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MGG

Nutritional Control of the Frequency of MNNG-Induced True Branching Habit in *Anabaena doliolum**

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Summary. MNNG survival and mutagenesis were studied in *Anabaena doliolum* Bharadwaja. The survival curves of germinating spores were exponential while those of resting spores and filament-fragments were sigmoidal. Blue mutants with much higher phycocyanin contents than the parent were recovered in varying frequencies; the frequency (1.1%) was the highest with 2 mg/ml MNNG for 15 min. Some of the blue mutants sporulated normally; while others did not. A nonsporulating blue mutant (M16) showed branched filaments in liquid cultures. In nitrogen free medium, M16 had a high frequency (70%) of branched filaments during the early phase of growth. In some filaments, the branch arose from a heterocyst showing three polar nodules. There was no other difference between the parent and the mutant in cell morphology and cellular differentiation. High light intensity adversely affected phycocyanin and chlorophyll *a* pigments and nitrogen fixation in the mutant. Frequency of mutant branched character appears to be a factor of inorganic nitrogen nutrition.

Introduction

Mutational analysis of biochemical or morphological traits has proved very useful in understanding the basic processes involved. Singh and Singh (1964a, b) reported isolation of some UV-induced biochemical mutants in two species of filamentous, N_2-fixing blue-green algae. Subsequently, N-methyl-N′-nitro-N-nitrosoguanidine (MNNG) was found to be a potent mutagen in blue-green algae (Van Baalen, 1973). Previous reports on UV-mutagenesis in *Anabaena doliolum* suggested that only nonsporulating mutants could be recovered in this system (Singh, 1967). However, MNNG induces a variety of mutations in *A. doliolum*, suggesting this system to be as mutable as any other blue-green alga (Sinha and Kumar, 1973; Singh and Dikshit, 1976). The present paper examines quantitative aspects of MNNG mutagenesis in *A. doliolum* with regard to some morphological markers affecting pigment ratio or inducing true branching.

Material and Methods

Algal Strain

An axenic culture of *Anabaena doliolum* Bharadwaja was employed in the present study. The alga had unbranched filaments with intercalary and/or apical heterocysts (Fig. 3A), and synchronously produced characteristic doliform spores (akinetes). Clonal cultures were derived from single spores.

Culture Media and Growth Conditions

Stock culture of the alga was maintained on a mineral medium (Allen and Arnon, 1955) without combined nitrogen (AA-N), at 25° C in a culture room and illuminated with fluorescent strip light (approx. 1,000 lux) for 14 h per day.

Mutagenesis with MNNG

Spores were collected from stationary phase cultures, washed thrice and suspended in distilled water. Spores germinated in the basal medium after 30 h to produce 2–4 cell germlings. Freshly prepared stock solution of MNNG (Aldrich, Chemical Co., Milwaukee, Wisc.) was filter sterilized. The homogeneous suspensions of resting spores, germinating spores and filaments consisting of 3–5 cells were treated with 2 mg/ml MNNG at 35° C in TM buffer (pH 8.0). Aliquots were withdrawn at regular intervals of 5 min and immediately diluted hundred-folds in the basal medium; 0.2 ml samples were spread on plates of $AA+NO_3$ ($AA+10$ mM KNO_3) medium with 1.0% Difco-bacto agar. The plates were incubated in a light cabinet and survival was scored after 10–15 days. For mutagenesis,

* Dedicated to the memory of my revered teacher the late Prof. R.N. Singh

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treated populations were washed, free of mutagen with buffer by repeated centrifugation. They were allowed to grow in AA+NO_3 medium for 3–4 generations and subsequently used for screening the mutants.

Blue coloured colonies appearing among dark brown wild type colonies were scored as blue mutants. These mutants were isolated independently and tested in basal medium for sporulation.

Growth and Heterocyst Frequency

Growth was estimated as an increase in protein content measured by the Folin-reagent (Lowry et al., 1951), using egg albumin (Sigma Chemical Co. USA) as a standard. For determination of heterocyst frequency algal filaments grown on nitrate were fragmented, washed and inoculated into flasks containing the basal AA-N medium. The cultures were daily examined microscopically and the frequency of heterocyst was calculated on the basis of number of heterocyst per hundred vegetative cells.

Determination of Pigment Content and Total Nitrogen

From intact filaments, chlorophyll *a* and carotenoids were extracted in 80% v/v acetone. From the remaining residue phycobilin pigments were separated in water after cold sonication. The absorption spectra of the pigments were determined with the 'Unicam' spectrophotometer SP600. Amount of chlorophyll *a* and phycocyanin pigments in the extracts was calculated using specific absorption coefficients as suggested by Allen and Smith (1969). Total fixed nitrogen in 100 ml AA-N medium was estimated by micro-Kjeldahl method after 15 days of growth.

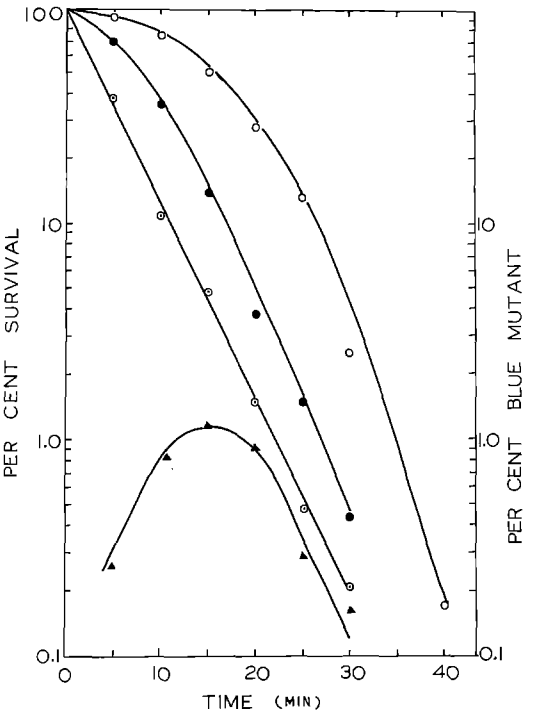

Fig. 1. Sensitivity of vegetative cells (–○–), resting spores (–●–) and germinating spores (–⊙–) of *A. doliolum* and induction of blue-mutants (–▲–) after treatment with 2 mg/ml MNNG at pH 8.0

Results

The inactivation of germinating spores by MNNG exhibited an exponential pattern; the inactivation was more pronounced in germinating spores than in resting spores or vegetative cells (Fig. 1). Filament fragments showed greater resistance than resting spores. Morphological variants were maximum in the populations from treated germinating spore. Distinct blue colour colonies, never found in the parent population, appeared at a frequency of 0.26% after 5 min MNNG treatment and reached the maximum (1.15%) after 15 min, beyond this the frequency declined sharply (Fig. 1). In other words, the mutation frequency for blue colony appears to a function of the MNNG-dose.

Initially, 185 colonies of blue mutants were isolated and tested for sporulation; only 73 clones showed sporulation in basal medium after 15–20 days.

One nonsporulating blue mutant (M16) showed true-branched filaments in liquid culture. This novel character of M16 was maintained after nearly seventy subcultures. M16 grew and produced heterocysts in nitrogen free medium with an efficiency comparable to the parent; however, differentiation of heterocysts was delayed (Fig. 2). The growth rate of the mutant in AA+NO_3 medium was less than the parent, and some cells of trichomes developed into giant spherical cells frequently (Fig. 3B, D). In addition, few fila-

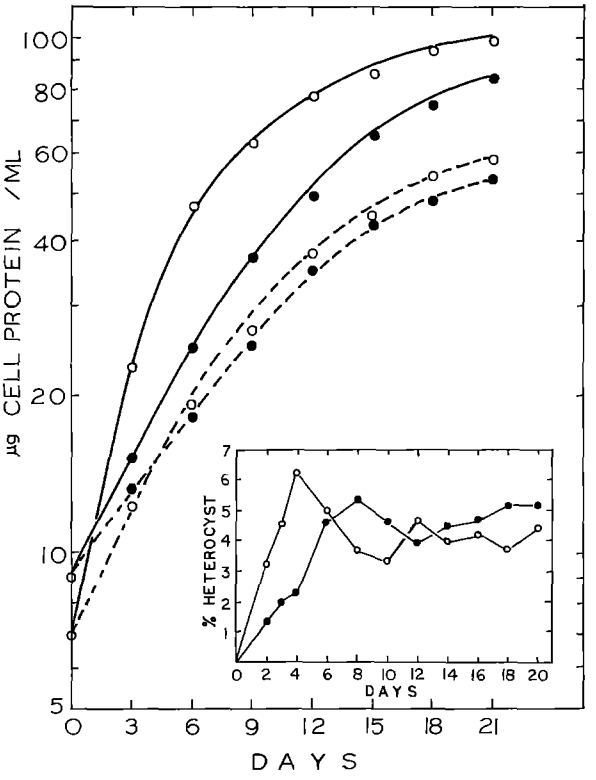

Fig. 2. Growth of wild type (○) and mutant M16 (●) of *A. doliolum* in nitrogen free (-----) and nitrate (——) containing media. Inset: heterocyst frequency of wild (–○–) and mutant (–●–) clones

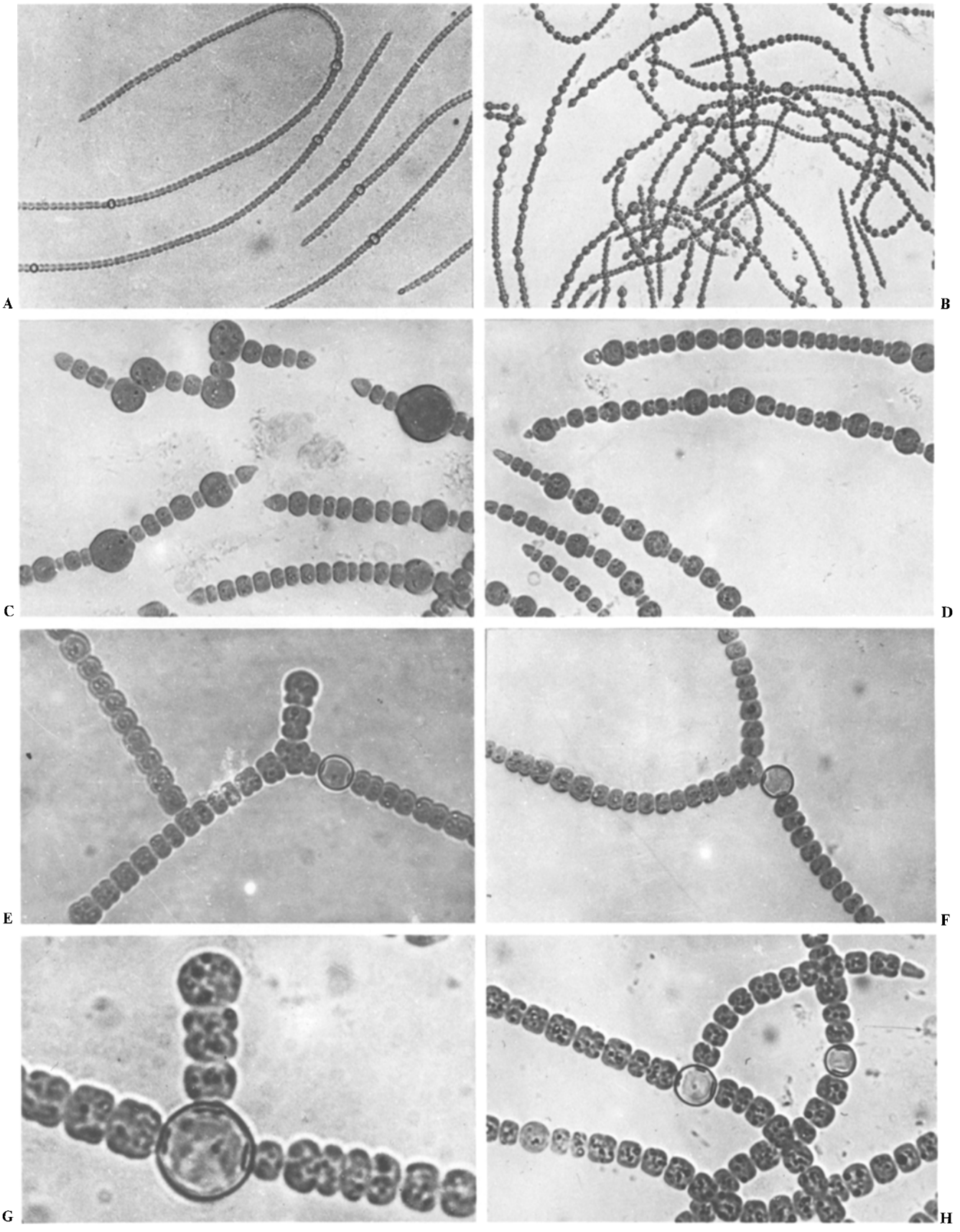

Fig. 3A–H. Photo-micrographs of the blue-green alga *Anabaena doliolum* grown in nitrogen-free medium (**A** × 150) and its mutant M16 showing frequent giant cells (**B** × 150, **D** × 460) and some zigzag trichome (**C** × 615) in nitrate medium. In nitrogen-free medium, M16 showing branching from vegetative cells (**E** × 690, **F** × 615) and from heterocysts (**G** × 1,650, **H** × 800)

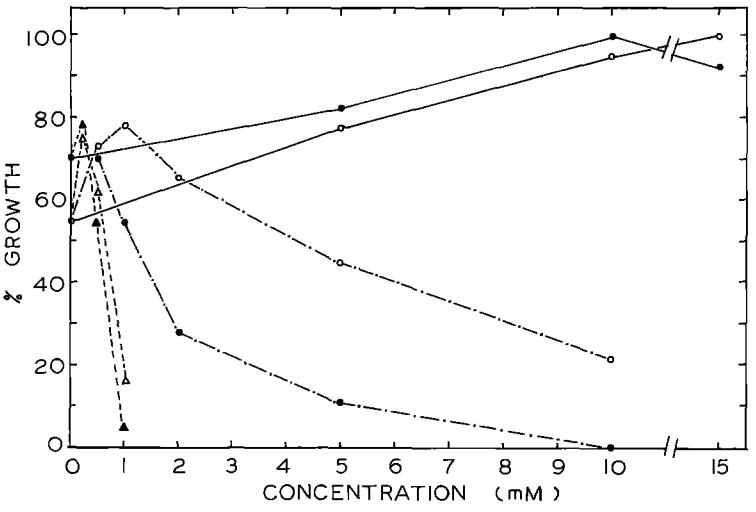

Fig. 4. Growth of wild type (○ or △) and mutant M16 (● or ▲) of *A. doliolum* in different concentration of KNO_3 (——), urea (-----) and NH_4Cl (·—·—·)

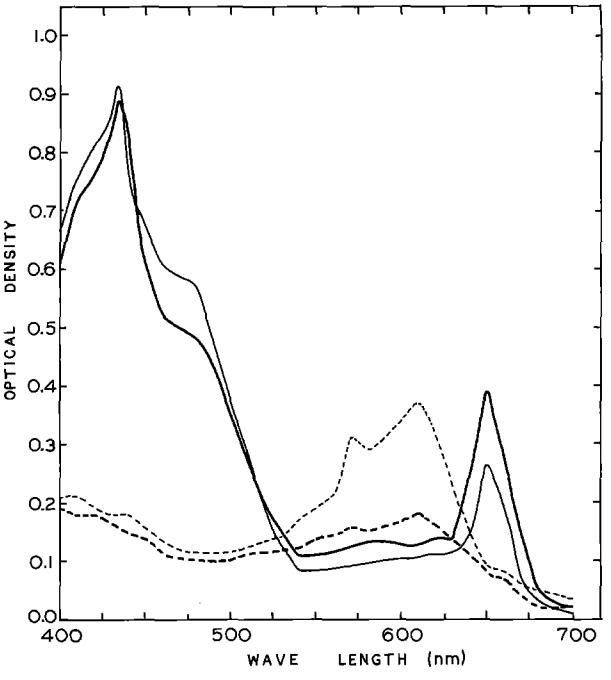

Fig. 5. Absorption spectra of acetone (——) and water soluble (-----) pigments of wild type (thick line) and mutant M16 (thin line) of *A. doliolum*

ments had branching from these cells (Fig. 3C). Such abnormal cells were formed in low frequency in cultures grown either in ammonium, nitrite or urea. On transfer of nitrate-grown filaments to nitrogen free medium, these giant cells divided abnormally giving rise to true-branching in most cases (Fig. 3E, F). In some filaments, the branch arose from a heterocyst and such a heterocyst had three polar nodules (Fig. 3G, H) which indicate that it is a case of true branching. The apical cells of the lateral and main

Table 1. Quantitative variation in pigment content and total nitrogen fixation in parent and mutant M16 of *A. doliolum* at different light intensity

Light intensity (× lux)	Parent (mg/100 ml)			Mutant (mg/100 ml)		
	Chlorophyll *a*	Phycocyanin	Total nitrogen	Chlorophyll *a*	Phycocyanin	Total nitrogen
500	0.21	0.004	3.83	0.06	0.005	4.13
1,000	0.27	0.005	4.76	0.11	0.009	4.65
1,500	0.34	0.007	5.22	0.19	0.015	4.24
2,000	0.40	0.009	5.55	0.22	0.016	3.77
3,000	0.52	0.006	5.74	0.10	0.006	2.18

filaments were conical like the parent. The persistence of branching after several subcultures suggests that it is a genetic change. There was no other difference between the parent and M16 with regard to characteristics of heterocysts and vegetative cells.

The growth of M16 was studied along with the parent in media without nitrogen or with various nitrogen sources in different combinations (Fig. 4). Ammonical nitrogen had toxic effect on growth of the mutant strain; 1 mM NH_4Cl reduced growth of M16 from 70% to 55% whereas growth of the parent was increased from 55% to 78%. There was no sporulation in M16 in any of the growth conditions after long incubation.

The absorption spectrum of both acetone and water soluble pigments of M16 did not show any additional absorption peak as compared to the parent (Fig. 5). However, the peak at 610 nm was much sharper in M16 than the parent which is characteristic of phycocyanin. The data in Table 1 clearly indicate that the mutant had nearly four times higher

phycocyanin-chlorophyll *a* ratio than the parent and fillaments of the mutant were sensitive to higher illumination (3000 lux). In spite of the difference in the pigment content, M16 showed nitrogen fixation comparable to the parent at low light intensity (Table 1).

Discussion

MNNG has two major characteristics: it is very potent in inducing mutations and is very weak in inactivation (Adelberg et al., 1965), although the reasons underlying these are not clearly understood (Drake, 1969). *A. doliolum* appears to tolerate much higher doses of MNNG than other blue-green algae (Asato and Folsome, 1969; Stevens and Van Baalen, 1969; Singh and Kashyap, 1977). It is not clear whether the greater tolerance of the alga is due to a difference in basic mechanism of action of MNNG or due to minor differences in cellular organization of the organism, or due to difference in permeability to the mutagen. In the case of germinating spores, the higher sensitivity to MNNG and the higher mutation frequency may be related to the presence of replicating DNA during germination, since replicating DNA is preferentially attacked by MNNG (Cerda'-Olmedo et al., 1968). Some of the blue branched mutants isolated from nitrate containing medium showed significant increase in the frequency of branching when transferred to nitrogen free medium. Most of the enlarged cells observed in nitrate medium in a dormant state, start dividing in a plane prependicular to the main axis in the nitrogen free medium, this giving rise to the true branching habit. This appears to be a case of nitrate controlled expression of branched character in *A. doliolum*. Nitrogen-nutrition control of mutagenesis in *A. doliolum* has been postulated (Singh, 1973).

This is the first report of true branching in *Anabaena*. A similar true branching mutant with lateral heterocysts was isolated from *Nostoc linckia* (Singh and Tiwari, 1969). Presence or absence of branching is one of the chief criteria to distinguish between order Nostocales and Stigonematales (Fritsch, 1942). But, the findings of this study together with those of Singh and Tiwari (1969) suggest that this character is governed by a single or atmost a few gene(s). Thus, the validity of using this character in classification of Cyanophyeae is open to question.

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