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Sowmya Nagarajan

Sanvesh Srivastava

Louis A. Sherman
Purdue University, lsherman@purdue.edu

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Essential role of the plasmid *hik31* operon in regulating central metabolism in the dark in *Synechocystis* sp. PCC 6803.

Sowmya Nagarajan [1], Sanvesh Srivastava [2], and Louis A. Sherman [1*]

Department of Biological Sciences [1] and Department of Statistics [2], Purdue University, West Lafayette, Indiana 47907.

Running Title: P3 regulates dark growth and metabolism

*Corresponding Author:
Prof. Louis Sherman
Purdue University
Dept. of Biological Sciences
915 W. State St.
West Lafayette, IN 47907
Phone: 765-494-8106
Fax: 765-494-8106
lsherman@purdue.edu

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Summary-

The plasmid *hik31* operon (P3, slr6039-slr6041) is located on the pSYSX plasmid in *Synechocystis* sp. PCC 6803. A P3 mutant (Δ*P3*) had a growth defect in the dark and a pigment defect that was worsened by the addition of glucose. The glucose defect was from incomplete metabolism of the substrate, was pH dependent, and completely overcome by the addition of bicarbonate. Addition of organic carbon and nitrogen sources partly alleviated the defects of the mutant in the dark. Electron micrographs of the mutant revealed larger cells with division defects, glycogen limitation, lack of carboxysomes, deteriorated thylakoids and accumulation of polyhydroxybutyrate and cyanophycin. A microarray experiment over two days of growth in light-dark plus glucose revealed downregulation of several photosynthesis, amino acid biosynthesis, energy metabolism genes; and an upregulation of cell envelope and transport and binding genes in the mutant. Δ*P3* had an imbalance in carbon and nitrogen levels and many sugar catabolic and cell division genes were negatively affected after the first dark period. The mutant suffered from oxidative and osmotic stress, macronutrient limitation, and an energy deficit. Therefore, the P3 operon is an important regulator of central metabolism and cell divison in the dark.

Introduction

Cyanobacteria grow in the natural environment in alternating light- dark (LD) cycles. Such a growth pattern requires sensing the change in light intensity and adjusting to this change through regulation of genes in various pathways for utilization of appropriate energy sources for metabolism. *Synechocystis* sp. PCC 6803 is a model cyanobacterium for many studies that involve photosynthesis, responses to environmental changes and biofuel production. This organism can grow either in autotrophic conditions using light and CO_2 from the air or on an organic source of carbon such as glucose (Singh and Sherman 2005). Central metabolism in the light with glucose involves the photosynthetic electron transport chain and the Calvin cycle for carbon fixation, the storage of carbon in the form of glycogen granules, glycolysis and a TCA cycle with N assimilation through the Glutamine Synthase-Glutamate Synthase (GS-GOGAT) pathway. In the dark, glycogen can be metabolized through the upper half of glycolysis, the Oxidative Pentose Phosphate (OPP) pathway, lower half of glycolysis, the oxidative and reductive branches of the TCA cycle and the C4 cycle (Steuer *et al*., 2012; Yang *et al*., 2002a). Thus, *Synechocystis* can grow on glucose under i) continuous light and LD cycles through mixotrophic (MT) growth; ii) light activated heterotrophic growth (LAHG) that mainly consists of dark periods with brief pulses of light; and iii) heterotrophic (HT) growth in continuous dark (DD) as shown by Yang *et al*., 2002b.

The regulatory schemes that control glucose utilization in alternating LD cycles are very complex due to the large number of genes, metabolites and pathways involved. In this condition, there is catabolism and anabolism of storage compounds like glycogen, polyhydroxybutyrate, polyphosphate and cyanophycin. The means by which cells coordinate these metabolic processes and the regulatory genes responsible for controlling targets in the central metabolic pathways are not completely known. Over 25 mutants with phenotypic defects in glucose metabolism in *Synechocystis* have been described previously (Nagarajan, 2013). Many of these mutants show defective expression of glucose catabolic genes or to a lesser extent in photosynthesis (PS) and carbon fixation genes and some have defects when grown in glucose in continuous low light (LL) or high light (HL). Only a few mutants in regulatory genes; e.g. *hik8* (Singh and Sherman, 2005), *rre37* (Tabei *et al*., 2007), *sigE* (Osanai *et al*., 2005), the double mutants *sigBsigE* and

sigDsigE, the triple mutant *sigBsigDsigE* (Summerfield and Sherman 2007); and the aquaporin mutant *aqpZ* (Akai *et al*., 2011), have been found to have defects when grown on glucose in alternating LD cycles or LAHG conditions. A *hik31* double mutant has a glucose defect in LL and in the dark when grown in high CO_2 (HC) due to lower expression of *icfG*, constitutive Glc-6-P dehydrogenase activity and no glucokinase activity (Kahlon *et al*., 2006). We have begun an intensive study of the *hik31* operons (Nagarajan *et al*., 2012) and are focusing on the dark-associated glucose defect.

The genomes of cyanobacteria are quite diverse and the majority of the sequenced strains do not contain plasmids. *Synechococcus elongatus* PCC 7942 and *Synechococcus elongatus* PCC 6301, each contain two plasmids, and six other strains contain between 3-9 plasmids. The few studies undertaken on the functions of individual plasmid genes in cyanobacteria have revealed roles in antibiotic and metal resistance, production of toxins and gas vacuoles, sulfur metabolism, signal transduction and thermal tolerance (Chen *et al*., 2008; Kimura *et al*., 2002 and references therein; Steinmuller *et al*., 1991; Lopez-Maury *et al*., 2009). Some mutants in cyanobacterial plasmid genes have been lethal for the host cell suggesting that these genes encode some essential functions (Kimura *et al*., 2002; Nagarajan *et al*., 2012). Due to the availability of sophisticated microarray slides containing genes from all seven plasmids of *Synechocystis*, more studies are being undertaken that focus on the expression and importance of plasmid genes (Singh *et al*., 2008; Singh *et al*., 2009).

In a previous study, we showed that the plasmid operon mutant Δ*P3*, with a deletion of three genes in the plasmid pSYSX (slr6039-slr6041) grew similar to the WT in photoautotrophic (PA) LL, MT LL and PA HC conditions, slower than the WT in PA LD, MT LD, HT DD and MT HC conditions and poorly in PA HL conditions (Nagarajan *et al*., 2012). The cells for this mutant were also larger than the WT and showed varying degrees of division and shape defects after the 3rd day in all growth conditions. Metal stress experiments revealed a defective phenotype in cobalt chloride salts at a final concentration of 5 mM, suggesting that P3 may control the signal transduction of the neighboring cation transporter genes. The P3 operon was also shown to be important for low O_2 adaptation as only the mutants lacking P3 did not show increased growth in low O_2 conditions (Summerfield *et al*., 2011). Our results suggested that P3 encodes elements

that act as a positive regulator of PS, carbon breakdown, cell division and cation transporter genes. Although chromosomal homologs are found in many other cyanobacteria, plasmid homologs for the *hik31* operon are only found in *Anaebaena azollae* 0708 on plasmid pAzo01, *Anabaena* sp. PCC 7120 on plasmid Beta, and in *Synechococcus elongatus* PCC 7942 on plasmid pANL.

We also showed previously that the two duplicated *hik31* operons P3 (slr6039-6041) on the plasmid, and C3 (sll0788-sll0790) on the chromosome are not redundant, and are differentially regulated in the light and dark. In addition, mutants for both copies had similar phenotypes in PA LL and MT LL, but differed when exposed to high light, dark periods with and without glucose, or high CO_2 with glucose. This suggests that P3 and C3 are functionally divergent, have common targets in the light and separate targets in the dark, and also share a mutually beneficial regulatory relationship. The Δ*C3* mutant was similar to the WT, only slightly affected in glucose growth in LL and LD, but had defective growth in MT HL. P3 transcript expression was lower than C3 and like C3, was induced by glucose and high light in active growth conditions. Unlike C3, P3 expression was upregulated in the dark with or without glucose, indicating that it has an additional role in regulating metabolism in the dark.

The goal of this work was to identify the role of *hik31* and the neighboring response regulator in response to various environmental conditions utilizing the mutants described previously (Nagarajan *et al*., 2012). We chose to work primarily with the P3 mutant under MT LD conditions, since this generated the worst growth among all of the mutants. In this study, we explore in detail the role of P3 in regulation of LD and glucose metabolism through transcriptomics, functional phenotyping and metabolic complementation. Our results show that P3 is an important regulator in the dark that helps maintain and coordinate photosynthetic metabolism with carbon breakdown and nitrogen assimilation; and that P3 mediates macronutrient, redox and pH homeostasis as well as cell division. We also show a relationship between P3 and the previously studied regulators *hik8*, *hik34*, *rre37*, sll0822, sigma factors and their targets, thereby expanding on known signal transduction networks.

Results

Effect of glucose on growth and pigmentation in the light and the dark: We studied the effect of glucose on growth through viable count determination for the WT and the P3 mutant cells grown in PA LL, PA LD, MT LL and MT LD for 3 days and plated on media with and without glucose. WT and mutant PA LL cells grew better on plates without glucose than with glucose (1.7 and 2 fold respectively), suggesting that autotrophically grown cells were stressed on exposure to glucose and could not adapt as quickly to glucose media (Table 1). The WT MT LL and WT PA LD cells continued to grow well on media with glucose. WT PA LD cells grew more than 1000 fold better than Δ*P3* PA LD cells on plates with glucose. In addition, the WT MT LD grown cells grew 2.5 fold better on plates without glucose than with glucose, more than 100 fold better than the mutant on media with glucose, and more than 1000 fold better on media without glucose. The results indicated that growth under LD, especially in the presence of 5 mM glucose, led to severe problems of survival on plates, especially when glucose was present. Pigment content also was assessed for Chl a, phycbillins and carotenoids. The results shown in Fig. S1 demonstrated that there were pigment alterations in the mutant and that glucose worsened the pigment defect in LL and even more so in LD.

Growth with other sugars: Spotting results with various other sugars as carbon sources for the WT and Δ*P3* are presented in Figure S2. We compared the inhibitory effect of glucose on the mutant with the known effects of these other sugars. 3OMG is permeable, not phosphorylated by glucokinase and not metabolized; fructose is permeable and lethal; mannose is phosphorylated by glucokinase, but not further metabolized as it inhibits the production of G-6-P, NADPH and ATP; and mannitol and sucrose are non-permeable solutes (Hihara *et al*., 1998; Lee *et al*., 2005). Most of these sugars were tolerated by the cells (except for fructose and mannose that were lethal), so that they grew in the presence of these sugars similar to the control PA LD cells. The effect of glucose for the P3 mutant was in between that obtained for a permeable, not phosphorylated, not metabolized sugar effect (3OG), and a sugar that is permeable, phosphorylated, not further metabolized and inhibits energy production (mannose). Thus, we concluded that glucose is permeable, phosphorylated and partially metabolized by the mutant. These results showed that the

187 defect caused by glucose was unique from the other sugars and was due to insufficient
188 metabolism after entry.
189
190 *Effect of addition of glycolysis and TCA components*: Growth experiments were carried
191 out for the WT and the mutant in LD with glucose and supplemented individually with
192 six carbon sources from the TCA and glycolysis pathways (pyruvate, acetate,
193 oxaloacetate, succinate, fumarate and malate) to see if they could complement the growth
194 defect in glucose metabolism (Figure S3). For the WT, none of the additions increased
195 growth above the control. Growth for the plasmid operon mutant was improved by the
196 addition of all these compounds compared to growth with glucose alone, with the best
197 growth seen with acetate, succinate and fumarate. This suggested a defect in metabolism
198 for the plasmid operon mutant in both the glycolysis and TCA pathways, since addition
199 of pyruvate and TCA components partly complements the defect. Such improved growth
200 on addition of TCA metabolites has been seen previously for the *spkD* and *icfG*
201 regulatory mutants (Laurent *et al*., 2008; Beuf *et al*., 1994).
202
203 *Microarray experiment*- A microarray experiment was performed in MT LD to determine
204 the different genes affected by the removal of P3 that led to the defects in growth,
205 pigment content, and carbon breakdown in the dark. We also performed the same
206 experiment with the C3 operon mutant to assess the role of C3, since growth of ΔC3 was
207 hardly perturbed at all under similar growth conditions. The microarray time points were
208 chosen to identify genes more directly affected by P3 and C3 (Day 1, L1 and D1) and
209 those eventually affected (Day 2, L13 and D13) .The growth of the mutants was little
210 affected in day 1, and affected significantly for Δ*P3*, but not Δ*C3* in day 2. Δ*P3* cells
211 showed a 1.3 fold reduced growth at L13 and a 1.7 fold reduced growth at D13 compared
212 to the WT (data not shown). The transcriptional response of the two operon mutants in
213 the same time points was striking and reflected the growth patterns. Both strains had a
214 relatively similar number of differentially transcribed genes at L1, but even at D1, fewer
215 genes were affected in Δ*C3*. The major differences between the two mutants were seen in
216 the day 2, where Δ*C3* had very few differentially expressed genes (data not shown,
217 manuscript in preparation). On the contrary, Δ*P3* showed extreme changes in the

transcriptional patterns in the second day, with many hundreds of genes affected. We interpret this result to mean that Δ*C3* and Δ*P3* respond similarly to the addition of glucose in the light, but face different consequences once they initiate growth in the dark. By the second day, Δ*C3* had recovered and grew as well as WT, whereas Δ*P3* could not grow and a large host of metabolic pathways were affected. These results demonstrated the important role of the P3 operon in MT LD conditions that is not shown by C3. We will describe the metabolic and regulatory fate of gene transcription in Δ*P3* in detail, as cells attempt to compensate for the loss of the P3 operon under MT LD conditions.

Table 2 shows the gene categories that changed in the different time points and the large number of genes significantly upregulated and downregulated in the mutant compared to the WT. The full microarray dataset and adjusted P- values for all genes are provided in Table S1A and arranged according to general pathways. Subsequent Tables S2 to S5 have genes arranged in sections A to F and more based on functional categories. Gene categories that contained more genes upregulated in both days of the experiment included cell envelope and transport and binding proteins. Gene categories that were mainly downregulated included amino acid biosynthesis; central intermediary metabolism; energy metabolism; PS and respiration; and purines, pyrimidines, nucleosides and nucleotides (Figure S4). Certain gene categories in Table 2 had oppositely expressed genes in day 1 and day 2. Cellular processes and transcription genes were upregulated in day 1, but downregulated by day 2. Some genes in biosynthesis of cofactors, prosthetic groups, and carriers; DNA replication, restriction, modification, recombination and repair; other categories; and regulatory functions were downregulated in day 1 and upregulated by day 2.

Gene expression for both hik31 operons and validation of the microarray experiment: We tested the expression of the individual *hik31* operon genes in our microarray time points for both cultures through RT-PCR and restriction digests to differentiate the expression of both C3 and P3 operon copies. The plasmid copy P3 op and *hikP* were progressively upregulated in the WT from L1 to D13 and missing in the mutant (Figure 1A). We selected differentially expressed genes from the microarray in several categories and tested the correlation with RT-PCR. There was an excellent match between the two for

249 almost all the genes tested. A few selected gene results are included in Figure 1B and
250 Table S1B.
251
252 *Photosynthesis, pigment biosynthesis, redox and stress responses:* The genes referred to
253 in this section are listed in Table S2. Many Photosynthesis (PS) genes were
254 downregulated in L1 and day 2 in the P3 mutant, and this included the subcategories for
255 ATP synthase, cytochrome b6/f complex, photosystem I and II, and phycobilisomes
256 (Table S2A). Operons coordinately downregulated in Δ*P3* included *psaAB, psbCD1,*
257 *psbEFLJ, apcABC, cpcDC1C2AB,* and *atpIHGFDACBE*. The respiratory electron
258 transport terminal oxidases slr1136-slr1138 (*ctaC1D1E1*) and slr1379-slr1380 (*cydAB*)
259 were slightly upregulated in L1, possibly due to enhanced respiration based on glucose
260 addition, whereas most of the genes in the thylakoid were downregulated (Schultze *et al*.,
261 2009).
262 Pigment biosynthesis genes in the cobalamin, heme, phycobilin and porphyrins
263 group were mostly downregulated and cellular protection genes of the high light-
264 inducible polypeptide Hli proteins for PS were downregulated (Table S2B). General
265 protection responses in day 1 included the upregulation of many heat shock proteins and
266 chaperones, and photoprotection genes of the *flv* operon (sll0216-sll0219) in day 2 (Table
267 S2C).
268 Many genes that were affected by oxidative stress after H_2O_2 addition (Li *et al*.,
269 2004) were similary affected in the P3 mutant (Table S2D). This included many
270 downregulated PS and detoxification genes in day 1 and day 2 that affected responses to
271 redox or oxidative stress; e.g., sll5104 (arsenate reductase), peroxidases and glutathiones.
272 The V4R proteins, encoded within the gene cluster slr0144 to slr0151 were mainly
273 downregulated in peroxide stress, in HL and in L1 and day 2 (Table S2D), but
274 upregulated in D1 (see Summerfield and Sherman, 2008).
275 Specific genes upregulated in peroxide stress and in our study included heat shock
276 proteins and sll0247 (*isiA*) (Singh *et al*., 2004). slr1516 (*sodB*), the peroxiredoxins (*tpx*)
277 sll0755 and sll0221 (*prxQ*), thus indicating the presence of high levels of Reactive
278 Oxygen Species (ROS) in the P3 mutant. Correspondingly, genes encoding ROS
279 scavenging molecules (e.g. carotenoids) were activated in day 2 (Table S2D). Numerous

biosynthesis genes for riboflavin, pantothenate, quinolinate, folic acid, menaquinone, putative ubiquinone and cobalamine were upregulated in day 2 (Table S1A). Various *hyp* genes were also upregulated in either the first day or second day, and may be responding to changes in redox in the P3 mutant. The *hoxEFUYH* operon was downregulated in day 2 along with sll0359, an AbrB-like transcriptional regulator that has been known to activate this operon (Table S2E).

There is a close relationship between iron homeostasis and oxidative stress in cells (Shcolnick *et al*., 2009). Upregulation of the PerR regulator, slr1738 was seen in D1 and day 2 along with the adjacent and divergent sll1621 (*ahpC*) gene that encodes a peroxiredoxin (Table S2F). Iron uptake genes are upregulated in oxidative stress, and antioxidant genes are upregulated to repair Fe-S clusters that may be damaged due to ROS (Houot *et al*., 2007). Genes that encode ferric iron ABC transporters and related proteins, comprised of slr1295, slr0513, slr0327 and sll1878 (*futA1, futA2, futB* and *futC* respectively), and sll1404-sll1406 were upregulated mainly in day 1. Similarly, slr1319 (*fecB*), and ssl2250 (*bfd*), and sll0221 (*bcp*) were also upregulated in D1 and day 2. Together, these genes may serve to take up more iron for repair or sequester ferric iron (Katoh *et al*., 2001). On the other hand, slr1894 (*mrgA*), was downregulated in L1 and day 2. This gene product may be involved with iron storage and mobilization and an *mrgA* mutant was highly sensitive to peroxide stress (Li *et al*., 2004; Shcolnick *et al*., 2009). The downregulated bacterioferritins slr1890 (*bfrB*) and sll1341 (*bfrA*), along with the downregulated *mrgA* and sll0567 (*fur*) regulator could worsen the oxidative stress due to limited iron storage, scavenging and detoxification.

Excess metals have also been known to cause protein denaturation, oxidative stress and damage PS proteins (Blasi *et al*., 2012). The C3 operon (termed *copMRS* by Giner-Lamia *et al*., 2012) was previously shown to regulate the slr6042-slr6044 operon for copper efflux in the presence of copper. However, the authors could not detect an effect on plastocyanin sll0199 (*petE*), the complementary cytochrome sll1796 (*petJ*), or on genes for copper import into the thylakoid, slr1950 (*ctaA*) and sll1920 (*pacS*). *petE* has more copper content, is present in both photosynthetic and respiratory electron transport chains and is also essential in glucose growth whereas *petJ* has more heme content and operates primarily in the PS electron transport chain (Giner-Lamia *et al*.,

311 2012 and references therein). In our study, the copper efflux genes slr6042-slr6044 were
312 upregulated in most time points together with *pacS* and *ctaA* in day 2 in the P3 mutant
313 (Table S2G). P3 may repress slr6042-slr6044 and *petE* (upregulated in D1) and be
314 responsible for activating *petJ* (downregulated in day 1 and day 2). It is possible that
315 these differences are due to genotype differences in our lab strain from that used by
316 Giner-Lamia *et al*., 2012. We found no significant changes in growth for our operon
317 mutants compared to the WT when grown on $CuCl_2$ at 5 μM (data not shown). Our
318 results suggest a direct effect for P3 in regulating these genes, even though there was no
319 change in copper concentration in this study. Nonetheless, similar conditions may result
320 from the oxidizing environment found in the P3 mutant relative to those caused by copper
321 addition.
322 In a similar microarray experiment done with the C3 mutant, we found an
323 opposite effect on *petE* (downregulated in day 1) and *petJ* (upregulated in day 1 and day
324 2) and the slr6042- slr6044 operon (downregulated in day 1 and day 2, unpublished data)
325 compared to the P3 mutant microarray results. Thus, C3 and P3 and their oppositely
326 regulated *petE*, *petJ* and copper transport genes may be affected by redox imbalance of
327 the electron transport system and oxidative stress and not just stress caused by copper.
328 This would explain the large number of phenotypes we see for mutants in this system in
329 glucose, high light and in the dark in our lab strains. Furthermore, *hik31* was found in
330 both the thylakoid and plasma membranes (Giner-Lamia *et al*., 2012). The removal of P3
331 may therefore result in the early downregulation of many PS genes in this study. Another
332 cluster sll1783-sll1785 found to play a role in copper transport previously was
333 downregulated in Δ*P3* (Singh *et al*., 2010; Tottey *et al*., 2008).
334 Other metal transporter genes were activated in the P3 mutant and may indicate
335 metal limitations and a response to ROS (Table S2G). Nickel transporters, zinc
336 tranporters, manganese transporters and regulators, and a magnesium transporter were
337 also upregulated in day 2. The sll0797 (*nrsR*), regulator for nickel transport, may be
338 responding to changes in the plastoquinone redox poise as seen before (Li and Sherman,
339 2002). A possible cobalt regulon sll0381-sll0384 containing a cobalamine biosynthesis
340 protein *cbiM* was seen to be downregulated in D1 and day 2 and may serve to explain the
341 defective phenotype for the P3 mutant when grown in $CoCl_2$ salts (Nagarajan *et al*., 2012).

342 Thus, metal homeostasis for cobalt, nickel, zinc, magnesium and manganese was affected
343 in Δ*P3* and could be due to multiple reasons- to compensate for the downregulation of PS
344 genes, due to oxidative stress, or in order to export toxic metals that may accumulate in
345 the cell based on other defective processes. In all, these results suggest a decrease in
346 photosynthesis and pigment production in the P3 mutant and an increase in oxidative
347 stress that may affect iron and metal homeostasis for the cell.
348
349 *Regulatory, transcription factors, translation and cellular processes genes*: Regulatory
350 genes affected in the P3 mutant included many genes shown to be involved with
351 oxidative stress, motility or in the preservation of membrane integrity (Table S3). slr1285
352 (*hik34)* was upregulated in day 1 and L13, slr1147 (*hik2*) in day 2 and slr1783 (*rre1*) was
353 downregulated in day 2. Genes shown to be regulated by the *hik34/ hik2-rre1* system that
354 behaved similarly in the P3 mutant are presented in Table S3A (Paithoonrangsarid *et al*.,
355 2004; Shoumskaya *et al*., 2005). Hik34 is a negative regulator of heat shock proteins in
356 normal conditions, but a positive regulator under stress conditions, like in Δ*P3*, as both
357 *hik34* and its target chaperones were upregulated in day 1. The *hik31* operon genes were
358 all downregulated in the *hik34* mutant, but *hik34* was upregulated in in the P3 mutant,
359 thus suggesting a reciprocal impact of these genes on each other (Suzuki *et al*., 2005). We
360 conclude that *hik34* may activate P3, whereas P3 may repress *hik34*.
361 Some regulatory genes known to be affected in different stress conditions were
362 similarly affected in the P3 mutant (Table S3B). These genes were downregulated or
363 upregulated in response to oxidative stress, redox stress or in C, iron limiting and
364 oxidative stress conditions. The slr0947 (*rpaB*) regulator was downregulated in D1 and
365 D13 and so were the PSI, *apc* and *cpc* genes as possible targets (Seino *et al*., 2009). A
366 putative signaling protein slr6110 on the pSYSX plasmid was also downregulated in both
367 days and may be activated by P3.
368 Many sigma factors were induced in our study and their known targets were also
369 affected similarly (Table S3C, Los *et al*., 2010; Osanai *et al*., 2008). The sll0306 (*sigB*)
370 gene may regulate other genes in the P3 mutant in day 1 (Table S3D) (Osanai *et al*.,
371 2008; Singh *et al*., 2006; Imamura *et al*., 2003). *sigB* and sll1689 (*sigE*) are important for
372 gene regulation in mixotrophic conditions and mutants in both these genes did not grow

in 8L/16D (Summerfield and Sherman 2007). Both genes were downregulated by D13 and Δ*P3* could not grow in similar conditions. It is possible that P3 negatively controls the expression of *sigB*, *sigH, sigI, sigG* and *sigF* in glucose as these were all induced in day 1. The genes *hik31* and *rreC* were induced in the dark in the *sigB* mutant, decreased in the *sigD* mutant in the light and in the *sigBE* mutant in both light and dark (Summerfield and Sherman, 2007). Downregulated *sigB* and *sigE* could be contributing to the growth defect of the P3 mutant in MT LD from day 2 onwards. Numerous genes in most categories of translation also were downregulated in the P3 mutant and could lead to reduced protein synthesis and increased protein misfolding (Table S3E).

Type 4 pili are important for twitching motility, cell adherence and phototaxis. In Δ*P3*, some phototaxis genes and regulators were repressed (Table S3F). Downregulated sll1694 (*pilA1*) and sll1695 (*pilA2*) may affect chlorophyll biogenesis, assembly and delivery to new photosystems, competence, and motility (He and Vermaas, 1999; Yoshihara *et al*., 2001). Other downregulated genes in day 2 could affect motility, orientation and directional movement towards the light as well as membrane fluidity (Bhaya *et al*., 2001). Alternatively, other pilins were transiently upregulated in Δ*P3* like the *pilA4-pilA11* genes and may be a stress response. Many genes in the hexosamine pathway, murein sacculus and peptidoglycan genes were upregulated in Δ*P3* and may lead to more peptidoglycan synthesis (Singh *et al*., 2008, Table S3G). Overall, these results suggest a gradual slowing down of key metabolic processes in the P3 mutant by day 2 with reduced translation, motility and an increase in peptidoglycan synthesis that may worsen cell division.

Ultrastructure of Δ*P3 in MT LD and cell division genes*- Δ*P3* cells were larger in size and showed improper septation after the third day of growth in all conditions. Two doubling cells attached to each other resulted in a clover leaf-shaped tetrad appearance due to divison of each daughter cell even before separation from its twin (Nagarajan *et al*., 2012). Figure 2 shows the P3 mutant cells in comparison to the WT in MT LL and MT LD conditions. Notably, the mutant appeared less abnormal in MT LL and had the same number of carboxysomes as the WT.

In contrast, the mutant cells in MT LD demonstrated a dense cytoplasm with fewer and disorganized thylakoids. No lipid bodies and very few glycogen granules were seen. Importantly, no carboxysomes were found in over 200 mutant cells. Δ*P3* cells each had many storage organelles including 2-6 polyphosphate bodies, 1-4 cyanophycin granules and a few large PHB granules. In contrast, the WT cells had a normal size and shape, thylakoid arrangement and intracellular organelles like lipid bodies, glycogen granules, 1-3 carboxysomes, 2-3 polyphosphate bodies and no cyanophycin granules.

There have been relatively few studies on cell division in cyanobacteria. Table S3G shows the cell division genes downregulated in day 2 in the P3 mutant. For example, sll1833 (*ftsI/pbp4*) was downregulated in Δ*P3* in D13 and may affect the inward synthesis or incorporation of peptidoglycan in septation allowing the separation of daughter cells. Another gene slr0804 (*pbp8*) known to be important for complete septation and separation of daughter cells, was downregulated in D1 in Δ*P3*. A *pbp4* mutant had giant cells and both *pbp4* and *pbp8* mutants had septation defects and clover leaf-shaped cells (Marbouty *et al*., 2009).

The sll0202 (*gidA*) and sll0288 (*minC*) genes were also downregulated in Δ*P3* and *minC* was shown to have an important role in proper cell shape and size (Mazouni *et al*., 2004). *gidA* encodes a glucose inhibited division protein and may also contribute to the cell division defect of Δ*P3*, as seen for a *gidA* mutant in the rod-shaped strain *Salmonella enterica Serovar typhimurim* (Shippy *et al*., 2012). Finally, three *ftsH* genes associated with the thylakoid membrane were downregulated in day 2, and may have a role in cell division as seen in other bacteria. These downregulated genes could also contribute towards reduced photoprotection of the D1 protein of PSII (Muramatsu and Hihara, 2012). Overall, these downregulated cell division genes may lead to the division defects seen in the mutant.

Nitrogen metabolism and transporters: Table S4 lists selected genes from nitrogen metabolism that were affected in the P3 mutant. *Synechocystis* can use nitrogen in the preferred form of ammonium; or as nitrate, nitrite, and urea; or as amino acids glutamine, glutamate and arginine. After uptake, urea, nitrate and nitrite are converted to ammonium that is then assimilated into carbon skeletons through the GS-GOGAT pathway or using

434 glutamate dehydrogenase and incorporated into organic nitrogen compounds. These
435 processes need sufficient ATP, Mg^{2+} or Mn^{2+}, NADH, NADPH and ferredoxin for proper
436 functioning (Flores and Herrero, 2005; Muro-Pastor *et al*., 2005).
437 The main regulators affecting N metabolism and amino acid homeostasis include
438 NtcA, NtcB, PII, PphA, and PamA. In this study, sll1423 (*ntcA*), slr0395 (*ntcB*) and
439 sll0985 (*pamA*) were unchanged, and ssl0707 (*glnB*) encoding PII and sll1771 (*pphA*)
440 were downregulated in day 2 (Table S4A). Although the known targets for these genes
441 were not affected in agreement with their mode of function, these regulators could be
442 active in their stable protein forms in different states even if gene expression can vary, as
443 described previously (Forchhammer, 2004).
444 Nitrogen metabolism is fine-tuned by the careful coordination among the different
445 regulators that sense the levels of the different metabolites in a sophisticated manner. In
446 low/limited N conditions (high C to N ratio), the NH_4^+ or NO_3^- content inside the cells is
447 lower, the 2OG content is high, PII is phosphorylated and activates NtcA. Other N
448 transport and assimilation genes are then activated by NtcA, and *gifA* and *gifB* are
449 repressed. Importantly, the *amt1* and *amt2* genes, *urtC, ureF, nrtCDnarB* and *glnN* all
450 were upregulated in L1 and in some cases in D1 in the P3 mutant, thus indicating N
451 limitation in the initial day after glucose addition (Tables S4B and S4C).
452 Alternatively, in high/excess N conditions, (low C to N ratio), the 2OG content is
453 low, PII is dephosphorylated by PphA and inhibits NtcA so that other N transporters and
454 conversion genes are not activated by NtcA, and *gifA* and *gifB* are de-repressed and
455 inactivate Glutamine Synthase (GS) (Garcia-Dominguez *et al*., 1999). PamA binds PII,
456 *argB* is activated and increases arginine synthesis, and cyanophycin is produced. Low C
457 levels are likely to be present in the P3 mutant after D 1 and maybe caused by inhibited
458 CO_2 fixation and HT conditions with glucose as seen before (Kurian *et al*., 2006). The *icd*
459 gene for 2OG production was downregulated in the dark, possibly due to reduced carbon
460 fixation. A higher N content could be present from D1 onwards due to the upregulation of
461 *amt1, amt2,* and *amt3, urtD* and *ureE, ureF* genes that may bring in and convert N in
462 various forms, upregulated *argB* for arginine synthesis and *gifA* and *gifB* (Tables S4B,
463 S4C, and S4E). The expression of the *gif, glnA, glnN, glsF, gltB, gdhA* and slr0899 (*cynS*)
464 genes also suggest ammonium accumulation from D1 in Δ*P3* (Herrero *et al*., 2001). The

gif genes were upregulated at D1 and the other genes were downregulated at day 2 time points. Other gene responses that indicated reduced N demand and counter indicated N starvation in day 2 included downregulated *nblA1, nblA2*, *nblB2*, sll0783 and sll0784 (*merR*) (Li *et al*., 2002) (Table S4D).

The *nrtABCDnarB* operon genes were downregulated by day 2 (Table S4B). The molybdopterin biosynthesis genes slr0900-slr0903 and ssr1527 associated with *narB* were upregulated in day 1 in the P3 mutant and donregulated in day 2 like *narB* (Herrero *et al*., 2001; Flores and Herrero, 2005). slr0898 (*nirA*) was downregulated throughout all 4 time points and P3 may be needed to activate this gene for efficient conversion of nitrite to ammonium. Reduced *nirA* expression could also be a result of decreased nitrate transport or ferredoxin availability (Reyes *et al*., 1993). Downregulated GOGAT genes in the mutant may reflect the lack of 2OG, ferredoxin and NADH and the presence of NH_4^+ for conversion of glutamine to glutamate. Many ferredoxin genes were downregulated in the mutant, especially in day 2 (Table S1A). The gene for *gdhA* was downregulated and may lead to insufficient glutamate production in day 2 (Chavez *et al*., 1999).

Cyanophycin is a polymer made up of multi-L-arginyl-poly-L-aspartate and can be stored in granules. Cyanophcin synthetase slr2002 (*cphA*) was upregulated at L1 in the Δ*P3* mutant and this could lead to gradual accumulation of cyanopycin. Growth on media containing arginine with or without nitrate leads to round-shaped cyanophycin granules in the WT, largely reduced thylakoids, reduced pigment content and photosynthetic activity (Stephan *et al*., 2000). This is similar to what is seen for Δ*P3* in Figure 2 in both MT LL (similar to growth on arginine plus nitrate media with few cyanophycin granules and slightly reduced thylakoid content) and MT LD (similar to growth on arginine as a sole N source with many cyanophycin granules and much lower thylakoid content) (Stephan *et al*., 2000). Arginine is used for protein synthesis in the WT in MT LD, but in the Δ*P3* mutant, it may accumulate, and together with aspartate, become stored as cyanophycin. Thus, the behavior of the N metabolism genes suggests an initial N limitation that is followed by an accumulation of ammonia and cyanophycin and limitation of glutamate.

Amino acid, sulfur, phosphate metabolism and transporters: There are 3 main pathways for arginine metabolism in *Synechocystis* containing many different routes and interconnecting side branches with shared genes (Quintero *et al*., 2001; Schriek *et al*., 2007; KEGG database). Two major routes include the arginine deiminase and oxidase/dehydrogenase pathways and a minor pathway includes arginine decarboxylase and all were mainly affected in D1 and day 2 (Table S4E). Overall, these gene expression results indicated increased arginine production though upregulated slr1898 (*argB*), and reduced or incomplete arginine catabolism that could result in decreased conversion to succinate, fumarate and glutamate with possible accumulation of ornithine, citrulline, proline and ammonia in day 2 in the P3 mutant. Similarly, aspartate breakdown pathway branches were either downregulated or incomplete (Table S4F). Aspartate synthesis may be increased (upregulated slr1476 (*pyrB*) and slr1705) and there could also be reduced production of threonine and lysine and accumulation of pantetheine and oxaloacetate by day 2. Taken together, these gene expression changes indicated that aspartate and arginine accumulated in the P3 mutant to produce cyanophycin.

Other processes affected included synthesis and transport of other amino acids (downregulated genes, Table S4G), as well as sulfate and phosphate metabolism (upregulated genes, Tables S4H and S4I) (Wang *et al*., 2004; Juntarajumnong *et al*., 2007). The *hik31* operon genes were upregulated after S deprivation and may mediate S acclimation in day 2 in the P3 mutant (Zhang *et al*., 2008). Additionally, *sphR* could be negatively controlled by P3 as it was upregulated in day 1 in response to phosphate limitation for activating the pho regulon genes.

Organic carbon metabolism, energy and reducing sources: Table S5 lists the genes affected in central carbon metabolism in Δ*P3*, including glucose anabolic and catabolic pathways. Most genes in these pathways were downregulated in day 2. Studies to understand the metabolic flux and proteome through these pathways were explored previously for the WT in MT LL, HT DD and LAHG conditions, but a detailed study of MT LD conditions has not been performed as yet (Yang *et al*., 2002a; Yang *et al*., 2002b; Tabei *et al*., 2009). We compared the information in these studies with our microarray experiment to understand the extent of the deviations in Δ*P3*.

The glucose transporter sll0771 (*glcP*) was downregulated in L1 and D13 (Table S5C). Glucokinase activity is maximal in heterotrophic conditions (Knowles and Plaxton, 2003). Glucokinase slr0329 (*xylR*) was upregulated in day 2 and may help phosphorylate glucose in the P3 mutant in LD conditions (Lee *et al*., 2005). In Δ*P3*, downregulated PS and the *icd* gene may lead to lower NADPH. The *zwf* and *gnd* genes in the OPP were upregulated for NADPH production (Table S5B). Also, the *opcA* gene, essential for dark NADPH production, behaved similar to *zwf* in day 2 in Δ*P3*. The slr0400 (an NAD kinase) gene was upregulated in day 2 and may be another attempt to increase NAD(P)H for the P3 mutant (Gao and Xu, 2012). The OPP is important to generate ribose-5P as a precursor to nucleotides and nucleic acids, amino acid histidine synthesis and cofactors like folate and riboflavin (Knoop *et al*., 2010). Accordingly, we found upregulated slr0194 (*rpiA*) and ssl2153 (*rpiB*), and several histidine, riboflavin and folate genes in day 2 (Table S1A).

The P3 mutant in MT LD already behaved as if it was experiencing HT DD conditions in day 2, since it had reduced expression for genes in PS (Table S2), CO_2 fixation (Table S5J), gluconeogenesis (*gap2, fba2, fbp1*, Tables S5J and S5C), and glycogen formation and breakdown (*pgm, glgC, malQ* and *glgP2*; Tables S5A and S5C) that could cause problems in the dark as early as D1 (Kurian *et al*., 2006). Inorganic carbon fixation to 3PGA could be lower in the mutant leading to a reduced lower half of the glycolysis pathway with downregulated *gpmB*, *eno* and the *pdh* genes in Tables S5C and 5D (Osanai *et al*., 2006). Downregulation of *glgP2* could affect responses to Ci limitation and downregulation of thioredoxins slr0623 and slr1139 may cause adaptation problems for the mutant in LD and redox transitions (Table S5A, Fu and Xu, 2006). Downregulated *gap2* as the major GAPDH is significant to the P3 mutant phenotype in MT LD and also suggests insufficient levels of NADH and NADPH (Table S5J, Koksharova *et al*., 1998). Furthermore, the *icdA* gene was downregulated in D1 and D13 and maybe due to reduced carbon fixation. Other TCA cycle genes *sucC* and *fumC* were upregulated and *citH* was downregulated (Table S5E) and may compensate for the reduced production of succinate and fumarate and accumulation of oxaloacetate as suggested by the analysis of arginine and aspartate metabolism genes in Table S4.

556 The NADH dehydrogenase genes *ndhD1*, *ndhD2*, *ndhB* and *ndhF1* genes (Table
557 S5F) were downregulated by D13, and this could affect respiration, cyclic electon flow,
558 ATP production, as well as CO_2 uptake in Δ*P3* (Ma and Mi, 2009; Ohkawa *et al*., 2000;
559 Zhang *et al.,* 2004; Ogawa and Mi, 2007). The *ndbC* gene was upregulated and may help
560 to regulate the redox state of the PQ pool (Howitt *et al*., 1999). The ATPase operon may
561 be downregulated in the P3 mutant due to incomplete electron transport leading to
562 deficient H^+ translocation and cytosolic alkalinization (Lee *et al*., 2007; Table S2A).
563 Polyhydroxyalkanoate synthesis genes were upregulated in L1 and L13 but
564 downregulated by D13 (Table S5H); and this induction may be responsible for
565 production of PHB granules seen in Figure 2.
566 The sugar catabolic genes induced in our study were also induced in nitrogen
567 limitation and belong to the NtcA and SigE regulons (Osanai *et al*., 2006). Genes induced
568 in glycogen metabolism and the OPP in Table S5 could be due to a combination of N
569 depletion in the mutant in L1 when the OPP is used for reducing power, and an attempt to
570 use the glycogen reserves from PA LL growth for energy. The sll0750 (*hik8*) gene was
571 upregulated in day 2 and *hik8* activated genes *pfkB1*, *gap1, zwf, gnd* were upregulated in
572 L13 by > 1.4 fold (Singh and Sherman, 2005). These and additional genes *pyk1, tal*, *glgX,*
573 *glgP2*, *cph1*, and *glnN* were downregulated in D13 when *sigE* was downregulated,
574 suggesting that *sigE* takes precedence over *hik8* in D13 for overlapping regulated genes
575 (Osanai *et al*., 2005). Genes affected similarly to sll1330 (*rre37*) that may be regulated by
576 this gene include *fba2, fbp1* and *gpmB* in day 1 (Azuma *et al*., 2011). Genes *pfkB1* and
577 *pyk1* thought to be regulated by *rre37* were upregulated in L13 when *rre37* was
578 downregulated and this could be due to upregulated *hik8* and an unidentified regulator for
579 *pyk1*, normally induced in the dark and in N depletion (Tabei, *et al*., 2007). Thus, there
580 was a hierarchy of the known regulated genes in Δ*P3* in day 2 with *sigE* taking
581 precedence over *hik8,* and *hik8* in turn, over *rre37* (Table S5I). Taken together, these
582 results indicate that there is downregulation of organic carbon genes for glucose
583 catabolism by day 2 and an upregulation of glucosylglycerol genes (Table S5G) and *pha*
584 genes (Table S5H), leading to the production of sucrose and PHB respectively.
585

586 *Inorganic carbon metabolism and photorespiration*: The Carbon Concentrating
587 Mechanism (CCM) comprised of the carboxysome structural proteins, enzymes and
588 inorganic carbon (Ci) transporters, serves to enhance the cell's internal concentration of
589 Ci so that Rubisco is saturated. Otherwise, the oxygenase activity of Rubisco results in
590 accumulation of the toxic metabolite 2PG that inhibits enzymes in the Calvin cycle.
591 The carboxysome genes, sll1028-sll1032 (*ccmK2K1LMN*), comprise an operon
592 that was downregulated in Δ*P3*, in a similar fashion to many PS genes (Table S5J). The
593 negligible carboxysomes and reduced shell structure in the P3 mutant may lead to lower
594 content of enzymes for rubisco (downregulated slr0009-slr0012 *rbcLSX*) and carbonic
595 anhydrase, and their presence in the cytoplasm rather than being confined to the
596 carboxysome. This may cause the induction of periplasmic carbonic anhydrase slr0051
597 (*ecaB*) and slr0436 (*ccmO*) needed for carboxysyome assembly. Δ*P3* also has a strikingly
598 similar phenotype to the *ccmM* mutant (no carboxysomes) and similar gene expression
599 patterns. Impaired carbon fixation in the *ccmM* mutant led to excess N over C with time
600 in LC, less 2OG and other amino acids, and less N assimilation through genes in N
601 transport and GS-GOGAT (Hackenberg *et al*., 2012), similar to our analysis for Δ*P3*.
602 In the P3 mutant, high affinity Ci transporters were upregulated, whereas low
603 affinity systems *bicA* and *cupB* and the regulator sll1594 (*ndhR*) were downregulated
604 (Table S5K). In addition, *sbtA,* a sodium dependent bicarbonate transporter, and sodium
605 transporter genes were upregulated in the dark (Price *et al*., 2008). The slr1860 (*icfG)* and
606 sll0776 (*spkD*) regulatory genes coordinate the assimilation of Ci and are needed for
607 growth when external Ci levels are low (Table S5I). *icfG* was downregulated in day 2 and
608 *spkD* was upregulated by 1.4 fold in day 1 for adaptation to low Ci levels as seen before
609 (Beuf *et al*., 1994; Laurent *et al*., 2008).
610 The *ndhR* gene (downregulated; see Table S5K) is a negative regulator that
611 impacts many Ci transporters, slr2006-slr2013 (*mrp* cluster- putative cation/H^+
612 antiporters), and the sll0217- sll0219 *flv* operon that together are thought to be activated
613 by low Ci levels. Several genes affected in Ci limitation that play a role in coordinating
614 carbon metabolism, outer membrane and cell wall permeability, and stress responses,
615 were consistent with the P3 mutant microarray gene changes and previous results (Wang
616 *et al*., 2004, Eisenhut *et al*., 2007), suggesting a Ci limitation for the P3 mutant.

Both cyAbrB-like transcriptional regulators, sll0359 and sll0822 (Kaniya *et al.,* 2013), were downregulated at least 2-fold in day 2 (Table S5I). Gene sll0822 induces many N assimilation genes, and *nrtA, amt1, glnB, urtA*, and *sigE* were downregulated in day 2 (Ishii and Hihara, 2008, Table S4B). This gene can also repress Ci transporters *sbtA, ndhF3* and *cmpA* in HC, but these genes were all upregulated in mutant P3 in day 2 even in LC (Lieman-Hurvitz *et al*., 2009). Recent work has shown that cyAbrB2 (sll0822) is essential for transcription of genes involved with carbon and nitrogen metabolism after a shift to phtomixotrophic conditions (Kaniya *et al.,* 2013). Noteably, electron micrographs indicated some of the same ultrastructural changes in the cyAbrB2 mutant as in the P3 mutant (Kaniya *et al.,* 2013), but the P3 mutant changes were more severe. Taken together, the results of our work and those referenced above lead us to suggest that RreP is an important regulator that influences the expression of key C and N metabolic regulators sll0822 and PII.

More photorespiratory flux was found in LC conditions in an earlier study, and we find evidence of this in Δ*P3* (Huege *et al*., 2011, Table S5L). Pathway analysis for photorespiratory cycles in Δ*P3* suggested accumulation of oxalate, glycine, 2PG and glycolate; with reduced breakdown of glycine to ammonium and CO_2; and reduced serine, and glycerate content. This may reduce 3PGA production from glycerate in the absence of sufficient carbon fixation. Together, these results strongly support a Ci deficiency in Δ*P3* and indicate incomplete carbon fixation, despite the upregulation of the high affinity CCM transporters, and the existence of active photorespiratory cycles to detoxify 2PG (Eisenhut *et al*., 2008).

The effect of pH on the glucose defect of ΔP3: Some mutants have shown growth improvement at certain pH levels in the presence of glucose in *Synechocystis* (e.g. an a*qpZ* mutant, Akai *et al*., 2011). To determine if this is the case for Δ*P3,* we performed an experiment with buffered media with pH at 7.5, 8, 9 and 10 for WT and Δ*P3* in MT LD (Figure 3). The WT grew best at pH 7.5 and progressively worse with increasing pH. Δ*P3* on the other hand, grew better at pH 8 and had increased pigment content. The mutant grew almost 2-3 folds better at pH 8 than at pH 7.5, slightly better than the WT at pH 8, and grew worse at pH 9 and 10. This supports an inorganic carbon (Ci) defect in

the Δ*P3* mutant in pH 7.5 that is functionally complemented by growth at pH 8. It could be that the availability and transport of HCO_3^- ions by the CCM genes is ideal for the P3 mutant at pH 8 and enhances growth. An active photosynthetic metabolism, C/N status and redox state of the plastoquinone pool and increasing pH from 7 to 8 were found to activate glutamine synthase (Reyes *et al*., 1995; Muro-Pastor *et al*., 2001). The increased inorganic carbon availability at pH 8 would potentially lead to more carbon fixation, 2OG production and an active GS-GOGAT pathway to convert the excess ammonium to glutamate in Δ*P3*. Thus, the glucose defect of Δ*P3* is pH-dependent, as pH 8 helped partially reverse the growth defect and problems with pH homeostasis at pH 7.5.

Growth with added N sources and metabolites: Upon examining the N metabolism genes in Table S4, C metabolism genes in Table S5, the predicted nitrogen limitation in day 1, and Ci limitation in Figure 3, we reasoned that growth of Δ*P3* may improve on addition of N and C sources. We tested the growth of the mutant and WT in MT LD conditions for 3 days with addition of NH_4Cl, $NaHCO_3$, 2OG and sodium glutamate (Figure 4). 2OG is known to be important for many functions, including ammonia assimilation, nitrogen and carbon metabolic regulation, and many of the added nitrogen compounds lead to production of precursors for pigment synthesis. Figure 4 shows improved growth for the WT in $NaHCO_3$, followed by 2OG, and not much change in growth with NH_4Cl and glutamate. The growth for the mutant improved significantly with all of the added compounds with bicarbonate leading to the best growth (4 fold), followed by 2OG (3.5 fold), NH_4Cl (3 fold) and glutamate (2 fold). In fact, the mutant cells with bicarbonate grew almost as well as the WT in glucose. Pigment production improved for the mutant with all the added compounds, with ammonium addition resulting in double the pigment content (data not shown). Thus, these results support our analysis of the microarray results for C and N limitation in the P3 mutant and interestingly, reveal that the demand for inorganic C is more than that for N. These results also suggest that the addition of N compounds along with glucose may reduce the initial N limitation, leading to the recovery of growth, and may not lead to the detrimental N accumulation in day 2. The *hik31* operon genes were upregulated more than 2 fold in N limited growth (Aguirre von Wobeser *et al.,* 2011), suggesting a role in regulation of N metabolism.

679

680 **Discussion**

681 Glucose, the preferred organic carbon compound for *Synechocystis*, has many effects on
682 cell growth and physiology. Cells need both light and dark incubation periods to use
683 glucose fully as some important catabolic genes and regulatory genes are active only in
684 the dark and others only in the light (Tabei *et al*., 2009; Yang *et al*., 2002b). In this study,
685 we investigated the role of the P3 operon during growth in LD cycles in the presence of
686 glucose and examined in detail the effects of glucose on metabolism for the cell. Our
687 results showed that there was an incomplete metabolism of glucose, especially in the dark,
688 that affected the growth and survival properties for Δ*P3* (Table 1, Figure S1, and Figure
689 S2). Genes affected in the microarray experiment in LD cycles with glucose in the mutant
690 indicated reduced photosynthesis and increased oxidative stress that affected metal and
691 iron homeostasis; (Table S2); reduced protein synthesis, motility and defective cell
692 division (Table S3); a transition between N limitation in day 1 and excess in day 2 that
693 affected TCA metabolites, sulfur and phosphorus levels (Table S4); and decreased
694 organic carbon metabolism and inorganic carbon limitation (Table S5). The ultrastucture
695 of the mutant revealed reduced thylakoids; lack of carboxysomes; and the storage of
696 polyphosphate, PHB and cyanophycin in Δ*P3* (Figure 2). The results in Figures 3 and 4
697 indicated that Δ*P3* suffered from Ci and N deficiency that was overcome by addition of
698 supplemental C and N compounds.

699

700 *Physiological changes in day 1 vs day 2:* The microarray data in Table 2 and Figure S4
701 revealed two main phases in gene responses in day 1 and day 2 in our microarray study.
702 Sixteen groups of around 10 genes or more displayed various transcriptional temporal
703 patterns at different time points in the P3 mutant (Table S6). There were many more
704 genes downregulated in day 2 for the P3 mutant than in day 1. In day 1, PS genes and
705 carbon fixation genes were downregulated (groups 1, 3, and 4), and this was followed by
706 a downregulation of many N assimilation, transport and regulatory genes (groups 1, 5, 8
707 and 9), and eventually by decreased energy metabolism, amino acid biosynthesis,
708 transcription, translation, cellular processes, and central intermediary metabolism (groups
709 2, 4, 6, 7, and 10) in day 2. Consequently, there was increased transcription for genes

encoding biosynthesis of cofactors, regulatory, and transport genes (groups 12-16) in day 1 and day 2. Additionally, genes in the PHB and cyanophycin biosynthesis categories were upregulated (groups 11, 4 and 14). It appears that the mutant is trying to cope by upregulating the flavoprotein and transport and binding proteins for many high affinity transporters, the biosynthesis of cofactors genes, along with regulatory genes to relieve the stress caused by glucose. Upregulated chaperones were an indication of general stress responses. Nevertheless, the upregulated genes do not overcome the defective growth of the mutant. These gene expression patterns explain the reasons behind the growth defect, the defect in the dark, the pigment defect, and the cell division defect for the P3 mutant.

Many genes in PS and respiration were downregulated as early as L1 in the first day and continued to be downregulated in day 2, indicating that this is likely to be an inherent defect of the mutant even in autotrophic growth conditions. The P3 mutant may be defective in both linear electron flow required for bicarbonate transport and cyclic electron flow required for CO_2 uptake, and may have lower ATP levels. Some genes in the cell division, chemotaxis, glycogen metabolism, glycolysis, and OPP pathways were mainly downregulated in the second day, thus making this a delayed response of the mutant and a conditional defect that takes place in late log phase of growth. Such a delayed phenotypic defect in glucose conditions has been seen previously with other mutants, like the double *hik31* mutant and the *pamA* mutant (Haimovich-Dayan *et al*., 2011). The differential transcription results from the second day reflected the changing energy and nutrient status of the mutant as the cells exhausted the pre-existing pools from growth in PA LL before being subjected to MT LD conditions. It has been reported that cells grown in log conditions can better adapt to HT DD, even without light, as their glucose catabolic machineries are still active from PA LL growth (Tabei *et al*., 2009).

Growth defects and a role in heterotrophic metabolism in the P3 mutant may be generally explained by the large downregulation of amino acid biosynthesis, photosynthesis, energy metabolism, translation and other downregulated gene categories. Many high affinity Ci transporters along with sodium ion transporters were upregulated in the P3 mutant and could be due to downregulated *ndhR* regulator, sll0822 or the PII protein. Cyanobacterial Ci transporters known to be inactive in darkness were active in the dark in the P3 mutant, and may be depleting the cell of energy reserves quickly.

Together with downregulation of the *rbc* and *ccm* operons for the carboxysome, these results suggest a severe Ci limitation for the P3 mutant in MT LD conditions and that carbon fixation is of paramount importance to the cell even in the presence of glucose. The Ci limitation in Δ*P3* may increase the production of toxic metabolites of the photorespiratory pathways and 2 of the 3 photorespiratory cycles showed signs of being upregulated. The glycolate produced could also be the signal to increase the expression of high affinity CCM transporters by upregulated *cmpR* (Hackenberg *et al*., 2012).

Genes affected by the removal of P3: Genes that could be activated by P3 and that are downregulated in the mutant in day 1 and/or beyond in day 2 are highlighted in Figure 5 with green asterisks and also summarized in Table S7. Genes that were upregulated in Δ*P3* are indicated with red asterisks in Figure 5. The red and green arrows indicate enhanced or reduced processes. In Δ*P3*, glucose could be phosphorylated by enhanced expression of *xylR*, converted to 3PGA and channeled into sucrose (upregulated *sps, ggt, ggp, glp*) as an osmotic stress response, into PHB rather than glycogen, and into amino acid pools ornithine/arginine/citrulline (Huege *et al*., 2011). Key genes affected in day 1 that could be activated by P3 include *rbcLSX*, *ccmK2K1L*, *malQ, glgC, gap2, fbp1, fba2, gpmB, pdhA* and *icd*. Genes similarly affected in L1 and/or D1 and in day 2 as well are likely to be due to the removal of P3. Conversely, genes affected oppositely in day 2 are likely to be due to other secondary regulatory effects other than P3. Therefore, we have only selected those genes that were consistently downregulated in day 1 and/or day 2 for Table S7. P3 has a definite role in activating the *petJ* gene and repressing the slr6042-slr6044 operon for copper efflux and C3 has the opposite impact on these genes. Regulatory genes affected in the P3 mutant and downregulated could be downstream of the signal transduction cascade. These results strongly show the impact of the P3 operon as a potential activator of key metabolic genes in most important central metabolism pathways.

RreP regulates central metabolism in the dark: In this study, we analyzed the growth phenotype, functional complementation of the P3 mutant, and differentially expressed genes to determine the role of the P3 operon in regulating central metabolism in the dark.

MT conditions use many metabolic pathways like photosynthesis, nitrogen assimilation, carbon metabolism using both CO_2 through the Calvin cycle and glucose through OPP, glycolysis and the TCA cycle so that the constant switching between these pathways caused problems due to downregulated genes in the P3 mutant (Figure 5, Table S6). The *hik31* operon genes were induced in growth with HL (Nagarajan *et al*., 2012), high salt and sorbitol and in peroxide stress supporting a role for this operon in mediating the redox, electron transport, osmotic and oxidative stress responses (Kanesaki *et al*., 2002). Hence, *rreP* may be involved in the signal transduction of multiple stresses like dark, HL, N limitation, glucose and redox. P3/ RreP is an important multifunctional system involved in integrating photosynthetic, N and C metabolism; pH and metal homeostasis; redox and osmotic balance; and cell division in the dark. Therefore, we conclude that an operon on a plasmid in the cyanobacterium *Synechocystis* regulates a number of key metabolic pathways when cells are grown on an organic carbon source in typical diurnal conditions.

Experimental Procedures

Bacterial strains and culture conditions- The *Synechocystis* WT glucose-tolerant strain and mutant Δ*P3* (constructed as described previously in Nagarajan *et al*., 2012) were grown in triplicate at 30° C in 12h light 12h dark (12LD) cycles for 3 days in BG-11 medium buffered with 25 mM Hepes-NaOH (pH 7.5) in 100 ml flasks and shaken at a constant speed of 125 rpm. The starting cell density for these experiments was around 1 x 10^7 cells ml $^{-1}$. A light intensity of about 30-40 µE m^{-2} s^{-1} was used for all studies. 5 mM glucose was used for both cultures and 25 µg ml^{-1} kanamycin was added for the Δ*P3* mutant. Both absorbance at 730 nm and doubling times from cell counts using a Petroff-Hausser counting chamber were used to measure growth. Pigment content was estimated from whole-cell absorption values and normalized at 800 nm. We compared the peaks for the pigments for chlorophyll a at 436 nm and 686 nm, carotenoids at 480 nm and 530 nm and phycocyanin at 630 nm. Growth on plates for both cultures was carried out by spotting 5 µl of 4-fold serial dilutions of cultures on appropriate duplicate unbuffered BG-11 plates with 5 mM glucose and antibiotics that were incubated in LL or 12LD conditions for 8 days. For the viable count growth experiment, cells were grown for 3

803 days in PA LL, PA LD, MT LL or MT LD conditions in liquid media and then serially
804 diluted and spread- plated on to BG-11 plates, with and without glucose, and incubated in
805 LL for 10 days. 5 mM of the following sugars were added to monitor growth of cells
806 spotted on BG-11 plates to compare and assess the effect of glucose in LL and 12LD - 3
807 Ortho Methyl D-Glucose (3OMG), fructose, sucrose, mannose and mannitol. Spotting
808 with TCA intermediates was performed in LL and 12LD and incubated for 8 days using
809 BG-11 plates supplemented with 5 mM glucose and 5 mM of each of the following
810 glycolysis or TCA components (sodium salts) in separate plates- pyruvate, acetate,
811 oxaloacetate, succinate, fumarate and malate. Cells were also grown in liquid media for 3
812 days in LL and 12LD to test the growth with 5 mM glucose and one of the above
813 glycolysis or TCA compounds. Additional liquid growth experiments were carried out
814 with BG-11 supplemented with 5 mM glucose along with 5 mM of one of the following
815 in separate flasks- NH_4Cl, 2-OG sodium salt, $NaHCO_3$ and sodium glutamate for 3 days
816 in 12LD. pH sensitivity experiments were carried out in liquid BG-11 media with 5 mM
817 glucose and buffered with Hepes-NaOH pH 7.5 and 8 and CAPS-NaOH pH 9 and 10 and
818 grown for 3 days in LL and 12LD.
819
820 *Growth conditions for the microarray experiment*- Starter WT and Δ*P3* cells from plates
821 were grown in liquid BG-11 for 4 days in PA LL conditions. They were re-suspended to
822 about $1x10^7$cells ml^{-1} in 100 ml of BG-11 with 25 mM Hepes-NaOH pH 7.5, then grown
823 for 1.25 days in photoautotrophic conditions till the cell count reached around the mid-
824 log level. 5 mM glucose was then added and the cultures were grown for another 2 days
825 in 12LD cycles. Samples for RNA extraction were taken after adding glucose at 1 h in
826 day 1 in the light (L1) and in the dark (D1), and 1h in day 2 in the light (L13), and in the
827 dark (D13). Two biological replicates and four technical replicates were used per time
828 point per culture including a dye swap. Cells from the four time points were spun down
829 and the pellet was frozen in STET buffer at -80°C. RNA was extracted using Tri-reagent
830 (Ambion), Dnase-treated, and column purified (Zymo research). Labeling of the purified
831 RNA and hybridization was carried out at MOgene, LLC in St. Louis, MO. as previously
832 described (Toepel *et al*., 2008). The arrays used were Agilent 2x11K 60 oligomer arrays.
833 RNA from both biological replicates mixed in equal concentrations for each culture were

834 used. Dye biases were taken into account by using the dye swap replicates for each time
835 point in this dual color microarray. The slides were scanned and feature extracted using
836 an Agilent scanner. Data analysis was initially done by the Rosetta Luminator software
837 package that included p-values for fold changes of gene expression.
838
839 *Statistical analysis of microarray data*- The differential gene expression analysis was
840 done using open-source software, R (R Development Core Team, 2012) and
841 Bioconductor (Gentleman *et al*., 2004). For the differential gene expression analysis,
842 microarray data was normalized using the Variance Stabilizing Normalization (VSN)
843 method (Huber *et al*., 2002) using the Bioconductor package. VSN uses a robust
844 statistical model that controls for any systematic background, experimental, or technical
845 errors that affect microarray data and minimizes the chances of detecting false positives
846 in the differential gene expression analysis later. We used an analysis of variance
847 (ANOVA) for detecting differentially expressed genes between mutant and wild type
848 separately across four time points in the experiment; the baseline treatment effect was the
849 wild type. Specifically, at each time point, we used the moderated two sample *t*-test
850 implemented in Bioconductor package *limma* (Smyth, 2004), which is a robust extension
851 of the two sample *t*-test targeted for microarray data for testing the null hypothesis that
852 there is no difference in a gene's expression between the mutant and wild type. The
853 differentially expressed genes at each time point are determined from the p-values of
854 these hypotheses tests after controlling the false discovery rate (FDR) at1% (Benjamini
855 and Hochberg, 1995). The stringent FDR cutoff bounds the number of genes that are
856 falsely declared as differentially expressed by the statistical analysis. To make the results
857 more reliable and practically significant, we filtered only those differentially expressed
858 genes that had a fold change ≥ 1.5 or ≤ 0.7. The microarray data contained 8635 probes
859 for 3494 genes. We selected a gene only if all its probes satisfied the aforementioned
860 filtration criterion. 2020 genes were differentially expressed in at least 1 time point. For
861 our data analysis of the microarray results, we also included genes changed by 1.3 fold or
862 more and 0.8 fold or less for genes that were in an operon, or in the same pathway for
863 related functions. The transcriptome data from this study was submitted to the

ArrayExpress database at the European Bioinformatics Institute (accession no. E-MTAB-1539).

RT-PCR primers and conditions- RNA was Dnase-treated with DNase I Amplification grade (Invitrogen) for 15 minutes. The treatment was checked with PCR using standard PCR conditions and the *rnpB* control primer set. Dnase-treated RNA was used to make cDNA using random primers and Superscript II using the manufacturer's instructions. RT-PCR was carried out at 94°C for 1 min and 20-40 cycles of 94°C for 15s, 54°C for 30s and 68-72°C for 60-180s depending on size of the amplified region and level of gene expression. Primers for testing the expression of the C3 and P3 operon genes and the restriction digests to differentiate between both copies were described previously (Nagarajan *et al*. 2012). Selected genes from different categories were chosen for microarray data validation with RT-PCR and the primers for these genes and their amplicon sizes are listed below in 5'to 3' direction- *atpG* (310 bp) F-TGATGCCACCCTGCCCCTGAT and R- CGCCGCCGTTTCCTTTTGCC , *ccmK1* (176 bp) F- GCGGCCGAGTCACCGTGATT and R- CACCGCCTCGGTGTAGCGAA, *psaL* (208 bp) F- TGGGCATGGCCCACGGTTACT and R-TGGCTCCAACCATCGGCTGT, *psbO* (363bp) F-AAGCGCCAAAAGGCTGAGTACGT and R- ATCCGCCGCTGAAGGCAGAG, *apcA* (418 bp) F- ATGCTGATGCAGAAGCCCGCT and R-GCTTCGGCGGCATCATCGGA, *ftsH3* (195 bp) F- ATCCCAGCGGTGGTCCTCCC and R- TCTGGGCCCGACGGAAGAGG, *pilT2* (386 bp) F-TCTAGTGACGGGGCCCACCG and R- CCGTCCGCATGGCTGGTTGT, *rpe* (418 bp) F- GCCAAGGCCGGTGCTGACAT and R- TGCTGTTGCGTACTCCGGCG, *fbaII* (414 bp) F- TTCACCGCCTGCTGCCCAAC and R- CAACGGAGGTGCGGGAGGTG, *fumC* (395 bp) F- GGGGAGCGCAAACCCAACGT and R-CACCGCCGCAATGTGCATCG, *cmpA* (353 bp) F- AGCCAACTGGGCCTCCGCTA and R- CAGGATCAACGCCTCCGGCC, *czcB* (127 bp) F-GGTCAGCCAACGGGCCAGTC and R- TGGCGGTGCTCCAGGCAATG. The above genes were tested as indicated for the requisite number of cycles in figure 5.

Electron Microscopy- Cells were grown in MTLL and MT 12LD conditions in liquid media for 4 days before being processed for electron microscopy as preciously described (Nagarajan *et al*., 2012).

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Table 1- Viable counts for the WT and Δ*P3* grown for 3 days in PA and MT conditions

Growth condition	Strain	Viable Counts in cfu/ml ($x10^7$) [a]	
		Control, no glucose	Glucose (5 mM)
PA LL	WT	10.0	6.0
	Δ*P3*	10.0	5.0
MT LL	WT	6.0	10.0
	Δ*P3*	8.0	7.0
PA LD	WT	1.0	2.0
	Δ*P3*	0.8	<0.001
MT LD	WT	10.0	4.0
	Δ*P3*	0.06	<0.001

[a] Cells were grown from same starting cell density in labeled condition for 3 days and then serially diluted and spread plated onto control and glucose media. Plates were incubated in LL conditions for 8 days before counting colonies.

1246

1247 **Table 2**- Gene categories significantly changed (upregulated or downregulated) in the P3
1248 mutant (FDR ≤ 0.01, Fold change ≥ 1.5).

General Pathways	**Number of genes**	**Δ*P3*/WT**							
		L1		**D1**		**L13**		**D13**	
		↑	↓	↑	↓	↑	↓	↑	↓
Amino Acid Biosynthesis	97	2	6	1	13	16	28	20	26
Biosynthesis of cofactors, prosthetic groups, and carriers	125	4	10	3	11	28	25	34	21
Cell envelope	67	4	1	8	3	12	11	18	11
Cellular processes	77	6	3	11	2	6	26	8	26
Central intermediary metabolism	31	1	0	1	3	5	6	2	12
DNA replication, restriction, modification, recombination, and repair	73	0	0	1	5	23	7	25	7
Energy metabolism	93	3	4	4	5	17	18	15	26
Fatty acid, phospholipid and sterol metabolism	39	2	2	2	1	6	9	8	10
Hypothetical	1243	29	62	60	48	237	246	289	247
Other categories	253	10	14	12	13	40	46	48	44
Photosynthesis and respiration	142	7	39	4	16	6	84	6	88

Purines, pyrimidines, nucleosides, and nucleotides	43	0	3	1	6	7	11	9	10
Regulatory functions	153	2	10	8	9	38	20	43	26
Transcription	30	3	0	3	1	5	16	8	15
Translation	168	2	27	28	10	14	83	20	78
Transport and binding proteins	199	14	14	13	7	45	23	57	29
Unknown	611	24	31	60	29	92	117	114	146
total	3444	113	226	220	182	597	776	724	822
Total genes and percent of genome changed on slide		339, 9.8%		402, 11.7%		1373, 39.9%		1546, 44.9%	

Figure 1. Expression of the *hik31* operon genes and selected differentially transcribed genes from the microarray experiment for the WT and Δ*P3*.
A. Gels showing RT-PCR amplification of both *hik31* operons and individual genes. The RT-PCR products for both copies of the operons were separated using restriction digests and separate primers were used for the *hiks* as previously described (Nagarajan *et al*., 2012). 35 cycles were used for all the genes and *rnpB* was used as a control. B. Genes from different categories that showed altered behavior were selected for validation of the microarray experiment. The number of cycles used is indicated next to the gene name in the figure.

Figure 2. Electron micrographs of the WT (A and C) and Δ*P3* (B and D) grown in 5 mM glucose for 4 days in LL (A and B) and 12 L/ 12D (C and D). CG, cyanophycin granules; PHB, polyhydroxybutyrate granules. Magnification was 11,500 X to 21,000 X.

Figure 3. Growth of the WT and Δ*P3* at the end of 3 days in MT 12L/ 12D using liquid media of different pH (7.5, 8, 9 and 10 as indicated in the legend). Error bars for cell counts are for triplicate measurements.

Figure 4. Growth of the WT and Δ*P3* at the end of 3 days in MT 12L/ 12D with added nitrogen and carbon sources (NH_4Cl, 2OG, $NaHCO_3$ and sodium glutamate as indicated in the legend). Error bars for cell counts are for triplicate measurements.

Figure 5. Pathway map of central metabolism showing genes and processes upregulated (red asterisks, red arrows respectively) and downregulated (green asterisks, green arrows respectively) in Δ*P3* compared to the WT in day 1 (L1 and/ or D1). The majority of the genes shown were downregulated in the mutant by D13 and this included genes listed in Table S5- *glgX, glgP, malQ, glgC, pgm, zwf, pgl, gnd, rpe, talB, tktA, pgi, pfkB1, fbp1, fba1, fba2, gap1, pgk, gpmB, eno, pyk1, ppsA, pdhB, pdhD, icdA, citH, phaA, phaB phaC,* and *phaD*.

Figure S1. Whole cell absorption spectra of pigments for the WT and Δ*P3* grown for 3 days in A. PA LL, B. MT LL, C. PA LD and D. MT LD conditions. Values were normalized at 800 nm.

Figure S2. Growth of serially diluted cultures of the WT and Δ*P3* for 8 days on plates containing either 5 mM glucose or the same concentration of five other sugars in 12L/ 12D conditions. Cells were also grown on plates without any sugar and incubated in the control PA 12L/ 12D condition.

Figure S3. Growth of serially diluted cultures of the WT and Δ*P3* for 8 days on plates containing 5 mM glucose and 5 mM of one of the following sugars- pyruvate, acetate, oxaloacetate, succinate, fumarate and malate in 12L/ 12D conditions. Cells were also grown on control plates with only glucose and on control plates without any sugar and incubated in the PA 12L/ 12D conditions.

Figure S4. Number of genes in different gene categories on CyanoBase significantly FDR ≤ 0.01, Fold change ≥ 1.5) upregulated (positive numbers) or downregulated (negative numbers) in Δ*P3* compared to the WT in the microarray experiment. The percentage of the genome affected is indicated below the graph for each time point.

Supplemental Table S1A- Full microarray dataset.

Supplemental Table S1B- Selected genes tested for microarray validation using RT-PCR (Figure 1B).

Supplemental Table S2- Selected genes from photosynthesis, pigment biosynthesis, redox stress and cellular protection.

Supplemental Table S3- Selected genes from regulatory, transcription, translation and cellular processes.

Supplemental Table S4- Selected nitrogen, amino acids, sulfur and phosphate metabolism genes.

Supplemental Table S5- Selected organic and inorganic carbon metabolism genes.

Supplemental Table S6- Groups of co-expressed genes in different temporal transcriptional patterns.

SupplementalTable S7- Possible genes regulated and activated by P3 in day 1.