

Construction of a chassis for hydrogen production: physiological and molecular characterization of a *Synechocystis* sp. PCC 6803 mutant lacking a functional bidirectional hydrogenase

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Cyanobacteria are photosynthetic prokaryotes that are promising 'low-cost' microbial cell factories due to their simple nutritional requirements and metabolic plasticity, and the availability of tools for their genetic manipulation. The unicellular non-nitrogen-fixing *Synechocystis* sp. PCC 6803 is the best studied cyanobacterial strain and its genome was the first to be sequenced. The vast amount of physiological and molecular data available, together with a relatively small genome, makes *Synechocystis* suitable for computational metabolic modelling and to be used as a photoautotrophic chassis in synthetic biology applications. To prepare it for the introduction of a synthetic hydrogen producing device, a *Synechocystis* sp. PCC 6803 deletion mutant lacking an active bidirectional hydrogenase ($\Delta hoxYH$) was produced and characterized at different levels: physiological, proteomic and transcriptional. The results showed that, under conditions favouring hydrogenase activity, 17 of the 210 identified proteins had significant differential fold changes in comparisons of the mutant with the wild-type. Most of these proteins are related to the redox and energy state of the cell. Transcriptional studies revealed that only six genes encoding those proteins exhibited significant differences in transcript levels. Moreover, the mutant exhibits similar growth behaviour compared with the wild-type, reflecting *Synechocystis* plasticity and metabolic adaptability. Overall, this study reveals that the *Synechocystis* $\Delta hoxYH$ mutant is robust and can be used as a photoautotrophic chassis for the integration of synthetic constructs, i.e. molecular constructs assembled from well characterized biological and/or synthetic parts (e.g. promoters, regulators, coding regions, terminators) designed for a specific purpose.

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INTRODUCTION

Cyanobacteria are photosynthetic prokaryotes that are able to perform oxygenic photosynthesis, using water as an electron donor. They constitute one of the largest and most remarkable groups of bacteria on Earth, playing a key role in

Abbreviations: FDR, false discovery rate; RT-qPCR, real time quantitative PCR.

A set of supplementary methods, four supplementary figures and two supplementary tables are available with the online version of this paper.

both the carbon and the nitrogen cycle (Bothe *et al.*, 2010b; Moisaner *et al.*, 2010). Cyanobacteria are a prolific source of bioactive compounds (Burja *et al.*, 2001; Dembitsky, 2006), they have been used as biofertilizers and food supplements, and their strong potential in the fields of energy, bioremediation, bioplastics and medical diagnostics has been reported (Abed *et al.*, 2009; Devillers *et al.*, 2007). The simple nutritional requirements of these organisms, combined with their autotrophy and metabolic plasticity, as well as the availability of molecular tools for their genetic manipulation, makes them promising 'low-cost' microbial

cell factories (Abed *et al.*, 2009; Thajuddin & Subramanian, 2005).

Synthetic biology applies the standardization and hierarchical abstraction of engineering to biological systems, in order to redesign existing systems or design entirely new ones (Bashor *et al.*, 2010; Endy, 2005; Khalil & Collins, 2010; Yadav & Stephanopoulos, 2010). Following this philosophy, the BioModularH₂ project (www.biomodularh2.org) aimed at designing and constructing molecular parts/modules/devices that, once integrated into a cyanobacterial chassis, will redirect metabolic fluxes towards efficient hydrogen production. For this purpose, the unicellular non-N₂-fixing *Synechocystis* sp. PCC 6803 (hereafter referred to as *Synechocystis*) was chosen as the chassis. Biological chassis should be robust and well characterized organisms preferably capable of growing on minimal, inexpensive carbon sources considering their use for laboratory- or large-scale production of the desired molecule(s). Nonetheless, even in most robust hosts/chassis, foreign DNA instability can pose a problem, due to their ability to inactivate or dispose of the foreign genes (Keasling, 2008). In *Escherichia coli* this issue has been addressed by removing large sections of the chromosome, some containing mobile elements and cryptic virulence genes (Pósfai *et al.*, 2006; Sharma *et al.*, 2007a, b). Additionally, the presence of genes encoding proteins that may interfere and/or compete with those introduced should be eliminated. In this context, *Synechocystis* is a photoautotrophic organism with minimal nutritional requirements that uses CO₂ and sunlight as sources of carbon and energy. Its genome, the first to be sequenced among cyanobacteria, comprises a 3.6 Mb chromosome and seven plasmids (Kaneko *et al.*, 1996, 2003; Xu & McFadden, 1997; Yang & McFadden, 1993, 1994). This strain became the most studied cyanobacterium, for which plenty of physiological and molecular information is now available (Barrios-Llerena *et al.*, 2006; Bhaya *et al.*, 2000; Koksharova & Wolk, 2002; Ow & Wright, 2009; Schmitt & Stephanopoulos, 2003; Suzuki *et al.*, 2006; Zhang *et al.*, 2008). Moreover, it is naturally transformable with exogenous DNA, and a variety of molecular tools have been developed for its genetic manipulation. The vast amount of data, together with a relatively small genome, makes *Synechocystis* suitable for computational metabolic modelling and to be used as a photoautotrophic chassis.

Taking into account the aim of the BioModularH₂ project, and since a heterologous hydrogenase (hydrogen producing device) was going to be introduced, the native bidirectional hydrogenase of *Synechocystis* became a redundant part, and therefore should be removed. It has been reported that the bidirectional hydrogenase of *Synechocystis* could be involved in dark fermentation, act as an electron valve during photosynthesis, or even be part of the respiratory complex I (Antal & Lindblad, 2005; Appel & Schulz, 1996; Appel *et al.*, 2000; Gutthann *et al.*, 2007; Troshina *et al.*, 2002). The bidirectional hydrogenase of *Synechocystis* has been recently characterized as a truly bidirectional enzyme with a bias to H₂ production (McIntosh *et al.*, 2011); however, further

investigation is needed to clarify the role of this type of enzyme (Angermayr *et al.*, 2009; Bothe *et al.*, 2010a; Carrieri *et al.*, 2011; McIntosh *et al.*, 2011; Oliveira, 2008; Tamagnini *et al.*, 2002, 2007). In this work, a *Synechocystis* deletion mutant (Δ hoxYH), lacking an active bidirectional hydrogenase and without any selection marker, was produced and characterized. In addition, a strategy to construct vectors compatible with the BioBrick system (http://partsregistry.org/Main_Page) was developed to efficiently remove redundant genes and/or introduce DNA-based synthetic parts and circuits into the *Synechocystis* genome. The molecular tools (BioBrick compatible integrative vectors) and the photoautotrophic chassis produced in this work can be easily extended to other biotechnological applications aiming to exploit a light-driven biological system.

METHODS

Organisms and standard growth conditions. The unicellular non-N₂-fixing cyanobacterium *Synechocystis* sp. PCC 6803 (obtained from the Pasteur Culture Collection, Paris, France) was maintained in BG11 medium (Stanier *et al.*, 1971) at 25 °C and 12 h light (20 μ E m⁻² s⁻¹ with Osram L18W/765 cool white daylight bulbs)/12 h dark cycles, unless stated otherwise. Cosine-corrected irradiance was measured by using a quantum meter (Skye SKP 216 Quantum sensor with SKP 200 Measuring unit, Hansatech Instruments). For solid medium, BG11 was supplemented with 1.5% Noble agar (Difco), 0.3% sodium thiosulfate and 10 mM TES-KOH buffer (pH 8.2). For the selection of the mutants, BG11 medium was supplemented with kanamycin (Km, 10–50 μ g ml⁻¹) or sucrose [10% (w/v)]. For the proteomics analysis, standard growth conditions were used, with a scalar irradiance of 75 μ E m⁻² s⁻¹ (measured with a QSL-2100 Radiometer, Biospherical Instruments, and equivalent to the standard growth light intensity described above). Anaerobic conditions were induced by sparging with argon for 30 min in the dark. Cells were then kept in the dark for 2 h before being harvested. *E. coli* DH5 α (Stratagene) was used for cloning purposes. *E. coli* transformants were cultivated at 37 °C in Luria-Bertani (LB) medium supplemented with 100 μ g ampicillin ml⁻¹ or 50 μ g kanamycin ml⁻¹.

DNA extraction and recovery. Cyanobacterial genomic DNA was extracted using the phenol/chloroform method described previously (Tamagnini *et al.*, 1997). Agarose gel electrophoresis was performed by standard protocols using 1 \times TAE buffer (Sambrook & Russell, 2001), and the DNA fragments were isolated from gels using the GFX PCR DNA and Gel Band Purification kit (GE Healthcare), according to the manufacturer's instructions.

Plasmid construction. The plasmids used in this work, pGDYH and pGDYH.NS, were based on pGEM-T Easy (Promega) and contain the *Synechocystis* chromosomal regions flanking the *hoxYH* genes (encoding subunits of the bidirectional hydrogenase). The regions upstream from *hoxY* and downstream from *hoxH* were amplified by PCR using primers with tags containing restriction sites for cloning purposes (Table 1). Additionally, the primers 5-I and 3-I contain sites compatible with the BioBrick system: *MunI*, *XbaI*, *SpeI* and *PstI*. Each PCR mixture (50 μ l) contained 1.5 U *Pfu* DNA polymerase (Fermentas), 1 \times reaction buffer [10 \times reaction buffer: 200 mM Tris/HCl pH 8.8, 100 mM (NH₄)₂SO₄, 100 mM KCl, 1 mg BSA ml⁻¹, 1% (v/v) Triton X-100, 20 mM MgSO₄], 250 μ M of each deoxyribonucleotide triphosphate, 200 nM each primer (primer pairs 5-O/5-I or 3-O/3-I) and 7 ng *Synechocystis* genomic DNA. The PCR profile was 2 min at 95 °C followed by 35 cycles of 30 s at 95 °C, 45 s at 60 °C and

Table 1. Oligonucleotide primers used in this study

Name	Sequence (5'–3')	Purpose
5-O*	GAAG <u>CCCGGGT</u> TC C ATTC C ACCCTTTGCCATTTAG	Mutant construction/Southern probe
5-I*	CTGCAGACTAGTACGACCTTCTAGACAATTGGAGCCA TTCGTCCATATCAAGGAAGGACATATGACAGC	Mutant construction/Southern probe
3-O*	TAGTAAGCTTGCCTTTGATAACCACAGTGCCCGAG	Mutant construction
3-I*	CAATTGTCTAGAAGGTCGTA C CTAGTCTGCAGCC TGCCTTAGTTGTTCTACCCATGCAGCGGGACAAATG	Mutant construction
FO	AGAATATTGGATTGTGGATGTGAAGGC	Mutant confirmation
RO	CAGGGAGTGGAGTTAATTAGAACAGG	Mutant confirmation
NeosacB2F*	GCTGGAATTCAGGAAGCGGAACACGTAGAAAG	Selection cassette amplification
NeosacB3R*	CTACCAATTGCGTAACAGATGAGGGCAAGCGGATGG	Selection cassette amplification
U.atpC_2F	CTAGTGGCTCTATTTCGTATC	RT-qPCR
U.atpC_2R	ATAACTGGTTGGTGTGTGATC	RT-qPCR
U.lexA_F	GGTTACGCAATAAAGG	RT-qPCR
U.lexA_R	AGAACGGAGAATCAG	RT-qPCR
U.lrtA_F	GAAGACAAGCCAGTG	RT-qPCR
U.lrtA_R	TGAATAACGCCATAGC	RT-qPCR
U.petB_2F	TCAATGGTTCATGATCGTC	RT-qPCR
U.petB_2R	CATGATGTATTGGACAGAGG	RT-qPCR
U.psbY_F	ATTGGCGTGTAAATTGTAG	RT-qPCR
U.psbY_R	AACGTCCTGTAACCTGC	RT-qPCR
U.rplK_F	GATGATTATTCCTGTGG	RT-qPCR
U.rplK_R	GTCGTTGGCATTAAAG	RT-qPCR
U.sodB_F	CGCTGACTTTGGTAG	RT-qPCR
U.sodB_R	TCGGCAATGTAATCG	RT-qPCR
U.trxA_F	TTAGACAGCGAGTTAC	RT-qPCR
U.trxA_R	CACCACCATATCCAC	RT-qPCR
U.tufA_F	GAATTGGTGGAACTG	RT-qPCR
U.tufA_R	GTGATGGAGAATACG	RT-qPCR
D.ppa_2F	TAAATACGAGTTCGACAAAAG	RT-qPCR
D.ppa_2R	TCAATCATTCCAACATACC	RT-qPCR
D.pgk_2F	GAATTACAGTTCCTCCAAGG	RT-qPCR
D.pgk_2R	AGTCCAATTTGTCCTTCTTCC	RT-qPCR
D.slr2025_F	CAAGATAACGACCAG	RT-qPCR
D.slr2025_R	GAGAGCCACTAAATG	RT-qPCR
BD16SF1†	CACACTGGGACTGAGACAC	RT-qPCR
BD16SR1†	CTGCTGGCACGGAGTTAG	RT-qPCR

*Restriction enzyme recognition sites are underlined.

†Ferreira *et al.* (2009).

2.5 min at 72 °C, and a final extension at 72 °C for 7 min. The two purified PCR fragments were fused by 'overlap-PCR', and the reaction mixture (50 µl) contained 1.25 U *Taq* polymerase (GE Healthcare), 1 × reaction buffer (10 × reaction buffer: 100 mM Tris/HCl pH 9.0, 15 mM MgCl₂ and 500 mM KCl), 250 µM of each deoxyribonucleotide triphosphate, 0.2 µg BSA µl⁻¹, 130 nM each outer primer (5-O and 3-O) and 80 ng each purified DNA fragment. The PCR profile was 5 min at 95 °C followed by 35 cycles of 30 s at 95 °C, 45 s at 60 °C and 1.75 min at 72 °C, and a final extension at 72 °C for 10 min. The resulting product was purified and cloned into the vector pGEM-T Easy, according to the manufacturer's instructions, producing the pGDYH plasmid. A selection cassette, containing the *nptII* gene (conferring resistance to neomycin and kanamycin) and the *sacB* gene (sucrose sensitivity), was amplified by PCR from the plasmid pK18mobsacB (Schäfer *et al.*, 1994), using the primer pair NeosacB2F and NeosacB3R. The PCR mixture (20 µl) contained 0.5 U *Taq* polymerase (GE Healthcare), 1 × reaction buffer (10 ×

reaction buffer is 100 mM Tris/HCl pH 9.0, 15 mM MgCl₂ and 500 mM KCl), 200 µM of each deoxyribonucleotide triphosphate, 1 µM of each primer and 3 ng of each purified DNA fragment. The PCR profile was: 2 min at 95 °C followed by 40 cycles of 30 s at 95 °C, 30 s at 60 °C and 3 min at 72 °C and a final extension at 72 °C for 7 min. The purified PCR fragment was cloned into pGEM-T Easy, and the resulting vector was digested with *EcoRI* and *MunI* restriction enzymes (Fermentas), according to the manufacturer's instructions. Subsequently, the purified DNA fragment containing the selection cassette was cloned in the *MunI* restriction site of pGDYH, using the T4 DNA ligase (Fermentas) to form the plasmid pGDYH.NS. All the constructs were confirmed by DNA sequencing (STAB VIDA).

Generation of the *Synechocystis* sp. PCC 6803 *hoxYH* deletion mutant. *Synechocystis* was transformed using a method based on a procedure described by Williams (1988). Briefly, *Synechocystis* cultures were grown in BG11 medium at 30 °C, under continuous

light ($20 \mu\text{E m}^{-2} \text{s}^{-1}$), to $\text{OD}_{730} \sim 0.5$. Cells were harvested by centrifugation and resuspended in 1/10 volume of BG11. A 100 μl sample of these cells was incubated with the purified pGDYH.NS plasmid for 5 h, in light at room temperature, with a final plasmid DNA concentration of $6\text{--}20 \mu\text{g ml}^{-1}$. Cells were then spread onto Immobilon-NC membranes ($0.45 \mu\text{m}$ pore size, 82 mm, Millipore) resting on solid BG11 plates, grown at 30°C under continuous light, and were transferred to selective plates containing $10 \mu\text{g}$ kanamycin (Km) ml^{-1} after 24 h. Transformants were observed after 1–2 weeks. For complete segregation, Km-resistant colonies were grown at increasing Km concentrations (25 and $50 \mu\text{g ml}^{-1}$) and finally transferred into liquid medium. Mutants were then tested for sucrose sensitivity, and confirmed by PCR and Southern blot with a probe labelled with digoxigenin using the DIG DNA labelling kit (Roche Molecular Biochemicals) (Fig. 1). Subsequently, to remove the selection markers from the disruption mutant, cells were transformed as described above with the vector pGDYH, and the mutants were selected in solid BG11 containing 10% sucrose (w/v). These mutants were also screened for Km-sensitivity, and the full segregation was confirmed by PCR using primers external to the insertion sites (FO and RO, Table 1) and by Southern blot (Fig. 1). Southern blot was performed using genomic DNA that was digested with *Hind*III or *Hinc*II, and a probe covering the 5' flanking region of *hoxYH* genes (amplified by PCR using the primer pair 5-O and 5-I). A representative diagram of the mutant construction is depicted in Fig. 2. Moreover, the presence/absence of an active bidirectional hydrogenase in the wild-type and Δ *hoxYH* mutant was assessed by gas chromatography after placing the cells in dark and replacing air with argon.

Growth experiments. Cultures of *Synechocystis* wild-type and Δ *hoxYH* mutant were grown to $\text{OD}_{730} \sim 1.0$, and 24 h before the

beginning of the experiment, were transferred to fresh BG11 medium. Inocula of 200 μl were then added to sterile 4.5 ml cuvettes containing 1800 μl medium with or without 5 mM glucose and given concentrations of nitrate (0.1 or 1.5 g l^{-1}). Each experiment was performed in triplicate and under aseptic conditions. The cuvettes were closed with sterilized Parafilm and placed in acrylic racks specially designed for this experiment. The racks were placed in a chamber with constant temperature (25°C), under continuous light ($20 \mu\text{E m}^{-2} \text{s}^{-1}$) or a 12 h light ($20 \mu\text{E m}^{-2} \text{s}^{-1}$)/12 h dark regimen, and the OD_{730} was recorded daily.

Statistical analysis of the specific growth. The quantitative analysis of *Synechocystis* wild-type and Δ *hoxYH* mutant growth in various conditions was based on the optical density measurements (see above). For each bin, i , a specific growth parameter, representing the exponent of (assumed) exponential growth within the given time interval was calculated as

$$\mu_i = \frac{\log(x_{i+1}/x_i)}{t_{i+1} - t_i}$$

where t_i is time, in units of days running from day 0 to t_{imax} , and x_i is the corresponding measured optical density.

In order to work with a statistic that does not depend rather arbitrarily on test duration, particularly if under some particular conditions stationary growth is reached sooner than in others, we chose not to work with the mean specific growth rate but rather the maximum specific growth rate:

$$\mu_{\text{max}} = \max(\mu_i)_{i=0, \dots, i_{\text{max}}}$$

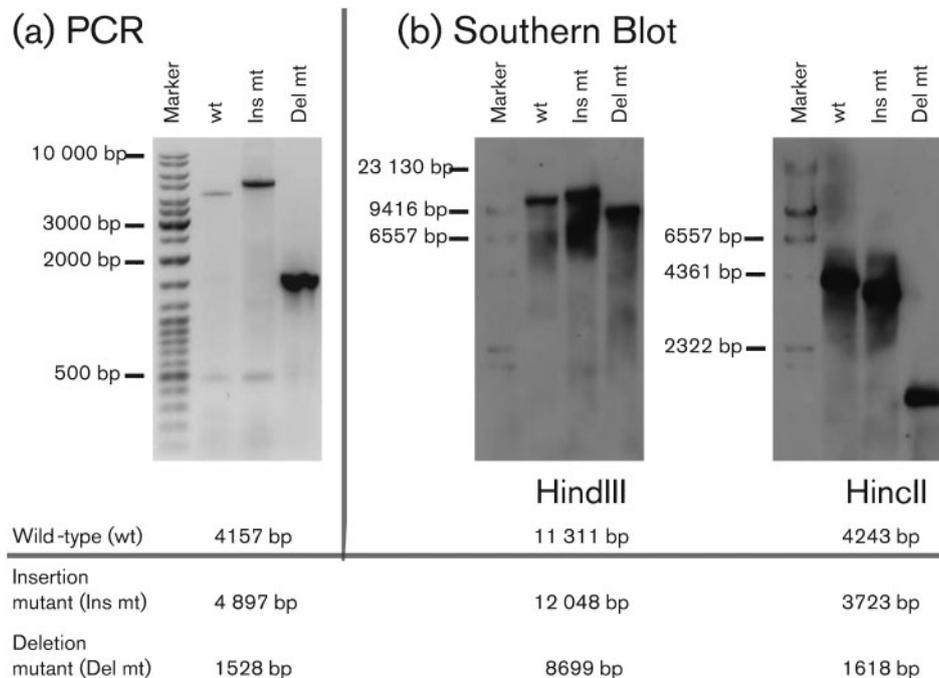


Fig. 1. Confirmation of the segregation of *Synechocystis* insertion (Ins mt) and deletion (Del mt) mutants by PCR amplification using a primer upstream of *hoxU* and another within *fabF* (a) and by Southern blot hybridization (b). Genomic DNA was digested with *Hind*III or *Hinc*II and hybridized with a probe covering the 5' flanking region of *hoxYH* genes. The expected sizes of the PCR products and restriction fragments are shown below the figure.

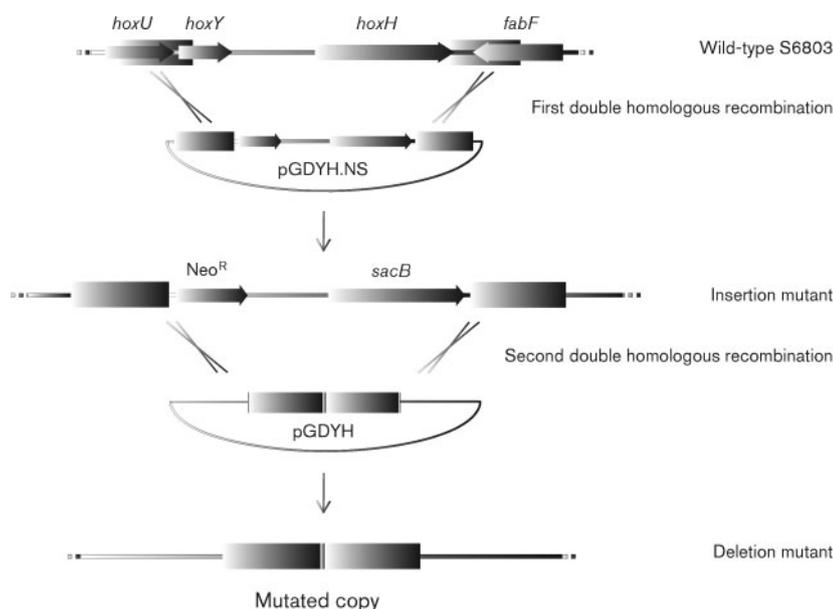


Fig. 2. Schematic representation of the experimental design used to generate the *Synechocystis* Δ *hoxYH* mutant.

In addition, we can obtain the time (t_{\max}) at which maximum specific growth is reached and the corresponding optical density recorded (x_{\max}).

From the three replicates of any of the experimental samples with a given level of light, nitrate and glucose, it was possible to calculate confidence intervals for the sample growth parameters, corrected using Student's *t*-test. The usual confidence level of 95% was selected and used to represent mean and error bars in Fig. 3.

Sample collection, protein extraction and iTRAQ labelling.

Cells were collected by centrifugation, the supernatant (medium) was removed, the pellets were snap-frozen in liquid nitrogen and preserved at -80°C prior to protein extraction. The protein extraction and quantification was performed as described by Gan *et al.* (2007). Protein samples (100 μg) were precipitated, prepared and digested as described by Chong *et al.* (2006). iTRAQ 8-plex labelling (ABSciex) was performed as described by Ow *et al.* (2009).

LC-MS and MS analysis. Two biological *Synechocystis* wild-type replicates were labelled with the 117 and 118 reagents and two biological Δ *hoxYH* mutant replicates were labelled with the 119 and 121 reagents (ABSciex). Peptide pre-fractionation of complex iTRAQ-labelled lysate was performed as described by Gan *et al.* (2007). Peptide detection was performed using LC-MS-MS iTRAQ compatible settings (see Gan *et al.*, 2007). Protein quantification and identification was carried out using Phenyx Software version 2.6 (Genebio). Spectra were searched against the Uniprot database (accessed August 2009), containing 3576 protein sequences of *Synechocystis*. The false discovery rate (FDR) was calculated using a reverse database generated with Phenyx (Reidegeld *et al.*, 2008), as described by Elias & Gygi (2007). Both the true and the reversed database were searched in the same run. The FDR was below 5% (Ow & Wright, 2009). Searches were performed in four steps in order to increase proteome coverage (see Supplementary Methods, available with the online version of this paper). Mass tolerances were set to 0.4 Da MS and 0.4 Da MS-MS. The peptide modifications were the same for each search (MMTS, fixed cysteine modification; oxidation, variable; iTRAQ_8plex_K, fixed; iTRAQ_8plex_N, fixed). The peptide lists generated by Phenyx were analysed as described by Ow *et al.* (2009). This list was used for further analysis as described by Pham

et al. (2010) to produce a list of differentially expressed proteins. Protein function and annotations were assigned according to CyanoBase (Nakamura *et al.*, 2000).

Pathway-level analysis of the proteomic data. The network modelling approach 'mixture model on graphs' (MMG) (Noirel *et al.*, 2008; Sanguinetti *et al.*, 2008) was used on the metabolic network of *Synechocystis* as given by the Kyoto Encyclopedia of Genes and Genomes (KEGG; accessed September 2009). This provides a pathway-level detection of changes based on the proteomics dataset, even when the changes are too small to pass the statistical test used to detect differential expression (for details see Noirel *et al.*, 2009). Briefly, this method classifies the genes' products into three categories: upregulated, downregulated or unregulated. This is based on assumed distribution of the log ratios (Gaussian distribution for unregulated proteins, exponential distribution for differentially regulated proteins) and the topology of the network: if an enzyme is surrounded in the metabolic network by other upregulated enzymes, it will be more likely to be classified as upregulated. The details are described by Sanguinetti *et al.* (2008).

An improvement with respect to the original implementation has been introduced here: instead of using a single variance parameter σ^2 for the unregulated proteins' Gaussian distribution, a protein-specific variance σ^2 is introduced. It is calculated as

$$\sigma^2 = \sigma_B^2 + (\sigma_{117}^2 + \sigma_{118}^2 + \sigma_{119}^2 + \sigma_{121}^2)/n$$

where the biological variation σ_B^2 is estimated from all eight-plex values; σ_{117}^2 etc. represent the peptide-level, technical variation which is adjusted by the number of MS-MS spectra, *n*. Proteins whose quantifications are more reliable will therefore be more reliably classified.

Sample collection and RNA extraction for transcriptional studies.

The sample collection for RNA extraction is depicted in Fig. 4. Briefly, three independent cultures of *Synechocystis* wild-type and Δ *hoxYH* mutant cells were grown under standard conditions (see above) and at the middle of the light phase, they were transferred to the dark and sparged with argon to induce anaerobiosis. Samples were collected 0, 60 and 120 min after anaerobiosis induction (t_0 , t_{60} and t_{120} , respectively). In addition, extra cultures were put back in light

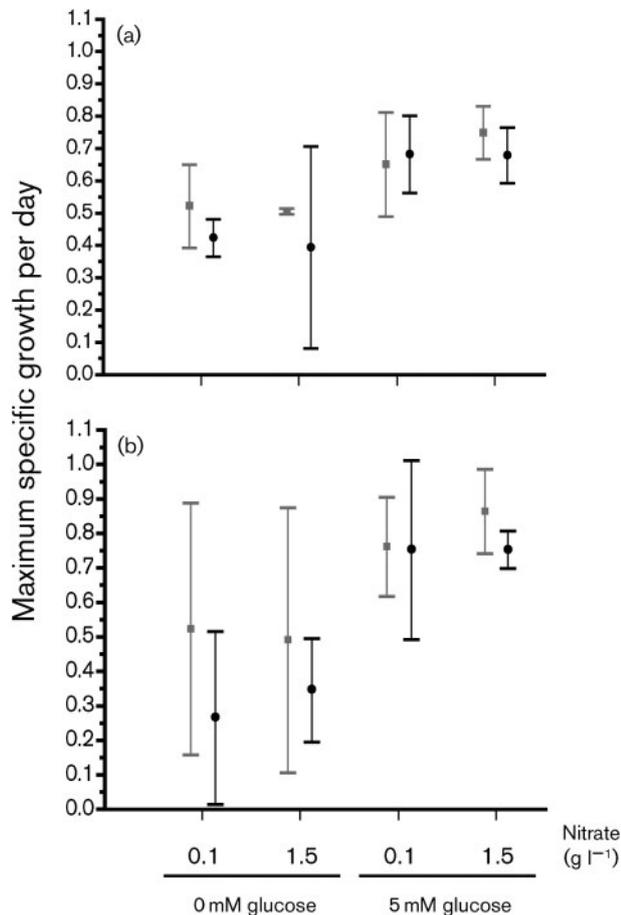


Fig. 3. Mean of the maximum specific growth of *Synechocystis* wild-type (■) and $\Delta hoxYH$ mutant (●), under different nitrate, glucose and light regimens: (a) 12 h light ($20 \mu\text{E m}^{-2} \text{s}^{-1}$)/12 h dark regimen; (b) continuous light ($20 \mu\text{E m}^{-2} \text{s}^{-1}$). Whiskers represent confidence intervals. Mean and confidence intervals limits were calculated and corrected using the Student's *t*-distribution.

60 min after anaerobiosis induction, and samples were collected 60 min later [$t_{120(60L)}$]. To inhibit RNA transcription, rifampicin was added to each sample upon harvesting at a final concentration of $300 \mu\text{g ml}^{-1}$. Cells were centrifuged (8 min at 4500 g , 4°C) and washed once with fresh BG11, and the cell pellets were frozen at -80°C . RNA was extracted using the TRIZOL Reagent (Invitrogen) according to the method described previously (Leitão *et al.*, 2006). RNA was quantified on a NanoDrop 2000 spectrophotometer (Thermo Scientific) and the quality was checked using the Experion RNA StdSens Analysis kit (Bio-Rad).

Transcription analysis by real time quantitative PCR (RT-qPCR). For cDNA synthesis, $1 \mu\text{g}$ total RNA was transcribed with the iScript Select cDNA Synthesis kit (Bio-Rad), using the random primers supplied and following the manufacturer's instructions. The primer pairs used for the gene transcription analysis are listed in Table 1 and Supplementary Table S1 (available with the online version of this paper), and were designed using Beacon Designer 6 software (PREMIER Biosoft International). For each analysis, the 16S rRNA was used for normalization. The real-time PCRs

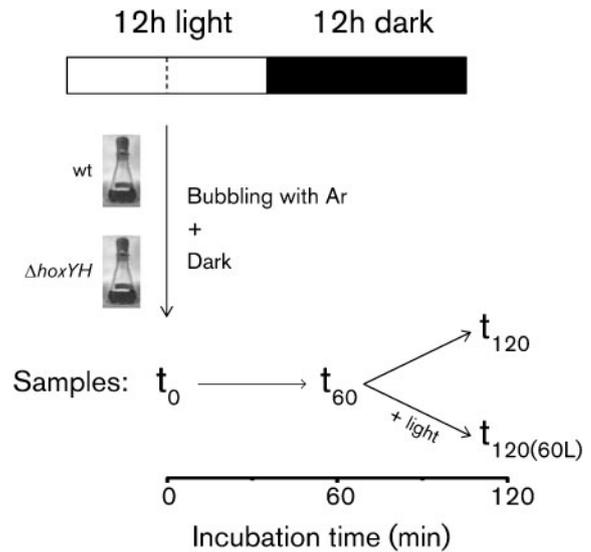


Fig. 4. Schematic representation of the experimental procedure for the collection of the samples for the RNA extraction used for RT-qPCR.

were performed using $0.25 \mu\text{M}$ each primer, $10 \mu\text{l}$ iQ SYBR Green Supermix (Bio-Rad) and $2 \mu\text{l}$ template cDNA, and technical triplicates were performed. The PCR profile was: 3 min at 95°C followed by 40 – 60 cycles of 30 s at 95°C , 30 s at 51°C and 30 s at 72°C ; exceptionally the annealing temperature for the study of *atpC* and *ppa* was 53°C and 54°C for *pgk*. Standard dilutions of the cDNA were used to check the relative efficiency and quality of primers. Negative controls (no template cDNA) were included in all assays. A melting curve analysis was performed at the end of each PCR program, to exclude the formation of non-specific products. Real time quantitative PCRs (RT-qPCRs) were carried out in the iCycler iQ5 Real-Time PCR Detection System (Bio-Rad). The data obtained were analysed using the Bio-Rad iQ5 Optical System Software v2.1 (Bio-Rad). The two-way ANOVA statistical analysis of the data obtained in the RT-qPCR experiment was performed using the software GraphPad Prism v5 (GraphPad Software), comparing *Synechocystis* $\Delta hoxYH$ mutant with the wild-type, under dark anoxic conditions (t_0 , t_{60} and t_{120}) and after cells were transferred back to light [t_{60} and $t_{120(60L)}$].

RESULTS AND DISCUSSION

Construction and physiological characterization of *Synechocystis* sp. PCC 6803 $\Delta hoxYH$ deletion mutant

The genes coding for the two subunits corresponding to the hydrogenase part of the bidirectional hydrogenase (*hoxYH*) were deleted from the *Synechocystis* genome in a two-step procedure: a first transformation was performed using the plasmid pGDYH.NS in order to replace *hoxYH* with a selection cassette conferring kanamycin resistance and sucrose sensitivity, followed by a second transformation, using the plasmid pGDYH, in order to remove the selection cassette. The complete segregation of both mutants was confirmed by resistance/sensitivity to kanamycin/sucrose,

and by PCR and Southern blot (Fig. 1). The absence of H₂ evolution (bidirectional hydrogenase activity) by the $\Delta hoxYH$ mutant was confirmed by gas chromatography (data not shown). To elucidate the possible effects of this mutation on the fitness of *Synechocystis*, the growth of both the wild-type and the $\Delta hoxYH$ mutant was compared under several physiological conditions (see Methods). In Fig. 3(a) the sample mean of maximum specific growth, μ_{max} , at different levels of nitrate and glucose, under 12 h light (20 $\mu\text{E m}^{-2} \text{s}^{-1}$)/12 h dark cycles for *Synechocystis* wild-type and $\Delta hoxYH$ mutant are shown. As can be observed, in the presence of 1.5 g nitrate l^{-1} and with glucose, the wild-type μ_{max} increases significantly compared with the same conditions in the absence of glucose. In Fig. 3(b), with continuous 20 $\mu\text{E m}^{-2} \text{s}^{-1}$ irradiance, glucose increases maximum specific growth, while nitrate decreases the variability of the mutant. In conclusion, these analyses show no significant difference in terms of specific growth for *Synechocystis* wild-type and $\Delta hoxYH$ mutant under the different levels of nitrate, glucose and irradiance used. These results are in agreement with what was previously reported for a disruption mutant (with a kanamycin resistance cassette within *hoxH*) growing in continuous light (100 $\mu\text{E m}^{-2} \text{s}^{-1}$) and bubbled with air (Appel *et al.*, 2000). However, differences in growth have been detected when cultures were bubbled with CO₂-enriched air [2 % (v/v) CO₂], suggesting that $\Delta hoxH$ cells need more time than the wild-type to reach the maximal growth rate (Appel *et al.*, 2000).

The role of the bidirectional hydrogenase in cyanobacterial metabolism is not fully understood (Bothe *et al.*, 2010a; Carrieri *et al.*, 2011; Tamagnini *et al.*, 2007). However, it was hypothesized by Ludwig *et al.* (2006) that the ancestor of cyanobacteria would have vertically transmitted the two hydrogenases genes (*hox* – bidirectional hydrogenase, *hup* – uptake hydrogenase), and that none, one or both enzymes were subsequently lost through evolution. Thus, the current distribution would be the result of cyanobacterial adaptation to various ecological niches. About 50 % of the

N₂-fixing strains do not possess the bidirectional hydrogenase (Ludwig *et al.*, 2006; Schütz *et al.*, 2004; Tamagnini *et al.*, 1997, 2000), and although most of the non-N₂-fixing strains contain it, this enzyme does not seem to be essential for cell survival (Appel *et al.*, 2000; Masukawa *et al.*, 2002). Accordingly, our results suggest that, under the conditions tested, the bidirectional hydrogenase has no significant effect on the fitness of *Synechocystis*. Altogether, and taking also into account the results obtained by Appel *et al.* (2000), the absence of this enzyme seems to have only a marginal effect.

Comparison of the proteomes of wild-type *Synechocystis* and of the $\Delta hoxYH$ mutant

The proteomes of *Synechocystis* wild-type and of the $\Delta hoxYH$ mutant were analysed using the iTRAQ methodology, under conditions in which the hydrogenase activity is favoured – dark anoxic conditions – and the effect of the mutation should be easily observed (Cournac *et al.*, 2002, 2004). In total, 210 proteins were identified using Phenyx v2.6, of which there were 145 with two or more MS/MS spectra; false positive discovery rate was estimated to be 1.7 %. None of the bidirectional hydrogenase subunits was detected, probably due to their low abundance in the cells. To date, only Gan *et al.* (2005) have been able to identify a bidirectional hydrogenase subunit in proteomics studies. Out of the 210 proteins identified, 192 proteins were quantified, of which there were 131 with two or more MS–MS spectra. The methodology for quantification was described by Ow *et al.* (2009). The peptide matches and the protein identifications and quantifications are included in Supplementary Table S2, as recommended by Noirel *et al.* (2011). The quantified proteins were further analysed to determine which ones had their levels significantly altered by the $\Delta hoxYH$ mutation, according to the method used by Pham *et al.* (2010). According to this analysis, 17 proteins were differentially expressed, with a 95 % confidence interval, with five being less abundant in the mutant (Fig. 5 and Table 2). Sixteen of these proteins

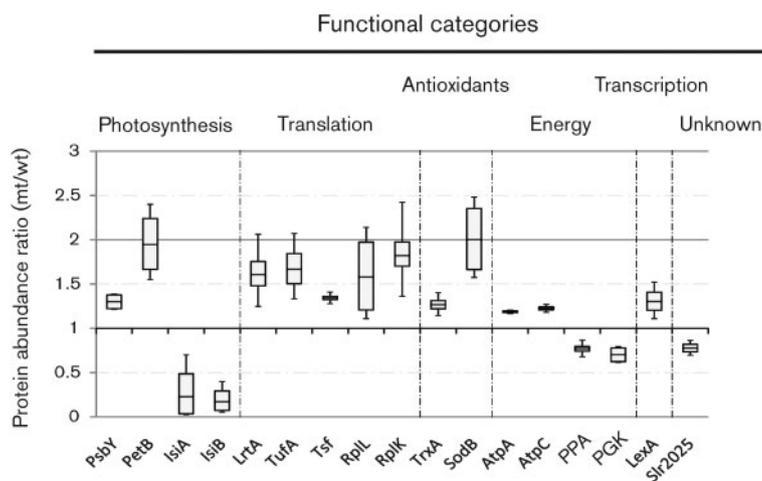


Fig. 5. Box-and-whisker plot of the abundance ratio between proteins showing significant differential fold changes in *Synechocystis* $\Delta hoxYH$ mutant and wild-type. The proteins are grouped according to their functional categories.

Table 2. List of proteins exhibiting significant differences in their abundance in *Synechocystis* Δ *hoxYH* mutant compared with the wild-type strain, after induction of dark anoxic conditions

Functional categories: PR, photosynthesis and respiration; TL, translation; AD, antioxidant defences; EM, energy metabolism; Tr, transcription; U, unknown.

ID	Gene*	Protein	Putative function/characteristics of the encoded protein*	Category
<i>sll0247</i>	<i>isiA</i>	IsiA	Iron-stress chlorophyll-binding protein, homologous to <i>psbC</i> (CP43)	PR
<i>sll0248</i>	<i>isiB</i>	IsiB	Flavodoxin	PR
<i>sll0947</i>	<i>lrtA</i>	LrtA	Light repressed protein A homologue	TL
<i>sll1099</i>	<i>tufA</i>	TufA	Elongation factor Tu	TL
<i>sll1261</i>	<i>tsf</i>	Tsf	Elongation factor Ts	TL
<i>sll1326</i>	<i>atpA</i>	AtpA	ATP synthase alpha chain	EM
<i>sll1327</i>	<i>atpC</i>	AtpC	ATP synthase gamma chain	EM
<i>sll1626</i>	<i>lexA</i>	LexA	Transcription factor	Tr
<i>sll1743</i>	<i>rpl11</i>	RplK	50S ribosomal protein L11	TL
<i>sll1746</i>	<i>rpl12</i>	RplL	50S ribosomal protein L12	TL
<i>slr0342</i>	<i>petB</i>	PetB	Cytochrome <i>b₆</i>	PR
<i>slr0394</i>	<i>pgk</i>	PGK	Phosphoglycerate kinase	EM
<i>slr0623</i>	<i>trxA</i>	TrxA	Thioredoxin	AD
<i>slr1516</i>	<i>sodB</i>	SodB	Iron superoxide dismutase	AD
<i>slr1622</i>	<i>ppa</i>	PPA	Soluble inorganic pyrophosphatase	EM
<i>slr2025</i>	<i>slr2025</i>	Slr2025	Hypothetical protein of unknown function DUF1821	U
<i>sml0007</i>	<i>psbY</i>	PsbY	Photosystem II protein Y	PR

*According to CyanoBase annotation.

fit into five functional categories: photosynthesis and respiration (IsiA, IsiB, PetB and PsbY), translation (LrtA, RplL, RplK, Tsf and TufA), antioxidant defences (SodB and TrxA), energy metabolism (AtpA, AtpC, PGK and PPA) and transcription (LexA). Additionally, a protein of unknown function was identified (Slr2025). The differential expression of each one of these proteins will be discussed in light of the putative roles of the bidirectional hydrogenase.

The association of the bidirectional hydrogenase activity with the photosynthetic and respiratory processes has been extensively discussed previously (e.g. Appel & Schulz, 1996, 1998; Appel *et al.*, 1999, 2000; Ludwig *et al.*, 2006; Schmitz & Bothe, 1996). In both cases, the bidirectional hydrogenase would serve as an important element for a proper redox balance of the electron-transport chains in transition states (Appel & Schulz, 1998). Our results revealed the higher abundance of a photosystem II (PSII) protein (PsbY) and of PetB in the Δ *hoxYH* mutant, indicating that both photosynthetic and respiratory machineries are being affected by the loss of hydrogenase activity. Moreover, the photosynthesis-associated proteins IsiA and IsiB, which are expressed under iron-deficiency conditions, were less abundant in the Δ *hoxYH* mutant, suggesting a higher bioavailability of this metal in the mutant. Since the cyanobacterial hydrogenases are metalloproteins possessing iron–sulfur clusters and nickel–iron active sites, it is likely that the absence of this enzyme could contribute to higher iron content in the mutant cells. It has been suggested that, in *Synechocystis*, the *isiAB* operon is under the control

of the ferric uptake regulator (Fur) (Kunert *et al.*, 2003; Vinnemeier *et al.*, 1998). The importance of Fur is not limited to its participation as a modulator in all the iron-dependent-regulation genes; it has been shown that Fur is involved in the oxidative stress response (Nunoshiba *et al.*, 1999) and in overcoming acidic stress situations (Hall & Foster, 1996). Therefore, the lower abundance of IsiA and IsiB in the Δ *hoxYH* mutant could be related to a stress condition that the cells have to cope with.

The higher abundance of the five proteins involved in translation in the Δ *hoxYH* mutant indicates that protein synthesis is altered. Two of these proteins are structural components of the ribosome (RplK and RplL) and two others are involved in the process of translation itself (TufA and Tsf), indicating a reinforcement of the translational machinery in the mutant. Additionally, LrtA belongs to the σ^{54} modulation protein/ribosomal protein S30EA family and is also more abundant in the Δ *hoxYH* mutant. Although no clear function has been attributed to this protein, its homologue in *E. coli* – the ribosome binding protein Y (YfiA) – putatively works as a stress-response protein that binds the ribosomal subunit interface and arrests translation (Agafonov *et al.*, 2001; Vila-Sanjurjo *et al.*, 2004). The increased levels of LrtA in the mutant suggest a tighter control of translation, as a response to the stress condition caused by the absence of the bidirectional hydrogenase. Moreover, according to CyanoBase, this protein is assigned to the functional category of ‘adaptations and atypical conditions’. Furthermore, a large-scale analysis of *Synechocystis* protein–protein interaction (Sato

et al., 2007) reported that LrtA interacts with a hypothetical protein encoded by *sll1773*, which is a Pirin-like protein induced under stress conditions (Hihara *et al.*, 2004).

Interestingly, even after oxygen has been removed from the medium, *Synechocystis* Δ *hoxYH* mutant cells still display increased levels of the enzymes SodB and TrxA, which act as antioxidant defences. SodB, the iron superoxide dismutase (Fe-SOD), catalyses the elimination of superoxide radicals (O_2^-). The higher levels of this protein may indicate a more reductive intracellular environment, leading to an increased concentration of toxic oxygen reduction products, and therefore, the necessity for higher levels of antioxidant defences. Furthermore, Nefedova *et al.* (2003) reported that mutants lacking SodB are non-viable under standard photoautotrophic conditions, emphasizing the importance of the Fe-SOD for providing resistance of *Synechocystis* cells to oxidative stress. Thioredoxins constitute a large family of redox-active enzymes, capable of reducing protein disulphides, and participate in a variety of processes, such as enzyme modulation, donation of reducing equivalents and signal transduction. Lindahl & Florencio (2003) have identified several proteins in *Synechocystis* likely to be thioredoxin (TrxA) substrates, mainly enzymes participating in anabolic processes. Among these is the elongation factor EF-Tu (TufA), which has been reported to interact with chloroplast thioredoxins (Motohashi *et al.*, 2001). Both TrxA and TufA levels are increased in the Δ *hoxYH* mutant compared with the wild-type. It has been suggested that TrxA is regulated by the photosynthetic electron transport (Navarro *et al.*, 2000; Pérez-Pérez *et al.*, 2009); therefore, the differential expression of TrxA protein may be due to a higher redox imbalance in the mutant. Again, significant changes in proteins related to defence mechanisms suggest the mutant is coping with a stress generated by the lack of the bidirectional hydrogenase, and this stress is likely to be related to the redox state of the cell.

Regarding the energy-metabolism-related proteins, two subunits of the ATP synthase (AtpA and AtpC) were more abundant in the Δ *hoxYH* mutant, while the phosphoglycerate kinase (PGK) and soluble inorganic pyrophosphatase (PPA) were less abundant. The ATP synthase is a ubiquitous membrane enzyme complex that functions to couple ATP synthesis to proton translocation across a membrane and, in cyanobacteria, this complex is present in both thylakoid and plasma membranes (Sherman *et al.*, 1994). The higher abundance of the two ATP synthase subunits suggests a higher requirement for this enzyme, under the conditions tested. Since the mutant lacks an active bidirectional hydrogenase, which is responsible for the reduction of protons to molecular hydrogen under anoxic conditions, one can hypothesize that the higher abundance of the ATP synthase is a cellular mechanism to cope with the excess of H^+ in order to maintain intracellular pH homeostasis. This mechanism has already been described for cell adaptations to changes in pH (Padan *et al.*, 2005; Summerfield & Sherman, 2008). PGK is the enzyme catalysing the reversible phosphorylation

of 3-phosphoglycerate to 1,3-diphosphoglycerate. It is involved in the Calvin cycle, in glycolysis and in the glycolytic degradation of glycogen (fermentation). In the filamentous cyanobacterium *Arthrospira* (*Spirulina*) *maxima*, H_2 evolution represents a major pathway for energy (ATP) production during fermentation by regenerating the NAD^+ essential for the glycolytic degradation of glycogen and the catabolism of other substrates (Ananyev *et al.*, 2008). The lower abundance of PGK in the mutant under dark anoxic conditions can, therefore, be related to a decrease in the glycogen catabolism, due to a lower availability of NAD^+ . PPA is an essential and ubiquitous metal-dependent enzyme that is thought to be involved in the removal of inorganic pyrophosphate (PPi), which is a by-product of many vital anabolic reactions (Kornberg, 1962). It has been shown that PPA function is essential for cell viability and that this protein is more abundant in *Synechocystis* cells subjected to phosphate deprivation (Gómez-García *et al.*, 2003). Accordingly, the lower abundance of PPA in the mutant suggests a higher availability of phosphate within the cells.

The transcription factor LexA is encoded in almost every bacterial group with a wide range of evolutionary distances and plays an important role in the SOS response in *E. coli* (Fernández de Henestrosa *et al.*, 2000; Little & Mount, 1982). Still, its precise functions in each group/species are largely unknown. It has been shown that in several cyanobacteria this protein interacts with the promoter region of the bidirectional hydrogenase (Antal *et al.*, 2006; Gutekunst *et al.*, 2005; Oliveira & Lindblad, 2005, 2009; Sjöholm *et al.*, 2007) and it has been suggested that LexA is a mediator of the redox state in *Synechocystis* cells (Antal *et al.*, 2006; Patterson-Fortin *et al.*, 2006). Interestingly, the LexA regulator was more abundant in the Δ *hoxYH* mutant. Since it was proposed that LexA acts as a transcriptional activator of the *hox* genes in *Synechocystis* (Gutekunst *et al.*, 2005), the higher abundance of LexA could be a cellular response to the lack of a functional bidirectional hydrogenase. It can be postulated that a feedback loop leads to an increased quantity of activator that in the wild-type would promote the transcription of the *hox* genes. Moreover, a predictive analysis of LexA binding sites on 26 sequenced cyanobacterial genomes (Li *et al.*, 2010) showed that this protein could regulate the expression of some of the genes reported in this study: *psbY*, *ppa*, *pgk*, *petB*, *isiB*, *tsf*, *trxA*, *rpl12*, *rpl11* and *lexA* itself.

According to a similarity BLAST analysis (Johnson *et al.*, 2008), the protein Slr2025 belongs to the DUF1821 protein family, which comprises uncharacterized proteins principally found in cyanobacteria with subunit and dimer structures similar to the 'Type III secretory system chaperone' from proteobacteria. In a large-scale protein-protein interaction analysis in *Synechocystis* (Sato *et al.*, 2007) using the yeast two-hybrid (YTH) system it was revealed that Slr2025 interacts with four other proteins: Slr2037 (unknown protein), Slr1444 (hypothetical protein), Sll1875 (HO₂, haem oxygenase) and notably Sll1226

(HoxH, hydrogenase large subunit of the bidirectional hydrogenase).

Overall, the iTRAQ results indicate that, under dark anoxic conditions, *Synechocystis* cells lacking a functional bidirectional hydrogenase have to cope with a more stressful intracellular environment. Nonetheless, the cellular responses triggered seem to be sufficient to avoid a major interference in its metabolism as a whole. These conclusions are consistent with our growth experiments that showed no significant differences between the wild-type and Δ *hoxYH* mutant, under the conditions tested. Moreover, the proteins with altered levels are mainly related to the redox and energy state of the cells, corroborating the previously attributed role of the bidirectional hydrogenase in avoiding an overload of low potential electrons in the electron transport chains (Appel & Schulz, 1998; Appel *et al.*, 2000; Carrieri *et al.*, 2011; Cournac *et al.*, 2002, 2004).

Pathway analysis

The analysis based on 'mixture model on graphs' (MMG) provided some insight into the biological pathways that may undergo subtler, yet wider regulation patterns (see Supplementary Fig. S1 and Supplementary Table S2, available with the online version of this paper). This reveals and predicts changes in the central metabolism that had not been detected using the traditional protein-to-protein analysis of the previous section. Although individual protein quantifications are questionable, overall the evidence at the pathway level seems to point at a general downregulation of the pentose phosphate pathway (PPP), of glycolysis/gluconeogenesis and of the glyceraldehyde-3-phosphate-to-pyruvate route (see Fig. 6). This is of particular interest if we consider the putative involvement of the bidirectional hydrogenase in fermentation. It has been proposed that during fermentation in *Cyanothece* sp. PCC 7822, protons may serve as terminal electron acceptors, being reduced to molecular hydrogen in a hydrogenase-catalysed reaction (van der Oost *et al.*, 1989). In addition, two possible fermentative pathways are: (i) the heterofermentative lactate fermentation, via the pentose phosphate pathway; and (ii) the CoA-dependent decarboxylation of pyruvate, via glycolysis (Stal & Moezelaar, 1997; van der Oost *et al.*, 1989). In a recent study, McNeely *et al.* (2010) reported the successful improvement of hydrogen production in *Synechococcus* sp. PCC 7002, by redirecting the reductant flux, through metabolic engineering. In their study, the gene encoding D-lactate dehydrogenase (*ldhA*), responsible for the formation of the main fermentation product (lactate) was inactivated, resulting in the increase of other metabolic by-products, namely hydrogen. Interestingly, the *ldhA* mutant did not exhibit significant differences in terms of photoautotrophic growth in aerobic respiration either. In our study, in the absence of an active hydrogenase, capable of producing hydrogen to remove the excess reducing equivalents produced during the fermentative degradation of carbohydrates, it is reasonable to expect (i) a downregulation of the

pathways originating this reducing power (namely glycolysis and PPP) and/or (ii) an increase in other metabolic by-products. This has to be further investigated, through experiments targeting the fermentative metabolism of the wild-type and the Δ *hoxYH* mutant.

Transcription analysis of genes encoding proteins with different abundance in *Synechocystis* wild-type and Δ *hoxYH* mutant

To assess if the changes observed at the protein levels were due to transcriptional regulation, RT-qPCR analyses were carried out. This experiment covers all gene clusters encoding the proteins showing different relative levels reported above, with the exception of *isiAB* and *tsf*, for which the PCR conditions could not be optimized (secondary amplification products were always detected in the melting curve analysis). For this experiment, three biological and three technical replicates were used (see Methods for further details). Fig. 7 shows the transcriptional analysis of the genes encoding proteins representative of each functional category (full analysis is shown in Supplementary Fig. S2, available with the online version of this paper; graphics were constructed based on one biological replicate representative of the biological variability observed). In all cases, a two-way ANOVA analysis was performed (Table 3), revealing significant differences between the wild-type and the Δ *hoxYH* mutant in the transcription of *psbY*, *petB*, *slr2025*, *ppa*, *pgk* and *lexA*. The transcription patterns of the genes *ppa*, *pgk*, *lexA* and *slr2025* seem to be in agreement with the differences found in the iTRAQ experiment, while for the other two genes (*psbY* and *petB*), the changes observed in protein abundance do not seem to be directly related to their transcriptional regulation and, most likely, the regulation is exerted at post-transcriptional and/or protein synthesis/degradation levels.

Additionally, this experiment revealed significant variations in the transcript levels of all genes under the physiological conditions tested (dark-anaerobiosis/light-aerobiosis). Overall, the genes exhibited one of the following transcription patterns: (i) induction under dark anoxic conditions and repression by light (*sodB*); (ii) repression under dark anoxic conditions and induction by light (*atpC*, *psbY* and *trxA*); (iii) induction under dark anoxic conditions and unaffected by light (*lexA*); (iv) repression under dark anoxic conditions and unaffected by light (*petB*, *pgk*, *ppa*, *slr2025* and *tufA*); and (v) transient induction under dark anoxic conditions and unaffected by light (*lrtA* and *rplK*). The transcription patterns of the genes grouped in (i), (ii) and (iii), which presented greater transcript levels variations, and *petB* that presented an inverse correlation with the proteomics analysis, will be discussed.

Interestingly, *sodB* transcript levels increased after anaerobiosis induction and decreased when cells were transferred back to the light. These results are unexpected, since it has been previously shown that, in *Synechocystis*, *sodB* expression is light-dependent, being downregulated in the

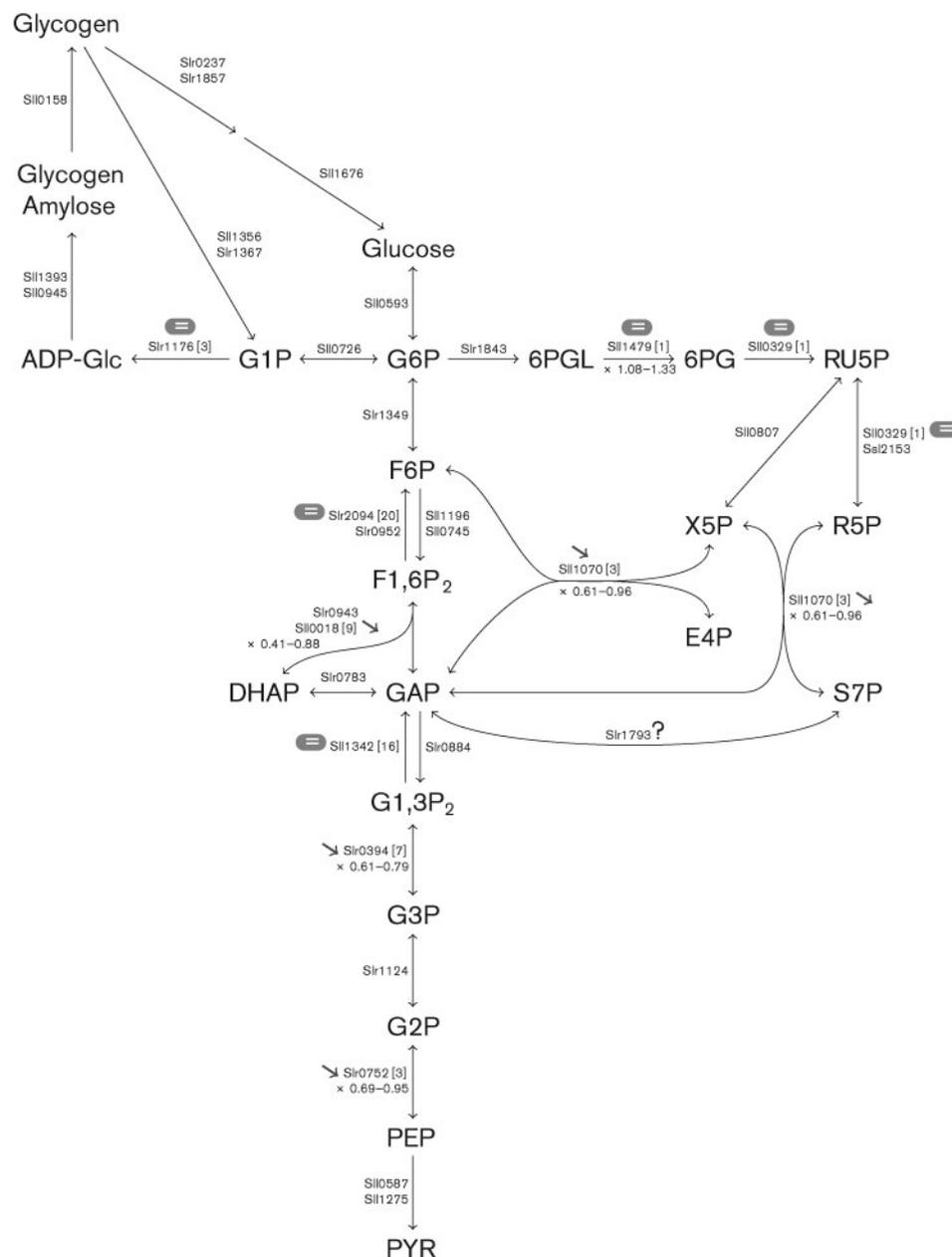


Fig. 6. Protein changes in the glycolysis/gluconeogenesis, the PPP pathway and glycogen metabolism in *Synechocystis* $\Delta hoxYH$ mutant according to the MMG analysis. The arrows indicate underexpression of enzymes in the mutant and the equals signs indicate expression levels that are not significantly different. The number of MS/MS spectra used for quantification is indicated in brackets (adapted from Osanai *et al.*, 2005). Abbreviations: G1P, glucose-1-phosphate; G6P, glucose 6-phosphate; 6PGL, 6-phosphogluconolactone; 6PG, 6-phosphogluconate; F6P, fructose-6-phosphate; F1,6P₂, fructose 1,6-bisphosphate; GAP, glyceraldehyde 3-phosphate; DHAP, dihydroxyacetone phosphate; G1,3P₂, 1,3-bisphosphoglycerate; G3P, 3-phosphoglycerate; G2P, 2-phosphoglycerate; PEP, phosphoenolpyruvate; PYR, pyruvate; RU5P, ribulose-5-phosphate; R5P, ribose 5-phosphate; S7P, sedoheptulose 7-phosphate; X5P, xylulose 5-phosphate; E4P, erythrose 4-phosphate.

darkness (Kim & Suh, 2005; Ushimaru *et al.*, 2002). Still, an increase in the transcript levels of *sodB* induced by anaerobiosis has already been described for other organisms, although SodB is known to act as an antioxidant defence (Dubrac & Touati, 2000; Kanematsu & Sato, 2008).

The increase in *sodB* transcript levels was attributed to the Fur regulator, which may play an important role in the oxidative stress response, as referred to above. This may be a mechanism for the cell to prepare and protect itself from an eventual O₂ burst and the concomitant production of

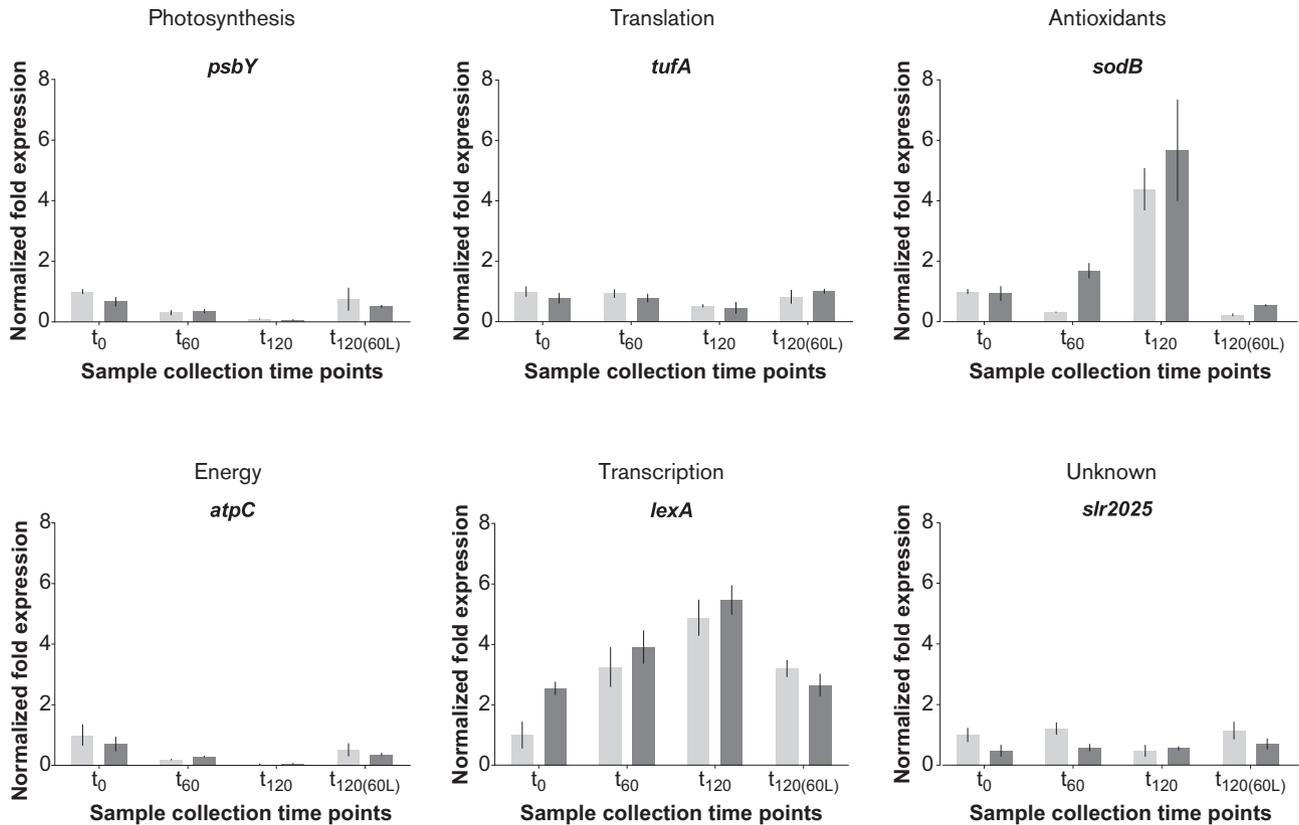


Fig. 7. Transcription profiles of selected genes encoding proteins (one of each functional category, see Fig. 5) showing significant differential fold changes in *Synechocystis* wild-type (light grey) and Δ *hoxYH* mutant (dark grey) and evaluated by RT-qPCR. The mean normalized expression was calculated relative to the transcription of the 16S rRNA gene and the reaction internal normalization was performed using the sample t_0 from *Synechocystis* wild-type. Error bars represent SD.

Table 3. Statistical significance of the data obtained in the RT-qPCR analysis, comparing *Synechocystis* Δ *hoxYH* mutant (mt) with the wild-type (wt) under dark anoxic conditions (Dark) and after transferring cells back to light (Light)

Gene	P-values mt/wt	
	Dark*	Light†
<i>psbY</i>	<0.05/<0.001	NS/<0.05
<i>petB</i>	<0.05/<0.01	NS/NS
<i>lrtA</i>	NS/<0.01	NS/NS
<i>tufA</i>	NS/<0.01	NS/NS
<i>rplK</i>	NS/<0.001	NS/NS
<i>trxA</i>	NS/<0.05	NS/<0.01
<i>sodB</i>	NS/<0.001	<0.001/<0.01
<i>atpC</i>	NS/<0.01	NS/<0.05
<i>ppa</i>	<0.01/<0.001	NS/NS
<i>pgk</i>	<0.05/<0.001	NS/NS
<i>lexA</i>	<0.05/<0.001	NS/NS
<i>slr2025</i>	<0.01/<0.05	<0.01/NS

*Two-way ANOVA analysis of t_0 , t_{60} and t_{120} .

†Two-way ANOVA analysis of t_{60} and $t_{120(60L)}$.

reactive oxygen species. However, SodB activity did not present major differences before and after light transition, as it was observed in both gel and spectrophotometric superoxide dismutase activity assays (data not shown).

Cyanobacteria switch immediately from photoautotrophy to fermentation when exposed to dark anoxic conditions and, under these conditions, it is expected that the ATP synthase transcription is downregulated. It is generally assumed that the energy yield of fermentation is minimal, thus sustaining only survival (Stal & Moezelaar, 1997). This implies that biosynthetic processes are switched off during anaerobic dark metabolism in order to decrease energy demand. When the cells are transferred back to light, photosynthesis is reactivated and the requirement for ATP synthase may induce the transcription of the genes coding for its subunits. Moreover, the gene *atpC* encodes the gamma subunit of the ATP synthase, and AtpC is believed to be involved in the regulation of the ATPase activity and the flow of protons through the membrane-embedded domain of the enzyme (CF_0 complex) (Krenn *et al.*, 1997). Similarly, when cells are transferred into dark conditions, there is a decrease in the transcription of *psbY*, which encodes a core component of PSII (Kawakami *et al.*, 2007).

This is probably due to the cessation of the photosynthesis and downregulation of the transcription of components of the photosystems. A small increase in *psbY* transcript levels can be observed when cells were exposed back to light. In accord with what has been previously described by Navarro *et al.* (2000) and by Pérez-Pérez *et al.* (2009), *trxA* transcription slightly decreases in the dark and increases when cells are transferred to light, exhibiting photosynthetic dependence.

LexA seems to have a broad effect on gene regulation in *Synechocystis*, and although its precise function is unknown, LexA does not seem to be a regulator of the SOS response in this organism, as has been proposed for other cyanobacteria (Li *et al.*, 2010). Several studies reported that *lexA* transcription patterns do not always follow the same trend as those of *hox* genes (Oliveira & Lindblad, 2008; Zhang *et al.*, 2008), indicating that additional factors may be involved in the regulation of the transcription of the bidirectional hydrogenase genes of *Synechocystis* (Oliveira & Lindblad, 2008, 2009). However, our results are in agreement with previous studies that reported an increase in *lexA* transcript levels under microaerobic conditions (Kiss *et al.*, 2009; Summerfield *et al.*, 2011). In fact, in our study, after induction of dark anoxic conditions, an increase in the transcription of *lexA* can be observed (see Fig. 7). The fact that the transcription of both *lexA* and *hox* genes increases under anaerobiosis (Oliveira & Lindblad, 2008) and that LexA interacts with the promoter region of the *hox* genes (Antal *et al.*, 2006; Gutekunst *et al.*, 2005; Oliveira & Lindblad, 2005, 2009; Sjöholm *et al.*, 2007), suggests that LexA activates the transcription of the *hox* genes particularly under dark anoxic conditions.

The *petB* gene encodes the cytochrome b_6 subunit of the cytochrome b_6f complex that mediates electron transfer between PSII and PSI, cyclic electron flow around PSI and state transitions (Cramer *et al.*, 1994; Kruip *et al.*, 1994; Stroebel *et al.*, 2003). As has been previously reported for *Synechococcus* sp. PCC 7002 (Brand *et al.*, 1992), *petB* transcripts decrease when cells are placed in dark conditions. Overall, the transcript levels of *petB* are lower in the Δ *hoxYH* mutant, in contrast with the results obtained at the protein level (which was approximately twofold higher in the mutant), again indicating post-transcriptional regulation. Our results on the transcription of both *psbY* and *petB* are in disagreement with a study performed using another hydrogenase mutant (Δ *hoxH*), where the amounts of the two transcripts were slightly higher than in the wild-type (Appel *et al.*, 2000). However, in that work, Northern blot hybridizations were performed using RNA isolated from 7-day-old cultures, grown under light and bubbled with air; therefore a direct comparison with the present study is not possible. Nonetheless, both studies concur that the hydrogenase has an effect on the regulation of the *petB* gene.

Interestingly, a recent study on *Synechocystis* gene expression under low oxygen conditions, revealed changes in a

number of genes coding for proteins putatively involved in assembling or stabilizing PSII, and an upregulation of the *hox* operon and *lexA* (Summerfield *et al.*, 2011). These responses, mostly focused on PSII and overall redox control, suggest an involvement of the bidirectional hydrogenase in the cell homeostasis under specific conditions or transition states.

A peculiar observation, regarding the genes coding for the proteins showing differential abundance (iTRAQ analysis), is that most of them are located in the same half of the *Synechocystis* chromosome, surrounding the *hox* cluster (see Supplementary Fig. S3, available with the online version of this paper), with a binomial test giving a *P*-value <0.013. However, the biological relevance (if any) of this occurrence needs to be further investigated.

In addition, the transcription of genes encoding proteins related to stress response and iron levels was studied: two ferric uptake regulation proteins – Fur (encoded by *sll0567* and *sll1937*) and the Fur-like peroxide-responsive repressor PerR (*slr1738*) (Kobayashi *et al.*, 2004), the putative glutamate-cysteine ligase GshA, which is the enzyme responsible for the first step in the glutathione biosynthesis pathway (*slr0990*), a small heat-shock protein, HspA, that stabilizes the thylakoid membrane proteins under oxidative stress (*sll1514*) (Sakthivel *et al.*, 2009) and the ferritin-type storage complex MrgA (*slr1894*), suggested to play a role in the coordination of iron homeostasis and oxidative stress response in *Synechocystis* (Scholnick *et al.*, 2009). The transcription of *mrgA* is suggested to be under the control of the PerR regulator and, therefore, induced under oxidative stress (Li *et al.*, 2004). Overall, the RT-qPCR results showed no significant differences between the wild-type and the Δ *hoxYH* mutant, with the exception of the Fur regulator genes. Significantly (*P*<0.05) lower levels of *sll0567* transcript (in the dark) and a transient decrease in *sll1937* transcript levels (60 min after dark anoxic condition induction) could be observed in the mutant (see Supplementary Fig. S4, available with the online version of this paper). Since *sll0567* and *sll1937* encode transcription factors involved in iron homeostasis, these results suggest differences in iron pools/availability in the wild-type and the Δ *hoxYH* mutant.

Conclusions

In conclusion, a *Synechocystis* mutant lacking an active bidirectional hydrogenase was produced by deleting the two genes – *hoxY* and *hoxH* – encoding the hydrogenase part of this enzyme. Physiological studies were performed by growing the wild-type strain and the Δ *hoxYH* mutant under different physiological conditions and assessing and comparing their growth behaviour. From this analysis, no significant differences were observed, and therefore it can be assumed that the mutant exhibits similar growth behaviour to the wild-type. Moreover, changes in protein content were analysed, in conditions where the hydrogenase activity is favoured, revealing that 17 proteins had significant differential fold changes. These proteins are

mainly related to the redox and energy state of the cells. Twelve proteins were more abundant in the mutant, including one of the possible transcription regulators of the bidirectional hydrogenase of *Synechocystis*. Overall, the functions of the proteins exhibiting different relative levels correlate with the proposed roles of the bidirectional hydrogenase in dark fermentation and photosynthesis (acting as an electron valve), as well as a role in the maintenance of cell homeostasis under specific conditions (transition states). In addition, transcription studies were performed to assess the patterns of 12 genes coding for proteins showing differential abundance. From this analysis, six genes exhibited significant differences in transcript levels following the same pattern as the protein abundance, with the exception of *psbY* and *petB* that showed an inverse trend. Altogether, these results indicate an adjustment in the metabolism of the Δ hoxYH mutant allowing growth at similar rates to the wild-type and reflecting the plasticity and metabolic adaptability of *Synechocystis*. Finally, this study suggests that the *Synechocystis* Δ hoxYH mutant is robust and can be used as a photoautotrophic chassis for the integration of synthetic devices to fulfil specific purposes, namely hydrogen production.

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