Gene expression under low-oxygen conditions in the cyanobacterium *Synechocystis* sp. PCC 6803 demonstrates Hik31-dependent and -independent responses

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We have investigated the response of the cyanobacterium *Synechocystis* sp. PCC 6803 during growth at very low O$_2$ concentration (bubbled with 99.9 % N$_2$/0.1 % CO$_2$). Significant transcriptional changes upon low-O$_2$ incubation included upregulation of a cluster of genes that contained *psbA1* and an operon that includes a gene encoding the two-component regulatory histidine kinase, Hik31. This regulatory cluster is of particular interest, since there are virtually identical copies on both the chromosome and plasmid pSYSX. We used a knockout mutant lacking the chromosomal copy of *hik31* and studied differential transcription during the aerobic–low-O$_2$ transition in this ΔHik31 strain and the wild-type. We observed two distinct responses to this transition, one Hik31 dependent, the other Hik31 independent. The Hik31-independent responses included the *psbA1* induction and genes involved in chlorophyll biosynthesis. In addition, there were changes in a number of genes that may be involved in assembling or stabilizing photosystem (PS)II, and the *hox* operon and the LexA-like protein (Sll1626) were upregulated during low-O$_2$ growth. This family of responses mostly focused on PSII and overall redox control. There was also a large set of genes that responded differently in the absence of the chromosomal Hik31. In the vast majority of these cases, Hik31 functioned as a repressor and transcription was enhanced when Hik31 was deleted. Genes in this category encoded both core and peripheral proteins for PSI and PSII, the main phycobilisome proteins, chaperones, the ATP synthase cluster and virtually all of the ribosomal proteins. These findings, coupled with the fact that ΔHik31 grew better than the wild-type under low-O$_2$ conditions, suggested that Hik31 helps to regulate growth and overall cellular homeostasis. We detected changes in the transcription of other regulatory genes that may compensate for the loss of Hik31. We conclude that Hik31 regulates an important series of genes that relate to energy production and growth and that help to determine how *Synechocystis* responds to changes in O$_2$ conditions.

INTRODUCTION

Cyanobacteria are a diverse and ancient phylum of phototrophic prokaryotes that thrive in terrestrial, marine and freshwater habitats (Rippka *et al.*, 1979). Their ability to adapt to fluctuating environments has contributed to their widespread distribution. Mechanisms involved in sensing and responding to many environmental parameters have been determined through DNA microarray experiments investigating genome-wide expression patterns. Transcriptional responses to numerous stimuli have been well characterized in the freshwater cyanobacterium *Synechocystis* sp. PCC 6803 (hereafter *Synechocystis*); stimuli included changes in light conditions, pH, nutrient availability, salt stress and temperature stress (Gill *et al.*, 2002; Hihara *et al.*, 2001; Huang *et al.*, 2002; Singh *et al.*, 2003, 2006; Summerfield & Sherman, 2008; Wang *et al.*, 2004a, b). In addition, we have examined the response to low-oxygen conditions in *Synechocystis* (Summerfield *et al.*, 2008). Cyanobacteria experience low oxygen concentrations in habitats such as hot springs, soils (e.g. rice paddies or estuarine mud) and eutrophic lakes (Stewart & Pearson, 1970). Where the rate of photosynthesis is low and/or oxygen consumption is high, low oxygen levels may occur intracellularly (Paerl...
et al., 2000). Key questions are: how did protocyanobacteria function in a primarily anoxygenic environment, and how did their metabolism change as they began adding oxygen into the atmosphere? An understanding of this anaerobic to aerobic transition may be important for understanding the development and regulation of photosynthesis.

In *Synechocystis*, the transition to low-oxygen conditions resulted in widespread changes in transcript abundance. This included increased mRNA levels of genes encoding the histidine kinase, Hik31 (Summerfield et al., 2008). A histidine kinase is one of two proteins comprising a two-component regulatory system that senses and responds to environmental fluctuations. For a typical two-component system, a specific environmental change first activates the histidine kinase; in turn, this kinase phosphorylates its cognate response regulator resulting in a specific output that frequently involves altered transcript abundance of a specific group of genes (Stock et al., 2000). The *Synechocystis* genome contains 47 genes that encode putative histidine kinases, and 45 genes that encode putative response regulators (Los et al., 2008). In *Synechocystis*, characterization of gene-interruption mutants has identified histidine kinases involved in sensing environmental stimuli, including high light, cold and oxidative stress (Hik33), heat (Hik34), salt stress and hyperosmotic stress (Hik33, Hik34, Hik2, Hik41, Hik16 and Hik10), and phosphate limitation (Hik7) (Hirani et al., 2001; Hsiao et al., 2004; Kanesaki et al., 2007; Mikami et al., 2002; Paithoonrangsarid et al., 2004; Shoumskaya et al., 2005; Suzuki et al., 2005). These data demonstrate that one histidine kinase may respond to more than one type of stimulus and that multiple histidine kinases may respond to any specific environmental cue.

The histidine kinase Hik31 (sll0790) is the only chromosomally encoded histidine kinase in *Synechocystis* to have a plasmid-encoded homologue (Str6041). These proteins share 95.6 % sequence identity, with the first 420 residues being identical, and they are predicted to contain three transmembrane helices supporting the idea that these are membrane-bound sensors. However, the signalling domains have yet to be identified (Ashby & Houmard, 2006). The *hik31* gene appears to be part of a duplicated three gene cluster encoding a hypothetical protein, a response regulator and a histidine kinase [chromosomal genes sll0788–sll0790 and plasmid (pSYSX) genes slr6039–slr6041]. Both of the gene clusters are located next to genes encoding probable cation efflux proteins and it has been suggested that these two-component systems may have a role in mediating cation transport (Kaneko et al., 2003).

In addition, Hik31 is involved in regulating glucose metabolism. A strain lacking both the chromosomal (sll0790) and plasmid (slr6041) *hik31* homologues was unable to grow in the presence of glucose (Kahlon et al., 2006). Data indicated that this mutant was able to take up glucose, but lacked glucokinase activity to convert glucose into glucose 6-phosphate, so that glucose could not be metabolized. In contrast, elevated glucose-6-phosphate dehydrogenase activity indicated that the mutant was able to convert glucose 6-phosphate to gluco-1,5-lactone phosphate. This led the authors to suggest that Hik31 is involved in both positive and negative regulation of functions involved in glucose metabolism that enable switching between photoautotrophic and mixotrophic growth.

Upregulation of *hik31* under low-oxygen conditions indicates that Hik31 has an additional role in the transition between aerobic and low-oxygen growth. We report on the growth of the wild-type and *hik31* mutants under low-O_2_ conditions and the way in which gene expression is altered by such growth conditions. We demonstrate that in the absence of Hik31, strains exhibited increased growth compared with the wild-type under low-oxygen conditions and that Hik31 is involved in negative regulation of genes involved in growth in low-oxygen conditions.

**METHODS**

**Cyanobacterial strains and growth conditions.** The glucose tolerant strain, *Synechocystis* sp. strain PCC 6803 (Williams, 1988) was grown at 30 ± 2 °C. Cells were grown in blue green algae medium BG11 with 25 mM HEPES pH 7.5, in an airlift bioreactor (BioFlo 3000, 6 l) or in 750 ml Cytolitivs (Kontes). The ΔHik31 strain in which the sll0790 gene was interrupted with a spectomycin resistance cassette at the Ncol site was provided by Julian Eaton-Rye, University of Otago. Thus, 25 µg spectomycin ml⁻¹ was added to the medium during the growth of this mutant. In addition, deletion mutants were constructed in the chromosomal copy of *hik31* (Δ*hik31c, Cm''), in the plasmid copy (Δ*hik31p, Sp'') and in both copies (Δ*hikcP*) (S. Nagarajan and L. A. Sherman, unpublished observations). The culture was illuminated by two panels using alternating arrays of orange (640 nm) and blue (430 nm) light-emitting diodes, yielding an intensity of ~100 µmol photons m⁻²s⁻¹ inside the bioreactor or Cytolit (underwater quantum light meter, LI 192, Li-Cor). For microarray experiments, cultures were grown in the bioreactor to a cell density of ~ 5 × 10⁶ cells ml⁻¹ and then bubbled with 99.9 % nitrogen/0.1 % carbon dioxide to achieve low-oxygen conditions, as described by Summerfield et al. (2008).

**Whole cell spectra.** Spectra were collected on a Perkin-Elmer spectrophotometer (Lambda 40). To correct for scattering, Scotch tape was placed on the front and back of the reference and sample cuvette holders. Spectra were normalized to the peak at 682 nm.

**RNA extraction.** Total RNA was extracted and purified using phenol/chloroform extraction and CsCl gradient purification as described previously (Reddy et al., 1990; Singh & Sherman, 2002).

**Semi-quantitative RT-PCR.** DNase I treatment and reverse transcription were performed as described by Summerfield et al. (2008). PCR was carried out at 94 °C 1 min, 20–30 cycles of 94 °C 30 s, 54 °C 30 s and 68 °C 30–120 s (depending on amplicon size), to amplify regions of the genes listed below. Primers (F 5’-gatgccatgc- caatgga and R 5’-gggtagtcatacttgg) were used to amplify the three-gene cluster containing *hik31*. These primers were a 100 % match to the chromosomal and plasmid copies of these genes with the forward primer binding at 116–133 bp of sll0788/slr6039 and the reverse primer binding at 698–681 bp of sll0790/slr6041. This enabled amplification of both the chromosomal and plasmid clusters, resulting in product of 1865 bp. This PCR product was digested with Nhel that has a restriction site at nt 144 in the plasmid response regulator gene *slr6040*, but not in its chromosomal homologue.
Semi-quantitative RT-PCR was used to validate the microarray data. The genes amplified, the primers used and PCR product size and number of PCR cycles were: sll1875 (F 5'-ggcttatgccggtct, R 5'-ttcacacactattgag, 367 bp, 25 cycles), sh1181 (F 5'-ggctatcagaaaccagc, R 5'-aggggcacattgg, 1040 bp, 30 cycles) sh1311 (F 5'-cgctgcgtatcctagtcgc, R 5'-tgtcacagggaatctgagg, 450 bp, 20 cycles), sll1514 (F 5'-gtaatctcagacagcag, R 5'-gtcaagatgtagacagc, 350 bp, 25 cycles), srr2595 (F 5'-agtgtctgcctgcacaca, R 5'-agaggacaaacacccac, 195 bp, 25 cycles), sll1577 (F 5'-tcctcaagcttgctgc, R 5'-agactgtcagacacagc, 439 bp, 20 cycles) and mpRB (F 5'-ttgctacagagacttgtagc, R 5'-agagagagcaaccaaccac, 405 bp, 30 cycles). The mpRB gene was included as this transcript is frequently used as a constitutively expressed control for gene expression. Amplification of chromosomal hik31 (sll0790) was performed (primers: F 5'-gtggtataactctggc and R 5'-ggtttgtgc); the forward primer differed from the plasmid hik31 sequence at three nucleotide positions. This was sufficient to enable PCR of only the chromosomal copy and resulted in a PCR product of 567 bp.

**Microarray design.** The microarray platform and construction was as described by Postier et al. (2003) and the cDNA labelling, prehybridization and hybridization protocols are described in detail in Singh et al. (2003). The microarray experiment involved a loop design that compared the wild type and the ΔHik31 strain under aerobic conditions and 1 h following a transition to low oxygen by using an ANOVA model (Li et al., 2004; Singh et al., 2003). The microarray was deposited in the NCBI Gene Expression Omnibus Series under accession number GSE24882.

Data acquisition included an ANOVA model approach to test the null hypothesis that a particular gene's expression level did not differ between the treatments and to calculate a P-value (Singh et al., 2003). This experiment contained two genotypes (wild-type and the ΔHik31 strain) and two stimuli (aerobic growth and transition to low-oxygen conditions) for a total of four treatment combinations. The effects of interrupting Hik31 and the transition from aerobic to low-oxygen conditions were examined in an ANOVA essentially as described by Kerr & Churchill (2001a, b), Li et al. (2004) and Singh et al. (2003). We used the false discovery rate (FDR) of 5% to control the proportion of significant results that are type I errors (false rejection of the null hypothesis) as described by Summerfield & Sherman (2007). Genes with an FDR=0.05 (corresponding to 5% expected false positives) and that exhibited a change of at least 1.5-fold were considered interesting and retained for further analysis. The P-value of these genes ranged from 3.2 × 10^-2 to 9.6 × 10^-15.

**RESULTS AND DISCUSSION**

**Both the chromosomal and plasmid hik31 copies are in operons upregulated under low-oxygen conditions**

The chromosomal and plasmid hik31 genes (sll0790 and slr6041, respectively) are part of homologous three gene clusters. The other two genes in each of these clusters encode response regulators sll0789 and slr6040 and hypothetical proteins sll0788 and slr6039. The homologous gene clusters encode proteins that differ by 19/458, 10/232 and 2/196 amino acids, for the Hik31, Rre34 and hypothetical protein, respectively. Using RT-PCR with a forward primer to the hypothetical genes sll0788 and slr6039 and a reverse primer to hik31 (both sll0790 and slr6041), we demonstrated that this three gene cluster is co-transcribed and that this operon is upregulated under low-oxygen conditions (Fig. 1a). DNA microarray data indicated that the hypothetical gene was upregulated only 1.3-fold on transfer to low-oxygen conditions, but this is likely to be an underestimate as the abundance of this transcript is at the top of the dynamic range for our detection system. The high level of nucleotide sequence identity between the chromosomal and plasmid genes meant they could not be distinguished in our microarray experiment or by using RT-PCR.

To determine whether the RT-PCR products were derived from the chromosomal and/or plasmid transcripts, we utilized an NheI restriction site that was present in the gene encoding the plasmid response regulator, but was not present in the gene encoding the chromosomal response regulator. Digestion with NheI would result in cleavage of the RT-PCR products derived from the plasmid gene, but not the RT-PCR products from the chromosomal gene. The NheI digestion of RT-PCR products from the wild-type resulted in cleavage of the plasmid-derived RT-PCR product to give a PCR product of ~1.51 kb (and a ~0.35 kb fragment that is not shown in Fig. 1a), and an uncut ~1.86 kb PCR product corresponding to the RT-PCR product from the chromosomal genes. These data indicated both the plasmid and chromosomal copies of the genes are expressed and are upregulated under low-oxygen conditions (Fig. 1a).

Kahlon et al. (2006) used a strain lacking both the plasmid and chromosomal hik31 to examine the role of this histidine kinase in glucose metabolism. Their data indicated that Hik31 is involved in switching between photoautotrophic and mixotrophic growth by acting as both a positive and a negative regulator of functions involved in glucose metabolism. However, they reported that investigating the role of Hik31 using a mutant lacking both the plasmid and chromosomal copies presented complications, including serious difficulty with the stability of phenotypes. We first examined a strain (ΔHik31) lacking only the chromosomal copy of hik31, sll0790. As expected, only the plasmid copy of the gene was detected in the ΔHik31 strain. However, expression of this copy was altered in the mutant compared with the wild-type, as levels of the plasmid transcripts were reduced in the mutant at 1 h compared with the wild-type, but increased by 2 h (Fig. 1a). These data indicated that chromosomally encoded Hik31 regulates plasmid hik31 expression.

**Growth and whole-cell spectra of the wild-type and ΔHik31 strains**

We constructed strains lacking each of the hik31 genes and also a double deletion mutant lacking both the chromosomal and plasmid hik31 genes. These strains have been tested for growth under diverse photoautotrophic and
mixotrophic conditions and these results will be described separately (S. Nagarajan and L. A. Sherman, unpublished results). In addition, each strain was grown under identical conditions in the bioreactor (bubbled with 99.9 % nitrogen/0.1 % carbon dioxide) and cultures were sampled at regular intervals for cell number and absorption spectra for 2 days prior to the low oxygen shift and 2 days thereafter (Table 1). These data indicated that strains lacking the chromosomal \( hik31 \) (\( D_{hik31c} \)), the plasmid \( hik31 \) (\( D_{hik31p} \)) and both \( hik31 \) copies (\( D_{hikcp} \)) all grew better than the wild-type under low-O2 conditions. This type of experiment has been repeated over 12 times and, in each case, the \( hik \) mutant grew as well as, or better than, the wild-type. The \( D_{hik31c} \) and \( D_{hikcp} \) strains always grew better than the wild-type. We concluded that the original \( D_{Hik31} \) mutation was representative of the expression changes that would occur during the transition from aerobic to low-oxygen conditions and that the double mutant would only lead to further quantitative, but not qualitative, differences in gene expression. Therefore, we used the chromosomal \( D_{Hik31} \) strain to investigate the role of \( Hik31 \) in the response to low-oxygen conditions. In addition, use of the \( D_{Hik31} \) strain avoided any potential problems of phenotype stability of the double mutant that have previously been reported (Kahlon et al., 2006), the stable phenotype of the \( D_{Hik31} \) strain under low oxygen conditions is shown in Fig. 1(b).

The wild-type and \( \Delta Hik31 \) strain had similar doubling times under aerobic conditions in flasks (13 ± 1 h), consistent with the growth of the \( Hik31 \) mutant reported by Kahlon et al. (2006). Generation times were typically slower at low cell density in the 6 l bioreactor and aerobic doubling times were 20–30 h for all of the strains (Table 1). The mutants all grew faster than the wild-type under low-O2 conditions with doubling times up to 50 % faster (Table 1). Cultures of the wild-type grown in a 750 ml Cytolift for 30 h under low-oxygen conditions showed a decreased growth rate (19 h) compared with the \( \Delta Hik31 \) strain (12 h) (Fig. 1b). The whole-cell spectra of the two strains differed in cells grown under aerobic conditions, as the peak with absorption maxima at 630 nm, originating from the phycobilins, was higher in the wild-type (Fig. 1c and d). Incubation under low-oxygen conditions altered the whole-cell spectra of both strains. The wild-type had a decreased 440 nm chlorophyll \( a \) peak relative to the 680 nm peak (Fig. 1c), whereas the \( \Delta Hik31 \) strain showed an increased phycobilin peak relative to the chlorophyll \( a \) peaks (Fig. 1d).

### Differential transcript abundance in the wild-type following transition to low-oxygen conditions

Based on the growth data, \( \Delta Hik31 \) was used to investigate the role of \( Hik31 \) in modulating gene expression under
Table 1. Growth of *Synechocystis* sp. PCC 6803 wild-type and hik31 mutants with air bubbling for 48 h (aerobic) followed by 99.9 % N₂ and 0.1 % CO₂ (low O₂) for a subsequent 48 h

Cultures were grown in a 6 l bioreactor and cells were counted in a hemocytometer. The data presented are for individual experiments representative of three or more experiments for each strain.

<table>
<thead>
<tr>
<th>Strain</th>
<th>10⁻⁷ × Cells ml⁻¹</th>
<th>Low O₂ growth mean fold increase*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0 h</td>
<td>48 h, air</td>
</tr>
<tr>
<td>Wild-type</td>
<td>1.2</td>
<td>3.5</td>
</tr>
<tr>
<td>Δhikc†</td>
<td>0.9</td>
<td>3.8</td>
</tr>
<tr>
<td>Δhikp‡</td>
<td>0.9</td>
<td>4.3</td>
</tr>
<tr>
<td>Δhikc§</td>
<td>1.5</td>
<td>4.8</td>
</tr>
</tbody>
</table>

*Mean growth increase for three or more experiments for each strain under low O₂, low growth increase was reproducible within ± 15 % of the mean for each strain.
†Strain lacking chromosomal hik31.
‡Strain lacking chromosomal genes for the wild-type and ΔHik31 strain, respectively. These differentially expressed genes were divided into functional categories, according to the Cyanobase designation (http://genome.kazusa.or.jp/cyanobase/) and the number of these genes in each functional category (excluding hypothetical, other or unknown genes) is shown in Fig. 2, all gene categories are shown in Supplementary Fig. S1 (available with the online version of this paper).

In the wild-type, a similar number of genes were upregulated and downregulated following transition to low oxygen. There were 211 downregulated genes, more than a third of these belonged to either the hypothetical, other or unknown gene categories (81 genes). Additional functional categories containing large numbers of downregulated genes included translation (36 genes), photosynthesis and respiration (20 genes), regulatory functions (15 genes) and energy metabolism (14 genes) (Fig. 2). A total of 209 genes were upregulated in the wild-type under low-oxygen conditions, half of which were designated hypothetical, other or unknown genes (115 genes) (data not shown). Additional genes with increased mRNA levels under low-oxygen conditions included those encoding transport and binding proteins (28 genes), regulatory proteins (16 genes) and energy metabolism proteins (11 genes).

A major component of the transcriptional response to low oxygen was the decreased transcript levels for many genes essential for growth; e.g. genes encoding ribosomal proteins, ATP synthase and phycobilisome components. Decreased mRNA levels of these genes were observed in conditions associated with decreased growth; e.g. during the linear growth phase, after the transition from light to dark, under sulfur deprivation and during acclimation to decreased inorganic carbon (Foster *et al.*, 2007; Summerfield & Sherman, 2007; Wang *et al.*, 2004a; Zhang *et al.*, 2008). Response to low-oxygen conditions involved the upregulation of genes that encoded proteins involved in sensing and responding to environmental change and maintaining cellular homeostasis, including some stress-responsive proteins, regulatory proteins and transport and binding proteins.

A comparison with previous microarray data generated similar results (Supplementary Fig. S1) and especially included all of the downregulated genes with functions in translation, and photosynthesis and respiration. In addition, semiquantitative RT-PCR was used to validate a subset of microarray data and we observed good agreement between these two techniques (see Supplementary Fig. S2, available with the online version of this paper).

The absence of Hik31 alters transcriptional response to low oxygen conditions

We compared the transcriptional response of the wild-type and ΔHik31 strain to the transition from aerobic to low-oxygen conditions (see Tables 2 and 3 and Fig. 2). Following this transition, only 32 genes were downregulated in ΔHik31 compared with >200 genes downregulated in the wild-type. The two strains had similar numbers of genes with increased mRNA levels under low-oxygen conditions (209 and 230 genes in the wild-type and ΔHik31 strain, respectively), approximately half these genes were common to both strains.
The major difference in the transcriptional response of the two strains to low-oxygen conditions involved genes associated with growth that were downregulated in the wild-type and/or upregulated in the ΔHik31 strain. This included genes categorized as cellular processes, energy metabolism, photosynthesis and respiration, regulatory functions, and translation (Fig. 2), as well as 69 genes belonging to hypothetical, other and unknown categories that were downregulated in the wild-type, but not in ΔHik31 (Supplementary Fig. S1). These differences in gene expression may explain the enhanced growth of the ΔHik31 strain compared with the wild-type under low-oxygen conditions. Genes involved in the response to low-oxygen conditions were divided into two categories: those that responded similarly in the two strains and those that responded differently in the two strains. Representatives of both of these categories are discussed in more detail below.

**Genes showing similar expression patterns in the wild-type and the ΔHik31 strain under low-oxygen conditions (Hik31-independent changes)**

Transcriptional responses shared by both strains involved adaptation to low oxygen conditions that included unique changes to the photosynthetic electron transport chain. This included a low-oxygen-induced gene cluster containing a divergent copy of the photosystem II (PSII) psbA gene (psbA1) and an alternative copy of the gene encoding the Rieske iron–sulfur protein of the cytochrome b$_6$f complex (petC2) that we previously reported to be upregulated in the wild-type (Summerfield et al., 2008). Other genes involved in photosynthetic electron transport were upregulated e.g. ctpA (slr0008), a gene that encodes a protease that processes the D1 protein into its mature form during turnover of PSII (Anbudurai et al., 1994; Janssen et al., 2003) and ctpB (slr0257) encoding a related carboxyl-terminal endoprotease thought to have a minor role in processing D1 (Komenda et al., 2007). Transcripts of both of these proteases are elevated under stress conditions (e.g. high-light and high-salt) where PSII damage is increased (Janssen et al., 2003; Komenda et al., 2007). The gene encoding ClpB1 (Slr1641) was upregulated, this molecular chaperone has a role in acquisition of thermotolerance in the cyanobacterium *Synechococcus* sp. strain PCC 7942 (Eriksson & Clarke, 1996).

In addition, genes sll0217–sll0220 (including two genes encoding A-type flavoproteins, sll0217, flv4 and sll0219, flv2) and a third flavoprotein (slr0550, flv3) were upregulated in both strains. The sequences of these proteins are indicative of functions in electron transfer (Helman et al., 2003). The proteins Flv2 and Flv4 are thought to protect PSII from photoinhibition (Zhang et al., 2009) and our results may suggest that these proteins have a greater role in PSII assembly. In contrast, Flv3 appears to be involved in the flux of electrons from photosystem I (PSI) to O$_2$ in the Mehler reaction (Helman et al., 2003). Genes downregulated under low-oxygen conditions included str0074–0077, genes homologous to the *suf*BCDS operon of *Synechococcus* sp. PCC 7002, and the *suf* operon of *Escherichia coli* that functions in iron–sulfur cluster biogenesis (Takahashi & Tokumoto, 2002; Wang et al., 2004b) (Table 2). In *Synechococcus* sp. PCC 7002, this operon is believed to have a role in PSI biogenesis (Wang et al., 2004b).

Low-oxygen conditions altered the transcript levels of genes involved in chlorophyll synthesis. In both strains, we observed a large increase in transcript abundance for ho2 (sll1875) and hemN1 (sll1876), genes that encode an oxygen-independent haem oxygenase (HO) and coproporphyrinogen III oxidase (CPO), respectively. Upregulation of these genes is thought to enable maintenance of haem
Table 2. Selected genes showing similar regulation in *Synechocystis* sp. strain PCC 6803 wild-type and a ΔHik31 strain on transition from aerobic to low-oxygen condition

Genes (grouped in the table) were considered differentially regulated when FDR = 0.05, fold change > 1.5 (bold type). The fold changes were calculated by dividing the normalized mean intensities obtained for: the wild-type under low-oxygen conditions by those for the wild-type under aerobic conditions (WT); the ΔHik31 strain under low-oxygen conditions by those for the ΔHik31 strain under aerobic conditions (ΔHik31); the wild-type under aerobic conditions by those for the ΔHik31 strain under aerobic conditions (WT); and the wild-type under low-oxygen conditions by those for the ΔHik31 strain under low-oxygen conditions (WT/ΔHik31, Low O₂).

<table>
<thead>
<tr>
<th>Gene no.</th>
<th>Name</th>
<th>WT/D</th>
<th>ΔHik31</th>
<th>WT/ΔHik31</th>
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<tr>
<td>sll0219</td>
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</tr>
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<td>sll0220</td>
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<td>5.9</td>
<td>5.6</td>
</tr>
<tr>
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<td>fba3</td>
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</tr>
<tr>
<td>Photosynthesis cluster</td>
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<td></td>
</tr>
<tr>
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<td>−1.3</td>
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<tr>
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<td>mfs</td>
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<td>−1.6</td>
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</tr>
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</table>

and chlorophyll *a* biosynthesis under low-oxygen conditions (Minamizaki *et al.*, 2008). In addition, we observed upregulation of *chlG* (sll0056), encoding chlorophyll *a* synthase and downregulation of *chlN* (sll0750) and *por* (sll0506) in both strains (Table 2).

Uregulation of genes encoding transport and binding proteins constituted a major response to low-oxygen conditions in both strains. Transcript abundance was increased for 20 genes that encoded a diverse range of transport and binding proteins, including cation transporters and metal permeases in both strains. Only three genes in this category were downregulated in both strains, these encoded the two ferric iron-binding subunits of the iron transport system, *Fut* (futA1, srl1295 and futA2, srl0513) and a putative ferrichrome-iron receptor (*srl1490*) (Badarau *et al.*, 2008; Katoh *et al.*, 2001).

Nine regulatory genes were upregulated in both strains, including four histidine kinases, two response regulators, a hybrid sensor–regulator, a transcription regulator and the LexA-like protein (sll1626). The LexA-like protein binds two sites in the hydrogenase (*hox*) operon and may function as a transcription activator (Gutekunst *et al.*, 2005; Oliveira & Lindblad, 2005). Consistent with this, we observed that transcript levels of the *hox* operon (*sll1220–sll1226*) increased in both strains under low oxygen (Table 2).

**Table 2. cont.**

<table>
<thead>
<tr>
<th>Gene no.</th>
<th>Name</th>
<th>WT/D</th>
<th>ΔHik31</th>
<th>WT/ΔHik31</th>
</tr>
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<tr>
<td>Photosynthesis cluster</td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>srl1181</td>
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<td>2.9</td>
<td>−1.3</td>
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<td>3.6</td>
<td>1.1</td>
</tr>
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<tr>
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<td>−1.5</td>
</tr>
<tr>
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<td>2.4</td>
<td>1.3</td>
</tr>
<tr>
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<td>1.5</td>
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<tr>
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<td></td>
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</tr>
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<tr>
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<td>mfs</td>
<td>−1.6</td>
<td>−1.6</td>
<td>1.2</td>
</tr>
</tbody>
</table>

**Genes with altered expression in the ΔHik31 strain compared with the wild-type under low-oxygen conditions (Hik31-dependent changes)**

Low-oxygen conditions altered transcript abundance of many genes involved in photosynthesis and respiration (26 and 36 genes in the wild-type and mutant, respectively). Twenty genes had decreased mRNA levels in the wild-type and one had decreased mRNA levels in the ΔHik31 strain. Many of the genes downregulated in the wild-type and
Table 3. Selected differentially regulated genes in Synechocystis sp. strain PCC 6803 in the wild-type and ΔHik31 strain on transition from aerobic to low-oxygen conditions

Genes were considered differentially regulated when FDR = 0.05, fold change > 1.5 (bold type). The fold changes were calculated by dividing the normalized mean intensities obtained for: the wild-type under low-oxygen conditions by those for the wild-type under aerobic conditions (WT); the ΔHik31 strain under low-oxygen conditions by those for the ΔHik31 strain under aerobic conditions (ΔHik31); the wild-type under aerobic conditions by those for the ΔHik31 strain under aerobic conditions (WT/ΔHik31, Air); and the wild-type under low-oxygen conditions by those for the ΔHik31 strain under low-oxygen conditions (WT/ΔHik31, Low O2). unk, Unknown; reg, regulatory function.

Table 3. cont.

<table>
<thead>
<tr>
<th>Gene no.</th>
<th>Name</th>
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<th>ΔHik31</th>
<th>WT/ΔHik31</th>
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<td></td>
<td></td>
<td>Air</td>
<td>Low O2</td>
<td></td>
</tr>
<tr>
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<td>1.4</td>
</tr>
<tr>
<td>slr1655</td>
<td>psaF</td>
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<td>1.1</td>
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</tbody>
</table>

upregulated in the mutant encoded proteins involved in light harvesting and photosynthetic electron transport. For example, genes encoding phycobilisome components were upregulated in the ΔHik31 strain and downregulated in the wild-type (Table 3). This parallels the changes observed in phycobilisome content in the whole cell spectra shown in Fig. 2. In addition to the genes encoding HO and CPO that were dramatically upregulated in both strains, a second copy of genes encoding HO (ho1, slr1184) and CPO (hemF, slr1185) had increased mRNA levels in the mutant strain, but not in the wild-type.

Genes encoding core proteins of PSII were upregulated in ΔHik31, but not in the wild-type under low-oxygen conditions.
conditions (Table 3). The gene *psbZ*, encoding a PSII protein involved in modulating electron transfer (Bishop et al., 2007) had decreased mRNA levels in the wild-type, but not in the mutant. Similarly, genes encoding cytochrome b6f subunits (*petB*, *slr0342 and petD*, *slr0343*) were upregulated in the mutant and the gene encoding subunit PetA (*sll1317*) was downregulated in the wild-type. Several genes encoding PSI subunits, as well as soluble electron carriers ferredoxin NADP-oxidoreductase (*petH*, *slr1643*), plastocyanin (*petE*, *sll0199*) and ferredoxin I (*petF*, *ssl0020*) had increased mRNA levels in the ΔHik31 strain but not the wild-type (Table 3). In addition, under low-oxygen conditions, the genes encoding ATP synthase subunits were downregulated in the wild-type (Table 3).

In ΔHik31, the gene *slr0228*, encoding an FtsH metalloprotease, was upregulated; this protein is involved in the maintenance of PSII activity under stress conditions (Komenda et al., 2006). Two genes downregulated in the wild-type, *desA* (*srl1350*) and *desD* (*ssl0262*), encode acyl-lipid desaturases that introduce double bonds at defined positions in fatty acids that form membrane glycerolipids (Wada & Murata, 1990). The presence of double bonds in membrane lipids has been implicated in protecting PSII from photo-inhibition under stress conditions (Allakhverdiev et al., 2001; Tasaka et al., 1996). These data suggest that there were modifications to photosynthetic performance under low oxygen and that these modifications were mediated by Hik31.

Molecular chaperones play important roles in preventing the misfolding and aggregation of proteins and increased transcript levels of these genes are frequently associated with stresses, such as heat, high light or oxidative stress (Hihara et al., 2001; Li et al., 2004; Rupprecht et al., 2007; Singh et al., 2006). Low-oxygen conditions did not induce this stress response in the wild-type, in fact four chaperones (*groEL2*, *groES*, *groEL1* and *htpG*) exhibited decreased transcript abundance under low-oxygen conditions (Table 3). In contrast, in ΔHik31, transcript levels of several chaperones were increased (*htpG*, *dnaK1*, * dnaK2 and groES*) or unchanged (*groEL2* and *groEL1*) following the transition to low-oxygen conditions (Table 3). In addition, *sll1514*, encoding the chaperone HspA, exhibited substantially higher mRNA levels in the ΔHik31 strain than the wild-type under both aerobic and low oxygen conditions.

Twelve regulatory genes had decreased mRNA levels in the wild-type, but not the mutant. These genes included *sll0797* and *sll0798* that encode a two-component system with multiple regulatory roles responding to Ni^{2+}, cellular redox and inorganic carbon availability (Li & Sherman, 2000; López-Maury et al., 2002; Wang et al., 2004a). The gene *srl1285* that encodes Hik34 was also downregulated in the wild-type. This histidine kinase mediates the response to numerous stresses, including upregulation of transcript abundance of a number of chaperone-encoding genes. This is consistent with our observation of downregulation of *hik34* and genes encoding chaperones (*groEL2*, *htpG*, *groES* and *groEL1*) in the wild-type under low oxygen conditions (Table 3). A further four response regulators (*sll0039*, *srl0312*, *srl1042* and *srl1693*) and one hybrid sensor-regulator (*srl0322*) were downregulated in the wild-type.

Under low-oxygen conditions, genes encoding ribosomal proteins showed decreased mRNA abundance in the wild-type (23 genes) and increased mRNA levels in the ΔHik31 strain (20 genes). Downregulation of ten genes involved in tRNA synthesis and modification provided a further indication that translation is suppressed in the wild-type (Supplementary Table S1, available with the online version of this paper). This response appeared to be accompanied by a decrease in transcription, as we observed the down-regulation of seven genes involved in transcription in the wild-type, including genes encoding RNA polymerase subunits, and increased mRNA level of the sigma factor, SigH, associated with stationary-phase growth (Foster et al., 2007).

Kahlon et al. (2006) reported that the absence of Hik31 resulted in glucose sensitivity. This sensitivity was likely to result from accumulation of glucose due to decreased glucokinase activity and not due to transcriptional changes. In fact, the authors reported that the presence of glucose resulted in few transcriptional changes for genes involved in glucose metabolism in the wild-type compared with a Hik31 mutant. Similarly, our data show little change in transcripts involved in glucose metabolism, or the oxidative pentose phosphate pathway (Supplementary Table S1). However, several genes encoding proteins involved in pyruvate metabolism were altered in the wild-type, but not in the mutant, following transition to low oxygen (Table 3). This included downregulation of genes *phdB* (*sll1721*) and *phdD* (*slr1096*) involved in converting pyruvate to acetyl-CoA and upregulation of genes encoding proteins phosphoenolpyruvate synthase (*ppmA*, *srl0301*) and phosphoenolpyruvate carboxylase (*ppc*, *sll0920*) that catalyse the conversion of pyruvate to phosphoenolpyruvate (PEP) and PEP to oxaloacetate, respectively. Oxaloacetate is a precursor in numerous biosynthetic reactions (e.g. production of amino acids) and it has been suggested that PEP carboxylase plays a role in maintaining the availability of carbon compounds for many pathways (Owttrim & Colman, 1986). The role of oxaloacetate under low-oxygen conditions is not clear, but our data indicate that Hik31 is involved in regulating its production.

The gene cluster *srl1852–srl1860* was downregulated in the wild-type, but not in the mutant. The gene *icfG* (*srl1860*) encodes a protein, serine/threonine phosphatase, that is vital for growth in the presence of glucose and this gene is upregulated in the wild-type in the presence of glucose, but not in a ΔHik31 strain (Beuf et al., 1994; Kahlon et al., 2006; Shi et al., 1999). In our experiment, *icfG* was part of an eight-gene cluster that exhibited low transcript levels in both strains, but was more abundant in the wild-type than the mutant under aerobic conditions. Other genes in this cluster may have roles in regulation, including *srl1856* and...
slr1859 that encode proteins with similarities to phosphoprotein substrates for protein kinases (Shi et al., 1999). Our data indicated that Hik31 is involved in regulating this igrG cluster and this was suggested to be one of the roles of Hik31 in glucose metabolism (Kahlon et al., 2006).

**Conclusion**

*Synechocystis* sp. PCC 6803 demonstrated a low-oxygen-specific response that included upregulation of the *psbA1* gene cluster, genes involved in chlorophyll biosynthesis, and the hydrogenase operon, as well as the transient upregulation of *hik31* (Summerfield & Sherman, 2008). The interruption of the chromosomal copy of *hik31* resulted in increased growth under low-oxygen conditions. However, the wild-type and ΔHik31 strain shared some transcriptional responses to low oxygen conditions. These responses indicated that additional regulators are involved in the response to low-oxygen conditions. One correlation is between the gene encoding the LexA-like protein (Sll1626) and the *hox* operon, these genes were upregulated after the transition to low O2. This was consistent with the finding that Sll1626 interacts with the *hox* promoter and positively regulates its expression (Oliveira & Lindblad, 2005). The genes encoding Sll0359 and Sll0882 are also regulators of the *hox* operon (Ishii & Hihara, 2008; Oliveira & Lindblad, 2008), these genes are not upregulated under low-oxygen conditions, but may play a role in regulation.

Differences in the transcriptional response of the wild-type and mutant to low-oxygen conditions included: (i) the downregulation of many genes associated with decreased growth in the wild-type, but not in the ΔHik31 mutant and (ii) the upregulation of many stress responsive genes in the mutant, but not the wild type. These data suggest that the chromosomally encoded Hik31 is involved in negatively regulating gene expression in response to low oxygen. This is consistent with the growth observed under low-oxygen conditions where, in the absence of Hik31, there was no downregulation of genes involved in growth and the ΔHik31 strain grew faster than the wild-type (Fig. 3). It is unclear whether Hik31 is sensing low-oxygen conditions or responding to intracellular changes resulting from low-oxygen conditions, for example changes in cellular redox, which have been shown to induce transcription of *hik31* (Hihara et al., 2003). The plasmid-encoded Hik31 may be involved as well, but the growth data suggest that the two Hik31 copies have similar effects under low-oxygen conditions and that any gene expression changes will only show quantitative alterations. However, the role of Hik31 in switching between photoautotrophic and mixotrophic growth suggests a wider role of this histidine kinase. We will identify other conditions in which the Hik31 proteins control different, but related, processes and highlight the overall importance of this two-component regulator (S. Nagarajan and L. A. Sherman, unpublished results).

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