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 (ΔhoxYH) 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* ΔhoxYH 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.

**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 both the carbon and the nitrogen cycle (Bothe et al., 2010b; Moisander 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 (Pósai 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 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; Toshina 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 (ΔhoxYH), 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 integrating vectors) and the phototrophic 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 μE 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 μg ml⁻¹) or sucrose [10% (w/v)]. For the proteomics analysis, standard growth conditions were used, with a scalar irradiance of 75 μE 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 DH5α (Stratagene) was used for cloning purposes. E. coli transformants were cultivated at 37 °C in Luria–Bertani (LB) medium supplemented with 100 μg ampicillin ml⁻¹ or 50 μg 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× 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 pGDYHNS, 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 hoxYH 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 for PCR DNA and Gel Band Purification kit (GE Healthcare), according to the manufacturer’s instructions.
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 ml⁻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 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).

**Table 1. Oligonucleotide primers used in this study**

<table>
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<th>Name</th>
<th>Sequence (5’–3’)</th>
<th>Purpose</th>
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<tbody>
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<td>5-O*</td>
<td>GAAGCCCGGTGTTCCCATTCCTCCACCTTTGCGATTTAG</td>
<td>Mutant construction/Southern probe</td>
</tr>
<tr>
<td>5-I*</td>
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<td>Mutant construction/Southern probe</td>
</tr>
<tr>
<td>3-O</td>
<td>TGGTCTGCTTGAAGCTTGGGTTGAGCAAGCAAGGC</td>
<td>Mutant construction</td>
</tr>
<tr>
<td>3-I</td>
<td>TGGTCTGCTTGAAGCTTGGGTTGAGCAAGCAAGGC</td>
<td>Mutant construction</td>
</tr>
<tr>
<td>FO</td>
<td>AGAATATGGTGGATTGATGAGGAGGGC</td>
<td>Mutant confirmation</td>
</tr>
<tr>
<td>RO</td>
<td>GAGGGATGGAGGTTATAGAAGAAGAGG</td>
<td>Mutant confirmation</td>
</tr>
<tr>
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<td>Selection cassette amplification</td>
</tr>
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*Restriction enzyme recognition sites are underlined.
†Ferreira et al. (2009).
light (20 \( \mu \text{E} \text{ m}^{-2} \text{ s}^{-1} \)), to OD\(_{730} \approx 0.5\). Cells were harvested by centrifugation and resuspended in 1/10 volume of BG11. A 100 \( \mu \)l 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 6–20 \( \mu \text{g ml}^{-1} \). 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 \( ^\circ \text{C} \) under continuous light, and were transferred to selective plates containing 10 \( \mu \)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 \( \text{HindIII} \) or \( \text{HincII} \), and a probe covering the 5’ flanking region of \( \text{hoxYH} \) 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 \( \Delta \text{hoxYH} \) mutant was assessed by gas chromatography after placing the cells in dark and replacing air with argon.

**Growth experiments.** Cultures of \( \text{Synechocystis} \) wild-type and \( \Delta \text{hoxYH} \) mutant were grown to OD\(_{730} \approx 1.0\), and 24 h before the beginning of the experiment, were transferred to fresh BG11 medium. Inocula of 200 \( \mu \)l were then added to sterile 4.5 ml cuvettes containing 1800 \( \mu \)l 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 \( ^\circ \text{C} \)), under continuous light (20 \( \mu \text{E} \text{ m}^{-2} \text{ s}^{-1} \)) or a 12 h light (20 \( \mu \text{E} \text{ m}^{-2} \text{ s}^{-1} \))/12 h dark regimen, and the OD\(_{730} \) was recorded daily.

**Statistical analysis of the specific growth.** The quantitative analysis of \( \text{Synechocystis} \) wild-type and \( \Delta \text{hoxYH} \) 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 \( t_{\text{max}} \), 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:

\[
\mu_{\text{max}} = \max(\mu_i)_{i=0,...,t_{\text{max}}}
\]

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**Fig. 1.** Confirmation of the segregation of \( \text{Synechocystis} \) insertion (Ins mt) and deletion (Del mt) mutants by PCR amplification using a primer upstream of \( \text{hoxU} \) and another within \( \text{fabF} \) (a) and by Southern blot hybridization (b). Genomic DNA was digested with \( \text{HindIII} \) or \( \text{HincII} \) and hybridized with a probe covering the 5’ flanking region of \( \text{hoxYH} \) genes. The expected sizes of the PCR products and restriction fragments are shown below the figure.
In addition, we can obtain the time ($t_{\text{max}}$) at which maximum specific growth is reached and the corresponding optical density recorded ($x_{\text{max}}$).

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 $-80\,^\circ\text{C}$ 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 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 $\sigma^2$ for the unregulated proteins’ Gaussian distribution, a protein-specific variance $\sigma^2$ is introduced. It is calculated as

$$\sigma^2 = \sigma_0^2 + \left(\sigma_{117}^2 + \sigma_{118}^2 + \sigma_{119}^2 + \sigma_{121}^2\right)/n$$

where the biological variation $\sigma_0^2$ is estimated from all eight-plex values; $\sigma_{117}^2$ 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

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**Fig. 2.** Schematic representation of the experimental design used to generate the *Synechocystis ΔhoxYH* mutant.
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 \(\mu\)g \(\text{mL}^{-1}\). Cells were centrifuged (8 min at 4500 \(g\), 4 \(\degree\)C) and washed once with fresh BG11, and the cell pellets were frozen at \(-80\degree\)C. RNA was extracted using the TRIZOL Reagent (Invitrogen) according to the method described previously (Leitao 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 \(\mu\)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 were performed using 0.25 \(\mu\)M each primer, 10 \(\mu\)l iQ SYBR Green Supermix (Bio-Rad) and 2 \(\mu\)l template cDNA, and technical triplicates were performed. The PCR profile was: 3 min at 95 \(\degree\)C followed by 40 – 60 cycles of 30 s at 95 \(\degree\)C, 30 s at 51 \(\degree\)C and 30 s at 72 \(\degree\)C; exceptionally the annealing temperature for the study of \(\text{atpC}\) and \(\text{ppa}\) was 53 \(\degree\)C and 54 \(\degree\)C for \(\text{pgk}\). 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 \(\text{Synechocystis}\ \Delta\text{hoxYH}\) mutant with the wild-type, under dark anoxic conditions \([t_0, t_{60}\) and \(t_{120(60L)}]\) and after cells were transferred back to light \([t_{60}\) and \(t_{120(60L)}]\).

**RESULTS AND DISCUSSION**

**Construction and physiological characterization of Synechocystis \(\Delta\text{hoxYH}\) deletion mutant**

The genes coding for the two subunits corresponding to the hydrogenase part of the bidirectional hydrogenase \(\text{hoxYH}\) were deleted from the \(\text{Synechocystis}\) genome in a two-step procedure: a first transformation was performed using the plasmid pGDYH.NS in order to replace \(\text{hoxYH}\) 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 ΔhoxYH 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 ΔhoxYH mutant was compared under several physiological conditions (see Methods). In Fig. 3(a) the mean sample of maximum specific growth, μₘₓ, at different levels of nitrate and glucose, under 12 h light (20 μE m⁻² s⁻¹)/12 h dark cycles for *Synechocystis* wild-type and ΔhoxYH mutant are shown. As can be observed, in the presence of 1.5 g nitrate l⁻¹ and with glucose, the wild-type μₘₓ increases significantly compared with the same conditions in the absence of glucose. In Fig. 3(b), with continuous 20 μE m⁻² 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 ΔhoxYH 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 μE m⁻² 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 ΔhoxH 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 ΔhoxYH mutant**

The proteomes of *Synechocystis* wild-type and of the ΔhoxYH 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 Phenylx 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 ΔhoxYH 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

![Figure 5](image-url)
Table 2. List of proteins exhibiting significant differences in their abundance in *Synechocystis ΔhoxYH* mutant compared with the wild-type strain, after induction of dark anoxic conditions

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

<table>
<thead>
<tr>
<th>ID</th>
<th>Gene*</th>
<th>Protein</th>
<th>Putative function/characteristics of the encoded protein*</th>
<th>Category</th>
</tr>
</thead>
<tbody>
<tr>
<td>sll0247</td>
<td>isiA</td>
<td>IsiA</td>
<td>Iron-stress chlorophyll-binding protein, homologous to psbC (CP43)</td>
<td>PR</td>
</tr>
<tr>
<td>sll0248</td>
<td>isiB</td>
<td>IsiB</td>
<td>Flavodoxin</td>
<td>PR</td>
</tr>
<tr>
<td>sll0947</td>
<td>lrtA</td>
<td>LrtA</td>
<td>Light repressed protein A homologue</td>
<td>TI</td>
</tr>
<tr>
<td>sll1099</td>
<td>tufA</td>
<td>TufA</td>
<td>Elongation factor T</td>
<td>TI</td>
</tr>
<tr>
<td>sll1261</td>
<td>tfS</td>
<td>TfS</td>
<td>Elongation factor T</td>
<td>TI</td>
</tr>
<tr>
<td>sll1326</td>
<td>atpA</td>
<td>AtpA</td>
<td>ATP synthase alpha chain</td>
<td>EM</td>
</tr>
<tr>
<td>sll1327</td>
<td>atpC</td>
<td>AtpC</td>
<td>ATP synthase gamma chain</td>
<td>EM</td>
</tr>
<tr>
<td>sll1626</td>
<td>lexA</td>
<td>LexA</td>
<td>Transcription factor</td>
<td>Tr</td>
</tr>
<tr>
<td>sll1743</td>
<td>rpl11</td>
<td>RplK</td>
<td>50S ribosomal protein L11</td>
<td>TI</td>
</tr>
<tr>
<td>sll1746</td>
<td>rpl12</td>
<td>RplL</td>
<td>50S ribosomal protein L12</td>
<td>TI</td>
</tr>
<tr>
<td>sdr0342</td>
<td>petB</td>
<td>PetB</td>
<td>Cytochrome bs</td>
<td>PR</td>
</tr>
<tr>
<td>sdr0394</td>
<td>pgk</td>
<td>PGK</td>
<td>Phosphoglycerate kinase</td>
<td>EM</td>
</tr>
<tr>
<td>sdr0623</td>
<td>trxA</td>
<td>TrxA</td>
<td>Thioredoxin</td>
<td>AD</td>
</tr>
<tr>
<td>srl1516</td>
<td>sodB</td>
<td>SodB</td>
<td>Iron superoxide dismutase</td>
<td>AD</td>
</tr>
<tr>
<td>srl1622</td>
<td>ppa</td>
<td>PPA</td>
<td>Soluble inorganic pyrophosphatase</td>
<td>EM</td>
</tr>
<tr>
<td>srl2025</td>
<td>slr2025</td>
<td>Slr2025</td>
<td>Hypothetical protein of unknown function DUF1821</td>
<td>U</td>
</tr>
<tr>
<td>sml0007</td>
<td>psbY</td>
<td>PsbY</td>
<td>Photosystem II protein Y</td>
<td>PR</td>
</tr>
</tbody>
</table>

*According to CyanoBase annotation.

The higher abundance of the five proteins involved in translation in the *ΔhoxYH* 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 σ54 modulation protein/ribosomal protein S30EA family and is also more abundant in the *ΔhoxYH* 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 ΔhoxYH 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 ΔhoxYH 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 ΔhoxYH 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 ΔhoxYH 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
Characterization of a Synechocystis $\Delta$hosYH mutant

(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 $\Delta$hosYH 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; Carriéri 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 glycerdehyde-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 Cyanobacteria 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 & Moezelaa, 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 phototrophic 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 $\Delta$hosYH mutant.

Transcription analysis of genes encoding proteins with different abundance in Synechocystis wild-type and $\Delta$hosYH 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 $istAB$ 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 $\Delta$hosYH 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
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 oxygen species.

**Fig. 6.** Protein changes in the glycolysis/gluconeogenesis, the PPP pathway and glycogen metabolism in *Synechocystis ΔhoxYH* 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.
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), trcA 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 b6 subunit of the cytochrome b6f complex that mediates electron transfer between PSII and PSI, cyclic electron flow around PSI and state transitions (Cramer et al., 1994; Kruijff et al., 1994; Stroebele 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 ΔhoxYH 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 (ΔhoxH), 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 slr0567 and slr1937) 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 (slr1514) (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* (Shcolnick 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 ΔhoxYH mutant, with the exception of the Fur regulator genes. Significantly (P<0.05) lower levels of slr0567 transcript (in the dark) and a transient decrease in slr1937 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 slr0567 and slr1937 encode transcription factors involved in iron homeostasis, these results suggest differences in iron pools/availability in the wild-type and the ΔhoxYH 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 ΔhoxYH 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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