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ORIGINAL PAPER

Growth-phase dependent differential gene expression in *Synechocystis* sp. strain PCC 6803 and regulation by a group 2 sigma factor

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Abstract Cyanobacteria must continually alter their physiological growth state in response to changes in light intensity and their nutritional and physical environment. Under typical laboratory batch growth conditions, cyanobacteria grow exponentially, then transition to a light-limited stage of linear growth before finally reaching a non-growth stationary phase. In this study, we utilized DNA microarrays to profile the expression of genes in the cyanobacterium *Synechocystis* sp. PCC 6803 to compare exponential and linear growth. We also studied the importance of SigB, a group 2 sigma factor in this cyanobacterium, during the different growth phases. The transcription of approximately 10% of the genes in the wild type were different in the linear, compared to the exponential phase, and our results showed that: (1) many photosynthesis and regulatory genes had lowered transcript levels; (2) individual genes, such as *sigH*, *phrA*, and *isiA*, which encode a group 4 sigma factor, a DNA photolyase, and a Chl-binding protein, respectively, were strongly induced; and, (3) the loss of SigB significantly impacted the differential expression of genes and modulated the changes seen in the wild type in regard to photosynthesis, regulatory and the unknown genes.

Keywords Cyanobacteria · Light-limited growth · Sigma factors · Gene regulation · Microarrays · *IsiA* · *phrA*

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Introduction

Cyanobacteria are a physiologically and ecologically diverse group of microbes that occupy a wide range of habitats (Potts 1999; Taton et al. 2003). The competitive success of these dominant phototrophs depends heavily on continuous fine-tuning of growth rate in order to exploit the changing nutritional environment. To cope with depleted nutrients and exploit those that are plentiful, microbes undergo transitions from exponential to arithmetic (linear) growth into non-growth physiological states (Gerhardt and Drew 1994). As a required nutrient becomes exhausted, there is a cessation of growth and the bacteria are said to enter the stationary state (Nystrom 2004). The duration of the exponential and linear growth phase in culture depends upon the size of the inoculum, growth rate, environmental conditions, and capacity of the medium to support microbial growth. Physiological changes that occur as microbes transition through different

phases include decreases in cell volume (Huisman et al. 1996; Makinoshima et al. 2002, 2003), changes in cell wall composition (Makinoshima et al. 2003), accumulation of inhibitory metabolites (Siegele and Kolter 1992; Huisman et al. 1996; Nystrom 2004), increases in RNA and protein turnover (Mandelstam 1960; Nystrom and Kjelleberg 1989) and changes in gene expression (Tani et al. 2002).

In cyanobacteria, growth is also dependent on light intensity. Most cyanobacteria grow optimally in the range of 15–75 μE m^{-2} s^{-1} and batch cultures progress from a lag phase into an exponential growth phase. This is typically followed by a period of linear growth that continues until the culture reaches the non-growing stationary phase. Linear growth in bacteria occurs when there are perturbations in the environment such that a critical nutrient is regulated arithmetically; e.g., the limited diffusion of air through the cotton plug of a test tube (Gerhardt and Drew 1994). In cyanobacteria, linear growth is most often associated with light limitation caused by self-shading of cells as cultures reach a certain cell density (Tandeau de Marsac and Houmard 1993; Gerhardt and Drew 1994; Sakamoto and Bryant 1998). This linear growth phase can continue for days (Singh and Sherman 2006) before the culture finally enters the stationary phase.

In this study, we examine the global transcriptional changes that occur during the linear compared to the exponential growth phase. Experiments have been performed for individual genes as a function of light intensity or growth phase (Lepp and Schmidt 1998; Tonk et al. 2005), but much of what is known regarding gene expression during slower growing phases, including linear and stationary phase, results from studies in non-photosynthetic organisms, specifically *Escherichia coli* (Nystrom 2004) and *Bacillus subtilis* (Britton et al. 2002; Hoper et al. 2005; Koburger et al. 2005). One key group of regulatory genes involved in growth phase transitions encodes the RNA polymerase holoenzyme (Gruber and Gross 2003; Imamura et al. 2003b) and associated initiation factors (sigma factors) which are now classified into four groups (Gruber and Gross 2003; Murakami and Darst 2003; Paget and Helmann 2003). Group 1 sigma factors are proteins that recognize the promoters of housekeeping genes and are essential for cell viability (σ^{70}), whereas the structurally-similar Group 2 sigma factors are non-essential for growth. *Synechocystis* sp. PCC 6803 (*Synechocystis* 6803), a model organism for the study of cyanobacterial photosynthesis and other metabolic processes, has four closely related group 2 sigma factors, termed SigB, SigC, SigD and SigE (Cyanobase: http://www.kazusa.or.jp/cyano/cyano.html). *sigB* and *sigC* are up-regulated during stationary phase (Boylan et al. 1993; Imamura et al. 2003b) and are essential for gene regulation under environmental conditions such as low nitrogen (Asayama et al. 2004), heat shock (Imamura et al. 2003b; Li et al. 2004) and oxidative stress (Mostertz and Hecker 2003; Li et al. 2004). SigB and SigD demonstrated antagonistic light/dark-induced expression via changes in redox potential (Imamura et al. 2003a).

In this report, we also compared differential gene expression in the wild-type organism to a knock-out mutant defective in the group 2 sigma factor SigB. Based on these experiments, we have developed an overview of changes in gene regulation in *Synechocystis* 6803 in a change from exponential to linear growth and the resulting alterations in the physiological state.

Materials and methods

Strains and culture conditions

Synechocystis 6803 wild-type (WT) and Δ*sigB* were grown in chemically defined liquid BG-11 medium (Rippka et al. 1981) at 30°C, with shaking at 125 rpm under fluorescent cool white lights (30 μE m^{-2} s^{-1}). Growth was monitored by spectrophotometric measurement at A_{750} and direct cell counting with a Petroff-Hausser cell counter. Cells were inoculated into fresh media to a starting density of ~1 x 10^6 cells/ml. The doubling times of the laboratory cultures *Synechocystis* 6803 WT and Δ*sigB* under standard conditions were approximately 12 h. No statistical difference between the growth rates of WT and Δ*sigB* were observed at the time points (72 h and 120 h) chosen for this transcriptional comparison (Fig. 1). Exponential and linear growth phases in the present study were defined as cultures with cell density of 1.0×10^8 cells/ml (≈3 days), and 4.0×10^8 cells/ml (≈5 days), respectively (Singh and Sherman 2006). It should be noted that these conditions were quite different than those in the circadian microarray experiment of Kucho et al. (2005) in which cells were first dark adapted for 12 h and then placed in continuous light. The optical density was always maintained at approximately 0.35 OD_{730} by dilution with fresh medium. Our goal was to elucidate the global transcription as cells grew in batch culture and determine the effects of increased cell density versus light intensity.

Mutant construction

The construction of the *sigB* mutant was performed as described previously (Singh et al. 2006). The mutant

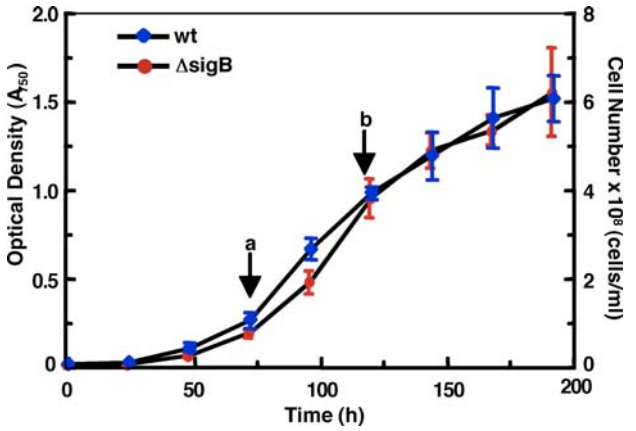

Fig. 1 Growth curve for *Synechocystis* sp. PCC 6803 wild-type (*WT*, *diamond*) and Δ*sigB* strains (*circle*) over the first 200 h of growth. Cells enter early exponential growth at approximately 72 h (**a**) and continue logarithmic growth until approximately 120 h (**b**) where the cells appear to enter a linear growth phase for at least another 3 days. Note the *y*-axis is arithmetic and not logarithmetic. *Experimental error bars* are shown for $n = 3$

could be complemented by the wild type gene (data not shown) and the complete segregation of the mutant was confirmed by PCR and southern blotting (data not shown). The resulting Δ*sigB* mutant did not show any definable phenotype and had the same growth rate as wild type under normal growth conditions. In addition, it did not show growth differences from the wild type under heat shock conditions (Singh et al. 2006).

DNA microarray hybridization

Total RNA was isolated from three biological replicates of either WT or Δ*sigB* cultures of *Synechocystis* 6803 during exponential and linear growth using methods described previously (Singh and Sherman 2000). The expression profile of *Synechocystis* 6803 during the transition between exponential and linear growth phases was examined using a DNA microarray that contained the 3,165 genes in the annotation of the *Synechocystis* sp. PCC 6803 genome prior to May 2002 (Postier et al. 2003). Fluorescently labeled cDNA was synthesized and hybridized to the *Synechocystis* 6803 microarray as described in (Singh et al. 2003). Briefly, 20 μg total RNA was used for cDNA synthesis using a reverse transcriptase reaction that incorporated an aminoallyl-modified deoxynucleotide (aadUTP) (Ambion, Austin, TX, USA). A fluorescent dye (CY3 or CY5, Amersham Pharmacia, Piscataway, NJ, USA) was chemically coupled to the aadUTP-modified cDNA. Prior to hybridization with the labeled cDNA probe, genome-printed microarray slides were washed twice with 0.1% SDS and once with deionized (DI) water for 5 min each in order to remove unbound materials. The slides were then boiled in DI water for 5 min to denature the printed DNA and then rewashed in 0.1% SDS for 5 min. Slides were incubated for 1 h at 42°C in a pre-hybridization solution containing 25% formamide, 5× SSC, 0.1% SDS, 1% BSA and 0.1 mg salmon sperm DNA. After prehybridization, slides were then rinsed with DI water, dried and pre-warmed in a hybridization chamber (Corning, Acton, MA, USA) to 42°C. A coverslip (Fisher Scientific, Hampton, NH, USA) was placed on the dried microarray slide and labeled cDNA probe was pippetted onto the slide through capillary action. The hybridization chamber was incubated at 42°C for 16 h in the dark. After hybridization the microarray was washed in a 2× SSC solution containing 0.1% SDS for 5 min at 42°C then rinsed in a solution containing 0.1× SSC and 0.1% SDS for 5 min. The slide was then rinsed twice in 0.1× SSC and once in distilled water for 5 min each rinse. Following washing, slides were dried and scanned immediately. The expression of genes of interest was independently confirmed with northern blotting using methods described below.

RNA isolation and northern blotting

Total RNA was isolated from three biological replicates of either wild-type (WT) or Δ*sigB* cultures of *Synechocystis* 6803 during exponential and linear growth using methods described in Singh and Sherman (2000). Five micrograms of total RNA was fractionated on a denaturing 1% agarose gel and transferred to a nylon membrane following the protocol of Sambrook et al. (1989). Membranes were then hybridized with DNA probes labeled with the chemiluminescent marker BrightStar Psoralen-Biotin (Ambion) and visualized using the BrightStar BioDetect kit (Ambion).

Statistical analysis

Microarray experiments were performed using a loop design procedure in which six slides (each slide contains three independent copies of the genome) were used, thus providing nine replicates for each gene (Fig. 2). Slides were scanned using the Scanarray 4000 (Perkin Elmer, Shelton, CT, USA) and spot intensities of the images were quantified by using Quantarray 3.0 (Perkin Elmer). Data were then collated into two sets (one for each experiment) by using SAS (version 8.02; SAS Institute, Cary, NC, USA). For each replicate block on a slide, there were 422 empty spots. We examined the distribution of spot intensities for these empty spots and declared data from a non-empty spot

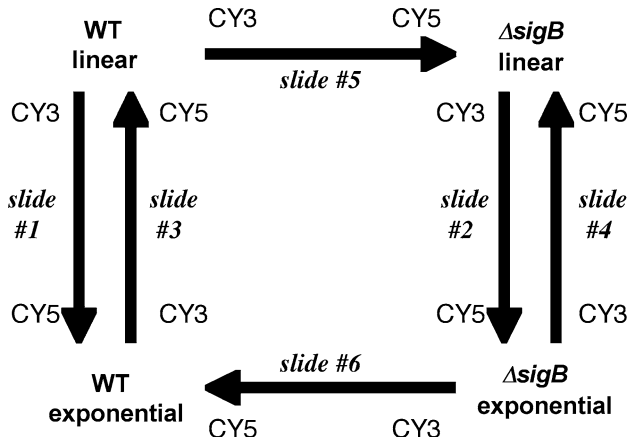

Fig. 2 Diagrammatic representation of the experimental loop design utilized for identification of differentially expressed genes during the transition from exponential to linear growth. A total of six slides was used with dye swaps between linear and exponential growth for both WT and Δ*sigB* cells. The results were analyzed with the ANOVA model as described in detail (Singh et al. 2003; Li et al. 2004)

to be detected if the background-corrected intensity of the spot was greater than that for 95% of the empty spots. If all the spots for a given gene were not detected on all the slides in an experiment, then the gene was considered to be ''off''and was not analyzed further (n = 1,346 genes). We then calculated the log of the background-corrected signals that were normalized to the slide median (i.e., the median for all non-control spots detected). Each experiment contained two genotypes (Δ*sigB* and WT) and two growth conditions (exponential vs. linear) for a total of four treatment combinations (Fig. 2). The effects of the mutant and the growth transition were examined in an analysis of variance (ANOVA) as described in (Singh et al. 2003; Li et al. 2004). Once the analysis was completed, we focused our attention on statistically significant and interesting genes (P value < 0.001) that exhibited a change of at least 1.4-fold (Singh et al. 2003; Lin and Wu 2004). In some cases, we included genes with a P value of 0.05 because they augmented or completed a functional category. Our objective was to identify genes that exhibited differential expression for further experimentation. Thus, we bracketed our interpretation of the results with a conservative (Bonferroni) threshold ($0.05/1819 = 2.7 \times 10^{-5}$) and a liberal 0.001 criterion, and we used a fold change filter to focus our efforts. The raw P values are shown in Table S1 in the supplemental materials.

Results

Overview of differential gene expression during exponential and linear growth

The determination of a suitable cell density (and/or time) to define exponential and linear growth was based on the growth and survival curves for both the WT and the Δ*sigB* strains (Fig. 1). Under our experimental growth conditions, we observed that the cell density reached over 1×10^9 cells/ml when the culture was allowed to grow up to 2 weeks (Singh and Sherman 2006). In this report, we limit our focus to transcriptional changes, and the effect of *sigB* on transcription, that occurred near to the transition from

Table 1 Functional categories of differentially expressed genes during the transition between Exponential (Ex) and linear (Ln) growth in wild-type (WT) and Δ*sigB* *Synechocystis* 6803

General pathway	Number of genes	$\frac{\text{WT Ln}}{\text{WT Ex}}$	$\frac{\Delta sigB\ \text{Ln}}{\Delta sigB\ \text{Ex}}$	$\frac{\Delta sigB\ \text{Ex}}{\text{WT Ex}}$	$\frac{\Delta sigB\ \text{Ln}}{\text{WT Ln}}$
Amino acid biosynthesis	97	1 (1)[a]	0 (0)	2 (1)	1 (1)
Biosynthesis of cofactors prosthetic groups, and carriers	124	11 (1)	7 (2)	2 (1)	5 (3)
Cell envelope	67	8 (2)	3 (2)	0 (0)	1 (0)
Cellular processes-chaperones cell division, chemotaxis	76	9 (4)	4 (0)	7 (7)	4 (4)
DNA replication/repair	60	3 (1)	2 (1)	0 (0)	1 (0)
Energy metabolism	132	6 (1)	4 (1)	1 (0)	4 (3)
Fatty acid synthesis	31	7 (3)	2 (1)	0 (0)	6 (3)
Hypothetical	1,076	24 (17)	18 (8)	9 (9)	16 (3)
Other categories	306	18 (8)	7 (3)	5 (4)	9 (5)
Photosynthesis	141	46 (2)	26 (1)	4 (2)	32 (29)
Purines, pyrimidines	41	0 (0)	0 (0)	1 (0)	0 (0)
Regulatory functions	146	32 (3)	23 (2)	2 (0)	16 (13)
Transcription	30	6 (4)	3 (2)	2 (1)	6 (1)
Translation	168	13 (4)	11 (3)	2 (0)	8 (3)
Transport and binding	196	12 (7)	5 (1)	5 (3)	7 (1)
Unknown	474	132 (98)	66 (46)	32 (18)	99 (16)
Total number of genes	3,165[b]	328 (156)	181 (73)	74 (46)	215 (85)

Genes were considered differentially regulated when $p < 0.001$ and the fold change was > 1.4-fold

[a] Number of up-regulated genes in each functional category

[b] Total number of genes based on Kazusa annotation prior to May 2002

exponential to linear growth. DNA microarray analysis of WT cells showed that some 328 genes were differentially regulated (P value < 0.001). (Table 1; Fig. 3). A number of genes in several pathways with important functions were differentially expressed in the linear compared to exponential growth (Table 2), and the absence of SigB had a significant impact on the differential expression of genes. In the Δ*sigB* , only 181 genes were differentially regulated, with 73 genes significantly up-regulated. The major functional groups affected were those genes associated with photosynthesis and regulatory functions and these functional categories will be discussed in greater detail.

Photosynthesis and respiratory genes

Of the 141 photosynthesis and respiratory genes present in *Synechocystis* 6803, 46 and 26 of the genes were differentially expressed in WT and Δ*sigB*, respectively. Of the 46 photosynthesis genes differentially expressed in WT, 44 genes representing proteins of all photosynthetic complexes were down-regulated. This trend of decreased photosynthesis gene transcription was similar in Δ*sigB*, although fewer genes were down-regulated. A notable exception to this pattern was the *isiAB* operon which was induced during linear growth. In particular, *isiA* was one of the most highly induced genes at 8.7-fold (P < 1.9E-08). The transcription of *isiA* demonstrated sensitivity to the loss of SigB and only increased 4.5-fold in the mutant (Table 2). These results were independently confirmed by northern blotting (Fig. 4). The *psbA* was relatively unchanged, as seen both by the northern blot (Fig. 4) and by the microarray results (see Supplemental Table). Thus, *psbA* represented a good control for genes, like *isiA*, that demonstrated significant changes in transcript levels in the two growth phases.

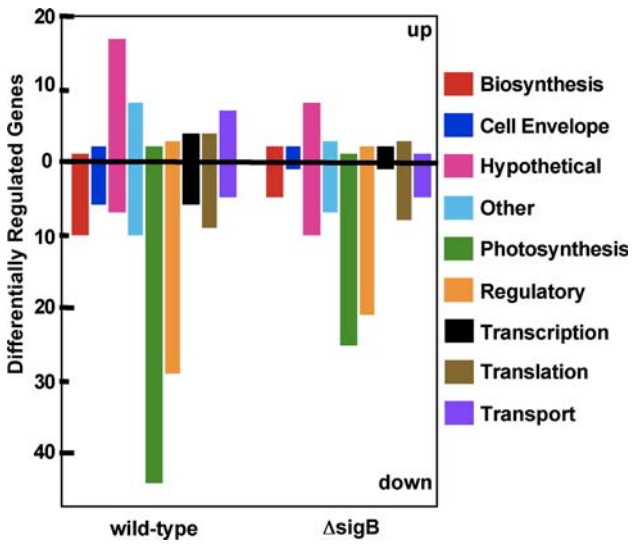

Fig. 3 *Histogram* of genes differentially expressed during the onset of linear growth divided into color-coded functional categories (unknown genes not included). Of the different gene categories, photosynthesis and regulatory functions contained the most number of genes that were differentially regulated, with most genes down-regulated

The changes in transcript levels of genes encoding photosynthesis proteins were profound and included all specific functions (Table 2; Fig. 3). Most of the genes that encoded proteins associated with photosynthesis including ATP synthase, CO_2 fixation, NADPH dehydrogenase, photosystem I and II, and phycobilisome were down-regulated by about 2-fold in the 5-day-old culture. (Table 2). The pattern is similar in Δ*sigB*, although fewer genes were down-regulated.

One- and two-component regulatory systems

Differential transcription of genes encoding regulatory proteins was also significant. These genes demonstrated a similar trend in *Synechocystis* 6803 WT and Δ*sigB* cells, and very few genes had enhanced transcription in the linear phase relative to exponential phase (Table 1). The genes categorized under Regulatory Functions in Table 1 include histidine kinases (*hik* genes) and response regulators (*rre* genes) of two-component regulatory systems and other uncategorized and little-studied regulatory genes, some of which are now categorized as one-component systems (Ulrich et al. 2005). The only regulatory proteins to show enhanced transcript levels in the linear phase were *hik37* (*sll0094*) and response regulator *rre30* (*sll0485*) both of which increased 2.9-fold (Table 2). However, at least 8 *hik* genes demonstrated transcript levels that were reduced two- to threefold. This includes *hik8* (*sasA*, sll0750), a gene that encodes a protein that is homologous to one that interacts with the KaiC circadian clock complex in *Synechococcus* sp. PCC 7942 (Iwasaki et al. 2000; Ditty et al. 2003; Golden 2003, 2004; Nishiwaki et al. 2004; Kucho et al. 2005), and involved in the regulation of carbohydrate metabolism in *Synechocystis* 6803 (Singh and Sherman 2005). Two other histidine kinases that had reduced linear phase transcript levels over two-fold were hik24 (*slr1969*), which is thought to be a circadian clock input sensor (Ditty et al. 2003), and Hik3 (*plpA*, *sll1124*), a phytochrome-like protein that was also reduced over twofold. These results suggested that proteins with phytochrome-like domains are down-regulated as cells become nutrient and light limited. Another important protein that was significantly reduced during was *slr0593* (–2.6-fold), a protein with presumptive cAMP-binding and kinase motifs.

Table 2 Selected genes from *Synechocystis* sp. PCC 6803 that are differentially regulated during the transition from exponential (Ex) and linear (Ln) growth phase in wild type and Δ*sigB*

Gene[a]	Gene function	Fold change[b]		*P* value
		WT(Ln/Ex)	Δ*sigB* (Ln/Ex)	
Biosynthesis of cofactors				
Thioredoxin, glutaredoxin				
slr0233	Thioredoxin M (*trxM*)	–	–1.4	8.9E–05
ssr0330	Ferredoxin-thioredoxin reductase (*ftrV*)	–2.6	–2.0	8.9E-06
slr0623	Thioredoxin (*trxA*)	–1.6	1.6	7.2E-04
Cell envelope				
Membranes, lipoproteins				
slr0423	Lipoprotein A (*rplA*)	3.3	2.6	2.8E-07
Cellular processes				
Chaperones				
sll0403	Heat shock protein (*htpG*)	2.3	–	6.2E-12
sll1514	Heat shock protein (*hsp17*)	2.0	–	1.4E-05
Detoxification				
slr1516	Superoxide dismutase (*sodB*)	2.3	–	7.2E-04
Central intermediary meabolism				
Polysaccharide and glygcoproteins				
slr1857	Glycogen operon protein (*glgX*)	–2.7	–2.0	6.0E-07
DNA replication, restriction				
sll0377	Transcription-repair coupling factor (*mfd*)	–1.7	–	3.9E-04
slr0854	Deoxyribopyrimidine photolyase (*phrA*)	10.8	5.4	5.1E-08
Photosynthesis and respirations				
ATP synthase				
sll1322	ATP synthase A subunit (*atpI*)	–2.4	–2.0	9.0E-08
sll1324	ATP synthase β subunit (*atpF*)	–1.8	–2.0	7.1E-05
sll1325	ATP synthase δ subunit (*atpD*)	–2.0	–2.0	1.0E-07
sll1326	ATP synthase α subunit (*atpA*)	–2.7	–2.0	6.1E-06
sll1327	ATP synthase γ subunit (*atpC*)	–1.7	–	4.1E–04
slr1330	ATP synthase ε subunit (*atpE*)	–1.6	–	5.7E-04
Carbon dioxide fixation				
sll1028	CO_2 concentrating mechanism (*ccmK*)	–2.2	–2.0	6.7E-05
sll1029	CO_2 concentrating mechanism (*ccmK*)	–1.9	–2.0	4.2E-07
sll1031	CO_2 concentrating mechanism (*ccmM*)	–2.3	–2.0	2.4E-05
sll1342	Glyceraldehyde-3-phosphate dehydrogenase	–1.5	–	2.5E-04
slr0009	Ribulose bisphosphate carboxylase (*rbcL*)	–1.6	–	9.8E-05
slr0051	Carbonic anhydrase (*icfA*)	–1.6	–	4.3E-04
NADP dehydrogenase				
sll1732	NADH dehydrogenase subunit 5 (*ndhF*)	–1.4	–	4.7E-04
sll1733	NADH dehydrogenase subunit 4 (*ndhD3*)	–3.2	–2.0	3.8E-05
slr2007	NADH dehydrogenase subunit 4 (*ndhD*)	–1.5	–	2.5E-05
Photosystem I and II				
sll0247	Iron-stress chlorophyll binding protein (*isiA*)	8.7	4.5	1.9E-08
sll1194	Photosystem II extrinsic protein (*psbU*)	–1.6	–	2.4E-04
slr0737	Photosystem I subunit II (*psaD*)	–1.4	–	8.6E-06
sml0001	Photosystem II PsbI protein (*psbI*)	–	–1.8	1.4E-04
sml0002	Photosystem II PsbX protein (*psbX*)	–1.5	–	8.5E-04
smr0001	Photosystem II PsbT protein (*psbT*)	–2.3	–2.0	2.9E-08
smr0007	Photosystem II PsbL protein (*psbL*)	–1.5	–	4.7E-04
Phycobilisome				
sll1577	Phycocyanin b subunit (*cpcB*)	–1.6	–	1.3E-09
sll1579	Phycocyanin associated linker protein (*cpcC*)	–2.0	–2.0	5.0E-21
sll1580	Phycocyanin associated linker protein (*cpcC*)	–2.1	–2.0	4.5E-04
slr0335	Phycobilisome LCM core membrane linker polypeptide (*apcE*)	–1.5	–	3.0E-04
slr1459	Phycobilisome core component (*apcF*)	–1.7	–	3.0E-04
slr1986	Allophycocyanin β chain (*apcB*)	–1.7	–	4.9E-07

Table 2 continued

Gene[a]	Gene function	Fold change[b]		*P* value
		WT(Ln/Ex)	Δ*sigB* (Ln/Ex)	
slr2067	Allophycocyanin α chain (*apcA*)	–2.0	–2.0	3.1E-05
ssl0452	Phycobilisome degradation protein (*nblA*)	–1.7	–	8.9E-05
ssl3093	Phycocyanin associated linker protein (*cpcD*)	–2.7	–2.0	4.9E-09
Soluble electron carriers				
sll0199	Plastocyanin (*petE*)	–2.0	–2.0	5.7E-05
sll0248	Flavodoxin (*isiB*)	2.2	–	5.9E-07
slr0150	Ferrodoxin (*petF*)	–2.7	–2.0	2.3E-07
slr1643	Ferredoxin-NADP oxidoreductase (*petH*)	–1.9	–2.0	2.0E-04
slr1828	Ferrodoxin (*petF*)	–2.5	–2.0	7.2E-07
ssl0020	Ferrodoxin (*petF*)	–2.2	–2.0	5.7E-06
Regulatory functions				
sll0030	Transcription regulator-one-component system[c]	–1.8	–2.0	2.8E-04
sll0094	Sensory transduction his kinase (*hik37*)	2.9	–	1.2E-06
sll0396	Response regulator OmpR subfamily (*rre28*)	–2.2	–2.0	8.7E-05
sll0485	Response regulator NarL subfamily (*rre30*)	2.9	1.4	1.0E-08
sll0750	Sensory transduction his kinase (*hik8*, *sasA*)	–1.7	–	1.7E-04
sll0776	Protein kinase (*pknA*)- One-component system[c]	–2.4	–2.0	7.7E-06
sll0789	Response regulator OmpR subfamily (*rre34*)	–2.0	–2.0	3.8E-06
sll1003	Sensory transduction histidine kinase (*hik13*)	–2.2	–2.0	5.7E-04
sll1005	MazG homologue	–2.9	–2.0	2.2E-06
sll1124	Sensory transduction histidine kinase (*hik3*)-phytochrome-like protein (*plpA*)	–2.2	–2.0	9.2E-05
sll1229	Receiver/transmitter (*hik41*)	–2.5	–2.0	3.0E-07
sll1708	Response regulator NarL subfamily (*rre17*)	–2.2	–2.0	2.8E-05
sll1888	Sensory transduction histidine kinase (*hik5*)	2.2	1.5	7.4E-05
slr0447	Periplasmic ABC-type (*urtA*, *amiC*)	–2.5	–2.0	4.0E-04
slr0593	cAMP binding membrane protein (*samp*)	–2.6	–2.0	1.1E-06
slr1214	Response regulator PatA subfamily (*rre15*)	–2.9	–2.0	1.3E-07
slr1871	Transcriptional regulator-One-component system[c]	–2.3	–2.0	9.2E-06
slr1969	Transmitter/receiver (*hik24*)	–2.0	–2.0	7.2E-05
slr2024	Response regulator CheY subfamily (*rre13*)	–2.3	–2.0	1.6E-05
slr2031	Sigma factor regulation protein (rsbU)	–1.9	–2.0	7.4E-04
Transcription				
RNA synthesis, modification, DNA				
sll0184	RNA polymerase sigma factor (*sigC*)	2.5	–	9.9E-05
sll0306	RNA polymerase sigma factor (*sigB*)	4.0	–	3.0E-05
sll0856	RNA polymerase sigma factor (*sigH*)	11.0	4.9	3.3E-09
sll1818	RNA polymerase alpha subunit (*rpoA*)	–1.6	–	4.6E–04
Translation				
Ribosomal protein synthesis				
sll0767	50S ribosomal protein (*rpl20*)	–	–1.4	8.7E-05
sll1767	30S ribosomal protein (*rps6*)	–1.4	–	1.4E-07
sll1799	50S ribosomal protein (*rpl3*)	–1.8	–2.0	5.3E-04
sll1800	50S ribosomal protein (*rpl4*)	–1.6	–	5.4E-04

[a] Genes were considered differentially regulated when the fold change was >1.4-fold and $P < 0.001$

[b] The fold changes for wild type and Δ*sigB* were calculated by dividing the normalized mean intensities of the linear growth phase by that of exponential phase

[c] One-component regulators as defined by Ulrich et al. (2005)

Expression profiles of selected genes

DNA microarray results revealed that several individual genes and certain gene categories exhibited dramatic changes in gene expression (Table 2). One such gene, *slr0854* (*phrA*), a deoxyribopyrimidine photolyase gene associated with DNA repair, was up-regulated 10.8-fold in wild type as cellular growth became linear, whereas it was only up-regulated 5.4-fold in Δ*sigB* ($P < 5.1E-8$). In the transcription gene category, three RNA polymerase sigma factors, *sigC* (sll0184), *sigB* (sll0306) and *sigH* (sll0856) were significantly up-regulated in the wild type, by 2.5-fold ($P < 9.9E-05$), 4-fold ($P < 3.0E-05$), and 11-fold ($P < 3.3E-09$), respectively. The transcript level of all three sigma factors was strongly affected by the lack of SigB and only *sigH* was up-regulated (4.9-fold) in Δ*sigB*. Interestingly, *sigH* and *phrA* are located near each other on the genome (Fig. 5a). The up-regulation of *phrA* and *sigH* during linear growth was independently confirmed with northern blotting. By 5 days, there was a dramatic increase in transcript levels in

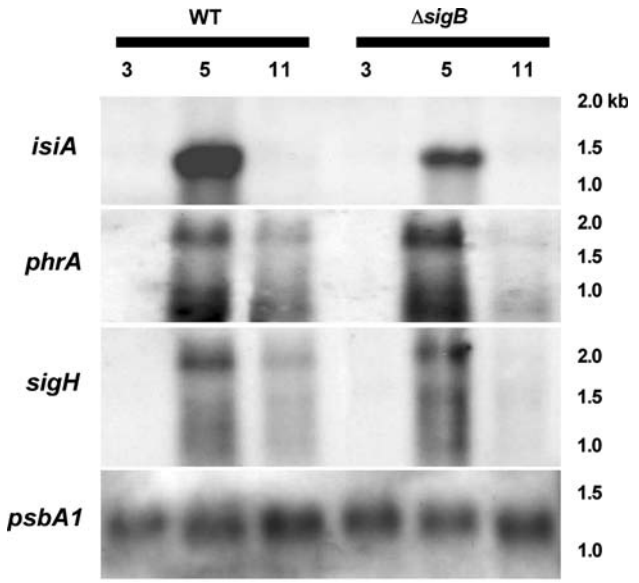

Fig. 4 Northern blots that validate the differential transcript levels of *isiA*, *phrA*, *sigH* genes during exponential (3 days) and linear (5 days) and stationary (11 days) phase in WT and Δ*sigB* cells. The gene encoding the photosystem D1 protein (*psbA1*) also was examined and used as a control, since it demonstrated essentially equal transcript levels at 3 and 5 days, with a slightly increased expression at 11 days in both WT and Δ*sigB* cells

both wild type and Δ*sigB* cells compared to 3 days (Fig. 4). Although by 11 days the transcripts of both genes have decreased, the differential expression of *phrA* and *sigH* in wild type and Δ*sigB* cells was still clearly visible (Fig. 4). A third gene, *sll0858* is also up-regulated (8.2-fold). This gene encodes a protein that is listed as hypothetical, but it has strong homology to the class of proteins that includes P pilus assembly/Cpx signaling pathway, periplasmic inhibitor/zinc-resistance associated proteins (Ruiz and Silhavy 2005).

Another key set of genes down regulated during linear growth were those encoding ribosomal proteins. Again, transcription of these genes was reduced ~2-fold in both strains. When we used $P < 0.05$ as a cut-off, over 25 ribosomal-protein genes showed a ~2-fold reduction (data not shown). These results are consistent with the cell regulating ribosome production as cell division is reduced.

The above analysis was mostly concerned with genes that demonstrated reduced transcript levels in the transition into linear growth. Not surprisingly, the classes containing the greatest number of up-regulated genes were hypothetical and unknown (i.e., those genes that have not yet been identified with a function or a functional domain). A total of 115 of the 156 up-regulated genes in the WT (74%) and 54/74 (73%) in Δ*sigB* were in these amorphous categories. Thus, many genes that become important under linear growth conditions have now been delineated, although their functions are mostly unknown. We do have information on some of these genes that seem especially germane for linear growth. In particular, the gene cluster, sll1722 to sll1726 (Fig. 5b), which contains three unknown genes and one poorly identified gene, encodes proteins that have strong homology to *E. coli* enzymes involved with the production of exopolysaccharide (e.g., colanic acid) (Rick and Silver 1996; Whitfield and Roberts 1999; Potrykus and Wegrzyn 2004).

Comparison of differentially expressed genes of wild type and Δ*sigB*

Differences in the gene expression pattern between wild type and Δ*sigB* cells are listed in Tables 1 and 3. Of the 3165 genes in the *Synechocystis* 6803 microarray, 78 differed between wild type and Δ*sigB* during exponential growth, whereas 214 differed during linear growth (Table 1). Once again, the two gene functional categories that demonstrated the most extensive differences between wild type and Δ*sigB* were the photosynthesis and regulatory categories (Tables 1, 3). In exponential phase, there were only two photosynthesis genes (fold > 1.4) differentially expressed between the wild type and Δ*sigB* mutant, whereas in linear growth 32 genes were different in the Δ*sigB* mutant (see Supplemental Table S1). The pattern was similar for regulatory genes and 16 genes were differentially regulated in the mutant relative to wild type in linear growth (Table S1). For both categories, 80–90% of

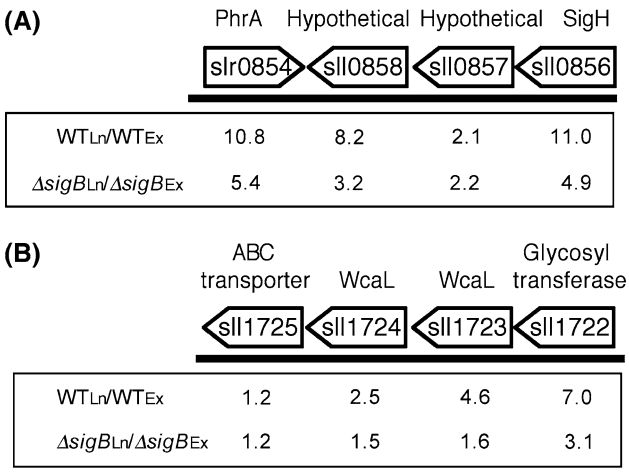

Fig. 5 Regions in the *Synechocystis* sp. PCC 6803 genome in which clusters of genes were up-regulated during the transition from exponential to linear growth. **a** The region that includes slr0854 (*phrA*) and sll0856 (*sigH*). **b** The region that includes genes that demonstrated homology to *E. coli* genes involved with exopolysaccharide production

Table 3 Selected photosynthetic and regulatory genes from *Synechocystis* sp. PCC 6803 differentially regulated in Δ*sigB* compared to wild type during exponential and linear growth

Gene	Gene function	Fold change [a,b]		*P* value
		Exponential	Linear	
Photosynthesis and respiration				
ATP synthase				
sll1322	ATP synthase A subunit (*atpI*)	–	2.0	9.0E-08
sll1325	ATP synthase δ subunit (*atpD*)	–	1.6	1.0E-07
sll1326	ATP synthase α subunit (*atpA*)	–1.7	1.7	6.1E-06
sll1327	ATP synthase γ subunit (*atpC*)	–	1.6	4.1E-04
Carbon dioxide fixation				
sll1031	CO_2 concentrating mechanism (*ccmM*)	–	1.7	2.4E-05
slr0009	Ribulose bisphosphate carboxylase (*rbcL*)	–	1.5	9.8E-05
slr0012	Ribulose bisphosphate carboxylase (*rbcS*)	–	1.5	2.5E-05
Photosystem I and II				
sll0247	Iron-stress chlorophyll binding protein (*isiA*)	–	–2.2	1.9E-08
sll1867	Photosystem II D1 protein (*psbA3*)	–	1.4	2.1E-05
slr1834	p700 apoprotein subunit (*psaA*)	–	1.6	5.9E-04
slr1835	p700 apoprotein subunit (*psaB*)	1.4	1.6	1.2E-06
slr0737	Photosystem I subunit II (*psaD*)	–	1.5	8.6E-06
sml0001	Photosystem II PsbI protein (*psbI*)	1.7	–	1.4E-04
sml0003	Photosystem II PsbM protein (*psbM*)	–	–2.0	9.0E-06
smr0001	Photosystem II PsbX protein (*psbX*)	–1.4	1.6	8.5E-04
Phycobilisome				
sll1577	Phycocyanin β subunit (*cpcB*)	–	2.0	1.3E-09
sll1579	Phycocyanin associated linker protein (*cpcC*)	–	2.2	5.0E-21
sll1580	Phycocyanin associated linker protein (*cpcC*)	–	2.1	4.5E-04
slr2067	Allophycocyanin α chain (*apcA*)	–	1.7	3.1E-05
ssl3093	Phycocyanin associated linker protein (*cpcD*)	–	1.8	4.9E-09
Soluble electron carriers				
sll0248	Flavodoxin (*isiB*)	–	–1.8	5.9E-07
slr0150	Ferrodoxin (*petF*)	–	2.2	2.3E-07
slr1828	Ferrodoxin (*petF*)	–	2.0	7.2E-07
ssl0020	Ferrodoxin (*petF*)	–	1.7	5.7E-06
Regulatory functions				
sll0030	Transcription regulator-one-component system[c]	–	1.5	2.8E-04
sll0094	Sensory transduction his kinase (*hik37*)	–	–1.9	1.2E-06
sll0485	Response regulator NarL subfamily (rre30)	–	–2.2	1.0E-08
sll0750	Sensory transduction his kinase (*hik8*, *sasA*)	–	1.5	1.7E-04
sll0776	Protein kinase (*pknA*)-one-component system[c]	–	1.5	7.7E-06
sll1003	Sensory transduction histidine kinase (*hik13*)	–	1.6	5.7E-04
sll1005	MazG homologue	–1.8	1.5	2.2E-06
sll1353	Sensory transduction histidine kinase (*hik15*)	–	1.5	9.1E-04
sll1888	Sensory transduction histidine kinase (*hik5*)	–	–1.6	7.4E-05
slr0447	Periplasmic ABC-type (*urtA*, *amiC*)	–	1.8	4.0E-04
slr0593	cAMP binding membrane protein (*samp*)	–	1.7	1.1E-06
slr0947	Response regulator-phycobilisoms to photosystems (*rpaB, ycf27*)	–	1.5	3.2E-05
slr1214	Response regulator PatA subfamily (*rre15*)	–1.5	1.5	1.3E-07

[a] Genes were considered differentially regulated when the fold change was >1.4-fold and $P < 0.001$

[b] The fold changes for exponential and linear growth were calculated by dividing the normalized mean intensities of Δ*sigB* by that of wild type

[c] One-component regulators as defined by Ulrich et al. (2005)

these genes were up-regulated in the mutant relative to the wild type, suggesting that the lack of SigB resulted in an increase of gene transcription in these genes.

A selected list of those photosynthesis ($n = 29$) and regulatory ($n = 13$) genes that had enhanced transcript levels in Δ*sigB* versus wild type under linear growth conditions are presented in Table 3. The photosynthesis category had a simple common theme in that a gene for at least one major protein in each complex was affected; e.g., rubisco and the carbon-concentrating mechanism; the major ATP synthase operon; PSI reaction center genes (*psaAB*); one PSII reaction center gene (*psbA3*, *sll1867*); and the major phycocyanin operon (*sll1577–sll1580*). Thus, SigB may be responsible for transcriptional control of an important sub-set of photosynthesis genes as the cells go into the linear growth phase. The lack of SigB also leads to enhanced transcription of some histidine kinases and response regulators including *hik8* (*sll0750*). These results suggest that SigB may be involved with negatively

regulating the transcription of a sub-set of genes involved with carbohydrate metabolism and circadian functions.

Discussion

The results of this study provide evidence that widespread differential gene regulation occurs during the physiological change from exponential to linear growth in the cyanobacterium *Synechocystis* 6803. Our results showed that: (1) many photosynthesis and regulatory genes had lowered transcript levels; (2) individual genes such as *sigH*, *phrA*, and *isiA* which encode a group 4 sigma factor, a DNA photolyase, and a Chl-binding protein, respectively, were strongly induced; (3) several genes currently categorized as hypothetical or unknown were up-regulated; and, (4) the loss of SigB significantly impacted the differential expression of genes; this included the up-regulation of the photosynthesis and specific regulatory genes as well as a general down-regulation of the hypothetical and unknown genes. Thus, SigB appeared to function as both a positive and negative regulator of gene transcription.

Cells respond to perturbations in their surrounding environment by continually adjusting their growth rates and physiological states. Here, we have examined cyanobacteria undergoing a change from exponential to linear growth. Few studies have examined this change as cells move from logarithmic to linear growth (Wyman and Fay 1987; Sakamoto and Bryant 1998). For *Synechocystis* 6803, we simply define the transition from exponential to linear growth states as the initial reduction of growth during the early stages of nutrient and light limitation. Optimal growth rates typically occur at 50–60 μmol m^{-2} s^{-1} (Wyman and Fay 1987), and we find linear growth began at ~4×10^8 cells ml^{-1} under our nutrient and light conditions (Fig. 1). Previously, it has been shown that during light-limited growth, the variation in cell Chl and phycobiliprotein content is coordinated with cell growth, and the Chl/phycobilisome ratio is constant (Wyman and Fay 1987). These results are consistent with our spectral results (Singh and Sherman 2006) and our global transcription analysis (Tables 1, 2) that showed that the transcription of most photosynthesis genes decreased a comparable level.

The nature of the changes in the photosynthesis genes is deserving of mention. Genes associated with the PSI and PSII core complexes demonstrated no significant changes in expression during the onset of linear growth. Therefore, the cells responded to an increase in cell density (and a net decrease in light intensity absorbed per cell) by down-regulating the transcription of certain genes needed for photosynthesis. As phototrophs go into low light conditions, they often adapt by producing larger antennae around the photosystems in order to collect more light (Tandeau de Marsac and Houmard 1993). The opposite appeared to occur during linear growth of *Synechocystis* 6803; thus, although the reaction center components are little changed, the peripheral components of the photosystems appear to be down-regulated, as are the energy producing complexes (i.e., ATP synthase, NADH dehydrogenase and the carbon concentrating mechanism) (Fig. 6). Decreases in photosynthesis gene

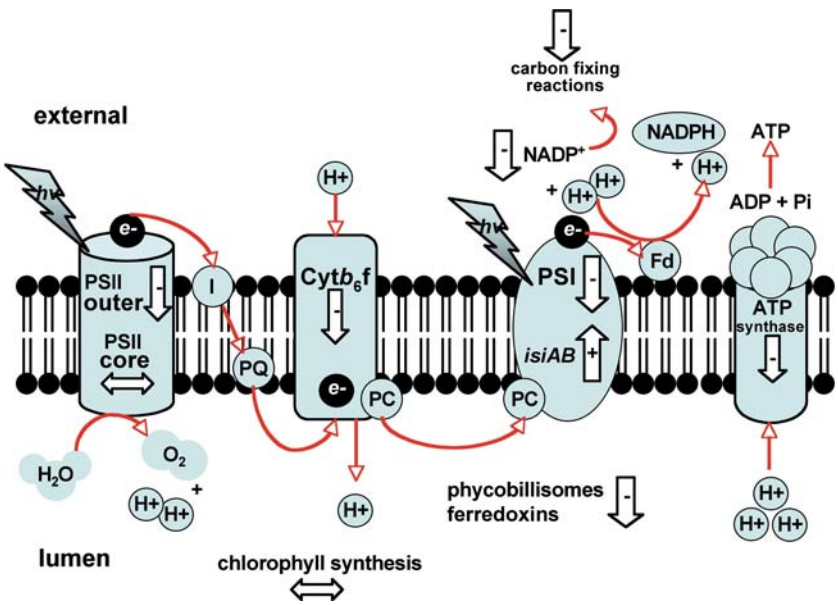

Fig. 6 A model of gene expression changes in photosynthesis in *Synechocystis* sp. PCC 6803. The figure is a diagrammatic representation of the four major photosynthesis complexes, along with a summary of the differential transcription results. The *arrows* indicate whether or not the genes for the complex showed enhanced transcript levels (*up arrow* +), showed decreased transcript levels (*down arrow*, –) or showed little or no change (*double-headed horizontal arrow*)

transcription have also been detected under other environmental stresses, such as elevated CO_2, UV stress and low nutrient conditions (Sinha and Hader 2002; Temperton et al. 2003; Wu et al. 2003).

However, a major exception to the decrease expression of photosynthesis genes was the enhanced transcription of *isiA* and *isiB*, which encode the iron-stress Chl-binding protein and flavodoxin, respectively. We have shown recently that the IsiA protein is produced as the cells become light-limited and eventually transition into stationary phase and that the typical IsiA spectrum (with a Chl peak at 672 nm) became evident by the time the culture was 5–6 days old (Singh and Sherman 2006). Several key studies have shown that *isiA* and *isiB*, organized as a dicistronic operon, facilitate the protection of PSI or PSII under nutritional stress conditions, specifically low iron (Park et al. 1999; Singh et al. 2003). In cyanobacteria, IsiA has also been shown to protect cells under other forms of environmental stress such as high light and oxidative stress (Park et al. 1999; Yousef et al. 2003; Havaux et al. 2005). Numerous papers have implicated IsiA as a non-radiative dissipator of light energy that would protect PSII against photooxidative stress (Sandstrom et al. 2001). More recently, IsiA has been shown to form multimers around the trimeric form of PSI, as well as with monomeric PSI and with other IsiA molecules (Bibby et al. 2001; Yeremenko et al. 2004). At a minimum, these studies indicated that IsiA functioned as an antenna for PSI, but the previous results involving PSII and our current results suggest a more complex role for IsiA. Our results suggest that a primary function of IsiA may be in the assembly/disassembly or protection of photosystem complexes during the transition to linear growth and may function as an antennae during conditions like low iron (Singh et al. 2003), salt stress (Vinnemeier et al. 1998; Vinnemeier and Hagemann 1999) or oxidative stress (Li et al. 2004).

In addition to *isiA*, two other genes were up-regulated greater than 10-fold during linear growth, including the DNA photolyase, *phrA* (10.8-fold) and the alternative sigma factor *sigH* (11-fold). Genome analysis has determined that *Synechocystis* 6803 has two DNA photolyase genes responsible for the repair of DNA photoproducts induced by UV radiation, *phrA* (*slr0854*) and *phrB* (*sll1629*). Of the two, only *phrA* was significantly up-regulated during linear growth in *Synechocystis* 6803 (Table 2). Previous studies have shown that *phrA* is the major photoreactivating factor in *Synechocystis* 6803 (Ng et al. 2000; Ng and Pakrasi 2001) and shares sequence similarities to eukaryotic cryptochromes known to function as transcriptional repressors (Ng and Pakrasi 2001; Brudler et al. 2003). In this study, the batch cultures of *Synechocystis* 6803 were grown in artificial light with no UV radiation, suggesting that the protection from UV-induced DNA damage was not the primary function of *phrA*. In addition, *phrA* transcription was virtually negligible under exponential growth conditions and decreased sharply as cells moved into stationary phase (Fig. 4). One function for PhrA may be to repair DNA damage (and the resulting mutations) that tend to increase as cells enter slower growth phases (Nystrom 2004).

The second gene up-regulated greater than 10-fold was the alternative group 4 sigma factor *sigH* (sll0856). Alternative sigma factors, although nonessential for growth (Missiakas and Raina 1998), have been shown to act as transcriptional regulators in response to environmental stresses, including heat shock and oxidative stress (Manganelli et al. 2002). In *Synechocystis* 6803, *sigH* was the only alternative sigma factor up-regulated during linear growth, and it was modulated twofold (but still up-regulated overall about fivefold) when SigB was missing. As the cells transition to stationary phase (11 days) the expression of *sigH* dropped significantly in both WT and Δ*sigB* (Fig. 4). These results suggest that SigH transcription is not the result of light limitation, but may be particularly important to the cell in linear growth when SigB is absent. Two genes with unknown function, located directly downstream of *sigH*, were also significantly up-regulated in linear growth suggesting a common promoter. Genes *sll0857* and *sll0858* were expressed 2.1-fold and 8.2-fold, respectively (Fig. 5a), indicating a possible role of these uncharacterized genes during linear growth. In fact, *sll0858* has a strong relationship to CpxP, a periplasmic protein in *E. coli* that acts as a negative regulator of the histidine kinase, CpxA (Ruiz and Silhavy 2005). The Cpx system (along with σ^E) monitors the cell envelope and changes in the cell envelope may be a critical function in the transition of cyanobacteria between the exponential and stationary phases. This feature is emphasized by the strong induction of the gene cluster that may be responsible for the production of capsule-like expolysaccharide (sll1722 to sll1726, Fig. 5b). These results, along with the finding that so many hypothetical and unknown genes are up-regulated during linear growth, strongly suggest that *sigH* may be involved in gene regulation under these transitional growth phases.

Cyanobacteria are also the only bacterial species that are known to have a circadian clock (Ditty et al. 2003; Golden and Canales 2003; Bell-Pedersen et al. 2005). Many cyanobacterial functions, such as cell division, amino acid uptake, carbohydrate metabolism and nitrogen fixation may be controlled by the

circadian clock, even in strains such as *Synechococcus elongatus* that are obligate photoautotrophs (Ditty et al. 2003; Golden and Canales 2003; Bell-Pedersen et al. 2005). The circadian clock has a significant effect on transcription and was shown to regulate some 800 genes in *Synechococcus elongatus* (Liu et al. 1995). Related studies have been performed on *Synechocystis* sp. PCC 6803, which is capable of heterotrophic growth, and this strain also demonstrated circadian oscillations of gene transcription in both the light and the dark (Aoki et al. 1997; Aoki et al. 2002; Kucho et al. 2005). A global analysis of circadian expression in *Synechocystis* sp. PCC 6803 demonstrated that the transcription of a subset of genes oscillated in a circadian fashion (Kucho et al. 2005). When these authors applied a very conservative statistical filter to their results, they identified 54 cycling genes and another 183 genes if they relaxed the statistical filter (Kucho et al. 2005). This number is actually less than what was estimated from promoter trap experiments reported earlier (Aoki et al. 2002), presumably due to differences in the level of resolution between the two experiments. In particular, the promoter trap experiments identified rhythms with very low amplitude in *Synechocystis* and these were likely below the level of detection using microarray analysis. We compared our dataset to that of Kucho et al. (2005) on circadian expression and found very little overlap between the two datasets (data not shown). The *kaiABC* genes showed no change in transcript level in our microarray experiments and *cikA* (slr1969) and *sasA* (sll0750, *hik8*) demonstrated modest decreases in transcription during the linear growth phase (Table 2). Genes in our experiment that were differentially regulated as the cells went into light-limited growth ($P < 0.001$) included only 8/54 of the genes that exhibited circadian rhythms under stringent filtering conditions and only 15/183 under relaxed filtering conditions (Kucho et al. 2005). Additionally, these genes did not represent any specific functional category or circadian periodicity. Since our cultures were grown in continuous light without a dark synchronization period, we suggest that transcription of the circadian-regulated genes was damped and that we detected the genes that were differentially regulated as the culture density increased and light intensity per cell decreased.

Our results strongly implicate the group 2 sigma factor, SigB, as an important regulatory element during the transition from exponential to linear growth. SigB acts to negatively regulate genes encoding major components of the photosynthetic apparatus and as a positive regulator of genes with functions that are poorly understood, but which certainly include genes involved with synthesizing material intended for the outer compartment (i.e., cytoplasmic membrane, the periplasm, and the cell wall). Some studies have examined the relationship between cyanobacterial growth-phase dependent gene activation and group 2 sigma factors (Gruber and Bryant 1998; Sen et al. 2000). More recently, Imamura et al. (2006) studied the functions of group 2 sigma factors in NtcA-dependent, nitrogen-related gene expression in *Synechocystis* 6803. They demonstrated that SigB and SigC mutually regulate the transcriptional level of each other and do so somewhat differently under exponential or stationary phase conditions. Similarly, Lemeille et al. (2005) studied crosstalk among the group 2 sigma factors, using conditions such as nitrogen deprivation and long-term growth.

The idea of sigma cascades in prokaryotic regulatory networks has recently been discussed by Fang (2005) and there is some evidence for such cascades in cyanobacteria (Caslake et al. 1997; Chan et al. 1998). Related and consistent models for transcriptional regulation among sigma factors in *Synechocystis* 6803 have been published recently (Lemeille et al. 2005; Imamura et al. 2006; Singh et al. 2006). Both Lemeille et al. (2005) and Singh et al. (2006) demonstrated that SigE and SigB represented the central pair of sigma factors and positively regulate each other under different conditions. These authors agreed on the regulation of SigC by SigB, but had somewhat different schemes for the relationship of SigB and SigD. Imamura et al. (2006) used growth in nitrate-deficient medium and the relationship between exponential and stationary-phase cultures to demonstrate that SigB and SigC both negatively and positively control each other. Their work involved in vitro transcription analysis and their model emphasized a greater role for SigC under stationary phase conditions. These authors also suggested that SigB might be a sensor for the status of PSII and phycobilisomes and our results are consistent with this idea. In this study, we see that SigB and SigH are both induced as cells transition to linear growth and it is certainly possible that they function cooperatively or antagonistically to induce or repress specific gene categories. As more experiments are performed under additional environmental conditions, it can be expected that the sigma factor cascade in *Synechocystis* 6803 will become more complete. To this end, we are constructing multiple sigma factor mutants so that we can understand more thoroughly the nature of their mutual regulatory control.

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