

An L-methionine–D,L-sulfoximine-resistant mutant of the cyanobacterium *Nostoc muscorum* showing inhibitor-resistant γ -glutamyl-transferase, defective glutamine synthetase and producing extracellular ammonia during N_2 fixation

H.N. Singh, R.K. Singh and R. Sharma

School of Life Sciences, University of Hyderabad, Hyderabad-500 134, India

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The role of γ -glutamyl-transferase in regulation of N_2 -fixation and ammonia assimilation in *Nostoc muscorum* was examined by isolating mutants in which the enzyme was resistant to L-methionine–D,L-sulfoximine. Mutant and wild-type were compared with respect to nitrogenase activity, extracellular production of nitrogenase-catalysed ammonia, γ -glutamyl-transferase activity and growth in N_2 and glutamine media. While the production of inhibitor-resistant enzyme with defective γ -glutamyl-transferase activity fully explains the inhibitor-resistant growth phenotype, the present results also suggest close metabolic linkage between γ -glutamyl transferase, nitrogenase and assimilation and extracellular production of N_2 -derived ammonia.

Glutamine synthetase

*γ -Glutamyl-transferase
Nitrogen fixation*

*Cyanobacterium
Ammonia excretion*

L-Methionine–D,L-sulfoximine

1. INTRODUCTION

The glutamine synthetase–glutamate synthase is the main primary route of ammonia assimilation in free-living aerobic, N_2 -fixing, heterocystous cyanobacteria [1–5]. The inhibitor of glutamine synthetase (GS), L-methionine–D,L-sulfoximine (MSX), has been observed to cause excretion of ammonia resulting from N_2 -fixation and the release of heterocyst and nitrogenase production from ammonia inhibition by a mechanism involving inhibition of glutamine synthetase activity [2]. The GS inhibitor is growth toxic and causes derepression of heterocyst production in ammonium medium in *Nostoc muscorum* and *Nostoc linckia* [6].

GS catalyses glutamine formation from ammonia and glutamate and the MSX inhibition of GS activity results in the inhibition of glutamine formation in *Anabaena cylindrica* and in other

heterocystous N_2 -fixing cyanobacteria [4,7]. The isolation of mutants of cyanobacteria with MSX-resistant GS-activity would be highly informative in understanding the role of GS in regulation of ammonium assimilation, heterocyst differentiation and N_2 -fixation in these oxygenic photosynthetic prokaryotes.

Here we report the results of studies on an MSX-resistant mutant of *Nostoc muscorum* which show that mutation to MSX resistance involves production of an MSX-resistant GS severely altered in its γ -glutamyl-transferase activity and that this mutant excretes a considerable amount of ammonia during photoautotrophic N_2 -fixation. We also show the production of MSX-resistant strains as a useful technique for isolating ammonia excreting mutants of N_2 -fixing cyanobacteria with potentials for biological production of ammonia under complete photoautotrophic conditions.

2. MATERIALS AND METHODS

The axenic clonal culture of *N. muscorum* was grown and maintained under the conditions as in [8]. Since 0.05 mM MSX was found to be completely growth toxic, MSX-resistant clones were isolated on combined-nitrogen-free solid medium, designated here as N₂-medium, containing 0.25 mM MSX as in [9]. The N₂ medium supplemented with 1 mM glutamine and henceforth called glutamine medium, was used for maintaining the stock cultures of MSX-resistant clones, one of which was used for study of growth, heterocyst frequency, acetylene reducing activity, GS activity and estimation of extracellular ammonia by the methods in [2].

The variously treated cultures were harvested, washed with 50 mM Tris-HCl buffer, pH 7.5 (buffer A), then suspended in buffer B (buffer A supplemented with 5 mM MgCl₂, 10 mM sodium glutamate, 5 mM 2-mercaptoethanol and 1 mM EDTA) for sonication at 4°C in a MSE MK-2 sonicator. Cell debris of the sonicated samples was removed by centrifugation at 35000 × *g* for 30 min and the supernatant thus obtained was assayed for the estimation of the level of GS biosynthetic and γ -glutamyl-transferase activity in a Gilford spectrophotometer as in [10,11]. The effect of 0.5 mM MSX on in vitro GS activities was studied by preincubating the reaction mixture extract with the inhibitor for 15 min before adding the required substrates (200 μ mol NH₄Cl, 60 μ mol sodium glutamate for biosynthetic activity and 90 μ mol glutamine for γ -glutamyl-transferase activity in 3 ml).

The air gas phase and argon gas phase of N₂ cultures were obtained by inoculating the glutamine-grown cultures into N₂-medium containing flasks with rubber stoppers, each with two inserted glass tubes one serving as inlet for continuous bubbling by the desired gas and the other serving as an outlet as in [12].

Protein and chlorophyll were estimated following the methods of [13] and [14], respectively.

3. RESULTS AND DISCUSSION

The results with the parental and MSX-resistant strains on growth, heterocyst and extractable GS biosynthetic activity under different treatment

Table 1

Growth (turbidity at 663 nm), heterocyst frequency (%) and extractable GS biosynthetic activity of the parent strain and MSX-resistant strain

Condition	Parent strain	MSX-resistant strain
Growth in		
N ₂ medium	0.62 ± 0.031	0.12 ± 0.042
glutamine medium	0.81 ± 0.092	0.83 ± 0.095
Heterocyst frequency in		
N ₂ medium	4–5	4–5
glutamine medium	0.0	0.0
Extractable GS biosynthetic activity in N ₂ medium without MSX		
(– MSX)	6.3 ± 1.5	7.5 ± 2.1
with MSX		
(+ MSX)	0.5 ± 0.04	6.2 ± 2.5

Growth and heterocyst frequency were estimated with 6-day-old cultures. Cultures incubated for 24 h with 0.1 mM MSX under growth conditions were used for extracting GS and measuring the GS biosynthetic activity (nmol NADH oxidized .mg protein⁻¹ .min⁻¹)

conditions are given in table 1. While both strains grew in N₂-medium, producing heterocyst with identical frequency, the parental strain showed nearly 5-fold higher growth than the MSX-resistant strain in N₂-medium. The observed difference in growth in N₂-medium disappeared following glutamine supplementation which induced non-heterocystous filaments. Evidently, the mutation to MSX-resistant phenotype has resulted in impairment of the cyanobacterial growth at the expense of aerobic N₂-fixation. Both MSX-treated and untreated N₂ growing cultures of the MSX-resistant strain showed a level of extractable GS biosynthetic activity almost equal to that shown by similar MSX-untreated cultures of the parental strain, but when such parental cultures were examined for extractable GS biosynthetic activity following MSX treatment, it was found considerably reduced to almost negligible level. Thus it appears that the basic difference between the two strains is the presence of a mechanism in MSX-resistant strain which protects its in vivo GS from inactivation by MSX, thereby enabling it to grow in the presence of growth-toxic concentration of the inhibitor.

The *in vivo* protective mechanism of GS activity against MSX inactivation could be a permeability barrier to MSX or a mutant GS itself resistant to MSX inhibition. This problem was investigated by examining the inhibitory effect of MSX directly on *in vitro* GS biosynthetic as well as γ -glutamyl-transferase activity in extracts from N_2 grown cultures. The GS of the mutant strain, whose activity was found resistant to the inhibitor, showed severe reduction in its γ -glutamyl-transferase activity (fig.1). However, both the *in vivo* GS and *in vitro* GS of the parental strain were sensitive to inhibition by MSX but the observed degree of *in vitro* GS inhibition by 0.5 mM MSX was much

lower as compared to that of *in vivo* inhibition in the presence of 0.1 mM MSX. These results suggest that presence of MSX-resistant γ -glutamyl-transferase defective GS is the apparent cause of MSX-resistant phenotype in the cyanobacterium and that MSX is possibly activated *in vivo* to a more potent form of GS inhibitor in the parental strain.

The parent and MSX-resistant strain both showed nominal acetylene reducing activity under dark incubation with air or argon as gas phase but considerable acetylene reducing activity under light incubation with either gases as the gas phase (table 2). The light-dependent acetylene reducing activity was nearly 1.5-fold more in MSX-resistant strains as compared to that in parental strain. This means that both the strains fix nitrogen vigorously only under photoautotrophic condition. Interestingly, the extracellular ammonia production in large quantity remained confined to only MSX-resistant strain under photoautotrophic N_2 -fixing conditions as the parental cultures under similar growth conditions showed insignificant level of extracellular ammonia. Since the MSX-resistant strain grew very slowly in N_2 medium despite having higher level of nitrogenase activity, as compared to parental strain, the main reason for its slow growth in N_2 -medium appears to be its newly acquired ability to excrete significant proportion of ammonia derived from N_2 -fixation into the growth medium. This could happen if the ammonia assimilating pathway and ammonium transport pathway are interlinked by some sort of a common regulatory mechanism which is disrupted in the mutant but not in the parental strain.

MSX being an analogue of glutamate, competes with it for a common binding site on the catalytic part of the enzyme, and once firmly and irreversibly bound, causes irreversible inhibition of the enzyme activity [15]. The first available report of a mutant GS showing resistance to the glutamate analogue with a defective γ -glutamyl-transferase activity has been made in *Salmonella typhimurium* which, on purification and further kinetic characterization, showed affinity for its substrates ammonia and glutamate several fold lower than that of the normal native GS [16].

The novel properties of the mutant strain of *N. muscorum* while fixing and assimilating N_2 are:

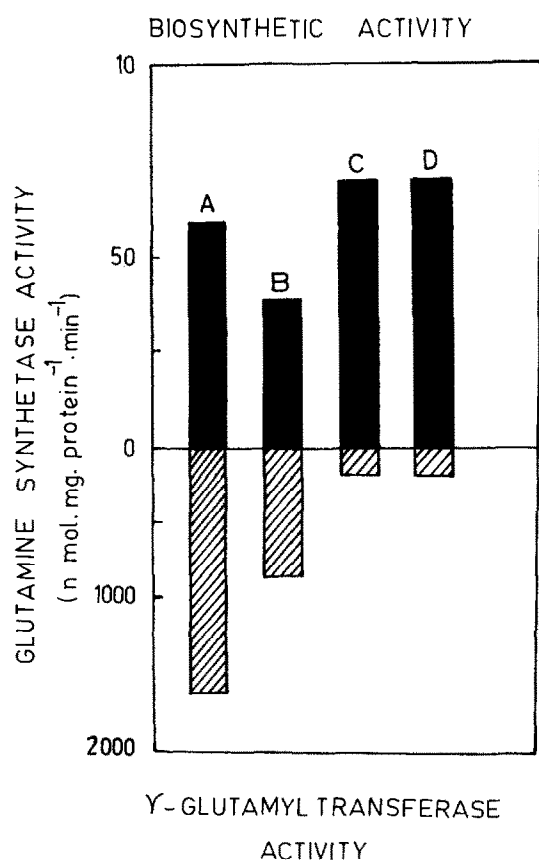


Fig.1. *In vitro* glutamine synthetase (GS) activities: (■) Biosynthetic (nmol NADH oxidized · mg protein⁻¹ · min⁻¹); (▨) γ -glutamyl-transferase (nmol γ -glutamyl-hydroxamate formed · mg protein⁻¹ · min⁻¹); (A) parent GS without MSX; (B) parent GS with MSX; (C) mutant GS without MSX; (D) mutant GS with MSX.

Table 2

Estimation of the acetylene reducing activity and production of extracellular ammonia in N₂-fixing cultures of the parental strain and MSX-resistant strain

Incubation conditions	Parent strain		MSX-resistant strain	
	Ethylene	Ammonia	Ethylene	Ammonia
Light:				
Air	10.5 ± 1.2	0.86 ± 0.073	15.5 ± 1.8	85.0 ± 3.2
Argon	12.6 ± 1.5	0.92 ± 0.085	16.0 ± 2.1	0.63 ± 0.25
Dark:				
Air	0.82 ± 0.13	0.65 ± 0.11	0.0	0.53 ± 0.16
Argon	0.65 ± 0.15	0.56 ± 0.08	0.0	0.33 ± 0.091

The cultures were grown in air (aerobic condition) in N₂ medium at 25000 lux and 28°C. The samples were transferred to 50 ml batch cultures in N₂ media and then incubated in light (phototrophic growth conditions) and dark (non-phototrophic conditions) under air or argon as gas phases for 24 h, at the end of which they were analysed for acetylene reduction to ethylene (nmol C₂H₄ · μg chl a⁻¹ · g⁻¹) and extracellular ammonia (nmol NH₃ · μg chl a⁻¹ · 24 h⁻¹)

- (i) Significantly higher in vivo nitrogenase activity;
- (ii) Considerably reduced growth;
- (iii) Substantial production of extracellular ammonia;
- (iv) The presence of a MSX-resistant GS showing nearly normal biosynthetic activity but defective γ-glutamyl-transferase activity under in vitro conditions.

In view of the reported observations that in vivo inhibition of GS activity in cyanobacteria results in enhancement of in vivo nitrogenase activity and extracellular ammonia production [2,7], the present results suggest a close metabolic link between the in vivo activity of the mutant GS and the remaining 3 properties of the mutant strain. Since the exogenous glutamine is able to restore the mutant growth nearly to the level of parental growth, the intracellular level of glutamine appears to be the most likely metabolic link regulating growth, nitrogenase activity and production of extracellular ammonia during N₂-fixation. This view is consistent with the recent reports showing modification of in vivo GS of N₂-grown cultures of *Anabaena* to a less active form by mechanism(s) involving fresh protein synthesis [17] and glutamine regulation of ammonium transport in heterotrophic N₂-fixing *Klebsiella pneumoniae* [18].

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