Proteomic and cellular views of *Arthrospira* sp. PCC 8005 adaptation to nitrogen depletion

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Cyanobacteria are photosynthetic prokaryotes that play a crucial role in the Earth’s nitrogen and carbon cycles. Nitrogen availability is one of the most important factors in cyanobacterial growth. Interestingly, filamentous non-diazotrophic cyanobacteria, such as *Arthrospira* sp. PCC 8005, have developed survival strategies that enable them to adapt to nitrogen deprivation. Metabolic studies recently demonstrated a substantial synthesis and accumulation of glycogen derived from amino acids during nitrogen starvation. Nevertheless, the regulatory mechanism of this adaptation is poorly understood. To the best of our knowledge, this study is the first proteomic and cellular analysis of *Arthrospira* sp. PCC 8005 under nitrogen depletion. Label-free differential proteomic analysis indicated the global carbon and nitrogen reprogramming of the cells during nitrogen depletion as characterized by an upregulation of glycogen synthesis and the use of endogenous nitrogen sources. The degradation of proteins and cyanophycin provided endogenous nitrogen when exogenous nitrogen was limited. Moreover, formamides, cyanates and urea were also potential endogenous nitrogen sources. The transporters of some amino acids and alternative nitrogen sources such as ammonium permease 1 were induced under nitrogen depletion.

Intriguingly, although *Arthrospira* is a non-diazotrophic cyanobacterium, we observed the upregulation of HetR and HglK proteins, which are involved in heterocyst differentiation. Moreover, after a long period without nitrate, only a few highly fluorescent cells in each trichome were observed, and they might be involved in the long-term survival mechanism of this non-diazotrophic cyanobacterium under nitrogen deprivation.

INTRODUCTION

Cyanobacteria are photosynthetic prokaryotes (Herrero & Flores, 2008; Luque et al., 1994) of crucial importance to the Earth’s nitrogen and carbon cycles, and in the conversion of solar light into chemical energy (Flores et al., 2005; Montoya et al., 2004; Nomsawai et al., 1999). In addition, their role in the primary production of biomass and oxygen has led to their being intensively studied, especially in regard to their nitrogen metabolism (Muro-Pastor et al., 2005). Because of their environmental tolerance, cyanobacteria are able to grow on different nitrogen sources, such as nitrate, nitrite, ammonium, urea or some amino acids (Flores & Herrero, 1994; Muro-Pastor et al., 2005; Walsby & Jüttner, 2006). Although nitrate is the predominant form in the natural environment, ammonium appears to be the preferred nitrogen source (Muro-Pastor et al., 2005). Whatever the source, nitrogen passes through the cytoplasmic membrane into the cyanobacterial cell. Its uptake from the environment into the cytoplasm is catalysed by specific permeases. Whereas nitrate and nitrite are taken up by ATP-binding cassette (ABC) type (Flores et al., 2005; Kobayashi et al., 1997) or major facilitator superfamily (MFS) type (Aichi & Omata, 1997; Aichi et al., 2006; Flores et al., 2005; Sakamoto et al., 1999) transporters, the transfer of ammonium involved a potential-dependent active channel called the ammonium transporter (Amt) (Muro-Pastor et al., 2005). In the

Abbreviations: 2-OG, 2-oxoglutarate; APC, allophycocyanin; chla, chlorophyll a; CP, cyanophycin; GOGAT, glutamine oxoglutarate aminotransferase; GS, glutamine synthetase; GV, gaseous vesicle; PC, phycocyanin; RuBisCO, ribulose-1,5-bisphosphate carboxylase/oxygenase; SI, sinking index.

Four supplementary figures and two supplementary tables are available with the online version of this paper.
cytoplasmic space, all assimilated nitrogen sources are reduced to ammonium (e.g. nitrate is converted into nitrite and then into ammonium by nitrate reductase and nitrite reductase, respectively) (Flores & Herrero, 1994; Herrero et al., 1981; Muro-Pastor & Florencio, 2003; Muro-Pastor et al., 2005). The ammonium that is available in the cell is then consumed through the glutamine synthetase/glutamine oxoglutarate aminotransferase (GS/GOGAT) cycle as operated by the enzymes GS and GOGAT, which ultimately requires 2-oxoglutarate (2-OG) produced during the TCA cycle (Muro-Pastor et al., 2005; van den Heuvel et al., 2004). The 2-OG is an important organic molecule in nitrogen metabolism for monitoring the intracellular nitrogen pool, and it represents a functional link between the carbon and nitrogen cycles (Herrero et al., 2001).

Nitrogen deprivation has been widely studied among members of the phylum Cyanobacteria. This nutrient depletion is known to induce many different effects, such as morphological, physiological and molecular modifications (Schwarz & Forchhammer, 2005). Cyanobacteria respond to nutrient starvation, including nitrogen starvation, by decreasing their photosynthetic pigments, phycobiliproteins and chlorophyll a (chl a) contents, resulting in an obvious bleaching of cultures from blue-green to yellow (Collier & Grossman, 1992; Göl et al., 1998; Krasikov et al., 2012; Richaud et al., 2001; Sauer et al., 2001). In nitrogen-depleted cultures, cyanobacteria (e.g. Oscillatoria rubescens and Anabaena flosaquae) are known to lose the buoyancy resulting from cellular density modification by collapsing their gas vesicles or increasing their ballast-like carbohydrates (Schwarz & Forchhammer, 2005; Walsby, 1994).

When the C-to-N ratio is unbalanced (e.g. during nitrogen starvation), 2-OG influences the activity of several proteins, including nitrogen metabolism regulators such as NtcA (Herrero et al., 2001; Laurent et al., 2005; Llácer et al., 2010; Muro-Pastor et al., 2001; Vázquez-Bermúdez et al., 2002; Zhao et al., 2010) and P11 (Fokina et al., 2010; Forchhammer & Tandeau de Marsac, 1995a; Lee et al., 1998). NtcA then upregulates the expression of genes encoding GS orAmt and other NtcA-dependent genes such as glnB, pipX or ntcB (Aichi & Omata, 1997; Aichi et al., 2001; Espinosa et al., 2007; Herrero et al., 2001; Llácer et al., 2010; Muro-Pastor et al., 2001). During nitrogen deprivation, the phosphoprotein P11 induces cellular responses by interacting with protein targets (e.g. PipX) or by regulating gene expression (e.g. glnA, which encodes GS) (Fokina et al., 2011; Forchhammer et al., 2004; Forchhammer, 2008). Cyanobacteria synthesize cyanophycin (CP), which is a highly nitrogen-rich polymer composed only of arginyl and aspartyl residues (Maheswaran et al., 2006; Picossi et al., 2004). Under nitrogen deprivation, CP is catabolized as an internal nitrogen source, balancing the nitrogen deficiency (Kolodny et al., 2006; Picossi et al., 2004). An in vivo labelling study recently revealed that under conditions of nitrogen depletion, cyanobacterial glycogen (Arthrospira platensis) is biosynthesized with the carbon derived from amino acids that are released from proteins via gluconeogenesis (Hasunuma et al., 2013). Moreover, a Synechocystis sp. PCC 6803 study showed an increase not only in glycogen, but also in metabolites downstream of sugar catabolism under nitrogen starvation (Hasunuma et al., 2013).

A typical physiological modification induced by nitrogen deprivation concerns the differentiation of vegetative cells into heterocysts, in which oxygen-sensitive nitrogenase is protected from oxidative inhibitions (Berman-Frank et al., 2003, 2007; Fay, 1992; Tamagnini et al., 2002; Zhang et al., 2006). This type of specialized cell allows diazotrophic cyanobacteria to fix and reduce dinitrogen when the given nitrogen sources are limiting (Berman-Frank et al., 2003; Flores & Herrero, 1994). Although dependence on this differentiation process has been shown to rely on HetR and PatS (Kumar et al., 2010; Maldener & Muro-Pastor, 2010; Zhang et al., 2009), it is still poorly characterized. Interestingly, Zhang and collaborators mentioned that the hetR and patS genes were also upregulated under nitrogen starvation in the non-heterocyst filamentous cyanobacterium A. platensis (Zhang et al., 2009).

Arthrospira sp. PCC 8005, which belongs to the order Oscillatoriales, appeared as non-heterocyst forming colonies called trichomes that are not able to fix dinitrogen (Janssen et al., 2010). Although some cyanobacteria cause concern because of their toxin production, this strain is a food supplement because of its high protein content and vitamin B12 (Markou et al., 2012; Nomsawai et al., 1999). Arthrospira sp. PCC 8005 has been selected by the European Space Agency as one of the key members of an artificial ecosystem called MELiSSA (micro-ecological life support system alternative) to supply the ecosystem with oxygen and edible biomass (Hendrickx et al., 2006).

In this study, we monitored changes in the Arthrospira sp. PCC 8005 proteome under nitrogen deprivation. We also analysed cellular modifications in response to nitrogen starvation. These data contribute to our understanding of cellular adaptation mechanisms during nitrogen depletion in Arthrospira sp. PCC 8005.

**METHODS**

**Bacterial strain and growth conditions.** Arthrospira sp. strain PCC 8005 was kindly provided by the Expert Group for Molecular and Cellular Biology MCB. The strain was incubated on an orbital shaker (160 r.p.m.; Heidolph Unimax 1010) and illuminated with a photosynthetic photon flux density (Sanyo Fl40SS-W/37) of ±43 μmol s⁻¹ m⁻² (Li-193SA; Li-Cor BioSciences) and 5.6 Watt m⁻² (Li-200SA; Li-Cor BioSciences) at 30 °C in Zarrouk medium (Zarrouk, 1966) with nitrates as the sole usable nitrogen source in the medium. (Zarrouk medium: 13.6 g NaHCO₃ L⁻¹; 0.5 g K₂HPO₄ L⁻¹; 3.5 g Na₂CO₃ L⁻¹; 2.5 g NaNO₃ L⁻¹; 1 g K₂SO₄ L⁻¹; 0.2 g MgSO₄·7H₂O L⁻¹; 0.04 g CaCl₂·H₂O L⁻¹; 0.01 g FeSO₄·7H₂O L⁻¹; 0.08 g Na₂EDTA L⁻¹; 4 ml solution A L⁻¹; 4 ml solution B L⁻¹. Solution A: 2.86 g H₃BO₃ L⁻¹; 1.81 g MnCl₂·4H₂O L⁻¹; 0.22 g ZnSO₄·7H₂O L⁻¹; 0.08 g CuSO₄·3H₂O L⁻¹. Solution B: 0.048 g NiSO₄·7H₂O L⁻¹; 0.049 g (NO₃)₃·Co·6H₂O L⁻¹; 0.018 g Na₂MoO₄·2H₂O L⁻¹.) Nitrogen deprivation was induced at an OD₇₅₀ of 0.8. The cyanobacteria were washed twice with Zarrouk medium lacking nitrogen sources and reincubated under the same incubation conditions as mentioned above.

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Four biological replicates were assessed, and the controls were treated in the same way except that the washing and incubation steps were carried out in Zarrouk medium containing nitrate. Samples were collected and centrifuged for each time period (15 min, 1 × 10^4 r.p.m., 4 °C). The resulting pellets were kept frozen for further analysis.

**Growth and sinking analysis.** Bacterial growth and sinking were monitored by optical density at 750 nm (Helios Zeta UV-Vis; Thermo Scientific). The change in sinking was monitored as a sinking index (SI) (%) describing the decrease in the optical density of an undisturbed culture caused by cell sinking. The SI was calculated according to the following formula: [(OD at t0 − OD at tsi)/ OD at t0] × 100. To achieve this measurement, 1 ml culture was placed in a regular spectrophotometer cell and the OD_750 was recorded immediately (t0) and after 16 min (tsi), without any disturbance.

**Photosynthetic pigment analysis.** A differential extraction of photosynthetic pigments was performed on the basis of an existing method (Brown, 1996). In brief, cellular aliquots (4 ml) were centrifuged (15 min, 1 × 10^4 r.p.m., 4 °C), and the resulting pellets were submitted to three freeze/thaw cycles with liquid nitrogen. The pellets were then incubated with 0.4 % (w/v) lysozyme/Na2HPO4 (50 mM, pH 7.0) solution at 37 °C for 2 h. The supernatants containing phycobiliproteins were recovered by centrifugation (15 min, 1 × 10^4 r.p.m., 4 °C) and the optical densities at 650 and 620 nm were measured for allophycocyanin (APC) and phycocyanin (PC), respectively. The resulting pellets were washed three times with 50 mM (w/v) Na2HPO4 (pH 7.0) and then resuspended in 100 % methanol, sonicated for 1 min (Elma; Transonic T460/H) and incubated overnight at 4 °C in a dark room. The supernatants containing chla were recovered by centrifugation (15 min, 1 × 10^4 r.p.m., at room temperature) and used to measure the OD_665.

To evaluate pigment evolution, the optical density was measured at 650, 620 and 665 nm for APC, PC and chla, respectively. Normalization was performed with the OD_750 of the corresponding culture.

**Determination of CP concentration.** CP was extracted from cell aliquots (20 ml) by using a method modified from Allen et al. (2005). In brief, the samples from each time period were broken twice in a French press (Thermo) and centrifuged (15 min, 2 × 10^6 r.p.m., 4 °C) in a 50 Ti rotor (Beckman L7 Ultracentrifuge). The pellets containing CP granules were washed once with distilled H2O and then extracted twice in 200 μl 0.1 M HCl for 30 min at room temperature. After centrifugation in the 50 Ti rotor (15 min, 1 × 10^6 r.p.m., 4 °C), the resulting supernatants were analysed as described by Simon (1973).

**Statistical analysis.** To highlight a significant difference in the bacterial growth rate depending on the culture conditions (with or without nitrogen source), an unpaired t-test was performed after verification with respect to the application conditions (Shapiro–Wilk test P value >0.05). In the negative, a non-parametric test called the Wilcoxon–Mann–Whitney test was performed. Both the t-test and the Wilcoxon–Mann–Whitney test were performed on bacterial growth rate μ as calculated according to the following formula: ln(OD at t2)−ln(OD at t1)/ t2−t1 (Powell, 1956).

Two-way repeated ANOVAs were performed to evaluate the impact of N-deprivation on cellular sinking, the photosynthetic pigment content and CP content. When the residual distribution did not have a normal distribution, the data were transformed to normalize them (by square root or logit(x) function). If no transformation was efficient, the data were transformed to rank with the rank(x) function to establish a non-parametric model. In addition, to elucidate whether there was any significant increase in chla contents under control conditions, supplementary t-tests were applied to data obtained at 48 and 72 h of culture.

These analyses were performed by R software (2.2.1 version) coupled to the SciViews R console (0.9.2 version).

**Urease activity.** To monitor the evolution of urease activity, a urease assay was carried out at 8 and 24 h of culture. Cell aliquots (5 ml) were centrifuged (30 min, 4500 r.p.m., 4 °C) and pellets were suspended in 2 ml PBS (BupH PBS; Thermo Scientific) on ice. The samples were then broken three times in a French press (Thermo) and centrifuged (10 min, 1 × 10^6 r.p.m., 4 °C). The supernatants were then recovered and submitted to the assay according to the instructions from the manufacturer (urease assay kit; Abnova). The results were normalized by the protein quantities as determined by a protein assay (Bradford, 1976).

**Phase-contrast microscopy.** The *Arthrospira* strain was incubated on an orbital shaker (160 r.p.m.; Heidelph Unimax 1010) and illuminated with a photosynthetic photon flux density (Sanyo FL40SS-W/37) of ±43 or 150 μmol s^-1 m^-2, at 30 °C in Zarrouk medium (Zarrouk, 1996) with or without nitrate in the medium. The cultures under 150 μmol s^-1 m^-2 were used as a positive control (Nomisawai et al., 1999). The samples were examined under a phase-contrast microscope (Carl Zeiss; Axiosvert 25) after 24 h of culture.

**Proteomic analysis.** After the centrifugation of the cellular aliquots (5 ml), proteins were extracted from the pellet using 6 M guanidine chloride pH 8.5 [lysis buffer from the ICPL kit (Serva)] combined with ultrasonication (3 × 10 s, 20 % amplitude; U50 IKA Technik). The supernatants containing proteins were recovered by centrifugation (1.32 × 10^4 r.p.m., 15 min, 4 °C) and a protein assay was performed (Bradford, 1976). Protein (25 μg) was submitted to label-free differential proteomic analysis. Proteins were reduced and alkylated according to the manufacturer’s instructions (Serva). Proteins were then recovered with acetone, dissolved in 20 μl 25 mM (w/v) NH4HCO3 (pH 8.5) containing 1 μg trypsin (Promega V511) and incubated at 37 °C overnight. Trypsinization was halted with formic acid (0.1 %, v/v, final).

Tryptic peptides were separated on a reverse-phase column (length 25 cm, diameter 75 μm, particles 3 μm, outlet 300 nl min^-1; PepMap C18; Dionex) equilibrated at 4 % (v/v) ACN and submitted to an ACN gradient (4 to 35 %, v/v) for 120 min. The column was previously equilibrated with 4 % ACN for 20 min. The peptide elution was followed by a wash step performed with 90 % (v/v) ACN for 10 min.

An online MS analysis was performed with an AB Sciex TripleTOF 5600. Peptide mass spectra were acquired in data-dependent acquisition (DDA) mode. During each acquisition cycle, 1 MS spectrum (m/z 400–1500; acquisition time 0.5 s) was acquired followed by 50 MS/MS spectra under high sensitivity (HS) mode (m/z 100–1800, acquisition time 0.05 s) leading to a duty cycle time of 3 s. These 50 precursors were selected according to their intensity peak (minimum 200 counts). The selected precursors were submitted to a collision-induced dissociation (CID) with nitrogen gas and then actively excluded after 1 MS/MS spectrum for 30 s. The TOF analyser was automatically calibrated with the tryptic peptides of β-galactosidase from *Escherichia coli* after every three samples, allowing for the maintenance of a mean mass error below 10 p.p.m. across all injections.

Raw spectral data were converted to mzXML format with MSConvert (64 bit, MS level, no filters) and treated with Progenesis LC-MS (version 4.0). The resulting peak list was used as the input for Mascot MS/MS ions searches by using an in-house Mascot 2.2 server (Matrix Science) against the *Arthrospira* sp. PCC 8005 protein database (*Arthrospira* sp. PCC 8005 v3) deduced from the genome sequence (Janssen et al., 2010). The search parameters included the following: trypsin as enzyme, carbamidomethylation (C) as fixed modifications,
oxidation (M) and deamination (N, Q) as variable modifications, peptide tolerance ± 15 p.p.m., MS/MS tolerance ± 0.05 Da, peptide charges 2+ and 3+, and instrument ESI-Q-TOF. Only proteins identified with a protein score above the Mascot calculated ion score, which was defined as a 95% confidence level, were considered.

Progenesis LC-MS (version 4.0) was used to realign the retention profile based on a multi-vectorial realignment algorithm and to determine the extracted ion chromatogram for the top five most abundant peptides. The quantitative data were evaluated by ANOVA, and only the quantitative data exhibiting a P value < 0.05 were reported. Data treatment returned a false discovery rate that characterized the set of studied proteins. The fold changes are the ratios of the abundance of proteins between nitrogen-starvation and control conditions after 8 and 24 h of culture. Protein classification was performed by following the COG automatic classification obtained from Genoscope, and the specific activity of considered proteins was elucidated with a KEGG database.

**Long-term nitrogen starvation.** Red autofluorescence was observed and profiled under nitrogen starvation over time by epifluorescence microscopy. After 30 days of nitrogen starvation, cyanobacteria from four parallel cultures were centrifuged (10 min, 4 × 10^3 r.p.m., room temperature) and the corresponding pellets were washed twice with Zarrouk medium containing nitrate. After the washing step, the final pellets were incubated in Zarrouk medium containing nitrate as the sole usable nitrogen source under standard incubation conditions.

Growth was monitored by measuring the optical density at 750 nm and fluorescence observations were carried out by epifluorescence microscopy (Zeiss Axio Scope A1) under white light and fluorescence (λ excitation 560 ± 40 nm, λ emission 630 ± 75 nm). Picture acquisition was performed by Axiovision Rel (version 4.7), and the red profile was analysed with Pro Plus (version 6, tools Line Profile & Free Form).

To assess their long-term viability and determine the reactivation of surviving cells rather than the multiplication of surviving cells, aztreonam (Sigma Aldrich) was used according to the method of Göril et al. (1998). In brief, 30 day starved aliquots of 10 ml were transferred into new sterile 25 ml flasks containing 17.6 mM sodium nitrate and 25 µg aztreonam ml⁻¹ and incubated under standard conditions (30 °C, ± 43 µmol m⁻² s⁻¹, 160 r.p.m.). Aztreonam was added again after 3 and 5 days. Cell growth and autofluorescence recovery were observed as described above.

**RESULTS**

**Effect of nitrogen depletion on Arthrospira sp. survival**

*Arthrospira* sp. cells were pre-cultivated at approximately 43 µmol s⁻¹ m⁻² light at 30 °C in Zarrouk medium, which contained almost 30 mM sodium nitrate as the sole exogenous nitrogen source. The cells were washed twice and transferred to Zarrouk (control) or nitrate-free Zarrouk medium at an initial OD₇₅₀ of 0.8. The cells were then cultivated under pre-culture conditions. As shown in Fig. 1, *Arthrospira* sp. was able to grow during ± 30 h under nitrate-depleted conditions. During this period, growth curves were not significantly different (t-test; P value > 0.1) (Fig. 1).

**Nitrogen-starvation induced changes in the phycobilisome content and the degradation of CP**

In the absence of nitrate, the cellular protein content decreased to 15.4% of the dry cell weight after 48 h (Fig. 2). In parallel, rapid trichome sinking was observed. Bacterial sinking was quantified as an SI (%) corresponding to the decrease in the OD₇₅₀ of an undisturbed culture over 16 min (Fig. 3). Although no major sinking phenomenon occurred in the control medium, the SI reached 80% after 20 h of nitrogen deprivation, leading to significant differences between the conditions (P value < 1 × 10⁻²). Interestingly, we detected a decrease in the number of gas vesicles observed by phase-contrast microscopy during nitrogen depletion (Fig. S1, available in the online Supplementary Material).
Moreover, during the first 48 h of culture under nitrogen depletion, a significant decrease in the APC and PC was also observed, indicating a decrease in the phycobilisome content (P values <0.001) (Fig. 4a, b). No significant variation in the chl \( \text{a} \) content was observed in nitrogen-free medium over time (Fig. 4c). By contrast, a significant increase in the chl \( \text{a} \) content (t-test at 48 h P value <0.05 and t-test at 72 h P value <0.05) was observed in the control medium after 48 h.

Following the transfer of cells to nitrate-free Zarrouk medium, the CP content gradually decreased (P value <0.05), most likely because of the rapid degradation of this polymer, which is exclusively made of arginyl and aspartyl residues (Fig. 5). By contrast, under complete Zarrouk medium, this reserve of energy and nitrogen increased during the first 24 h of bacterial growth.

**Proteome modification during nitrogen depletion**

The molecular response to nitrogen starvation was investigated at the proteomic level by using a quantitative label-free MS approach. In this context, three biologically independent replicates were starved of nitrogen for 8 and 24 h, analysed and compared with the control. Nine hundred and thirty-five different proteins were identified with a false discovery rate at peptide levels of 2.09 and 2.24 % for 8 and 24 h of culture, respectively (Tables S1 and S2). In our experimental conditions, changes in the proteome after nitrogen depletion were greater after 24 h compared with 8 h. Although 42 and 157 proteins were significantly downregulated (ANOVA, \( P \) value \( \leq 0.05 \)), 22 and 116 proteins were significantly upregulated at 8 and 24 h of culture, respectively. Nitrogen depletion induced downregulation in most cellular functions such as photosynthesis, the Calvin cycle, and the synthesis of some amino acids and sugars. By contrast, nitrogen starvation also induced an increase of the abundance of proteins that are known to play a crucial role in glutamine and carbohydrate synthesis and the TCA cycle.

**Downregulation of photosynthetic activities.** Nitrogen depletion induced downregulation in enzymes with a
crucial role in porphyrin, cytochrome and chlorophyll anabolic pathways [coproporphyrinogen III oxidase HemF (ARTHROv3_1130040), uroporphyrinogen decarboxylase HemE (ARTHROv3_640017), δ-aminolevulinic acid dehydratase HemB (ARTHROv3_1050040), magnesium chelatase H subunit ChlH (ARTHROv3_1090006) and aerobic magnesium protoporphyrin IX monomethylster (oxidative) cyclase AcsF (aerobic Mg-protoporphyrin IX monomethylester coproporphyrinogen III oxidase HemF (ARTHROv3_1130040), uroporphyrinogen decarboxylase HemE (ARTHROv3_640017), δ-aminolevulinic acid dehydratase HemB (ARTHROv3_1050040), magnesium chelatase H subunit ChlH (ARTHROv3_1090006) and aerobic magnesium protoporphyrin IX monomethylster (oxidative) cyclase AcsF (aerobic Mg-protoporphyrin IX monomethylester oxidative cyclase) (ARTHROv3_210008) (oxidative) cyclase AcsF (aerobic Mg-protoporphyrin IX monomethylester oxidative cyclase) (ARTHROv3_210008)] after 8 and/or 24 h of culture (Fig. 6). In addition, other enzymes or proteins that are directly or indirectly involved in photosynthesis were also downregulated after 8 h [ATP synthase AtpF (subunit I) (ARTHROv3_630063), Ycf4 (ARTHROv3_1210004)] or 24 h of bacterial culture [Ycf 3 (ARTHROv3_300032), D2 protein from photosystem II (protein Qa) PsbD1 (ARTHROv3_1210065), NAD(P) H-quione oxidoreductase subunit I (ARTHROv3_840044)] and K (ARTHROv3_630050), APC subunit β ApCB (ARTHROv3_6210004), formatting thyhalokid proteins Thf1-like (ARTHROv3_1130036), SqdB UDP-sulfoquinovose synthase (ARTHROv3_670004) and orange carotenoid-binding protein (ARTHROv3_1420023) (Table S1). All this downregulation could indicate decreased photosynthesis concomitant with the observed decrease in PC and APC contents under nitrogen deprivation.

**Upregulation of carbohydrate synthesis and downregulation of glycogenolysis.** After 24 h nitrogen deprivation, the enzymes malate dehydrogenase MaeB (oxaloacetate decarboxylating enzyme) (ARTHROv3_750044), phosphoenolpyruvate synthase Pps (ARTHROv3_810107), enolase Eno (ARTHROv3_960030), fructose 1,6-bisphosphatase (ARTHROv3_850006) and putative phosphoglycerate mutase GpmB-like (ARTHROv3_3200002) were significantly more abundant, suggesting an upregulation of carbohydrate synthesis (Fig. 6). In parallel, we observed a significant downregulation of glycogenolysis after 8 h of nitrogen deprivation. In fact, glycogen phosphorylase GlgP (ARTHROv3_1320010), fructose bisphosphate aldolase FbaA (ARTHROv3_320004), phosphoglycerate mutase (ARTHROv3_14900038), transaldolase TalB (ARTHROv3_1070005) and triose phosphate isomerase TpiA (ARTHROv3_10093) were less abundant after 8 and/or 24 h (Table S1). As previously observed (Aikawa

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**Fig. 5.** Changes in the CP content in nitrogen-starvation (▲) and control (■) media. The mean was taken from four biologically independent replicates and the error bars indicate the SEM.

**Fig. 6.** Molecular response of *Arthrosira* sp. PCC 8005 to nitrogen starvation. Molecular pathways were predicted according to the Genoscope and *kEGs* databases. Although the left square corresponds to protein abundance modification at 8 h (nitrogen starvation versus control), the right square represents protein abundance after 24 h of culture under starvation (nitrogen starvation versus control). All cultures were incubated at 30 °C on an orbital shaker with artificial light (± 43 μmol s⁻¹ m⁻²) in control and nitrate-depleted media. The data presented here were taken from three biologically independent replicates. ND, Not determined; pep, number of peptides used for the quantification.
et al., 2012; Hasunuma et al., 2013; Osanai et al., 2011; Page-Sharpe et al., 1998; Yoo et al., 2007), an increased glycogen content was observed at 24 h after nitrogen depletion (data not shown). In addition, glycogen synthase GlgA (ARTHROv3_300003) showed a significant increase at 8 h under nitrogen depletion. Together, these results suggested the potential downregulation in glycogen synthesis as opposed to an upregulation of glycogen synthesis.

**Downregulation of the inorganic carbon fixation pathway.** Interestingly, a significantly lower abundance of both ribulose bisphosphate carboxylase Cbbi (the large subunit of RuBisCO) (ARTHROv3_420092) and the potential Calvin cycle regulator CP12-like (ARTHROv3_810066) also occurred under nitrogen deprivation at 24 h (Table S1). Moreover, the GlpX d-fructose 1,6-bisphosphatase class II (ARTHROv3_810066), a key enzyme of the Calvin cycle of photosynthetic CO₂ assimilation, also showed a significant lower abundance.

**Upregulation of glutamine synthesis, the key molecular nitrogen donor.** Nitrogen starvation induced significant overregulation in the key GS enzyme at 24 h of culture, indicating a potential increase in the biosynthesis of the amino acid glutamine (Fig. 6). In addition, the enzymes aspartate aminotransferase Aat2 (ARTHROv3_1510038), carbamoylphosphate synthase CarA (ARTHROv3_10118), ornithine carbamoyltransferase ArgI (ARTHROv3_1260011), argininosuccinate synthase ArgG (ARTHROv3_1440061) and argininosuccinate lyase ArgH (ARTHROv3_1490046) were primarily upregulated after 24 h. These enzymes are involved in arginine and aspartate catabolism, most likely by promoting an increase in the production of amino acids glutamate and glutamine under nitrogen starvation.

By contrast, some enzymes that play a role in the metabolism of other amino acids were less abundant under nitrogen depletion for 8 and/or 24 h [cysteine synthase Cysk1 (ARTHROv3_1380045), 5-methyltetrahydropteroyltriglutamate-homocysteine S-methyltransferase MetE (ARTHROv3_1870001), 2-isopropylmalate synthase LeuA1 (ARTHROv3_760026), 3-isopropylmalate dehydrogenase LeuB (ARTHROv3_210023) and ketol acid reductoisomerase (ARTHROv3_2030001)].

Moreover, MS analyses showed that formamidase FmdA (ARTHROv3_1380031) and cyanase CynS (ARTHROv3_2180001) were also upregulated after 24 h, indicating the potentially enhanced degradation of formamides and cyanates, respectively. The enzyme nitrilase/cyanide hydrolase (ARTHROv3_1490024) was shown to be upregulated after 8 h of culture. Moreover, the nitrite hydratase [subunit α (ARTHROv3_520006), subunit β (ARTHROv3_520007)] and the putative SpeB-like agmatine ureohydrolase (ARTHROv3_100009) exhibited higher abundance at 8 and 24 h, respectively, indicating a potential upregulation of aliphatic amide synthesis from nitrite and agmatine degradation leading to urea production. Although urease was not significantly upregulated under nitrogen starvation, the ureolytic activity exhibited a significant increase at 24 h (Fig. S2).

Amino acid transporters [the putative aspartate/glutamate transporter (ARTHROv3_1420029), Livl-like transporter (branched chain amino acid transporter) (ARTHROv3_810056), etc.] and the alternative nitrogen source transporter ammonium permease l (ARTHROv3_1050035) were upregulated (Table S1), most likely indicating an optimization of intracellular nitrogen content.

These results indicated an upregulation in the biosynthesis of glutamine in addition to an optimization of endogenous nitrogen stock catabolism.

**Upregulation of the nitrogen metabolism regulator PII and kinases.** Proteomic analysis demonstrated the upregulation of PII (ARTHROv3_420013) under nitrogen deprivation. The biological response at the protein level also involved an increase in the abundance of a kinase protein (ARTHROv3_690062) and a serine/threonine kinase (ARTHROv3_840030) (Table S1). Taken together, these results suggested the adaptation of *Arthrosira* sp. to nitrogen deprivation stress by cellular signalling.

**Upregulation of key proteins involved in heterocyst differentiation.** In this study, we report that both HetR and HglK proteins are widespread among filamentous cyanobacteria that do not form heterocyst or fix N₂. Indeed, after 24 h, nitrogen limitation induced a significant upregulation in the proteins HetR (ARTHROv3_1530027) and HglK (ARTHROv3_420073) (Table S1).

**Long-term survival under nitrogen starvation for *Arthrosira* sp. PCC 8005**

The capacity of *Arthrosira* sp. PCC 8005 to face long-term nitrogen deprivation was analysed. In this context, cyanobacteria were cultivated over a long period in nitrate-free Zarrouk medium. As mentioned in the literature (Kebede, 1997; Wang & Zhao, 2005), two *Arthrosira* sp. phenotypes were observed, namely the helix and the straight form, under both conditions. After 30 days, the cells were transferred to nitrate-replete Zarrouk medium and then incubated under standard conditions (30 °C, ±43 μmol s⁻¹ m⁻²). After being transferred into nitrate medium, the OD₇₅₀ increased after 100 h of culture and exhibited a plateau phase until 350 h, suggesting a capacity for long-term survival (Fig. 7). The capacity of *Arthrosira* sp. PCC 8005 to endure long-term nitrogen starvation was investigated by using epifluorescence microscopy (Figs 8 and 9). As shown in Fig. 8, only a few highly fluorescent cells in trichomes were observed after 30 days of exposure to N-starved culture. The reappearance of red autofluorescence was observed after adding nitrate, indicating the regeneration of cyanobacterial pigments (Fig. 9). All the trichomes homogeneously recovered their fluorescence after ± 24 h of culture (Fig. 9). This phenomenon could be explained by two hypotheses, as follows: (i) all trichomes progressively

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**Microbiology 160**

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recovered a homogeneous fluorescence or (ii) a few cells that survived the nitrate removal gave rise to new trichomes with homogeneous fluorescence. Aztreonam, an inhibitor of cell division, was used to assess the origin of fluorescence recovery (see Methods). Interestingly, no growth was observed in presence of aztreonam while the red autofluorescence reappeared (Figs S3 and S4). These results supported the recovery of all the trichomes (hypothesis i) rather than the derivation of new homogeneous-fluorescence trichomes from a few surviving cells.

DISCUSSION

In general, non-diazotrophic and diazotrophic cyanobacteria have the capacity to adapt to nitrogen limitation. Under nitrogen depletion, *Arthrospira* sp. was able to grow and survive for over 50 h as also demonstrated in *Synechococcus* sp. (Görl et al., 1998), *Synechocystis* sp. (Hasunuma et al., 2013; Krasikov et al., 2012) and *A. platensis* NIES-39 (Hasunuma et al., 2013). Recently,
a metabolite profile analysis of nitrogen depletion in A. platensis NIES-39 and Synechocystis sp. PCC 6803 demonstrated the biosynthesis and accumulation of glycogen from carbon derived from amino acids as released by protein turnover and/or hydrolysis. In accordance with these studies, proteomic analysis showed the reprogramming of the carbon metabolism took place during nitrogen deprivation, but we also demonstrated the uptake optimization of endogenous nitrogen sources by the GS/GOGAT cycle.

In most cyanobacteria, CP is used as a nitrogen- and possibly carbon-storage compound (Kolodny et al., 2006; Picossi et al., 2004). The CP content decreased under nitrogen depletion, corresponding to a probable degradation of this highly nitrogen-rich polymer, thereby releasing arginine and aspartate residues.

In parallel, the arginine and aspartate catabolism associated with the GS/GOGAT cycle seemed to be upregulated. Higher synthesis of the key enzymes GS and Aat2 was observed. Moreover, arginine was catabolized by a combination of the urea cycle and arginase pathway as demonstrated by a higher abundance of carbamoylphosphate synthase CarA, ornithine carbamoyltransferase ArgI, argininosuccinate synthase ArgG and argininosuccinate lyase ArgH. Similar results were observed at the mRNA level in Synechococcus sp. strain PCC 7002 and Synechocystis sp. Indeed, transcriptomic studies showed that the glnA gene encoding GS was upregulated when Synechococcus sp. strain PCC 7002 and Synechocystis sp. were shifted into nitrogen-free medium (Krasikov et al., 2012; Ludwig & Bryant, 2012). A previous study indicated that nitrogen deprivation induced the upregulation of aspC, argG and argH genes in Synechococcus sp. (Miller et al., 2002).

Ammonium needed for glutamine synthesis as catalysed by GS might also be supplied by formamide, nitrile and cyanate catabolism, involving the nitrilase/cyanide hydratase NCH, FmdA and CynS enzymes, which were upregulated under nitrogen deficiency. Transcriptomic investigations of Synechocystis sp. and Synechococcus sp. partially corroborate our results, demonstrating the enhanced expression of three genes encoding CynS (Krasikov et al., 2012; Miller et al., 2002), the nitrilase MerR and a homologue of another nitrilase (Miller et al., 2002) under nitrogen deprivation. The nitrilase/cyanide hydratase (ARTHROv3_1490024) shares 19.27 % identity and 42.77 % similarity with MerR encoded by sll0784, which is upregulated under nitrogen starvation in

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*Fig. 9.* After 30 days of nitrogen starvation, the cyanobacteria were shifted into nitrate-replete medium and incubated at 30 °C on an orbital shaker under artificial light (± 43 μmol s⁻¹ m⁻² and 5.6 Watt m⁻²). Microscopy observations under white light (a) and fluorescence (λ excitation 560 ± 40 nm, λ emission 630 ± 75 nm) (b) were performed at 0 (i) and 24 h (ii) after shifting into nitrate-replete medium.
**Synchocystis sp.** PCC 6803 (Schlebusch & Forchhammer, 2010). This putative nitrilase was biochemically characterized and demonstrated to possess nitrilase activity (Heinemann et al., 2003). Taken together, these data suggest the potential involvement of these nitrilases in adapting to combined nitrogen starvation.

Proteomic results also showed an increase in the abundance of the putative agmatine ureohydrolase SpeB-like that hydrolyses agmatine into urea and carbon dioxide. Although urease was not significantly upregulated under nitrogen starvation, the ureolytic activity showed a significant increase at 24 h. Thus, urea produced from agmatine degradation could be hydrolysed by urease, producing ammonium to supply the GS.

Finally, an uptake optimization of external nitrogen sources was also observed with an increase of several transporters, such as the putative glutamate and aspartate amino acid transporter, branched-chain amino acid or ammonium transporter 1.

Furthermore, nitrogen starvation induced greater synthesis of regulator protein PII after 24 h of growth without nitrate. The phosphoprotein PII is known to be required under nitrogen deprivation (Görl et al., 1998), and the glnB gene appeared to be upregulated under the same conditions (Krasikov et al., 2012; Miller et al., 2002). PII signals the intracellular nitrogen pool to other molecular regulators, transporters or transcription factors through its phosphorylation/dephosphorylation on a seryl residue (Fokin et al., 2010, 2011; Forchhammer & Tandeau de Marsac, 1995b; Irmler & Forchhammer, 2001). In addition, the PphA that dephosphorylates PII was downregulated, suggesting that PII might not be dephosphorylated anymore. Moreover, the ppHA gene was shown to be downregulated in *Synechocystis* sp. during nitrogen deprivation (Krasikov et al., 2012). To date, the specific kinases of PII are still unknown (Fokin et al., 2011; Sant’Anna et al., 2009; Osanai & Tanaka, 2007).

Recently, metabolic intermediate profiling of targeted cyanobacteria, such as *A. platensis* NIES-39 and *Synechocystis* sp. PCC 6803, demonstrated a global carbon metabolic reprogramming that involved transient increases in the levels of some amino acids during the glycolysis production induced by nitrogen deprivation (Hasunuma et al., 2013). Normally, glycolysis is synthesized from the CO2 that has been assimilated during light exposure. Hasunuma and collaborators suggested that under nitrogen depletion conditions, the carbon skeleton of glycolysis was synthesized with carbon derived from amino acids that had been released from proteins via gluconeogenesis (Hasunuma et al., 2013). In this work, we observed protein degradation that might release free amino acids, which could be used for gluconeogenesis. First, a decrease in the protein content was also observed as shown by Hasunuma et al. (2013). Second, some proteases were upregulated after nitrogen depletion, such as metallopeptidase, peptidase C1 and carboxypeptidase. Finally, the well-known rapid bleaching phenomenon was observed under nitrogen deprivation. These results indicated a significant decrease in the PC and APC contents after 24 h. Proteomic analysis also showed a significant decrease in the abundance of APC subunit beta and other components of phycobilisomes (e.g. protein D2 and CpcC1 linker) after 24 h.

In parallel, we observed the potential downregulation of glycolysis and glycogen degradation (e.g. GltP, FbaA and TalB). By contrast, some enzymes involved in carbohydrate synthesis (e.g. Pps, Eno, Maeb, Fbp and GpmB-like) were upregulated, suggesting a potential upregulation of glycolysis biosynthesis. In this context, Rubisco catalyses carbon fixation, which involves the condensation of CO2 and ribulose-1,5-bisphosphate (RuBP). This process yields two molecules of 3-phosphoglycerate (3PGA). Through the Calvin cycle, 3PGA is converted into triose phosphates, which serve as initial carbon skeletons for the synthesis of intracellular metabolites. In our experiments, a significantly lower abundance of the large subunit of Rubisco and potential Calvin cycle regulator CP12-like was observed under nitrogen deprivation. In accordance with Hasunuma et al. (2013), these data suggested that the carbon skeleton of glycolysis was synthesized from amino acids released from proteins via gluconeogenesis and not from CO2 through the Calvin cycle. Clearly, the response of *Arthrospira* sp. strain PCC 8005 to the nitrogen stress is completely different from that of heterocystous bacteria (Bai et al., 2004; Stensjö et al., 2007).

A loss of cell buoyancy was observed during nitrogen deprivation. Gaseous vesicles (GVs) are hollow protein structures filled with several gases. The shell of these proteins is primarily made of gas vesicle protein A and C, GvpA and GvpC, respectively (Buchholz et al., 1993; McMaster et al., 1996; Mlouka et al., 2004; Walsby, 1994). Proteomic analysis did not indicate a significant variation in the GvpA and GvpC abundance under nitrogen starvation, suggesting that the sinking did not originate from a loss of GvpA and GvpC. Cyanobacterial sinking might also be related to processes such as an increase in the turgor pressure and/or an increase in cellular components such as carbohydrates (Schwarz & Forchhammer, 2005; Walsby, 1994). Indeed, the increased glycolgen contents could act as ballasts, which could explain the trichome sinking observed under nitrogen starvation. Nevertheless, the total protein content was decreased during nitrogen depletion. Because the proteomic analyses were normalized by the same quantity of proteins (2 µg for each sample), a decrease in the GV proteins could not be observed. A decrease in the GV proteins and thus of the gas vesicles could be another reasonable explanation for the sinking observed during nitrogen depletion. Microscopic analysis by phase contrast showed a significant decrease in gas vesicles (Fig. S2).

In this work, the survival of *Arthrospira* sp. strain PCC 8005 was also investigated after long-term nitrogen depletion. The results indicated a return of growth (+ 100 h) from cultures submitted to long-term nitrogen starvation (30 days). This ability to survive was already noted for *Synechococcus* sp. strain PCC 7942 after 12 months of nitrogen deficiency.
(Sauer et al., 2001). The observed accumulation of glycogen as a depot of intracellular energy and/or carbon source by cyanobacteria could be advantageous during periods of starvation. Interestingly, epifluorescence microscopy revealed that some cells in the trichomes maintained a high-fluorescence level after long-term nitrogen deprivation as already mentioned by Zhang et al. (2009). The number of these highly fluorescent cells increased after shifting the cyanobacterium into nitrate medium. All cells exhibited fluorescence after 96 h. The return of fluorescence suggested that the recovery of a long-term starved culture might arise from the reactivation of all cells rather than the multiplication of a few cells that had survived during starvation. Intriguingly, proteomic analyses showed proteins HetR and HglK, which are known to be involved in heterocyst formation, were significantly upregulated after 24 h. The other heterocyst-specific proteins, namely HetF-like (ARTHROv3_1370003) and PatS (ARTHROv3_1530028), were not identified in our data. Although the Arthrospira genus has no ability to form heterocysts, it seemed to be able to produce heterocyst-related molecular actors. These results suggested an additional role for HetR and HglK, not to exclude a possible role in cellular survival under nitrogen starvation. Moreover, we showed the presence of potential spores or dormant cells coming from the differentiation of vegetative cells (Görl et al., 1998; Sauer et al., 2001), and we also provided new insight into the evolutionary origin of heterocyst cells, which could derive from surviving cells. Nevertheless, the reappearance of red autofluorescence in N-starved trichomes in the presence of a septum-formation inhibitor suggests that the recovery of a long-term starved culture could arise from the reactivation of cells rather than the multiplication of a few cells that had survived starvation.

In conclusion, the proteomic analysis combined with the cellular view described here has confirmed the reprogramming of carbon metabolism during nitrogen depletion, but we also demonstrated the uptake optimization of endogenous nitrogen sources. This study also revealed some specific cells that could help maintain the viability of entire filaments during long periods of nitrogen depletion through an unknown mechanism.

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