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Isolation and Characterization of a Chlorate-Resistant Mutant (Clo-*R*) of the Symbiotic Cyanobacterium *Nostoc* ANTH: Heterocyst Formation and N_2-Fixation in the Presence of Nitrate, and Evidence for Separate Nitrate and Nitrite Transport Systems

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Abstract. *Nostoc* ANTH is a filamentous, heterocystous cyanobacterium capable of N_2-fixation in the absence of combined nitrogen. A chlorate-resistant mutant (Clo-*R*) of *Nostoc* ANTH was isolated that differentiates heterocysts and fixes N_2 in the presence of nitrate, but not in the presence of nitrite or ammonium. The mutant lacks nitrate uptake and thereby also lacks induction of nitrate reductase activity by nitrate. However, this mutant is able to transport and assimilate nitrite, indicating that there is a transport system for nitrite that is distinct from that for the nitrate. The lack of inhibitory effect of nitrate on N_2-fixation was owing to lack of nitrate uptake and not to lack of enzymes for its assimilation (nitrate reductase and glutamine synthetase) or the lack of an ammonium transport system for retention of ammonia. The mutant has potential for use as a biofertilizer supplementing chemical nitrate fertilizer in rice fields, without N_2-fixation being adversely affected.

N_2-fixing cyanobacteria are considered as potential biofertilizers, particularly for rice paddies [24]. However, owing to application of chemical nitrogen fertilizers, the N_2-fixing capacity of cyanobacteria is adversely affected in rice paddies. Nitrate is the most common form of combined inorganic nitrogen nutrient available for growth. Nitrate assimilation involves active transport of nitrate into the cell, followed by reduction of nitrate to ammonium by sequential action of ferredoxin-dependent nitrate reductase and nitrite reductase, and finally the incorporation of ammonium into amino acids by the GS-GOGAT pathway [6].

Heterocysts are the sites of N_2-fixation in filamentous cyanobacteria [6]. In *Anabaena* PCC 7120, both heterocyst formation and nitrogenase is repressed in the presence of ammonium and nitrate, but nitrate repression is effective only after reduction of nitrate to ammonium [17]. The presence of exogenous ammonium represses the proteins involved in nitrate/nitrite uptake and assimilation [3, 4, 7, 8, 16], glutamine synthetase [6, 7] and ammonium transport [18, 19], whereas in the absence of ammonium, the proteins are derepressed [3, 4, 7, 8, 16, 18, 19]. The actual repressor is thought to be a product of ammonium assimilation via GS and/or ammonium *per se* [6]. Nitrate is required as an inducer of nitrate uptake and assimilation [3, 4, 8, 16].

Nitrate uptake- and assimilation-defective mutants of cyanobacteria have been physiologically and genetically characterized [2, 8, 13]. The genes encoding nitrite reductase (*nirA*), a multicomponent transporter common for nitrate and nitrite (*nrtABCD*), and nitrate reductase (*narB*) have been identified [6, 11]. These genes are arranged in a cluster and are transcribed as a single operon (*nirA-nrtABCD-narB*) [8, 10]. However, recently a nitrite transport system quite distinct from the common nitrate/nitrite transporter has also been reported in *Synechococcus* sp. strain PCC 7942 [14].

In this study we describe the isolation of a spontaneous chlorate-resistant (Clo-*R*) mutant of symbiotic cyanobacterium *Nostoc* ANTH, using the nitrate analog chlorate as a selection agent. We report here that this mutant is capable of heterocyst formation and N_2-fixation in the presence of nitrate. The mutant lacks nitrate

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uptake and induction of nitrate reductase activity by nitrate. However, this mutant is able to transport and assimilate nitrite, indicating that there is a transport system for nitrite that is distinct from that for nitrate. Such mutants are likely to be more useful in biofertilizer technology, since they will have the advantage of N_2-fixation in the presence of nitrate fertilizers.

Materials and Methods

Strains and culture conditions. Axenic clonal cultures of the diazotrophic cyanobacterium *Nostoc* ANTH and its chlorate-resistant (Clo-*R*) mutant were grown in batch cultures with $BG11_0$ (N_2 medium) at 25°C with a photon fluence rate of 50 μmol m^{-2} s^{-1} [20]. N_2 media supplemented with 5 mM $NaNO_3$, 5 mM KNO_2 or 2 mM NH_4Cl are referred to as NO_3^- medium, NO_2^- medium, and NH_4^+ medium, respectively. The NO_3^-, NO_2^-, and NH_4 media were always buffered with equimolar concentration of HEPES (pH 7.5).

Isolation of the Clo-*R* mutant. As a first step towards isolation of Clo-*R* mutants, the survival of cyanobacterium *Nostoc* ANTH was checked at increasing concentrations of potassium chlorate. A concentration of 60 mM chlorate was found to be the lethal dose for *Nostoc* ANTH, both in liquid as well as solid media. Spontaneously occurring Clo-*R* mutants of cyanobacterium *Nostoc* ANTH were obtained by plating approximately 2.4×10^7 colony-forming units on solid N_2 medium containing 60 mM chlorate. After 3 weeks of incubation, the few surviving colonies that remained were picked up and transferred to plates containing 60 mM chlorate. The Clo-*R* mutants arose at a frequency of 1.3×10^{-7}. One such mutant was further characterized with regard to growth, heterocyst frequency, N_2-fixation, and uptake and assimilation of nitrate, nitrite, and ammonium.

Growth, heterocyst frequency, and protein measurement. Growth was measured as the increase in concentration of Chl *a* [12]. Heterocyst frequency was calculated as the percentage of total cells by light microscopic observations after 96 h of incubation in different nitrogen media. Protein concentration was determined by the method of Lowry et al. [9].

Nitrate and nitrite uptake assays. The N_2-, NO_3^--, and NH_4^+-grown cultures of the parent and mutant strains were harvested during the exponential growth phase, washed, and resuspended in N_2 medium adjusted to pH 8.6 with 1 mM HEPES-NaOH buffer. Uptake of nitrate and nitrite was measured by determining the rates of their depletion from the external medium. Uptake was started by addition of $NaNO_3$ (100 μM) or KNO_2 (100 μM) to the cell suspension. The choice of 100 μM external concentration was based on earlier studies in *Anabaena* sp. PCC 7120 and *Synechococcus* sp. strain PCC 7942 [8, 14]. Where needed, dicyclohexylcarbodimide (DCCD, 10 μM) was added to the cell suspension 15 min prior to the addition of nitrate or nitrite. Samples were withdrawn after 3 h of incubation, the cells were removed by rapid centrifugation, and the cell-free supernatants were analyzed for residual nitrate or nitrite. Nitrate concentration was measured by its absorbance at 210 nm in acid solution [5], and nitrite concentration was measured by the method of Snell and Snell [22].

Enzyme activities. The acetylene reduction assay was used to measure nitrogenase activity [23]. Glutamine synthetase (transferase), nitrate reductase, and nitrite reductase activities were measured in alkyltrimethylammoniumbromide (CTAB)-permeabilized cells. GS activity was as described by Sampio et al. [21]. Nitrate reductase activity and nitrite reductase activity were measured with dithionite-reduced methyl viologen as reductant [1, 15].

Ammonium transport assays. This was done by using the radioactive analog of ammonium, ^{14}C-methylammonium ($^{14}CH_3NH_3$) [19]. The NO_3^--grown cultures of the parent and mutant strains were harvested during the exponential growth phase, washed in N_2 medium, and then transferred to N_2, NO_3^-, and NH_4^+ media. After 48 h, cells were harvested, washed, and resuspended in 10 mM HEPES-NaOH buffer (pH 7.0) to a concentration of 5 μg Chl *a* ml^{-1}. After equilibrating for 10 min at 25°C and a photon fluence rate of 50 μmol m^{-2} s^{-1}, ^{14}C-methylammonium was added to a final concentration of 50 μM (sp. activity 172 kBq μmol^{-1}). At different time intervals, 400 μl samples were taken out rapidly, and the cells were separated from their bathing medium by centrifugation through silicon-oil DC 550/dinonyl phthalate (40/60 vol/vol) into perchloric acid/water (15/85 vol/vol) [19]. The ^{14}C in the perchloric acid fraction was measured in a liquid scintillation counter (model 1801, Beckman Instruments). Nonspecific binding of ^{14}C-methylammonium was determined by measuring its incorporation into toluene-treated cells [19]. This value was always subtracted from the value obtained for a toluene-untreated sample.

Results

The growth of the parent strain and that of its Clo-*R* mutant were relatively similar in NO_2^-, NH_4^+, and N_2 medium. Growth was accompanied by production of heterocysts and nitrogenase activity in N_2 medium, but not in NO_2^-- or NH_4^+-containing media, thus suggesting that the two strains had similar abilities to utilize N_2, NO_2^-, and NH_4^{+4} as sole sources of nitrogen (Table 1). However, the two strains differed in their response to growth in NO_3^- medium. While the growth of the parent strain increased on transfer from N_2 to NO_3^- medium, the growth of the mutant strain remained similar to that in N_2 medium. Thus, in NO_3^- medium, the Clo-*R* mutant grew at a slower rate than its parent strain. Furthermore, in contrast to the parent strain, the mutant developed heterocysts and nitrogenase activity in NO_3^- medium (Table 1). This slower growth of the mutant and its ability to form heterocysts and fix N_2 in NO_3^- medium may have resulted from an impairment of nitrate uptake and/or its assimilation as a nitrogen source.

In order to determine the cause of nitrogenase activity remaining derepressed in NO_3^- medium, nitrate uptake and reductase activities were measured in the parent and mutant strains (Table 2). The ammonium-repressible nitrate uptake activity of the parent strain, when grown with nitrate as a nitrogen source, was higher than its N_2-grown cultures. The ATPase inhibitor, DCCD, strongly inhibited nitrate uptake, suggesting that the nitrate uptake system is energy dependent. In contrast, the mutant showed negligible levels of nitrate uptake activity, suggesting that the mutant is defective in nitrate uptake. Ammonium also caused a strong repression of nitrate reductase activity in both the parent and its Clo-*R* mutant. The nitrate reductase activity of the parent strain was 60% higher in the presence of nitrate than in N_2-grown cells, indicating that the enzyme may be in-

Table 1. Growth (Gr), heterocyst frequency (HF), and nitrogenase activity (N_2ase) of *Nostoc* ANTH and its Clo-*R* mutant as a function of nitrogen sources

	Parent			Clo-*R*		
Growth medium	Gr	HF	N_2ase	Gr	HF	N_2ase
$BG11_0$	2.0 ± 0.1	5.8 ± 0.3	11 ± 0.8	1.9 ± 0.3	5.6 ± 0.2	10 ± 0.7
$BG11_0$ + 5mM NO_3^-	2.2 ± 0.1	0.0	0.0	1.9 ± 0.1	5.2 ± 0.5	11 ± 1.3
$BG11_0$ + 5mM NO_2^-	2.3 ± 0.1	0.0	0.0	2.4 ± 0.2	0.0	0.0
$BG11_0$ + 2mM NH_4^+	2.6 ± 0.2	0.0	0.0	2.5 ± 0.1	0.0	0.0

Six-day-old N_2-grown cultures were used as the initial source of inoculum. The growth (μg Chl *a* ml^{-1}), frequency of heterocysts (per 100 vegetative cell), and nitrogenase activity (nmol C_2H_4 formed μg^{-1} Chl *a* h^{-1}) were determined after 96 h of incubation in media containing different nitrogen sources as indicated. Chl *a* concentration at the start of incubation (0 time) was 0.27 μg Chl *a* ml^{-1}. The values presented are means ± standard error (SE) from two independent experiments, each with two replicates.

Table 2. Nitrate uptake and nitrate reductase activities of *Nostoc* ANTH (parent) and its Clo-*R* mutant in different nitrogen media

	Nitrate uptake				Nitrate reductase	
	Parent		Clo-*R*			
Growth medium	−DCCD	+DCCD	−DCCD	+DCCD	Parent	Clo-*R*
$BG11_0$	2.8 ± 0.2	0.0	0.2 ± 0.1	0.0	1.9 ± 0.1	1.8 ± 0.1
$BG11_0$ + 5mM NO_3^-	3.6 ± 0.1	0.0	0.3 ± 0.1	0.0	4.7 ± 0.6	1.9 ± 0.1
$BG11_0$ + 2mM NH_4^+	0.4 ± 0.1	0.0	0.0	0.0	0.8 ± 0.1	0.4 ± 0.2

For nitrate uptake experiments, exponentially growing cultures of both parent and mutant strains were washed and resuspended in N_2 medium (5–7 μg Chl *a* ml^{-1}). Nitrate was added to a final concentration of 100 μM, and its disappearance from the medium was measured in the presence and absence of DCCD (10 μM). See Materials and Methods for more details. The uptake rates are expressed as μmol nitrate taken up min^{-1} mg^{-1} Chl *a*.
The nitrate reductase (nmol NO_2^- formed min^{-1} mg^{-1} protein) activity was determined after 48 h of incubation in different media, as indicated. The values presented are means ± SE from two independent experiments, each with two replicates.

duced/stabilized by nitrate. N_2-grown cultures of the Clo-*R* mutant showed a nitrate reductase activity similar to that in the N_2-grown parent strain. However, there was no change in activity of nitrate reductase when the mutant cells were transferred to the nitrate medium, implying that the mutant lacked the induction of nitrate reductase by nitrate because nitrate could not enter the cells.

Since the proteins involved in transport of nitrate have been suggested to transport nitrite also [11], it was of interest to characterize further the relationship between nitrate and nitrite transport in this nitrate uptake-defective mutant. Both the parent and its Clo-*R* mutant, possessed nitrite uptake activities, and these activities were ammonium repressible and sensitive to DCCD (Table 3). However, the nitrite uptake activity of the mutant was 40% less than that in the parent. Furthermore, since the assays were done under alkaline conditions (pH 8.6), the uptake could not be attributed to passive diffusion of nitrous acid [14, 16]. Since the nitrate uptake-defective mutant showed significant levels of nitrite uptake activity, it is suggested that in the cyanobacterium *Nostoc* ANTH there is an uptake system for nitrite quite distinct from that for nitrate. The ammonium-repressible nitrite reductase activity was similar in the parent and its mutant strain, suggesting that nitrite assimilation is intact in the mutant.

The status of ammonium uptake was studied in *Nostoc* ANTH and its mutant as a function of inorganic nitrogen sources, by using ^{14}C-methylammonium (an analog of ammonium) [18, 19]. As shown in Fig. 1A and B, N_2-grown parent and mutant strains showed a biphasic pattern of methylammonium uptake. Such a methylammonium uptake pattern was marked by an initial rapid phase lasting for 60 s, followed by a slower second phase, which remained linear during the next 8 min of the experimental period. The rates of methylammonium uptake were similar in the parent and mutant strains. The methylammonium uptake activities during the first and second phase were 55 and 7.3 nmol mg^{-1} Chl *a* min^{-1}, respectively. However, the NH_4^+- and NO_3^--grown cells of the parent strain and the NH_4^+-grown cells of the mutant strain showed negligible methylammonium uptake activity. These results are consistent with the ammonium-repressible nature of the methylammonium up-

Table 3. Nitrite uptake and nitrite reductase activities of *Nostoc* ANTH (parent) and its Clo-*R* mutant in different nitrogen media

Growth medium	Nitrite uptake: Parent −DCCD	Nitrite uptake: Parent +DCCD	Nitrite uptake: Clo-*R* −DCCD	Nitrite uptake: Clo-*R* +DCCD	Nitrite reductase: Parent	Nitrite reductase: Clo-*R*
$BG11_0$	24 ± 1.1	0.5 ± 0.2	14 ± 0.8	0.4 ± 0.1	540 ± 22	516 ± 10
$BG11_0$ + 5mM NO_3^-	29 ± 0.9	1.3 ± 0.2	17 ± 1.0	0.7 ± 0.2	Nd[a]	Nd
$BG11_0$ + 2mM NH_4^+	0.2 ± 0.1	0.0	0.0	0.0	230 ± 13	211 ± 6

For nitrite uptake experiments, exponentially growing cultures of both parent and mutant strains were washed and resuspended in N_2 medium (5–7 μg Chl *a* ml^{-1}). Nitrite was added to a final concentration of 100 μM, and its disappearance from the medium was measured in the presence and absence of DCCD (10 μM). See Materials and Methods for more details. The uptake rates are expressed as μmol nitrite taken up min^{-1} mg^{-1} Chl *a*.
The nitrite reductase (nmol NO_2^- consumed min^{-1} mg^{-1} protein) activity was determined after 48 h of incubation in different media, as indicated. The values presented are means ± SE from two independent experiments, each with two replicates.
[a] Nd = not determined.

take in *Nostoc* ANTH [18]. In contrast to the parent strain, the Clo-*R* mutant had a fully derepressed methylammonium uptake activity under NO_3^--grown condition. The pattern and rate of methylammonium uptake in the NO_3^--grown mutant were quite similar to that in N_2-grown cells. This is as expected, since the Clo-*R* mutant was defective in nitrate uptake and assimilation; hence, nitrate was unable to affect the methylammonium uptake activity. Also, these results suggest that the ammonium transport system is intact in the mutant, and its activity is unaffected by nitrate.

The activity of the primary ammonium-assimilating enzyme GS was determined in N_2-, NO_3^--, and NH_4^+-grown cells of the parent and mutant strains (Table 4). The N_2- and NO_3^--grown cells of both the parent and mutant strains showed comparable levels of GS activity. Thus, the acquisition of resistance to chlorate had no effect on GS activity. However, in NH_4^+-grown cells of both the parent and mutant strains, GS activity was repressed by 50%. These results on *Nostoc* ANTH are consistent with the earlier findings of the ammonium-repressible nature of glutamine synthetase in other cyanobacteria [6, 7].

Discussion

The results presented in this study, indicating the ammonium-repressible nature of nitrate uptake and reductase, with nitrate uptake being energy dependent in *Nostoc* ANTH, are consistent with earlier reports in cyanobacteria [2, 3, 4, 8, 16].

On transfer to NO_3^- medium, *Nostoc* ANTH ceased to fix N_2 and switched over to use NO_3^- as a nitrogen source. However, its chlorate-resistant mutant continued to use N_2 as a nitrogen source even when transferred to NO_3^- medium. This was why the growth rate of the parent strain increased on transfer from N_2 to NO_3^- medium but not of the mutant strain. That the Clo-*R* mutant was nitrate uptake defective is suggested by the following facts: 1) lack of nitrate uptake, 2) lack of induction of nitrate reductase by nitrate, 3) lack of increase in growth when transferred to NO_3^- medium even though nitrate reductase, nitrite reductase, and glutamine synthetase activities were intact, 4) lack of repression of heterocyst and N_2-fixation by nitrate, and 5) lack of repression of ammonium transport by nitrate. The loss of nitrate uptake ability in the mutant without affecting its nitrite assimilation ability argues against the possibility of a common gene that regulates nitrate/nitrate uptake and assimilation being affected (compare Tables 1 and 3) [8]. These characteristics of the mutant were similar to the *Synechococcus* sp. strain PCC 7942 (FM16) mutant [13] in the sense that both lacked nitrate uptake activity but retained significant levels of nitrate reductase activity. However, in contrast to *Synechococcus* sp. strain PCC 7942 (FM16) in which ammonium did not repress nitrate reductase [13], the nitrate reductase activity of the Clo-*R* mutant was ammonium repressible.

The nitrate uptake-defective mutants (*nrtABCD* and *nrtA* or *nrtD* deleted) of *Synechococcus* sp. strain PCC 7942 have been employed in the past to understand the functional relationship between the nitrate and nitrite transport systems [11, 14]. The nitrate uptake-defective Clo-*R* mutant showed significant levels of nitrite uptake activity, suggesting that in the cyanobacterium *Nostoc* ANTH there is an uptake system for nitrite quite distinct from that for nitrate (Table 3). These results are in contrast to the observations of Luque et al. [11] and in agreement with Maeda and Omata [14], suggesting a nitrite transport system quite distinct from the transport system common for nitrate and nitrite in *Synechococcus* sp. strain PCC 7942. It is suggested that the *ntcA*-medi-

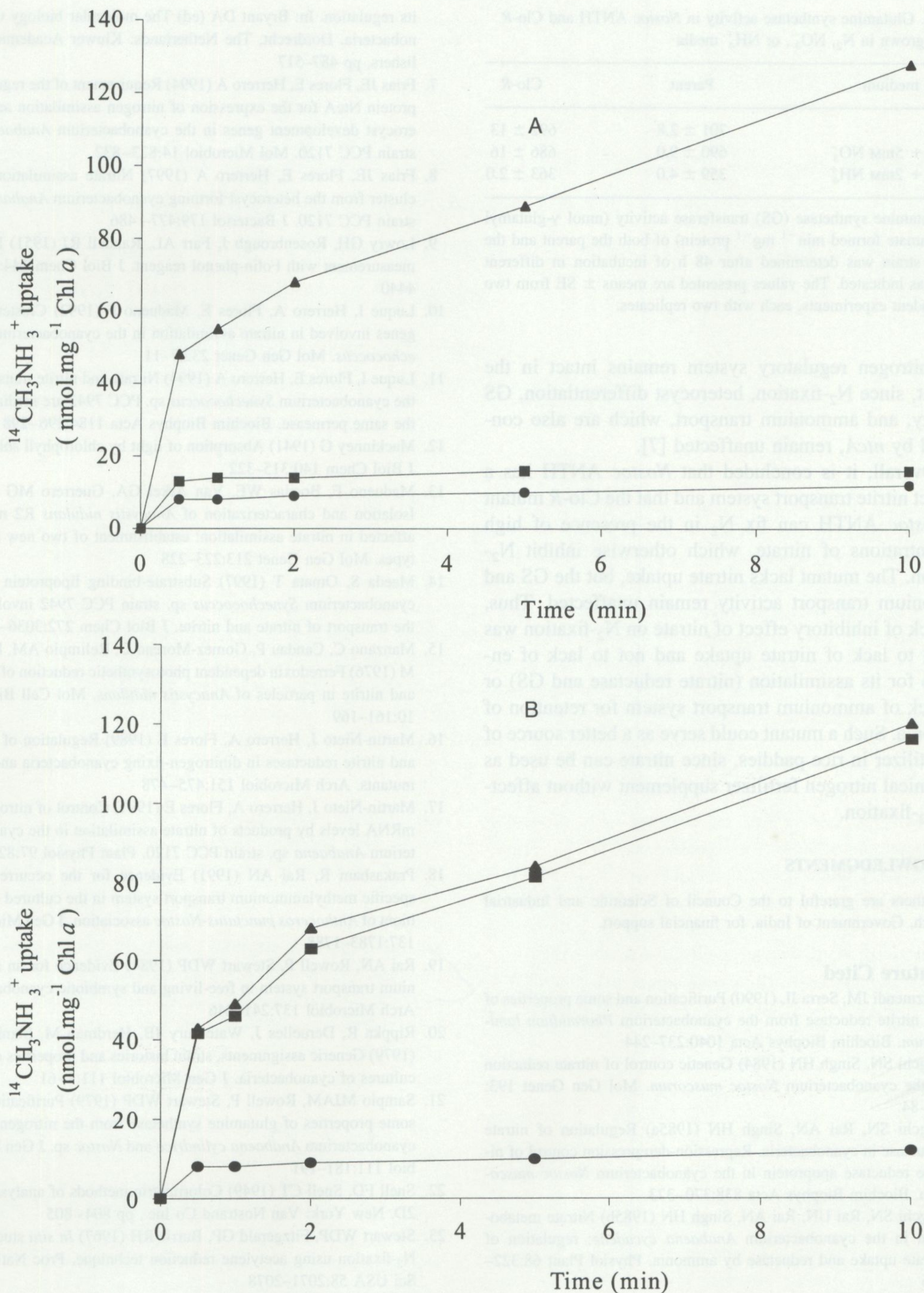

Fig. 1. ^{14}C-methylammonium uptake in *Nostoc* ANTH (A) and its Clo-*R* mutant (B) under N_2- (▲), nitrate- (■), and ammonium-grown conditions (●). Nitrate-grown exponential cultures were washed and transferred to different nitrogen media and incubated for 48 h. Thereafter, the cells were washed, resuspended in HEPES buffer, and used for ^{14}C-methylammonium uptake as described in Materials and Methods. The values presented are means from two independent experiments, each with two replicates.

Table 4. Glutamine synthetase activity in *Nostoc* ANTH and Clo-*R* mutant grown in N_2, NO_3^-, or NH_4^+ media

Growth medium	Parent	Clo-*R*
$BG11_0$	701 ± 2.8	692 ± 13
$BG11_0$ + 5mM NO_3^-	690 ± 9.0	686 ± 16
$BG11_0$ + 2mM NH_4^+	359 ± 4.0	363 ± 2.0

The glutamine synthetase (GS) transferase activity (nmol γ-glutamyl hydroxamate formed min^{-1} mg^{-1} protein) of both the parent and the mutant strain was determined after 48 h of incubation in different media, as indicated. The values presented are means ± SE from two independent experiments, each with two replicates.

ated nitrogen regulatory system remains intact in the mutant, since N_2-fixation, heterocyst differentiation, GS activity, and ammonium transport, which are also controlled by *ntcA*, remain unaffected [7].

Overall, it is concluded that *Nostoc* ANTH has a distinct nitrite transport system and that the Clo-*R* mutant of *Nostoc* ANTH can fix N_2 in the presence of high concentrations of nitrate, which otherwise inhibit N_2-fixation. The mutant lacks nitrate uptake, but the GS and ammonium transport activity remain unaffected. Thus, the lack of inhibitory effect of nitrate on N_2-fixation was owing to lack of nitrate uptake and not to lack of enzymes for its assimilation (nitrate reductase and GS) or the lack of ammonium transport system for retention of ammonia. Such a mutant could serve as a better source of biofertilizer in rice paddies, since nitrate can be used as a chemical nitrogen fertilizer supplement without affecting N_2-fixation.

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