Mol Gen Genet (1984) 195:180–185

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Isolation and characterisation of nitrate reductase mutants and regulation of nitrate reductase and nitrogenase in the cyanobacterium *Nostoc muscorum*

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Summary. Chlorate resistant mutants of the cyanobacterium *Nostoc muscorum* isolated after N-methyl-N′-nitro-N-nitrosoguanidine (MNNG) mutagenesis were found to be defective/blocked in nitrate reductase (NR).

The parent strain possessed active NR in the presence of nitrogen as nitrate and only basal levels of activity in ammonia and N-free grown cultures. Addition of ammonia suppressed the NR activity in the parent strain whereas addition of L-methionine DL-sulphoximine (MSX) restored NR activity. A similar repression by ammonia, glutamine and derepression with MSX were also observed for nitrogenase synthesis.

One class of mutants lacked NR activity (*nar*$^-$) whereas the specific activity of NR was low in another class of mutants (*nar*^{def}). Unlike the parent, the mutants synthesized nitrogenase and differentiated heterocysts in the presence of nitrate nitrogen. Uptake studies of nitrite and ammonia in mutants revealed that they possessed both nitrite reductase and glutamine synthetases (GS) at low levels, and the same level respectively in comparison with the parent.

Introduction

The cyanobacteria reduce N_2 and nitrate to ammonia via two distinct metabolic pathways, i.e. nitrogen fixation and nitrate reduction, catalysed by nitrogenase and the nitrate and nitrite reductase enzymes. Ammonia, the end product, behaves as an effective antagonist in both processes and its inhibitory action on nitrate reductase synthesis and activity has been reported (Stevens and Van Baalen 1974; Herrero et al. 1981). Ammonia was also claimed to inhibit nitrate uptake in *Anabaena cylindrica* (Ohmori et al. 1977). A common genetic determinant was suggested for NR and nitrogenase in *Anabaena doliolum* (Singh and Dikshit 1976) and *N. muscorum* (Singh and Sonie 1977) following the isolation of mutants defective/blocked in nitrogen assimilatory pathways. Unlike many cyanobacteria, it has been reported in an isolate of *Anabaena* sp. that both heterocyst differentiation and nitrogenase synthesis, although repressed by nitrogen in the form of nitrate, differentiated heterocysts when grown in medium supplemented with NH_4Cl. Thus a distinct trigger for heterocyst development from primary ammonia assimilatory mechanism was suggested in this organism (Bottomley et al. 1979).

In the present investigation we have attempted to isolate chlorate-resistant mutants of the N_2-fixing, filamentous cyanobacterium *Nostoc muscorum* showing partial or complete loss of the nitrate reductase enzyme, to determine the role of nitrate and ammonia in the regulation of nitrate reductase and nitrogenase. L-methionine DL-sulphoximine, an inhibitor of glutamine synthetase was used to clarify the action of ammonia in the repression of enzyme synthesis.

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Materials and methods

Cyanobacteria and culture conditions. The cyanobacterium *Nostoc muscorum* (Iowa State University Strain) was grown in modified Chu-10 nitrogen-free medium (Safferman and Morris 1964) with trace elements (Allen and Arnon 1955). The cultures were incubated photoautotrophically under cool fluorescent illumination (2.5 klx) for 10 h a day at $25 \pm 1°$ C in a culture room. For combined nitrogen supplementation, nitrate as KNO_3 (5, 10 mM), nitrite as $NaNO_2$ (5 mM) ammonia as NH_4Cl (0.5, 1 mM) and glutamine (0.5, 1 mM) were used.

Growth measurements. Growth was recorded by measuring the transmittance of cultures at 650 nm with the help of a Spectronic-20 and also in terms of protein values estimated by Lowry's procedure as modified by Herbert et al. (1971).

Nitrogen fixation. Nitrogen fixation by parent and mutants was determined by micro-kjeldahl and acetylene reduction assay techniques. The experiments were conducted in 100 ml flasks containing 50.0 ml medium with freshly inoculated cultures of equal protein content. For estimation of total nitrogen, growth was stopped at different periods by adding 3.0 ml concentrated H_2SO_4. The samples were digested and distilled with micro-kjeldahl apparatus. A nitrogenase assay was performed with whole cells by the acetylene reduction technique (Schollhorn and Burris 1967). For this purpose 5.0 ml aliquots of culture were placed in 15 ml capacity tubes fitted with air tight rubber stoppers into which 0.5 ml acetylene was injected after removing an equal volume of air. The reaction mixture was incubated in the light in a culture room. Samples of 0.5 ml gas mixture

were withdrawn from the vial, injected into a Gas Chromatograph and the nitrogenase activity was determined as nmol ethylene/mg protein per min. To observe the effect of MSX on ammonia and glutamine repression of nitrogenase, 25 µM MSX was added to the respective nitrogen sources and a nitrogenase assay was performed after 48 h.

Uptake of nitrogen sources. The experiments on uptake of nitrate, nitrite and ammonia were conducted in 100 ml Corning glass flasks containing 25.0 ml media supplemented with 100 µM KNO_3 or $NaNO_2$ or NH_4Cl. Nitrate was determined by the brucine method (Nicholas 1953) and nitrite was estimated using the azocoupling method (Snell and Snell 1949) while ammonia was assayed by Nessler's reagent (Burris and Wilson 1957) after the removal of cells.

Determination of nitrate reductase activity. The cellular NR activity was determined in an in situ assay where 40.0 µl toluene was added to a 1.0 ml cell suspension, shaken for 3 min vigorously to break the cells and then 0.5 ml samples were added to 1.0 ml reaction mixture, consisting of $NaHCO_3-Na_2CO_3$ buffer, pH 10.5 (500.0 µM), KNO_3 (100.0 µM), methyl vilogen (20.0 µM) and $Na_2S_2O_4$ (50.0 µM) in 0.1 ml 0.3 M $NaHCO_3$ (after modification of concentrations of constituents used by Manzano et al. 1976). The reaction mixture containing cell extract was incubated at 30° C for 10 min, nitrite formed during the period was determined and the activity (U) was expressed as µmol nitrite produced/mg protein per min (Herrero et al. 1981).

To study the effect of chloramphenicol on NR, cells grown in ammonia medium were transferred to a medium containing 10.0 mM KNO_3 and incubated for 20 h under growth conditions, with 25.0 µg/ml chloramphenicol. To observe the effect of L-methonine DL-sulphoximine (MSX) on ammonia repression of NR, cells grown in ammonia were transferred to a medium containing 1.0 mM NH_4Cl+ 10 mM KNO_3, incubated for 4 h and then divided into two halves, to one of which MSX was added at a final concentration of 10.0 µM.

Mutagenesis and isolation of NR-less mutants. To exponentially growing cultures of *N. muscorum* containing 1×10^6 cells/ml, N-methyl-N′-nitro-N-nitrosoguanidine (MNNG) was added to a final concentration of 100.0 µg/ml followed by incubation in a culture room for 2 h. After the removal of mutagen the treated population was grown in C+NO_3 culture medium for 20 days under growth conditions. The mutants were selected on the basic of chlorate resistance (25 mM) on nitrate-supplemented agar plates. Mutation frequency was estimated by counting non-heterocystous and heterocystous colonies of mutagenised and control populations on C+NO_3 agar plates. The developing colonies devoid of heterocysts on C+NO_3 agar medium were counted to determine the reversion frequency.

Results

Isolation of mutants

Mutants with complete or partial loss of nitrate reductase were isolated among the chlorate-resistant population. Twenty-nine cultured isolates from single colonies differentiated heterocysts in C+NO_3 medium, out of which 16 isolates liberated less nitrite into the medium than did the parent strain. One representative of each class, i.e. *nar*$^-$ (NR completely blocked) and *nar*^{def} (NR partially blocked) were selected for a detailed study on the basis of maximum growth and heterocyst differentiation in nitrate-supplemented medium. These were characterised with respect to growth, heterocyst differentiation, release of nitrite into the medium, total nitrogen fixation, nitrogenase assay, development of NR activity and regulation of NR and nitrogenase.

Growth characteristics

Growth curves in C–N medium of the parent and mutants have a similar pattern except in the stationary phase (Fig. 1 A). After 16 days the *nar*$^-$ mutant turned yellowish and showed slow growth (measured in terms of optical density) whereas the protein contents of parent and *nar*$^-$ mutant were the same during this period (Fig. 1 B). A nitrate concentration of 10 mM was found to suppress the growth of parent and mutants in comparision to N-free medium, although the inhibition was comparatively less in the parent than in the mutants (Fig. 1 A, B). Though both mutants followed the same pattern of growth due to nitrate inhibition the *nar*$^-$ mutant grew slowly and its cultures turned to yellowish from blue-green in the medium.

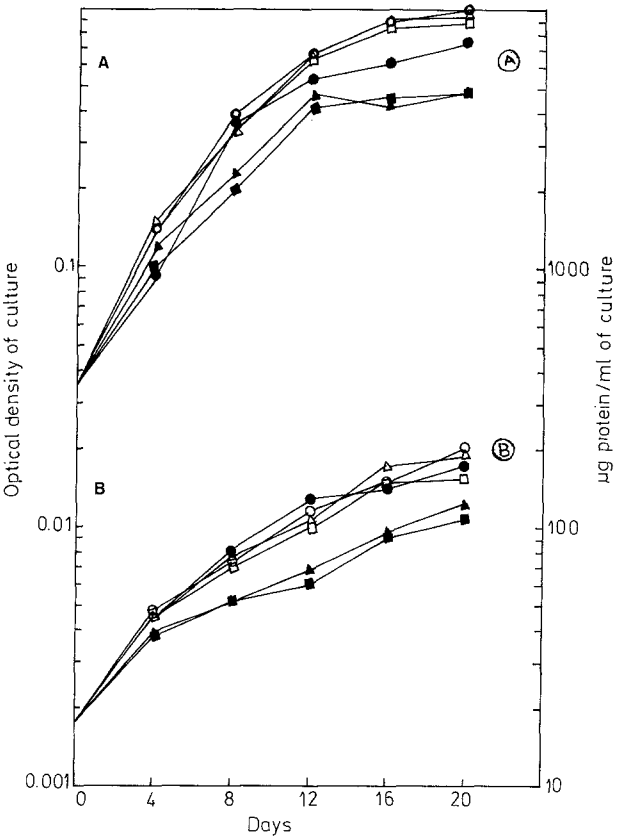

Fig. 1. A Growth measured in terms of the optical density of cultures of mutants and parent *N. muscorum*. N-free medium: ○ parent, □ *nar*$^-$ and △ *nar*^{def}. Medium containing 10 mM KNO_3: ● parent, ■ *nar*$^-$ and ▲ *nar*^{def}. **B** Growth measured in terms of the protein content of mutants and parent *N. muscorum*. N-free medium: ○ parent □ *nar*$^-$ and △ *nar*^{def}. Medium containing 10 mM KNO_3: ● parent, ■ *nar*$^-$ and ▲ *nar*^{def}

Heterocyst differentiation, nitrogen fixation and nitrogenase activity

The parent strain formed heterocysts with a frequency of 5.0%–6.5% and fixed molecular nitrogen in N-free medium but both functions were repressed in the presence of 10.0 mM nitrate. The *nar*$^-$ and *nar*^{def} mutants also differentiated heterocysts (5.0%–7.0%) and fixed N_2 in N-free medium. The amount of N_2 fixed by parent and *nar*$^-$ mutant was similar in N-free medium whereas the *nar*^{def} mutant fixed less nitrogen 12 days after inoculation (Fig. 2). The *nar*^{def} mutants differentiated heterocysts in nitrate-containing medium, as observed in N-free medium, and reduced nitrate to nitrite (Fig. 3B).

The acetylene reducing activities of parent and mutants support the results of total nitrogen estimation. Maximum nitrogenase activity was recorded in the *nar*$^-$ mutant in N-free medium followed by the parent and *nar*^{def} mutant. Nitrate repressed nitrogenase activity in the parent whereas the *nar*$^-$ and *nar*^{def} mutants exhibited activity though suppression was observed with an increasing concentration of nitrate ions. Ammonia suppressed nitrogenase activity in both parent and mutants. Addition of MSX to the nitrate-grown parent and to the ammonia or glutamine-grown parent, *nar*$^-$ and *nar*^{def} mutants resulted in derepression of nitrogenase activity to some extent (Table 1). The nitrogenase activity in N-free medium was also found to be affected adversely in parent and mutants by the 25 µM MSX used in experiment.

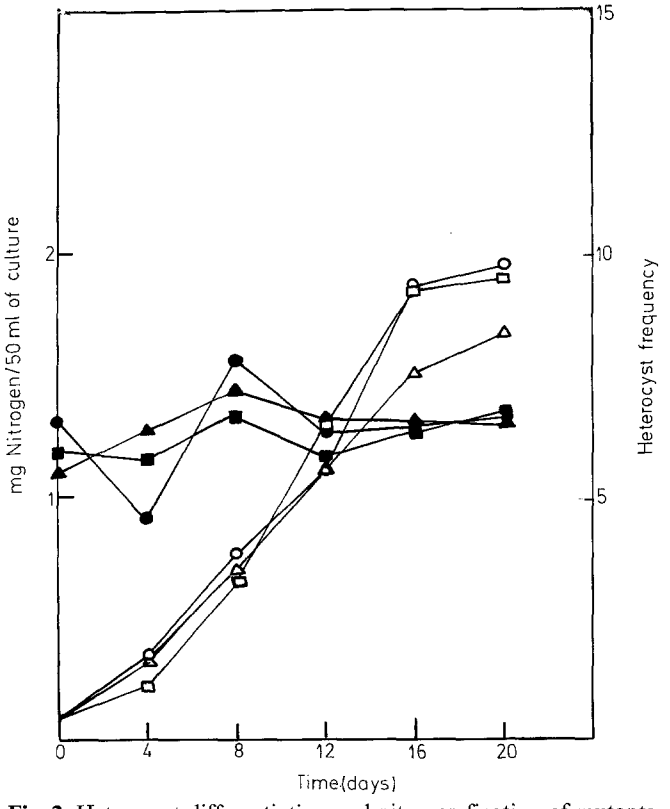

Fig. 2. Heterocyst differentiation and nitrogen fixation of mutants and parent *N. muscorum* in N-free medium. Heterocyst differentiation: ● parent, ■ *nar*$^-$ and ▲ *nar*^{def}. Nitrogen fixation: ○ parent, □ *nar*$^-$ and △ *nar*^{def}

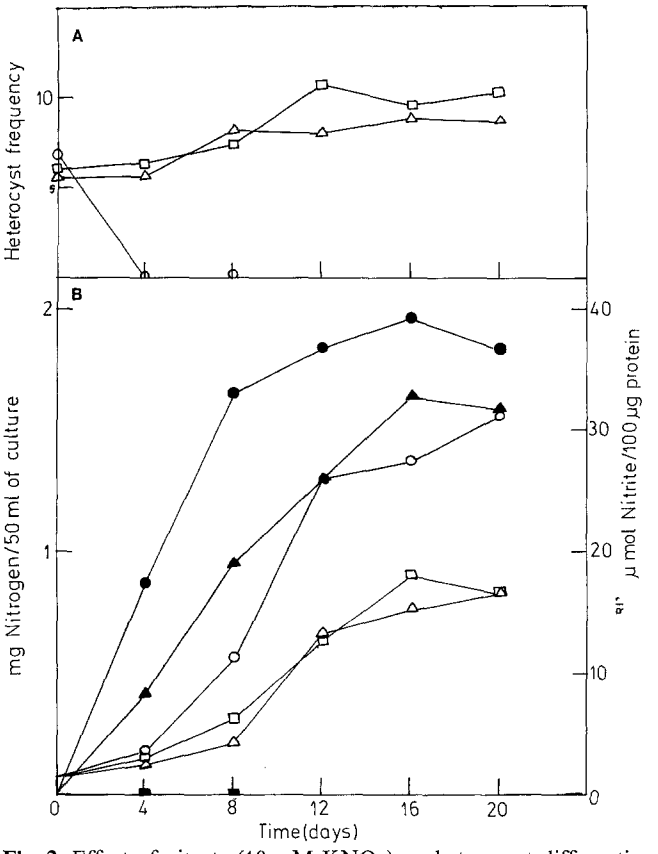

Fig. 3. Effect of nitrate (10 mM KNO_3) on heterocyst differentiation and nitrite liberation in the medium of mutants and parent *N. muscorum*. (A) Heterocyst differentiation: ○ parent, □ *nar*$^-$, △ *nar*^{def}. (B) nitrite liberation: ● parent, ■ *nar*$^-$, ▲ *nar*^{def}; N-content: ○ parent, □ *nar*$^-$ and △ *nar*^{def}

Table 1. Acetylene reducing activity (nitrogenase assay) of parent and chlorate-resistant mutants of *Nostoc muscorum* in the presence or absence of combined nitrogen sources and MSX (25 µM) in the medium

Nitrogen sources	Nitrogenase activity[a]		
	Parent	*nar*^{def}	*nar*$^-$
N-free	19.5	17.6	21.3
N-free + MSX	2.4	1.6	3.5
Nitrate			
5 mM	0.0	5.2	5.5
10 mM	0.0	2.1	3.0
20 mM	0.0	1.7	1.9
5 mM + MSX	1.4	0.7	1.7
10 mM + MSX	0.8	0.7	0.9
20 mM + MSX	0.1	0.5	0.3
Ammonia			
0.5 mM	0.0	0.0	0.0
1.0 mM	0.0	0.0	0.0
0.5 mM + MSX	0.4	0.6	0.8
1.0 mM + MSX	0.3	0.2	0.6
Glutamine			
0.5 mM	1.2	0.1	0.0
1.0 mM	0.0	0.0	0.0
0.5 mM + MSX	12.9	1.4	5.1
1.0 mM + MSX	7.1	0.0	0.2

[a] nmol C_2H_4 formed/min/per mg protein

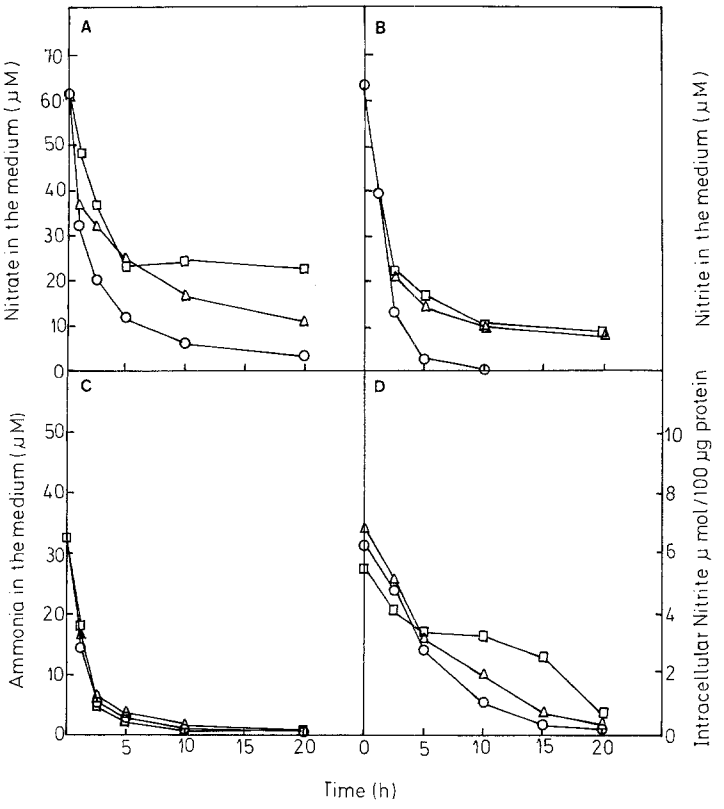

Fig. 4A–D. Nitrate (**A**), nitrite (**B**), ammonia uptake (**C**) and intracellular nitrite (**D**) of □ *nar*$^-$, △ *nar*^{def} and ○ parent *N. muscorum*

Uptake of nitrogen sources

The time course of nitrate, nitrite and ammonia uptake by parent, *nar*$^-$ and *nar*^{def} mutants was studied (Figs. 4A, B and C). The nitrate uptake was faster in the early hours and no uptake in the *nar*$^-$ mutant was observed 5 h after inoculation. Nitrate uptake was rapid in the parent and *nar*^{def} mutant and a small amount of nitrate remained in the medium 20 h after inoculation, although the rate of uptake in the parent strain was comparatively greater. The nitrite uptake by parent and mutants was faster than the nitrate uptake as two-thirds of the nitrite was utilized 2.5 h after inoculation. The *nar*$^-$ and *nar*^{def} mutants showed a similar pattern of nitrite uptake although slower than in the parent strain. Ammonium was taken up at a similar rate by parent and both mutants and in comparision to other nitrogen sources the rate of its uptake was higher, ammonium being exhausted from the medium within 10 h incubation.

The nitrite-grown cultures of parent and mutants were transferred to N-free medium for the measurement of intracellular nitrite. The reduction of nitrite to ammonia as shown by intracellular nitrite levels was almost similar in the parent and *nar*^{def} mutant whereas it was less in the *nar*$^-$ mutant and within 15–20 h incubation cells became nitrite starved in all three isolates (Fig. 4D).

Nitrate reductase activity

The cellular activity levels of NR in *N. muscorum*, *nar*^{def} and *nar*$^-$ mutants grown in nitrate-supplemented media were 88.0, 26.0 and 0.0 mU/mg protein whereas cultures grown in ammonia or N-free media exhibited only basal activity levels of 5.1, 4.0 and 0.0 mU/mg protein, respectively (Fig. 5A). An increase in NR activity in the parent and *nar*^{def} mutant was found in nitrate supplemented medium whereas the *nar*$^-$ mutant did not exhibit any activity. The development of NR specific activity of the parent and *nar*^{def} mutant was found only in the presence of nitrate and no induction at cellular level of nitrate reductase was observed in nitrogen-free and ammonia-containing medium from zero to 20 h incubation. Thus the results indicate that nitrate is a necessary requirement for induction of NR in the parent and its mutant partially blocked in nitrate reductase.

Effect of chloramphenicol and MSX on NR activity

The inhibitory action of chloramphenicol (25 μg/ml) on NR was observed in the parent and *nar*^{def} mutant where NR activity values were found to be similar to those occurring in ammonia, indicating NR repression by this antibiotic.

The NR specific activity levels decreased at 20 h incubation from 88.0 to 18.0 in the parent and 25.0 to 8.0 mU/mg protein in the *nar*^{def} mutants when these were transferred from nitrate to ammonia-containing medium (Fig. 5B). When ammonium-grown cultures of *nar*^{def} and parent strain were transferred to ammonia + nitrate-supplemented medium no change was exhibited in NR basal level but the addition of 10 μM MSX resulted in the derepression of NR activity after a 5 h lag period in which the values of NR specific activity were 22.5 and 11.5 mU/mg protein in the parent and *nar*^{def} mutant, respectively (Fig. 6).

Mutation and reversion frequencies

The mutation frequency of *nar*$^-$ mutants was 1.3×10^{-3} whereas it was 1.6×10^{-3} for *nar*^{def} mutants and of the two classes, of mutants *nar*$^-$ mutants were more stable. Both the mutants reverted to parental type with reversion

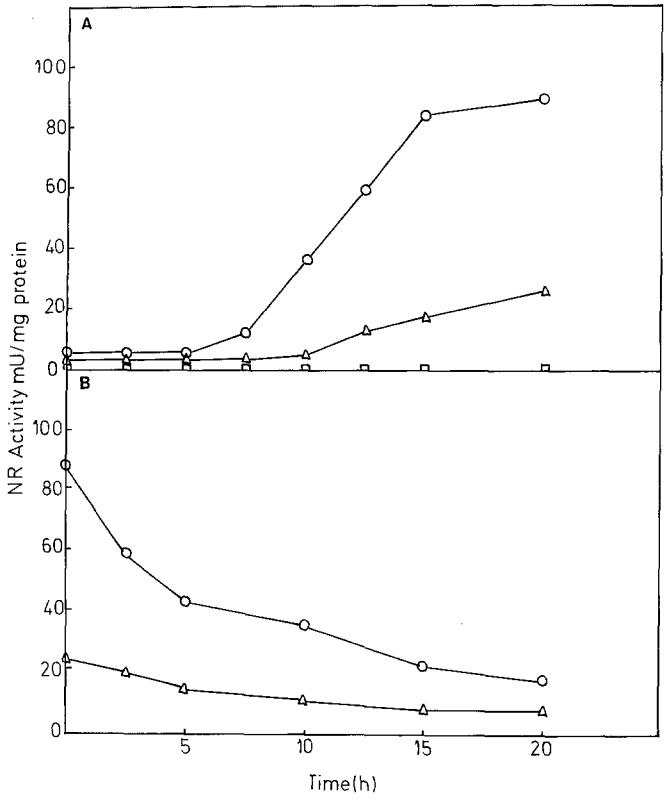

Fig. 5A, B. Induction of nitrate reductase (NR) activity by nitrate (**A**) and NR repression by ammonia (**B**) in □ *nar*$^-$, △ *nar*^{def} and ○ parent *N. muscorum*

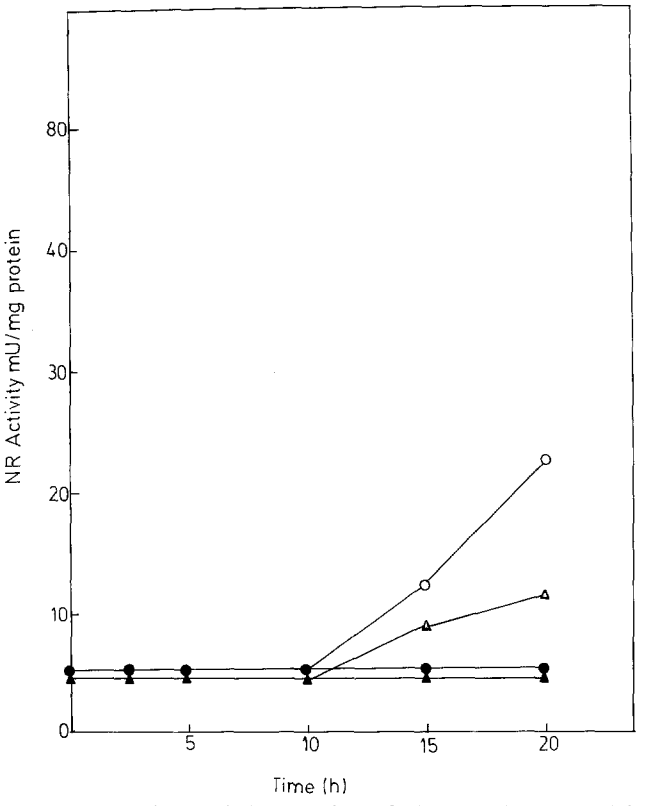

Fig. 6. Repression and derepression of nitrate reductase activity of a mutant and parent *N. muscorum*. Medium containing nitrate + ammonia: ● parent and ▲ *nar*^{def}. Medium + MSX: ○ parent and △ *nar*^{def}

frequencies of 3.7×10^{-7} and 2.8×10^{-6} for *nar*$^-$ and *nar*^{def} mutants, respectively.

Discussion

Chlorate (a structural analogue of nitrate) resistant mutants lacking in active nitrate reductase are reported in cyanobacteria (Singh et al. 1972; Singh and Sonie 1977). In this study the *nar*^{def} mutant formed one-third active NR whereas the *nar*$^-$ mutant lacked the active NR needed for nitrate reduction and therefore repressor of nitrogenase synthesis and heterocyst differentiation is not synthesised in the presence of nitrate in the medium. NR in the parent and *nar*^{def} mutant was also found to be inducible since its activity increased several fold in the presence of substrate (NO_3^-) whereas only a basal level of activity was noticed in N-free and ammonia-containing media, as reported in *Anabaena* (Herrero et al. 1981).

The rapid rate of nitrate uptake and further block after reaching a certain level confirmed that the *nar*$^-$ mutant possesses an active permease system and is defective in NR gene(s). The slower uptake rate of nitrite from the medium in comparison to the parent suggests that resistant mutants are also affected in nitrite reductase. The similar uptake rate of ammonia by mutants in comparison to the parent showed that ammonia metabolism in the mutants was not affected.

Ammonia has no inhibitory effect on NR of *Anabaena cylindrica* (Ohmori et al. 1977). Herrero et al. (1981) reported that NR is an inducible enzyme by its substrate in *Anabaena* sp. strain 7119 and *Nostoc* strain 6719. The study conducted here with MSX on the parent and a *nar*^{def} mutant suggests that ammonia behaves as a nutritional repressor of NR but repression occurs as a result of its metabolism. Thus, ammonia should be metabolised through the GS for its repression of the NR system as reported by Herrero et al. (1981) in *Anacystis nidulans* and *Anabaena* sp. Nitrate inhibition of nitrogenase activity in mutants appears to be a result of direct interaction of nitrate ions with nitrogenase. Singh and Sonie (1977) also observed growth inhibition by nitrate ions. The inhibition of nitrogenase activity due to the addition NH_4^+ to the N_2-fixing cultures of parent and mutants was prevented to some extent by addition of MSX. The direct or indirect involvement of GS in nitrogenase was suggested by the fact that the inhibition of nitrogenase activity occurring due to addition of NH_4^+ could be prevented by adding MSX to the cultures (Stewart 1980). Glutamine was also found to supress the nitrogenase activity, which could be derepressed in the presence of MSX and thus there appears to be a role for GS in glutamine metabolism in this organism. Our observations suggest a common regulatory mechanism for both processes, although *nar* and *nif* genes may not be closely associated since the *nif* gene was functional in *nar* mutants. Mutants with loss of nitrogenase but with functional NR activity are reported in *Cylindrospermum* sp. (Singh 1976a), *Wollea* sp. (Singh 1976b) and *N. muscorum* (Padhy and Singh 1978).

Acknowledgements. We are grateful to Dr. H.K. Pande, Director, Central Rice Research Institute, Cuttack for providing facilities and encouragement. The research was supported by a grant from Indian National Science Academy and Indian Council of Agricultural Research, New Delhi.

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Communicated by D. Goldfarb

Received August 1 / December 5, 1983