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Identification and Cloning of a Regulatory Gene for Nitrogen Assimilation in the Cyanobacterium *Synechococcus* sp. Strain PCC 7942

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Twenty-seven mutants that were unable to assimilate nitrate were isolated from *Synechococcus* sp. strain PCC 7942. In addition to mutants that lacked nitrate reductase or nitrite reductase, seven pleiotropic mutants impaired in both reductases, glutamine synthetase, and methylammonium transport were also isolated. One of the pleiotropic mutants was complemented by transformation with a cosmid gene bank from wild-type strain PCC 7942. Three complementing cosmids were isolated, and a 3.1-kilobase-pair DNA fragment that was still able to complement the mutant was identified. The regulatory gene that was cloned (*ntcA*) appeared to be required for full expression of proteins subject to ammonium repression in *Synechococcus* sp.

Synechococcus sp. is a strict photoautotrophic cyanobacterium which is able to use nitrate, nitrite, or ammonium as a nitrogen source. Nitrate assimilation involves nitrate uptake into the cell (8) and its intracellular reduction, via nitrite, to ammonium, which is the inorganic nitrogen source incorporated into carbon skeletons (10). In *Synechococcus* sp., a nitrate transport element has been identified as a ca. 48-kilodalton (kDa) cytoplasmic membrane protein (19, 23, 30), and nitrate reductase (a molybdenum-containing enzyme) and nitrite reductase have been isolated and characterized and represent single-polypeptide enzymes of about 75 and 50 kDa, respectively (10). An ammonium transport system has been characterized in *Synechococcus* sp. by means of studying methylammonium transport (1). Ammonium, which either results from the reduction of nitrate or nitrite or is taken up from the external medium, is mainly assimilated via glutamine synthetase (10).

In *Synechococcus* sp., ammonium acts as a repressor of a number of activities related to nitrogen nutrition, including nitrate reductase (13), nitrite reductase (15), the 48-kDa nitrate transport protein (19, 23, 30), glutamine synthetase (7), and methylammonium transport (1, 2). The cellular levels of these proteins are higher in nitrate- than in ammonium-grown cells. However, nitrate is not required as an inducer, since the increase in the activities and proteins takes place in response to ammonium deprivation (2, 13, 15, 19). This behavior is similar to that of nitrogenase synthesis and heterocyst development in filamentous, nitrogen-fixing cyanobacteria (31). In the case of nitrate reductase and nitrite reductase, it has been established that ammonium metabolism through glutamine synthetase is required for ammonium repression to take place (13, 15). However, the genetic mechanism underlying ammonium repression in these organisms had not been investigated to date.

Some mutants of *Synechococcus* sp. that are impaired in nitrate assimilation have been reported previously (4, 16–18, 32). Most of those mutants lacked nitrate reductase activity, and from complementation analysis, at least three genes have been shown to be involved in nitrate reduction (16, 17). In addition, two mutants that lack nitrite reductase have been reported, one from strain PCC 7002 (formerly known as *Agmenellum quadruplicatum* [32]) and another from strain PCC 7942 (*Anacystis nidulans* R2 [18]). The latter exhibits a complex phenotype, since it expresses constitutively both nitrate reductase (18) and the 48-kDa nitrate transport protein (19). Finally, a mutant has been isolated from strain PCC 7942 which might be affected in the transport of nitrate into the cell (18).

With the aim of gaining insight into the genetics of nitrate assimilation in a *Synechococcus* sp., we isolated, from strain PCC 7942, a new set of mutants impaired in nitrate assimilation and characterized them by looking for new phenotypes. Here we report the identification and cloning of a gene involved in the regulation of the synthesis not only of the nitrate assimilatory system but also of glutamine synthetase and of the ammonium (methylammonium) permease.

MATERIALS AND METHODS

Strains and growth conditions. The unicellular cyanobacterium *Synechococcus* sp. strain R2 (PCC 7942) and its derivative strain M4$^-$, which lacks plasmid pUH24, were used in this study. Cells were grown photoautotrophically at 39°C in the synthetic medium described previously (13). The medium was sparged with 5% CO_2 in air, and KNO_3 or $(NH_4)_2SO_4$ was used as the nitrogen source. Alternatively, cells were grown photoautotrophically at 30°C with shaking in BG11 medium (26) supplemented with 12 mM $NaHCO_3$ (medium BG11C). When ammonium was substituted for nitrate in medium BG11 (rendering medium $BG11_0$ + NH_4^+), it was supplied as NH_4Cl (4 mM) in the presence of 8 mM *N*-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid (TES)–NaOH buffer (pH 7.5). $BG11_0C$ + NO_2^- medium was medium BG11C without nitrate and supplemented with 2 mM KNO_2. For growth on plates, the medium was solidified with separately autoclaved 1% agar (Difco Laboratories, Detroit, Mich.). The plates were incubated at 30°C in the light.

Isolation of nitrate assimilation mutants. Nitrate-grown cells of strain M4$^-$ were mutagenized with nitrosoguanidine (3) or UV light (11, 34) as described previously. After mutagenesis, portions of the cell suspensions were processed separately in order to favor the recovery of indepen-

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dent mutants. After growth of the mutants in $BG11_0$ + NH_4^+ medium to permit segregation and expression of mutations, the cells were incubated in BG11C medium and, after an incubation of ca. 48 h, were subjected to ampicillin (200 μg/ml) enrichment. Survivors were plated onto $BG11_0$ + NH_4^+ solid medium. Colonies appeared after 1 to 2 weeks, and their ability to grow on BG11C or $BG11_0C$ + NO_2^- medium was determined. Colonies that were unable to grow on nitrate were purified by restreaking them onto $BG11_0$ + NH_4^+ medium, and their phenotypes were then rechecked.

Enzyme activities and glutamine synthetase protein. Nitrate reductase (14) and nitrite reductase (15) were determined by using dithionite-reduced methyl viologen as the reductant. Glutamine synthetase (transferase assay) was determined as described previously (5). In every case, cells were made permeable with mixed alkyltrimethylammonium bromide. The concentrations of chlorophyll in an enzymatic assay were 5, 25, and 2.5 μg/ml for nitrate reductase, nitrite reductase, and glutamine synthetase, respectively. One unit of enzyme activity corresponded to 1 μmol of product that formed or substrate that disappeared (nitrite reductase) per min.

The relative amount of glutamine synthetase protein was determined in cell homogenates by rocket immunoelectrophoresis, which was performed essentially as described previously (29). Cell homogenates were prepared by grinding cells that had been frozen with liquid air. After three freeze-thaw cycles in the presence of 2% Triton X-100 and 1 mM phenylmethylsulfonyl fluoride, the cell homogenates were centrifuged in an Eppendorf centrifuge, and samples from the supernatants (68 μg of protein) were applied to a 1% agarose gel slab containing anti-glutamine synthetase antibody. The antibodies were generated against glutamine synthetase protein isolated from *Synechococcus* sp. strain PCC 6301 (S. Marqués, Ph.D. thesis, Universidad de Sevilla, Seville, Spain, 1988).

Methylammonium transport. Ammonium-grown cells that were derepressed (derepression was achieved by incubation for 20 h in $BG11_0$ medium supplemented with TES buffer) or not were washed and suspended (2) in 10 mM $NaCO_3H$–20 mM $NaPO_4H_2$–NaOH buffer (pH 7.1) at 10 μg of chlorophyll per ml. After 15 to 25 min of incubation at 23°C under white light, [^{14}C]CH_3NH_3Cl (9 μM, 48 μCi/μmol) was added, and after 45 s the cells were separated from their incubation medium by centrifugation through silicon into perchloric acid (25). Samples of the perchloric acid fraction were mixed with scintillation cocktail and counted. The radioactivity that bound to boiled cells was used as a blank.

Genetic techniques. Plasmid DNA isolation from *Escherichia coli*, endonuclease restriction, ligation, and transformation of competent *E. coli* JM109 cells were carried out by standard methods (21).

Total DNA from the *Synechococcus* sp. was isolated as previously reported (18). Transformation of the *Synechococcus* sp. was carried out essentially as described previously (12). To obtain nitrate-assimilating transformants from nitrate assimilation-defective mutants, transformation mixtures (10^9 cells per ml, 1 to 20 μg of DNA per ml) were incubated in the dark for 2.5 h and 0.1-ml samples were spread onto BG11 plates.

RESULTS

Isolation and characterization of nitrate assimilation mutants. Twenty-seven mutants that were unable to grow on nitrate as the nitrogen source were isolated from *Synechococcus* sp. strain M4$^-$ (Table 1). Fourteen of those mutants were also unable to grow on nitrite. All of the mutants which were able to grow on nitrite specifically exhibited low levels of nitrate reductase (Table 1). The mutants that were unable to grow on either nitrate or nitrite showed undetectable levels of nitrite reductase, and seven of them also exhibited low levels of nitrate reductase (Table 1). The spontaneous reversion frequency of all the mutants was in the range of 10^{-9} to 10^{-7} (Table 1).

TABLE 1. Phenotypic characterization of nitrate assimilation mutants of *Synechococcus* sp.

Strain	Mutagen[a]	Growth on nitrite[b]	Enzyme activity (mU/mg of protein)[c]				Reversion frequency (CFU/cell)[d]
			Nitrate reductase		Nitrite reductase		
			NH_4^+	−N	NH_4^+	−N	
M4$^-$ (wild type)		+	5	33	0	12	
CS3[e]	NTG	+	2	1	1	11	4×10^{-9}
CS8[e]	UV	+	2	2	0	9	2×10^{-9}
CS2[f]	NTG	−	1	17	0	0	2×10^{-8}
CS6[f]	UV	−	3	30	0	0	3×10^{-7}
CS1	NTG	−	3	3	0	0	2×10^{-9}
CS7	UV	−	3	3	0	0	3×10^{-9}
CS13	NTG	−	4	2	0	0	1×10^{-9}
CS14	NTG	−	3	4	0	0	6×10^{-10}
CS16	NTG	−	3	4	0	0	2×10^{-9}
CS17	NTG	−	4	3	0	0	9×10^{-10}
CS18	NTG	−	6	4	0	0	1×10^{-8}

[a] NTG, *N*-Methyl-*N'*-nitro-*N*-nitrosoguanidine; UV, UV light.
[b] Growth on $BG11_0C$ + NO_2^- plates. Symbols: +, growth was observed; −, no growth was observed.
[c] Enzyme activities were measured in cells that were grown at 39°C as described in the text. Abbreviations: NH_4^+, cells grown with $(NH_4)_2SO_4$ as the nitrogen source; −N, ammonium-grown cells incubated for 6 h under growth conditions (39°C) in the absence of any added nitrogen source.
[d] Cells that were grown on $BG11_0$ + NH_4^+ liquid medium were washed and plated at ca. 5×10^8 cells per plate on BG11C solid medium. Colonies appeared after 5 to 7 days of incubation at 30°C.
[e] These strains are representative of 13 mutants that exhibited the same phenotype.
[f] These strains are representative of seven mutants that exhibited the same phenotype.

Because the mutants that were impaired in both nitrate reductase and nitrite reductase represented a new phenotype and may have been affected in a regulatory gene (see below), they were investigated in detail. The regulation of nitrate reductase and nitrite reductase synthesis was studied in a number of revertants. The results presented in Table 2 indicate that the revertants exhibited both normal levels and patterns of regulation of both enzymes which, like in the wild-type strain, were repressed in ammonium-grown cells.

In the wild-type *Synechococcus* sp., the levels of both glutamine synthetase activity (7) and glutamine synthetase protein present in ammonium-grown cells were about 50% of the levels obtained in cells that were grown on nitrate or incubated in the absence of a nitrogen source (Table 3). The pleiotropic mutants exhibited, irrespective of the nitrogen status of the medium, glutamine synthetase (activity or protein) levels similar to those of ammonium-grown cells of the wild-type strain, whereas the revertants showed glutamine synthetase levels that were regulated like they were in the wild-type strain (Table 3).

The transport of methylammonium is subject to repression

TABLE 2. Regulation of nitrate reductase and nitrite reductase in revertants of the nitrate assimilation pleiotropic mutants

Strain[a]	Enzyme activity (mU/mg of protein)[b]			
	Nitrate reductase		Nitrite reductase	
	NH_4^+	NO_3^-	NH_4^+	NO_3^-
M4$^-$ (wild type)	5	30	2	8
CS1.1	6	19	3	8
CS7.2	6	22	3	9
CS13.1	6	46	1	7
CS14.1	5	27	0	10
CS16.3	6	31	0	8
CS17.1	5	24	4	15
CS18.1	4	24	0	10

[a] Revertants that were able to grow on nitrate were named after the mutant from which they were isolated (e.g., CS1.1 was a revertant of mutant CS1).
[b] Enzyme activities were measured in cells that were grown at 30°C in BG11 medium supplemented with 12 mM $NaHCO_3$ or in medium $BG11_0$ + NH_4^+.

by ammonium in *Synechococcus* sp. strain R2 (2). Table 4 shows that the pleiotropic mutants were also impaired in [^{14}C]methylammonium transport. The levels of transport in derepressed cultures of those mutants were similar to those of ammonium-grown cells of the parental or revertant strains.

Glutamate synthase (6, 22) and leucine transport are not repressed by ammonium in cyanobacteria. Similar levels were found in representative pleiotropic mutants and in the wild-type strain, about 40 nmol of glutamate was formed (measured as described previously [6]), and 0.4 nmol of leucine was taken up (measured with 10 μM L-[^{14}C]leucine as described previously [9]) per min per mg of protein.

Cloning of the regulatory gene. All of the mutants derived from strain M4$^-$, as well as our clone of M4$^-$ itself, turned out to be nontransformable. (It is known that individual clones of *Synechococcus* sp. strain R2 frequently lose the ability to act as recipients in transformation.) To transfer the pleiotropic mutation to a transformable background, wild-type strain R2 was transformed with total DNA from strain CS1. After enrichment with ampicillin in nitrate-containing medium, plating onto permissive medium, and testing for the ability to grow on nitrate, 2 clones (of 300 clones that were tested) that were unable to grow on nitrate were identified (strains CS31 and CS32). These clones exhibited levels of nitrate reductase, nitrite reductase, glutamine synthetase,

TABLE 3. Glutamine synthetase activity and protein levels in *Synechococcus* sp. and in some pleiotropic mutants and revertants[a]

Strain	% Glutamine synthetase:			
	Activity		Protein	
	NH_4^+	−N	NH_4^+	−N
M4$^-$ (wild type)	59	100	54	100
CS1	30	31	32	36
CS13	51	66	51	45
CS1.1	60	119	52	89
CS13.1	51	114	51	88

[a] Cells that were grown at 30°C in $BG11_0$ + NH_4^+ medium were incubated for 45 h in the same medium (NH_4^+) or in $BG11_0$ medium supplemented with TES buffer (−N). Glutamine synthetase activity of 100% was equal to 622 mU/mg of protein. In the case of glutamine synthetase protein, relative measurements are presented by taking as 100% the area of immunoprecipitation (rocket) obtained with extracts from the wild-type strain incubated in nitrogen-free medium.

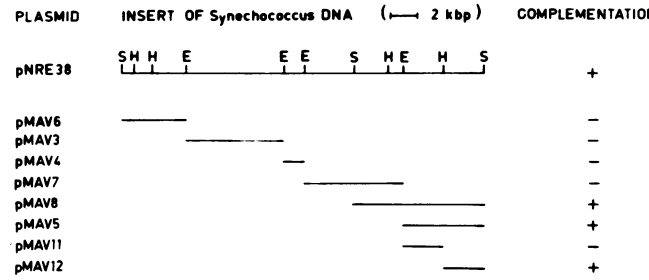

FIG. 1. Restriction map of the insert of pNRE38 and location of the complementation ability. Plasmid pNRE38 contained a 28-kilobase-pair (kbp) insert of strain 7942 DNA in cosmid vector pPUC29. pMAV plasmids contained the *Synechococcus* DNA fragment indicated in each case inserted into the polylinker of vector pUC19, except for pMAV5, which was the result of a total *Eco*RI digestion of pNRE38 followed by recircularization of the plasmid. The capacity of the different plasmids to complement strain CS31 is shown to the right of the figure. Symbols: +, positive complementation; −, no complementation. Abbreviations: E, *Eco*RI; H, *Hin*dIII; S, *Sal*I.

and methylammonium transport similar to those found in the pleiotropic mutants. Therefore, it appeared that strains CS31 and CS32 gained the mutation that was originally present in CS1. This was further supported by the fact that total DNA from strain CS1 did not complement strain CS31 (see below).

Transformation of strain CS31 with a gene bank of strain R2 that was constructed in the shuttle cosmid pPUC29 by Kuhlemeier et al. (16) rendered colonies able to grow on nitrate with a frequency of 10^{-6} to 10^{-5} per CFU. This indicates the presence in the gene bank of a wild-type copy of the gene that was mutated in strain CS31. A total of 1,000 clones from the library were tested for their ability to complement strain CS31. For this, plasmid DNA was isolated from successively smaller groups of clones, and after transformation and selection for growth on nitrate, three clones that were able to complement strain CS31 were identified. Restriction analysis of plasmid DNAs from those clones showed that two of them contained identical cosmids and that the third one contained a cosmid that was 2.6 kilobase pairs smaller than the cosmids in the other two clones, but that was otherwise identical to them. Figure 1 shows a restriction map of the insert of the smallest cosmid (pNRE38).

Several different restriction fragments from the insert of pNRE38 were subcloned into pUC19. These subclones were tested for their ability to complement strain CS31, which was found to be located on a 3.1-kilobase-pair *Hin*dIII-*Sal*I fragment (Fig. 1). Because pUC19 does not replicate in

TABLE 4. Methylammonium transport in *Synechococcus* sp. and in some pleiotropic mutants and revertants[a]

Strain	[^{14}C]CH_3-NH_3^+ transport (nmol/min · mg of protein)	
	NH_4^+	−N
M4$^-$ (wild type)	0.07	0.77
CS1	0.03	0.04
CS13	0.02	0.03
CS1.1	0.07	1.10
CS13.1	0.04	1.33

[a] Cells that were grown on $BG11_0$ + NH_4^+ medium were derepressed (−N) or not (NH_4^+) and were used in methylammonium transport assays as described in the text.

TABLE 5. Transformation studies with strain CS31 and some other nitrate assimilation mutants[a]

Recipient strain	Donor DNA[b]	Transformation (no. of transformants/cell)
FM2	pNRE38	–[c]
FM4	pNRE38	–
FM6	pNRE38	–
FM12	pNRE38	–
CS31	pNRE38	3×10^{-5}
CS31	pNR631	–
CS31	pNR1211	–
CS31	pNR1934	–
CS31	Total DNA from strain FM2	7×10^{-5}
CS31	Total DNA from strain FM16	5×10^{-5}
CS31	Total DNA from strain CS1	–

[a] Transformation was carried out as described in the text; selection was for growth on nitrate.

[b] The following plasmids contained the indicated genes: pNRE38, *ntcA*; pNR1211, *narA*; pNR631, *narB*; pNR1934, *narC*. The following strains used in this study were mutated in the indicated genes: FM4, *narB*; FM6, *narA*; FM12, *narC*; CS31, *ntcA*. Strains FM2 and FM16 are mutated in genes that have not yet been cloned (18).

[c] –, The number of colonies that appeared was in the range of the reversion frequency of the mutants.

Synechococcus sp., transformation of CS31 to growth on nitrate should have relied on homologous recombination between the insert and the chromosome.

Cosmid pNRE38 was used in transformation experiments with mutants of previously identified genes involved in nitrate assimilation in *Synechococcus* sp. (18), namely, strains FM2, FM4, FM6, and FM12 (Table 5). None of these mutants was complemented by cosmid pNRE38. In turn, plasmid DNAs containing the three nitrate reduction genes which were cloned previously (16, 17) did not complement strain CS31. Finally, chromosomal DNA from strains FM2 and FM16, which are mutated in nitrate assimilation genes that have not been cloned (18), transformed strain CS31 to growth on nitrate. Taken together, these results indicate that the gene that was mutated in strain CS31 is different from the five genes that are involved in nitrate assimilation in *Synechococcus* sp. which were identified previously (16–18).

DISCUSSION

A set of mutants that were unable to grow on nitrate was isolated from *Synechococcus* sp. strain 7942. In addition to mutants that lacked nitrate reductase or nitrite reductase, a new type of mutant that was simultaneously impaired in nitrate reductase, nitrite reductase, glutamine synthetase, and methylammonium transport was obtained. We have previously reported (19) that these pleiotropic mutants are also impaired in the synthesis of a component of the nitrate transport system. All of these nitrogen assimilation elements, which were altered in the pleiotropic mutants, were subject to ammonium repression in the wild-type *Synechococcus* sp. In contrast, two activities related to nitrogen metabolism which were not subject to ammonium repression in *Synechococcus* sp. (namely, leucine transport and glutamate synthase) were not found to be altered in those mutants. The levels of the nitrate assimilation elements (the cytoplasmic membrane protein and the reductases) and of glutamine synthetase and methylammonium transport in the pleiotropic mutants were similar to those found in ammonium-grown cells of the wild-type strain, irrespective of the nitrogen content in the medium. In every case the phenotype appeared to be the result of a single mutation, since revertants were obtained with a relatively high frequency and every revertant analyzed showed all the wild-type characteristics. The wild-type version of the gene that was mutated in one of the pleiotropic mutants was cloned by complementation. Transformation studies corroborated the fact that this gene is distinct from any of the genes involved in nitrate assimilation by cyanobacteria which have been reported to date. We refer to this gene as *ntcA* (*ntc* for nitrogen control).

The product of the *ntcA* gene seems to be a positive-acting element that is required for the expression of some genes that are involved in nitrogen assimilation. The alternative hypothesis, that it is a repressor, is not favored by our results, which showed that the revertants of the pleiotropic mutants exhibit normal regulation of the synthesis of nitrate reductase and nitrite reductase (Table 2), glutamine synthetase (Table 3), methylammonium transport (Table 4), and the 48-kDa nitrate transport element (19). In the case of a repressor, the mutants that we isolated (which were unable to derepress ammonium-regulated proteins) should correspond to superrepressors, from which it follows that most of their revertants should have been constitutive.

The mechanism by which ammonium represses a number of genes involved in nitrogen assimilation in cyanobacteria is unknown. As is the case in *Synechococcus* sp. (see above), in filamentous, nitrogen-fixing cyanobacteria glutamine synthetase is regulated and its levels are lower (about 50%) in ammonium-grown than in N_2-grown cells (22, 24, 27). In *Anabaena* sp. strain PCC 7120, the *glnA* gene has been shown to be transcribed from different promoters during growth on N_2 or ammonium (33). Promoter P_I, which is used in cells that fix nitrogen and which shows homology to the *Anabaena nifHDK* promoter (28, 33), is not recognized by either *E. coli* RNA polymerase or RNA polymerase isolated from ammonium-grown, vegetative cells of *Anabaena* sp. (28). Therefore, it is possible that a system analogous to that of the enterobacterial *ntr* genes, which involves a modified RNA polymerase assisted by an activator protein (20), is required for the expression of some nitrogen assimilation genes in cyanobacteria. *ntcA*, the gene that we identified which appears to be required for the expression of genes in response to nitrogen starvation in *Synechococcus* sp., can represent an element of such a regulatory system for nitrogen control in cyanobacteria.

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