FUNCTIONS OF THE DUPLICATED HIK31 OPERONS IN CENTRAL METABOLISM AND RESPONSES TO LIGHT, DARK AND CARBON SOURCES IN SYNECHOCYSTIS SP. STRAIN PCC 6803.

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Running Title: Non-redundant functions for duplicated Hik31 operons

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ABSTRACT

There are two closely related \textit{hik31} operons involved in signal transduction on the chromosome and the pSYSX plasmid in the cyanobacterium \textit{Synechocystis} sp. PCC 6803. We studied the growth, cell morphology and gene expression in operon and \textit{hik} mutants for both copies, in different growth conditions, to examine whether the duplicated copies have the same or different functions and gene targets, and whether they are similarly regulated. Phenotype analysis suggested that both operons regulated common and separate targets in the light and the dark. The chromosomal operon was involved in negative control of autotrophic events, whereas the plasmid operon was involved in positive control of heterotrophic events. Both the plasmid and double operon mutant cells were larger and had division defects. The growth data also showed a regulatory role for the chromosomal \textit{hik} in high CO\textsubscript{2}, and the plasmid operon in low O\textsubscript{2} conditions. Metal stress experiments indicated a role for the chromosomal \textit{hik} and operon in mediating Zn and Cd tolerance, the plasmid operon in Co tolerance, and the chromosomal operon and plasmid \textit{hik} in Ni tolerance. We conclude that both operons are differentially and temporally regulated. We suggest that the chromosomal operon is the primarily expressed copy and the plasmid operon acts as a backup to maintain appropriate gene dosages. Both operons share an integrated regulatory relationship and are induced in high light, glucose and in active cell growth. Additionally, the plasmid operon is induced in the dark with or without glucose.
INTRODUCTION

Bacteria use several devices to monitor their environment and coordinate appropriate adaptive changes to maximize survival. These include chemotaxis receptors, sigma factors, Ser/Thr protein kinases and Two-Component Systems (2CS) (25). The prototypical 2CS consists of a Histidine kinase (Hik) sensor that is a transmembrane protein and a Response regulator (Rre) that usually binds to DNA, and acts as a transcription factor either activating or repressing the target genes or both. Each protein has two or more domains that perform the various functions and participate in phosphotransfer reactions, and can be classified into different types (7, 42, 8, 3). Higher order 2CSs can have a more complex interaction with combinations of domains and cross-talk between different partner 2CS (7).

Signal transduction systems in the freshwater model cyanobacterium Synechocystis sp. PCC 6803 (hereinafter Synechocystis) are important for sensing, responding and adapting to different environmental changes. The Synechocystis genome includes about 47 Hiks and 45 Rres and these comprise > 2.5% of the genome. Although most of these are located on the chromosome, 3 each of the Hiks and the Rres are found on the plasmids pSYSX and pSYSM. Unlike other bacteria, the positions of these genes are scattered throughout the genome, and only 14 sets or 32 ORFs are in close proximity to each other. The domains of these 2CS proteins have been compiled and the functions of some of these have been determined, but the partners for many Hiks have not been identified. The previously studied 2CSs have involved the chromosomally-located...
This study will be concerned with the closely related hik31 operons (sll0788-sll0790) on the chromosome (C3) and on the plasmid (P3) pSYSX (slr6039-slr6041). They each contain a Histidine Kinase sensor (Hik), a Response Regulator (Rre) and a Hypothetical protein (Hypo) in the same order, hinting at segmental duplications. The Hiks and the Rres are identical in sequence at the protein level by about 96%, and the Hypos by 99%. The Hypos have two DUF305 (Domain of Unknown Function) domains that contain a double histidine (HH) motif that is presumed to be functionally important. Some of these proteins in other bacteria can bind to Fe\(^{2+}\), Zn\(^{2+}\), ethanediol and Cl\(^{-}\) (33). This is the only duplicated 2CS present in Synechocystis, and the only cluster to be present on both the chromosome and the plasmid among the cyanobacteria that have been sequenced. The plasmid localization of this 2CS gene duplication is unusual, and to our knowledge, this is the only such system studied in bacteria.

The presence of two copies of this gene cluster raises interesting questions as to their function and the selective pressures that allow both copies to be stably maintained in the same cell. There are four main outcomes arising from gene duplications that explain the fate of duplicated genes—nonfunctionalization or loss of the duplicate, subfunctionalization to retain complementary functions, neofunctionalization to develop new functions, and differential regulation of duplicates with similar sequence and function (19). We consider this last possibility the most likely for the hik31 2CSs, since the
upstream regions of the hik31 operons are only identical for 72 bp and there are many differences further upstream. Such an arrangement might enable both coordinated, as well as differential, regulation of both operons. Several studies conclude that changes in regulation are more common than biochemical changes, and that non-essential duplicate genes are retained by the cell. The duplication and location of the plasmid copy may also ease selection pressure allowing gradual diversity in function for the two copies (20, 26). Varied environmental conditions could enable retention by altering gene dosages and lead to non-redundant network interactions with other proteins (15, 21).

These genes were first identified because they were actively expressed in several growth and stress conditions in microarray studies. The genes were up-regulated in salt and osmotic stress (13), redox stress with DBMIB (10), pH 10 (35, 37), in the dark (38), and down-regulated in heat shock (39), peroxide stress (14) and stationary phase (Singh, A.K. and Sherman, L.A. unpublished data) in the Wild Type (WT) and specific mutants. The arrays for these experiments only had the chromosomal gene probes; however, once the plasmids were sequenced, and with the realization that the two operons were nearly identical, it was not known which copy was expressed. In one experiment with WT cells and the new Agilent microarrays that contained probes for both operon gene copies, it was determined that growth under low O2 conditions (36; Summerfield, T.C. and Sherman, L.A. unpublished data) led to the transcription of all 6 genes in the dark, but only the chromosomal genes in the light. Thus, there may be temporal regulation of these genes with one of the copies sufficient in light, but both copies
needed in the dark for increased protein dosage. A putative double hik31 mutant
has been implicated in the response to glucose (11). Also, a chromosomal
Δhik31 mutant has implicated Hik31 as a transcriptional repressor in low O2
conditions affecting photosynthetic and ribosomal genes (36). Additionally, both
sets of operons are close to genes predicted to be Zn\(^{2+} /\) Co\(^{2+}\) cation transporters,
suggesting that these 2CSs may control the transduction of cation signals (12).
The presence of the Hypo closely associated with the 2CS genes indicates
that it could be involved in signal transduction as an extra non-kinase receptor
activating the Hik, making the operon a Three Component System (3CS).
Topology and domain predictions (using the SMART domain prediction tool and
TOPCONS) indicate that both the DUF305 domains of the hypothetical protein
and the N-terminal part of Hik31 are periplasmic. 3CSs are rare and they
enhance sensitivity, range of detecting signals and adaptability for the cell (4).
Domain analysis of the hik31 operons against the well studied EnvZ/ OmpR
proteins in *Escherichia coli* indicated that the operon arrangement, length of the
proteins, domains and secondary structures, as well as the topology were very
similar. It is thought that EnvZ, which does not have a defined sensor region,
may sense osmotic changes indirectly through interactions with other integral
membrane proteins or monitor membrane tension (42). Since neither Hik has
domains for detecting light, O\(_2\), redox or metals, it is possible that Hik31 may
sense changes in a similar manner or through interactions with the Hypo.
The occurrence of two copies of the Hik and Rre raises questions on how
specificity is maintained in phosphotransfer for both cognate sets of proteins.
Both Hik31 proteins are identical in their functional domains and mainly differ in the β7 sheet and beyond by 19 residues on the C-terminus of the proteins. The Rres differ in the receiver α1 region by one residue, in the output domain between the β1-β4 by 7 residues, and the recognition helix by one residue. The α1 residue has also been shown to be a co-evolving residue for the EnvZ-OmpR pair that affects specificity for the 2CS (5). Taken together, these changes may affect the way that both receiver and output parts of the Rre protein fold together so that the Hiks can bind selectively or in a hierarchical manner to transfer the ~P to their own partner Rre first. Moreover, homologs for the hik31 operon genes exist in 14 other cyanobacteria with these genes conserved and unique to cyanobacteria. Many of these homologs have the chromosomal duplicates only, some do not contain the Hypo, and only two of them have plasmid copies.

In this study, we have explored the role of both hik31 operons in metabolism and tested for functions in response to different growth conditions using mutants in both copies. We examined the growth, ultrastructural properties and gene expression to environmental parameters involving light, dark and carbon sources. Our results indicate that both operons are involved in common and separate functions, are temporally and differentially regulated and also share an integrated regulatory relationship.

MATERIALS AND METHODS

Cyanobacterial strains and growth conditions. WT and mutants of the glucose-tolerant Synechocystis sp. strain PCC 6803 were grown in BG-11 medium at 30°C under different conditions of light, dark, air composition and
metal salts. During the course of these studies, cells were grown under photoautotrophic (PA), mixotrophic (MT), and heterotrophic (HT) conditions with different durations of light and dark (LD) and in high continuous light (HL) for 6 days. High CO₂ (HC) growth was performed in continuous light (LL) both with and without glucose for 2 days. Light intensities of 30-200 µE m⁻² s⁻¹ were used for different growth conditions and the medium was supplemented with 5 mM glucose for mixotrophic and heterotrophic growth. Liquid cultures were grown in BG-11 media buffered with 25-40 mM HEPES-NaOH pH 7.5 in 250 ml Erlenmeyer flasks. Low O₂ growth experiments were performed in the 6 L bioreactor (BioFlo 3000, USA) and high CO₂ experiments in 750 ml Cytolifts (Kontes, Inc. USA) (36). Spectinomycin and kanamycin (25 µg/ml), and chloramphenicol (10 µg/ml) were added to the medium for specific mutant strains. Growth was assayed by both absorbance at 730 nm (Perkin-Elmer UV-VIS λ 40 Spectrophotometer, USA) and cell counts in a Petroff-Hauser counting chamber. Doubling times were calculated for 3 day grown cultures in PA, MT, and HT conditions and for 2 days for the HC and low O₂ conditions. Growth on solid media was performed in duplicate by spotting 5 µl of 4-fold serial dilutions on BG-11 plates, containing appropriate antibiotics and 5 mM glucose as needed. Plates were incubated for 8-11 days in LL, 12L/12D and 6L/18D with and without glucose and exposed to both 30 and 60 µmol photons/m²/s of light. Light activated heterotrophic growth (LAHG) was tested on plates with 5 mM glucose and exposed to 15 minutes of light (60 µmol photons/m²/s) per day for 15 days (31). These plates were maintained in the dark otherwise. For the metal
tolerance experiment, 20 µM of NiCl₂, 10 µM each of CoCl₂, ZnCl₂, and CdCl₂ were used. Spectral analysis of pigment composition was estimated from whole cell absorption values (17). Statistical analysis of Table 1 doubling times was carried out through Tukey’s test of comparison using SAS software and a significance level of \( \alpha = 0.05 \) in collaboration with Zhuo Chen and Dr. Thomas Kuczek of the Statistical Consulting Service at Purdue.

**Construction of mutants.** Six deletion mutants that involved *hik31* were constructed. These included the entire *hik31* operons located on the chromosome (\( \Delta C3 \)), the plasmid (\( \Delta P3 \)), as well as both operons (\( \Delta C3P3 \)). In addition, *hik31* genes were also deleted from the chromosome (\( \Delta hikC \)), the plasmid (\( \Delta hikP \)) and from both positions (\( \Delta hikCP \)). *Synechocystis* sp. strain PCC 6803 genomic DNA was used to amplify the plasmid operon (2634 bp) along with flanking regions using the primers F: 5'-CTTCATGATGCTGACTGTC-3' and R: 5'-ATGACAATGGTGCCATCG-3' to yield a 4524 bp PCR product that was cloned into the pGEM-T vector. Similarly, the chromosomal operon (2658 bp) was amplified with primers F: 5'-CGGGATCCACTAACATGCTCTTGACTGCAGACTCG-3' and R: 5'-CGGGATCCATCCCATTCCACTCCTCATCCATTGC-3' containing the *BamH*I site (as underlined) to yield a 4180 bp PCR product that was then cloned into the pUC19 vector. Specific deletions were made using restriction sites in the coding region of the operons and the *hik* genes as outlined in Fig.1A. The deleted portions were then replaced with various antibiotic cassettes—spectinomycin (\( \Delta C3 \) and \( \Delta hikP \)), kanamycin (\( \Delta P3 \)) and chloramphenicol (\( \Delta hikC \)) and used to transform WT *Synechocystis* sp. strain PCC 6803. The double
mutants had different antibiotic cassettes (spectinomycin and kanamycin for ΔC3P3 and chloramphenicol and spectinomycin for ΔhikCP) replacing each deleted portion. Transformed colonies were selected on antibiotic plates and transferred over 2-4 months for segregation. Full segregation of the mutants was confirmed using PCR (Fig.1B), and at regular intervals thereafter.

**Cell morphology and electron microscopy.** Cell sizes and shapes were evaluated by light microscopy with a VWR Vista Vision camera. Cells for electron microscopy were prepared by microwave chemical fixation and sectioned, stained and imaged in a FEI Phillips CM-100 electron microscope as previously described (34). Cell sizes were measured by selecting cells in Photoshop and calculating the area on an Apple Macintosh computer using Ivision software.

**RNA extraction and semi-quantitative RT-PCR.** The experiments depicted in Figs. 6 and 7 were performed independently with different batches of WT and mutant cells. For monitoring growth phase induction of the hik31 operon genes, the WT cells were grown in photoautotrophic 24L/0D for 6 days and cells from the end of each day were stored in STET buffer. For testing induction of the hik31 operon genes by glucose in continuous light, WT cells were grown till about mid-log (30 hours) and 5 mM glucose was added. Cells grown for 2, 6, 18, 24 and 48 hours after the addition of glucose were stored in STET. For checking induction of the hik31 operon genes by glucose in 12 L/12D conditions, WT cells were grown till about mid-log (30 hours) and 5 mM glucose was added. Cells grown for 2 and 6 hours in the light and in the dark after the addition of glucose were stored in STET. To evaluate transcription of the hik31 operon genes, the
WT and mutants were grown for about 3 days in photoautotrophic 24L/0D and 12L/12D, as well as in mixotrophic 24L/0D and 12L/12D, centrifuged at 8000 × g, and stored in STET buffer at -80°C. Cells were grown for 2 days in high CO₂ conditions before being stored as above. For the high light experiments, cells were grown till about mid-log (26 hours) at 30 µE m⁻² s⁻¹ and then exposed to 150-200 µE m⁻² s⁻¹ of light both with and without 5 mM glucose for 2 hours before being treated as above. Total RNA from two biological replicates for each condition was extracted and purified using Tri-reagent (Ambion, USA). RNA was treated using DNase I (Invitrogen Amplification grade, USA) for 15 minutes and confirmed for reaction success through PCR. RNA was subsequently reverse transcribed using Superscript II (Invitrogen, USA) and random primers. RT-PCR was then performed to amplify the transcripts of the C3/P3 operons (2464 bp), the rre C/P genes (511 bp) and the hypo C/P genes (454 bp) using the following common primers for both copies on the chromosome and the plasmid listed in 5' to 3' orientation: C3/P3 operon - F: CAGCGGCTGGGGTAACAGCG and R: TGGC AAGGCCTAATCCTGCC; rre C/P- F: GGGTGCAGGACGGCAAACCTA and R: AAACGCACCTGGGCCGCTAC; hypo C/P- F: CAGCGGCTGGGGTAACAGCG and R: TCCATCTCCGGCCGTTCCGT. In order to measure the transcript levels of both individual operons, we used the scheme in Fig. 1C. Because the two operons are so similar, we could not use qRT-PCR to analyze their individual transcription patterns and thus used the more qualitative RT-PCR. However, we took great care to identify appropriate conditions for amplification and separated the transcripts for both operons.
through restriction digests. The operon and *rre* amplicons was digested with KpnI at a unique restriction site in the plasmid *rre* to result in two fragments of 1749 bp and 715 bp, and 425 bp and 86 bp respectively. The *hypo* transcripts were differentiated by digestion with MlyI which cuts the plasmid *hypo* at a unique site to result in two fragments of 353 bp and 101 bp (Fig. 1C). These sites are not present on the chromosomal genes and consequently, we could separate the chromosomal and plasmid transcripts for these genes. For simplicity, we have only shown the larger of the two digest pieces in the gel pictures in Figs. 7 and 8. We used sufficiently different primers listed in 5' to 3' orientation for both *hiks* (567 bp) to tell their transcripts apart. *hikC-F*: GCTGGATCAAGAGCTTAC and *hikC-R*: GGTGTACGTAATTCGTGG as well as *hikP-F*: GCTGGATCAAGAATTAAC and *hikP-R*: GGTGTACGTAATTCGTGG. RT-PCR was performed at 94°C for 1 minute, for 30-40 cycles of 94°C, 54°C for 30 seconds and 68-72°C for 60-180 seconds depending on the amplicon size and abundance. The *rnpB* gene was used as a positive control as previously described (36).

**RESULTS AND DISCUSSION**

**Growth in liquid media- cell doubling and pigmentation**

The mutants described in Fig. 1 were characterized for growth and morphology in liquid medium and on plates. Under LL conditions, *ΔhikC* grew somewhat better than the WT, whereas under LD conditions, all 3 *hik* mutants grew slower (Table 1). The mutant *ΔC3P3* was also the slowest growing strain in
PA and grew additively worse than both ΔC3 and ΔP3. The operon mutants ΔP3 and ΔC3P3 grew much slower than the WT under the 6L/18D reduced light conditions. ΔC3 was the best growing strain in both PA LD and PA HL conditions. Under PA HL, all mutants except ΔP3 and ΔC3P3 grew fairly well, whereas these two strains were incapable of growth and displayed signs of photoinhibition.

Under MT conditions, the operon mutants grew poorly in different light regimes, whereas all the hik mutants grew nearly as well or better than the WT. ΔC3P3 was found to grow less than both individual operon mutants in LL, but intermediate to both individual operon mutants in LD and HL. ΔP3 grew less than half as well as the WT under LD cycles and this suggests that ΔP3 has defects in dark metabolism involving the processes of sugar catabolism and respiration.

ΔC3 grew poorly in LL, the worst under HL conditions and ΔC3P3 also grew slower resembling ΔC3. It was observed that after 4 days of growth, two of the cultures had increased cell concentrations reaching high cell numbers of $3 \times 10^8$ cells/ml (ΔhikP in MT HL) and $4 \times 10^8$ cells/ml (ΔhikCP in MT 12L/12D) compared to about $2 \times 10^8$ cells/ml for the WT. When cultures were grown heterotrophically, both ΔP3 and ΔC3P3 grew poorly and ΔhikCP grew the fastest.

The hik mutants demonstrated significant growth defects when grown with 3% CO2 in the presence or absence of glucose. ΔhikP and ΔhikCP grew poorly in the absence of glucose and ΔhikC and ΔhikCP grew poorly in the presence of glucose. The defect exhibited by ΔhikCP in MT LL and HL became more severe in HC. Furthermore, when grown in high CO2 with glucose, ΔC3 reached a high cell density of $3.5 \times 10^8$ cells/ml and ΔhikP of $2.8 \times 10^8$ cells/ml compared to $1.7 \times 10^8$ cells/ml.
10^6 cells/ml for the WT. Thus, ΔC3 is sensitive to HL conditions, but benefits from HC when grown in the presence of glucose. ΔC3P3 grew better in MT LL than PA LL, in PA HC than PA LL conditions, and intermediate between both operon mutants in MT HC conditions, indicating that both glucose and high CO2 can overcome the growth defect of the ΔC3P3 strain.

Finally, low O2 growth was monitored by growth of cultures in PA LL for 2 days and then bubbled with 99.9% N2 and 0.1% CO2 for 2 additional days as previously described (36). ΔC3, ΔhikC, ΔhikP and ΔhikCP all showed a mean growth increase in low O2. Moreover, the 3 hik mutants also had a higher growth increase compared to the WT with the ΔhikCP strain exhibiting the highest level after the first day of low O2. Both ΔP3 and ΔC3P3 grew poorly in low O2 conditions and did not show an increase in growth like the other strains, suggesting that the plasmid operon, and especially the plasmid Rre and/or Hypo, may be important in regulating the growth increase and adapting to low O2 conditions (data not shown). Based on these results, we concluded that the various mutants had alterations in their growth caused by light or dark, by the presence or absence of carbon sources, and by low O2. A Tukey’s test was used to measure the significance of the different growth results of the mutants under the various conditions and those results shown to be statistically significant at α=0.05 are marked with an asterisk in Table 1. Based on this analysis, the main growth defects from the growth experiments for each strain are summarized in Table 2. Changes in pigmentation reflected the alterations in growth fairly closely (data not shown, indicated as bolded font in Table 1). The main changes in
chlorophyll and phycobilisome content were in ΔP3 and ΔC3P3 with the strongest influence under glucose and high light conditions in the presence and absence of glucose (a 2- to 5-fold reduction in pigment content under most conditions).

**Growth on plates**

The mutants were also grown on BG-11 agar plates in order to analyze cultures that might grow well at high density, but would manifest defects when diluted. This was an excellent way to differentiate the impact of light and dark cycles and glucose, either separately or together, and to determine the effect of metals on mutant growth. The main changes in growth patterns seen on plates that were different from the liquid growth are shown in Supplemental Fig. 1.

An alternative plating approach consisted of cell growth in MT either for 4 days in LL, followed by 4 days in DD (38), or 4 days in DD, followed by 11 days in LL. We also carried out light activated heterotrophic growth (LAHG) for all the cultures on glucose plates with regular BG-11 containing sodium nitrate as well as ammonium chloride as previously described (31). The light dark treatments were particularly useful in eliciting growth changes in ΔP3 under all of these conditions, and for demonstrating that all mutants had growth problems if plates were first incubated in complete darkness in the presence of glucose for 4 days, or in LAHG conditions (data not shown). ΔP3 grew better under the 4 days LL, followed by dark, than under continuous cycles of LD. This result also reveals that our glucose-tolerant WT strain is different from other reports in the literature in that it grows heterotrophically without requiring light in liquid, and does not grow at all on plates exposed to LAHG. We also tested all strains for phenotypes...
in NaCl and sorbitol stress, but did not find significant differences from the WT. The most interesting plating experiments were those involving metal stress using concentrations that permitted reasonably good growth of the WT (Supplemental Fig. 2). There are putative transporter genes (efflux pumps) for these metals adjacent to both the chromosomal and plasmid operons. The genes next to C3 may provide tolerance to Ni, Co and Zn, whereas the genes next to P3 can potentially control Zn and Cd. None of the mutants that were missing hikC could grow on plates with Zn or Cd. These results indicated a role for hikC and C3 in mediating Zn and Cd tolerance. Ni tolerance seems to be dependant on both C3 (specifically, the two genes other than hikC) and hikP. ΔP3 alone has a growth defect in CoCl2. Based on the phenotypes above, it is possible that there is cross-regulation between both operons that may control the genes adjacent to the other copy.

**Cell morphology and ultrastructure- light and electron microscopy**

All cultures were also carefully analyzed by phase microscopy and electron microscopy. Both of these indicated that the ΔhikC, ΔhikP and ΔhikCP were similar in overall size and shape to the WT (data not shown), whereas mutants that contained a deletion of the plasmid operon (ΔP3 and ΔC3P3) had some morphological alterations under most growth conditions after 3 days of growth for both individual cells and cells that were in the process of doubling. Cells of ΔC3P3, and to a lesser extent ΔP3, demonstrated a slight asymmetry in septum formation even under PA conditions (LL and LD, Fig.2) and had varied sizes and shapes.
Synechocystis mutants that involve genes not directly known to have a function in cell division, but demonstrate division defects in certain growth conditions have been reported (1, 6, 18). To our knowledge, this is the first report of mutants (ΔP3, ΔC3P3) that had division defects in all conditions and among the first published micrographs of Synechocystis in MT LD and HT conditions. Importantly, when ΔP3 and ΔC3P3 were grown in PA HL, significant changes in cell division were noted (Fig.2, H and I vs. G). This inability to divide led to cell clusters, which were the reason for the large sizes of cells in the process of doubling. Both kidney-bean shaped, mushroom-shaped and clover leaf-shaped tetrad cells were seen for these mutants. Tetrad cells appeared to be formed by two daughter cells dividing even before they completely separated from their twin. Some cells were large at one end and tapered at the other. ΔP3 and ΔC3P3 cells were up to 3 times larger than the WT in all studied conditions of light, dark and glucose studied (data not shown) and always had fewer glycogen granules. In the WT, there were many fewer cells with the dividing septa as seen for the mutants (Fig.3, C, F and I) indicating slower or arrested cell division for the mutants. A summary of cell division defects for each mutant is presented in Table 2.

The most intriguing morphology was demonstrated by ΔP3 and by ΔC3P3, during growth in MT 12L/12D or HT in the dark. These cells had few, if any photosynthetic membranes (Fig. 3E and 3F), and had one or two large storage granules, typically cyanophycin granules (Fig. 3H and 3I) and to a lesser extent polyhydroxybutyrate (Fig.3H). In addition, the cytoplasm became opaque and this
opacity obscured other typical features. Although these cells doubled at about half the rate of WT, the cells were clearly altered when grown in glucose. ΔP3 in MT 12L/12D resembled the WT in HT (Fig. 3, E vs G) indicating that this mutant is unable to adapt to changing light-dark conditions and switch its metabolism.

Finally, ΔC3 demonstrated numerous morphological changes when grown in MT HL (Fig. 4). Once again, cell division defects could be seen in the accumulation of excess material at the dividing septum (Fig. 4B) and by clusters of cells that cannot separate properly (Fig. 4C). The main difference between the division defects shown by ΔP3 and ΔC3 is that, while ΔP3 shows shape and division defects in all conditions, ΔC3 only has these defects when grown in the presence of glucose and high light. Cells respond to HL stress by reducing the amount of photosynthetic pigments and reaction centers to minimize the oxidative damage. Some of the ΔC3 cells were almost filled with thylakoid membranes, suggesting that the balance between photosynthesis and carbon catabolism is disturbed in this cell in MT HL. All the operon mutants showed many cells that had thylakoid membranes with branches running across the middle of the cell in conditions of altered cell structure (Table 2). On the other hand, WT cells contained thylakoids arranged in neat rings around the periphery.

Model for the functions of C3 and P3

Integration of the data on the growth, pigment content, morphology and ultrastructure results of these mutants, suggested separate, but interacting, effects between photosynthetic metabolism (light-dark) and carbohydrate metabolism (glucose). Both ΔC3 and ΔP3 grew similarly in PA LL and HC.
conditions, but showed different phenotypes in 10 other growth conditions involving high light, dark, all conditions with glucose and low O₂ (Table 1). These results suggested that both operon copies were involved in regulating targets in similar connected pathways in the light, but different ones in the dark (Table 2; Fig.5). Importantly, both operons appear to have a regulatory role in high light. P3 also appears to be responsible for regulating cell division, cell shape and photosynthesis and *rreP* and *hypoP* are important in regulating growth and adaptation to low O₂.

The *hik* mutants differed in their results compared to the corresponding operon mutants when grown in HC conditions. The similarity in the growth defects for Δ*hikC* and Δ*hikCP* in high CO₂ with glucose and for Δ*hikP* and Δ*hikCP* in high CO₂ without glucose suggests that there is a regulatory switch when glucose is added that is mediated by HikC. This result led us to hypothesize that it is high CO₂ that affects the growth of Δ*hikCP* more than glucose. In this respect, our results differ from the report by Kahlon et al (11). In our liquid studies, we found only a slight defect for the Δ*hikCP* mutant with glucose alone. Δ*hikCP* displayed growth defects in HC conditions even without glucose, thus differing from the report by Haimovich-Dayan et al (9). This may be due to slightly different growth conditions and genetic composition of the host strain. The growth conditions we used were 5 mM glucose with 3% CO₂ and their conditions were 10 mM glucose and 1-5% CO₂. The ability of their mutant to grow well in HC may also be due to the unstable phenotypes caused by suppressor mutants. Our deletion mutants were very stable and gave us
reproducible phenotypes and we have taken carefully repeated measurements over a period of three years.

Growth in both liquid and solid media suggested that HikC and RreP and/or HypoP may be dominant and have a hierarchy over their counterpart on the other operon. C3 and HikC seem to be involved in responses to light with or without glucose and high CO₂, and P3 and HikP together are involved in responses to light and dark with or without glucose and high CO₂. Similarly, C3 is involved in MT HL growth and P3 in PA HL growth, whereas HikC is involved in MT HC growth and HikP in PA HC growth. These data indicated that for ΔC3 and ΔP3, light and dark take precedence over glucose, which in turn takes precedence over high CO₂. However, in the ΔhikC and ΔhikP mutants, it appears that high CO₂ takes precedence over glucose. Such interrelationships are schematically depicted in Fig. 5 with the functional targets of the chromosome and the plasmid operons shown as overlapping circles. We hypothesize that the protein products for both copies regulate major common and separate metabolic processes in the light and dark. They display both negative (C3) and positive (P3) control. The chromosomal copy C3 is involved in autotrophic growth in light (targets in carbon fixation, photosynthetic electron transport and energy metabolism). The plasmid copy P3 is involved in heterotrophic growth in the dark (targets in carbon breakdown, respiratory electron transport, and cell division). Both copies control certain shared targets in photosynthesis, glycolysis and shared transporters including metal transporters. P3 could be responsible for activating C3 and C3 may, in turn, repress P3.
Growth phase and glucose-dependent expression of hik31 genes

In order to test the model in Fig. 5, we analyzed the expression of the individual hik31 operons from the WT and mutants under the same physiological conditions. We first studied the expression for all 3 genes on both the plasmid and the chromosome in the WT in PA LL (Fig.6A), MT LL (Fig.6B) and MT LD (Fig.6C) conditions. In PA LL, the starter cells at t=0 showed both operon transcripts. For cells grown from days 1 to 6, we observed a distinct trend in expression of the individual operons. Both operons were expressed in the active growth phase of cells for 2 to 5 days corresponding to log and linear growth. However, P3 was down-regulated at 1 and 6 days which correspond to lag/early log and stationary phase, respectively. P3 was also transcribed less than C3 in a majority of the cases. Both operon copies were present in days 3 and 4, suggesting a high-demand that made one copy insufficient. Thus, the C3 operon represented the genes that were primarily expressed, with P3 expressed as a back-up under faster growth conditions.

Both operons were strongly induced in MT LL and their expression levels increased until 6 h and then reduced as cells adapted to the changed conditions. P3 was down-regulated at t=0, but increased gradually along with C3, and was maintained at one-third to two-thirds of the C3 levels. However, in MT LD, both operons were induced and remained high in the dark. The expression of hikC was nearly constant in all the points in Fig. 6, but hikP increased gradually in LL, reached a peak at 3 days and 6 hours and then declined. In LD, hikP remained high in the dark at both 2D and 6D (Fig.6C). This indicated that both operons...
were induced by glucose and that both P3 and hikP were induced by glucose in the dark. There were no changes in the re and hypo gene expression results in these experiments (data not shown). Both copies of these genes were expressed and the plasmid re and hypo were found to be of lower abundance, similar to what was found for P3. This could indicate a weaker plasmid operon promoter, or be due to structural differences in the plasmid like super-coiling or post-transcriptional changes from asRNA (32, 28).

Expression of both operon genes in various growth conditions

The experiments in Fig. 6 revealed the expression of the hik31 operon genes in the WT for both initial and acclimated growth conditions. We observed that 3 to 4 days of continuous growth in PA LL gave us strong expression of both operons. Hence, we decided to use WT and mutant cells grown for ~ 3 days to monitor changes in expression in different growth conditions, especially since the phenotypes recorded in Table 1 were also noted on or after 3 days. The salient findings from Fig. 7A (1-7 below) and 7B (8-11 below) are: 1. Both copies were co-transcribed as operons under all conditions (Fig. 7A) and C3 was always expressed more than P3 in the WT. 2. C3 was active in the light and P3 in the dark: C3 was up-regulated in PA LL and MT LL in the WT, whereas P3 was up-regulated in PA LD. 3. C3 may down-regulate P3 in glucose and P3 may up-regulate C3 in LL: ΔC3 had up-regulated expression of P3 in MT LL and MT LD compared to the WT, suggesting that C3 may normally down-regulate P3 in glucose conditions. C3 was down-regulated in ΔP3 in PA LL and MT LL, compared to the WT, suggesting that P3 may up-regulate C3 in these LL
conditions. This could mean that RreC represses P3 and RreP activates C3 in
the WT. 4. hikC may be important for the expression of P3: ΔhikC had reduced
expression of reP in PA LL and P3 in MT LL. Thus, hikC could be important for
the expression of P3 in MT LL and may have primacy over P3. 5. It is possible
that in the absence of hikC, reC still down-regulates P3/hikP, since ΔhikC and
ΔC3 are different in MTLL, with the P3 operon down-regulated in ΔhikC. hikP
was down-regulated, even when reP and hypoP were transcribed. 6. Regulatory
connection between hikP and hypoP: ΔhikP and ΔhikCP had reduced expression
of hypoP, suggesting a connection between both. 7. Induction in HL: In high light
growth conditions, expression of both operons was induced after 2 hours in all
the strains (Fig. 7B). 8. Effect of glucose and HL: Glucose and HL together
causd an up-regulation of C3 in all strains containing C3 (WT, ΔP3, ΔhikP),
whereas P3 only increased in the WT and was constant with or without glucose
in mutants containing P3 (ΔC3, ΔhikC). This may indicate the C3 is needed for
the induction of P3 in MT HL. 9. Role of C3 in growth of ΔhikP in HL: ΔhikP may
grow better in PA HL and MT HL after 3 days due to the constitutive expression
of C3 in all 3 time points. 10. Expression of both hiks: hikC was expressed in all
time points for both the WT and ΔP3 and constitutively expressed in ΔhikP,
whereas hikP levels varied at t=0 for ΔC3 and ΔhikC. Both hiks were up-
regulated in HL. 11. Expression of the rres and hypos: reP was down-regulated
only in ΔC3 at t=0, whereas reC and both hypos were expressed and
unchanged for all the mutants containing these genes (data not shown).

These data suggest that high light and glucose are sensed by the cell as
independent stimuli and had non-overlapping effects on transcription of these
genes. The expression results in Figs.6 and 7 demonstrate differential and
temporal regulation for both operons in response to the growth phase of the cell
as well as the growth condition. The full-length operon transcripts were down-
regulated sometimes even as the individual genes are expressed. The different
locations for these genes on the chromosome and plasmid could also lead to
structural and spatial regulation in the cell as shown for some other paralogous
genes for specificity control (24, 16). We also monitored the expression of these
genes after growth in high CO2 conditions for 2 days, but did not observe any
significant expression changes. Notably, both the operons were down-regulated,
suggesting that high CO2 may be a low-demand condition, as this is the only
condition tested where we find no operon transcripts for any of the strains.

Model of the regulatory relationship between C3 and P3

Fig. 8 is a working model to explain the relationship between both hik31 operons
and hypotheses as to the effect of the Hiks on the Rres under different growth
conditions. The combination of phenotypic characterization and transcription
allowed us to construct a working model for the interrelationships between the
two operons that incorporates positive and negative regulation, the role of both
operons in the light and the dark, and the effect of glucose, thereby extending the
scheme in Fig. 5. The direction of transcription (thin arrows) and the promoter
(thick arrows) are marked for both copies. The model suggests that HikP has
both negative effects in the light and positive effects in the dark on RreP, which in
turn, has positive effects on heterotrophic targets. RreP also acts to activate C3
in LL. HikC has a negative effect on RreC, which in turn, has negative effects on
its autotrophic targets. RreC may inhibit transcription of P3 in all conditions, as
P3 is always up-regulated in ΔC3. This model provides an explanation as to why
the plasmid operon mutant grows poorly (heterotrophic targets down-regulated),
but the plasmid hik mutant grows better (HikP cannot inhibit RreP, so targets are
up-regulated). Similarly, ΔC3 mostly grows well (RreC cannot down-regulate
autotrophic targets), but ΔhikC grows poorly in HC (RreC still inhibits targets).

We suggest that the RreC acts in negative regulation and the C3 promoter
is a high-level promoter expressing a low-demand product (41). In contrast, RreP
is involved in positive regulation and the P3 promoter is a low-level promoter
expressing a high-demand product. The RT-PCR experiments demonstrated that
both high light and glucose are high demand environments inducing the
expression of both operons (41). The numerous phenotypic defects displayed by
the double operon and hik mutants (Table 2) may be a result of both positive and
negative regulation as the cell attempts to fine-tune the levels of both copies.

There are also exceptions; e.g., cultures that grow poorly under many
conditions, but grow well in certain conditions or vice versa. This behavior could
be due to the effects of the other copy. For example, ΔP3 grows well in MT HL,
probably due to HikC down-regulating RreC so that autotrophic targets are
active. ΔhikC grows better in LL (HikP or some other protein inhibits RreC, so
that targets are de-repressed). ΔhikP grows extremely well in MT HL or high CO₂
with glucose and we suggest that this is due to both autotrophic (HikC down-
regulates RreC) and heterotrophic targets being active (RreP is not down-
regulated as HikP is missing). Interestingly, high CO2 with glucose did not affect
ΔC3, but MT HL did, suggesting that autotrophic targets are active in ΔC3 and
this caused photo-inhibition in MT HL.

We hypothesize that the Hypo for both copies activates the corresponding
Hik which may alter the effect on the Rre. Both Hiks have ATP-lids that closely
match the ATP-lid of the bifunctional protein EnvZ (2). This would make them
bifunctional as well, and both Hiks would be able to act as kinases and
phosphatases for their partner Rres. However, because the operons maybe a
3CS (e.g. the NRI/NRII system in *E.coli*), and due to the diversity of signal
responses seen, the Hiks would switch from being bifunctional to monofunctional
when needed (2). This would allow them to both transduce primary signals, like
light and dark, as well as to integrate secondary signals like high light and
glucose as needed. Also, like the EnvZ-OmpR system, there are weaker
ribosome binding sites (RBS) between the Rre and the Hik. This leads to lower
protein levels for EnvZ compared to OmpR (about 100 EnvZ to 3500 OmpR
molecules per cell of *E.coli*). The larger OmpR concentrations are considered
important for the dual role of activation and repression on the target porins (23).
The *hiks* have lower transcript levels compared to *rres* in our experiments,
suggesting similar behavior that could lead to lower translation of the Hiks and
degradation of the full length operon transcripts.

The transcriptional regulation for the well-studied EnvZ-OmpR system is
quite complex with four levels of control and 11 transcription factors that directly
or indirectly have been shown to regulate expression (24). It is possible that a
similar regulatory control exists in *Synechocystis* to maintain the two *hik31*
operon copies and appropriate gene dosages OmpR-P is thought to undergo a
conformational change that relieves self- inhibition of DNA binding. It is possible
that both Rres differ in their ability to relieve self- inhibition and bind DNA on
phosphorylation due to the sequence changes between both receiver and output
domains. OmpR can both activate and repress its target porins based on its
phosphorylation level and 2 molecules of OmpR-P have been shown to bind to
as many as 7 different promoter sites in a hierarchical manner (43). RreC and
RreP also have differences in their output domain that may enable them to bind
to adjacent regions of the promoters of their common target genes, as well as
different regions for different targets in a hierarchical manner. The model we
have presented in Fig. 8 is a simple one, serving to provide a hypothesis to
explain a more complex and unique system combining many previously classified
mechanisms of gene fixation after duplication. Nonetheless, it is evident that the
right balance between C3 and P3 needs to be maintained in the cell for proper
regulation of central metabolic processes.

ACKNOWLEDGEMENTS

We would like to acknowledge the help of Dr. Tina Summerfield (now at
University of Otago, Dunedin, NZ) throughout the initial stages of this project.
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DOE-BER (DE-FC02-07ER64694).
TABLE 1. Growth properties of the WT, operon and hik mutants under different trophic conditions of light, dark and carbon.

<table>
<thead>
<tr>
<th>Strain</th>
<th>PA</th>
<th>MT</th>
<th>HT</th>
<th>HC</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>24L/0D</td>
<td>12L/12D</td>
<td>6L/18D</td>
<td>24L/0D</td>
</tr>
<tr>
<td>WT</td>
<td>24L/0D</td>
<td>12L/12D</td>
<td>6L/18D</td>
<td>24L/0D</td>
</tr>
<tr>
<td>ΔC3</td>
<td>24L/0D</td>
<td>12L/12D</td>
<td>6L/18D</td>
<td>24L/0D</td>
</tr>
<tr>
<td>ΔP3</td>
<td>24L/0D</td>
<td>12L/12D</td>
<td>6L/18D</td>
<td>24L/0D</td>
</tr>
<tr>
<td>ΔC3P3</td>
<td>24L/0D</td>
<td>12L/12D</td>
<td>6L/18D</td>
<td>24L/0D</td>
</tr>
<tr>
<td>ΔhikC</td>
<td>24L/0D</td>
<td>12L/12D</td>
<td>6L/18D</td>
<td>24L/0D</td>
</tr>
<tr>
<td>ΔhikP</td>
<td>24L/0D</td>
<td>12L/12D</td>
<td>6L/18D</td>
<td>24L/0D</td>
</tr>
<tr>
<td>ΔhikCP</td>
<td>24L/0D</td>
<td>12L/12D</td>
<td>6L/18D</td>
<td>24L/0D</td>
</tr>
</tbody>
</table>

Doubling times for all strains are means ± standard errors of cell counts in a Petroff-Hauser counter for n ≥ 30. µE m⁻² s⁻¹ light was used for all conditions except high light and high CO₂.

* indicates that α=0.05, Tukey’s comparison for each column separately using WT as standard.

5 mM glucose was added.

3% CO₂ and 50 µE m⁻² s⁻¹ light were used for HC (High CO₂) growth.

Bold font indicates a defect in pigment content.
TABLE 2. Growth defects of the mutants compared to the WT

<table>
<thead>
<tr>
<th>Strain</th>
<th>Growth defects in liquid</th>
<th>Cell division defects</th>
<th>Metal sensitivity</th>
</tr>
</thead>
<tbody>
<tr>
<td>ΔC3MTLL, MT HL</td>
<td>Yes</td>
<td>NiCl2, ZnCl2, CdCl2</td>
<td></td>
</tr>
<tr>
<td>ΔP3PA LD, PA HL, MT LD, HT DD, Low O2</td>
<td>Yes</td>
<td>CoCl2</td>
<td></td>
</tr>
<tr>
<td>ΔC3P3PA LL, PA LD, PA HL, MTLL, MTLD, MTHL, HT DD, Low O2</td>
<td>Yes</td>
<td>ZnCl2, CdCl2</td>
<td></td>
</tr>
<tr>
<td>ΔhikCMT HC</td>
<td>No</td>
<td>ZnCl2, CdCl2</td>
<td></td>
</tr>
<tr>
<td>ΔhikPMT HC</td>
<td>No</td>
<td>NiCl2</td>
<td></td>
</tr>
<tr>
<td>ΔhikCPA LD, MT LL, MT LD, MT HL, PA HC, MT HC</td>
<td>No</td>
<td>NiCl2, ZnCl2, CdCl2</td>
<td></td>
</tr>
</tbody>
</table>

a Based on statistically significant differences for liquid growth (Table 1)
b Via light and electron microscopy (Figs. 2-4)
c Growth on solid plates (Supplemental Fig. 2)
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FIGURE 1. Construction of the operon and hik mutants and separation of transcripts for both operons

1A. Diagrammatic representation of the construction of hik31 mutants. We constructed deletion mutants that lacked all three genes in the operon, on either the chromosome (ΔC3) or the plasmid (ΔP3), and on both the chromosome and the plasmid (ΔC3P3). The dotted portions were deleted and replaced with various antibiotic cassettes- Spectinomycin (Sp'), Kanamycin (Kn') and Chloramphenicol (Cl'). Similarly, we also made deletion mutants that lack hik31 alone on the chromosome (ΔhikC), on the plasmid (ΔhikP) and on both the chromosome and the plasmid (ΔhikCP). The figure describes the portions of the genes that were replaced (dotted clear area), deleted extra (+) and left behind (-) as well as the restriction enzymes used. The promoter and the direction of transcription are indicated for each construct.

1B. Gels showing PCR confirmation of the mutations and complete segregation. Separate primers were used to selectively amplify the chromosomal and plasmid copies. Sizes of the bands are indicated in the figure for the WT and mutants.

1C. PCR primer and restriction digest design to differentiate the expression of both operons. The restriction enzymes KpnI and MlyI cut at unique sites on the plasmid rre and hypo respectively.

FIGURE 2. TEM under photoautotrophic conditions. Transmission electron micrographs of Synechocystis sp. PCC 6803 WT (A, D and G), ΔC3P3 (B, E and
H) and ΔP3 (C, F and I) grown under photoautotrophic (PA) conditions under continuous light (LL; A, B and C), 12 hours light/12 hours dark (LD; D, E and F), or under high light (HL; G, H and I). Details are described in Materials and Methods. PM, photosynthetic membranes; S, septum. Magnification, 28,500 to 29,400X.

FIGURE 3. TEM under mixotrophic and heterotrophic conditions. Transmission electron micrographs of Synechocystis sp. PCC 6803 WT (A, D and G) and ΔP3 (B, C, E, F, H and I) grown under mixotrophic (MT) conditions with 5 mM glucose under continuous light (LL; A, B and C), 12 hours light/12 hours dark (LD; D, E and F) and under heterotrophic (HT) conditions in continuous dark (DD; G, H and I). CG, cyanophycin granules; PHB, polyhydroxybutyrate granules; S, septum. Magnification, 20,000 to 21,000X.

FIGURE 4. TEM under mixotrophic high light conditions. Transmission electron micrographs of Synechocystis sp. PCC 6803 WT (A) and ΔC3 (B and C) in mixotrophic (MT) conditions under high light (HL). Magnification, A, 18,600X; B and C, 12,000X.

FIGURE 5. Regulatory functions of the chromosomal and plasmid hik31 operons. Regulatory relationship of the hik31 operons as shown by a Venn diagram representing the different and overlapping functions that are regulated by the hik31 operon on the chromosome (C3) and the plasmid (P3). The operons are
represented as bars on the chromosome and plasmid. Both operons regulate
major metabolic processes in the light and the dark and show both positive (P3 to
C3) and negative control (C3 to P3) to their targets and to each other.

FIGURE 6. Transcription of the operon and hik genes of both hik31 operons in
the WT in various growth conditions.

A. Expression of the hik31 operon genes in different stages of growth. WT cells
were grown in PALL conditions for 6 days. RNA was extracted just before (t=0)
and after each day (1 to 6) of growth for RT-PCR.

B. Expression of the hik31 operon genes after addition of glucose in continuous
light. WT cells were grown for about a day (30 hours) in PALL conditions and
then 5 mM glucose was added and the labeled time points (2, 6, 18, 24 and 48
hours) were analyzed in continuous light. Dotted lines above sample times
indicate those taken after the addition of glucose. The 30 hour time point
represents RNA from cells just before the addition of glucose (t=0).

C. Expression of the hik31 operon genes after addition of glucose in light-dark
conditions. WT cells were grown for about a day (30 hours) in PALL conditions
and 5 mM glucose was added and the labeled time points (2L, 6L, 2D and 6D)
were analyzed in 12 hours light, 12 hours dark. Dotted lines above sample times
indicate those taken after the addition of glucose. The 30 hour time point
represents RNA from cells just before the addition of glucose.
FIGURE 7. Transcription of the *hik* and *rre* genes of both *hik31* operons in the WT and mutants in various growth conditions.

A. Expression of *hik31* operon genes in the WT, operon and hik mutants. Cells were grown for about 3 days in 1-PA LL, 2-PA LD, 3-MT LL and 4-MT LD conditions. Photoautotrophic (PA), Mixotrophic (MT), 24 hours light (LL) and 12 hours light/12 hours dark (LD) conditions.

B. Expression of the operon and *hik* genes in all strains after growth in high light conditions. Cells were grown for 1 day in PALL, and then exposed to high light of 150 µE m⁻² s⁻¹ for 2 hours in the presence (MT) and absence (PA) of 5 mM glucose. The 0 time point was taken after growth for about 1 day (26 hours) in PALL conditions to serve as the control. The RnpB and -RTase controls were similar to the experiments in Fig. 7A (data not shown).

FIGURE 8. Working model of the regulatory relationship between both *hik31* operons. The direction of transcription, expression conditions and promoters are indicated for each operon, along with the effect of the Hiks on the Rres, and in turn, the Rres on the targets. The plasmid operon promoter is shown smaller to represent the lower expression results.
Chromosomal *hik31* operon

Plasmid *hik31* operon

- **C3**
  - *hik*  *rre*  *hypo*

- **P3**
  - *hypo*  *rre*  *hik*

- **ΔC3**
  - *hik*  *rre*  *hypo*

- **ΔP3**
  - *hypo*  *rre*  *hik*

- **ΔhikC**
  - *hik*  *rre*  *hypo*

- **ΔhikP**
  - *hypo*  *rre*  *hik*

- **WT**
  - *hypo*  *rre*  *hik*

- **ΔP3**
  - *hypo*  *rre*  *hik*

- **ΔC3P3**
  - *hypo*  *rre*  *hik*

- **Δhik**
  - *hypo*  *rre*  *hik*

- **ΔhikP**
  - *hypo*  *rre*  *hik*

- **ΔhikCP**
  - *hypo*  *rre*  *hik*

---

**deleted 2947/2658 bases**

**deleted 2605/2634 bases**

**deleted 1158/1376 bases**

**deleted 961/1352 bases**

---

**plasmid primer**

**chromosomal primer**

---

**KpnI**

- **WT**  **ΔP3**  **ΔC3P3**  **Δhik**  **ΔhikP**  **ΔhikCP**

- **WT**  **ΔC3**  **ΔC3P3**  **ΔhikC**  **ΔhikCP**

---

**2464 bp C3/P3 operon**

**567 bp hik**

---

**511 bp rre**

---

**454 bp hypo**