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Expression of Genes Involved in Nitrogen Assimilation and the C/N Balance Sensing in *Prochlorococcus* sp. Strain SS120

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The expression of five genes involved in nitrogen assimilation in cyanobacteria, namely *glnA*, *glsF*, *icd*, *ntcA*, and *glnB*, encoding three key enzymes from that pathway (glutamine synthetase, glutamate synthase, isocitrate dehydrogenase) and two regulatory proteins (NtcA and P_{II}), was studied in this work. Their changes under different conditions were analyzed by quantitative real-time RT-PCR. Nutrient limitation induced clear modifications on the expression of most studied genes: lack of nitrogen provoked an initial increase, followed by a marked decrease; in the cases of phosphorus and iron starvation, a general, stronger expression decrease was observed, particularly striking in the case of iron. Darkness and addition of the photosynthethic inhibitors DCMU and DBMIB also had a strong effect on gene expression. Methionine sulfoximine and azaserine, inhibitors of glutamine synthetase and glutamate synthase, respectively, provoked a sharp increase in *icd* expression. These results, together with previous studies, suggest that 2-oxoglutarate could be the molecule utilized by *Prochlorococcus* to sense the C/N balance. Besides, our results confirm the different regulation of nitrogen assimilation in *Prochlorococcus* with regard to other cyanobacteria.

Key words: Carbon and nitrogen assimilation; Cyanobacteria; Gene expression; Metabolic regulation

INTRODUCTION

Prochlorococcus is a cyanobacterium (4,5) of central importance in marine ecology (6,36). It is the smallest and most abundant photosynthetic organism on earth, contributing for a significant fraction of global primary production (17,25). The occurrence in the field of several ecotypes (30,50) adapted to different conditions is a key for the ecological success of *Prochlorococcus*. The comparative analysis of an increasing number of *Prochlorococcus* genomes (7,9,10, 21,43) is providing an outstanding body of knowledge on the large degree of diversity among either high-irradiance- or low-irradiance-adapted ecotypes.

Yet, in spite of this wealth of genomic data, there is a clear lack of physiological information to complement those studies (8), and to fully understand the meaning of the revealed differences among ecotypes. This is particularly true if we consider the nutrition metabolism in *Prochlorococcus*, and specifically the nitrogen and carbon pathways. Our group has been studying some key enzymes of the nitrogen assimilation in several *Prochlorococcus* strains during the last years (11,12,15,16,18,19,26). Lately, we have started as well the study of carbon metabolism pathways, because comparative genomics (15) and experimental evidence (20) suggested that they could also be involved in the adaptive mechanisms conferring *Prochlorococcus* its extraordinary ecological success in marine oligotrophic ecosystems. In order to complement our work on the regulation and characterization of enzymes, we decided to study the expression of several genes involved in nitrogen assimilation and its link to carbon metabolism, which are regulated by the NtcA and P_{II} proteins.

Given that detailed transcriptomic studies are available under different stress conditions (28,45,47) in the *Prochlorococcus* strains MED4 and MIT9313

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(adapted to grow at surface and at depth, respectively), we focused our attention on another model strain adapted to grow at depth, SS120. We addressed the effect of nutrient limitation, in particular those that are most important in the oceans: nitrogen, phosphorus, and iron, which are often found at very low concentrations in oligotrophic areas (35). We also analyzed the effect of darkness and that of inhibitors of the photosynthetic electron transport at the level of the plastoquinone pool. Finally, we studied the effect of blocking the nitrogen assimilation, by using specific inhibitors of the two key enzymes, glutamine synthetase (GS) and glutamate synthase (GOGAT), by methionine sulfoximine (MSX) and azaserine, respectively. Our goal was to understand the response of those genes to different key changes. 2-Oxoglutarate is the molecule utilized by cyanobacteria to sense the balance between the C and N metabolisms (27, 32,46,49), so we were particularly interested in the possible role of this metabolite in *Prochlorococcus*. However, no specific study has been carried out thus far addressing the role of 2-oxoglutarate in marine cyanobacteria.

Taking into account the simplification of the regulatory networks observed in *Prochlorococcus* (16,43), it seems plausible to expect changes in the perception of the C/N balance. The inhibition of GOGAT by azaserine provokes a very significant increase in the concentration of 2-oxoglutarate (29), because it cannot be incorporated into glutamate by means of GOGAT and cannot be further metabolized by 2-oxoglutarate dehydrogenase (an enzyme lacking in cyanobacteria). Previous studies on glutamine synthetase regulation in *Prochlorococcus* showed that azaserine addition has a strong effect on GS activity, inducing a large increase in both the activity and the concentration of the enzyme (11). This work summarizes our findings regarding the gene expression changes observed under the above described conditions; together with previous studies (11,12) they suggest that 2-oxoglutarate is also used in *Prochlorococcus* to perceive the C/N balance. However, our results show different regulatory responses with regard to other cyanobacteria.

MATERIALS AND METHODS

Chemicals

All chemicals were of reagent grade, obtained from Merck or Sigma.

Prochlorococcus *Strains and Growth Conditions*

Prochlorococcus strain SS120 (low-irradiance-adapted, nonaxenic) was cultured in polycarbonate Nalgene flasks (10 L) using PCR-S11 medium as described previously (41). The seawater used as basis for this medium was kindly provided by the Instituto Español de Oceanografía (Spain). Cells were grown in a culture room set at 24°C under continuous blue irradiance (4 $\mu E\ m^2\ s^{-1}$). Cells were collected during the exponential phase of growth. Growth was determined by measuring the absorbance of cultures at 674 nm.

Cell Collection

Cells were centrifuged at $30{,}100 \times g$ for 5 min in an Avanti J-25 Beckman centrifuge equipped with a JA-14 Beckman rotor. When large volumes were required, centrifugations were performed at $18{,}600 \times g$ for 8 min in a JLA-10.500 Beckman rotor. After pouring most of the supernatant and carefully pipetting out the remaining medium, the pellet was resuspended in 10 mM sodium acetate (pH 4.5), 200 mM sucrose, and 5 mM EDTA, then quickly frozen at −80°C until used for RNA isolation.

In the experiments studying nutrient starvation, cultures were centrifuged as described above. The pellets were washed with nutrient-free PCR-S11 medium, and finally diluted with the same original volume of medium, by using standard PCR-S11 for controls, or medium without addition of the corresponding nutrient supplementation (either nitrogen, phosphorus, or iron); therefore, the media were under limited nutrient condition, but not completely deprived of it, because they were made with natural seawater. Two aliquots were prepared from each culture (control and nutrient depleted), which were subjected to standard conditions of light and temperature. Samples were taken at the indicated times following the protocol described above.

For experiments requiring darkness, culture bottles were completely covered with two layers of aluminum foil, and the sampling was performed in the dark. For experiments with inhibitors, DBMIB (2,5-dibromo-3-methyl-6-isopropyl-*p*-benzoquinone) was dissolved in ethanol prior to addition to the cultures, while DCMU [diuron, 3-(3-4-dichlorophenyl)-1,1-dimethylurea] was dissolved in culture media. Cells from samples where inhibitors had been added were washed with PCR-S11 medium prior to freezing. The concentration of inhibitors and their effect on glutamine synthetase (11,12) and isocitrate dehydrogenase (López-Lozano et al., unpublished) activity and enzyme concentration were tested in previous studies.

RNA Isolation

RNA was extracted by using the Aurum kit (Bio-Rad), according to the manufacturer's instructions.

This kit includes a DNAase step removing any remaining genomic DNA from RNA samples, to ensure that all DNA (later quantified in the iCycler system) is produced by reverse transcription from mRNA.

Determination of Gene Expression by Quantitative Real-Time RT-PCR (qRT-PCR)

Specific primers to amplify fragments of the genes *glnA*, *glsF*, *icd*, *ntcA*, *glnB*, and *rnpB* from the *Prochlorococcus* strain SS120 were designed using the software Oligo 4.0.5 (Molecular Biology Insights, Inc.), on the basis of corresponding genes from the genome of *Prochlorococcus* SS120 (10). The primers used are listed in Table 1.

After quantitation of RNA samples by UV spectrophotometry, 400 ng of total RNA was reverse transcribed using the iScript cDNA Synthesis kit (Bio-Rad), following the manufacturer's instructions. The cDNA obtained was used as template for the qRT-PCR reactions. The efficiency of amplifications for each couple of primers was analyzed by preparing serial dilutions of samples to test qRT-PCR reactions, in order to reach optimal, linear amplification. Each reaction mixture contained the following components: 2.5 µl *Taq* buffer 10-fold concentrated (including 20 mM $MgCl_2$; Biotools); 0.5 µl 10 mM dNTPs mixture; 1–3 µl forward primer (20 ng/µl); 1–3 µl reverse primer (20 ng/µl); 1 µl cDNA template solution; 0.6 µl SYBR-Green I/Fluorescein solution (10^{-4} diluted in DMSO; Molecular Probes Inc.); 0.4 µl *Taq* polymerase (1 U/µl; Biotools); 14–18 µl sterilized water. A master mix containing all components except cDNA templates and primers was prepared for all tubes, including positive and negative controls (containing living cells and no cDNA, respectively). All reactions were performed in triplicate, including controls. The qRT-PCR reaction was monitorized in a BioRad iCycler system for 40 cycles (95°C for 15 s, 58°C for 30 s, 72°C for 30 s). At the end, reactions were checked to discard false amplifications by verifying the melting point of PCR products, determining the fluorescence between 60 and 100°C, with increases of 0.5°C, measured each 10 s. During the optimization of qRT-PCR reactions, products were checked for correct, single product amplification by agarose gel electrophoresis.

TABLE 1
PRIMERS USED IN THE QUANTITATIVE RT-PCR AMPLIFICATIONS

Gene	Primer	Sequence
glnA	SGF	GCGTCTTGTTCCTGGCTTC
	SGR	AGCATCTCCTGACCTGAACTC
glsF	FALL-3	CGTGGGAGTTGGGCTTG
	RALL-3	TTAAACCTCCATCTGCTCTAAG
icd	SIF	AATTGTTGGAGGATTAGGAATGG
	SIR	ACTGAACCTGGATTAATTCTATCG
ntcA	FALL-1	AGCTCCTGCTGGCTCAGTTA
	RALL-1	GAGAAGTAGCCCAACCCCAC
glnB	FALL-2	TTTGGGCGACAAAAAGGA
	RALL-2	TCAACACTTTCATCAGCAACAA
rnpB	SRF	CTCTCGGTTGAGGAAAGTC
	SRR	CCTTGCCTGTGCTCTATG

Gene expression was determined by monitoring the PCR amplification of double-stranded DNA with SYBR-Green I (Molecular Probes Inc.), using an iCycler system (BioRad). Relative expression of *glnA*, *glsF*, *icd*, *ntcA*, and *glnB* after standardization to *rnpB* expression, was calculated by following the method previously described (37). In this way, we show the changes in expression for each gene, with respect to its own expression under standard conditions (growing under light with no inhibitors) at the same sampling time; expression values of 1 mean no change.

Statistical Analysis

Three different biological samples were used in each experiment, and each sample was measured in triplicate. Standard deviation is shown for every determination of gene expression. Significance of data was evaluated by performing the Student's *t*-test.

RESULTS AND DISCUSSION

In our previous studies on glutamine synthetase, we observed that several conditions known to cause marked effects on its regulation in other cyanobacteria lacked such effect in *Prochlorococcus* (11,12); therefore, we were interested to check whether this might be due to a general streamlining on regulatory networks or to some specific response of glutamine synthetase.

Effect of Key Nutrients Starvation

Nitrogen Starvation. Ammonium is the preferred nitrogen source by cyanobacteria (13,31), and the only one being readily assimilated by all *Prochlorococcus* strains thus far studied, although other nitrogen sources (i.e., nitrite, amino acids) can be assimilated by specific strains (16). In order to analyze the impact of N stress on the nitrogen assimilation pathway, we studied gene expression in *Prochlorococcus* SS120 cultures subjected to nitrogen starvation (Fig. 1). The expression of *icd* decreased until 24 h, while that of *glnA* increased markedly (ca. five-

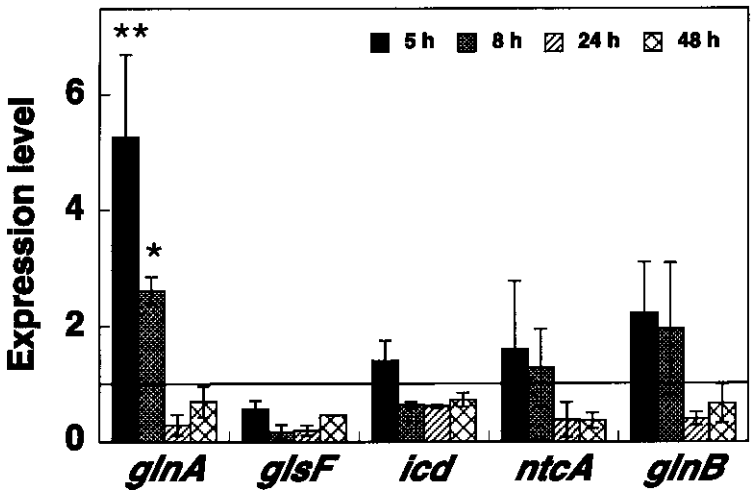

Figure 1. Effect of nitrogen starvation. Expression of *glnA*, *glsF*, *icd*, *ntcA*, and *glnB* measured by qRT-PCR in *Prochlorococcus* SS120 cultures. Data are relative values (N starved vs. control cells). Values are the average of at least three biological samples, each of them measured in triplicate. Error bars correspond to the SD. *A confidence of 95% ($p < 0.05$) according to the Student's *t*-test; **the level of confidence is 99% ($p < 0.01$). The horizontal grid line corresponds to expression level = 1 (no change compared to expression at time 0).

fold) after 5 h to decrease later on, reaching a level close to the initial at the end of the experiment (48 h). This contrasts with the results of Muro-Pastor and coworkers, who observed a fivefold increase in the expression of *icd* in *Synechocystis* PCC 6803 cells subjected to nitrogen starvation (33). Besides, the response of both regulatory genes, *ntcA* and *glnB*, in *Prochlorococcus* SS120 was similar, with an initial increase (after 5 and 8 h) followed by a strong decrease at 24 h.

In a global expression study on two *Prochlorococcus* strains (47), the authors observed diverging results of N starvation on *icd* expression, depending on the studied strain, because they reported a 2.46 increase in the expression of *icd* for the strain MED4 versus a 3.03 decrease for the strain MIT9313. Regarding the remaining genes studied here, their results on MIT9313 were rather similar to ours (i.e., the largest increase was observed in the expression of *glnA*, while *glsF* showed similar changes). *ntcA* and *glnB* expressions also increased, more markedly in the case of MED4. However, the sampling times were different in both strains, MED4 and MIT9313, and additionally both are genotypically different from the strain used in this study (SS120), so we cannot make direct extrapolations.

Expression studies carried out with the closely related marine cyanobacterium *Synechococcus* strain WH8103 (3) have shown that nitrogen deprivation induced an upregulation in the levels of both *glnA* and *ntcA* expression, which were also in good agreement with our results. In the *Synechococcus* strain WH7803, *ntcA* expression also increased upon nitrogen starvation (24). Consequently, it seems that, in the case of *ntcA*, marine cyanobacteria have a physiological response similar to that found in other freshwater cyanobacteria.

Phosphorus Starvation. Another key nutrient in oligotrophic oceans is phosphorus. The lack of phosphorus induced an almost general decrease in gene expression after 5 and 24 h (Fig. 2), which was especially strong in the case of *glnA* and *ntcA* after 24 h. The results on *glnA* fit nicely with our previous results on the decrease of GS activity (11,12), mentioned above. In the case of *icd*, we observed a recovery to values close to the initial ones after 48 h. Finally, studies on *ntcA* expression in the strain PCC 9511 showed that expression of *ntcA* did not change significantly after 48 h (23), in contrast with the increase reported here; this could suggest a regulatory difference between both strains. Our results suggest that phosphorus starvation had a clearer effect on gene expression than nitrogen starvation. In another global expression study focused on the effects of phosphorus starvation in the MED4 and MIT9313 strains (28), all described changes were observed in sets of genes directly related with phosphorus metabolism, but no information is provided regarding any of the genes here analyzed. In studies performed with *Synechococcus* WH7803, *ntcA* expression was not clearly affected by phosphorus starvation (24).

Iron Starvation. Finally, we studied the effect of iron starvation, another element whose concentration is limiting in many oligotrophic oceans. As previously reported (12), the lack of iron had a most striking effect, inducing a marked decrease in the number

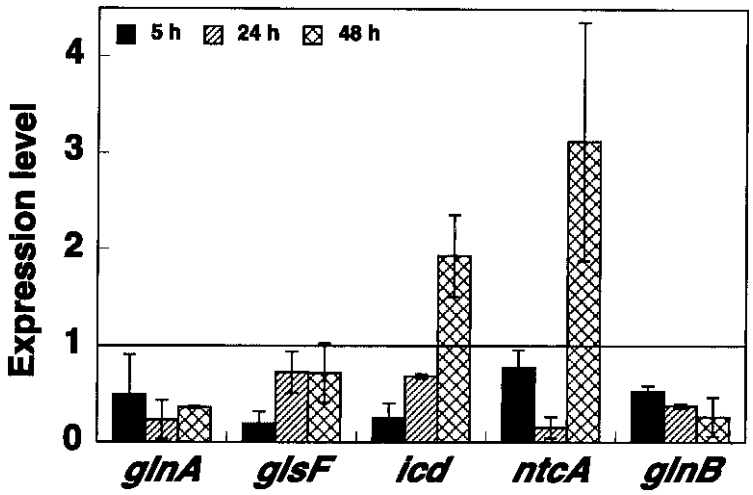

Figure 2. Effect of phosphorus starvation. Expression of *glnA*, *glsF*, *icd*, *ntcA*, and *glnB* measured by qRT-PCR in *Prochlorococcus* SS120 cultures. Data are relative values (P starved vs. control cells). Values are the average of at least three biological samples, each of them measured in triplicate. Error bars correspond to the SD. The horizontal grid line corresponds to expression level = 1 (no change compared to expression at time 0).

of cells. We observed a general downregulation in gene expression, with respect to the control cultures (Fig. 3). This was particularly strong after 24 and 48 h, finding a decrease of ca. eight times in the expression of *icd*. Once more, the low expression of *glnA* was in good agreement with our results on GS activity in the PCC 9511 strain subjected to iron starvation (12). Although iron starvation in cyanobacteria has received considerable attention, no global expression study on its effect in *Prochlorococcus* has been published thus far; however, in studies carried out on *Synechocystis* PCC 6803 (44), a general effect inducing the decrease in the expression of many genes belonging to different metabolic pathways has been described, and our results fit nicely in this context. In studies focused on marine cyanobacteria, the expression of *ntcA* showed no clear effects when cells were deprived of iron (24).

Effect of Darkness and Inhibitors of the Photosynthetic Electron Flow

Darkness. Light is one of the most important factors in the metabolic regulation of photosynthetic organisms, and it has been shown to be involved in the control of gene expression and enzyme activity

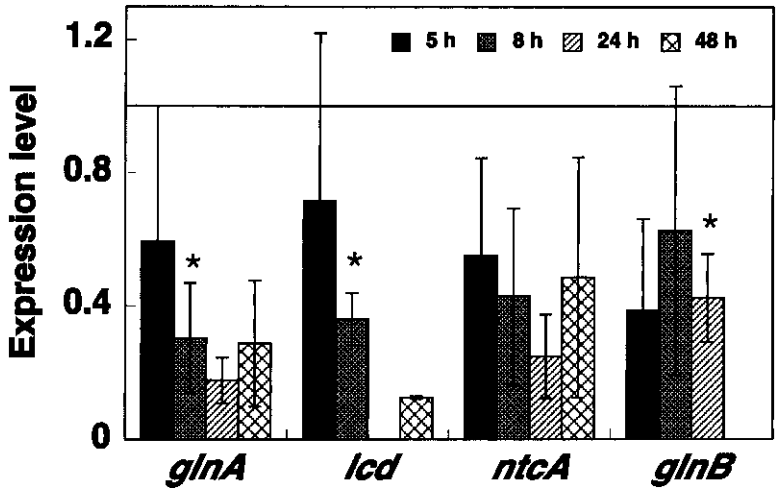

Figure 3. Effect of iron starvation. Expression of *glnA*, *icd*, *ntcA*, and *glnB* measured by qRT-PCR in *Prochlorococcus* SS120 cultures. Data are relative values (Fe starved vs. control cells). Values are the average of at least three biological samples, each of them measured in triplicate. Error bars correspond to the SD. *A confidence of 95% ($p < 0.05$) according to the Student's *t*-test. The horizontal grid line corresponds to expression level = 1 (no change compared to expression at time 0).

in cyanobacteria. We detected a clear effect on the expression of both *icd* and *glnA* genes, which decreased ca. three and seven times, respectively, after 24 h under darkness (Fig. 4). The strong decrease in *glnA* expression contrasts with the lack of effect of darkness on the GS activity, reported previously in the *Prochlorococcus* PCC 9511 strain (11). Although no information is available regarding *icd* under these conditions in other cyanobacteria, it is well established that the expression of *glnA* decreases (39). Besides, we observed some important changes in the expression of *ntcA* and *glnB*, indicating that darkness is indeed participating in the regulation of gene expression in *Prochlorococcus*. It is striking the contrast between the darkness-induced decrease in the expression of *glnB* in *Synechocystis* PCC 6803 (14) and the clear upregulation observed in our studies (ca. fourfold). In other cyanobacteria, it has been established that the regulation of N metabolism is affected by darkness, because the expression of the global nitrogen regulator, *ntcA*, is decreased under darkness in *Synechocystis* PCC 6803 (1). Our results were consistent with this model, because we observed a decrease of ca. threefold in the expression of *ntcA* after 24 h of darkness.

Inhibitors of the Photosynthetic Electron Flow. DCMU and DBMIB are inhibitors of the photosynthetic electron flow, blocking the electron transfer before and after the plastoquinone pool, respectively (40,48). DCMU, as darkness, provokes the oxidation of the plastoquinone pool and the decrease in the NADPH intracellular levels, which in turn induces a decrease in the respiratory activity in *Synechocystis* PCC 6803 (2). When 0.3 μM DCMU was added to *Prochlorococcus* cultures (Fig. 5), the expression of *icd* was mostly unchanged, but that of *glnA* and *glsF* followed different patterns: the expression of *glsF* increased almost ninefold after only 1 h after DCMU addition, to progressively recover levels close to the initial ones. Meanwhile, *glnA* expression decreased markedly at 8 h, before coming closer to control values at 24 h. Similar effects were observed in *Synechocystis* PCC 6803 for *glnA* (39) and *glnB* (14). In studies focused on the regulation of GS in the *Prochlorococcus* strain PCC 9511, we found that DCMU induced a decrease in GS activity, in good agreement with the observations reported here (11).

In the case of DBMIB (Fig. 6), the response was completely different: strikingly the *icd* expression showed an increase after 8 h (fourfold) to decrease markedly after 24 h. *glnA* expression decreased during the experiment, reaching a minimum of ca. 12-fold in the last sample, while *glnB* decreased to a lesser extent. The expression of both genes (*glnA* and *glnB*) was also found to decrease in *Synechocystis* PCC 6803 (14,39). We have previously described a strong decrease in GS activity after DBMIB addition to cultures of both *Prochlorococcus* PCC 9511 and SS120 (11).

Hihara and coworkers performed a global expression of redox-responsive genes in *Synechocystis* PCC 6803, by analyzing the effects of both DCMU and DBMIB (22); they observed that the NtcA-regulated genes (*glnA*, *glnB*, *ntcA*) were repressed under the effect of both inhibitor; this contrasts with the clear increase we observed in *ntcA* levels after DBMIB addition (Fig. 6). On the other hand, a global transcrip-

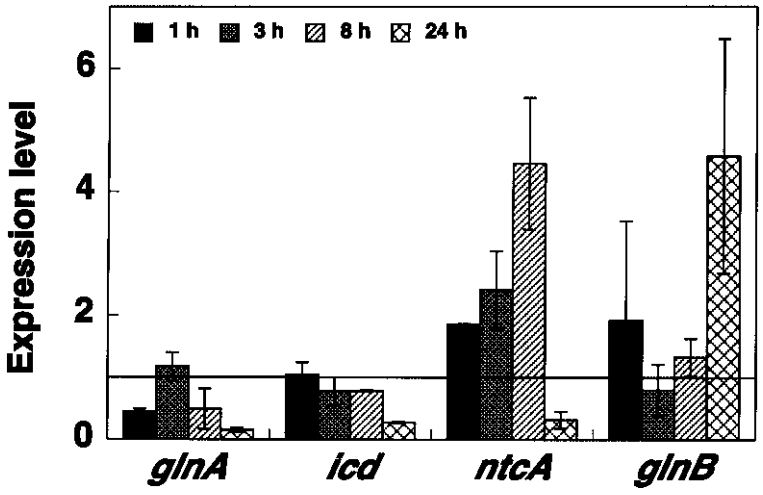

Figure 4. Effect of darkness. Expression of *glnA*, *icd*, *ntcA*, and *glnB* measured by qRT-PCR in *Prochlorococcus* SS120 cultures. Data are relative values (cells in the dark vs. control cells). Values are the average of at least three biological samples, each of them measured in triplicate. Error bars correspond to the SD. The horizontal grid line corresponds to expression level = 1 (no change compared to expression at time 0).

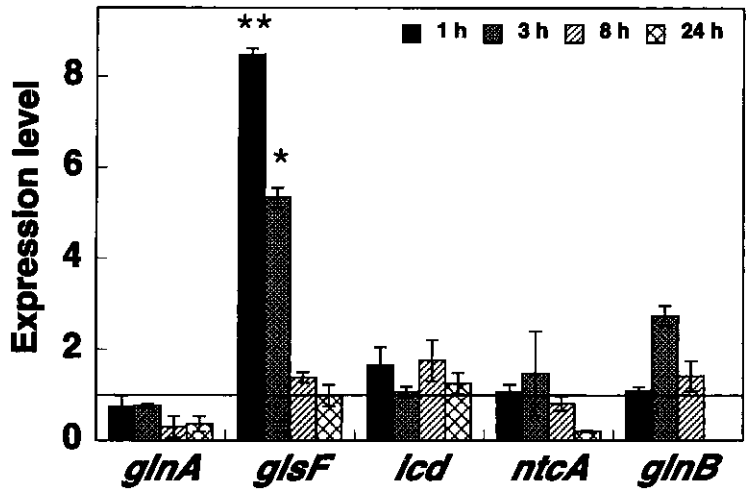

Figure 5. Effect of DCMU. DCMU (0.3 μM) was added to cultures at time 0. Expression of *glnA*, *glsF*, *icd*, *ntcA*, and *glnB* was measured by qRT-PCR in *Prochlorococcus* SS120 cultures. Data are relative values (DCMU-treated vs. control cells). Values are the average of at least three biological samples, each of them measured in triplicate. Error bars correspond to the SD. *A confidence of 95% ($p < 0.05$) according to the Student's *t*-test; **the level of confidence is 99% ($p < 0.01$). The horizontal grid line corresponds to expression level = 1 (no change compared to expression at time 0).

tome analysis on light sensing in *Prochlorococcus* (45) addressed the effects of darkness and DCMU, but reported no particular changes in the genes here studied.

Effect of Nitrogen Assimilation Inhibitors

We analyzed *Prochlorococcus* cultures after addition of 100 μM MSX (specific inhibitor of glutamine synthetase) (34) and 100 μM azaserine (specific inhibitor of glutamate synthase) (38). The results are shown in Figures 7 and 8, respectively.

MSX addition induced a quick upregulation in the expression of *glnA*, a logic response given that this compound inhibits GS. In the next hours, *glnA* and *icd* expression recovered levels similar to those found at the begining of the experiment. *ntcA* and *glnB* expression showed a different pattern: *ntcA* showed a sharp decrease after 5 h, when *glnB* was upregulated. However the expression of *glnB* was also reduced at 24 h. *glsF* expression levels were, in general, much lower than that of the control cultures at all sampling points. In contrast with those results, *Synechococcus*

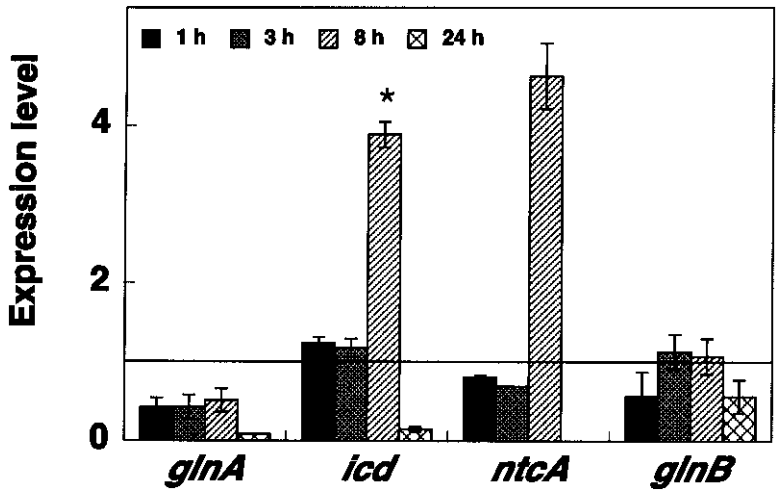

Figure 6. Effect of DBMIB. DBMIB (0.06 μM) was added to cultures at time 0. Expression of *glnA*, *icd*, *ntcA*, and *glnB* was measured by qRT-PCR in *Prochlorococcus* SS120 cultures. Data are relative values (DBMIB-treated vs. control cells). Values are the average of at least three biological samples, each of them measured in triplicate. Error bars correspond to the SD. *A confidence of 95% ($p < 0.05$) according to the Student's *t*-test. The horizontal grid line corresponds to expression level = 1 (no change compared to expression at time 0).

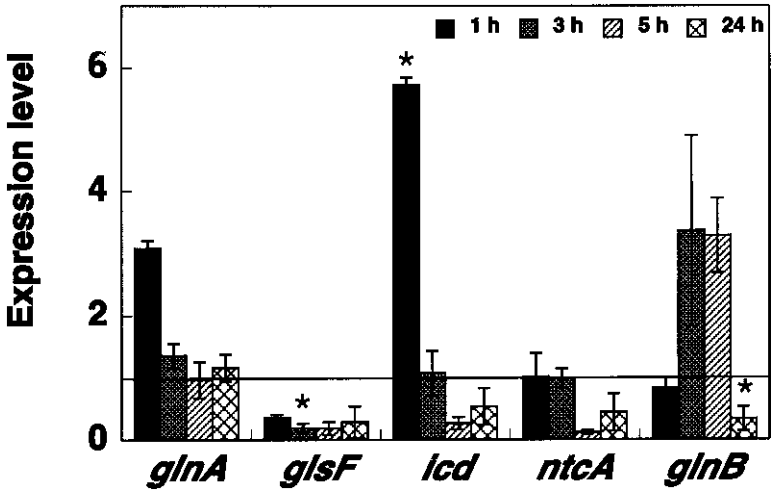

Figure 7. Effect of MSX. MSX (100 μM) was added to cultures at time 0. Expression of *glnA*, *glsF*, *icd*, *ntcA*, and *glnB* was measured by qRT-PCR in *Prochlorococcus* SS120 cultures. Data are relative values (MSX-treated vs. control cells). Values are the average of at least three biological samples, each of them measured in triplicate. Error bars correspond to the SD. *A confidence of 95% ($p < 0.05$) according to the Student's *t*-test. The horizontal grid line corresponds to expression level = 1 (no change compared to expression at time 0).

strain WH7803, MSX was shown to strongly increase the expression of *ntcA* (*24*), but there is no further information in the literature regarding the effect of this inhibitor on the other genes studied here.

Interestingly, in the experiments with azaserine, among the three enzyme-coding genes studied, only the expression of *icd* was increased (ca. ninefold) after azaserine addition, while *glnA* and *glsF* showed minor changes. In the case of the regulatory genes, *ntcA* expression was upregulated during the first hours, decreasing strongly after 24 h, while *glnB* expression decreased significantly along the experiment.

The results of our experiments with azaserine provide interesting information regarding the system of sensing the C/N balance in *Prochlorococcus*. First, the strong increases observed after azaserine addition in the activity of two key enzymes of the C and N metabolism, GS (11) and ICDH (López-Lozano, Gómez-Baena, Rangel, Diez, and García-Fernández, unpublished) indicate that this inhibitor (blocking GOGAT, and thus presumably inducing a sharp in-

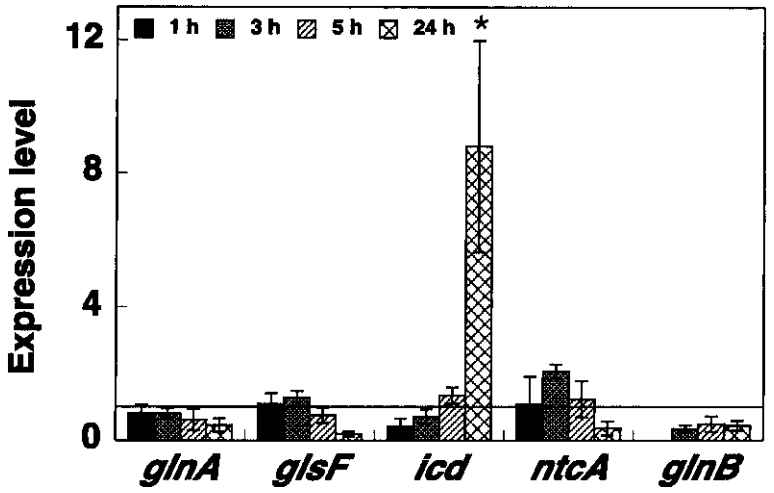

Figure 8. Effect of azaserine. Azaserine (100 μM) was added to cultures at time 0. Expression of *glnA*, *glsF*, *icd*, *ntcA*, and *glnB* was measured by qRT-PCR in *Prochlorococcus* SS120 cultures. Data are relative values (azaserine-treated vs. control cells). Values are the average of at least three biological samples, each of them measured in triplicate. Error bars correspond to the SD. *A confidence of 95% ($p < 0.05$) according to the Student's *t*-test. The horizontal grid line corresponds to expression level = 1 (no change compared to expression at time 0).

crease in the intracellular concentration of 2-oxoglutarate) is acting on one of the keys of the metabolic regulation in *Prochlorococcus*. In fact, azaserine addition has been shown to strongly increase the intracellular levels of 2-oxoglutarate in *Synechocystis* PCC 6803 (29). This, taken together with our results, strongly suggests that 2-oxoglutarate is also the molecule utilized by *Prochlorococcus* to monitorize the C/N balance. However, the fact that we did not detect significant changes in GS activity under N starvation (11,12) could indicate that *Prochlorococcus* is adapted to live under low N concentration conditions, so that stronger increases in 2-oxoglutarate concentration (by addition of azaserine) are required in order to give rise to the metabolic responses concerning gene expression and enzyme activity. This could suggest that an increased threshold in 2-oxoglutarate concentrations to trigger the nitrogen starvation regulatory responses could be an adaptive metabolic modification in *Prochlorococcus*, derived from the standard conditions of its oligotrophic habitat.

Second, the lack of response in *glnA* expression to azaserine (Fig. 8) was surprising given the high increase in GS activity and protein concentration in the strains PCC 9511 and SS120 (11,12); this might be caused by some kind of posttranscriptional mechanism of regulation, enabling the production of bigger amounts of GS protein from similar amounts of mRNA derived from *glnA*.

Third, the comparatively minor responses observed in *ntcA* and *glnB* (especially in the case of *ntcA*) point to possible regulatory differences in *Prochlorococcus* with regard to freshwater cyanobacteria. This topic deserves further attention and will be addressed in future works.

CONCLUSIONS

The present is the first study analyzing in detail the changes observed in the expression of five genes related with the nitrogen/carbon metabolism, under a number of conditions representative of the actual challenges faced by natural *Prochlorococcus* populations. Among the key nutrients in the ocean, the strong effects of iron limitation (reinforced by previous observations from our team) (12) are surprising, as it is commonly considered that *Prochlorococcus* is adapted to low iron concentration. These results indicate that iron is a paramount nutrient for *Prochlorococcus*, and its complete absence cannot be compensated even by the adaptive mechanisms developed by this group of cyanobacteria along its evolution.

Besides, our results suggest that the metabolic regulation of *Prochlorococcus* shares some of the core components with the rest of cyanobacteria, as the sensing of C/N balance by 2-oxoglutarate. However, there exist some key differences: regulation by light is not working in a standard manner in *Prochlorococcus* (11) (Fig. 4). In addition, the comparatively slight effect of nitrogen starvation (11,12) (Fig. 1) points to a lower threshold for N concentration in the environment, required to trigger the response through the NtcA regulatory system.

Finally, the differences in gene expression observed in *Prochlorococcus* sp strain SS120 with respect to other model strains, as MED4 and MIT9313, suggest that the regulatory mechanisms involved in the balance of the C/N metabolism in *Prochlorococcus* have been modified along the evolution of this genus, leading to specific responses depending on the strain. This is in good agreement with the significant changes observed also between the strains MED4 and MIT9313 in global transcriptomic studies addressing the availability of different nitrogen sources by *Prochlorococcus* (47).

Furthermore, the fact that such responses are also different between strains adapted to live at depth in the oceans, such as MIT9313 (47) versus SS120 (this work) point to the development of specific responses in both strains, probably related to their very different genomic backgrounds: MIT9313 has been described as one of the oldest *Prochlorococcus* ecotypes (42, 43), while SS120 seems to have evolved more recently and possesses a more streamlined genome. Recent studies from our team (Gómez-Baena, Rangel, Lopez-Lozano, García-Fernández, and Diez, unpublished) reinforce this hypothesis. Further comparative transcriptomic and physiological studies between strains adapted to similar habitats are needed to unveil how the different evolution of *Prochlorococcus* ecotypes is reflected in their response to environmental changes.

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