. Cell growth was monitored by measurement of the optical density at 730 nm on a BioTek μQuant plate reader (BioTek, VT). Cultures were sampled for microarrays in exponential phase and twice during stationary phase (Fig. 1) and analyzed as described previously (20). Briefly, 2 replicate microarrays were analyzed for each of 2 replicate cultures for each nutritional condition. Data were LOWESS normalized by using the MATLAB bioinformatics toolbox. Normalized probe intensities were grouped by genes and *t* tested to determine significant up- or downregulation (*P* < 0.05).

SPPS. To quantify the activity of potential promoters at stationary phase, we calculated a stationary-phase promoter score (SPPS) for each open reading frame (ORF), based on the following equation: SPPS = log₂(fold change) + log₂(normalized expression).

The changes were averaged across nutritional conditions. Nor-

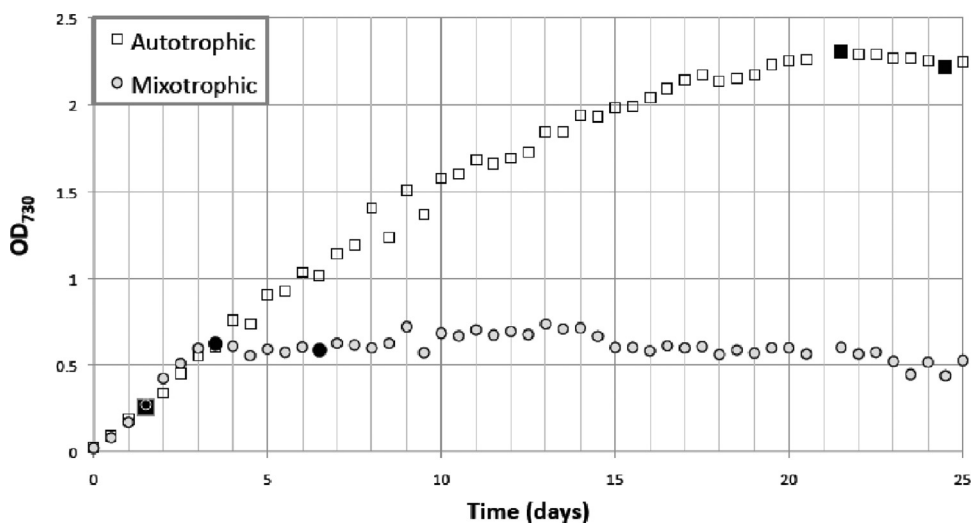


FIG 1 Growth curves. Time points sampled for nucleic acid analysis are indicated with filled symbols.

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TABLE 1 Top genes, ranked by stationary-phase promoter score

ORF	Replicon	Annotation	Stationary-phase promoter score	Normalized expression level	Fold changes (autotrophic/mixotrophic)
<i>slr9003</i>	pCC5.2	Unknown	8.53	7.46	1.76/0.38
<i>pSysA_116</i>	pSysA	Unknown	7.37	3.82	4.19/2.90
<i>slr9002</i>	pCC5.2	Unknown	6.87	4.41	0.44/4.47
<i>sll9006</i>	pCC5.2	Unknown	6.41	4.24	1.02/3.31
<i>pSysA_145</i>	pSysA	Unknown	6.40	3.53	4.02/1.72
<i>sll1982</i>	Chromosome	Putative transposase	6.32	4.85	2.28/0.66
<i>slr9101</i>	pCA2.4	Replication protein A	6.31	6.55	0.28/−0.77
<i>ssr9005</i>	pCC5.2	Unknown	6.03	3.46	−0.14/5.28
<i>pSysA_39</i>	pSysA	Unknown	5.97	3.44	2.89/2.17
<i>sll5036</i>	pSysM	Sulfide-quinone reductase	5.94	2.40	3.61/3.47
<i>pSysA_27</i>	pSysA	Unknown	5.92	3.32	2.96/2.24
<i>ssl9001</i>	pCC5.2	Unknown	5.90	3.58	0.05/4.59
<i>sll8019</i>	pSysG	Unknown	5.84	4.42	2.03/0.81
<i>pSysA_25</i>	pSysA	Unknown	5.83	3.17	2.99/2.33
<i>slr0915</i>	Chromosome	Putative endonuclease	5.68	3.81	1.93/1.81
<i>pCA24_1</i>	pCA2.4	Unknown	5.61	3.88	−0.01/3.47
<i>pSysA_24</i>	pSysA	Unknown	5.60	3.11	2.81/2.18
<i>ssr9004</i>	pCC5.2	Unknown	5.44	3.07	−0.48/5.22
<i>pSysA_34</i>	pSysA	Unknown	5.44	2.52	3.10/2.72
<i>pSysA_22</i>	pSysA	Unknown	5.24	2.37	3.03/2.72

malized expression was the mean normalized intensity of all microarray spots corresponding to a gene at stationary phase divided by the mean normalized intensity for all genes at stationary phase.

Many of the genes with the highest SPPS are located on endogenous plasmids, especially pSysA, pCA2.4, and pCC5.2 (Table 1). The upstream sequences of these genes are listed in Table 2. The genome of *Synechocystis* PCC 6803 includes 1 circular chromosome of 3.57 Mb, 4 larger plasmids of 44 to 120 kb (pSysA, pSysG, pSysM, and pSysX), and 3 smaller plasmids of 2.4 to 5.2 kb (pCA2.4, pCB2.4, and pCC5.2) (10, 11, 22, 24, 25). Although the plasmids of *Synechocystis* PCC 6803 have undergone limited study, plasmid-borne genes are required for glucose tolerance (9) and carry genes for a 2-component system responsive to low oxygen (21). The 3 smaller plasmids contain only 10 ORFs, and *repA* on pCA2.4 is the only one with an annotated function (16).

Most genes on pSysA, pSysG, and pSysM were upregulated during stationary phase under either nutritional condition. Under mixotrophic conditions, nearly all genes on the smaller plasmids (12/14) were also upregulated (Table 3).

In terms of function, our results agree with those of previous studies of the exponential-to-linear growth transition in *Synechocystis* PCC 6803 (2) and *E. coli* (6), which found that photosynthesis (in *Synechocystis*) and energy production processes (in both strains) were downregulated. However, the largest category of regulated genes in our study was that of unknown and hypothetical genes. Despite their unknown functions, the promoters of these genes are expected to serve useful roles in synthetic biology studies (15, 19).

Plasmid copy numbers. Because plasmid copy numbers often increase during stationary phase, we were interested in testing whether this phenomenon might explain the observed upregulation of plasmid genes (Table 4). We measured plasmid copy numbers per chromosome via quantitative PCR (12). The 3 smaller plasmids had higher copy numbers, in the range of ~3 to 7 at stationary phase under autotrophic conditions and at both exponential and stationary phases under mixotrophic growth conditions. The copy numbers of the 4 larger plasmids ranged from

~0.3 to 1.2 per chromosome and varied less with growth phase. Copy numbers of pSysA, pSysM, and pSysX were about twice as high during mixotrophic growth as during autotrophic growth, but copy numbers were only slightly higher for pSysG.

Copy numbers of pSysA, pCA2.4, and pCC5.2, the plasmids containing the highest-scoring SPPS genes, did not increase at stationary phase under any of the nutritional conditions, indicating that expression levels of such genes are controlled both at the gene dosage and transcriptional levels. For synthetic biology applications, the flexibility afforded by a range of available gene copy numbers and promoter specificities will serve as a benefit, since higher-copy-number plasmids have been associated with growth deficits, lower productivity, and lower inducibility (8). High-copy-number plasmids from *E. coli* have been modified for use in *Synechocystis* PCC 6803 (7) and have copy numbers between ~1 (14) and ~3 (17) per chromosome (10 to 30 per cell). These plasmids can be maintained with antibiotics, in contrast to endogenous cyanobacterial plasmids, which have higher copy numbers and can be modified to contain heterologous genes and maintained based on essential sequences they carry (23).

Thus, we have identified genes upregulated during the transition to stationary phase under various nutritional conditions in *Synechocystis* PCC 6803. These genes are mostly carried on plas-

TABLE 3 Genes up- or downregulated at stationary phase by replicons

Replicon	Total no. of ORFs in replicon	No. of up- or downregulated genes			
		Autotrophic		Mixotrophic	
		Up	Down	Up	Down
Chromosome	3,239	792	1,278	658	1,245
pSysA	134	105	2	123	2
pSysG	52	48	1	44	1
pSysM	141	104	9	95	13
pSysX	114	51	31	42	14
pCA2.4	4	1	2	3	1
pCB2.4	4	0	4	4	0
pCC5.2	6	3	2	5	0

TABLE 2 Upstream sequences of high-scoring SPPS genes

ORF	Upstream sequence
<i>slr9003^a</i>	TCCAACAATAAAAGCTTTTCAGGAGGGAATTAAGATTGCTGCAGTAAACACGTAAGAAAGTTTGTAGCGTAAACAACTTACCTACAGACAATAACCCGGGCCCAAAA AGCCAAACAATACTTCAAAAATATTTCTACTGTAGCTTAAATAATCCCAAAAGAAAAGGGTCAACTTTGAAACCCGAGACCCCT
<i>pSysA_116</i>	ATATTTCTGGACGTGGAATGACTTTGTTCAGGTTTATGACACTGTACGAAATGAGATACTTTTGTGTAGCTTTTGTGTGAAAGAGGGGTGTTTGAAC GAGCCATGGGTACTATTGACCACTCAGCGCGTTCAGTCCCTCAGCGGAAAGAGGGTGTGAACCTTGAAGAAAGTTTCCCTTT ACGCAGATACGATGACCCCTTTTAGCACGCTAGGAGCGGTGATATCTTCTGCAACACCTATATAGTATTTGCGATCGCGAGCGTATGACCGGCAATAA GCTACACTGGCCGATTCACGCAAAAGATAATCCCTTAAGCAACGCAATAATCTTCTGGAACTTATATAAGGTCTGCAATATAACGCACG
<i>slr9002</i>	TTGGTCAGGAAATCCTTGGAAAATTTCCCTATCCCGTAAAGAAATTTCAAAGCCCAATTTTAAGAAATGAAAGCTTAAAGAAATGAAAGCTTCCGGGTAGCATCT TGGCAGCAAACTTTCCCATCTTTCCCGCCCAATTTTGGAGTTCCTTGGCTGCTTGCCTGCTTCAAAAATTTCCACCCGCTGCTGAAGTAATA ATATTTCTGGACGTGGAATGACTTTGTTCAGGTTTATGACACTGTACGAAATGAGATACTTTTGTGTGAACTTTCAGTCCCTAACGGGAAAGAGGGTGTGTTGAAC
<i>pSysA_145</i>	GAGCCATGGGTACTATTGACCACTCAGCGCGTTCAGTCCCTCAGCGGAAAGAGGGTGTGAACCTTGAAGAAAGTTTCCCTTT TCCCTGGCAATACGCTCAAAACGCAACTTCGATTTGTTCAAACAGAGTTGATAAACTGCTCATCGGAAAGGATAGGCTGTCAAAGTTGACGGTTATGGCGGGCGGC ATTGGCTTTACCCCAAGGCAATGATGTTGGCTCCCAATCTAAGTGTGATTTACGGTGACAGATACTCTATCGCTCTCTCTCAT
<i>slI1982</i>	AAATATGGCAATTTCAATTTTTCAGGTTTCCCAAGTTTCAAATTTCCCTTATATTTTACTGAGGATAAAGTGGGGATGACAAAATTTGCTGAAACCCCTTACCAGA TAAGGATGAATGACAGGCAATTTGACCGCTACAGGAAAGACTTACTGGCAAGGAGACTTCCCAAGATTTCCCAAGTCTCTACGAGGCTCAAAACGGCGGATCGCCAT
<i>ssr9005^a</i>	TCTGACGGAGGCACTAGGCTTAAAGCAATGCGGTGGGCCATCAAAGCAAAAATCAGAGAAAGCCCTAGTATCTTTGGAAAGGAACTGAA GGATTAGTAAAGGAAAGATTGCCACCGTTCAAGCAATATGCTGGAGCGATGTTTCACTCCGATCGCCGGATAGTAAAGGAAAGCTTTGGCGAAACAGG AAAAATCTAGTAAACAGAAAGATTGACGCTCCCGGATTAGTAAAGGAAAGAAATTAAGAGGATTTGGGGGCTGGCTAGTAAACA
<i>pSysA_39</i>	ATGCCCTTTTGTAGGAAATTCCTTACTAATCCGATAGCTTCAATCCCAAGTTTAACTTCAAGTGTGCTTCAATCCCAAGTGTGCTGAACTTATTTAATGAACTAGT CCTTCCCAATTCGAAACGCAATATTAAGAAATATAACATACATAGTGTGATCTGCTCAATAGTTTACTTAAAGTAAATPACATTTTATCTCTGATTTTATTTGAACCTAGT
<i>slI5036</i>	CTTACAGCAATTCAGTCCCGATCGCGGATTTAGTAAAGAAAGTAGACATTTGTAAGAACTTGGACTGTGAAAGAAAGTTTTCAGTCCCGATCGCCGGGATAGTAG AAGGAAAGAAAGGGCTGGTCTGAAACCGGAATTCGTTCTATTGTTTTCAGTCCCGATCGCGGGATTAGTAAAGGAAAGTCAAGTCTT TGGGTGACCAACAGACTATAGTGTTCAGTAGATTATCGCGTTCCTACCGTACTAGTGTCTGATAGGCTCAATGTTGTTTTCCCGGTCATACCTTCATAA CGCTTTTCTAGGGGATACCTTAGGTAGTGCAGTAGATTATCGTCTCCAGCGTGTAAACTGCTTGTAGGCGCAATGTAGG
<i>pSysA_27</i>	TTAAGAGAGTAACTTAACTTAAACAAGCAATTCGAGTCTTAAACGTACACCCAGAAAAGTTAAAGCCACTGGCAAGAGCGTGTCTCAGGCACGCCACAGGT AGCTACACAGACTAAATCTTATGTTGTAGTAGCATGCCAATTTGCCGGTAGCTCTCTGGGAAATTTAGGAGAGTATCTAAGCA CTTACAGCAATTCAGTCCCGATCGCGGATTTAGTAAAGAAAGTAGACATTTGTAAGAACTTGGACTGTGAAAGAAAGTTTTCAGTCCCGATCGCCGGGATAGTA GAAAGAAAGAAAGGGCTGGTCTGAAACCGGAATTCGTTCTATTGTTTTCAGTCCCGATCGCGGGATTAGTAAAGGAAAGTCAAGTCTT GTTGTACCAATTCAGTAAAGTACCGCCGTATCTCGAAATCCCTAAAATCTTACTTTCAGTGCATAGACTGGGATGAACTGCCCTAAAATAAAGTTTGG CAAAAATTTCCCGGATCAGTTATGATATTCGAAAGGACGGGGATAGAGCAGTCTGGTAGCTCGGGCTCAGGTCGCAAGATGTAAGCC
<i>slI8019</i>	TATGTCATTCAAAGCCCTTAAATGTCCTAAACCTTGCCAAATATGCAITTTCAITCTTTCAGTCCCGATCGCGGGATTAGTAAAGGAAAGTCAAGTCTT GGATAAGTCGGGATGACAAAATTTGTTGAAACCTTACCAGATAAGCCATAGAGCCATTTGACAAAAGTAGAAAACCCCTCAGTAAAGCT GGATTAGTAAAGGAAAGCCACCACCCAGTAAATCAGCAATTTATTAGTTCAGTCCCGATCGCGGGATTAGTAAAGGAAAGTCAAGTAAAGCTGATAGATCGTAGCGGAA TGCTATGGATGCCCTTAGTGTTCAGTCCCGATCGCGGGATTAGTAAAGGAAAGTTCGAGGTTCTTCTAAAATCTTCCCTATAT
<i>pSysA_25</i>	ACTAAGGCAATTTAGCCCGCAATTTGACCCCGATGGCGGATCGGTATTTTCCCGGTGTTGAGGGGATCTATTTTGTATCACTGATACCGTATCCCA CTGAACTCCTTATTAATCAAGCTTAGAACTGGATACCAAAACAGGGAGTTATGATGGAAATATAATCCCGTTAACAGGCTAAACCC TCGCCGGATTAGTAAAGGAAAGCAACACTCAGATCAGATTTCAATCAATCAAGCAAGTTTTCAGTCCCGATCGCGGGATTAGTAAAGGAAAGTCAAGTCCGAGG CCCAACAAAAGCCGTTGGTAGGATTTTCAGTCCCGATCGCGGGATTAGTAAAGGAAAGCCGACTTTGGATCGATAGAAATCCCGACG CCTTAGTGTTCAGTCCCGATCGCGGGATTAGTAAAGGAAAGTTCGAGGTTCTTCTAAAATCTTCCCTATATTTTGGTTTCAGTCCCGATCGCGGGATTAGT AGAAAGGAAAGCCACATTCATCGCTACAGACTTAGAAAGGATAGTACGAGTTTTCAGTCCCGATCGCGGGATTAGTAAAGGAAAGTCAAGTCAAGCT
<i>slr0915</i>	
<i>pCA24_1^b</i>	
<i>pSysA_24^c</i>	
<i>ssr9004^a</i>	
<i>pSysA_34</i>	
<i>pSysA_22^c</i>	

^a This ORF appears to be in an operon, with *slr9003* at the 5' end.

^b The predicted ORFs *slr0910* and *pCA24_1* overlap, and the latter includes an additional 42 nucleotides at its 5' end.

^c This ORF appears to be in an operon, with *pSysA_24* at the 5' end.

TABLE 4 Effects of nutritional condition and growth phase on plasmid copy number per chromosome

Replicon	Copy no./chromosome (mean \pm SD)			
	Autotrophic		Mixotrophic	
	Exponential	Stationary	Exponential	Stationary
pSysA	0.34 \pm 0.00	0.33 \pm 0.00	0.64 \pm 0.01	0.60 \pm 0.01
pSysG	0.64 \pm 0.01	0.54 \pm 0.01	0.72 \pm 0.02	0.83 \pm 0.01
pSysM	0.33 \pm 0.00	0.31 \pm 0.00	0.69 \pm 0.01	0.49 \pm 0.01
pSysX	0.65 \pm 0.01	0.66 \pm 0.01	1.24 \pm 0.02	1.09 \pm 0.01
pCA2.4	0.75 \pm 0.01	5.41 \pm 0.10	6.26 \pm 0.06	7.39 \pm 0.09
pCB2.4	0.40 \pm 0.00	2.46 \pm 0.02	3.74 \pm 0.04	2.68 \pm 0.02
pCC5.2	0.93 \pm 0.01	3.72 \pm 0.04	6.02 \pm 0.05	7.33 \pm 0.07

mids, whose copy numbers range between \sim 0.4 and 7 per chromosome. The transcriptional behaviors of the promoters of these genes may make them useful for synthetic biology applications where expression is desired only at stationary phase, to maximize production while not interfering with cell growth during the exponential phase. The higher copy numbers of these plasmids relative to the chromosome may also make them useful insertion sites for heterologous genes.

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