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A Mutant Lacking the Glutamine Synthetase Gene (*glnA*) Is Impaired in the Regulation of the Nitrate Assimilation System in the Cyanobacterium *Synechocystis* sp. Strain PCC 6803

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The existence in the unicellular cyanobacterium *Synechocystis* sp. strain PCC 6803 of two genes (*glnA* and *glnN*) coding for glutamine synthetase (GS) has been recently reported (J. C. Reyes and F. J. Florencio, J. Bacteriol. 176:1260–1267, 1994). In the current work, the regulation of the nitrate assimilation system was studied with a *glnA*-disrupted *Synechocystis* mutant (strain SJCR3) in which the only GS activity is that corresponding to the *glnN* product. This mutant was unable to grow in ammonium-containing medium because of its very low levels of GS activity. In the SJCR3 strain, nitrate and nitrite reductases were not repressed by ammonium, and short-term ammonium-promoted inhibition of nitrate uptake was impaired. In *Synechocystis* sp. strain PCC 6803, nitrate seems to act as a true inducer of its assimilation system, in a way similar to that proposed for the dinitrogen-fixing cyanobacteria. A spontaneous derivative strain from SJCR3 (SJCR3.1), was able to grow in ammonium-containing medium and exhibited a fourfold-higher level of GS activity than but the same amount of *glnN* transcript as its parental strain (SJCR3). Taken together, these finding suggest that SJCR3.1 is a mutant affected in the posttranscriptional regulation of the GS encoded by *glnN*. This strain recovered regulation by ammonium of nitrate assimilation. SJCR3 cells were completely depleted of intracellular glutamine shortly after addition of ammonium to cells growing with nitrate, while SJCR3.1 cells maintained glutamine levels similar to that reached in the wild-type *Synechocystis* sp. strain PCC 6803. Our results indicate that metabolic signals that control the nitrate assimilation system in *Synechocystis* sp. strain PCC 6803 require ammonium metabolism through GS.

Nitrate assimilation takes place in cyanobacteria by a two-step process: (i) transport of nitrate inside the cells and (ii) its further reduction to ammonium by the sequential action of nitrate and nitrite reductases (Nar and Nir, respectively) (7, 8). Ammonium exerts a negative effect on the nitrate assimilation system at two different levels: (i) addition of ammonium provokes a short-term inhibition of nitrate uptake by the cells (6, 12), and (ii) ammonium represses de novo synthesis of the proteins involved in nitrate assimilation (9–11, 32).

In the non-nitrogen-fixing cyanobacteria, the presence of nitrate is not required for Nar or Nir induction, while in the filamentous nitrogen-fixing strains, nitrate is required as an inducer for the synthesis of both enzymes (9–11).

Recently, it has been reported that the genes coding for Nir (*nirA*) and Nar (*narB*) and four other genes involved in nitrate transport (*nrtA*, *nrtB*, *nrtC*, and *nrtD*) are clustered in the genome of the non-nitrogen-fixing unicellular cyanobacterium *Synechococcus* sp. strain PCC 7942, forming a transcriptional unit (13, 24).

Ammonium is incorporated into carbon skeletons in cyanobacteria by the sequential action of glutamine synthetase (GS) and glutamate synthase (GOGAT) (19). GS plays a central role in the regulation of nitrogen assimilation in cyanobacteria, since inhibition of GS with the glutamate analog L-methionine DL-sulfoximine (MSX) leads to expression of Nar and Nir even in the presence of ammonium, suggesting that metabolism of ammonium through GS is required for repression of the syntheses of both reductases (9–11). Thus, treatment of *Synechococcus* sp. strain PCC 7942 cells with MSX leads to the expression of the *nirAnrtABCD-narB* operon in the presence of ammonium (32).

Expression of the *glnA* gene (encoding GS) is subject to regulation by the nitrogen source in cyanobacteria, usually being lower in ammonium- than in nitrate- or dinitrogen-grown cells (5, 22, 25, 34, 37). An ammonium-promoted GS inactivation in the unicellular non-nitrogen-fixing cyanobacterium *Synechocystis* sp. strain PCC 6803 has also been reported (20, 21). This mechanism does not involve the classical adenylylation of the enzyme, as in enterobacteria (20, 21).

Recently we have reported the presence in *Synechocystis* sp. strain PCC 6803 of a gene (*glnN*) coding for a second GS, which has been characterized as a new type of GS (GS type III) in cyanobacteria (26). In cells growing in nitrate, the *glnA* gene product (GS type I) is responsible for 97% of the total GS activity, while the *glnN* product accounts for approximately 3%. However, GS type III was able to support nitrogen assimilation in a *Synechocystis* sp. strain PCC 6803 *glnA* mutant (SJCR3) (26). In this study we have shown that in a *glnA* insertional mutant, the nitrate assimilation system is deregulated. In addition, we have obtained a spontaneous derivative (SJCR3.1) which recovered the wild-type (WT) regulation pattern while retaining the *glnA* mutation. The study of both strains (SJCR3 and SJCR3.1) suggests that glutamine or some derivative of glutamine mediates the metabolic signals promoted by ammonium. Furthermore, our results suggest that nitrate acts as an inducer of Nar and Nir syntheses in this non-nitrogen-fixing cyanobacterium, similar to the model for nitrogen-fixing cyanobacteria.

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MATERIALS AND METHODS

Strains and growth conditions. *Synechocystis* sp. strain PCC 6803 was grown photoautotrophically at 35°C on BG11 medium (27) (with 18 mM nitrate as a nitrogen source) under continuous fluorescent illumination (50 $W \cdot m^{-2}$; white light). The cultures were bubbled with 1.5% (vol/vol) CO_2 in air. $BG11_0$ medium was BG11 medium lacking a nitrogen source. When ammonium was used as a nitrogen source, $BG11_0$ medium was supplemented with 10 mM NH_4Cl and the medium was buffered with 20 mM *N*-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid (TES) buffer. Strains SJCR3 and SJCR3.1 were grown on the same media supplemented with kanamycin at a final concentration of 50 μg/ml.

Growth curves. For growth determination, cells cultured in BG11 medium were harvested, washed with $BG11_0$ medium, and inoculated at a final concentration of 20 μg of protein per ml into the culture media BG11, $BG11_0$ plus 10 mM NH_4Cl, and BG11 plus 10 mM NH_4Cl. Samples were taken at various times, and growth was estimated by protein determination.

DNA manipulation. All DNA manipulations were performed according to standard procedures (1, 30). Total DNA from *Synechocystis* sp. strain PCC 6803 cells was isolated as described previously (3). For Southern hybridizations, DNA was digested and fragments were electrophoresed in 0.7% agarose gels in a Tris-borate-EDTA buffer system (30). Transfer of DNA to nylon Z-Probe membranes (Bio-Rad) and Southern blot hybridizations were performed as described previously (1). DNA probes were ^{32}P labelled with a nick translation kit (Boehringer Mannheim), using [α-^{32}P]dCTP.

RNA isolation and Northern (RNA) blot analysis. Total RNA from the WT, SJCR3, and SJCR3.1 strains was isolated by the hot-phenol method as described by Mohamed and Jansson (23) with the modification described in reference 26. Separation of RNA on formaldehyde gels, transfer to nylon membranes (Hybond N-plus; Amersham), and prehybridization and hybridization conditions were as described in instruction manuals from Amersham. A 30-μg sample of total RNA was loaded per lane.

Determination of enzyme activities. GS activity was determined in situ by using the Mn^{2+}-dependent γ-glutamyl-transferase assay with cells permeabilized with mixed alkyltrimethylammonium bromide (20). Nar and Nir were determined in situ by using dithionite-reduced methyl viologen as the reductant, as previously described (9, 11). Nitrite was determined by the method of Snell and Snell (31). One unit of enzymatic activity corresponds to the amount of enzyme that catalyzes the synthesis of 1 micromole of product per minute.

Nitrate uptake experiments. Nitrate uptake experiments were done as described previously (6) with the following differences. Cultures in mid-exponential growth phase (0.1 to 0.2 mg of protein per ml) were harvested by centrifugation, washed with 20 mM Tricine-NaOH buffer (pH 8.3) supplemented with 10 mM $NaHCO_3$, and finally resuspended in the same buffer at 0.45 mg of protein per ml. Uptake assays were carried out with continuous shaking in white light (100 $W \cdot m^{-2}$) at 35°C in 50-ml Erlenmeyer flasks and were started by addition of $NaNO_3$. Nitrate uptake activity was determined following the disappearance of the nitrogen ion from the outer medium. Nitrate was determined by measurements of A_{210} in acid solution as described by Cawse (4).

Analytical methods. For the determination of intracellular pools of glutamate and glutamine, cell lysates were obtained by addition of 0.9 ml of a culture in exponential phase (0.1 to 0.2 mg of protein per ml) to 0.1 ml of 2 N HCl, followed by vigorous shaking and centrifugation at 12,000 × *g* for 5 min at 4°C. The amino acid concentration in the supernatants was determined by high-pressure liquid chromatography as previously described (18). Data are given as nanomoles of amino acid per milligram of protein. Protein in whole cells was determined by the method of Lowry et al. as modified by Markwell et al. (17). Chlorophyll was determined as described by MacKinney (15).

RESULTS

Isolation of pseudorevertants of SJCR3. We have previously reported that *Synechocystis* strain SJCR3 is a *glnA* mutant generated by disruption of the *glnA* gene with a kanamycin resistance cassette (26). This strain was not auxotrophic for glutamine, since the product of the *glnN* gene was able to support growth in medium with nitrate as a nitrogen source at a rate similar to that of the WT strain (doubling time, 9 h) (26). However, strain SJCR3 was not able to grow in ammonium-containing medium (Fig. 1). Spontaneous revertants of SJCR3 that had recovered the ability to grow with ammonium as a nitrogen source were isolated at a frequency of 10^{-6} by direct selection on plates with 10 mM ammonium as a nitrogen source. One of these revertants, named SJCR3.1, was analyzed. Strain SJCR3.1 retained the insertional *glnA* mutation, as was determined by Southern hybridization (Fig. 2), but its doubling time in medium containing ammonium was only slightly higher than that of the WT strain (11 and 8 h, respectively) (Fig. 1).

Since SJCR3 and SJCR3.1 are *glnA* mutants, the GS activity observed in both strains corresponds to the second GS of *Synechocystis* sp. strain PCC 6803, encoded by the *glnN* gene. GS activity in the presence of ammonium was extremely low in strain SJCR3, but the GS activity in SJCR3.1 was 2- to 12-fold higher than that of SJCR3 in ammonium-containing medium (Table 1). To investigate whether this increase in GS activity in the SJCR3.1 strain was due to a higher steady-state level of the *glnN* mRNA, WT, SJCR3, and SJCR3.1 cells grown with nitrate as a nitrogen source were transferred to ammonium-containing medium for 24 h, and total RNA was isolated. Total RNA was subjected to Northern blot analysis as described in Materials and Methods. Two bands were detected: a 2.3-kb transcript that corresponds to the complete *glnN* mRNA and a 1.2-kb transcript which is a degradation product of the complete transcript (26a). The *glnN* transcript level was extremely low, as previously described for the WT strain (26), and was approximately the same in the SJCR3.1 and SJCR3 strains (Fig. 3). These data suggest that the mutation creating strain SJCR3.1 did not affect accumulation of the *glnN* transcript in ammonium-containing medium. On the other hand, GS type III activity induction by nitrogen starvation (26) was observed in both the SJCR3 and SJCR3.1 strains (Table 1).

Regulation of Nar and Nir in *Synechocystis* sp. strain PCC 6803 *glnA* mutants. *Synechocystis* sp. strain PCC 6803 cells grown in medium containing nitrate as a nitrogen source had Nar and Nir specific activities of 65 ± 5 mU/mg of protein and 40 ± 5 mU/mg of protein, respectively, whereas cells grown on ammonium-containing medium had specific activities of 10 ± 5 mU/mg of protein for both reductases, regardless of the presence of nitrate in the culture medium. When ammonium-grown cells were transferred to medium containing nitrate as a nitrogen source, induction of Nar and Nir activities was observed; these activities reached the maximum specific levels mentioned above after about 5 h. However, a small increase in enzyme levels was seen after transfer to nitrogen-free medium, especially in the case of Nir (data not shown).

Since SJCR3 was unable to grow in ammonium-containing

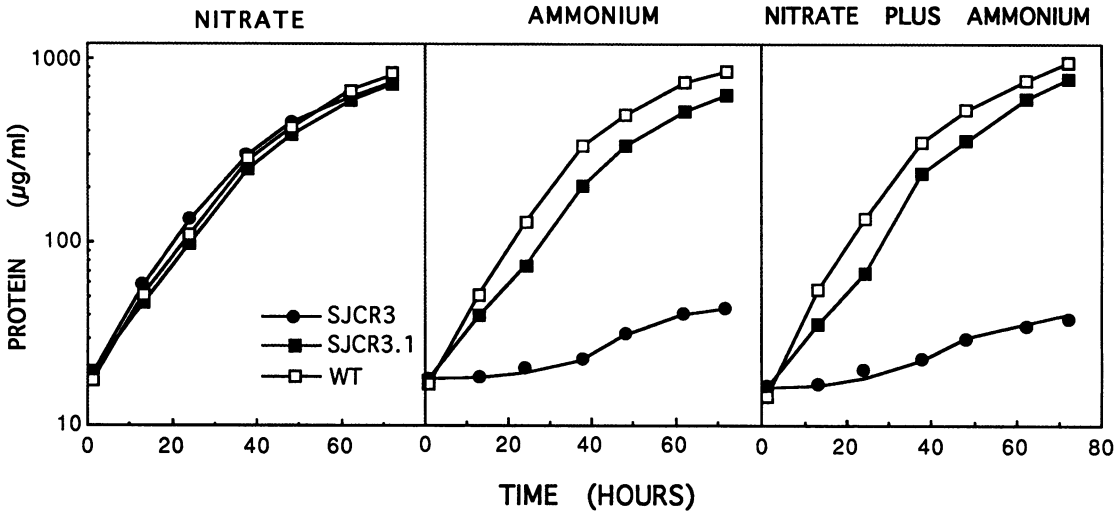

FIG. 1. Growth curves of *Synechocystis* sp. strains PCC 6803 (WT), SJCR3, and SJCR3.1 in medium containing nitrate, ammonium, or nitrate plus ammonium as a nitrogen source. Data are the means of three independent experiments, and standard errors were never higher than 5%.

medium, it was impossible to carry out induction experiments. However, we could determine the time courses of Nar and Nir activities after transfer of nitrate-grown SJCR3 cells to ammonium-containing medium.

Levels of Nar and Nir specific activities 24 h after transfer of nitrate-grown cells to fresh medium containing either no nitrogen source, nitrate, ammonium, or both forms of nitrogen are shown in Table 2. Nar and Nir specific activities of *Synechocystis* sp. strain PCC 6803 (WT) decreased about three- and fourfold, respectively, after 24 h in ammonium-containing medium, either in the absence or in the presence of nitrate. By contrast, the decrease in the Nar and Nir specific activities of strain SJCR3 (2.5-fold) was detected only in the absence of nitrate from the culture medium. In fact, Nar and Nir were not repressed in the ammonium-containing medium, but development of both activities was possible only when nitrate was present. The behavior of Nar and Nir enzyme activities in strain SJCR3.1 was similar to that in the WT strain, and both reductases showed the ammonium-promoted repression in ammonium-containing medium with or without nitrate. Nar and Nir specific activities of the three strains showed the same development, after 24 h, in medium lacking any nitrogen source; both activities decreased, and the decrease was more drastic in the case of Nar activity in both *glnA* mutants (Table 2).

The time courses of Nar and Nir total activities after transfer of nitrate-grown cells to medium containing either nitrate, ammonium, or nitrate plus ammonium are shown in Fig. 4. The increase in Nar and Nir total activities in nitrate medium observed in the three strains was a consequence of culture growth. The presence of ammonium caused total activities to remain constant in the WT and SJCR3.1 strains, indicating that the decrease in specific activities shown in Table 2 was due only to the increase in total protein of the cell culture during the time of the experiment, and suggested a dilution effect rather than a specific degradation of Nar and Nir proteins. The repression of Nar and Nir syntheses by ammonium was independent of the presence of nitrate in these two strains. In contrast, in the SJCR3 mutant, Nar and Nir syntheses were not repressed by ammonium when nitrate was present in the culture medium (Fig. 4).

Short-term inhibition of nitrate uptake by ammonium in

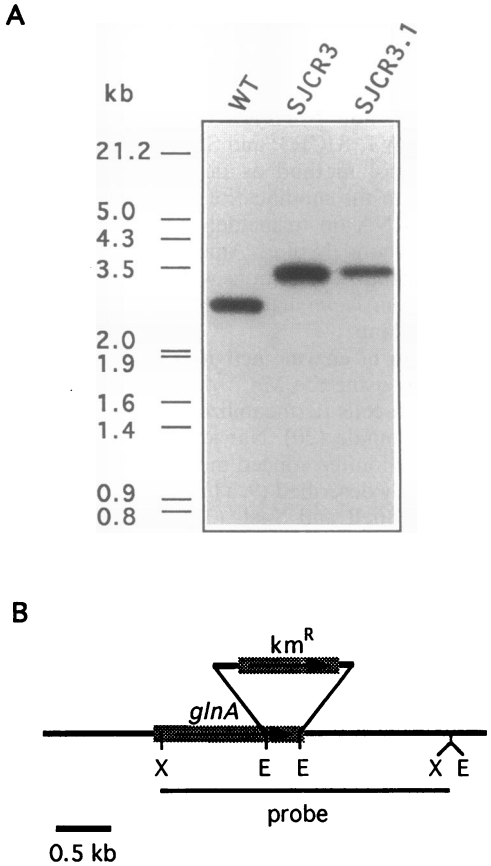

FIG. 2. (A) Southern blot of genomic DNA from *Synechocystis* WT, SJCR3, and SJCR3.1 strains. Genomic DNA was digested with *Xmn*I and hybridized by using the *Xmn*I-*Xmn*I 2.66-kb fragment indicated in panel B as a ^{32}P-labelled probe. (B) Structure of the *glnA* region in the WT strain and *glnA*::*npt* mutants. In both *glnA* mutant strains, a 315-bp internal *Eco*RI fragment of *glnA* is replaced by a 1.3-kb fragment containing an *npt* (Km^r) gene. Restriction site abbreviations: E, *Eco*RI; X, *Xmn*I.

TABLE 1. GS activities of *Synechocystis* sp. strain PCC 6803 (WT) and its *glnA* mutants[a]

Strain	GS sp act (mU/mg of protein) with the following nitrogen source:			
	NO_3^-	NH_4^+	NO_3NH_4	None
WT	2,020 ± 115	70 ± 13	67 ± 12	2,823 ± 130
SJCR3	53 ± 7	5 ± 3	5 ± 2	502 ± 25
SJCR3.1	58 ± 6	20 ± 4	21 ± 4	513 ± 27

[a] *Synechocystis* cells grown in BG11 medium were harvested at the mid-exponential growth phase, washed with $BG11_0$ medium, and transferred to fresh medium containing the indicated nitrogen source. After 24 h, samples were taken for determination of GS activity. Values are the averages of three independent experiments ± standard error.

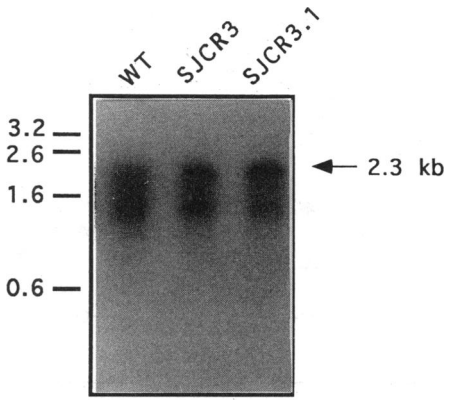

FIG. 3. Northern blot of total RNA isolated from *Synechocystis* WT, SJCR3, and SJCR3.1 cells grown in nitrate and transferred to ammonium-containing medium for 24 h. Total RNA was denatured, electrophoresed in a 1% agarose gel, blotted, and hybridized with a 666-bp *Eco*RI-*Eco*RI internal *glnN* probe. Thirty micrograms of total RNA was loaded per lane. The film was exposed for 14 days. Transcript sizes were estimated by comparison with 23S rRNA (3.2 kb), a cleavage product of 23S rRNA (2.6 kb), 16S rRNA (1.6 kb), and 5S rRNA (0.6 kb) (23). Sizes in kilobases are indicated.

***glnA* mutants.** *Synechocystis* SJCR3 cells grown in nitrate-containing medium showed the same rate of nitrate utilization as the WT or SJCR3.1 strain, but after 24 h of incubation with nitrate plus ammonium, nitrate utilization in SJCR3 cells was 3.5 times higher than that in the WT or SJCR3.1 strain (0.44 ± 0.05 $\mu mol \cdot h^{-1} \cdot mg^{-1}$ versus 0.12 ± 0.03 $\mu mol \cdot h^{-1} \cdot mg^{-1}$, respectively). These data suggest that synthesis of nitrate transport proteins was not inhibited by ammonium in the SJCR3 mutant.

The short-term ammonium inhibition of nitrate utilization observed in the WT strain (Fig. 5) did not occur in the SJCR3 strain. In fact, addition of 0.5 mM ammonium to SJCR3 cells grown in nitrate medium did not inhibit the nitrate uptake (Fig. 5). Results for strain SJCR3.1 were similar to those obtained for the WT strain (Fig. 5).

Alteration of amino acid pools in *glnA* mutants. Addition of ammonium to nitrate-grown *Synechocystis* sp. strain PCC 6803 (WT) cells produces a change in the intracellular concentrations of amino acids related to the GS-GOGAT pathway (22). Thus, in the first 30 s after ammonium addition, the pool of glutamate decreased dramatically, while the glutamine level increased reciprocally. Levels of both amino acids were restored to values close to the initial ones after 1 h (Fig. 6A). In order to find a possible reason for the deregulation of the nitrate assimilation system in strain SJCR3, we determined the intracellular concentrations of glutamate and glutamine before and after the addition of 2 mM ammonium. Figure 6 shows changes observed in glutamate and glutamine pools after addition of 2 mM ammonium to *Synechocystis* WT, SJCR3, and SJCR3.1 strains growing in nitrate. The intracellular glutamate concentration in SJCR3 decreased to about 50% at 30 s after ammonium addition, but initial levels were recovered in about 3 min. At the same time, the glutamine pool showed a small initial increase that was followed by a drop to an undetectable level after 20 min (Fig. 6B). The change in the glutamate and glutamine pools after ammonium addition in SJCR3.1 cells was similar to that found for SJCR3; however, glutamine levels remained at about 4 ± 1 nmol/mg of protein for at least 2 h after ammonium addition, a value similar to that observed in the WT strain (5.5 ± 0.5 nmol/mg of protein) (Fig. 6C).

DISCUSSION

We have recently described the existence of two different GSs in the unicellular cyanobacterium *Synechocystis* sp. strain PCC 6803, one encoded by the *glnA* gene (GS type I) and another encoded by the *glnN* gene (GS type III) (26). In this work we have characterized for the first time for cyanobacteria the nitrate assimilation system of an insertional *glnA* mutant (strain SJCR3), in which the only GS activity present corresponds to the *glnN* product. The lack of GS type I in this strain together with the extremely low activity of GS type III in ammonium-containing medium led to intracellular glutamine depletion and, as a consequence, to the deregulation of the nitrate assimilation system.

SJCR3 grew at the same rate as the WT strain in medium with nitrate as the sole nitrogen source, but it was unable to grow in ammonium-containing medium. The very low GS activity of strain SJCR3 in the presence of ammonium (Table 1) suggests that in this mutant glutamine biosynthesis is impaired in ammonium-containing medium, which is probably the cause of the absence of growth under this condition. Our

TABLE 2. Nar and Nir activities of *Synechocystis* sp. strain PCC 6803 (WT) and its *glnA* mutants[a]

Strain	Sp act (mU/mg of protein) with the indicated nitrogen source							
	Nar				Nir			
	NO_3^-	NH_4^+	NO_3NH_4	None	NO_3^-	NH_4^+	NO_3NH_4	None
WT	65 ± 5	18 ± 3	25 ± 4	52 ± 5	40 ± 5	9 ± 2	8 ± 2	39 ± 7
SJCR3	88 ± 7	38 ± 6	110 ± 11	47 ± 8	59 ± 6	24 ± 5	69 ± 7	44 ± 5
SJCR3.1	83 ± 9	24 ± 3	38 ± 6	50 ± 7	54 ± 6	15 ± 3	22 ± 6	39 ± 5

[a] *Synechocystis* WT and mutant cells growing in BG11 medium were harvested at the mid-exponential growth phase, washed with $BG11_0$ medium, and transferred to fresh medium containing the indicated nitrogen source. After 24 h, samples were taken to determine Nar and Nir activities as described in Materials and Methods. Values are the averages of three independent experiments ± standard error.

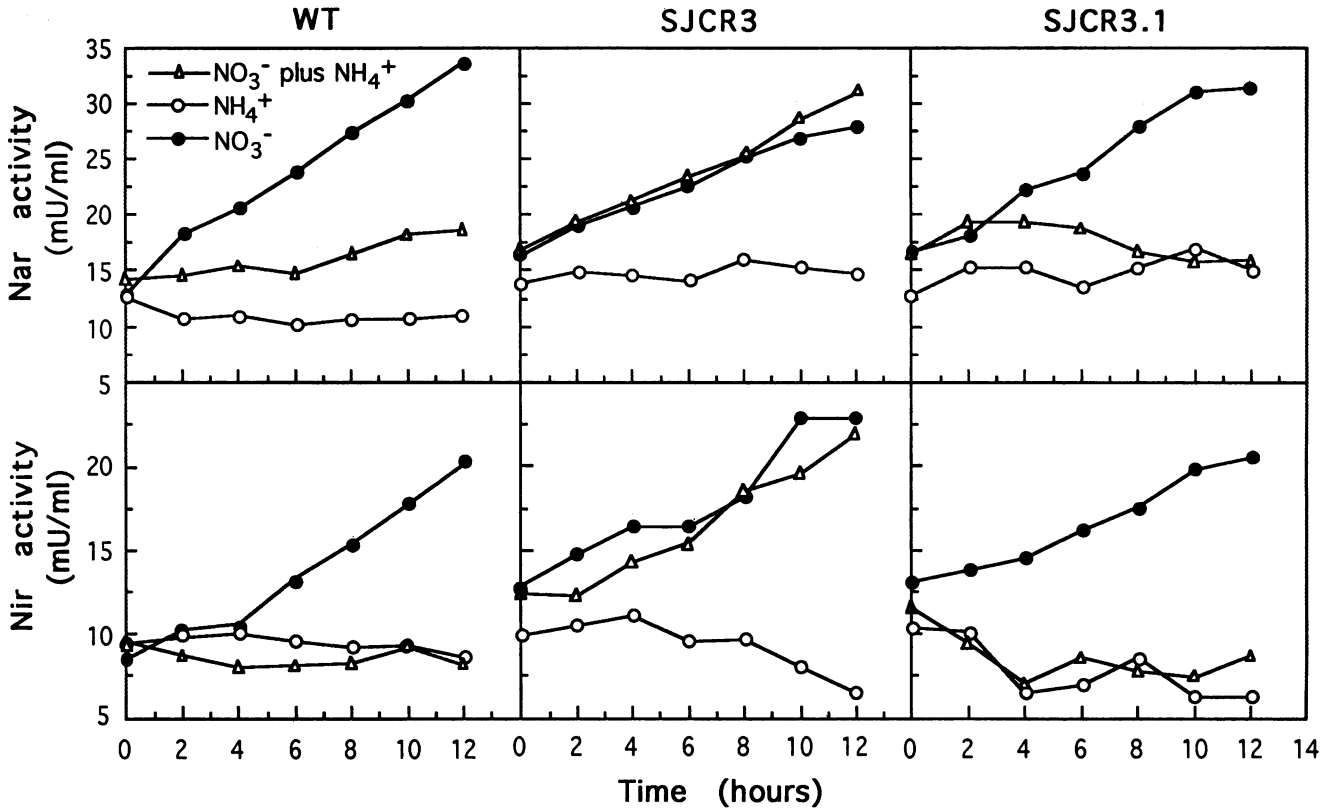

FIG. 4. Time courses of Nar and Nir total activities after transfer of nitrate-grown cells of WT, SJCR3, and SJCR3.1 strains to various media. Cells grown on BG11 medium (nitrate-containing medium) were harvested at the mid-exponential growth phase, washed with $BG11_0$ medium, and transferred to fresh medium containing the indicated nitrogen source. At the indicated times, samples were taken for enzyme determination as described in Materials and Methods. Data are the means of three independent experiments, and standard errors were never higher than 4%.

results are in contrast to those recently reported for a *glnA* mutant of *Agmenellum quadruplicatum* PR-6 (*Synechococcus* sp. strain PCC 7002), for which no differences in growth rates with different nitrogen sources were found (37).

A phenotypic revertant, SJCR3.1, which was able to grow in ammonium-containing medium (Fig. 1) was isolated by direct selection from SJCR3. The level of GS activity in strain SJCR3.1 in ammonium-containing medium was 2- to 12-fold higher than that in the parental strain (SJCR3) (Table 1). The increase in the level of GS activity seems to be sufficient to support growth of this strain in the presence of ammonium. This increase in GS activity in ammonium-containing medium was not due to a higher level of gene expression, since SJCR3.1 cells show roughly the same *glnN* transcript level as do SJCR3 cells (Fig. 3), suggesting that SJCR3.1 is a mutant affected in the posttranscriptional regulation of the GS encoded by *glnN*. We have previously reported that expression from the *glnN* promoter, as measured by *glnN-cat* fusions, is undetectable in ammonium-grown cells. However, we were able to detect *glnN* mRNA of ammonium-grown cells in Northern blot experiments after a long exposure of the filters. This discrepancy could be attributed to the sensitivity of the colorimetric chloramphenicol acetyltransferase assay, which would not allow for detection of very low levels of chloramphenicol acetyltransferase activity.

We have used the SJCR3, SJCR3.1, and WT strains to study the regulation of the nitrate assimilation system in *Synechocystis* sp. strain PCC 6803. The regulation of Nar and Nir has been studied for many cyanobacteria (7). Ammonium-promoted repression of Nar and Nir syntheses has been shown to take place in every cyanobacterium analyzed. However, two models have been described for the role of nitrate in the regulation of Nar and Nir syntheses. It has been reported that nitrate is not required for the synthesis of Nar and Nir in the non-nitrogen-fixing cyanobacteria tested so far; this view is supported by the observation that Nar and Nir activities develop in the absence of a combined form of nitrogen in the medium. On the other hand, in the dinitrogen-fixing species, nitrate is required as an inducer of the syntheses of both reductases (9).

Ammonium repression of the syntheses of both enzymes was also observed in the *Synechocystis* WT strain in medium with or without nitrate (Table 2 and Fig. 4). In contrast, Nar and Nir activities were not repressed by ammonium when nitrate was present in the SJCR3 strain, suggesting that in this strain the repression of the syntheses of both reductases in medium containing ammonium as the sole nitrogen source was due to the absence of nitrate (Table 2 and Fig. 4) rather than to the presence of ammonium. Therefore, our data clearly indicate that strain SJCR3 escaped the repression by ammonium of Nar and Nir syntheses and also that in *Synechocystis* sp. strain PCC 6803, a non-nitrogen-fixing strain, nitrate was required for Nar and Nir syntheses, acting as inducer at the transcriptional level or posttranscriptionally and thus stabilizing the mRNA or the proteins.

Ammonium-treated SJCR3 cells, which had extremely low levels of GS activity, lacked the ammonium-promoted repression of nitrate assimilation, indicating that repression by ammonium requires the incorporation of ammonium into

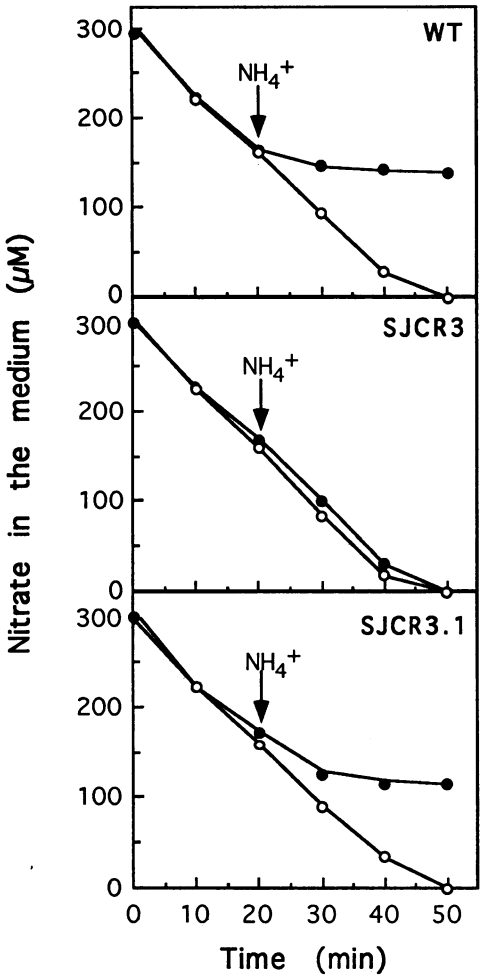

FIG. 5. Effect of the addition of ammonium on nitrate uptake by *Synechocystis* WT, SJCR3, and SJCR3.1 strains. Cells grown on BG11 medium were harvested at the mid-exponential growth phase, washed in 20 mM Tricine-NaOH buffer (pH 8.3)–10 mM $NaHCO_3$, and resuspended in the same solution at a final concentration of 0.45 mg of protein per ml. The uptake assay was started by addition of nitrate at a final concentration of 300 μM. ●, ammonium at a final concentration of 0.5 mM was added at the time indicated by the arrows. ○, control without ammonium addition.

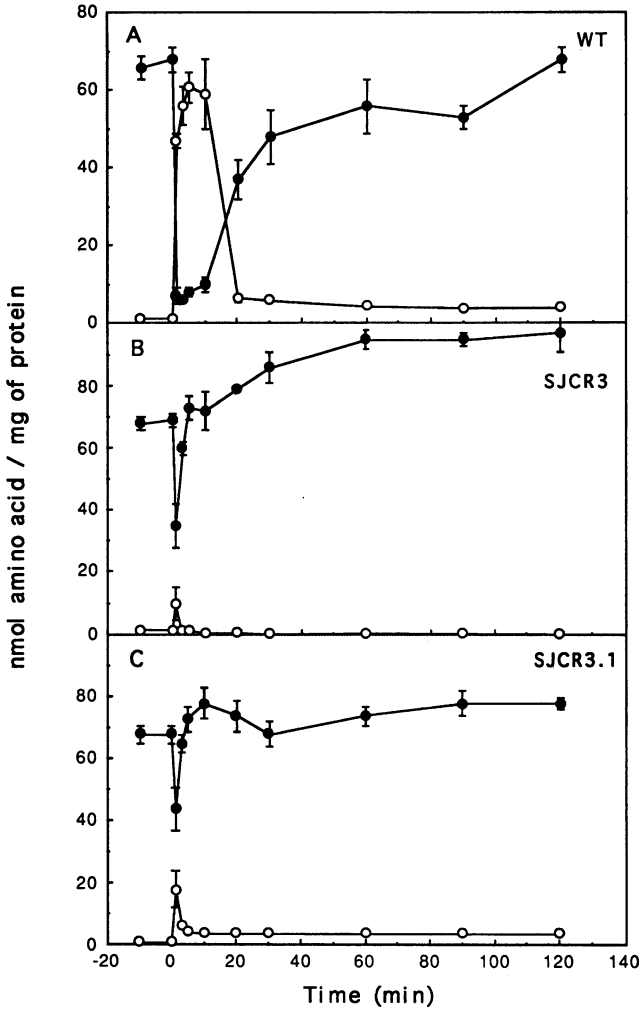

FIG. 6. Effect of addition of ammonium on intracellular glutamine (○) and glutamate (●) pools in *Synechocystis* WT, SJCR3, and SJCR3.1 strains. Ammonium at a final concentration of 1 mM was added to cells growing with nitrate at time zero, and samples were taken at the indicated times as described in Materials and Methods. Data are the means of three independent experiments, and standard errors are represented by bars.

carbon skeletons through the GS-GOGAT pathway. However, the SJCR3.1 mutant, which maintains a level of GS activity higher than that of SJCR3 after ammonium addition, exhibited normal regulation of Nar and Nir syntheses, suggesting that this level of GS activity (Table 1) was sufficient to originate the metabolic signals involved in control of the expression of the nitrate assimilation system.

From experiments with MSX, an irreversible inhibitor of GS, it has been proposed for other cyanobacterial strains that the ammonium-promoted repression of the nitrate assimilation system requires ammonium metabolism through the GS-GOGAT pathway (9, 32). In fact, inhibition of GS with MSX leads to the expression of Nar and Nir in the presence of ammonium (32) in a way similar to that seen in the *glnA* mutant of *Synechocystis* sp. strain PCC 6803.

Regulation by ammonium of nitrate uptake is exerted at two different levels: (i) repression of the synthesis of transport proteins and (ii) a short-term inhibition of the transport activity (7, 12). Our results indicate that ammonium-promoted short-term inhibition of nitrate uptake did not occur in the SJCR3 mutant (Fig. 5), suggesting that inhibition of nitrate transport by ammonium also requires the metabolism of ammonium through GS.

In other bacterial genera, such as *Azotobacter* and *Klebsiella*, the expression of Nar and Nir is under the control of the NtrB-NtrC system (2, 14, 33). In cyanobacteria, the transcriptional activator NtcA controls the expression of the *nirAnrtABCDnarB* operon (35, 36). However, little is known about the metabolic signals implicated in that regulation. The intracellular ratio of glutamine to 2-oxoglutarate has been shown to be a sensor of the ammonium content of the environment in enterobacteria, mediating the regulation of several Ntr (nitrogen-regulated) operons (16). For cyanobacteria, either glutamine or a glutamine derivative has also been proposed as a regulatory signal for the ammonium-promoted repression of nitrogenase or the nitrate assimilation system (6, 28, 29). We

have used the *glnA* mutants SJCR3 and SJCR3.1 to investigate the intracellular signals involved in the regulation of nitrate assimilation. The drastic alteration in glutamate and glutamine intracellular pools after addition of ammonium observed in the WT strain was not detected in either *glnA* mutant (SJCR3 or SJCR3.1) (Fig. 6), in which only a small and rapid change in both amino acid pools occurred. These data indicated that the GS encoded by the *glnA* gene is responsible for the changes in concentration observed in the WT strain. The time courses for glutamate and glutamine levels detected in both *glnA* mutants were similar; however, the steady-state level of glutamine reached 20 min after ammonium addition in strain SJCR3.1 was similar to the level reached in the WT strain, in contrast with the SJCR3 mutant, in which glutamine was undetectable (Fig. 6). Taking into account the fact that the SJCR3.1 strain exhibited a normally regulated expression of the nitrate assimilation proteins, our data show clearly that the glutamine level or the levels of derivatives of glutamine metabolism are or impinge on the signals responsible for the regulation of nitrate assimilation in this cyanobacterium.

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