Physiological and Biochemical Analysis of the Glutamine Synthetase-Impaired Mutants of the Nitrogen-Fixing Cyanobacterium *Nostoc muscorum*

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**Abstract.** Three types of glutamine synthetase (GS)-impaired mutants (*gin*) of *Nostoc muscorum* were isolated as ethylenediamine (EDA)-resistant phenotypes and characterized with respect to heterocyst development, nitrogen fixation, ammonium metabolism, photosynthetic characteristics, and glutamine synthetase activity. The criterion for categorizing the mutants was the extent of loss of GS activity (both in transferase and biosynthetic assays) compared with wild type; it was 70% in EDA-1, 30% in EDA-2, and more than 90% in EDA-3 strains. The level of nitrogenase activity in mutant strains was proportionate to heterocyst frequency and was found refractory to ammonium and EDA repression. In EDA-resistant strains, development of heterocysts and their spacing pattern remained unaffected and did not respond to treatment of amino acid analogues, drugs, and ammoniacal compounds which otherwise either stimulated or suppressed the number and altered the spacing pattern in wild type. A biphasic pattern of ammonium uptake indicating two transport systems was observed in all the strains except that the Km values for both high- and low-affinity systems were altered in mutant strains. In vivo treatment with MSX or EDA significantly inhibited the GS activity in wild type, whereas mutant strains did not respond to these treatments and were able to liberate NH₄⁺ continuously into the medium without MSX treatment. During NH₄⁺ uptake, percentage inhibition of O₂ evolution and changes in increase of fluorescence intensity were low in EDA strains compared with wild type. Assessment of GS protein with antibodies against GS and quantitative polyacrylamide gel electrophoresis (PAGE) suggested that loss in specific activity of GS per milligram of extractable protein in EDA mutants was owing to low production of GS-specific protein. SDS-PAGE of purified GS enzyme from all the strains revealed only one polypeptide band of molecular weight of about 51.28 kDa.

Cyanobacteria are O₂-evolving photosynthetic prokaryotes, and the filamentous heterocystous strains are capable of fixing molecular dinitrogen and metabolizing the combined nitrogen sources for growth and differentiation. However, ammonium being the essential nitrogenous compound for amino acid and protein biosynthesis is, therefore, the preferred nitrogen source for growth and development in higher plants and microorganisms including cyanobacteria [30]. In nitrogen-fixing cyanobacteria, NH₄⁺ produced in vivo by nitrogenase or assimilated from the external medium is metabolized and diverted to the biosynthetic pool for amination primarily by the ammonium-assimilating enzymes glutamine synthetase (GS) and glutamate synthase (GOGAT), since the physiological level of glutamate dehydrogenase (GDH) is either very low or negligible in cyanobacteria [32]. Interestingly, the *in vivo* concentration of NH₄⁺ pool in N₂-fixing cyanobacteria is involved in regulation of nitrogenase synthesis and heterocyst development by the GS–GOGAT pathway [8, 9].

1 Abbreviations: GS, glutamine synthetase; GOGAT, glutamate synthase; EDA, ethylene-diamine; MSX, L-methionine-DL-sulfoximine; RIF, rifampicin; AZT, 7-azatryptophan; BTA, β-thienylalanine; CAN, canavanine; AZA, azaserine; ETH, ethionine; CHL, chloramphenicol; PAGE, polyacrylamide gel electrophoresis; SDS, sodium dodecylsulfate; 2ME, β-mercaptoethanol.

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investigate the regulatory role of glutamine synthetase in ammonium metabolism, heterocyst development and nitrogenase activity in N$_2$-fixing cyanobacteria, and to exploit the possibilities of engineering the cyanobacterial system for photoproduction of ammonium and ammoniacal compounds, we attempted to isolate mutants of a cyanobacterium, *Nostoc muscorum*, impaired in GS activity.

In nitrogen-fixing microorganisms, selection of mutants resistant to analogues of ammonium and dinitrogen has been the strategy for isolation of mutants for derepressed nitrogenase synthesis and nitrogen fixation [5, 17, 33, 34]. Several studies in cyanobacteria have been conducted in the past by either blocking or partially suppressing the enzymatic activities of the GS-GOGAT pathway to investigate their role in the control of nitrogen metabolism and regulation of nitrogen fixation (nif) gene expression [8, 9, 28, 32]. However, studies based on the use of either inhibitors or under a specific set of conditions are not the true reflection of metabolic functions, because these conditions simultaneously affected other cellular functions. Perhaps use of mutants selectively impaired in specific enzymatic pathway seems a promising tool towards deciphering the regulation of nitrogen metabolism in cyanobacteria, as demonstrated in several N$_2$-fixing bacteria [7, 19]. Comparatively little work has been done to isolate and characterize the mutants altered in N$_2$ utilization or in regulatory properties in N$_2$-fixing cyanobacteria [4, 6, 22]. Therefore, the genetic and physiologic analyses of ammonium metabolism and the regulation of N$_2$-fixation are still lacking in cyanobacteria.

In this paper we report the isolation of ethylenediamine-resistant mutants precisely impaired in GS activity and describe their properties with respect to ammonium metabolism, nitrogen fixation, and other metabolic functions.

**Materials and Methods**

**Organism and culture conditions.** Cultures of *Nostoc muscorum* UW (ATCC 29151) and its mutant strains were grown in modified N-free Chu No. 10 medium at 25 ± 1°C and at a photon flux density of 40 µmol m$^{-2}$s$^{-1}$ at the surface of the culture vessel [4].

Growth of cultures was estimated spectrophotometrically at 660 nm or by increase in total protein content and the specific growth rate ($k$) expressed as log$_{10}$ unit/day.

**Isolation of mutants.** The exponentially growing clonal cultures of *N. muscorum* (10$^7$–10$^8$ cfu/ml) were plated onto the agar medium containing 100 µg ml$^{-1}$ ethylenediamine (EDA) and allowed to grow for 4–5 days until the resistant colonies were apparent. These colonies were further exposed to a top agar layer containing 200 µg ml$^{-1}$ EDA in order to be certain of obtaining the EDA-resistant colonies. Several discrete colonies appearing on the second layer were rescreened on agar medium with EDA, and finally their clonal population was raised in liquid medium containing EDA.

**Ammonium estimation.** Ammonium in the culture suspension was estimated by phenol-hypochlorite method [26]. Aliquots of 2–5 ml were withdrawn periodically from the culture suspension and either centrifuged or filtered. About 1 ml of the supernatant was employed for ammonium estimation.

**Protein determination.** Total protein content was determined by the procedure of Lowry et al. [12], and cell-free protein by that of Bradford [2].

**Determination of photosynthetic pigments.** Chlorophyll $a$ was extracted in methanol and determined by the extinction coefficient given by Mackinney [13]. Phycocyanin was extracted in 20 mM phosphate buffer, pH 7.0, and quantitated as described earlier [4].

**Measurement of photosynthetic O$_2$ evolution.** Rates of oxygen evolution in light during NH$_4^+$ or NO$_3^-$ uptake were measured polarographically with Gilson oxygen electrodes (Gilson Oxygenograph 5/6). Whole cells or spheroplast preparations of cultures were incubated in the chamber coupled with Clark type electrode at 30°C, and the rate of O$_2$ evolution in light (200 µmolites m$^{-2}$ s$^{-1}$) was determined. The rate of photosynthesis is expressed as nmol O$_2$ evolved min$^{-1}$ µg$^{-1}$ chla.

**Estimation of fluorescence.** Changes in fluorescence were monitored by Shimadzu spectrofluorometer RF-540 (Japan) by exciting the spheroplast suspension (7.25 µg chla ml$^{-1}$) with blue light (440 nm) and simultaneously recording the emission spectrum from 620 to 750 nm.

**Preparation of spheroplast.** Exponentially growing cells were harvested and resuspended in the medium containing 0.5 % sucrose, 10 mM MES-NaOH, 5 mM K$_2$HPO$_4$, 10 mM MgCl$_2$, 2% BSA (w/v), pH 6.9, and lysozyme 0.1% (w/v). The suspension was incubated in light for 60 min at 35°C, washed twice with the same buffer, and resuspended in the same medium supplemented with 0.5% (w/v) BSA without lysozyme. Formation of spheroplasts was examined microscopically and by osmotic lysis.

**Preparation of cell-free extract.** Cell-free extracts of the exponential cultures were prepared as described earlier [1].

**Enzyme assays.** Nitrogenase (EC 1.18.2.1) was estimated by the acetylene reduction technique [31]. Five milliliters of samples were withdrawn periodically from exponentially growing cultures and incubated in 25-ml bottles, under a gas phase of 10% (v/v) acetylene in air at 26°C and at a photon flux density of 60 µmol m$^{-2}$ s$^{-1}$. The rate of nitrogen fixation was measured by gas chromatography (CIC, Ahmedabad, India) and expressed as nmol acetylene reduced µg$^{-1}$ chla h$^{-1}$.

**Glutamine synthetase.** The Mn$^{2+}$-dependent glutamyl transferase activity of glutamine synthetase (EC 6.3.1.2) was measured in permeabilized or cell-free extracts by the procedure of Shapiro and Stadtman [23]. For biosynthetic activity, reduction of NADH