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EVIDENCE FOR AMMONIA AS AN INHIBITOR OF HETEROCYST AND NITROGENASE FORMATION IN THE CYANOBACTERIUM ANABAENA CYCADEAE

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SUMMARY: Growth and regulation of heterocyst and nitrogenase by fixed nitrogen sources were studied comparatively in parent and glutamine auxotrophic mutant of Anabaena cycadeae. The parent strain grew well on N_2, NH_4^+ or glutamine while the mutant strain grew on glutamine but not on N_2 or NH_4^+. The total lack of active glutamine synthetase in the mutant strain thus appears to be the reason for its observed lack of growth in N_2 or NH_4^+, which explains why it is a glutamine auxotroph and at the same time shows glutamine synthetase to be the sole primary ammonia assimilating enzyme. NH_4^+ repression of heterocyst and nitrogenase in the mutant and the parental strains and their derepression by L-methionine-DL-sulfoximine suggest that NH_4^+ *per se* and not glutamine synthetase mediated pathway of ammonia assimilation is the initial repressor signal of heterocyst and nitrogenase in A. cycadeae.

INTRODUCTION: A clear understanding of the regulatory mechanism of aerobic N_2-fixation by oxygenic photosynthetic cyanobacteria is highly desirable in view of their potential as a photobiological source of ammonia production at the expense of N_2. There is evidence that heterocysts are the loci of aerobic nitrogenase synthesis, aerobic nitrogenase activity and glutamine synthetase (EC.6.3.1.2) (GS) activity thus leading to formation of glutamine from N_2-generated ammonia (1-5). In an early study, ammonia or its close derivative was suggested to be the intracellular inhibitor of heterocysts differentiation (6) and this situation continued until Stewart and Rowell (7) concluded from studies based on the use of L-methionine-DL-sulfoximine (MSX), the

Abbreviations: L-methionine-DL-Sulfoximine : MSX
Tris (hydroxymethyl) aminomethane : Tris

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inhibitor of glutamine synthetase and glutamate synthase (GOGAT) in Anabaena cylindrica that not NH_4^+ per se but glutamine synthetase, glutamate synthase or their reaction product is the repressor of heterocyst and nitrogenase. There is also evidence for glutamine to be the repressor signal of heterocyst and nitrogenase (8).

Studies with glutamine auxotrophs of heterocystous N_2-fixing cyanobacteria will be extremely helpful not only in providing a clear understanding of the specific role of glutamine synthetase in assimilation of ammonia but also in clarifying the relative role of NH_4^+, glutamine synthetase or glutamine as a repressor signal of heterocyst and nitrogenase. We have isolated for the first time such mutants of Anabaena cycadeae, the endophyte from Cycas coralloid root, capable of good photoautotrophic growth with N_2, NO_3^- or NH_4^+ as a nitrogen source (9), and in this paper we present evidence to show that NH_4^+ inhibition of heterocyst and nitrogenase is not mediated by catalytically active glutamine synthetase, which is contrary to the conclusion of Stewart and Rowell (7), that NH_4^+ per se appears to be the repressor signal of heterocyst-nitrogenase and that catalytic glutamine synthetase is the sole primary pathway of ammonia assimilation.

MATERIALS AND METHODS: Anabaena cycadeae was isolated freshly from coralloid root of Cycas and grown in fixed nitrogen-free medium henceforth called N_2-medium in pure clonal culture according to the method of (9). The cultures were grown in air in an airconditioned growth room at light intensity of 2500 lux and temperature of 28±2°C as described previously (10). Since MSX is an inhibitor of growth in inorganic nitrogen media (11) as well as of glutamine synthetase activity (7), the spontaneously occurring glutamine auxotrophic mutants in N_2-fixing parental cultures were sought by incubating such cultures with growth inhibitory concentration of MSX (50 μM) for a week in the growth chamber followed by overlaying with glutamine (1mM) medium. This technique led to the successful isolation of a dozen glutamine auxotrophic clones, one of which has been used in the present study. The estimation of growth, heterocyst frequency and acetylene reducing activity was done as described (12). Ammonia was assayed according to (13). The cell-free extracts for assaying glutamine synthetase γ-glutamyl transferase activity were pre-

pared as follows: The cultures grown in different nitrogen media were harvested separately, washed with 50mM Tris-HCl buffer, pH 7.5,(buffer A) and then suspended in buffer B for sonication in a MSE MK-2 Sonicator at 4°C. The sonicated samples were centrifuged at 35,000 x g for 30 min and the resulting supernatant was assayed for the transferase activity spectrophotometrically at 540 nm in a Gilford-250 Spectrophotometer according to (14). Protein and chlorophyll-a were estimated respectively as described (15,16).

L-methionine-DL-sulfoximine (MSX) and glutamine supplied by Sigma Chemical Co., USA, were freshly prepared as required and sterilized by filtration. Other chemicals were used at highest purity available from BDH, Poole.

RESULTS AND DISCUSSION: As shown in Table 1, the mutant strain grew with glutamine but not with N_2 or NH_4^+ as nitrogen source, produced heterocyst and nitrogenase activity in N_2-medium but not in NH_4^+ medium and showed complete lack of glutamine synthetase γ-glutamyl transferase activity. In comparison, the parent strain grew with N_2, NH_4^+ or glutamine as nitrogen

TABLE 1 Growth, Heterocyst frequency, nitrogenase activity and glutamine synthetase γ-glutamyl transferase activity of the parent and the glutamine auxotrophic mutant in N_2, NH_4^+ (5mM NH_4Cl) and glutamine (2 mM) media.

	Parent			Mutant		
	N_2	NH_4^+	Glutamine	N_2	NH_4^+	Glutamine
Growth	0.42	0.45	0.56	0.0	0.0	0.53
Heterocyst frequency	6-8	0.0	0.0	3-4	0.0	0.0
Nitrogenase activity	11.66	0.0	0.0	9.12	0.0	0.0
Glutamine synthetase γ-glutamyl-transferase activity	412	334	301	0.0	0.0	0.0

The inoculum source for all cultures was glutamine grown samples. Growth (measured as OD changes at 663 nm) was estimated with 6 days old cultures. Heterocyst frequency (as percentage of total vegetative cells per filament), nitrogenase activity (n mol. C_2H_4 μg Chl a^{-1} h^{-1}) and glutamine synthetase transferase activity (n mol. γ-glutamyl hydroxymate mg $protein^{-1}$ min^{-1}) were assayed in 48h old cultures. The results are the average of three independent readings.

source, and showed normal level of glutamine synthetase γ-glutamyl transferase activity. The heterocyst frequency and nitrogenase activity levels of the mutant strain were respectively nearly 50% and 75% of the parental strain. The most likely reason for lower heterocyst frequency and nitrogenase activity of the mutant strain appears to be the limited availability of cellular nitrogen reserve for fresh protein synthesis required for the production of new heterocyst and nitrogenase. However, the parent and the glutamine auxotrophic mutant both appeared similar in respect of NH_4^+ or glutamine repression of heterocyst and nitrogenase and their derepression in N_2-medium. Since the mutant strain completely lacked glutamine synthetase

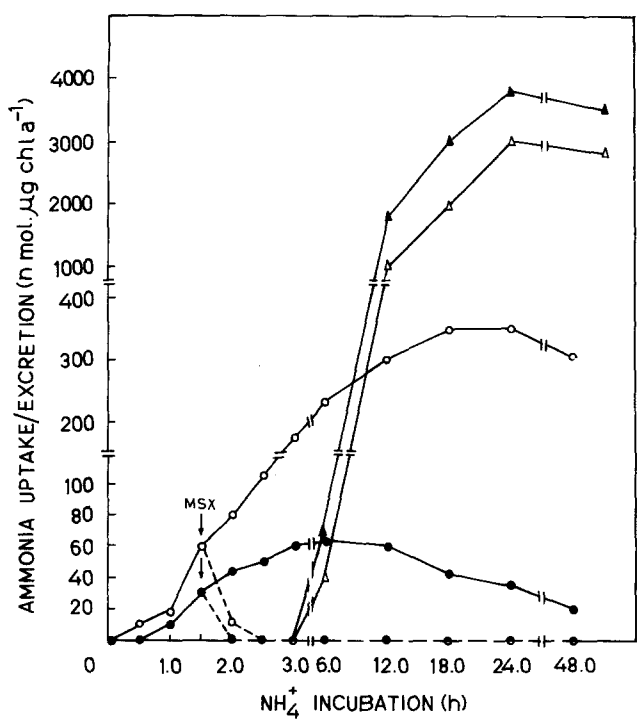

Fig. 1 Effect of adding MSX (10 µM) at the time arrowed on NH_4^+ uptake/excretion by cultures incubated in 5 mM NH_4Cl medium. NH_4^+ uptake in the absence of MSX:(o-o) parent; (●-●) mutant. NH_4^+ uptake in the presence of MSX: (o--o) parent; (●---●) mutant. NH_4^+ excretion in the presence of MSX: (Δ—Δ) parent; (▲—▲) mutant. No excretion was observed in the absence of MSX during the entire course of the experiment.

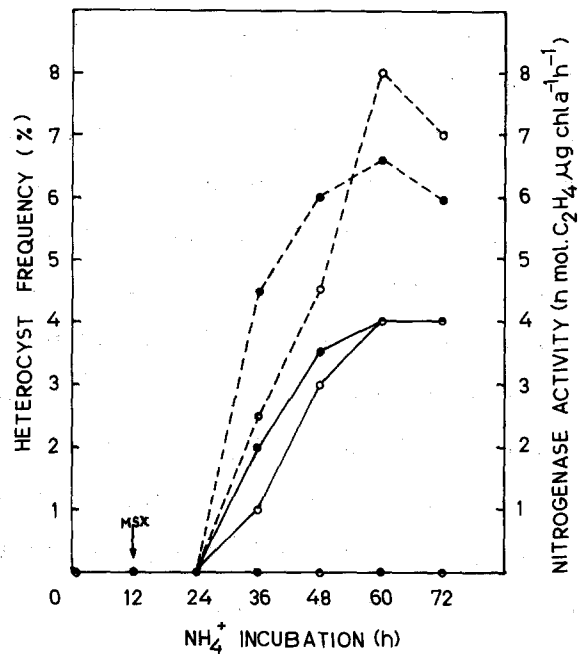

Fig. 2 Effect of adding MSX (10 µM) at the time arrowed on heterocyst and nitrogenase production in NH_4^+ (5mM NH_4Cl) media.
Strains:(o) parent; (●) mutant.
Features:(-) heterocyst; (--) nitrogenase activity.

transferase activity, catalytic glutamine synthetase protein *per se* is not the requirement for derepression of heterocyst and nitrogenase. Similarly NH_4^+ *per se* and not glutamine synthetase mediated pathway of ammonia assimilation appears to be the heterocyst-nitrogenase repressor signal in the mutant strain. The observed glutamine repression of heterocyst and nitrogenase could be either due to intracellular generation of ammonia from glutamine through the action of glutaminase or due to an entirely different mechanism. In this connection, it is important to point out the conclusion of Singh et al (11) that mechanism of ammonia inhibition of heterocyst formation is different from that involved in proheterocyst or heterocyst inhibition of new heterocyst formation in *Nostoc linkia*. NH_4^+ as a partial and KNO_3 as a complete repressor of nitrogenase

synthesis have been demonstrated in Anabaena sp. strain CA (17) and one of its mutants has been found to grow on N_2 but not on NH_4^+ despite having active glutamine synthetase (18). These findings merely emphasise the nitrogen-source dependent nature of various regulatory mechanisms involved in control of heterocyst and nitrogenase formation.

Our conclusion based on studies with the glutamine auxotrophic mutant that the NH_4^+ per se is the repressor signal is further supported by studies with MSX in NH_4^+ incubated cultures of the parental and mutant strains (Figs. 1-2). The glutamine (2mM) grown cultures completely devoid of heterocyst and nitrogenase activity were used as source materials for studies on NH_4^+ uptake and NH_4^+ excretion (Fig. 1) and on heterocyst formation and nitrogenase activity (Fig. 2) in the presence and absence of MSX in the ammonium (5mM NH_4Cl) medium. Before adding such cultures to the NH_4^+ medium, they were partially starved for nitrogen by incubating them in N_2-media for 0.5 h under growth conditions. As shown in Fig. 1, parent appeared considerably more efficient in NH_4^+ uptake than its mutant strain although both strains showed almost complete inhibition of NH_4^+ uptake by MSX treatment of 0.5-1 h duration. The MSX treatment also induced substantial level of NH_4^+ excretion in the two strains but induction of NH_4^+ excretion required minimum MSX treatment of nearly 3-4h, while complete inhibition of NH_4^+ uptake occurred following only 0.5-1 h treatment with the inhibitor. More prolonged MSX treatment caused proportionately more excretion of NH_4^+. Thus the initial rapid inhibitory action of MSX appears to be primarily on NH_4^+ uptake system followed by induction and elevation of NH_4^+ excretion in the given temporal sequence. Recently MSX has been shown to be a strong inhibitor of NH_4^+ transport in Klebsiella pneumoniae (19).

NH_4^+ incubated cultures of the mutant strain dies without differentiating heterocyst and nitrogenase activity a finding which suggest highly stable nature of the NH_4^+-generated repressor signal. Parent also did not produce heterocyst and nitrogenase in NH_4^+ medium, although it grew at the expense of NH_4^+. Treatment of 18-24 h duration with MSX caused both the strains to produce heterocyst and nitrogenase whose level increased to optimum during the next 24 h of treatment. Thus MSX is not only an inhibitor of glutamine synthetase activity as shown by (7) but also causes inhibition of NH_4^+ uptake and induction of NH_4^+ excretion in later possibly from intracellular nitrogenous pool. Since the mutant strain lacks active glutamine synthetase while still retaining the MSX-sensitive mechanism of NH_4^+ repression of heterocyst and nitrogenase, NH_4^+ *per se* appears to be the initial repressor signal of heterocyst and nitrogenase. This view is further corroborated by the results of inhibitor studies with ammonium cultures of the parental strain. Thus inhibition of NH_4^+ uptake rather than inhibition of glutamine synthetase activity by MSX at the moment appears to be the reason for derepression of heterocyst and nitrogenase in *Anabaena cycadeae*.

In view of the present findings, studies on MSX regulation of NH_4^+ transport in *A. cylindrica* is now required before accepting the conclusion of Stewart and Rowell (7) that NH_4^+ *per se* is not the repressor signal of heterocyst and nitrogenase in this Cyanobacterium.

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