CfrA, a Novel Carbon Flow Regulator, Adapts Carbon Metabolism to Nitrogen Deficiency in Cyanobacteria

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Cyanobacteria unable to fix atmospheric nitrogen have evolved sophisticated adaptations to survive to long periods of nitrogen starvation. These genetic programs are still largely unknown—as evidenced by the many proteins whose expression is regulated in response to nitrogen availability, but which belong to unknown or hypothetical categories. In *Synechocystis* sp. PCC 6803, the global nitrogen regulator NtcA activates the expression of the *sll0944* gene upon nitrogen deprivation. This gene encodes a protein that is highly conserved in cyanobacteria, but of unknown function. Based on the results described herein, we named the product of *sll0944* carbon flow regulator A (CfrA). We analyzed the phenotypes of strains containing different levels of CfrA, including a knock-out strain (ΔcfrA), and two strains overexpressing CfrA from either the constitutive *Ptrc* promoter (Ptrc-cfrA) or the arsenite-inducible promoter *Pourb* (Pars-cfrA). Our results show that the amount of CfrA determines the accumulation of glycogen, and affects the synthesis of protein and photosynthetic pigments as well as amino acid pools. Strains with high levels of CfrA present high levels of glycogen and a decrease in photosynthetic pigments and protein content when nitrogen is available. Possible interactions between CfrA and the pyruvate dehydrogenase complex or PII protein have been revealed. The phenotype associated with CfrA overexpression is also observed in PII-de deficient strains; however, it is lethal in this genetic background. Taken together, our results indicate a role for CfrA in the adaptation of carbon flux during acclimation to nitrogen deficiency.
The 2-OG plays a central role in a complex regulatory network as the effector of different regulators, including NtcA or the widely distributed PII signal transduction protein. 2-OG directly binds NtcA linking its function with nitrogen status but maximal activation of NtcA also requires the binding of the coactivator PII interacting protein (PipX), which mutually exclusively binds to either NtcA or PII (Forchhammer and Selim, 2020).

In the absence of combined nitrogen, the GS-GOGAT cycle is not able to cope with the supply of 2-OG, which results in increased levels of this metabolite (Muro-Pastor et al., 2001). This increase reports an imbalance in the nitrogen state that activates NtcA, which triggers the metabolic adaptation to nitrogen starvation known as “chlorotic response” or “bleaching” (Giner-Lamia et al., 2017). This process includes a rapid degradation of phycobilisomes (cyanobacterial light-harvesting complexes), which releases amino acids required for the synthesis of stress acclimation proteins (Forchhammer and Schwarz, 2019). Another immediate response to nitrogen starvation is the accumulation of glycogen, which serves as carbon sink for the fixed CO₂ (Gründel et al., 2012). When nitrogen starvation persists, cells gradually enter a quiescent state with minimum photosynthetic activity and arrested cell growth, which allows them to survive long periods of time in these conditions (Spät et al., 2018). This process is reversible, and chlorotic cells can efficiently return to vegetative growth upon nitrogen availability (Klotz et al., 2016).

Many high-throughput studies have addressed the process of adaptation to nitrogen deficiency in different cyanobacteria, and many genes and proteins of unknown function have been identified as possibly involved in this sophisticated adaptation strategy (Krasikov et al., 2012; Hasunuma et al., 2013; Osanai et al., 2014a; Spät et al., 2015, 2018; Choi et al., 2016; Carrieri et al., 2017; Giner-Lamia et al., 2017). A global study of the NtcA regulon of *Synechocystis* provided an overview of the genes whose expression depends on this regulator (Giner-Lamia et al., 2017), although many of them belong to hypothetical categories. One of these genes is *sll0944*, whose expression is positively regulated by NtcA and significantly induced during the first stages of acclimation to nitrogen starvation (Osanai et al., 2006, 2014a; Giner-Lamia et al., 2017). Recently it has also been linked to the C/N regulatory protein PII (Watzer et al., 2019). This gene currently lacks annotation and belongs to the group of conserved hypothetical genes with a wide phylogenetic distribution.

In this work we have carried out a characterization of the protein encoded by *sll0944*, which, based on our results, we have named “carbon flow regulator A” (CfrA). A physiological and metabolic study of *Synechocystis* strains with different levels of CfrA has allowed us to conclude that it is involved in the modulation of carbon flow that takes place during adaptation to nitrogen starvation.

**RESULTS**

**CfrA Is a Highly Conserved Protein Exclusive of Cyanobacteria**

A search of CfrA homologs revealed that this protein is widely distributed among cyanobacteria and seems exclusive to this phylum. Figure 1A shows an alignment of CfrA homologous sequences from some cyanobacteria. This alignment shows a highly conserved region that constitutes the unknown function domain DUF1830 followed by a variable region. In the case of *Synechocystis* CfrA, the DUF1830 domain extends between Cys-18 and Ile-83. This analysis, together with the previous one of the promoter region of *sll0944* (Giner-Lamia et al., 2017), reveals a misannotation of the CfrA protein in the Uniprot database (https://www.uniprot.org/uniprot/P77971). The protein has a molecular mass of 12 kDa and a theoretical pl of 4.2. A representation of the identity of the residues for each of the positions of the DUF1830 domain in all the available sequences using WebLogo (Crooks et al., 2004) is shown in Figure 1B, and a compilation of these sequences is provided (Supplemental Dataset S1). We have also carried out ab Initio models of CfrA structure using the prediction tool ROSETTA (Song et al., 2013). All the generated models were quite consistent in the
region corresponding to the DUF1830 domain. Images of tertiary structure and surface hydrophobicity of CfrA, obtained using the program UCSF Chimera (Petersen et al., 2004), are shown in Figure 1, C and D, respectively.

**CfrA Is Expressed during Early Acclimation to Nitrogen Starvation**

*slt0944*, encoding CfrA, was identified as a member of the NtcA regulon whose transcription is upregulated after nitrogen depletion (Giner-Lamia et al., 2017). This expression pattern has been observed for CfrA homologs in other cyanobacteria (Mitschke et al., 2011; Choi et al., 2016). To monitor the cellular level of CfrA, we produced specific antibodies against this protein (Supplemental Methods S1). Cells of the *Synechocystis* wild-type strain were grown under standard conditions until exponential growth phase, and then incubated in nitrate-free medium. As shown in Figure 2A, CfrA was undetectable in nitrate-growing cells and accumulated early after nitrogen removal. This accumulation reached its maximum in the first 24 h of nitrogen deficiency and decreased after 48 h in these conditions, with minimum levels at the end of the analyzed period (144 h). Then, sodium nitrate was added at the standard concentration in BG11C. CfrA accumulated again during the first 24 h in these conditions, and then it progressively decreased to the undetectable level characteristic of nitrate growth.

To investigate the effect of CfrA accumulation under nitrogen sufficiency, in which it is not naturally expressed, we generated a construct where the *cfrA* open reading frame (ORF) was placed under the control of *Ptrc*, a nitrogen-independent constitutive promoter in *Synechocystis* (Guerrero et al., 2012; Table 1; Supplemental Fig. S1). The *Ptrc-cfrA* strain was analyzed in the same conditions described above for the wild type. As expected, the *Ptrc-cfrA* strain accumulated high levels of CfrA in the presence of nitrate (Fig. 2B), and this accumulation was maintained during the first 48 h of nitrogen deficiency although it subsequently decreased to minimum levels in chlorotic cells (120 to 144 h of nitrogen deficiency). When nitrate was re-added to chlorotic cells, CfrA protein accumulated again at high levels that were maintained throughout the analyzed period. The accumulation of the housekeeping protein GSI used as control did not experience substantial changes throughout the chlorosis process and its reversion. However, its accumulation appears to be slightly less in the CfrA-overexpressed strain.

**CfrA Expression Levels Influence the Rate of Chlorosis Reversion**

Because CfrA expression is induced during adaptation to nitrogen deficiency (Fig. 2A), we analyzed in more detail the process of chlorosis and its reversion in strains with different levels of CfrA. Wild-type, *ΔcfrA*, and *Ptrc-cfrA* strains were compared. As shown in Figure 3A, after 13 d of nitrogen starvation, absorption spectra of the three strains were almost identical and showed the characteristic degradation of the photosynthetic pigments of the chlorotic cells, demonstrating that the CfrA protein is not required for the bleaching process. However, when nitrate was added, a clear difference was observed in the awakening of the three strains, indicating that CfrA expression levels affect resuscitation after chlorosis. In fact, the *cfrA* knock-out strain (*ΔcfrA*) regreened faster than the wild-type, whereas the overexpressing strain (*Ptrc-cfrA*) did it more slowly (Fig. 3, B–D). The maximum difference in pigmentation was observed 48 h after the addition of nitrate to the chlorotic cells (Fig. 3, C and D). Additionally, it was observed that the time required for greening was shorter in all the strains when the chlorosis period decreased (Supplemental Fig. S2).

**Glycogen Concentration Is Dependent on CfrA Levels in *Synechocystis***

Because in *Synechocystis* glycogen begins to accumulate almost immediately upon nitrogen depletion (Luan et al., 2019), we wanted to assess whether CfrA expression levels affected the accumulation of this polymer. We placed the *cfrA* gene under the nitrogen-independent, arsenite-inducible promoter of the *arsBHC* operon of *Synechocystis*, which shows high ON/OFF ratio (López-Maury et al., 2003) and constructed the *Pars-cfrA* strain (Table 1; Supplemental Fig. S1). To analyze this strain, it was first propagated without arsenite in BG11C medium and then flasks were inoculated at 0.3 OD$_{750}$ in the presence of increasing arsenite concentrations (1, 5, 10, 20, 50, 200, and 500 μM). A control culture without arsenite was also included. After 24 h in the presence of arsenite, samples were collected and the accumulation of CfrA and glycogen was analyzed. As shown in Figure 4A, an arsenite-dependent CfrA accumulation was observed in this strain. While *cfrA* overexpression in these conditions did not affect cell growth (Fig. 4B), the glycogen concentration increased proportionally to the amount of CfrA (Fig. 4C). We quantified up to a 12-fold increase of glycogen content in the presence of 500 μM of arsenite compared to the control sample. As an indication of the C/N balance and of carbon flux toward the tricarboxylic acid cycle, we also measured glutamine synthetase (GS) activity, which slightly decreased with increasing arsenite concentrations (Fig. 4D). To ensure maximum induction of CfrA in the experiments that follow, 1 mM of arsenite was used.

**The Level of CfrA Affects Glycogen Content without Altering the Accumulation of the Glycogen Synthesis Enzymes GlgC and GlgA1**

Once it was determined that the amount of CfrA affects the accumulation of glycogen, we compared the wild-type, *ΔcfrA*, and *Pars-cfrA* strains to test whether...
Figure 1. Sequence and structural analysis of CfrA. A, Sequence alignment of several CfrA homologous proteins from representative cyanobacteria. The asterisks denote identical residues in all aligned sequences, shaded in dark gray; the colons denote residues with a strong conservation, shaded in medium gray; and the periods denote weakly conserved residues, shaded in light gray. B, WebLogo representing the identity of amino acid residues for each of the positions of the DUF1830 domain in all the sequences available in the databases. C and D, Ab initio structural model of CfrA generated by the program ROSETTA (Song et al., 2013). Images of CfrA structure, using the program UCSF Chimera (Pettersen et al., 2004), are represented as both tertiary structure ribbon representation, colored from blue (N terminus) to red (C terminus; C), and surface hydrophobicity map from blue for the most hydrophilic, to white, to orange-red for the most hydrophobic (D).
the observed phenotype for the overexpressing strain (Fig. 4) was maintained or enhanced in the presence of additional carbon supply. The strains were propagated in BG11C at 0.3 OD750, after the elimination of the nitrogen source (NO3−), after and readdition of this source (+NO3−) at the indicated times. Total protein crude extracts were obtained from cells corresponding to 2 OD750 in each case. Equal volumes of each extract were loaded, resolved on SDS-PAGE, blotted, and incubated with anti-CfrA antibodies. The samples from 144 h of nitrogen starvation (~N) were loaded in both gels as a control. Membranes were incubated also with anti-GSI antibodies as control of a housekeeping protein.

The strains used in this work

Table 1. Synechocystis strains used in this work

| Name                   | Description                                      |
|------------------------|--------------------------------------------------|
| Wild type              | *Synechocystis* sp. PCC 6803 wild type           |
| ΔcfrA                  | ΔcfrA::aadA−, SmSp'                               |
| Ptc-cfrA               | ΔcfrA::aadA−, nrdD::Pptc-cfrA::kan, SmSp', Km'   |
| Pars-cfrA              | ΔcfrA::aadA−, nrdD::Pars-cfrA::kan, SmSp', Km'   |
| ΔglnB                  | ΔglnB::cm, Cm'                                   |
| ΔglnB/Pars-cfrA        | ΔglnB::cm, nrdD::Pars-cfrA::kan, Cm', Km'        |

Photosynthetic Pigment Concentrations Are Also Affected by CfrA Accumulation

A clear change in the color of the Pars-cfrA strain was noted after arsenite addition. These cultures appeared less blue-green than the wild-type or the ΔcfrA cultures. We analyzed and compared the absorption spectra of the three strains before and after 24 or 72 h of arsenite addition. We could observe a notable decrease in absorbance at 678 and 625 nm in the case of the overexpressing strain (Fig. 6A). Quantification of the differences in absorbance of the 678-, 625-, and 485-nm peaks, which are indicative of chlorophyll, phycobilin, and carotenoid contents, respectively, is shown in Figure 6, B to D. A significant decrease in chlorophyll content was observed in the case of Pars-cfrA strain, while a very subtle increase was observed for the ΔcfrA strain. As shown in Figure 6C, there was a significant decrease of phycobilins with CfrA overexpression, while the deletion of cfrA caused a small increase in the content of this pigment (Fig. 6C). With respect to carotenoids, there was a first decay 24 h after CfrA induction in Pars-cfrA, but carotenoid content returned to wild-type levels after 72 h. However, no significant changes were observed for ΔcfrA (Fig. 6D).

Using confocal laser microscopy, a notable decrease of auto-fluorescence (shown in red) was noticed in the Pars-cfrA strain respect to the others. Interestingly, we could also observe that cells of this strain were slightly larger than those of the other two strains, although due to the small size of *Synechocystis* cells (~2 μm), a more detailed study would be required to confirm whether these differences are significant (Fig. 6E).

CfrA Accumulation Affects Amino Acid Content

To analyze the set of amino acids in the Pars-cfrA strain, we proceeded as in previous experiments.
(Fig. 5), but by comparing cultures of Pars-cfrA strain in the absence (−) or presence (+) of 1 mM of arsenite. We quantified the 20 amino acids in both cultures and compared their levels at different times after arsenite addition. A control of the already described phenotype of Pars-cfrA in this experiment is shown in Supplemental Figure S3.

As shown in Figure 7, we observed a significant increase in the Ala and Ser pools 24 h after the induction of CfrA. However, after 48 h the content of these amino acids started to decline. Arg and Trp contents in Pars-cfrA (+) experienced a reduction of 73% and 70%, respectively, when compared to Pars-cfrA (−). CfrA overexpression also reduced the levels of Thr, Lys, and Glu ∼50%. There was a small reduction of Cys, His, Phe, Gln, Asp, and Asn pools, while the contents of the remaining amino acids were not significantly different in both cultures.

Figure 3. Evolution of pigmentation during resuscitation of chlorotic cells. A to C, Absorption spectra (400 to 750 nm) of Synechocystis cultures from wild-type, ΔcfrA, and Pt-cfrA strains during resuscitation. After 13 d of nitrogen starvation (−N), sodium nitrate (17.6 mM) was added to cultures and spectra were performed at the indicated times (+NO₃⁻). Spectra were normalized to the same optical density at 750 nm of 1. D, Image of wild-type (WT), ΔcfrA, and Pt-cfrA Synechocystis strains after 48 h of sodium nitrate addition. A representative experiment of several independent replicates is shown.

Figure 4. Analysis of Pars-cfrA strain with increasing arsenite concentrations. A, Western-blot analysis of CfrA and GSI in Pars-cfrA after 24-h growth with increasing arsenite (AsO₂⁻) concentrations (0, 1, 5, 10, 20, 50, 200, and 500 μM). Total protein crude extracts were obtained from cells corresponding to 2 OD₇₅₀ in every case. Equal volumes of each extract were loaded. Coomassie Brilliant Blue (CBB) staining is shown as a protein loading control. B, Growth analysis of Pars-cfrA cultures grown with increasing arsenite concentrations. C and D, Glycogen content (C) and GS activity (D) of Pars-cfrA cultures after 24-h growth with increasing arsenite concentrations. A representative experiment of several independent replicates is shown.
Figure 5. Comparative analysis of wild-type (WT), \( \Delta \text{cfrA} \), and Pars-cfrA strains. A, Western-blot analysis of CfrA in wild-type and Pars-cfrA strains at 0, 6, 24, and 48 h after 1 mM of arsenite addition. B, Growth analysis of wild-type, \( \Delta \text{cfrA} \), and Pars-cfrA before and after the addition of 1 mM of arsenite for CfrA overexpression. C, GS activity of wild-type, \( \Delta \text{cfrA} \), and Pars-cfrA at 0, 24, and 72 h after 1 mM of arsenite addition. D, Western-blot analysis of GSI, GSIII, and NtcA in wild-type, \( \Delta \text{cfrA} \), and Pars-cfrA strains at 0, 24, and 48 h after 1 mM of arsenite addition. E, Glycogen content of wild-type, \( \Delta \text{cfrA} \), and Pars-cfrA at 0, 24, and 72 h after 1 mM of arsenite addition. F, Western-blot analysis of GlgC and GlgA1 in wild-type, \( \Delta \text{cfrA} \), and Pars-cfrA strains at 0, 24, and 48 h after 1 mM of arsenite addition. In A, D, and F, total protein crude extracts were obtained from cells corresponding to 2 OD_{750} in every case. Equal volumes of each extract were loaded onto the gels, and Coomassie Brilliant Blue (CBB) staining is shown as a protein loading control. Error bars in B, C, and E represent SD of the mean values from three independent experiments. Asterisks indicate significance difference using ANOVA test (**P < 0.005 and ****P < 0.0001).
CfrA Overexpression Alters the Inner Structure and Morphology of *Synechocystis* Cells

As mentioned before, Pars-cfrA cultures showed slightly higher OD$_{750}$ after arsenite addition than those of the wild-type or ΔcfrA strains (Fig. 5B; Supplemental Fig. S3A). We wondered whether these differences revealed faster growth of these cells or were instead due to changes in cell morphology or cell size. In addition, the decantation time of the cultures seemed to depend on the amount of CfrA, being shorter in the cultures of the Pars-cfrA strain compared to the wild type and slightly higher in the cultures of the ΔcfrA strain. (Supplemental Fig. S4).

We compared cells of the Pars-cfrA strain grown for 24 h in the presence or absence of arsenite using flow cytometry and could detect a small but significant increase in cell size and complexity of cells grown in the presence of arsenite. Again, a decrease in chlorophyll a content was associated to CfrA overexpression (Supplemental Fig. S5).

Cells of the wild-type, ΔcfrA, and Pars-cfrA strains were also analyzed using transmission electron microscopy to study the accumulation of storage compounds as well as the status of the cell cytoplasm and thylakoid membranes. As shown in Figure 8, and consistent with the results previously described, induction of CfrA expression in Pars-cfrA strain resulted in a high increase of glycogen accumulation, while the deletion of *cfrA* caused some decrease in glycogen content in...
A significant but modest increase in glycogen content was observed in the cultures of the wild-type and ΔcfrA strains 24 h after the addition of arsenite. This increase is related to the age of the cultures and was quantified after 24 and 72 h of arsenite addition (Fig. 5E).

**The Phenotype Associated with CfrA Overexpression Is Reversible**

Considering the characteristics of the P_{arsB} promoter, the transcription of the cfrA gene in the Pars-cfrA strains would be stopped by removing the inducer. To analyze...
the evolution of CfrA in the cultures upon arsenite removal, we proceeded as in previous experiments and after 72 h of arsenite addition, it was removed. The control culture was also washed to equalize the effects of biomass loss. Samples were collected for the analysis of cell growth, GS activity, chlorophyll, and glycogen content. As shown in Supplemental Figure S6, the phenotype associated with CfrA overexpression had reverted and CfrA was undetectable 72 h after removing the inducer.

CfrA Expression Levels Affect the Rate of Glycogen Consumption during the Reversal of Chlorosis

We compared glycogen accumulation levels of wild-type, ΔcfrA, and Pars-cfrA strains after addition of arsenite for 24 h and subsequent nitrogen starvation for 5 d. Evolution of this polymer during recovery of the chlorosis by nitrate addition was also analyzed, maintaining the presence of arsenite throughout the process. As shown in Figure 9A, the three strains accumulated high levels of glycogen under nitrogen starvation. This accumulation was only 10% lower in the knockout strain compared to the wild type, and 10% higher in the overexpressing strain. However, a clear difference could be observed in the mobilization of glycogen after the addition of nitrate to chlorotic cells. The knockout strain consumed glycogen faster than the wild-type strain, coinciding with its rapid regreening, while the Pars-cfrA strain showed a long delay in the reversal of chlorosis and a low mobilization of glycogen (Fig. 9).
Various Components of the Pyruvate Dehydrogenase Complex and PII Protein Coimmunoprecipitated with CfrA

To identify possible CfrA interactors, cells of the Pars-cfrA strain cultivated in the presence of arsenite for 24 h were used to obtain crude extracts. CfrA was immunoprecipitated using anti-CfrA antibodies coupled to protein-A superparamagnetic beads. A control with preimmune serum was carried out in parallel. Precipitated material in both cases was analyzed by liquid chromatography with tandem mass spectrometry (LC-MS/MS) using a time-of-flight quadrupole and the software ProteinPilot v5.0.1 (Sciex) for protein identification (Supplemental Methods S2). A list of the proteins identified ordered by their score, based on the number of distinctive peptides identified and the false discovery rate, is available (Supplemental Dataset S2). One of the proteins identified only with the immune serum with highest score was dihydrolipoamide dehydrogenase, a component of the pyruvate dehydrogenase (PDH) complex and other 2-oxo acid dehydrogenase complexes (Engels and Pistorius, 1997). Interestingly, two other specific components of the PDH complex also coimmunoprecipitated with CfrA, dihydrolipoamide acetyltransferase, and pyruvate dehydrogenase E1 beta subunit. These results point to an interaction of CfrA with the PDH complex that could modulate its activity, thus regulating the flow of carbon toward the tricarboxylic acid cycle. The signal transduction PII protein was also identified in the immunoprecipitation with anti-CfrA serum. The fact that this protein is a regulator of the C/N homeostasis prompted us to further investigate their relationship with CfrA. Additionally, in the course of this work the presence of CfrA in pull-down experiments using PII-FLAG protein was reported (Watzer et al., 2019).

Overexpression of CfrA Causes High Glycogen Accumulation Irrespective of the Presence of PII

To study if the phenotype associated with CfrA overexpression was somehow dependent on PII, the cfrA version controlled by ParsB was introduced in a strain lacking PII (ΔglnB). The resulting strain, ΔglnB/Pars-cfrA, was analyzed together with its parental strain ΔglnB. The Pars-cfrA strain was included as a control of CfrA expression and glycogen accumulation. Comparative phenotypic analysis in an induction experiment with arsenite is shown in Figure 10. Addition of arsenite to ΔglnB/Pars-cfrA strain stopped growth of this strain in ~24 h, indicating that overexpression of CfrA in the absence of PII is lethal under the conditions tested (Fig. 10A). Accumulation of CfrA 24 and 48 h after arsenite addition was considerably lower in the ΔglnB/Pars-cfrA strain compared to the Pars-cfrA strain (Fig. 10B). PII protein was also analyzed to verify the ΔglnB strain as well as to test whether the overexpression of CfrA in the Pars-cfrA strain entails any modification in PII accumulation. A slight increase in the amount of PII could be observed in the Pars-cfrA strain after the addition of arsenite (Fig. 10B). This increase did not take place in the wild type, so it cannot be attributed to the addition of arsenite (not shown). Similar to strains with an intact glnB locus, CfrA overexpression in a ΔglnB genetic background leads to a decrease in the total protein content as well as in the activity and amount of GS, but these effects were somewhat more drastic than those described in strains with PII (Fig. 10, B and C). With respect to glycogen, accumulation of this polymer dependent on the addition of arsenite was observed in both strains, ΔglnB/Pars-cfrA and Pars-cfrA, but this accumulation was significantly higher in the ΔglnB/Pars-cfrA strain (Fig. 10D). As in Pars-cfrA strain, overexpression of CfrA in a ΔglnB genetic background caused a decrease in photosynthetic pigments (Fig. 10E).

DISCUSSION

Glycogen is the main carbon sink in cyanobacteria, and adaptation to nitrogen deficiency in nondiazotrophic cyanobacteria requires correct glycogen metabolism. In fact, mutants defective in glycogen synthesis are unable to acclimate and survive the nitrogen starvation (Gründel et al., 2012). Our results indicate that the CfrA protein begins to accumulate in Synechocystis early after the removal of the nitrogen source (Fig. 2), and that this accumulation is maintained at high levels during the first 48 h of nitrogen starvation. This period matches the early response to nitrogen deficiency in which the progression of chlorosis and glycogen synthesis take place simultaneously (Krasikov et al., 2012; Klotz et al., 2016). Consistent with this expression pattern, the analysis of strains with different amounts of CfrA shows that the amount of glycogen that accumulates in cells clearly depends on the quantity of CfrA, even if the protein is expressed independently of the nitrogen conditions (Figs. 4C, 5E, and 8). These results indicate that CfrA controls the amount of carbon stored as reserve, supporting the hypothesis that CfrA is involved in redirection of the carbon excess toward glycogen synthesis under conditions of nitrogen starvation. As shown in Figure 2, the amount of CfrA reaches minimum levels in both the wild-type and the Ptrc-cfrA strains after a prolonged period of nitrogen deficiency. This corresponds to a state of fully developed chlorosis in which the translational machinery must be reduced to the minimum. The transitory increase in CfrA accumulation after readdition of nitrate could correspond to the translation of pre-existing cfrA transcripts because an increase in mRNA stability in chlorotic cells has been proposed (Klotz et al., 2016). Interestingly, this period coincides with the reported first resuscitation phase of chlorotic cells, energetically supported by respiration and before the de novo synthesis of the photosynthetic machinery (Klotz et al., 2016).
Figure 9. Glycogen consumption and evolution of pigments during the reversal of chlorosis. A, Absorption spectra (400 to 750 nm) and glycogen content of Synechocystis cultures from wild-type (WT), ΔcfrA, and Ptrc-cfrA strains during the reversal of chlorosis. After 5 d of nitrogen starvation (120 h −N), sodium nitrate (17.6 mM) was added to cultures and spectra were performed at the indicated times (+ NO₃⁻). Spectra were normalized to the same optical density at 750 nm of 1. On the right, the glycogen accumulated by each strain at each stage of the process is shown. B, Evolution of relative glycogen content with respect to that of chlorotic cells. The glycogen content of each strain is shown during the reversal of chlorosis relative to the accumulated amount before the addition of nitrate (100%). Error bars represent SD of the mean values from two independent experiments.
Figure 10. Comparative analysis of ΔglnB, ΔglnB/Pars-cfrA, and Pars-cfrA strains. A, Growth analysis of ΔglnB and ΔglnB/Pars-cfrA before and after the addition of 1 mM of arsenite for CfrA overexpression. B, Western-blot analysis of CfrA, PII, and GSI in ΔglnB, ΔglnB/Pars-cfrA, and Pars-CfrA strains at 0, 24, and 48 h after 1 mM of arsenite addition. Total protein crude extracts were...
The delay in the chlorosis reversion observed in the Ptrc-cfrA and Pars-cfrA strains (Figs. 3 and 9; Supplemental Fig. S2) could indicate that the presence of high levels of CfrA during the awakening process would partially prevent the flow of carbon toward protein synthesis, thus delaying the reconstitution of the photosynthetic apparatus and the recovery as a whole. In line with this, the strain lacking CfrA presents a faster reversal of chlorosis with respect to the wild-type strain, and rapidly mobilizes the glycogen accumulated in the period of nitrogen deficiency (Fig. 9).

The accumulation of glycogen depends on the amount of carbon available, which is a function of the availability of light and CO2 or an extra source such as Glc (Zavrel et al., 2017). Accordingly, the observed glycogen accumulation in the Pars-cfrA strain was dependent on carbon supply. This can be seen by comparing the cultures in BG11C (Fig. 4C) with those made in BG11C supplemented with CO2 (Fig. 5E). With similar levels of CfrA, the amount of accumulated glycogen was greater under conditions of higher carbon availability. These results confirm that it is the photosynthetic capacity that ultimately controls the global carbon flux.

Some studies in *Synechocystis* have linked a higher content of glycogen with larger cell size (Yamauchi et al., 2011; Zavrel et al., 2017). Accordingly, the slight increase in size observed in the Pars-cfrA strains (Fig. 6E; Supplemental Fig. S5) and their faster decantation (Supplemental Fig. S4) could be related to their glycogen content.

A remarkable phenotypic feature associated with the induction of CfrA expression in Pars-cfrA strains was the decrease in photosynthetic pigments (Figs. 6 and 10E; Supplemental Fig. S3F). This partial chlorosis took place under conditions of nitrogen deficiency and is different from that associated with the chlorotic response to nitrogen starvation, because it did not imply the characteristic almost-complete loss of the photosynthetic apparatus. Our hypothesis is that the overexpression of CfrA causes a decrease in carbon flux toward the tricarboxylic acid cycle and therefore toward the production of carbon skeletons for protein synthesis, namely 2-OG. A decrease in total protein synthesis results in a progressive loss of the content of phycobiliproteins with the consequent change in the coloration of the culture as growth progresses. This decrease in the amount of total protein could be observed in the crude extracts of the strain Pars-cfrA analyzed by SDS-PAGE and Coomassie staining (Figs. 5, A, D, and F, and 10B). Accordingly, the ΔcfrA strain presented a slight but significantly greater quantity of phycobilins with respect to the wild type (Fig. 6C), consistent with the small difference in the amount of CfrA between these two strains under conditions of nitrogen sufficiency. As occurs in the Pars-cfrA strains, a reduced amount of photosynthetic pigments is associated with a higher amount of glycogen in other *Synechocystis* strains, as is the case of CyAbrB mutants (Yamauchi et al., 2011).

If CfrA overexpression leads to much of the assimilated carbon being destined for the synthesis of glycogen to the detriment of its use for anabolic processes, the assimilation of nitrogen through the GS-GOGAT cycle should decrease with this overexpression. In fact, a decrease in GS activity could always be observed when the amount of CfrA increases (Figs. 4D, 5C, and 10C; Supplemental Figs. S3D and S6C). Several factors could contribute to this downregulation of the GS activity. Low levels of 2-OG would have a double effect, decreasing the transcription of the *glnA*, *glnN*, and *ntcA* genes (activated by NtcA depending on the concentration of 2-OG; Giner-Lamia et al., 2017) and decreasing nitrogen assimilation and general protein synthesis as a substrate of the GS-GOGAT cycle. A decrease in the amount of GSII, GSIII, and NtcA was associated with overexpression of CfrA (Fig. 5D).

The expected direct consequence of a decrease in the activity of the GS-GOGAT cycle would be the decrease in its product Glu, which is by far the most abundant amino acid in *Synechocystis* (Kiyota et al., 2014) and the major source of nitrogen for cellular metabolism (Flores and Herrero, 2004). As shown in Figure 7, the intracellular Glu pool decreased significantly (~50%) in the Pars-cfrA strain when CfrA expression was induced. This reduction resulted also in a drastic decrease in the Arg pool, an amino acid synthesized from Glu, which is a nitrogen reservoir. The levels of other amino acids were also reduced, to a greater or lesser extent, with CfrA overexpression. The synthesis of most of these amino acids involves the participation of Glu or Gln as donors of the amino group, and therefore their homeostasis will be influenced by the intracellular level of these nitrogen distributors. Interestingly, the amounts of several of these amino acids (Glu, Gln, Arg, Asp, Trp, and Asn) also decrease during early acclimation to nitrogen starvation, conditions in which CfrA is naturally expressed and protein synthesis is limited by the absence of nitrogen. Also, in agreement with what happens under nitrogen deficiency (Kiyota et al., 2014;...
Osanai et al., 2014b), some amino acids synthesized from pyruvate or 3-phosphoglycerate (Ala and Ser) experienced a moderate increase with the over-expression of CfrA (Fig. 7). These data lead us to propose that CfrA could limit the carbon flow toward acetyl-CoA synthesis during adaptation to nitrogen limitation, increasing the amount of some glycolytic intermediates and redirecting the flow toward glycogen synthesis while decreasing the flow toward tricarboxylic acid cycle and protein synthesis (Fig. 11). The identification of various components of the PDH complex as possible CfrA interactors (Supplemental Dataset S2) suggests that it could modulate the activity of this complex, which would agree well with the hypothesis. In line with this, studies of metabolic flux changes under nitrogen starvation demonstrated minimal flux into acetyl-CoA via PDH in these conditions (Qian et al., 2018). In fact, a large-scale study of protein–protein interaction identified a possible interaction between sll0944 ORF and the E1 subunit of the PDH complex (Sato et al., 2007).

Our results, along with others recently published (Watzer et al., 2019), show that the PII regulatory protein also interacts with CfrA. Accumulation of CfrA from the ParsB promoter in the PII-deficient strain ΔglnB/Pars-cfrA was lower than that observed in the Pars-cfrA strain. An effect of PII deficiency on ParsB activity is not expected, but rather we hypothesized a stabilizing effect of PII on CfrA perhaps mediated by their interaction. Accordingly, a slight increase of PII was observed in the Pars-cfrA strain by inducing CfrA expression. Interestingly, although the PII protein is very abundant (Forcada-Nadal et al., 2018), its expression increases in conditions of nitrogen deficiency (Giner-Lamia et al., 2017), in which CfrA is physiologically expressed. These results could indicate a possible buffering or modulating role of PII on CfrA activity. In this sense, in the absence of PII, the action of CfrA would be enhanced.

Regarding the lethality of CfrA overexpression in the absence of PII under conditions of nitrogen sufficiency, some hypotheses can be raised. The PII protein has been described to interact with acetyl-CoA carboxylase (ACCase), negatively regulating fatty acid synthesis based on available carbon. This interaction is inhibited by 2-OG in a dose-dependent manner (Hauf et al., 2016). It has been proposed that PII, by controlling ACCase, could modulate the distribution of the carbon flux toward either the tricarboxylic acid cycle and GS-GOGAT cycle or toward lipid biosynthesis (Forchhammer and Selim, 2020). In addition, the interaction of the PII protein with phosphoenolpyruvate carboxylase (PEPC), an enzyme that plays an important role in the flow of carbon to the tricarboxylic acid cycle, has recently been reported. This interaction increases the PEPC activity and therefore the oxaloacetate synthesis when the 2-OG concentration is low (Scholl et al., 2020). The overexpression of CfrA, which leads to a decrease in the carbon flux toward 2-OG (probably acting on PDH), in a genetic background lacking PII and therefore without the negative control of the ACCase and the positive action on PEPC, could...
lead to the observed lethality of the ΔglnB/Pars-cfrA strains by depletion of the 2-OG and Glu pools. In fact, PII-deficient mutants have been reported to have greatly reduced levels of 2-OG and Glu (Schwarz et al., 2014; Scholl et al., 2020). Our data indicate that CfrA overexpression also causes a considerable reduction in the Glu pool (Fig. 7). The combination of both effects could have lethal consequences.

In the proposed model for the wild-type strain under nitrogen limitation (high 2-OG), both CfrA and PII would contribute to reduce flow to the tricarboxylic acid cycle and 2-OG synthesis via its control of PDH (inhibition) and PEPC (absence of activation), respectively (Fig. 11). However, understanding the mechanistic details and metabolic consequences of the functional relationship among CfrA, PDH, and PII requires further investigation, as well as some other possible interactions of CfrA (Supplemental Dataset S2).

A deeper knowledge of metabolic circuits is needed to successfully address different biotechnological strategies in cyanobacteria. Carbon metabolism and its plasticity are key points of interest to redirect the fixed CO2 toward the synthesis of valuable compounds (Xiong et al., 2017). Due to the advantages of glycogen over lipocellulosic biomass (Möllers et al., 2014), the possible development and use of cyanobacterial strains with high glycogen content has been considered on several occasions (Shimakawa et al., 2014; Gupta et al., 2020). Pars-cfrA strains have a high potential in biomass accumulation, unlike what occurred compared to the wild type (Figs. 5E and 10D). Furthermore, this accumulation, unlike what occurred on several occasions (Shimakawa et al., 2014; Gupta et al., 2020). Pars-cfrA strains have a high potential in biomass production due to the large amount of glycogen they accumulate compared to the wild type (Figs. 5E and 10D). Furthermore, this accumulation, unlike what occurs in the wild type, takes place in the presence of a nitrogen source in stably growing cultures.

## MATERIALS AND METHODS

### Culture Conditions

*Synechocystis* sp. PCC 6803 derivative strains were grown photoautotrophically at 30°C on BG11 medium (Rippka, 1988), supplemented with 1 g L−1 NaHCO3, (BG11C) and bubbled with 1% (w/v) CO2 in air, under continuous illumination (50 to 70 μmol of photons m−2 s−1; 4000 K LED lights), hereafter used as “standard” conditions. Nitrogen deficiency was induced by removal of combined nitrogen. For plate cultures, 1% (w/v) Bacto agar (Difco) and the 10D). Furthermore, this accumulation, unlike what occurred on several occasions (Shimakawa et al., 2014; Gupta et al., 2020). Pars-cfrA strains have a high potential in biomass accumulation, unlike what occurred compared to the wild type (Figs. 5E and 10D). Furthermore, this accumulation, unlike what occurs in the wild type, takes place in the presence of a nitrogen source in stably growing cultures.

### Preparation of Crude Extracts and Western-Blot Analysis

For the analysis of proteins abundance, 2 U OD750 were harvested and resuspended in 80 μL of 50 mM HEPES-NaOH buffer (pH 7.0), 50 mM of KCl, and 1 mM of phenylmethylsulfonyl fluoride. Crude extracts were prepared using glass beads as described in Reyes et al. (1995). For western-blot analysis, proteins were fractionated on 12% (w/v) SDS-PAGE (Laemmli, 1970) and transferred to nitrocellulose membranes (Bio-Rad). Blots were blocked with 5% (w/v) nonfat dry milk (AppliChem) in phosphate-buffered saline-TWEEN 20. Antisera were used at the following dilutions: Anti-CfrA (1:10,000; Supplemental Methods S1), anti-GSI (1:250,000; Marqués et al., 1992), anti-GSI (1:10,000; García-Dominguez et al., 1997), anti-ΔglnB (1:2,000; Giner-Lamia et al., 2017), anti-ΔglnB (1:2,000; Giner-Lamia et al., 2017), anti-GSI (1:10,000; Giner-Lamia et al., 2017), anti-ΔglnB (1:10,000; Giner-Lamia et al., 2017), anti-GSI (1:10,000; Giner-Lamia et al., 2017), anti-GSI (1:5,000; Diaz-Troya et al., 2014). The ECL Prime Western Blotting Detection Reagent (GE Healthcare) was used to detect the different antibodies with anti-rabbit secondary antibodies (1:25,000; Sigma-Aldrich). In all cases, at least three independent experiments were carried out and a representative western-blot is shown.

### GS Assay

GS activity was determined in situ by using the Mn2+-dependent γ-glutamyltransferase assay in permeabilized cells (Mérida et al., 1991).

### Photosynthetic Pigment Analysis

Absorption spectra (400 to 750 nm) of *Synechocystis* cultures were measured and peaks at 485, 625, and 678 nm were used for carotenoid, phycobilin, and chlorophyll content analysis, respectively. All spectra were normalized to 1 OD750 for the comparison of different samples. Autofluorescence was detected by laser confocal microscopy (FLUOVIEW FV 3000; Olympus) using the 488-nm line supplied by an argon ion laser. Fluorescent emission was monitored by collection across a window of 630 to 700 nm.
Glycogen Content Determination

Glycogen was determined as described in Xu et al. (2013) with some modifications. Two units of OD530 were harvested, washed twice with Milli-Q water (EMD Millipore), and resuspended in 300 μL of 30 mM of sodium acetate at pH 5.2. Cells were disrupted with glass beads (0.15 to 0.25 mm) in a bead beater, and the extract was recovered and boiled for 20 min. Two 100-μL aliquots were prepared; one of them was treated with 10 U amyloglucosidase from Aspergillus niger (Sigma-Aldrich) and the other with water as a control, incubating at 55°C overnight. Alternatively, glycogen was extracted as described in Klotz et al. (2016) and digested with amyloglucosidase under these same conditions. A calibration curve using commercial glycogen was also prepared. Released Glc was determined in all samples by the Glc oxidase/peroxidase method (cat. no. GAGO-20, Sigma-Aldrich). In all the glycogen determinations, two technical replications of each independent biological sample were carried out.

Amino Acid Pools Quantification

Amino acid pools were quantified by reverse-phase high performance liquid chromatography (Hjerkovsky and Meredith, 1984). Cells were harvested and lyophilized. Twenty milligrams of dry weight were resuspended in 400 μL of 0.1 M of HCl. After centrifugation to remove cell debris, 60-μL samples were derivatized by the Pico-Tag system (Waters; ethanol/water/triethylamine/water/triethylamine). One of these was treated with 10 U amyloglucosidase from Aspergillus niger Sigma-Aldrich) and the other with water as a control, incubating at 55°C overnight. Alternatively, glycogen was extracted as described in Klotz et al. (2016) and digested with amyloglucosidase under these same conditions. A calibration curve using commercial glycogen was also prepared. Released Glc was determined in all samples by the Glc oxidase/peroxidase method (cat. no. GAGO-20, Sigma-Aldrich). In all the glycogen determinations, two technical replications of each independent biological sample were carried out.

Electron Microscopy

Cells were fixed for 2 h at 25°C using 2.5% (w/v) glutaraldehyde in Na-cacodylate buffer (0.1 M at pH 7.4), washed five times with the same buffer at 25°C and incubated for 1 h at 4°C in the same buffer containing 1% (w/v) osmium tetroxide. Cells were then washed, immersed in 2% (w/v) uranyl acetate, dehydrated using serial concentrations of acetone (50, 70, 90 and 100% (v/v)), and finally embedded in Spurr resin. Three-hundred-nanometer sections were obtained and stained with 1% toluidine blue to orient and locate the cells in the samples using optical microscopy. Ultra-thin sections (70 nm), obtained using an ultramicrotome (model no. UC7; Leica) with diamond blade, were placed on 200-mesh copper grids. All samples were analyzed in a Libra 120 Transmission Electron Microscope (Zeiss) and digital images were obtained using an on-axis mounted TRS camera.

Accession Numbers

The accession numbers of the proteins with the DUF1830 domain and the proteins identified in the immunoprecipitation assays are indicated in Supplemental Datasets S1 and S2, respectively.

Supplemental Data

The following supplemental materials are available.

- Supplemental Figure S1. Scheme of gene structure of different CfrA Synecochystis strains.
- Supplemental Figure S2. Pigmentation of Synecochystis cells with different levels of CfrA during resuscitation of chlorosis.
- Supplemental Figure S3. Analysis of Pars-cfrA strain.
- Supplemental Figure S4. Decantation of wild-type, ΔcfrA, and Pars-cfrA cells in BG11C medium.
- Supplemental Figure S5. Flow cytometry characterization of Pars-cfrA strain.
- Supplemental Figure S6. Phenotypic analysis of the Pars-cfrA strain after the removal of arsenite.
- Supplemental Table S1. List and sequence of oligonucleotides used in this work.

Supplemental Dataset S1. Compilation of DUF1830-domain-containing sequences.

Supplemental Dataset S2. List of protein identified by LC-MS/MS in immunoprecipitation assays.

Supplemental Methods S1. CfrA-His, expression and purification and anti-CfrA antibody production.

Supplemental Methods S2. Immunoprecipitation assay and LC-MS/MS protein identification.

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