A putative sensor kinase, Hik31, is involved in the response of *Synechocystis* sp. strain PCC 6803 to the presence of glucose

Shira Kahlon,^{1†} Karen Beeri,^{1†} Hiroshi Ohkawa,^{2†} Yukako Hihara,³ Omer Murik,¹ Iwane Suzuki,⁴ Teruo Ogawa² and Aaron Kaplan¹

Correspondence

Aaron Kaplan

aaronka@vms.huji.ac.il

¹Department of Plant and Environmental Sciences, Hebrew University of Jerusalem, 91904 Jerusalem, Israel

²Bioscience Center, Nagoya University, Nagoya, Japan

³Department of Biochemistry and Molecular Biology, Saitama University, Saitama, Japan

⁴National Institute for Basic Biology, Okazaki, Japan

The reason(s) for glucose sensitivity in certain cyanobacterial strains is poorly understood. Inactivation of genes encoding the putative sensor kinase Hik31 in *Synechocystis* sp. strain PCC 6803 resulted in a mutant unable to grow in the presence of D-glucose. Sensitivities to D-glucose, its analogue 2-deoxy-D-glucose, and fructose, were alleviated in mutants in which *glcP*, encoding the glucose transporter, was inactivated. These data indicate that permeation of these substrates is required to inflict cell death. The mutant $\Delta hik31$, and the glucose-sensitive strain of *Synechocystis*, do not possess glucokinase activity, although a transcript originating from *glk*, encoding glucokinase, is present. Inactivation of *glk* led to severe sensitivity to glucose, indicating that the presence of glucose itself, within the cells, inflicted this sensitivity. On the other hand, sensitivity to 2-deoxy-D-glucose was lower in Δglk , thus distinguishing between the effect of glucose itself and that of its analogue, which, in the absence of glucokinase activity, may not be phosphorylated. Addition of glucose led to a small rise in glucose-6-phosphate dehydrogenase activity in the wild type, but constitutive activity was observed in the $\Delta hik31$ mutant regardless of the presence of glucose. Microarray analyses showed only small changes in the abundance of global transcripts in *Synechocystis* following glucose addition, but the transcription levels of several genes, including *icfG*, but not *glk*, were strongly affected by inactivation of *hik31*. The mechanism(s) whereby Hik31 is involved in glucose sensing and response is discussed.

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INTRODUCTION

Variations in the ability of cyanobacteria to consume carbon from their surroundings have been reported, ranging from obligate photoautotrophs, for which the sole carbon source is carbon dioxide, to photomixotrophic and heterotrophic growth (Rippka *et al.*, 1979; Stal & Moezelaar, 1997). Despite its importance to our understanding of cell metabolism, little is known about the mechanism(s) whereby cyanobacteria respond to the presence of glucose in their environment. Furthermore, while D-glucose-sensitive (GS) and D-glucose-tolerant (GT) strains of *Synechocystis* sp. PCC 6803 (henceforth referred to as *Synechocystis*) are available,

the mechanisms distinguishing their response to glucose are poorly understood.

Zhang *et al.* (1998) introduced *glcP*, which encodes the glucose transporter in *Synechocystis*, into the chromosome of the obligate autotroph *Synechococcus* sp. strain PCC 7942 (henceforth referred to as *Synechococcus*). This resulted in glucose sensitivity in the *Synechococcus* cells that expressed *glcP*; a glucose-tolerant subclone was found to have lost *glcP*. Integration of *glcP* in a replicative vector allowed glucose transport and photoheterotrophic growth of *Synechococcus*, but this could not be maintained. These data suggested that lack of glucose transport could partly explain obligate autotrophy, at least in *Synechococcus*, and that glucose uptake was essential to lead to glucose sensitivity by a mechanism that was unknown at that time.

Exposure of *Synechocystis* to glucose results in many changes at various cellular levels, including carbon metabolism (Knowles & Plaxton, 2003), pigmentation (Hsiao *et al.*,

[†]These authors contributed equally to this study.

Abbreviations: 2-D-Glc, 2-deoxy-D-glucose; G6P, glucose 6-phosphate; G6PD, glucose-6-phosphate dehydrogenase; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GS, D-glucose-sensitive; GT, D-glucose-tolerant; WT, wild type.

2004; Ryu *et al.*, 2004), phosphorylation patterns (Bloye *et al.*, 1992), carbon dioxide uptake (Kaplan & Reinhold, 1999) and redox state of the plastoquinone pool (S. Kahlon, I. Ohad & A. Kaplan, unpublished results). The mechanisms whereby *Synechocystis* senses glucose are not known. The transmembrane proteins Snf3, Rgt2 (Moriya & Johnston, 2004) and Gpr1 (Lemaire *et al.*, 2004) have been implicated in glucose sensing in the yeast *Saccharomyces cerevisiae*. Our analysis showed that the N-terminal of Snf3p is highly homologous to *glcP* from *Synechocystis*, but also that the cyanobacterium lacks the other half (the sensor?) of Snf3p. *Synechocystis* does not possess a homologue of Rgt2 or the G-protein Gpr1. In plants, glucose sensing is mediated by hexokinase (Moore *et al.*, 2003), but *Synechocystis* does not possess hexokinase, and phosphorylation of glucose is catalysed by glucokinase. Interestingly, despite the numerous responses of *Synechocystis* to the presence of glucose, and with the exception of cases such as carotene biosynthesis in the dark (Ryu *et al.*, 2004), the profiles of transcript abundance and protein levels during steady-state growth have not shown marked differences between control and glucose-supplied GT cells (Knowles & Plaxton, 2003; Tu *et al.*, 2004; Yang *et al.*, 2002). This may suggest that the response to glucose in *Synechocystis* rests mainly on alterations at the level of post-translational modifications, or modulation of enzyme activities.

Synechocystis mutants impaired in their growth in the presence of glucose have been isolated. A $\Delta icfG$ mutant cannot grow in the presence of glucose under low carbon dioxide conditions (Beuf *et al.*, 1994). The *icfG* (*slr1860*) gene, encoding a protein phosphatase, is part of a gene cluster that encodes a protein kinase, a protein phosphatase and two phosphoproteins (Shi *et al.*, 1999). A reduced level of *gap1* transcript (encoding glyceraldehyde-3-phosphate dehydrogenase, GAPDH) in a $\Delta hik8$ mutant of *Synechocystis* lowers the ability of the cells to degrade glucose, and grow in the dark (Singh & Sherman, 2005), thus implicating this sensor kinase in the response to glucose. PmgA, which shows homology with anti-sigma factor, is essential for the acclimation of *Synechocystis* to changing light intensity (Hihara *et al.*, 1998). Growth of a $\Delta pmgA$ mutant is impaired when glucose is provided in the light (Hihara & Ikeuchi, 1997), but the mechanism(s) involved is not known.

It is widely accepted that the oxidative pentose pathway is the main route of carbohydrate breakdown in cyanobacteria (Hagen & Meeks, 2001). However, inactivation of *zwf* encoding glucose-6-phosphate dehydrogenase (G6PD) in *Synechococcus* has little effect on the rate of oxygen uptake in the dark (Scanlan *et al.*, 1995), thus suggesting that alternative metabolic routes are present. The level of glucose-6-phosphate (G6P) increases following initiation of photosynthesis, whereas that of 6-phosphogluconate declines (Pelroy & Bassham, 1972), suggesting regulation of G6PD. Indeed, detailed kinetic studies have shown strict regulation of G6PD activity by G6P, ATP, NADP and NADPH, and a shift in the nature of the curve relating the rate of G6PD activity

to G6P concentration, from hyperbolic to sigmoidal, with a rising pH (Grossman & McGowan, 1975). Biochemical analyses have indicated that G6PD is present in various aggregation states that show variable kinetic properties (Gleason, 1996; Schaeffer & Stanier, 1978; Sundaram *et al.*, 1998). It has been suggested that OpcA is involved in the assembly of G6PD, and its oligomerization and activation, in various cyanobacteria (Min & Golden, 2000; Sundaram *et al.*, 1998). In contrast, detailed studies on G6PD from *Nostoc punctiforme* do not support involvement of OpcA in its aggregation, but show that the presence of OpcA strongly affects the activity of G6PD, and its affinity towards its substrate G6P (Hagen & Meeks, 2001).

The seminal studies by Shestakov and colleagues (Koksharova *et al.*, 1998) have shown the importance of *gap1* and *gap2*, which are closely related to the genes encoding cytosolic and chloroplast GAPDH, respectively, of higher plants. A mutant impaired in *gap1* is unable to grow on glucose, but retains autotrophic growth in carbon dioxide. On the other hand, the *gap2* mutant can perform photomixotrophic growth on glucose, but loses the ability to grow under photosynthetic conditions. Biochemical analyses indicate that Gap2 operates in the reductive photosynthetic (Calvin) cycle, and in non-photosynthetic gluconeogenesis, whereas Gap1 is essential for glycolytic glucose breakdown only (where the catabolic activity of Gap2 is repressed by a low-molecular-weight inhibitor of an unknown nature).

We show that inactivation of *slr0790* and its plasmid homologue *slr6041* (encoding sensory histidine kinase, Hik31) in the GT strain of *Synechocystis* results in a mutant unable to grow in the presence of glucose. These findings implicate Hik31 in the mechanisms that regulate between photo-autotrophic and photomixotrophic growth.

METHODS

Organism, mutants and growth conditions. GT and GS strains of *Synechocystis* were used in the experiments presented here. The GT strain was originally isolated by John Williams of DuPont, Wilmington, DE, USA; its DNA has been sequenced and placed in CyanoBase (<http://www.kazusa.or.jp/cyanobase>). The Pasteur Culture Collection of Cyanobacteria maintains the GS strain. In addition, we raised mutants of the GT strain, in which we inactivated genes encoding the chromosomal (*slr0790*) and plasmid (*slr6041*) copies of Hik31, the glucose transporter (*glcP*, *slr0771*), and a glucokinase (*glk*, *slr0593*). The methodology used to raise the mutants involved PCR amplifications of genomic regions in the 5' and the 3' ends of the relevant ORF, and ligations to an antibiotic-resistance-encoding cartridge, thereby deleting part of the ORF. This was followed by transformation of the host strain with the construct obtained, and selection in the presence of the relevant antibiotics. DNA from the mutant was examined by PCR and Southern analyses to confirm absence of the relevant genomic region from the wild type (WT), i.e. complete segregation. Both the chromosomal and the plasmid-located copies of *hik31* were inactivated by insertion of a spectinomycin-resistance cassette into the *NcoI* site (located at bp 3 064 298 of the chromosome, and at the homologous site in the plasmid). The $\Delta glcP$ mutant was obtained by ligating the PCR products from positions 18 to 542 (from the ATG codon), and 631 to 1133, with a

chloramphenicol-resistance-encoding cassette, thereby deleting nt 543–630. The Δglk mutant was obtained by ligating the PCR products from positions 75 to 625, and 809 to 1327, with a cassette encoding kanamycin resistance, thereby deleting nt 626–808. The double mutants $\Delta glk/\Delta hik31$ and $\Delta glcP/\Delta hik31$ were also prepared during the course of this study using the same constructs. All the mutants used here were fully segregated; we could not detect WT uninterrupted fragments in their DNA.

The *hik31* gene is located immediately downstream of *sll0788* and *sll0789* (<http://www.kazusa.or.jp/cyano/Synechocystis>). A $\Delta sll0788$ mutant was insensitive to glucose (not shown). We could not specifically inactivate *sll0789* encoding a putative two-component response regulator of the OmpR subfamily, which is likely to interact with Hik31 (Sato *et al.*, 2004), without affecting the transcription of *hik31*. Thus, at this time we cannot unequivocally conclude whether the GS phenotype observed in the $\Delta sll0789$ mutant was due to inactivation of *hik31*, or an effect on the abundance of its response regulator Sll0789.

The cells were grown in BG11 medium (Stanier *et al.*, 1971) at 30 °C, with continuous illumination of 60 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$; the medium was supplemented with 20 mM HEPES/NaOH, pH 7.8, and, where appropriate, the relevant antibiotics. Liquid cultures were bubbled with air enriched with 1–3 % carbon dioxide (high CO_2), or a mixture of 1:1 air and carbon-dioxide-free air (low CO_2); agar plates were placed in a plexiglas chamber aerated with one of these gas mixtures.

Western analysis and RT-PCR. Cultures were grown to a cell density of OD_{750} 0.5, transferred to fresh BG11 medium, either with or without 5 mM glucose, and harvested when required. For Western analyses, the cells were lysed mechanically, and the extracts placed onto polyacrylamide gels (7 %) in the presence (denaturing) or absence (native) of SDS. The sampling, but not the running buffer, contained 1.25 % β -mercaptoethanol. The proteins were transferred to nitrocellulose membranes, and probed with antibodies raised against G6PD and OpcA (kindly provided by Dr N. Mann, University of Warwick, UK, and Dr J. Meeks, University of California, Davis, CA, USA, respectively). Detection was with an ECL Western blot kit (catalogue no. RPN2106; Amersham). Analyses of transcript abundances encoding G6PD, Gap1, Gap2, GidA, PmgA, IcfG and 16S rRNA were performed using RT-PCR on RNA isolated from the GT strain of *Synechocystis* and mutant $\Delta hik31$. When required, the cells were exposed to 5 mM D-glucose for various time periods before RNA extraction; the cells were lysed, and total RNA was prepared as described by Katoh *et al.* (2001). A 100 ng quantity of total RNA was used in the reverse transcriptase reaction, using Super script II (catalogue no. 18064-014; Invitrogen) to synthesize cDNA, followed by PCR amplification using appropriate primers for 12–25 cycles, depending on the abundance of the transcript. Twelve

to fourteen cycles were sufficient in the case of 16S rDNA, whereas 25 cycles were required in the case of *gidA*. The specific primers used are shown in Table 1.

Glucokinase and G6PD activities. Enzyme activity assays, performed at 30 °C, were based on changes in the level of NADP, using a dual-wavelength-mode spectrophotometer ($A_{340}-A_{380}$). Cell extracts were produced by mechanical disruption on ice. The cells were harvested by centrifugation, suspended in 50 mM HEPES/NaOH, pH 7.5, and broken by glass beads in a Brown vortex. Glucokinase activity was measured in 50 mM HEPES NaOH, pH 7.5, in the presence of 5 mM MgCl_2 , 2 mM ATP, 0.5 mM NADP^+ , 2 U ml^{-1} G6PD, and 20 mM glucose. The activity of G6PD was measured in 50 mM Tris/malate, pH 7.8, in the presence of 10 mM MgCl_2 , 2 mM G6P, and 1 mM NADP^+ .

Microarray analyses. Isolation of RNA, and the analyses, were performed as described by Hihara *et al.* (2004).

Oxygen exchange. A Clark-type oxygen electrode (Kaplan *et al.*, 1980) and a membrane inlet mass spectrometer (Helman *et al.*, 2003) were used to measure the rate of oxygen uptake or evolution. Cell suspensions were placed in the measuring chamber at 30 °C, and provided with D-glucose as required.

RESULTS

Growth experiments

Synechocystis possesses two genes encoding the putative sensor kinase Hik31: the chromosome-located *sll0790*, and its plasmid-harboured copy *slr6041*. At the nucleotide level, the plasmid and the chromosomal genes are 94.8 % identical; the encoded amino acid sequences are 95.7 % identical. Differences between the amino acid sequences are observed downstream of position 420 only, out of 458 and 450 amino acids in the chromosomal- and plasmid-encoded sequences, respectively.

Addition of D-glucose to mutant $\Delta hik31$ (in which both genes are inactivated) arrested its growth within about 20 h (Fig. 1a, b). In the absence of glucose, the GT and GS strains of *Synechocystis*, and all the mutants raised on the background of GT during the course of this study, including $\Delta hik31$, grew equally well under the conditions used here in either solid (Fig. 1c) or liquid BG11 media (not shown). Addition of L-glucose or 3-O-methyl-D-glucose did not inhibit the growth of the WT or the $\Delta hik31$ mutant (Fig. 1b). On the other hand, addition of either 2-deoxy-D-glucose

Table 1. Specific primers used for PCR amplification reactions

Gene	Sense	Antisense
<i>zwf</i>	5'-TTCCCACTATTTACGCCGCCG-3'	5'-GTTTCATAGGTGTGGGCCCCAA-3'
<i>gap1</i>	5'-AAACATTATTCCTCATCCAC-3'	5'-CTAGACCAAACCGTCTTTGCT-3'
<i>gap2</i>	5'-GGATTGAGACGGATCGGA-3'	5'-GTGGTCATGGTGCCTTTGA-3'
<i>gidA</i>	5'-AATGCGCCCCGAACGTCTGAGT-3'	5'-TTCAGGGGAGCTTGTTCGGCCT-3'
<i>pmgA</i>	5'-AGCGGCCAGCACATTCAG-3'	5'-CCGGCGCTTAATTTCTTGC-3'
<i>icfG</i>	5'-AAGATCTTCAAAGCCGGGC-3'	5'-CCGATTGACAAGGGGAGATT-3'
16S rDNA	5'-AGACTCCTACGGGAGGCAGC-3'	5'-CGCGTTAGCTACGGCACGGCTCGG-3'

Fig. 1. Growth of *Synechocystis* sp. strain PCC 6803 and mutants thereof on glucose and its analogues. (a) The GT strain of *Synechocystis* (WT, ●) and mutant $\Delta hik31$ (○) grown in liquid BG11 medium supplemented with 5 mM D-glucose. After 24 h growth, the cells were diluted, and the growth experiment was continued under otherwise identical conditions. Light intensity was 50 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$, and the temperature was 30 °C. (b, c) The WT, and mutants $\Delta hik31$, $\Delta glcP$, Δglk , $\Delta hik31/\Delta glk$ and $\Delta hik31/\Delta glcP$ thereof, grown on solid BG11 medium supplemented with D-glucose (D-Glc), L-glucose (L-Glc), 2-D-Glc, 3-O-methyl-D-glucose (3OMG) or fructose (Frc), 5 mM each. Normal and high-P in (b) are standard BG11 medium, and $10 \times \text{Pi}$ in BG11, respectively. Cells grown in liquid BG11 were placed (at densities corresponding to OD₇₃₀ values 0.1, 0.01 and 0.001) on agar plates supplemented with the sugars indicated above. The plates were maintained in a closed plexiglas box aerated with 3% carbon dioxide in air, at 30 °C. Light intensity was 50 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$.

(2-D-Glc), which can undergo phosphorylation, or fructose was lethal for the GT and $\Delta hik31$ strains (Fig. 1b, c). Raising the level of Pi in the BG11 medium by 10-fold did not alter the response of the WT and the $\Delta hik31$ mutant to glucose or 2-D-Glc (Fig. 1b), suggesting that the response to 2-D-Glc was not due to a low internal Pi level, which could be caused by phosphorylation, but rather that the response was due to lack of further metabolism of this substrate.

To test whether the $\Delta hik31$ mutant was sensitive to internal or external glucose, we inactivated *glcP* encoding the glucose transporter. Mutants $\Delta glcP$ and $\Delta glcP/\Delta hik31$ grew like the WT in the presence or absence of glucose, in the light (Fig. 1c), indicating that glucose must enter the cells to induce sensitivity. As expected, mutants in which *glcP* was inactivated could not grow in the dark on glucose (not shown), and were not sensitive to the presence of 5 mM

2-D-Glc or fructose, unlike the WT (Fig. 1c; Flores & Schmetterer, 1986). These data also suggest that fructose enters the cells via the glucose transporter.

Glucokinase activity

The results presented in Fig. 1 suggest that phosphorylation of glucose or its analogues inside the cells could lead to the glucose sensitivity observed in this study. In *Synechocystis*, conversion of glucose to G6P is mediated by glucokinase, which is encoded by *glk*. Mutants Δglk and $\Delta glk/\Delta hik31$ could not grow in the presence of glucose (Fig. 1c), indicating that the entry of glucose itself into the cells inhibited growth. This observation raises the possibility that sensitivity of the GS strain and the $\Delta hik31$ mutant to glucose is due to impaired glucokinase activity. Measurements of enzyme activity on cell extracts (Table 2) confirmed that the GS and $\Delta hik31$ strains did not possess glucokinase activity. We were confronted with a very large variability in glucokinase activities in various cell extracts from the WT, despite the fact that, to the best of our knowledge, the growth and experimental conditions were identical. In addition, the methodology used to break the cells was identical for the measurements of G6PD (see below) and glucokinase activities, and we repeatedly examined these two activities on the same extracts; nevertheless, the reproducibility of the results obtained was much better for G6PD than glucokinase. Furthermore, the data in Table 2 are provided per milligram of protein in the cell extract, and thus breaking efficiency is normalized. Nevertheless, it was clear that glucokinase activity was essentially zero in all the experiments where extracts from GS or $\Delta hik31$ strains were examined.

As expected, heterotrophic growth in the dark on glucose was observed in the GT, $\Delta icfG$ and $\Delta pmgA$ strains that possess glucokinase activity (not shown). On the other hand, mutants $\Delta hik31$ and Δglk , lacking glucokinase activity, could not grow on glucose in the dark. Interestingly, inactivation of *glk* partly alleviated the sensitivity of the WT and $\Delta hik31$ mutant to 2-D-Glc (Fig. 1c), suggesting that phosphorylation by glucokinase is involved in the toxicity of 2-D-Glc.

Table 2. Glucokinase activity in the GT and GS strains of *Synechocystis*, and in the $\Delta hik31$ mutant

Experiments with the GT strain were repeated six times, and those with the GS and $\Delta hik31$ strains were repeated three times. Glucokinase activity at 0 and 5 h is given in $\mu\text{mol NADPH (mg protein)}^{-1} \text{ s}^{-1}$.

Strain	Glucokinase activity	
	0 h	5 h
GT	0.88 (0.24–1.44)	1.75 (0.23–3.2)
GS	0.00	0.00
$\Delta hik31$	0.00	0.00

G6PD

The G6P produced by glucokinase activity is further metabolized by G6PD. The activity of G6PD increased slightly in WT cells that were provided with glucose, but it was significantly higher in the $\Delta hik31$ mutant, irrespective of the presence of glucose (Fig. 2). To examine a possible effect of inactivation of *hik31* on the profile of G6PD, we extracted proteins from the WT and the mutant before and after addition of glucose, and separated them on a native (not shown) and a denaturing gel (Fig. 3). In agreement with the elevated G6PD activity in cell extracts from the $\Delta hik31$ strain, the levels of G6PD and of OpcA were also higher in the mutant than in the WT (Fig. 3). In both strains, the levels of OpcA and of G6PD were not affected by the presence of glucose (Fig. 3). Interestingly, a 105 kDa polypeptide was observed in protein extracts from the WT loaded onto the denaturing gel, but this was not observed in extracts from the $\Delta hik31$ mutant. The nature of this polypeptide is not known, but, since it was recognized by the G6PD antibody, we cannot discount the possibility that it is a dimer of G6PD that is resistant to the denaturing SDS conditions.

Transcript abundance

This was examined by microarray, and RT-PCR and Northern analyses. These analyses were performed on RNA isolated from the WT and $\Delta hik31$ mutant, with or without exposure to 5 mM D-glucose for 6 h. In general, our results confirmed earlier reports (see Introduction) that the profiles of transcript abundance do not show large changes in response to the addition of glucose; two exceptions are the *rpl* and *rps* genes involved in ribosome biosynthesis, which were upregulated two- to fourfold by glucose. In Table 3, we

Fig. 2. The activity of G6PD in *Synechocystis* (WT) and $\Delta hik31$, in the presence and absence of D-glucose. Where indicated, the cells were exposed to 5 mM D-glucose for 24 h before the extraction of proteins.

Fig. 3. Western analyses of protein extracts from *Synechocystis* (WT) and mutant $\Delta hik31$ separated on denaturing SDS-PAGE. The gels were loaded with 10 μ g protein. Where indicated, the cells were exposed to 5 mM D-glucose for 24 h prior to extraction of proteins. Antibodies raised against G6PD and OpcA were used.

provide the ratio (in the $\Delta hik31$ mutant versus that in the WT) of transcript abundances of genes that encode proteins directly involved in glucose metabolism, and that were also examined by RT-PCR and Northern analyses, during the course of this study. The analyses showed that the levels of the transcripts originating from *glk* (encoding glucokinase), *glcP* (encoding the glucose transporter), *opcA* (*slr1734*) and *zwf* (*slr1843*, encoding G6PD) hardly differed between the WT and the $\Delta hik31$ mutant (Table 3), and that the levels were not significantly affected by the addition of glucose (not shown). Among the transcripts examined by RT-PCR, that of *icfG* increased significantly after addition of D-glucose to the WT, but it was missing, or at least not detected, in RNA from the $\Delta hik31$ mutant (Fig. 4). The abundance of transcripts originating from *gap1*, *gidA* and *pmgA* was slightly affected by the addition of glucose, but the effect was much smaller than that observed for *icfG* (not shown).

DISCUSSION

The mechanism whereby *Synechocystis* senses (and responds) to the presence of glucose is poorly understood. Inactivation of the chromosomal and plasmid genes encoding Hik31, a putative sensor kinase in *Synechocystis*, resulted in a mutant unable to grow in the presence of D-glucose (Fig. 1). Since

inactivation of *glcP*, encoding the glucose transporter, alleviated the sensitivity of the $\Delta hik31$ mutant to glucose (Fig. 1), we conclude that entry of glucose into the cells is required to induce glucose sensitivity. The $\Delta hik31$ and GS strains of *Synechocystis* lack glucokinase activity (Table 2), and inactivation of *glk* (encoding glucokinase) in the GT strain resulted in sensitivity to glucose (Fig. 1c). These data suggest that glucose sensitivity is due to an inability to metabolize the compound. An ability of the GT strain to consume glucose is indicated, for instance, by a large rise in the rate of oxygen uptake (respiration) following the addition of glucose in the dark (Ohkawa *et al.*, 2000). This was not the case in the $\Delta hik31$ and Δglk strains, where the presence of glucose hardly affected the rate of oxygen uptake (not shown). Taken together, the data indicate that the presence of glucose itself, within the cells of strains unable to metabolize it, induces their sensitivity.

Since the abundance of a transcript originating from *glk* was not affected by inactivation of *hik31* (Table 3), we propose that downregulation of glucokinase activity in this strain is probably mediated by post-transcriptional regulation, the nature of which is being examined. In addition to *glk*, the genome of *Synechocystis* contains another ORF, *slr0329*, annotated as encoding glucokinase (<http://www.kazusa.or.jp/cyano/Synechocystis>). However, *glk* and *slr0329* are not

Table 3. Ratios of transcript amounts of certain *Synechocystis* genes in the $\Delta hik31$ mutant/WT, as indicated by microarray analysis

The cells were incubated for 6 h in the presence of 5 mM D-glucose, before the extraction of RNA. Ratios of transcript amounts are given in duplicate.

Gene	Protein	Ratio
<i>glk</i> (<i>slr0593</i>)	Glucokinase	1.37, 1.19
<i>glcP</i> (<i>slr0771</i>)	Glucose transporter	1.11, 0.97
<i>opcA</i> (<i>slr1734</i>)	OpcA	1.09, 1.10
<i>zwf</i> (<i>slr1843</i>)	G6PD	0.94, 1.10

Fig. 4. The abundance of transcripts of *icfG* and 16S rDNA in *Synechocystis* (WT) and mutant $\Delta hik31$ in the presence of D-glucose. The cells were provided with 5 mM D-glucose at time zero.

homologous, and, unlike the Δglk mutant, inactivation of *slr0329* did not result in sensitivity to glucose (not shown). Thus the role, function and annotation of *slr0329* remain to be elucidated.

Sensitivity to non-metabolized glucose analogues that can undergo phosphorylation (Fig. 1) has been observed in many organisms, including yeast (Rincon *et al.*, 2001) and plants (Kulma *et al.*, 2004). The ability to phosphorylate glucose is essential for the induction of cell death in nutrient-limited yeast (Granot & Dal, 1997). In contrast, we observed sensitivity to D-glucose in *Synechocystis* strains lacking glucokinase activity (GS, $\Delta hik31$ and Δglk), where the presence of D-glucose within the cells appeared to be lethal, but the mechanism involved is unknown. The *Synechocystis* genome contains genes homologous to *gidA* (*sll0202* and *sll0204*) and *gidB* (*slr0072*); the name *gid* (glucose-inhibited division) is derived from the observation that deletion of the gene in *Escherichia coli* results in a cell-elongation phenotype when glucose is provided (von Meyenburg *et al.*, 1982). *gidA* is widely distributed, and highly conserved, among prokaryotes and plants (including *Arabidopsis thaliana* and rice). Recent analyses of the structures of GidA (Iwasaki *et al.*, 2004) and GidB (Romanowski *et al.*, 2002), and characterization of mutants in various organisms in which *gidA* is inactivated (Kinscherf & Willis, 2002; Sha *et al.*, 2004), suggest that the latter is involved in the coordination of replication, and that it can act as a global regulator and moderator of translation. It is not known whether the *gid* genes function similarly in *Synechocystis*, and whether they are involved in its sensitivity to internal glucose. It should be noted that supply of glucose had little effect on the abundance of transcripts for *gidA* and *gidB* in *Synechocystis*, and that the number of these transcripts was very low (not shown).

Involvement of regulatory genes, including *hik8* and *icfG*, in the response of *Synechocystis* to glucose has been reported by others (Beuf *et al.*, 1994; Singh & Sherman, 2005). Unlike the $\Delta hik31$ mutant, a $\Delta hik8$ mutant can grow in the presence of glucose, but not in the dark, or an extended dark period, presumably due to an altered ability to express *gap1*, and thus to an altered ability to utilize glycogen in the dark (Singh & Sherman, 2005). In contrast, the $\Delta hik31$ mutant was found to possess a normal level of *gap1* transcript (not shown), but was unable to grow in the dark due to lack of glucokinase activity. It has been reported that inactivation of *icfG*, encoding a protein serine phosphatase in *Synechocystis* (Shi *et al.*, 1999), results in the inability to grow under limiting levels of inorganic carbon in the presence of glucose (Beuf *et al.*, 1994). In contrast, glucose sensitivity of the $\Delta hik31$ mutant was observed in cells exposed to either high or low levels of carbon dioxide, although the mutant was less sensitive to the presence of glucose under low CO₂ (not shown). The $\Delta icfG$ mutant was able to grow in the presence of glucose in the dark, whereas the $\Delta hik31$ mutant could not. A considerable rise in the level of *icfG* transcript was induced by glucose in the WT, but this transcript was not detected in

the $\Delta hik31$ mutant, regardless of the presence of glucose (Fig. 4). These data suggest that, in addition to activation of glucokinase, Hik31 is essential for the expression of *icfG*.

It is known that G6PD is present in various levels of aggregation (Gleason, 1996; Schaeffer & Stanier, 1978; Sundaram *et al.*, 1998), but the biological significance of the aggregation states is not known. The constitutive higher level and activity of G6PD exhibited by the $\Delta hik31$ mutant (Figs 2 and 3), and higher degree of aggregation (observed in native gels, not shown), suggest that Hik31 is also involved in the modulation of G6PD. The data also suggest that cells of the $\Delta hik31$ mutant are in an 'acclimated phase' to glucose, regardless of its presence. This may indicate a dual role for Hik31 as a repressor of some of the glucose-related functions, and as an inducer/activator of others, including the presence of the 105 kDa polypeptide, and the expression of *icfG*.

As indicated in the Introduction, the supply of glucose to *Synechocystis* cells imposes many changes in various and seemingly unrelated processes and activities. It is therefore surprising that analysis of global gene expression by means of DNA microarray (performed here, and by others; Singh & Sherman, 2005; Tu *et al.*, 2004) did not show marked changes in the transcript abundance of many genes. Nevertheless, detailed Northern, RT-PCR and biochemical analyses suggested changes in the expression of several genes, and the activity of certain enzymes, including glucokinase and G6PD, in response to deletion of *hik31* (Table 2; Figs 2, 3 and 4). A detailed proteomic approach, using some of the mutants raised here, is required to identify other components involved in the complex network whereby the cells regulate between photoautotrophic and photomixotrophic modes of growth.

Finally, while the data presented here support the suggestion that Hik31 plays an important role in the response of *Synechocystis* cells to glucose, the mechanisms involved, and the interactions with other components, are yet to be elucidated. Here, it is worth mentioning a serious difficulty that we faced during the course of this study – the stability of the phenotypes of some of the mutants used. It was apparent that addition of glucose caused an immense selection pressure, which led to the appearance of many suppression mutants. Analysis of these mutants may help to identify additional components in this complex system, and the interaction between them.

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