SPONTANEOUS MUTANTS AND THEIR USE IN STUDIES ON TRANSFORMATION IN CYANOBACTERIA

A THESIS SUBMITTED FOR THE DEGREE *Of*
DOCTOR OF PHILOSOPHY

BY

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DECLARATION

I hereby declare that the work presented in this dissertation has been carried out by me under the supervision of Prof. H.N. Singh, and that this work has not been previously submitted for any degree or diploma of any other university.

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CERTIFICATE

This is to certify that the Thesis entitled SPONTANEOUS MUTANTS AND THEIR USE IN STUDIES ON TRANSFORMATION IN CYANOBACTERIA is based on the results of the work carried out by Miss Shubhra Katiyar, M.Sc. for the degree of DOCTOR OF PHILOSOPHY under my supervision. This work has not been submitted to any degree or diploma in any other university.

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CONTENTS

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ABBREVIATIONS USED

ADP	Adenosine diphosphate
ATP	Adenosine triphosphate
CFU	Colony forming unit
DCIP	2,6-Dichlorophenol indophenol
DCMU	3-(3,4-dichlorophenyl)-1,1-dimethyl urea
DMSO	Dimethyl sulfoxide
DNA	Deoxyribose nucleic acid
DNase	Deoxyribonuclease
EDTA	Ethylene diamine tetraacetic acid
Fe Mo-Co	Iron-molybdenum-cofactor
xg	multiples of gravitational constant
GOGAT	Glutamine-oxo-glutamate amino transferase
GS	Glutamine synthetase
GSH	Reduced glutathione
HEPES	N-2-hydroxy ethyl piperazine-N'-ethanesulfonic acid
het	Gene coding for heterocyst differentiation
Het$^-$	Phenotype indicating absence of heterocysts
LDH	Lactate dehydrogenase
Mpa	Mega pascal
MV	Methyl viologen
NADH	Nicotinamide Adenine Dinucleotide (reduced)
nif	Genes coding for 'nitrogen fixation'
ntr	Genes coding for overall 'nitrogen regulatory system'
Nif$^-$	Phenotype indicating inability to fix nitrogen

Nar"	Phenotype indicating inability to utilise NO_3^- as nitrogen source
Nif$^+$	Phenotype indicating nitrogen fixing ability
Nar$^+$	Phenotype indicating ability to utilise nitrate
PS I	Photosystem 1
PS II	Photosystem II
Tricine	N-tris [hydroxymethyl]-methyl glycine
Tris	Tris-(hydroxymethyl) amino methane
TE	Tris EDTA buffer
v/v	Percent 'volume in volume' number of cubic centimeters of a constituent in 100 cm^3 of solution
w/v	Percent 'weight in volume' number of grams of constituent in 100 cm^3 of solution.

CHAPTER I

INTRODUCTION

Cyanobacteria are oxygenic photosynthetic prokaryotes a majority of which are diazotrophs as well. The group represents the culmination of evolution of morphological organisation characteristic of chlorophyceae (Fritsch, 1945; Smith, 1955; Gietler, 1960) N_2-fixing property of the group occur widely in unicellular, filamentous nonheterocystous and filamentous heterocystous forms. However the localisation of the N_2-fixing enzyme nitrogenase and the ways in which it is protected from oxygen are characteristically different in the three subgroups. Their ability to fix nitrogen phototrophically has made them very attractive candidates for photobiological production of ammonia from dinitrogen under aerobic growth conditions (Stewart et al., 1987). This similarity in structural and functional organisation of cyanobacterial photosynthetic apparatus, with that of chloroplasts have made them a model system for molecular analysis of photosynthetic process (Golden et ah, 1985). The great degree of similarity in cyanobacteria) photosynthetic apparatus and chloroplasts was noticed long back in the past and this fascinated Mereshkowsky in 1905 to suggest the origin of present day Chloroplast from photosynthetic prokaryotic ancestors like cyanobacteria, through the process of endosymbiosis.

Subsequent studies at molecular level have strengthened this notion and experiments are being attempted to examine the expression of E. coli plasmids in cyanobacteria as model system of analogous expression in chloroplasts (McFadden and Daniell, 1988).

In addition, the N_2-fixing cyanobacteria have been documented to contribute substantial amount of fixed nitrogen to many natural and artificial ecosystems and importance of such a role has been specially emphasised in tropical agriculture with reference to rice cultivation (Singh, 1961; Fogg, 1973).

The cyanobacterial group has attracted special attention for studies on N_2-fixation and its relation to photosynthesis and respiration. In fact the entire period of such studies can be broadly seggregated into three parts, the first part extending from 1889 to 1953 concerns mainly with the discovery of nitrogen fixation by various cyanobacteria. The second part extending from 1954 to 1966 was involved in understanding the physiology of the process under well defined laboratory conditions and the third period extending from 1967-1973 concerns with localisation and characterisation of nitrogenase enzyme activity under growth conditions (Stewart, 1974). Since 1973 until today molecular techniques of DNA analysis have gone into discovering a novel mechanism of gene rearrangement in N_2-fixing genes during heterocyst differentiation in cyanobacterial forms like Anabaena sps. (Haselkorn et al., 1986). A clear understanding of the process of N_2-fixation by itself or associated with heterocyst differentiation as well as that of oxygenic photosynthesis is a must to manipulate them for practical purposes. However, such an understanding at molecular level is not possible without understanding the genetics of the two major primary processes at molecular level.

The lack of study on genetics of cyanobacteria until the beginning of sixtys was mainly because of treating these groups alike to other algae by traditional phycologists, who **were** unfamiliar with the tools and genetics of bacteria. With the growing realisation that the group, like bacteria is prokaryotic, indicated of mutation and gene transfer system of bacterial nature in them. The introduction of techniques of bacterial genetics in the study of cyanobacterial genetics was introduced by Kumar in 1962, who found evidence for apparent genetic recombination of antibiotic resistance markers like Streptomycin and Penicillin. The work of Pikalek (1967) questioned the work of apparent genetic recombination by Kumar on the basis of instability of Penicillin resistance marker, however Bazin (1968) confirmed the occurrence of genetic recombination for stable antibiotic resistance markers in Anacystis nidulans. Mutation and recombination for antibiotic resistance markers were further demonstrated in Cyanobacteria by Singh et ah (1966).

Mixed cultures of genetically marked strains of Cylindrospermum majus also provided evidence for genetic recombination (Singh and Sinha, 1965). Singh (1967) had demonstrated genetic recombination for sporulating markers in mixed cultures of Anabaena doliolum. These studies merely indicated that genetic recombination do occur for certain markers in mixed cultures of cyanobacteria without specifying the nature of gene transfer system. These investigations were also of preliminary nature without being in any way adequate for mapping of cyanobacterial genes.

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Availability of wide range of mutants of stable nature is the basic requisite for understanding the structure and function of cyanobacterial gene as revealed by fine gene structure analysis through the method of genetic recombination. Occurrence of spontaneous as well as induced mutations in cyanobacteria has been fully established for antibiotic resistance, amino acid analogue resistance, morphological and auxotrophic markers and pigment markers (Ladha and Kumar, 1978). The chemical mutagens extensively used for generating variety of such mutants in unicellular and filamentous cyanobacteria include N-methyl-N-nitro-N-Nitrosoguanidine (NTG) and ultraviolet radiation. The frequency of spontaneous and induced mutants of cyanobacteria is reported to fall within the range characteristic of similar mutation frequencies for similar mutants in bacteria.

The biochemical and physiological characterisation of mutants affected in nitrogen metabolism have provided useful information about genetic linkage between heterocyst and nitrogenase and vegetative cells (Stewart and Singh, 1975; Ladha and Kumar, 1977; Singh and Singh, 1978; Singh et ah, 1983). Mutants defective in endogenous dark metabolism have similarly provided significance of oxidative pentose phosphate pathway in the physiology of Anacystis nidulans (Doolittle and Singer, 1974). Similarly physiological and biochemical analysis of various classes of chlorate-resistant mutants has provided the nature of functional interrelationship between nitrate reductase enzyme and nitrogenase enzyme (Bagchi and Singh, 1984).

Gene transfer system of nif and het genes have **been found** to operate in mixed cultures of Nostoc muscorum (Stewart and Singh, 1975; Singh and Padhy 1978; Singh, 1982). Such a gene transfer system in Nostoc muscorum might be mediated by DNA is indicated by the work of Trehan and Sinha (1981). However mode of gene-transfer system remains as ambiguous in such experiments as those reported for mixed cultures of unicellular forms. Similarly such recombinational studies have not much helped in analysing the structure and organisation of cyanobacterial genes.

Studies on transformation started in 1970 when Shestakov and Khyen found DNA extracted from Erythromycin and Streptomycin resistant mutants and from filamentous mutants of Anacystis nidulans was able to transform the appropriate genetic marker in antibiotic sensitive non-filamentous recipient cells respectively. Herdman and Carr (1971) confirmed the process of transformation in this strain and also described in it a novel system of transformation mediated by extracellular DNA/RNA complex. Herdman (1973) further reported that transformation of A. nidulans using both chemically extracted and extracellular nucleic acids, was associated with mutation. Orkwiszewski and Kanney (1974) confirmed transformation of Streptomycin marker and Phenylalanine and Ornithine markers in A. nidulans. These studies on transformation involved various mutant strains of the same species. The next attempt was to see whether intergeneric transformation is possible within unicellular cyanobacteria. Intergeneric transformation for Streptomycin resistance marker between Gloeocapsa alpicola and Anacystis nidulans has also been demonstrated. Devilly

and Houghton (1977), Stevens and Porter (1980) studied the process of DNA mediated transformation in Agmenellum quadruplicatum. Grigorieva and Shestakov (1976 and 1982) also further showed working of DNA mediated transformation as an efficient means of gene transfer in A. nidulans R2 and Synechocystis 6803 respectively. These studies confirm that unicellular cyanobacteria possess a natural system of transformation and that this mode of gene transfer system could be utilised in study of gene structure and functional relationship through modern tools of mutagenesis and recombination.

The gene transfer based methods of mutagenesis include transposon mutagenesis, recombinational mutagenesis and site directed mutagenesis. In such studies a plasmid with identifiable phenotype of antibiotic resistance nature is the basic requirement in addition to it being capable of replication in cyanobacteria and its alternate host. The cyanobacterial plasmids so far known are all cryptic lacking any identifiable phenotypic marker. This limitation has been overcome by construction of shuttle-plasmid vectors containing bacterial antibiotic resistance markers like Ampicillin, Chloramphenicol and Streptomycin and are capable of replication in cyanobacterial host and in E. coli. This method has been followed successfully and bacterial genes of antibiotic resistance nature have expressed themselves in cyanobacteria] system. However, the level of resistance expressed in cyanobacterial background is not as high as that in bacterial background (Van den Hondel et ah, 1980; Kuhlemeier et al., 1981; Tan deau de Marsac et al., 1982; Sherman and Van De Putte, 1982; Golden and Sherman, 1983; Chauvat et al., 1983). Conditions for optimising transformation

frequency have been worked out for unicellular forms (Chauvat et al., 1983; Williams and Szalay, 1983). Now transformation has become very powerful tool for cloning of cyanobacterial or bacteria! genes in either cyanobacterial or bacteria! host.

Transformation as a system of gene transfer has remained unreported until recently. Transformation for azide assimilation phenotype has however being recently shown in A. doliolum (Singh et al., 1987). Herbicide resistance gene of diazotrophic Gloeocapsa strain has been transformed to N. muscorum (Singh et al., 1987). Thus transformation system can also be effectively used in genetic analysis of heterocystous forms in a way it has been done for unicellular forms.

Wolk and his group have developed methods for conjugal transfer of gene from E. coli into cyanobacteria in a triparental cross using the broad host range conjugal plasmid RP4 (Wolk et al., 1984). The two basic requirements for a plasmid to be mobilised by a conjugal plasmid are, first presence in it of a bom site also called as Ori T region and second provision for mob gene which provides the protein for DNA nicking function. The success of Wolk et al., group in transfer of shuttle plasmids from E. coli to cyanobacteria, unicellular or filamentous is based on these considerations. An improved method of conjugal transfer of shuttle plasmid has been demonstrated by McFarlane et al. (1987) in which conjugation is biparentaJ and not triparental. Recently electroporation method has been used successfully in transforming heterocystous forms with shuttle-plasmid vector

containing **two** selectable antibiotic resistant markers. It will **not** be **too** long before transformation as a routine system of gene transfer becomes a common laboratory technique.

The development of heterologous hybridisation, gene probes have provided a very powerful tool for analysing gene structure and function in cyanobacteria without involvement of gene transfer method, a series of such heterologous nif probes and Chloroplast gene probes have helped in studying the organisation and distribution of nif genes in heterocystous and nonheterocystous cyanobacteria and various photosynthetic genes in unicellular and filamentous cyanobacteria (Rice et al., 1982; Haselkorn et al., 1987; Brusslan and Haselkorn, 1989).

Jn this thesis, conditions for successful transformation and the nature of markers used in monitoring the process in homologous and heterologous combinations have been described. They have lead to the following basic information for generating transformation in heterocystous forms like N. muscorum, A. doliolum and N. calcicola and in unicellular forms like Synechococcus sps. The source of genetic marker has been DNA from mutant strains of Nostoc muscorum in all cases except in the case of Ampicillin resistance, plasmid pBR322 derived from E. coli was used. The heterocystous cyanobacteria are not naturally competent organisms for transformation and their preparation of permeaplast population, induced artificially is the first basic requirement. DNA mediated genetic transformation for herbicide resistance and amino-acid analogue resistance in homologous and

heterologous combinations is (a) DNase sensitive, (b) DNA concentration dependent (c) DNA contact period dependent (d) photosynthetic light dependent. These phenotypic markers were scored by selecting spontaneously occurring mutants of N. muscorum in the presence of growth inhibitory concentration of the selective agent. Transformation for Streptomycin resistance marker was unsuccessful in homologous or heterologous combinations. These results suggest marker specific nature of transformation process in heterocystous forms. N. muscorum and N. calcicola were naturally resistant to Ampicillin, while A. doliolum and Synchococcus sps were sensitive. Ampicillin resistance marker of pBR322 could not be transformed to A. doliolum but Synechococcus sps was readily transformable with the plasmid marker. These results again suggest occurrence of a natural barrier for successful transformation with antibiotic resistance marker, whether chromosomal borne or plasmid borne in heterocystous forms.

Nature and number of genetic determinants required for nitrate assimilation in N. muscorum were analysed biochemically and physiologically in mutant strains resistant to growth inhibition like chlorate. Mutational analysis has led to identification of three distinct determinants, one controlling nitrate transport across the cell membrane, another controlling Mo-cofactor production necessary for nitrate reductase activity and the third mediating the level of apoprotein component of nitrate reductase.

The results of mutant studies and transformation studies and the conditions under which they have been scored and analysed have been discussed in detail in relevant chapters of the present thesis.

CHAPTER II

MATERIALS AND METHODS

Organisms and Maintenance

Parent Strains:

The parental strains of Nostoc muscorum, Nostoc calcicola and Anabaena doliolum are unbranched, filamentous cyanobacteria capable of growth at the expense of N_2 as nitrogen source under photoautotrophic growth conditions, in modified Chu-10 medium as described by Gerloff et al (1950). Under these conditions, 5-6% of the vegetative cells get differentiated into regularly spaced heterocysts along the filament. The combined nitrogen-free medium is represented as N_2-medium and when it is supplemented with a final concentration of 5 mM KNO_3, 5 mM $NaNO_2$ and 1 mM NH_4Cl (buffered with 50 mM HEPES-NaOH, pH 8.5), represented as NO_3^-, NO_2^- and NH_4^+ media respectively. Addition of NO_3^-, NO_2^- or NH_4^+ inhibits heterocyst formation and nitrogen-fixation under diazotrophic growth conditions (Stewart and Singh, 1975). Synechococcus sp is a unicellular non-diazotrophic cyanobacterium and is a natural isolate from paddy fields. It was maintained and grown always in Chu-10 nitrate medium.

Axenic batch cultures of these strains were grown in liquid medium in air-conditioned illuminated culture room at a photon fluence rate of 50 μ mol $m^{-2}s^{-1}$ and temperature of 28±2°C. Clonal cultures were maintained on agar slants which were prepared by adding 1.2% (v/v) Agar-Agar to the liquid medium before autoclaving.

Escherichia coli strain was grown in LB (Luria Bertani) medium. Most of the time it was maintained in stab cultures and for long-term storage

15% glycerol was added and stored at low temperatures. Before the experiment, culture from the stab was streaked on to Ampicillin containing plates and then from here a single colony was picked up and inoculated into 10 ml LB medium with Ampicillin.

Culture media:

The modified Chu-10 medium as described by Gerloff et al (1950), having the following composition was used for growing the cyanobacterial strains, Nostoc muscorum, Nostoc calcicola and Anabaena doliolum.

Concentration of Stock solutions ($g.l^{-1}$)

Macronutrients		Micronutrients	
1. Calcium chloride	11.0	1. Boric acid	2.86
2. Dipotassium hydrogen phosphate	**2.0**	2. Copper sulphate	0.08
3. Magnesium sulphate	**5.0**	3. Manganous chloride	1.81
4. Sodium carbonate	**4.0**	4. Sodium molybdate	0.02
5. Sodium silicate	**8.8**	5. Zinc sulphate	0.22
6.a.Citric acid	**0.6**		
b.Ferric citrate	**0.6**		

Preparation of final medium for use

5 ml each of the macronutrient and 1 ml of micronutrient solution was taken in a final volume of 1 1 of double distilled water and pH was adjusted to 8.5-9.0 before autoclaving. NO_3^-, NO_2^- and NH_4^+ were added to the above medium when required.

Escherichia coli HB101 was grown in LB medium and the composition as described in Maniatis et al (1982) is as follows:

LB medium ($g\text{-}l^{-1}$)

Bacto-tryptone	10.0
Bacto-Yeast extract	5.0
Sodium chloride	10.0

The pH of the medium was adjusted to 7.5 with sodium hydroxide before autoclaving. Solid medium was prepared by adding 1.2% of Agar-Agar to the medium.

Sterilisation:

Culture media were sterilised prior to inoculation by autoclaving at 15 $lbs/inch^2$ pressure and 121°C temperature for 15 minutes.

For plates:

The autoclaved medium was allowed to cool to 50°C or 48°C before the desired concentration of herbicide or antibiotic was added to the medium, just before pouring it into the plates. To the liquid medium 1.2% of Bacto-agar was added for solidifying the medium.

Growth measurements:

Cyanobacterial cultures are homogenous in liquid medium and therefore, a direct absorbance change at 665 nm is proportional to its multiplication and can be used for growth measurements, but growth also results in increase of biomass, therefore parameters like chlorophyll and protein will also facilitate measurement of growth in cyanobacteria. The growth in the present study was usually measured at regular intervals by estimating chlorophyll.

Estimation of Chlorophyll;

To determine **the** chlorophyll content, 5.0 ml samples of the culture were withdrawn and centrifuged at 2000 rpm for 15 mins and the supernatant was discarded. To the pellet equal volume of 100% methanol was added and shaken thoroughly. Then the tubes were kept in water bath maintained at 60°C for 10 mins. The solution was again centrifuged at 2000 rpm for 10 mins and optical density of the chlorophyll extract was measured at 665 nm. The chlorphyll content was estimated using the following formula:

$$13.42 \times A_{665} = \text{ug chlorphyll/ml}$$

(Mackinney, 1941)

Estimation of cellular protein:

Protein was estimated according to the procedure of Lowry et al (1951). The protein in the crude extracts was estimated after precipitating with an equal volume of 10% TCA (Bailey et al, 1967). The mixture was left overnight at room temperature and then centrifuged at 3000xg for 10 minutes. The precipitate was dissolved in 1N NaOH and an aliquot of it was withdrawn for estimating the protein content. A standard curve for protein estimation was prepared by using BSA as standard.

Measurement of Heterocyst frequency

Heterocyst frequency was calculated as the number of heterocysts per 100 vegetative cells by light microscopic observation of the filaments of the cyanobacterium used.

Cells were counted with the help of Haemocytometer.

Measurement of electron transport activities:

Cyanobacterial cells were harvested and washed twice with harvesting buffer consisting of 20 mM HEPES-NaOH, pH 7.5; 1 mM $CaCl_2$ and 7.5% PEG-4000 and resuspended in the same buffer. The cell suspension was centrifuged at 10,000xg for 10 min. Then, the cells were suspended in reaction buffer consisting of 25 mM HEPES-NaOH, pH 7.5, 20 mM NaCl. These cells were used for measuring PS1I activity.

Assay of electron transport activities

The photochemical activities (PS1I and PSI) were measured by following the method of Robinson et al (1982). Assays of electron transport activities were carried out using the thermostated (25±1°C) glass cuvette, fitted within a clark-type O_2 electrode (Yellow Spring Instrument Co., USA). The cells were illuminated with white saturating light (450 W m) at the surface of the vessel obtained from a projector. Thermostated water bath was used to maintain the temperature (25°C) of the reaction mixture constant during the illumination. The changes in the oxygen concentration were recorded.

Photosystem II assay

p-Benzoquinone (pBA) mediated electron transport activity of PSII (H_2O → pBQ) was used for intact cells. Being a lipophilic compound BQ enters into the intact cells and accepts electrons at the plastoquinone position (Trebst, 1974). The reaction mixture in a final volume of 3.0 ml

consisted of reaction buffer, 0.5 mM pBQ and the intact cells equivalent to 12-15 µg Chl a. The samples were illuminated with the help of a 300 W projector lamp which provided a saturating incandescent light (450 W m^{-2}) on the surface of the sample cuvette.

Preparation of spheroplasts

Spheroplasts for measurement of PS1 activity of Nostoc muscorum and herbicide-resistant mutant strains were prepared by following the method of Peter and Sherman (1978). The cyanobacterial cells in exponential growth phase were harvested and washed with 20 mM Tricine-KOH buffer (pH 7.5) containing 400 mM sucrose, 10 mM KCJ and 10 mM EDTA. The cells were suspended in the same buffer and incubated with 0.5 mg. ml^{-1} of Lysozyme for 15-20 mins at 37°C. After Lysozyme treatment, the cyanobacterial cells were centrifuged at 5,000xg for 15 min and used for measurement of photosystem I activity.

Photosystem I assay

Photosystem 1 (PSI) mediated electron transport activity was estimated as a measure of the oxygen uptake due to MV photoreduction and its subsequent autooxidation with 2,6-dichlorophenol indophenol (DCIP) and sodium ascorbate donor couple. The experimental set up was the same as described for PSII assay. Three ml of the reaction mixture consists of 25 mM HEPES-NaOH, pH 7.5; 100 uM, DCIP, 2 mM sodium ascorbate; 0.5 mM MV; 1 mM sodium azide and 10 uM DCMU. Spheroplasts equivalent to 15-20 ug Chl a, was used for each estimation (Robinson et al, 1982).

Photochemical activity estimated in terms of O_2 exchange has been expressed as µmol O_2 evolved or consumed mg Chl a^{-1} hr^{-1}. The amount of O_2 exchanged was calculated using the dissolved oxygen concentration of 235 n mol. ml^{-1} at 25°C (assay temperature).

Measurement of O_2 evolution

O_2 evolution was measured with a Clark-type O_2 electrode. The cyanobacterial cells were deposited on a flat platimum cathode that was polarised at 0.6V with reference to a large Ag/AgCl electrode. The electrodes were immersed in an electrolyte (consisting of 0.05 M phosphate buffer at pH 7.8, 0.1 M KC1). The electrode was separated from the magnetically stirred assay medium by a Teflon membrane. The difference between the output of the electrode in water in equillibrium with air and water in equilibrium with pure nitrogen was considered to represent 0.235 µmol. ml^{-1} in the assay medium. After injection of the sample into the assay medium, the medium was illuminated from opposite sides with projector lamps. The rate of O_2 evolution was determined from the initial slope of electrode output as a function of time.

Assay procedures for enzyme activities

Nitrogenase

Nitrogenase activity was measured using whole cells by estimating the acetylene reduction activity as described by Stewart & Lex (1970). Assay was done in test tubes fitted with serum stoppers. Air was removed from the tubes containing cyanobacterial cells (10-15 µg

Chl a) by evacuation **and replaced** by the desired gas phase which contained 10% (v/v) acetylene. These tubes were incubated with intermittent shaking at 28±2°C and a photon fluence rate of 50 $\mu mol\ m^{-2} s^{-1}$. After 1 hr of incubation, ethylene production was assayed gas chromatographically using a Perkin-Elmer Sigma 3B Gas Chromatograph fitted with a hydrogen flame ionization detector and a column of Porapak R. The nitrogenase activity was expressed in terms of u mol C_2H_4 formed g Chl $a^{-1}.hr^{-1}$.

Glutamine Synthetase assay

Glutamine synthetase can be assayed under in vitro conditions.

Cultures were harvested by centrifugation at 2000 rpm and washed with buffer A [Tris-HCl pH 7.5] followed by washing with buffer B [Buffer A+5mM $MgCl_2$+10mM Na glutamate + 5mM 2-mercapto ethanol and 1 mM EDTA pH 7.5].

The pellet was resuspended in buffer B and cells were broken using liquid nitrogen and the cell extract thus obtained was centrifuged at 30,000g using Rerm C-24 Model cooling centrifuge for 30 minutes.

Clutamine Synthetase (Transferase) assay

The enzyme was assayed using the method of Sampaio et al (1979).

The reaction mixture in a total volume of 1 ml contained the following concentrations:

40 mM	Tris-HCl (pH 7.0)
3 mM	$MnCl_2$
20 mM	K-arsenate
0.4 mM	Na-ADP
60 mM	Hydroxyl amine neutralised with 2N NaOH before use
30 mM	Glutamate
Enzyme	extract

The reaction was allowed to proceed for 10 minutes at 30°C in dark. Finally 2 ml of stop mixture [4 ml of 10% $FeCl_3$, 1 ml of 24% TCA + 0.5 ml of 6N HC1 + 6.5 ml of water] was added to the reaction mixture and the absorbance of the supernatant was measured at 540 nm after centrifugation for 10 minutes at 2000 rpm. The transferase activity was measured using the following formula:

$$\frac{\mu \text{ moles of Y-glutamyl hydroxymate} \times 1000}{\text{Mg of protein} \times 10} \sim \text{n moles of glutamyl hydroximate formed } \mu\text{g protein}^{-1} \text{ min}^{-1}.$$

Glutamine Synthetase (Biosynthetic) assay

Glutamine synthetase (biosynthetic) activity was measured by the method of Kingdon et. al (1968). Mg^{++} dependant production of ADP. is coupled to oxidation of NADH. Release of inorganic phosphate from Adenosine Triphosphate shows the presence of glutamine synthetase.

Reagents	µ moles
Na glutamate	60
ATP	3
Tris HC1 (pH 7.5)	150
NH_4Cl	200
KCl	150
$MgCl_2$	150
NADH	0.45
PEP	0.5
Lactate dehydrogenase	20 units
Pyruvate Kinase	8 units

1 ml of enzyme extract and 0.2 ml each of the reagents is added. Optical density was measured at 340 nm in Hitachi Spectrophotometer.

$$\text{Activity} = \frac{A_{340m}/\text{min}/100}{2.07 \times \mu g \text{ protein}} = \text{n moles of NADH oxidized} \mu g \text{ protein}^{-1} \text{ min}^{-1}$$

Chemicals

DCMU: Trade name of 3(3,4-dichlorophenyl)1,1 dimethyl urea and was obtained from Sigma Chemicals Limited. It was dissolved in isopropanol and the final concentration of the stock was made upto 1 mM with sterilized double distilled water.

Atrazine: Trade name of 2,chloro 4-(ethyl amino)-6-isopropyl amino-S-triazine, obtained from BASF India Limited. It was dissolved in dimethyl

sulfoxide and the final concentration was made upto 1 mM with double distilled water.

L-Ethionine, Ampicillin, Streptomycin and Chloramphenicol were obtained from Sigma Chemical Company, St. Louis, USA. All other chemicals used were either purchased from Sigma Chemical Company or were of analytical grade.

CHAPTER III

MUTATIONAL ANALYSIS OF NITRATE ASSIMILATION

INTRODUCTION

Diazotrophic and nondiazotrophic cyanobacteria are capable of efficient photoautotrophic growth at the expense of nitrate as nitrogen source. The enzymes involved in reduction of nitrate to ammonia are ferredoxin dependent nitrate reductase and nitrite reductase which occur together in particulate form in photosynthetic lamellae (Losada et al, 1981; Flores et al, 1983). The source of reductant for the reductive assimilation is oxygenic photosynthesis and it has been shown that the process of oxygenic photosynthesis is more directly linked to photo-assimilation of nitrate than to photoassimilation of CO_2 (Candau et al, 1976). The activity of assimilatory nitrate reductase is crucial for the efficiency of nitrate as nitrogen source. The processes limiting the rate of assimilatory nitrate reduction in intact cyanobacterial cells could be nitrate uptake or nitrate reductase.

Some basic information about the regulation of nitrate assimilatory system has been worked out in Nostoc muscorum. The enzyme is constituted of apoprotein and Mo-cofactor. Ammonia has been shown to cause repression of apoprotein level and synthesis of Mo-cofactor has been found constitutive occuring in cultures growing with N_2, nitrate or ammonia as nitrogen source. The apoprotein synthesis resumes under N_2-fixing conditions or nitrate assimilating conditions (Bagchi et al, 1985). N_2-fixing heterocystous filaments have been shown to contain active nitrate reductase in vegetative cells but not in heterocysts and the lack of reductase activity in heterocyst has been shown to be due

to absence of apoprotein level and not of Mo-cofactor level (Kumar et al, 1985). Ammonia was found repressible to nitrate uptake system as well and nitrate was required as an activator of the uptake system activity (Bagchi and Singh, 1984).

Recently newly developed techniques for gene cloning in Anacystis nidulans have been employed to investigate number of genes involved in nitrate reduction and this approach has resulted in identifying three distinct genes controlling nitrate reductase mediated nitrate assimilation in the unicellular cyanobacterium (Kuhlemeier et al, 1983 and 1984). Such an approach has not yet been tried in any other cyanobacteria. Here mutational method of genetic analysis has been used for identifying the possible number of genetic determinants controlling nitrate reduction in N. muscorum.

Chlorate the structural analogue of nitrate enters the cell and gets metabolised to toxic chlorite ion thus causing growth inhibition and cell lethality. Selection for chlorate resistance has thus become a standard practice and method for the isolation of mutants impaired in nitrate nutrition (Singh and Sonie, 1977; Bagchi and Singh, 1984). Molybdenum is an essential micronutrient for growth on N_2 or nitrate as nitrogen source (Bortels, 1930; Wolfe, 1954; Brill, 1975). Such a requirement of molybdenum lies in the very nature of N_2-fixing enzyme, nitrogenase and nitrate reducing enzyme, nitrate reductase both of which are molybdoproteins (Pienkos et al, 1977). Processing of molybdenum into a catalytically active form (Mo-cofactor) of nitrate reductase occurs by its specific association with a low molecular weight cofactor

of pterin nature and such a **Mo-cofactor,** which forms **an** integral, structural and functional component of active nitrate reductase from various phylogenetic sources has been isolated and characterised **in** detail (Arst *et al*, 1970; Amy and Rajagopalan, 1979). Nitrogenase, the other molybdoenzyme contains molybdenum in the catalytic active form of iron-molybdenum cofactor (FeMo-cofactor) (Shah and Brill, 1977; Kennedy and Taukdarian, 1987).

Mutations impairing molybdenum nutrition are expected to confer chlorate-resistant phenotype. Such mutants are likely to be defective in nitrogenase activity and nitrate reductase activity. Monogenic mutants of Rhizobium meliloti defective in N_2-fixation **(Nif** ~) and nitrate assimilation (*Nar*$^-$) has been reported, but without information about the molecular nature of defects in such mutants (Kondorosi *et al*, 1973). A class of chemically induced mutations in the *ntr* A gene of *Azotobacter vinelandii* conferring chlorate resistance has been isolated and found to be phenotypically *Nif*$^-$ *Nar*$^-$ thereby demonstrating a definite role of *ntr* A gene product in positive regulation of N_2-fixation and nitrate assimilation in this bacterium (Santero *et al*, 1986). Chlorate-resistant mutants of *Nostoc muscorum* defective in N_2-fixation or nitrate assimilation have been reported but without any information on the nature of biochemical defects in such mutants (Singh and Sonie, 1977; Bagchi and Singh, 1984).

We have isolated various phenotypic classes of chlorate resistant (Clr-R) mutants of the cyanobacterium *Nostoc muscorum* and analysed them biochemically to assess the nature of biochemical defects in such

mutants. We present here evidences to show that chlorate-resistant phenotype of the cyanobacterium results from mutations in four distinct types of genetic determinants controlling assimilation of N_2 or nitrate as nitrogen source.

EXPERIMENTAL PROCEDURE

Isolation of Spontaneous Chlorate-resistant (Clr-R) mutants:

Axenic clonal cultures of parental Nostoc muscorum were routinely grown in CHU-10 medium (Gerloff et al, 1950) free of combined nitrogen source (N_2-medium) or containing 5 mM KNO_3 (NO_3^--medium) or 1 mM NH_4Cl (NH_4^+-medium) supplemented with 1 ug ml^{-1} of Na_2MoO_4. Cultures for isolating spontaneous chlorate-resistant mutants were grown in liquid N_2-free medium also called N_2-medium.

Chlorate ($KClO_3$) at a concentration of 4 mM in N_2 medium was completely growth toxic to the parent Nostoc muscorum, therefore a chlorate concentration of 8 mM was used for selecting spontaneous mutants for chlorate resistance.

The N_2-grown cultures were harvested, washed, fragmented and spread onto chlorate-containing (8 mM) N_2 medium. The inoculated plates were incubated for growth for a week in the culture room at a temperature of 28 ± 2°C and a photon fluence rate of 50 µmol m^{-2} S^{-1}. Later they were divided into three sets : one set was overlayered with 5 mM KNO_3 containing agar medium, the second set with 1 mM NH_4Cl containing agar medium and the third set served as a control. The colonies growing on these three culture sets were all picked up

separately and transferred into 1 mM NH_4Cl containing medium for growth and stock cultures.

Measurement of growth, heterocyst frequency, nitrogenase activity and nitrate reductase activity:

Growth measured in terms of chlorophyll, heterocyst frequency and nitrogenase activity measured in terms of acetylene reducing activity (ARA) were estimated as described in Chapter II.

Nitrate reductase:

Cells of Nostoc muscorum in the exponential phase of growth were harvested by low-speed centrifugation, washed twice with 50 mM Tris-HCl, pH 7.5 buffer containing 0.1 M NaCl, 0.3 M sucrose and 1 mM EDTA and resuspended in the same buffer. This suspension was sonicated at *WC* for 6-8 mins. The broken material was centrifuged at 30,000xg for 30 min and the resulting supernatant was used as crude enzyme.

The method of Manzano et al (1976) as described in Bagchi and Singh (1984) was followed for estimating nitrate reductase activity. The enzyme activity was measured colorimetrically following the appearance of nitrite using methylviologen chemically reduced to dithionite as electron donor. The reaction mixture contained in a final volume of 1.0 ml : Glycine KOH buffer, pH 10.5, 100 µmoles; 20 µmoles KNO_3; 4µ moles methyl viologen; 2 mg $Na_2S_2O_4$ in 0.1 ml of 0.23 M $NaHCO_3$, and an appropriate amount of enzyme. This was incubated at 30°C for 10 min and the reaction was terminated by the

addition of 0.2 ml of 1.0 M zinc acetate and the nitrite thus formed was estimated by following the method of Snell and Snell (1966).

Growth of Escherichia coli Strain W:

Escherichia coli strain W was grown under aerobic conditions in minimal medium composed of the following in (g/1) : Na_2HPO_4 7.0 g; KH_2PO_4 3.0 g; NaCl 0.5 g; NH_4Cl 1.0 g and casamino acids 0.02%. The following components were autoclaved separately and added aseptically (per 1) : 1 M $MgSO_4$, 0.2 ml; 20% glucose, 1.0 ml: 1 M $CaCl_2$, 0.01 ml. The pH of the medium was adjusted to 7.4. The above minimal medium was supplemented with 1 mM $Na_2Mo\ O_4$. Cells were grown at 37°C with vigorous shaking on a rotary shaker. Bacteria were harvested during exponential growth phase and used for isolation of Mo-cofactor.

Preparation of Mo-cofactor from E.coli strain W:

Mo-cofactor was prepared from E.coli by following the method of Miller and Amy (1983) as given in Kumar et al. (198.5). E.coli cells were harvested by centrifugation, washed and resuspended in 100 mM potassium phosphate buffer, pH 7.4 containing 0.5 mM EDTA and 1 mM ß-mercaptoethanol. These cells were passed through French pressure cell at 110 MPa to break the cells. After centrifugation of the broken cells at 30,000xg for 15 mins, the resulting supernatant was used for Mo-cofactor assay.

Preparation of Nitrate reductase apoprotein (cofactor-free nitrate reductase:

Mo-cofactor free nitrate reductase (nitrate reductase apoprotein) was derived from W-resistant mutant (W^R) of Nostoc muscorum

as described by Bagchi et al (1985). Since W^R mutant was grown in tungsten (W) medium lacking Mo it synthesized W-cofactor instead of Mo-cofactor and W is more loosely bound and therefore easy to remove than Mo and therefore this mutant was used as a source for preparation of Mo-cofactor free nitrate reductase to be used in complementation test.

N.muscorum was grown in tungsten containing (50 µg/l) in nitrate medium and cells were harvested, washed and resuspended in 50 mM Tris-HCl buffer, pH 7.5 containing 100 mM NaCl, 300 mM sucrose, 1 mM EDTA and 5 mM $MgCl_2$. This suspension was sonicated for 10 mins at 4°C with 30 sec. bursts punctuated by 30 secs rest periods, followed by centrifugation at 30,000xg for 20 min at 4°C. The supernatant containing nitrate reductase was made free of Mo-cofactor by acid and subsequent neutral treatments. 200 µl of cell-free extract was incubated with 200 µl of molybdate-GSH solution (10 mM GSH and 5 mM sodium molybdate in 100 mM sodium acetate buffer, pH 4.5). This brought the pH of the reaction mixture to 4.8. After 30 sec-60 secs incubation at 30°C (acid incubation) the pH was adjusted to 7.2 by adding 200 mM K_2HPO_4 and incubated for 15-20 mins at 30°C (neutral incubation).

Preparation of Mo-cofactor from various strains of N.muscorum grown in different nitrogen sources:

The method described in Bagchi et al (1985) was followed for the isolation of Mo-cofactor. Cultures of various strains of N.muscorum grown in different nitrogen sources containing molybdenum were harvested

by centrifugation, washed and resuspended in Tris buffer (50 mM, pH 7.5) containing 100 mM NaCl, 300 mM sucrose, 1 mM EDTA and 5 mM $MgCl_2$. The cell free extract was prepared by sonicating the sample for 10 mins at 4°C with 30 secs bursts punctuated by 30 secs rest periods, followed by centrifugation at 30,000xg for 20 min at *WC.* Supernatant was used as source of Mo-cofactor for complementation analysis.

Complementation assay procedure for Mo-cofactor activity:

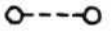
The method by Miller and Amy (1983) as described by Bagchi et al (1985) was followed for measuring reconstituted nitrate reductase activity by having mixed equal volumes of the nitrate reductase apoprotein preparation from W-containing, nitrate grown, W⁻⁻ mutant of N.muscorum and the Mo-cofactor prepared from various sources. This mixture was incubated for 10 mins at 30°C to achieve complementation and nitrate reductase activity was measured.

RESULTS

Growth pattern of parent Nostoc muscorum in the presence **and** absence of chlorate:

Figure 1 shows the growth of parent N.muscorum in the presence of 4 mM of chlorate and in the absence of chlorate in N_2-medium. Chlorate at this concentration was found to be growth inhibitory to N.muscorum parent, therefore, a higher concentration of 8 mM chlorate had been used for the isolation of chlorate resistant mutants. The frequency of mutation to chlorate resistance was found to be 1.7×10^{-7}.

Fig. 1 Growth pattern of parent Nostoc muscorum in N_2-medium, in the absence and presence of 4 mM of chlorate.



Parent grown with chlorate

Parent grown without chlorate

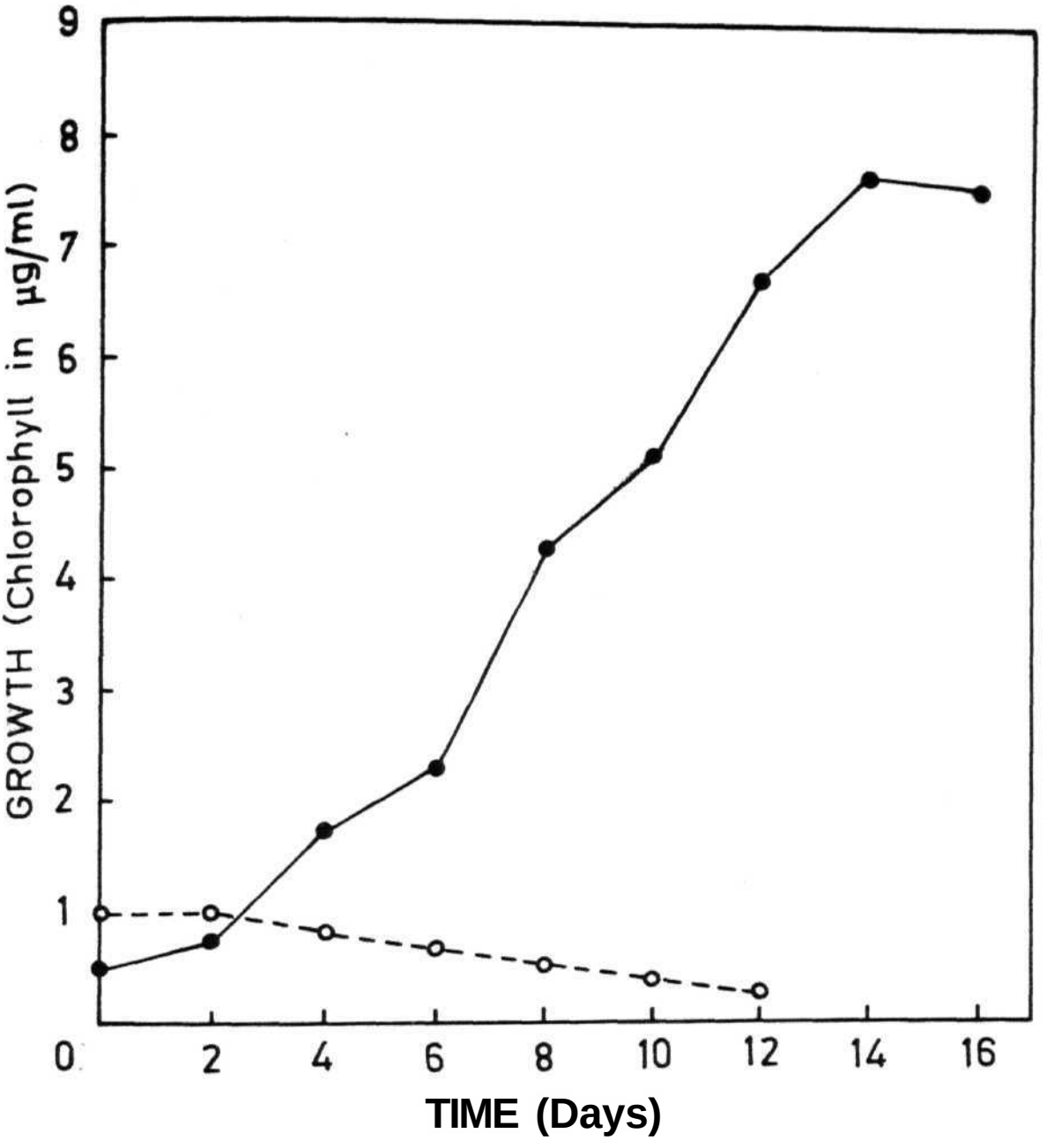

GROWTH (Chlorophyll in µg/ml)
0
1
2
3
4
5
6
7
8
9
2
4
6
8
10
12
14
16
TIME (Days)

Fig.1

Growth, Heterocyst frequency, Nitrogenase activity and Nitrate reductase activity:

Growth, heterocyst frequency (HF), nitrogenase activity (ARA) and nitrate reductase activity (NRA) of parent N.muscorum and its various phenotypic classes of chlorate-resistant (Clr-R) mutants are shown in Table 1. Parental strain showed nitrate reductase activity in N_2-grown and nitrate grown cultures and the activity was very low, almost negligible in ammonium grown cultures. The reductase activity was relatively more in nitrate assimilating cultures than in N_2-assimilating cultures. It showed the presence of heterocysts only in N_2-medium. The various Clr-R mutants were observed to fall into four distinct classes with respect to their ability to assimilate N_2 or nitrate as nitrogen source. Those mutants that could not fix N_2 but produced heterocysts were said to have Nif$^-$ phenotype and those defective in nitrate assimilation which maybe due to mutational inactivation of nitrate transport system or Mo-cofactor production or apoprotein production were described as having Nar" phenotype. Class 1 Clr-R type showed nitrogenase activity and grew diazotrophically like its parent, producing heterocyst but lacked the ability to assimilate nitrate as nitrogen source and produced heterocyst and showed nitrogenase activity in nitrate medium thus growing diazotrophically in nitrate medium. They are thus phenotypically Nif$^+$ Nar$^-$ producing heterocyst and nitrogenase in nitrate medium but not in ammonium medium. Class II Clr-R group was like Class I Clr-R type except that they showed in vitro nitrate reductase activity but failed to metabolise nitrate as nitrogen source. Thus it grew diazotrophically in nitrate medium and this maybe

Table 1

Growth, heterocyst frequency, nitrogenase activity and nitrate reductase activity of parent Nostoc muscorum and its various chlorate resistant mutants. Growth is measured in terms of chlorophyll in μg/ml. Heterocysts frequency (HF) in percentage, nitrogenase activity in terms of acetylene reducing activity (ARA) (nmol C_2H_4 formed μg^{-1} chl-a h^{-1}), nitrate reductase activity (NRA) (nmol NO_2^- formed mg^{-1} protein min^{-1}) by parental N. muscorum and its various chlorate-resistant (Clr-R) phenotypes grown in different nitrogen media. Stock culture of each strain was grown in NH_4^+ medium to serve as a source of inoculum for its growth in various nitrogen media. 6 days old different nitrogen source grown cultures were used in experiments and each value is an average of three independent results.

Strain	Growth in different nitrogen media			Heterocyst frequency in different nitrogen media			Nitrogenase activity (ARA) in different nitrogen media			Nitrate reductase activity in different nitrogen media		
	N_2	NO_3^-	NH_4^+	N_2	NO_3^-	NH_4^+	N_2	NO_3^-	NH_4^+	N_2	NO_3^-	NH_4^+
Parent strain with Nif$^+$ Nar$^+$ phenotype	7.5	9.0	5.6	5-6	0.0	0.0	8.5	0.0	0.0	3.9	4.5	0.10
Clr-R mutants with												
Class I Nif$^+$ Nar$^-$ phenotype	7.0	6.8	5.2	5-6	5-6	0.0	7.2	5.8	0.0	0.0	0.0	0.0
Class II Nif$^+$ Nar$^-$ phenotype	7.2	7.0	5.4	5-6	5-6	0.0	6.9	7.1	0.0	3.5	4.0	0.11
Class III Nif$^-$ Nar$^-$ phenotype	0.0	0.0	5.0	5-6	5-6	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Class IV Nif$^-$ Nar$^+$ phenotype	0.0	8.2	5.3	5-6	0.0	0.0	0.0	0.0	0.0	3.5	4.2	0.14

due to defect in nitrate uptake activity. This class was also Nif$^+$ Nar" phenotypically but for different genetical reasons. Class III type of Clr-R **strains** lacked **the** ability to utilise nitrogen or nitrate inspite **of** being able to produce heterocyst in both media but not in ammonium medium. This class is thus phenotypically Nif$^-$ Nar". Thus they were unable to grow in N_2-medium or nitrate medium but grew in ammonium medium. These findings suggested a genetic defect in a component common to both the enzymes, nitrogenase and nitrate reductase. Class IV Clr-R group of strains lacked nitrogenase and diazotrophic growth inspite of producing heterocyst in N_2-medium but contained nitrate reductase activity and the ability to grow with nitrate as nitrogen source. Such mutants were phenotypically Nif" Nar$^+$, which produced heterocyst under nitrogen-fixing condition and showed nitrate and ammonium repression of heterocyst formation.

Reconstitutive nitrate reductase activity of N_2-grown and nitrate-grown extracts of parent Nostoc muscorum and Clr-R-Nif$^-$ Nar" mutant after complementation with increasing amounts of Mo-cofactor from E.coli:

As shown in Table 2 parent grown at the expense of N_2 as the nitrogen source showed a level of nitrate reductase activity nearly double of that found in its nitrate grown cultures. In comparison Nif$^-$ Nar$^-$ mutant culture either from N_2 medium or from nitrate medium showed almost complete absence of nitrate reductase activity. The presence of nearly two fold increased level of nitrate reductase activity in cultures from nitrate medium does appear to suggest a role of nitrate nitrogen in maintaining increased level of nitrate reductase activity

Table 2

Reconstituted nitrate reductase activity in Mo-cofactor free N_2-grown and nitrate grown extracts of parent strain and Clr-R-*Nif*$^-$*Nar*$^-$ mutant strain after complementation with increasing amounts of Mo-cofactor from *E. coli*.

200 µl of each of various *N. muscorum* extracts was mixed with 200 µl of different dilutions of *E. coli* extract. After incubation for 10 min at 30°C, the reconstituted nitrate reductase activity was estimated. Values are an average three independent replicates within 10/variation limit.

E. coli extract (µ g protein)	Reconstituted nitrate reductase activity (n mol NO_2^- formed mg^{-1} protein min^{-1}) in N_2- extracts and NO_3^--extracts.			
	Parent strain		*Nif*$^-$ *Nar*$^-$ mutant	
	N_2-extracts	NO_3^- extracts	N_2-extracts	NO_3^--extracts
0.0	3.1	5.2	0.0	0.0
10.0	11.5	11.8	7.2	5.8
100.0	16.2	17.6	14.5	15.3
1000.0	43.8	40.6	45.5	42.2

in the cyanobacterium. However, the results of reconstituted nitrate reductase activity after complementation with increasing amounts of Mo-cofactor from aerobically growing E.coli cells suggested the following point : the nitrate reductase activity in N_2-grown and nitrate grown extracts of parent strain were Mo-cofactor limited and not apoprotein level limited as the activity showed continuous increase in both extracts with the increase in the amount of Mo-containing E.coli extracts added. The recovery and restoration of reductase activity in extracts of mutant strain following addition of Mo-cofactor containing E.coli extract showed clearly that the mutant strain is a result of mutational defect in functional production of Mo-cofactor and that it is as normal as its parental strain with respect to its apoprotein level. This experiment also clearly brings out limitation of Mo-cofactor as the reason for fifty percent less activity of nitrate reductase in N_2-grown cultures of parental strain as compared to its nitrate-grown culture. This also implies a role of nitrogenase enzyme activity in regulating Mo-nutrition of nitrate reductase in the cyanobacterium.

Mo-cofactor activity of parent Nostoc muscorum and its various classes of chlorate-resistant mutants in different nitrogen media and of E.coli strain W grown in Luria Broth:

The different classes of chlorate-resistant mutants of the cyanobacteria were analysed for their Mo-cofactor activity in cultures grown in different nitrogen sources. As shown in Table 3 Mo-cofactor activity was high in nitrate and ammonium assimilating cultures (nearly double) than in N_2-assimilating cultures of the parent strain. The Jack

of nitrate reductase activity but presence of very high level of Mo-cofactor activity in ammonium cultures of the parent strain suggested that Mo-cofactor activity is not regulated by fixed nitrogen source like ammonia. The results also suggest lack of nitrate reductase apoprotein to be the cause of lack of nitrate reductase activity in ammonium grown cultures. Nearly fifty percent less level of Mo-cofactor activity in cultures grown with N_2 than in cultures grown with nitrate suggest a role of diazotrophy in regulating the level of Mo-cofactor activity in the cyanobacteria. This pattern of Mo-cofactor activity was also characteristic of Class I and Class II type of chlorate-resistant (Nif$^+$ Nar$^-$ in both the cases) mutants. Since both the mutant classes were Nar" type the reason for apparent lack of nitrate assimilation by them is certainly not due to lack of Mo-cofactor. This is consistent with the findings reported in Table 1 on these mutants. The class III group of Nif$^-$ Nar$^-$ mutant phenotype showed almost negligible level of Mo-cofactor activity. This means that this class of mutant has a reason, which is the result of mutation in a genetic determinant controlling molybdenum nutrition common to both nitrogenase and nitrate reductase. The class IV (Nif$^-$ Nar$^+$) type of chlorate-resistant mutant appear to contain nearly as high a level of Mo-cofactor activity as its nitrate or ammonium grown cultures. Evidently mutation in this class has resulted in nearly fifty percent increase in the level of Mo-cofactor under N_2-fixing conditions. The lack of nitrogenase activity associated with the rise in Mo-cofactor activity level suggested a role of active nitrogenase in regulating the level of Mo-cofactor.

Table 3

Mo-cofactor activity of parent Nostoc muscorum and its various chlorate-resistant mutants in different nitrogen media and of E. coli strain in Luria broth.

The method of cofactor-free nitrate reductase (apoprotein) preparation from W-containing nitrate grown N. muscorum W^R-strain was reproducibily standardised with the extracts of aerobically grown E. coli which showed high level of Mo-cofactor with no nitrate reductase activity as described (Miller and Amy, 1983). The cofactor-free extract of N. muscorum W^R-strain was then complemented with Mo-cofactor from various other strains of N. muscorum grown in different nitrogen medium to determine the nitrogen source specific Mo-cofactor activity level.

Strain	Mo-cofactor activity in different nitrogen-sources expressed in U/mg protein.		
	N_2	NO_3^-	NH_4^+
Parent strain with:			
Nif$^+$ Nar$^+$ phenotype	52.5	118.5	108.3
Clr-R mutants with:			
Class I Nif$^+$ Nar$^-$ phenotype	49.2	109.5	104.5
Class II Nif$^+$ Nar$^-$ phenotype	54.5	124.2	107.3
Class III Nif$^-$ Nar$^-$ phenotype	1.5	1.6	1.8
Class IV Nif$^-$ Nar$^+$ phenotype	98.5	112.5	106.2

E. coli strain W grown in Luria broth was deficient in nitrate reductase activity but contained 382.5 units of Mo-cofactor per mg protein.

DISCUSSION

Active nitrate reductase in the cyanobacterium N.muscorum has been shown to consist of an apoprotein component and a Mo-cofactor (Bagchi et al, 1985). Mutant defective in nitrate assimilation are expected to result from mutational inactivation of nitrate transport system, Mo-cofactor production and apoprotein production. The consequence of any such mutation would be reflected in loss of nitrate repression control of heterocyst formation and nitrogenase activity. Nitrate repression control of diazotrophy in heterocystous cyanobacteria is an established fact (Singh et al, 1978; Haselkorn, 1978). The ability of nitrate reductase deficient mutant to produce heterocyst and nitrogenase activity in nitrate medium as efficiently as that produced under N_2-fixing condition, suggests that nitrate reductase mediated metabolic product of nitrate is the mechanism of generating nitrate repressor signal. A role of nitrate reductase activity in nitrate repression control of nitrogenase in Azotobacter vinelandii has also been demonstrated (Sorger, 1969). Since nitrate reductase deficient mutant with intact nitrate uptake system lacks nitrate repression control, it can be suggested that not nitrate itself but a close metabolic product of it generated by nitrate reductase activity is the repressor signal for heterocyst and nitrogenase in N.muscorum. Mutants defective in nitrate transport system are accordingly expected to lack nitrate repression control and such mutants of N.muscorum have already been reported (Bagchi and Singh, 1984).

Nitrogenase and nitrate reductase both are molybdoenzymes and mutations impairing molybdenum nutrition are likely to result in

lack of njtrogenase activity and nitrate reductase activity. The Mo-cofactor activity level in cultures growing with different inorganic nitrogen sources will also provide important information regarding the role of a fixed inorganic nitrogen source in regulating Mo-cofactor activity level. Presence of nearly two fold higher level of Mo-cofactor activity in nitrate or ammonium grown than in N_2-grown cultures of parental strain suggest a role of nitrogenase synthesis in regulating the cellular level of Mo-cofactor. This view is **further** supported by **the finding in** Nif$^-$ Nar$^+$ class **of chlorate-resistant** mutants in which loss of nitrogenase activity was found **associated with** fifty percent rise in its Mo-cofactor level. N.muscorum heterocystous, N_2-fixing filaments have been shown to contain nitrogenase activity within heterocysts and nitrate reductase activity within vegetative cells. The lower level of Mo-cofactor activity under N_2-fixing condition does reflect a sharing of available molybdenum by the two molybdoenzymes nitrogenase within heterocysts and nitrate reductase within vegetative cells. In this context, it is reasonable to suggest that heterocyst is not only an O_2 protection mechanism for nitrogenase but is also a mechanism for partitioning available molybdenum for production of active nitrogenase enzyme. Similar view has been expressed earlier (Bagchi et al, 1985 and Kumar et al, 1985). The Mo-cofactor activity has been demonstrated in isolated heterocyst preparation of N.mustocum lacking nitrate reductase activity (Kumar et al, 1985). The presence of active Mo-cofactor in heterocysts deficient in nitrate reductase activity does implicate a role of this molybdenum source in activity control of nitrogenase enzyme. The behaviour of Nif$^-$ Nar$^+$ type Clr-R mutant maybe the result of impairment of molybdenum

nutrition of heterocyst leading to loss in the activity of nitrogenase enzyme. Presence of nearly as much Mo-cofactor activity in nitrate reductase apoprotein deficient mutant strain as in its parental strain suggests no role of the apoprotein in regulation of Mo-cofactor activity level. Also the fact that Mo-cofactor occurs in substantial amount in cultures grown with N_2, nitrate or ammonium as nitrogen source indicates that synthesis of Mo-cofactor is constitutive and is not dependent on the nature of inorganic nitrogen source. This finding also confirms the earlier report that synthesis of Mo-cofactor is constitutive in the cyanobacteria (Bagchi et al, 1985). The virtual lack of Mo-cofactor activity in Clr-R **group** of Nif~ Nar⁻ mutants suggests that this mutant class arose as a result of mutational defect in Mo-nutrition, common to nitrogenase activity and nitrate **reductase** activity.

Biochemical analysis of Clr-R Nif⁻ Nar⁻ class of mutants suggested that they are deficient in active production of Mo-cofactor, leading to simultaneous absence of active nitrogenase and nitrate reductase. If that be so supplementation of mutant extract with Mo-cofactor from E.coli should restore and reconstitute the nitrate reductase activity. E.coli Mo-coiactor supplementation resulted in effective reconstitution of nitrate reductase activity in the mutant extract of the cyanobacterium. In fact addition of increasing amounts of Mo-cofactor from aerobically grown E.coli to extracts from parental and mutant strains caused progressive increase in the enzyme activity, reaching almost identical level in N_2-fixing and nitrate assimilating cultures. Evidently the Nif⁻ Nar" class of mutants is normal with respect to nitrate reductase apoprotein.

Since this mutant class is deficient in Mo-cofactor level it is suggested that Mo-cofactor does not play any significant role in the production of nitrate reductase apoprotein. The present findings also indicates that Mo-cofactor and not apoprotein limits the activity level of nitrate reductase in N_2-assimilating and nitrate assimilating cultures of the cyanobacteria.

As a result of this discussion the various chlorate-resistant mutants of **Nostoc muscorum** can be divided into four genetically distinct classes. Class I with phenotypes Nif$^+$ Nar" resulted from mutation in the genetic determinant controlling apoprotein activity level of nitrate reductase. Class II also with phenotype Nif$^+$ Nar$^-$ was most probably the result of mutation in the genetic determinant controlling nitrate uptake. Class **III** with Nif~ Nar' phenotype arose as a result of mutation in the genetic determinant involved in the production of Mo-cofactor required for functional production of nitrogenase and nitrate reductase. Class IV with Nif$^-$ Nar$^+$ phenotype was the result of mutation in the genetic determinant specific to nitrogenase activity. Lack of nitrate repression control of heterocyst and nitrogenase with mutant class specifically defective in nitrate reductase activity suggested a role of this enzyme activity in the process of nitrate repression control. Production of normal level of Mo-cofactor in the absence of nitrate reductase apoprotein and vice-versa suggested that neither component regulates the production of other component in the cyanobacterium.

CHAPTER IV

ISOLATION AND CHARACTERISATION OF MUTANTS RESISTANT TO ANTIBIOTICS, AMINO ACID ANAZOGUE AND PHOTOSYNTHETIC INHIBITORS

INTRODUCTION

A basic requirement for genetic studies in cyanobacteria is the availability of stable mutants that can be used as markers in studies involving genetransfer systems of transformation or conjugation nature. Transformation has been shown to be a very effective method of genetransfer in unicellular cyanobacteria. An effective system of conjugation between E.coli strain and cyanobacterial strains has been reproducibly worked out (Elhai and Wolk, 1988). Transformation for azide resistance and herbicide resistance has also been demonstrated in heterocystous, filamentous forms (Singh et al, 1987; a and b). With regard to heterocystous filamentous forms, transformation, has been reported to be successful for herbicide and azide resistance markers alone. The reported transformable herbicide resistance gene is not part of genetic system oi cyanobacterial photosynthetic machinery. In order to understand the specificity or generality of transformation process in heterocystous forms for a variety of resitance markers, it was necessary to first isolate the mutants of respective resitance classes. It was therefore, considered proper to isolate mutants of Nostoc muscorum showing resistance to antibiotics like Streptomycin, amino acid analogues like L-Ethionine and photosynthetic herbicides like 3-(3,4 Dichlorophenyl)-1, 1-Dimethyl urea (DCMU) and Atrazine.

Streptomycin is an inhibitor of protein synthesis in prokaryotes

because of its specificity of interaction with 30S subunit of 70S ribosome. Gene involved in conferring resistance against Streptomycin in cyanobacteria have been shown to be chromosomally located and there is no evidence so far for a plasmid gene to be responsible for determining this phenotype (Shestakov and Khyen, 1970; Stewart and Singh, 1975; Ladha and Kumar, 1978; Singh et al, 1987).

L-Ethionine is an analogue **of** methionine and its growth toxic affect is due to its interfering with methionine incorporation and utilisation in microbial systems. Spontaneous and chemically induced mutations to Ethionine resistance has been reported in the cyanobacterium Plectonema boryanum (Hentschel et al, 1978). Spontaneously occuring **Ethionine** resistant mutants of Nostoc muscorum were isolated and characterised for stability and growth characteristics in different nitrogen media.

DCMU and Atrazine both are inhibitors of photosystem II activity and the target of their action is the 32 kd protein also known as D1 protein or Q_B protein essential for functioning of photosystem II (PSI1) reaction centre. The techniques of heterocystous gene probe system and transformation system have identified psb A gene coding for Dl protein and it has been shown that in unicellular and heterocystous forms psb A gene is a member of multigene family, the full significance of it is not known (Golden et al, 1986). Brusslan and Haselkorn (1989) have shown that resistance to DCMU to be dominant over its sensitivity in the cyanobacterium Synechococcus sp. PCC 7942. In the present study spontaneously occurring mutants of Nostoc muscorum

resistant to DCMU and Atrazine were separately isolated and characterised for cross-resistance relationship and algal growth characteristics in different nitrogen media.

EXPERIMENTAL PROCEDURE

Alga

Axenic clonal culture of parental Nostoc muscorum was routinely grown in CHU-10 medium (Gerloff et al, 1950) free of combined nitrogen source (N_2-medium) or containing 5 mM KNO_3 (NO_3^--medium) or 1 mM NH_4Cl (NH_4^+-medium). Log-phase cultures were generally used for the isolation of various mutants after determining their lethal doses to the parent culture.

Isolation of spontaneous mutants resistant to various inhibitors

a) Streptomycin:

Streptomycin at a concentration of 0.60 µM was found to be extremely growth toxic and inhibitory to the parent Nostoc muscorum in N_2-medium, nitrate medium and ammonium medium. A Streptomycin concentration of 3.3 µM was used for selecting spontaneous mutants to Streptomycin resitance and it was nearly 6 times higher than the lethal dose of parent culture.

N_2-grown, log-phase cultures were harvested, washed, fragmented and approximately 10^9 colony-forming units (CFU) were spread on agar plates containing N_2-medium with 3.5 µM of Streptomycin. The inoculated plates were incubated for growth in the culture room

at a temperature of 28 ± 2°C and a photon fluence rate of 50 µmol m^{-2} s^{-1}.

b) L-Ethionine:

The parental strain of N.muscorum was tested for its sensitivity to L-Ethionine in different inorganic nitrogen media containing different concentrations of the inhibitor and 40 uM of Ethionine was found to be completely toxic and growth inhibitory to this strain in all inorganic nitrogen media. The inoculum for this was nitrate grown exponential-phase culture, used after centrifugation and washing the culture with sterile N_2-medium.

A washed inoculum of 10^9 colony forming units were spread on agar plates containing 130 uM of Ethionine in N_2-medium and these plates were incubated in the culture room at a temperature of 28 ± 2°C and a photon fluence rate of 50 µmol m^{-2} s^{-1} for a fortnight.

c) Photosynthetic inhibitors:

The parental strain of N.muscorum was tested for its sensitivity to DCMU and Atrazine in different inorganic nitrogen media containing different concentrations of the inhibitor and 0.4 µM DCMU, 0.6 uM of Atrazine were found to be inhibitory to the growth of the parental strain. The inoculum for this was N_2-grown log-phase cultures.

N_2-grown log-phase cultures were harvested, washed, fragmented and approximately 10^9 CFU were plated on agar-plates containing 1µM of DCMU and 2 µM of Atrazine in N_2-medium. The plates were

kept for incubation for **a** fortnight in the culture room at a temperature of 28 *t* 2°C and a photon fluence rate of 50 µmol m^{-2} s^{-1}.

Growth, heterocyst frequency, nitrogenase activity, glutamine **synthetase** (transferase and biosynthetic) activity and photochemical activities (PS II and PS I) were determined as described in Chapter II.

RESULTS

Effect of various inhibitors on cyanobacterial growth under diazotrophic growth conditions:

a) Streptomycin:

The growth inhibitory or lethal dose of Streptomycin was examined for N.muscorum growing in liquid or on solid medium with N_2, nitrate or ammonium as nitrogen source. The concentrations of Streptomycin used in the investigation were 0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.8 and 1.0 µM. The filter sterilised antibiotic was added to the autoclaved medium (sterile) after cooling the medium and just before it can be poured into plates. Such antibiotic treated cultures along with their respective controls were incubated in the culture room. Cultures fail to grow or survive with more than 0.6 PM of Streptomycin, the lethality of Streptomycin did not vary with the nature of nitrogen source in the growth medium. After having determined the lethal concentration, subsequent experiments for isolation of spontaneously occuring Streptomycin-resistant (Str^r) mutant were done on solid medium containing 3.5 µM Streptomycin. The spontaneous mutation frequency with which

Fig. 2 Growth pattern of parent Nostoc muscorum and its Streptomycin resistant mutant under diazotrophic growth conditions in the absence and presence of 20μM Streptomycin.

Parent grown in the presence of Streptomycin

Parent grown in the absence of Streptomycin

Mutant grown in the presence of Streptomycin

Mutant grown in the absence of Streptomycin

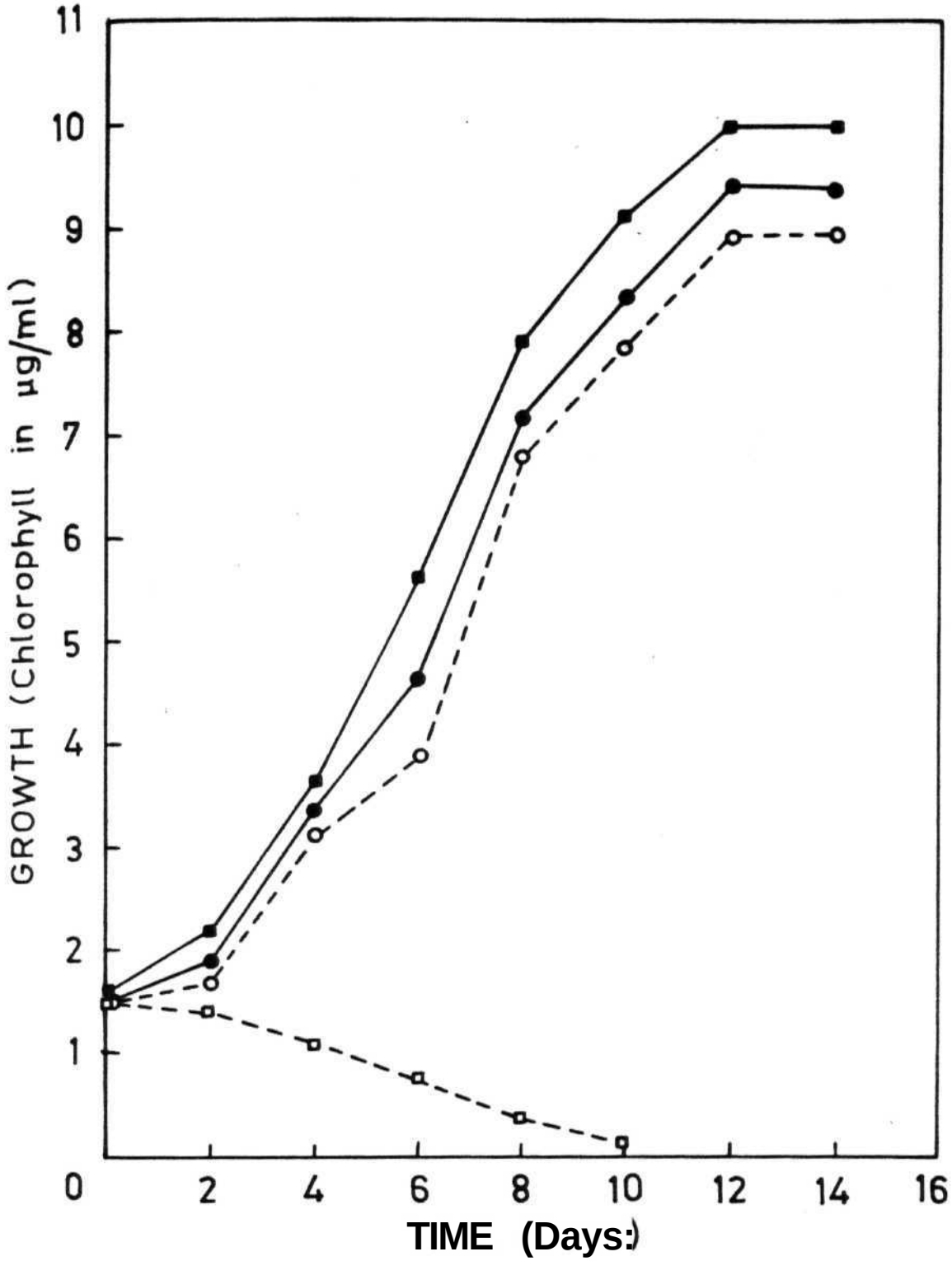

GROWTH (Chlorophyll in µg/ml)
TIME (Days)
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Fig.2

the **Streptomycin resistant mutant arose was in the range of 0.2-2.5 x 10^{-8}, a frequency characteristic of unicellular cyanobacteria and enterobacteria. A colony growing on 3.5 µM antibiotic containing medium was isolated and maintained on slants as stock cultures for subsequent use. The Streptomycin resistant mutant clone thus obtained on further examination was found to grow equally well in 68 µM of Streptomycin in the growth medium. Thus the mutation to Streptomycin resistance in the cyanobacterium appears to be a single step point mutation. The growth of parent N.muscorum and its antibiotic resistant mutant were compared in** N_2-grown medium lacking **or containing** Streptomycin (20 µM) **as shown** in Fig.2. **The** antibiotic free growth **of** parent strain was slightly better than similar growth of its mutant strain. Presence of antibiotic as expected caused an abrupt inhibition of cyanobacterial growth in parent strain and similar growth of mutant strain was found not to be much influenced by the presence of antibiotic in the growth medium. The stability of antibiotic resistance was checked by growing the mutant strain in antibiotic free medium for nearly 10 successive subcultures and then examining its growth ability in the same medium containing 68 µM Streptomycin. The presence of antibiotic did not cause any significant effect on cultures of mutant strain grown previously for 10 successive subcultures in the drug free medium. Evidently the mutation to antibiotic resistance is intrinsically a very stable phenotype in this cyanobacterium.

b) L-Ethionine:

L-Ethionine the analogue of methionine is a very well known

antimetabolite causing growth inhibition and lethality in microbial cultures. The parent N.mustorum was initially tested for its sensitivity to L-Ethionine as it was done in the case of Streptomycin. The appropriate millipore filter sterilized concentration of the analogue was added to the cyanobacterial cultures inoculated in N_2-medium, nitrate medium and ammonium medium. The range of concentrations of Ethionine used were 1 MM, 2 µM, 5 µM, 10 µM, 15 µM, 20 µM, 30 µM, 35 µM, 40 µM, 45 µM and 50 µM. The analogue treated cultures along with the untreated controls were incubated in the culture room for growth. None of the cultures could grow in different nitrogen media containing 40 µM of Ethionine. The visible pattern of change observed in treated cultures included gradual decrease in pigmentation of the cultures leading to their complete bleaching within a growth incubation period of one week. The spontaneously occurring mutants resistant to Ethionine were sought on N_2-medium containing 130 UM of the inhibitor. The colonies growing on the inhibitor containing medium were streaked separately on fresh inhibitor containing nutrient plates and those colonies surviving and growing on the second set of inhibitor containing nutrient plates were considered genuine resistant colonies. One such colony was isolated and maintained on the slant as stock culture for subsequent experiments. The calculated frequency of spontaneous mutation to Ethionine resistance was in the range of 1 x JO^{-7}. The colony isolated on 130 µM of inhibitor containing medium was tested for its ability to grow at higher concentration of the inhibitor. It was found to grow as effectively at 260 uM concentration as it grew at 130 *V* M conc.

Fig. 3 shows the growth response of parent strain in different

Fig. 3 **Growth pattern of parent Nostoc muscorum in different nitrogen media, Jacking or containing 40 μM of L-Ethionine**


Growth in N_2-medium containing L-Ethionine


Growth in N_2-medium Jacking L-Ethionine

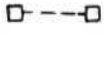

Growth in NO_3^--medium (5 mM) containing L-Ethionine

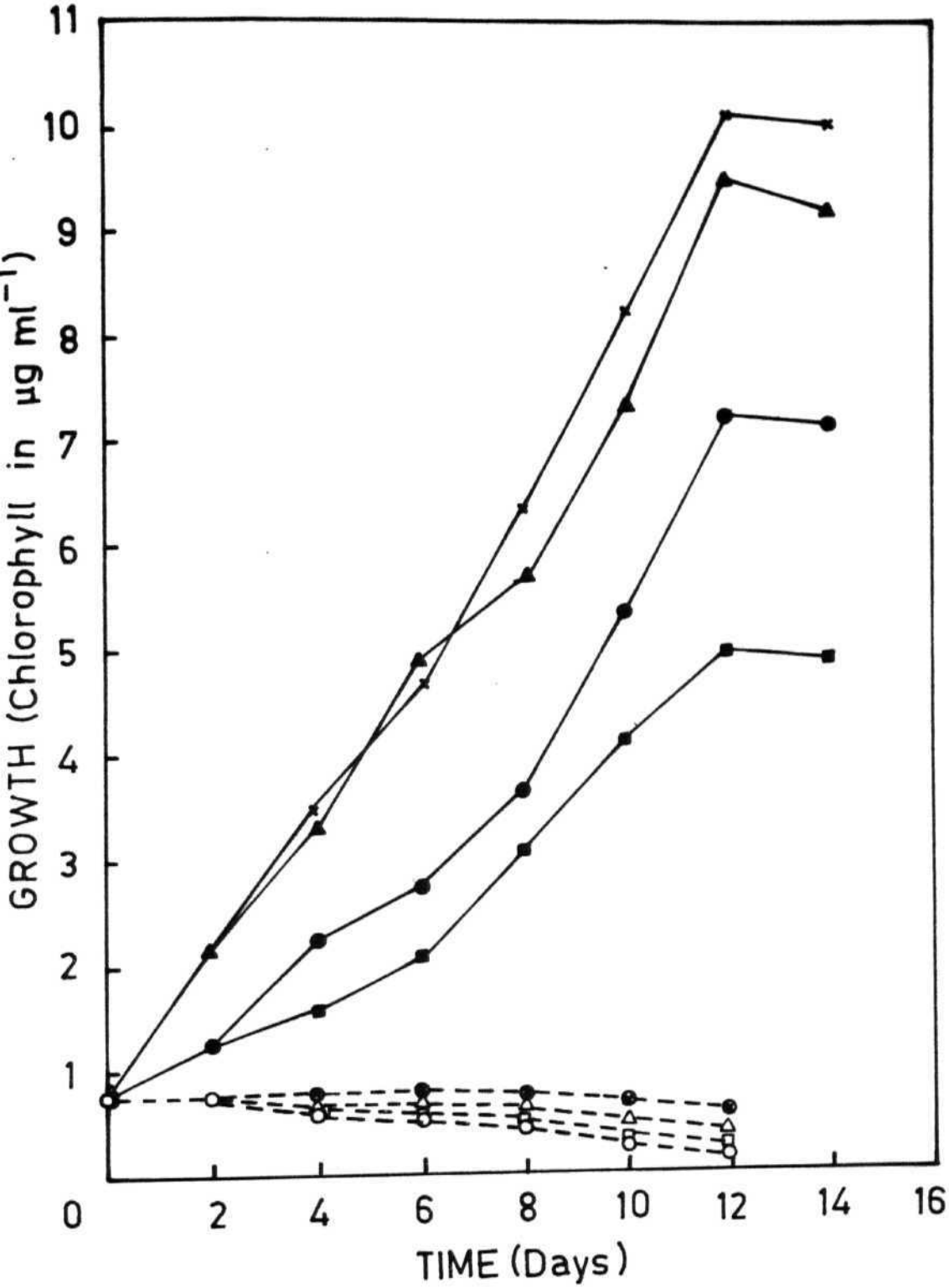

Growth in NO_3^--medium (5 mM) Jacking L-Ethionine


Growth in NO_2^--medium (5 mM) containing L-Ethionine

Growth in NO_2^--medium (5 mM) Jacking L-Ethionine

Growth in NH_4^+-medium (1 mM) containing L-Ethionine


Growth in NH_4^+-medium (J mM) lacking L-Ethionine


GROWTH (Chlorophyll in µg ml^{-1})
TIME (Days)
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16

Fig. 3

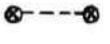
Fig. 4 Growth pattern of L-Ethionine resistant mutant of Nostoc muscorum in different nitrogen media Jacking or containing 130 μM of L-Ethionine

Growth in N_2-medium containing L-Ethionine

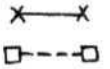
Growth in N_2-medium Jacking L-Ethionine

Growth in NO_3^--medium (5 mM) containing L-Ethionine

Growth in NO_3^--medium (5 mM) Jacking L-Ethionine



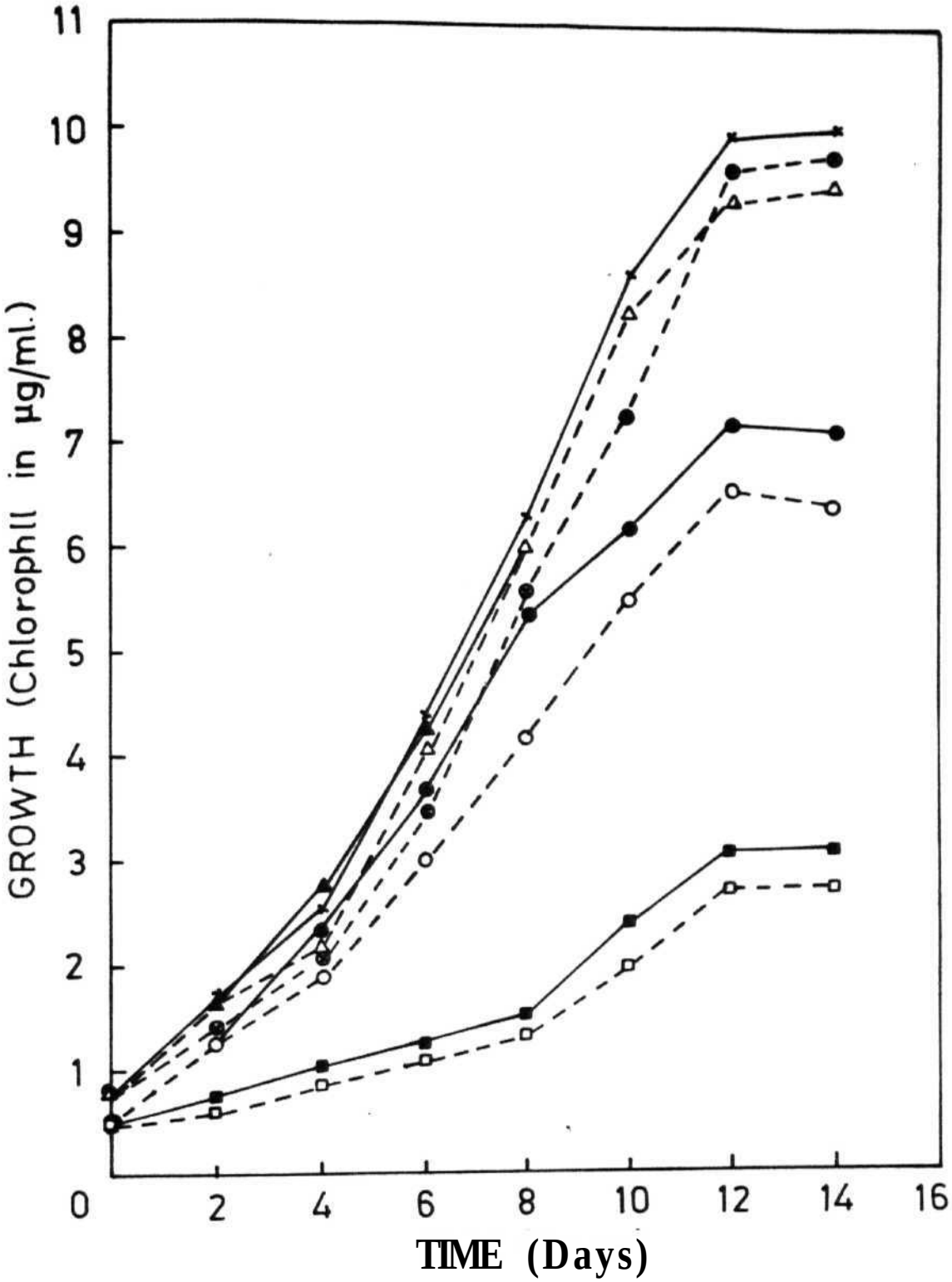
Growth in NO_2^--medium (5 mM) containing L-Ethionine



Growth in NO_2^--medium (5 mM) lacking L-Ethionine

Growth in NH_4^+-medium (1 mM) containing L-Ethionine

Growth in NH_4^+-medium (J mM) lacking L-Ethionine


GROWTH (Chlorophll in µg/ml.)
TIME (Days)
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16

Fig.4

nitrogen media lacking or containing 40 UM of Ethionine. The cyanobacterial culture grew well in different nitrogen media without the inhibitor but the growth was best with nitrite as nitrogen source followed by nitrate, N_2 and NH_4^+ in that order. Parent culture could not grow in the different nitrogen media containing 40 µM of Ethionine. The analogue resistant mutant growth lacking or containing 130 µM of the analogue is shown in Fig.4. The mutant grew nearly as well as the parental strain without or with the inhibitor. The most interesting noticeable point in the growth behaviour in mutant strain was in ammonium medium. It grew very slowly in comparison to its parent in ammonium medium without inhibitor over a period of nearly eight days and its subsequent growth was only slightly better. Spontaneous mutation to Ethionine resistance seem to have affected the ammonium growth response of the cyanobacteria.

c) Photosynthetic inhibitors:

Growth sensitivity of the parent strain to two photosynthetic herbicides DCMU and Atrazine in diferent inorganic nitrogen media was also examined. The photosynthetic: herbicides like other inhibitors were filter sterilised and added in appropriate amount to different nitrogen cultures of the cyanobacteria. The concentrations used for DCMU were 0.1, 0.2, 0.3, 0.4, 0.6 and 0.8 µM and for Atrazine 0.2, 0.4, 0.6, 0.8 µM. The treated cultures and their controls were incubated in the culture room and their growth was visually observed over a period of two weeks. The cultures of different nitrogen media could not grow in 0.4 µM of DCMU containing medium and 0.6 µM of Atrazine containing medium. The mutants resistant to DCMU were selected on 1 µM DCMU

Fig. 5a Growth pattern of parent Nostoc muscorum and its Atrazine resistant mutant strain under diazotrophic conditions in the absence and presence of 5μM Atrazine

Parent grown in presence of Atrazine

Parent grown in absence of Atrazine

Mutant grown in presence of Atrazine

Mutant grown in absence of Atrazine

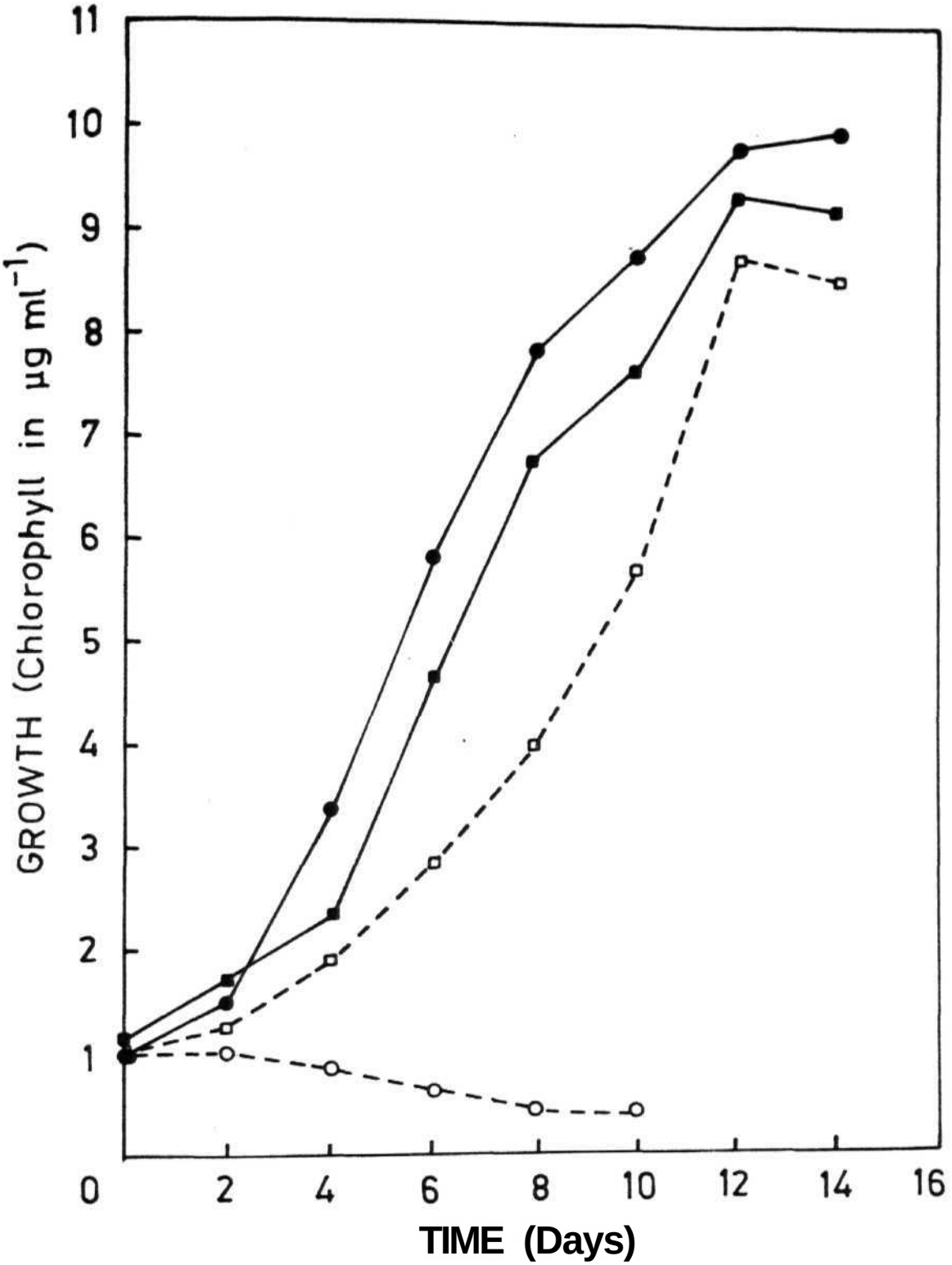
GROWTH (Chlorophyll in µg ml^{-1})
TIME (Days)
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Fig.5(a)

Fig. 5b **Growth pattern of parent Nostoc muscorum and its Atrazine resistant mutant strain under** diazotrophic **conditions in the absence and presence** of 6μM DCMU


Parent grown in presence of DCMU


Parent grown in absence of DCMU


Mutant grown in presence of DCMU


Mutant grown in absence of DCMU

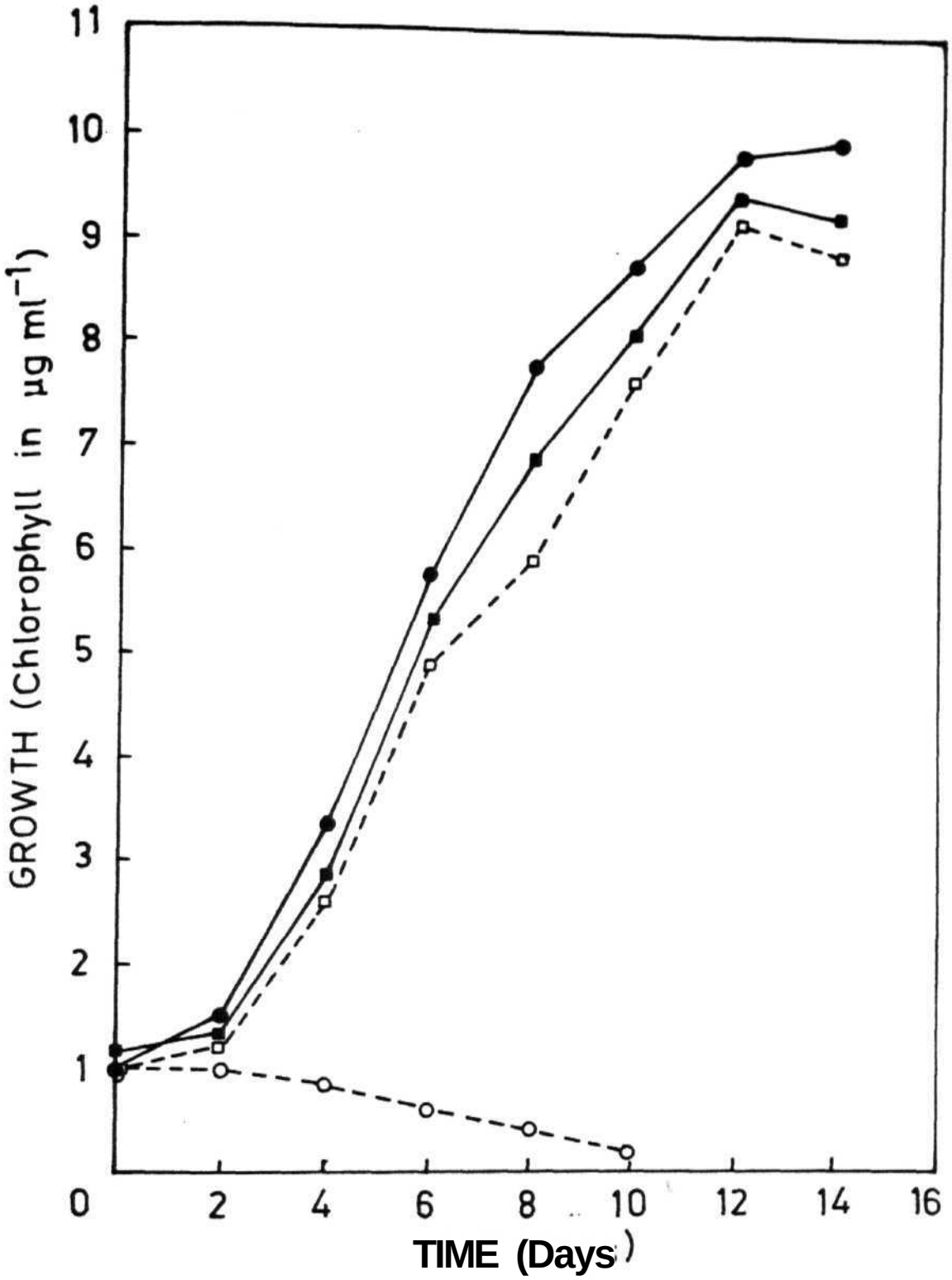
GROWTH (Chlorophyll in µg ml(-1))
TIME (Days)
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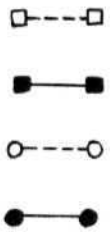
Fig.5(b)

containing N_2-medium and for Atrazine on 2 µM Atrazine containing N_2-medium. The attempt to isolate spontaneously occurring herbicide resistant mutants at higher doses were not successful. The mutants were tested for their stability as described earlier for Streptomycin resistance and the calculated frequency of their spontaneous mutation is in the range of 0.5-0.8 x 10^{-7}. A colony of DCMU resistant ($DCMU^r$) strain and a colony of Atrazine-resistant (Atr^r) strain were isolated and maintained separately on herbicide containing slants. The two herbicide resitant mutants were subsequently successively subcultured in increasing concentration of either herbicide and this lead to the two mutant strains to grow in much higher herbicide concentrations. The $DCMU^r$ strain thus subcultured was able to grow at 10µM DCMU and $Atrazine^r$ strain thus subcultured was found to grow in 10µ M of Atrazine.

Fig. 5a shows the growth response of parent and Atr^r-mutant strain in N_2-medium lacking or containing 5µM Atrazine. The parent grew better than mutant strain at the expense of N_2 in herbicide-free medium. However, as expected the parent strain did not grow in the herbicide containing medium while its mutant strain grew well in the herbicide containing medium; but its growth in herbicide medium was lesser than in medium without herbicide. The growth of parent and $Atrazine^r$ mutant strain in 6 PM of DCMU containing N_2-medium was also examined and the results are shown in Fig.5b. As expected the parent did not grow while $Atrazine^r$-mutant grew as efficiently with DCMU or without it. This finding clearly suggests $Atrazine^r$-mutant strain to be cross-resistant to DCMU as well. Parallel growth experiments were conducted with $DCMU^r$-mutant strain in 6 MM DCMU containing

Fig. 6a Growth pattern of parent Nostoc muscorum and its DCMU resistant mutant strain in N_2-medium in the absence and presence of 6µM DCMU



Parent grown in presence of DCMU

Parent grown in absence of DCMU

Mutant grown in presence of DCMU

Mutant grown in absence of DCMU

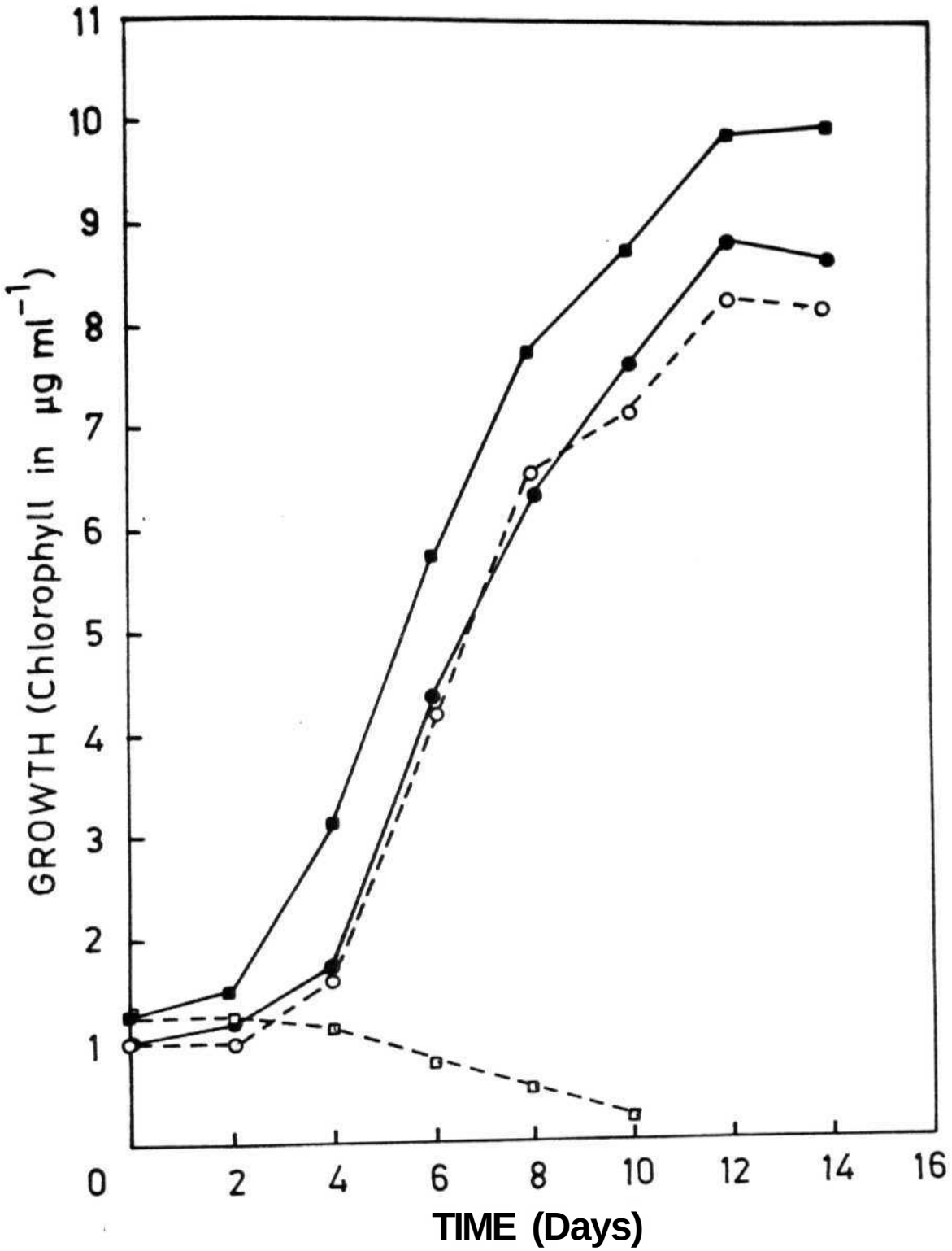

GROWTH (Chlorophyll in µg ml⁻¹)
TIME (Days)
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Fig.6(a)

Fig. 6b Growth pattern of parent Nostoc muscorum and its DCMU resistant mutant strain in N_2-medium in the absence and presence of 5 µM Atrazine


Parent grown in presence of Atrazine


Parent grown in absence of Atrazine


Mutant grown in presence of Atrazine


Mutant grown in absence of Atrazine

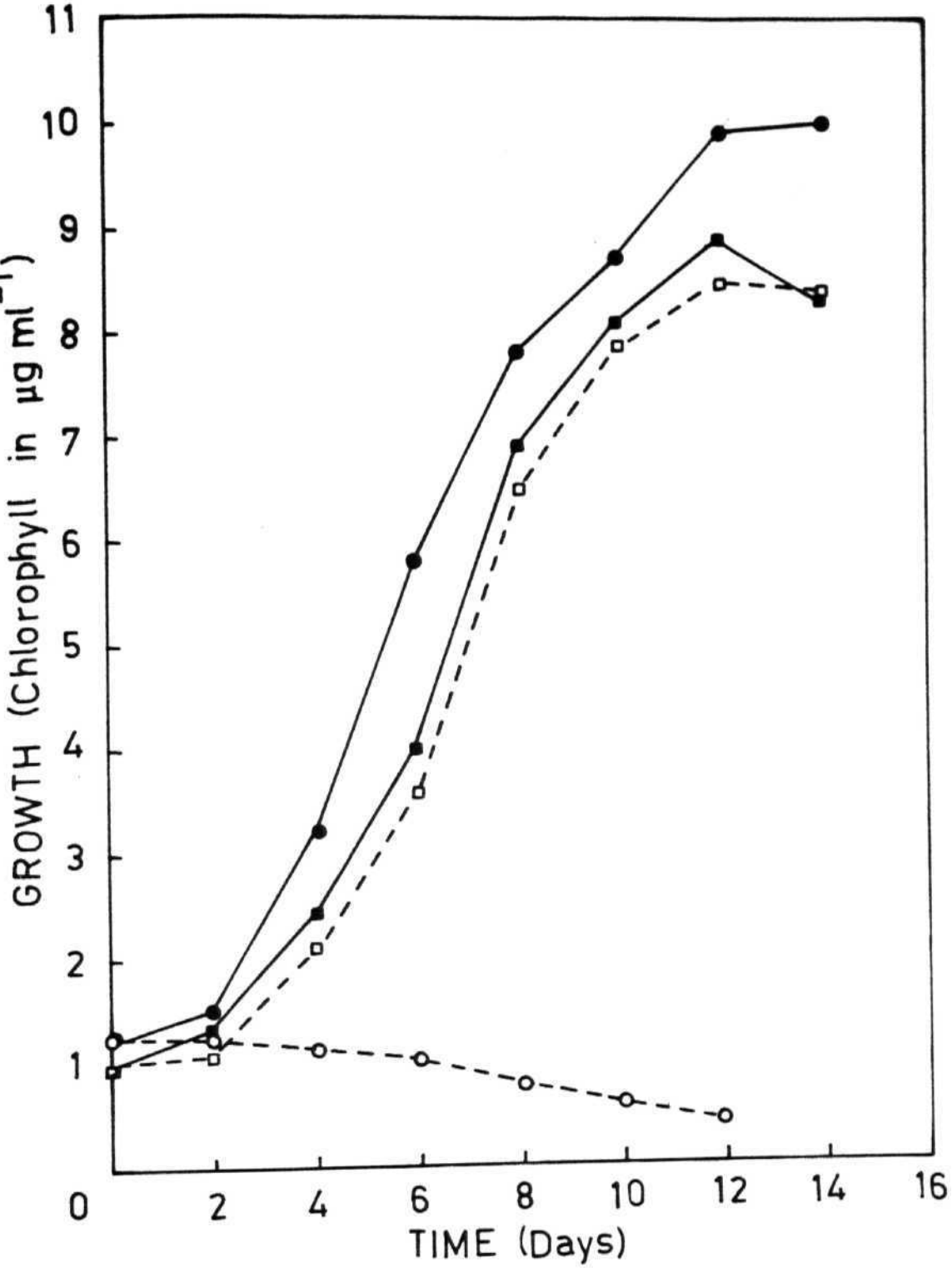
GROWTH (Chlorophyll in µg ml^{-1})
TIME (Days)
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Fig. 6 (b)

Fig. 7 Growth pattern of parent Nostoc muscorum and its DCMU resistant ($DCMU^r$) and Atrazine resistant (Atr^r) mutant strains under diazotrophic conditions in the absence and combined presence of 6 μM DCMU and 5 μM Atrazine


Parent grown in the presence of herbicides


Parent grown in the absence of herbicides


$DCMU^r$ mutant grown in the presence of herbicides


$DCMU^r$ mutant grown in the absence of herbicides


Atr^r mutant grown in presence of herbicides


Atr^r mutant grown in the absence of herbicides

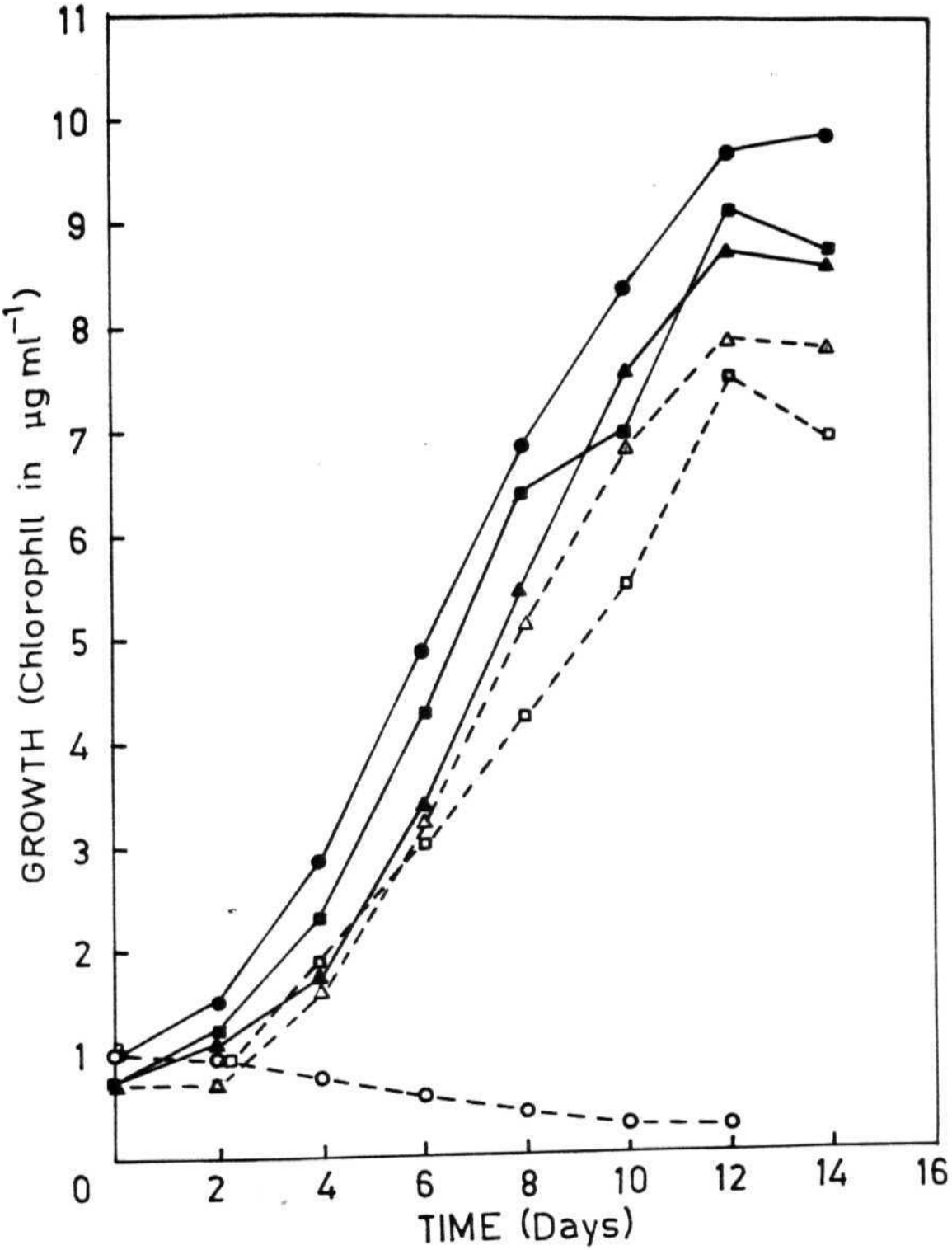
GROWTH (Chlorophll in µg ml^{-1})
TIME (Days)
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Fig.7

N_2-medium (Fig. 6a) and in 5 uM atrazine containing N_2-medium (Fig.6b). $DCMU^r$-mutant strain grew as efficiently with DCMU as without it suggesting it to be completely resistant to growth inhibition by 6 uM DCMU. $DCMU^r$-mutant strain was found growing as efficiently in 5 µM Atrazine containing medium as in 6 uM DCMU containing medium. These results also show that mutation to DCMU resistance also results in conferring resistance to growth inhibition by Atrazine. As shown in Fig.7, the combination of both the herbicides in the growth medium did not alter significantly diazotrophic growth of either herbicide resistant strain thus further suggesting that the present mutants are the result of a mutation in the common site leading simultaneously to development of resistance to both the herbicides.

Effect of inhibitors on heterocyst frequency **and** nitrogenase activity of parent **N.muscorum** and its various resistant mutants under diazotrophic conditions:

a) Streptomycin and **L-Ethionine:**

Streptomycin is an inhibitor of protein synthesis and the utilisation of Ethionine in place of methionine in protein synthesis leads to production of non-functional proteins. Nitrate grown, non-heterocystous, non N_2-fixing filaments of parent, Str^r-mutant, Eth^r-mutants were used to monitor the effect of Streptomycin and Ethionine on heterocyst differentiation and nitrogenase activity under N_2-fixing conditions. The nitrate grown cultures treated with either inhibitor in N_2-medium were examined after 3 days of such treatment and results are shown in Table 4. As expected Streptomycin or Ethionine treatment

Table 4

Effect of streptomycin and L-ethionine on heterocyst frequency and nitrogenase activity of parent N. muscorum and its Str^r-mutant and Eth^r-mutant strains under diazotrophic growth conditions.

Strain	Control		With Streptomycin (20 μM)		With Ethionine (130 μM)	
	Heterocyst frequency	Nitrogenase activity	Heterocyst frequency	Nitrogenase activity	Heterocyst frequency	Nitrogenase activity
Parent	5-6	8.9	0.0	0.0	0.0	0.0
Str^r-mutant	5-6	8.5	5-6	8.25	0.0	0.0
Eth^r-mutant	5-6	7.72	0.0	0.0	5-6	7.8

Nitrogenase activity is expressed as n mol C_2H_4 formed mg chl$\underline{a}^{-1}$ hr^{-1}.

Heterocyst frequency was calculated as number of heterocysts per 100 vegetative cells.

The source of inoculum was nitrate grown non heterocystous cultures.

All the above results are an average of three independent experiments which do not vary by more than 10%.

caused complete inhibition of heterocyst differentiation and nitrogenase activity, thus suggesting a role of novel proteins in induction of heterocyst formation and nitrogenase activity in the parent strain. Str^r-mutant **did** not show Streptomycin inhibition of heterocyst formation and nitrogenase activity but did show Ethionine inhibition of the two processes. Similarly Eth^r-mutant strain while continued production of heterocyst and nitrogenase in the presence of Ethionine, did not show heterocyst **formation** and nitrogenase activity in the presence of Streptomycin. These findings suggest that the mode of inhibition of heterocyst formation and nitrogenase activity by these two inhibitors is different and that there is no cross-resistant relationship between them for heterocyst formation and nitrogenase activity.

b) Photosynthetic inhibitors-DCMU and Atrazine:

We examined the effect of DCMU and Atrazine and the combined aflect of DCMU + Atrazine on heterocyst differentiation and nitrogenase activity in parent, $DCMU^r$-mutant and $Atrazine^r$-mutant strains. It is important to mention here that Eth^r-mutant and Str^r-mutant strains were as sensitive to growth, heterocyst formation and nitrogenase activity by DCMU and Atrazine as the parental strain. Nitrate grown non-heterocystous, non N_2-fixing cultures of the three strains were taken and transferred separately into fresh N_2-medium lacking or containing the herbicides. Results of such a study are shown in Table 5.

DCMU or Atrazine or combination of both blocked heterocyst formation and nitrogenase activity in the parent strain, however, neither herbicide by itself or in combination with the other blocked heterocyst

Table 5

Effect of DCMU, Atrazine or Both (5 μM each) on heterocyst frequency and nitrogenase activity of the parent *Nostoc muscorum*, $DCMU^r$-mutant and $Atrazine^r$-mutant strains under diazotrophic growth conditions.

Strain	Control		DCMU		Atrazine		DCMU + Atrazine	
	Heterocyst frequency	Nitrogenase activity	Heterocyst frequency	Nitrogenase activity	Heterocyst frequency	Nitrogenase activity	Heterocyst frequency	Nitrogenase activity
Parent	4.00	8.85	0.0	0.0	0.0	0.0	0.0	0.0
$DCMU^r$-mutant	4.5	5.8	3.85	2.87	3.9	3.20	4.0	5.50
$Atrazine^r$-mutant	4.6	6.01	3.7	2.95	4.0	3.8	4.0	5.85

Nitrogenase activity expressed as n mol C_2H_4 formed mg Chl $\underline{a}^{-1}$ hr^{-1}.

Heterocyst frequency calculated as number of heterocysts per 100 vegetative cells.

All the above results are an average of three independent experiments which do not vary by more than 10%.

formation and nitrogenase activity in $DCMU^r$-mutant or $Atrazine^r$-mutant. These results emphasise a role of photosystem II activity in control of heterocyst differentiation and nitrogenase activity. The inability of either herbicide separately or in combination, to inhibit heterocyst formation and nitrogenase activity in the herbicide resistant mutant strains suggests a role of the herbicide target in controlling heterocyst formation and nitrogenase activity-

Effect of inhibitors on Glutamine synthetase (transferase and biosynthetic) activity of parent N.muscorum and its various resistant mutants under different growth conditions:

a) Streptomycin and L-Ethionine:

The results of studies on Glutamine Synthetase activity in cultures treated with Streptomycin and Ethionine are given in Table 6. The parent was not treated with either inhibitor because of its extreme sensitivity to them. As the results indicate mutation to Streptomycin-resistance or Ethionine-resistance has not been accompanied by significant alteration in the activity of glutamine synthetase enzyme. The mutant strains like parent strain showed nearly 50% reduction in its glutamine synthetase activity when grown in ammonium medium than when growing in N_2-medium. Glutamine synthetase activity of nitrate assimilating cultures of the three strains were almost similar to the glutamine synthetase activity of their N_2-assimilating cultures. It is therefore, clear that mutation has not altered ammonium reduction of glutamine synthetase activity.

Table 6

Effect of Streptomycin and L-Ethiomine on Glutamine synthetase (transferase and biosynthetic) activity in Str^r-mutant and Eth^r-mutant strains under different growth conditions.

Growth conditions	Glutamine synthetase (transferase) activity					Glutamine synthetase (biosynthetic) activity				
	Parent strain	Str^r-mutant		Eth^r-mutant		Parent strain	Str^r-mutant		Eth^r-mutant	
		+Str	-Str	+Eth	-Eth		+Str	-Str	+Eth	-Eth
N_2-medium	1.6	1.5	1.5	1.4	1.4	0.16	0.14	0.14	0.14	0.14
NO_3^--medium	1.3	1.2	1.2	1.1	1.1	0.15	0.13	0.13	0.13	0.12
NH_4^+-medium	0.9	0.87	0.84	0.52	0.52	0.1	0.09	0.09	0.04	0.04

Glutamine synthetase (transferase) activity is expressed as μ mol product formed mg protein^{-1} min^{-1}.

Glutamine synthetase (biosynthetic) activity is expressed as μ mol of NADH oxidised mg protein^{-1} min^{-1}.

-Str and +Str indicates cultures lacking and containing Streptomycin (20 μM) respectively.

-Eth and +Eth indicates cultures lacking and containing Ethionine (130 μM) respectively.

All the results are an average of three independent experiments which did not vary more than 10%.

For other details please see text.

Effect of DCMU and Atrazine on Glutamine synthetase (transferase and biosynthetic) activity in parent Nostoc muscorum and its DCMU^r-mutant and Atrazine^r-mutant strains under different growth conditions.

Growth conditions	Glutamine synthetase (transferase) activity								Glutamine synthetase (biosynthetic) activity							
	Parent strain				DCMU^r-mutant		Atrazine^r-mutant		Parent strain				DCMU^r-mutant		Atrazine^r-mutant	
	+D	-D	+A	-A	+D	-D	+A	-A	+D	-D	+A	-A	+D	-D	+A	-A
N_2-medium	1.2	1.6	1.3	1.6	1.4	1.5	1.4	1.4	0.13	0.16	0.14	0.17	0.14	0.14	0.13	0.13
NO_3^--medium	0.9	1.2	1.0	1.3	1.1	1.1	1.2	1.2	0.1	0.15	0.1	0.16	0.13	0.13	0.12	0.13
NH_4^+-medium	0.7	0.9	0.5	0.9	0.8	0.8	0.8	0.8	0.04	0.1	0.04	0.1	0.09	0.09	0.09	0.09

Glutamine synthetase (transferase) activity is expressed as μmol product formed mg protein^{-1} min^{-1}.
Glutamine synthetase (biosynthetic) activity is expressed as μmol NADH oxidised mg protein^{-1} min^{-1}.

-D and +D indicates cultures lacking and containing DCMU (6 μM) respectively.
-A and +A indicates cultures lacking and containing Atrazine (5 μM) respectively.

All the results are an average of three independentexperiments which did not vary by more than 10%.

For other details please see text.

Table 8

Photochemical activities (PS II and PS I) in parent N. muscorum, DCMU^r-mutant and Atrazine^r-mutant strains under diazotrophic growth conditions.

Treatment	Parent Strain		DCMU^r-mutant Strain		Atrazine^r-mutant strain	
	$H_2O \rightarrow pBQ$	DCIP $\rightarrow$ MV	$H_2O \rightarrow pBQ$	DCIP $\rightarrow$ MV	$H_2O \rightarrow pBQ$	DCIP $\rightarrow$ MV
Control	289.0	292.16	136.0	294.12	178.0	301.27
+ DCMU (5 μM)	0.0	293.18	112.1	294.14	142.1	300.9
+ Atrazine (5 μM)	0.0	295.25	56.5	294.12	95.2	303.25

PS II activity ($H_2O \rightarrow pBQ$) expressed as μ mol O_2 evolved mg Chl$\underline{a}^{-1}$ hr^{-1}.

PS I activity (DCIP $\rightarrow$ MV) expressed as μ mol O_2 consumed mg Chl$\underline{a}^{-1}$ hr^{-1}.

All the results are an average of three independent experiments which did not vary by more than 10%.

b) Photosynthetic inhibitors : DCMU and Atrazine:

The effect of DCMU and Atrazine on glutamine synthetase activity was also studied as shown in Table 7. Neither DCMU nor Atrazine was able to cause significant variation in glutamine synthetase activity of **the** parental strain or DCMU or Atrazine resistant strains. In cultures treated with herbicides for 48 hrs. apparently the target of DCMU **and** Atrazine inhibitory action does not seem to be the activity of glutamine synthetase enzyme in cyanobacteria.

Photochemical activities (PS II and PS 0 in parent Nostoc muscorum, DCMU^r-mutant and Atrazine^r-mutant strains under diazotrophic growth conditions:

Parent and the two herbicide resistant strains were also examined for the sensitivity of their photosystem I (PS I) and photo-system II (PS 11) activity to DCMU or Atrazine under in vitro condition. The result of such studies are shown in Table 8. Mutation to DCMU or Atrazine resistance has resulted in decreased activity of PS 11. No such reduction or significant variation in PS I activity was found as a result of mutation to herbicide resistance. This is an evidence to suggest an involvement of herbicide sensitive target in control of only PS II activity in the cyanobacterium. PS II activity of parent was completely sensitive to DCMU or Atrazine. PS II activity of DCMU^r-mutant showed almost near complete resistance to DCMU inhibition, however, its activity was only partially resistant to Atrazine inhibition. Similarly, PS II activity of Atrazine^r-mutant was almost completely resistant to Atrazine but was partially resistant to DCMU inhibition. To conclude,

the *in vitro* results suggests that mutation to herbicide resistance is specifically associated with the acquisition of resistance of PS II activity to the herbicide and that in DCMU^r-mutant, PS II activity is partially resistant to **Atrazine and** PS **II** activity of Atrazine^r-mutant **strain is** partially resistant **to** DCMU **inhibition.**

DISCUSSION

Heterocystous filamentous cyanobacteria **show** nitrate **or** ammonium repression of heterocyst formation and nitrogenase activity. Nitrate or ammonium assimilating filaments of such cyanobacteria therefore remain nonheterocystous and non-nitrogen-fixing. On transfer of such nitrate and ammonium grown non-heterocystous, non-N_2-fixing filaments to N_2-medium, heterocyst and nitrogenase activity start appearing in parallel fashion. After nearly 24 hrs. of the transfer period they differentiate and reaches peak value by 48 hrs. of such transfer (Fogg *et al*, 1973). Heterocyst differentiation and nitrogenase activity is a function of N_2-starvation signal and during this process, a series of novel proteins including nitrogenase are synthesized along with laying down of new wall layers of polysaccharide nature and glycolipid nature in developing heterocyst (Haselkorn, 1978; Stewart, 1980). Any inhibitor of protein synthesis or any amino acid analogue leading to defective protein formation is expected to block heterocyst differentiation and N_2-fixation. It is therefore as expected that Streptomycin and Ethionine while inhibiting growth also inhibited heterocyst differentiation and nitrogenase activity. Mutants resistant to these inhibitors are expected to lack the growth inhibitory effect of the inhibitor, inhibiting heterocyst

differentiation and nitrogenase acvtivity. The results of the Streptomycin and Ethionine effect on growth, heterocyst differentiation, nitrogenase activity and glutamine synthetase activity are in full agreement with the similar studies reported earlier in cyanobacteria (Bazin, 1968; Shestakov and Khyen, 1970; Stewart and Singh, 1975; Ladha and Kumar, 1978).

In cyanobacteria, photosystem II is a pigment protein complex in the thylakoid membrane, consisting of atleast five membrane-internal proteins namely CP-47, CP-43, D1 (32 kd, herbicide binding protein), D2 (34 kd) and Cyt b_{559}. The herbicide binding protein or Dl protein is the target of action of DCMU or Atrazine in oxygenic, photosynthetic apparatus of Chloroplast or cyanobacteria (Curtis and Haselkorn, 1984; Golden and Haselkorn, 1985; Vermaas et al, 1987). Using the technique of heterologous gene probe Haselkorn and his group have demonstrated the occurance of three copies of psb A gene (psb AI, psb A 11, psb A III) in the cyanobacterium Anabaena 7120 (Curtis and Haselkorn, 1984). In subsequent extensive work on the molecular genetics of psb A gene they have shown that psb A system comprises a group of three genes occuring at different locations in the cyanobacterial genome, undergoing transcription at all stages of photoautotrophic growth and anyone being capable of supporting good photoautotrophic growth (Brusslan and Haselkorn, 1989).

psb A gene codes for 32 kd protein or D1 protein which is a component of PS II reaction centre in cyanobacteria. By using elegant techniques of molecular genetics involving gene deletion and gene inactivation, the group of Haselkorn has shown that mutation to Diuron resistance involves a change at 264 amino acid residue of Dl

protein from serine to alanine and that mutation in any one of the **three** psb A genes to Diuron resistance is dominant over their remaining **two** herbicide sensitive counterparts (Brusslan and Haselkorn, 1989). This is a very fortunate situation for easy isolation of herbicide resistant mutants in cyanobacteria.

The spontaneously occuring mutants of N. muscorum to DCMU or Atrazine resistance arose with a frequency characteristics of a single gene determinant. Growth, PS II activity, heterocyst differentiation and nitrogenase activity of $DCMU^r$-mutant or $Atrazine^r$-mutant strain were found to be simultaneously resistant to both the herbicides. Such findings imply a common target of inhibiting action of the two herbicides. Our mutant strains cannot be permeability mutants, as their in vitro PS II activity was also found resistant to herbicide inhibition. It is therefore, concluded that mutation in psb A gene is the cause of resistance phenotype to DCMU or Atrazine and that these strains become resistant to either herbicide by producing a mutant 32 kd protein resistant to both herbicides. Quantitatively DCMU is found more inhibitory to oxygenic photosynthesis than Atrazine, also $DCMU^r$-mutant is not as resistant to Atrazine as to DCMU, conversely Atr^r-mutant is not as resistant to Atrazine as to DCMU. The variability in the resistance level of the parent or mutant cyanobacterium to DCMU and Atrazine could result from occurance of differential herbicide binding sites in D1 protein.

Recently study of this problem has been done in considerable detail at molecular level by Kirilovsky et al (1989) who have shown a definite co-relation between difierent site mutations in psb A gene

and the **resulting amino-acid chain in D1 protein associated with acquisition of variable level of resistance to DCMU and Atrazine.**

The heterocyst differentiation and nitrogenase activity of the parent strain was found sensitive to inhibitory action of either herbicides. Since these herbicides inhibit PS II activity leading to inhibition of reductant, photo-reductant generation essential for CO_2 assimilation under autotrophic growth condition and since heterocyst development and **differentiation including nitrogenase activity requires abundant supply of photosynthetically generated organic carbon, it is therefore, natural that photosynthetic herbicides like DCMU or Atrazine should inhibit the process of heterocyst formation and nitrogenase activity.**

The glutamine synthetase activity of parent and its various **mutant** strains were also examined in the presence and absence **of their** respective inhibitors. Results suggest no inhibitor causes growth inhibitory effect due to their primary action on the glutamine synthetase activity. All the mutants reported here are stable **in** their respective phenotypes.

CHAPTER V

STUDIES ON GENETIC TRANSFORMATION

INTRODUCTION

Transformation is a mode of genetransfer system and has been used extensively in analysing and understanding the structure, function and regulation of native or foreign genes. Studies on gene-transfer system in cyanobacteria started with the report of apparent genetic recombination for antibiotic resistance markers in the mixed culture of unicellular cyanobacteria Anacystis nidulans. Pikalek (1967) questioned the conclusion of Kumar (1962) on the basis of unstable nature of Penicillin resistance marker used as a genetic trait in the recombination studies. Bazin (1968) provided convincing evidences for recombination of antibiotic resistance marker in Anacystis nidulans. Singh and Sinha (1965) following the method of Kumar (1962) provided evidence for genetic recombination in Cylindrospermum rnajus. Singh (1967) showed evidence of genetic recombination lor sporulation character in Anabaena doliolum. Stewart and Singh (1975) showed transfer of nif genes from parent Nostoc muscorum to its non N_2-fixing mutant strain. Padhy and Singh (1978) provided further evidence for the phenomenon of gene transfer and recombination in Nostoc muscorum. Singh and Singh (1982) went to analyse the nature of het gene, nif gene and their common regulatory gene by the recombination method and found evidence to conclude distinct classes of genes operating in organisation of heterocysts and nitrogenase enzyme. Trehan and Sinha (1981) also showed process of recombination in mixed cultures of mutant strains of N.muscorum and they analysed in a very preliminary way

the process oi genetransier preceeding recombination and concluded that genetransfer process occuring in mixed cultures is DNase sensitive. All these studies merely showed repeatedly the occurance of genetic recombination in unicellular non N_2-fixing and heterocystous filamentous N_2-fixing cyanobacteria, without specifying with unequivocal evidence, the nature of genetransfer process.

Transformation as a mode of genetransfer system leading to production of stable recombinants for antibiotic resistant marker in Anacystis nidulans 602 was shown by Shestakov and Khyen (1970). Subsequent studies have shown transformation taking place in Aphanocapsa 6714 (Astier and Espardellier, 1976). Gloeocapsa alpicola (Devilly and Houghton, 1977) Agmenellum quadruplicatum PR-6 (Stevens and Porter, 1980) and Synechocystis PCC 6803 (Grigorieva and Shestakov, 1982). In all these studies the genetic markers used have been chromosomal and therefore, they have provided evidence for naturally occuring chromosomal transformation system in cyanobacteria. Cyanobacteria are known to contain cryptic plasmids of various sizes. Demonstration of plasmid transformation in cyanobacteria would be expected to help development of plasmid cloning vehicles that can be used to produce cyanobacterial partial diploids or merodiploids, as well as to introduce foreign genes. One of the cryptic plasmids of A. nidulans pUH 24 has formed the basis for the development of plasmid shuttle vectors capable of replication in both, A. nidulans and Escherichia coli (Golden and Sherman, 1983). Van den Hondel et al (1979) for the first time constructed the pUH24::Tn 901 recombinant plasmids transformable to A. nidulans R2, thus for the first time showing evidence for expression

of β-lactamase gene of E. coli (transposon Tn 901). Subsequent workers have used genetically marked derivatives of pUH 24 to determine the optimal conditions for transformation in A. nidulans R2 (Chauvat et al, 1983). The two most important factors in this regard have been found to be the physiological state of the organism and the method employed to select for transformed genetic marker. The shuttle vector strategy have been used to examine the expression of E. coli lac Z gene in the unicellular cyanobacterium Agmenellum quadruplicatum PR-6 and the results show an efficient expression of this gene in cyanobacterial host as in E. coli host background (Buzby et al, 1985). Subsequent efforts have been made to construct better shuttle vectors with multiple restriction sites for efficient cloning of cyanobacterial gene in E. coli or E. coli gene in cyanobacteria (Gendel et al, 1983; Golden and Sherman, 1983; Friedberg and Seijffers, 1983). A further development in the ability of cyanobacteria to be transformable by an E. coli plasmid pBR 322 was successfully demonstrated by Daniell and McFadden (1986) thus opening the way lor cloning of a wide variety of prokaryotic genes in the cyanobacteriuni host and vice versa. Shuttle cosmid vector has also been used for cloning of nitrate reductase gene in A. nidulans R2 (Kuhlemeier et al, 1984). The development and establishment of efficient transformation system with chromosomal DNA or plasmid DNA in unicellular cyanobacteria have opened wide range of genetic analysis involving modern techniques of genetransfer based method of mutagenesis, such as transposon mutagenesis, recombinational mutagenesis, specific gene inactivation and site directed mutagenesis. These new methods have helped in identifying and cloning of specific cyanobacterial genes under a given set of genetic background (Tandeau de

Marsac et al, 1982; Williams and Szalay, 1983; Brusslan and Haselkorn, 1989). The increase in successful application of the tools of recombinant DNA technology in cyanobacteria, one expects a lot of exciting developments in understanding the genetics of cyanobacteria as a whole and of specific processes peculiar to cyanobacteria such as oxygenic photosynthesis, N_2-fixation, heterocyst differentiation, akinete formation and osmoregulation.

A series of studies have confirmed transformation as a mode of genetransfer system in homologous or heterologous nature of unicellular recipient cyanobacteria leading to recombination for chromosomal or plasmid markers. Although genetic recombination has been repeatedly demonstrated in mixed cultures of heterocystous cyanobacteria, nothing is known about the mode of genetransfer leading to recombination in such systems. Wolk et al (1984) have developed a triparental method of mating for filamentous cyanobacteria. In this process two E. coli strains are used, one bearing the broad host range plasmid RP4 and the other bearing the hybrid shuttle vector which is to be transferred to a recipient cyanobacterium and a helper plasmid to aid in its transfer. Such a method of conjugative genetransfer has succeeded in conjugal transfer of hybrid shuttle vectors from E. coli to the cyanobacterial cells carrying the drug resistance markers like Chloraniphenicol resistance, Kanamycin resistance, Streptomycin resistance and Erythromycin resistance. (Wolk et al, 1984; Flores and Wolk, 1985). McFarlane et al (1987) have succeeded in improving the method of conjugal transfer from triparental combination to biparental combination involving E. coli and Anabaena sps. The workers are working intensively to standardise

and optimise conjugal transfer of **genes** in cyanobacteria. Recently, **studies** have been done on the successful method of transformation in heterocystous forms. Singh et al (1987) demonstrated genetic transformation of azide-assimilating phenotype from N. muscorum to Anabaena doliolum. Subsequently herbicide resistance gene occuring naturally in the cyanobacterium Gloeocapsa sps has also been transformed to Nostoc muscorum (Singh et al, 1987). Thus it does seem that transformation as a system of genetransfer in heterocystous forms operates. The studies described here will reveal that transformation is not a naturally occuring process in the heterocystous forms and therefore one has to generate artificial conditions for the successful operation of this process in heterocystous forms. This chapter is divided into two sections, one dealing with conditions that are required for the successful transformation in heterocystous forms and the other section with genetic markers used for transformation in various combinations.

A. CONDITIONS FOR TRANSFORMATION

EXPERIMENTAL PROCEDURE

Total DNA isolation:

The method of Mazur, Rice and Haselkorn (1980) as described in Singh et al (1987) was followed for DNA isolation. Cyanobacterial cells, in their late exponential phase, were harvested, washed and resuspended in Tris.Cl-50mM, EDTA-100mM, Sucrose 25%, pH 8.0 (TES) at 1g (wet weight) per 10 ml of TES. Lysozyme (Sigma), in 0.25 M Tris, pH 8.0, was added to a final concentration of $10 \mu g\ ml^{-1}$. After

incubating the mixture at 37°C, for 60 min, the cells were gently lysed by adding sodium dodecyl sulphate (SDS) to a final concentration of 2%. Immediately, NaCl was added to a final concentration of 1.0 M, and the mixture was extracted once with phenol : chloroform : isoamyl alcohol (25:24:1) and then twice with chloroform : isoamyl alcohol (24:1). Chilled ethanol (2 volumes) was added to the acqueous phase, and the high molecular weight DNA was spooled onto a glass rod and dissolved in an appropriate volume of Tris.Cl-10 mM, EDTA-1mM, pH 8.0 (TE). The DNA was treated with ribonuclease (RNase) A at 100 µg ml^{-1} (predigested, at 70°C for 10 min) and then with pronase (at 50 µg ml^{-1}). DNA was again precipitated with ethanol and dissolved in TE. The integrity of the DNA was always checked by electrophoresis on agarose gel (0.8%) before purifying it. The buffers used in gel electrophoresis were electrophoretic buffer consisting of 0.089 M Tris, 0.089 M Boric acid and 0.002 M EDTA and gel-loading buffer with a composition of 0.25% Bromophenol blue and Sucrose 40% (Maniatis et al, 1982). Sterile glass ware and buffer solutions were used throughout.

Purification of isolated DNA:

The isolated DNA was purified on CsCl/Ethidium bromide density gradient centrifugation as described in Maniatis et al (1982). The volume of DNA solution was measured and for every millilitre of it lug of solid Cesium chloride was added and mixed gently until the entire salt dissolved in it. For every 10 ml of Cesium chloride solution, 800 Ml of Ethidium bromide solution (10 mg ml^{-1} in water) was added and mixed well. The final density of the solution was 1.55 g ml^{-1}

and the concentration of Ethidium bromide would be 600 ug ml"[1]. The CsCl/Ethidium bromide solution was then transferred to 4.5 ml capacity polyallomer tubes and centrifuged in a Beckman V T. 80 rotor at 60,000 rpm for 6 hrs at 18°C temperature. The upper chromosomal DNA band was collected and Ethidium bromide was extracted from it with TE-saturated n-Butanol. The acqueous phase was dialysed against TE (pH 8.0) for several hours with atleast three changes of buffer. The integrity of the DNA was always checked by electrophoresis on agarose gel (0.8%) before using it in transformation experiments.

Sensitivity to DNase:

The **DNA** isolated from cyanobacterial cells was treated with DNase (100 µg ml"[1]) for 15 mins at 37°C. Sensitivity of DNA to DNase was checked by gel electrophoresis.

Preparation of transformable recipient population and standardisation of conditions for optimum transformation:

Preparation of recipient cyanobacterial samples:

(a) Growth cycle and Transformation process:

The cyanobacteria used in transformation experiments were parental Nostoc muscorum, Anabaena doliolum and Nostoc calcicola, all heterocystous, filamentous, diazotrophic forms and unicellular, non N_2-fixing Synechococcus sp. The heterocystous forms were grown diazotrophically in modified Chu 10 medium (Gerloff et al, 1950) while Synechococcus sp was grown in nitrate medium. Parent Nostoc muscorum takes 15 days to complete its growth cycle, consisting of linear phase, exponential phase and stationary phase. JN. calcicola and A. doliolum

take nearly 20 to 22 days to complete their growth cycle. A. doliolum undergoes akinete formation in 20-22 days old culture. Synechococcus sp takes nearly 12-15 days to complete the growth cycle. L-Ethionine resistant phenotype of spontaneously occuring mutant of N. muscorum was used as a marker to study the process of transformation in homologous and heterologous, heterocystous filamentous forms. 5, 10 and 15 days old cultures of heterocystous forms were used as recipients in transformation experiments with purified DNA from L-Ethionine resistant Nostoc muscorum donor strain.

(b) Artificial induction of transformable state in the cyanobacterial population

1. $CaCl_2$ treatment;

Cultures from different growth stages were also subjected to treatment by $CaCl_2$ as described for Gloeocapsa alpicola by Devilly and Houghton (1977). Recipient cells were grown to a density of 2×10^8 CFU ml^{-1}, chilled quickly, sedimented and washed once in half the volume with 10 mM NaCl, centrifuged again and resuspended in half the original volume of chilled 30 mM $CaCl_2$. The suspension was held at 0°C for 20 min, then centrifuged and resuspended in one tenth the original volume with 30 mM $CaCl_2$ solution. $CaCl_2$-treated recipient cells were then used for further transformation experiments.

2. Permeaplast preparation:

Permeaplasts were made of recipient cells according to Daniell and McFadden (1986) with appropriate modifications. Recipient

cells were grown to a density of $2x10^8$ CFU ml^{-1} and washed with Tris-Cl-10mM EDTA-1mM, pH 7.5 (TE) buffer and resuspended in 1 ml of TE. 2 mg of Lysozyme (Sigma) was added and incubated for one hour at 36°C in the presence of photosynthetic light. After incubation the cells were washed with TE buffer and centrifuged and resuspended in 1 ml of TE buffer. Cells treated with lysozyme and EDTA were called permeaplasts. Permeaplasts are potentially viable structures of unusually high permeability and capacity for cell-wall regeneration and may therefore take up DNA at very high rates and subsequently repair and divide (McFadden and Daniell, 1988). Permeaplasts of 5, 10 and 15 days old culture were prepared and transformation experiments were done after different time intervals of making permeaplasts such as 1 hr, 2 hrs, 3 hrs, 4 hrs and 5 hrs.

Standardization of conditions:

a. Effect of washing on transformation:

Cyanobacterial cells of 10 days old were harvested by centrifugation and resuspended in 100 µl oi fresh N_2-medium to yield a final cell concentration of approximately $2x10^6$ CFU ml^{-1}. Two different sets of cells were taken, one was washed with N_2-medium and the other with TE buffer (pH 7.5). They were again resuspended in 1 ml of N_2-medium and TE buffer respectively and permeaplasts were prepared. **Washing with** TE (1X) buffer reduces the action of nucleases present in the culture on the transforming DNA.

Colony forming unit (CFU)

A filament of a healthy growing filamentous cyanobacterium in its late exponential phase of growth, contains hundreds of cells (>500). Even though every vegetative cell has the capacity to form a colony, the whole filament forms a single colony after plating on agar. Hence, for the sake of convenience in calculating transformation frequency, these long filaments were broken gently by vortexing (for a period of 30 to 60 s) in the presence of sterile glass beads. This process produced filaments with an average of 100 cells per filament. This unit was taken as CFU. The whole process was carried out aseptically and after spreading these CFU on agar plates, the percentage survival was found to be greater than 90%.

b. DNA contact period and DNA concentration versus transformation frequency:

The desired purified donor DNA extracted from Ethionine-resistant Nostoc musc or urn was added to the recipient whole cells or permea plasts ($2x10^6$ CFU) in a concentration range of 0.25, 0.5, 1.0, 1.5, 2.0, 2.5 $\mu g\ ml^{-1}$ and the transformation mixture was incubated at 28± 2°C under photosynthetic light (50ymol $m^{-2}\ s^{-1}$) for 60 min. The mixture was treated with DNase I to a final concentration of 100$\mu g\ ml^{-1}$ and the final Mg^{2+} concentration was 10 mM, at the end of incubation period. Samples were withdrawn from different mixtures and spread on non-selective agar plates ($2x10^4$ CFU per plate) and after 48 hrs the selective agent L-Ethionine to a final conc. of 76 μM was added. After the optimum concentration of DNA which gives maximum number

of transformants was determined, using that DNA concentration, the process of transformation was repeated and samples were withdrawn from the transformation mixture at diferent time intervals of 15 mins, 30 mins, 60 mins and 75 mins. The DNA uptake was stopped by the addition of DNase I at the end of different time intervals. Samples were plated on nonselective agar plates as described before and then transformants were scored.

c. Effect of Light and Dark on transformation efficiency;

The permeaplasts of recipient cells were treated with lug ml^{-1} of donor DNA **and incubated at** 28±2°C. One set was incubated in the presence of photosynthetic light and the other set was incubated in dark and after 60 min the process of DNA uptake was stopped by the addition of DNase 1. Samples were taken from both the sets and plated on non-selective agar medium and after 48 hrs. the selective agent L-Ethionine was added to a final concentration of 76 µM.

d. Method of scoring transformants;

Selection of drug-resistant or any other marker transformants on solid medium required that the potential transformants not be challenged with the selective agent until they have had time to express the relevant gene after its introduction into the cell. This can be accomplished by either of the methods mentioned here:

Underlaying:

The edge of agar was lifted and an appropriate volume of an acqueous solution of the selective agent was added under the agar.

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The edge of agar was lifted and an appropriate volume of an acqueous solution of the selective agent was added under the agar.

The selective agent was placed close to the middle of the plate. The whole process was done under aseptic conditions.

RESULTS

A basic step in the functioning of a transformation system in any microbe is the development of cellular competence for DNA uptake, depending on how it is developed, competence may be either physiological or artificial in nature. Physiological competence, defines the physiological state of some cells in the population which possess a natural ability to productively internalise exogenous DNA without any special treatment to the cells before their exposure to the donor DNA. Artificial competence means, occurance of DNA internalisation by cells as a result of treatment regimen that is never a part of the normal growth cycle. The results of transformation process in heterocystous cyanobacteria like N. muscorum, A. doliolum and N. calcicola for Ethionine resistance marker do not suggest heterocystous forms naturally to be competent for transformation (as revealed by the data presented in Table 9) because the frequency of transformants with recipient whole cells harvested from different stages of growth was almost zero i.e. it was in the range almost similar to spontaneous mutation frequency as given in the previous chapter. $CaCl_2$ treatment of the recipient cells also did not cause any significant increase in transformation frequency (data not given). The permeaplast preparation in TE buffer were found to be quite effective source of recipient population for the transformation process (Table 10) however, when one examines the efficiency of transformation process as a function of the age of

Table 9

Transformation frequency to Ethionine resistance at different stages of cyanobacterial growth cycle.

Different stages of growth (Days)	Transformation frequency*		
	Nostoc muscorum	Nostoc calcicola	Anabaena doliolum
5	0.1×10^{-6}	0.09×10^{-6}	0.07×10^{-6}
10	0.8×10^{-6}	0.6×10^{-6}	0.3×10^{-6}
15	0.05×10^{-6}	0.03×10^{-6}	0.03×10^{-6}
DNase treated DNA	0.01×10^{-7}	0.0	0.0

*Transformation frequencies are given as the number of transformants obtained in relation to viable colony forming units (CFU) in the transformation mixture. An average of 100 cells was taken as a CFU unit. The data presented here are an average of three independent experiments.

Recipient whole cells (2×10^6 CFU) from different stages of growth cycle were incubated with 1 μg ml^{-1} of donor DNA from Ethionine resistant N. muscorum in the presence of photosynthetic light, at a temperature of 28 ± 2°C and for 60 mins. The process was terminated by adding 100 μg ml^{-1} of DNase I to the incubation mixture. Incubation of recipient whole cells with DNase treated DNA was also carried out in the same way as described above.

Table 10

Effect of washing and resuspending medium on Transformation frequency of permeaplasts in Tris, Cl (10mM pH 7.5), EDTA (1mM) buffer (TE) of N. muscorum, N. calcicola and A. doliolum to Ethionine resistance.

Treatment (Nature of washing and permeaplast preparation)	Transformation frequency*		
	Nostoc muscorum	Nostoc calcicola	Anabaena doliolum
N_2 - medium	1.3×10^{-5}	1.1×10^{-5}	1.0×10^{-5}
TE buffer	14.8×10^{-4}	9.3×10^{-4}	7.2×10^{-4}
DNase treated DNA	0.03×10^{-6}	0.01×10^{-6}	0.02×10^{-6}

*Transformation frequencies are given as number of transformants obtained in relation to viable colony forming units (CFU) in the transformation mixture. An average of 100 cells was taken as a CFU unit. The data presented here are an average of three independent experiments.

Permeaplasts of the three strains were incubated with donor DNA from Ethionine resistant N. muscorum (1 μg/2x10^6 CFU of recipient cells) for 60 mins in the presence of photosynthetic light, at a temperature of 28 ± 2°C and the process was terminated by the addition of DNase I (100 μg ml^{-1}) to the incubation mixture. Incubation of permeaplasts in TE was also done with DNase treated DNA in the same way as described above.

permeaplasts, one finds that transformation frequency decreased with increase in the age of the permeaplasts. In fact 1 hr old permeaplast population were most effective in the process of transformation. Such 5 hour old permeaplasts were found inactive as a source of recipient cells in transformation experiments. The basic reason for inactivity of such 5 hr old permeaplasts was the loss of viability, as 5 hr old permeaplasts failed to regenerate the normal filament or colony. In addition permeaplasts of 5, 10 and 15 day old cultures gave almost similar frequency of transformation thus ruling out any role of cyanobacterial physiological state in regulating their transformation frequency. The results of the transformation studies described here are given in Table 11.

In order to examine the requirement of photosynthesis in control of transformation in permeaplasts, transformation experiments were conducted in photosynthetic light and dark respectively for 1 hr and then samples were withdrawn from the transformation mixture and plated on non-selective medium. After 48 hrs they were scored for transformants under selective conditions. The results of such light, dark studies on permeaplast transformation frequency were shown in Table 12. N. muscorum, N. calcicla and A. doliolum all showed nearly more than 100 fold rise in transformation frequency of samples, treated with photosynthetic light over dark treated samples. It is therefore, concluded that transformation frequency in heterocystous cyanobacteria is critically dependent on photosynthetic light.

We also examined the effect of DNA concentration and

Table 11

Age of permeaplasts in relation to Transformation frequency to Ethionine resistance in various cyanobacterial strains.

Age of Permeaplasts (hr)	Transformation frequency*								
	Nostoc muscorum			Nostoc calcicola			Anabaena doliolum		
	Days			Days			Days		
	5	10	15	5	10	15	5	10	15
1	14.95×10^{-4}	15.2×10^{-4}	13×10^{-4}	8.9×10^{-4}	9.5×10^{-4}	8.2×10^{-4}	6.8×10^{-4}	7×10^{-4}	6.75×10^{-4}
2	11.5×10^{-4}	12.2×10^{-4}	10×10^{-4}	6.12×10^{-4}	6.4×10^{-4}	5.9×10^{-4}	4.3×10^{-4}	4.9×10^{-4}	4.1×10^{-4}
3	4.2×10^{-5}	4.4×10^{-5}	3.9×10^{-5}	2×10^{-5}	2.45×10^{-5}	1.8×10^{-5}	1.1×10^{-5}	1.2×10^{-5}	0.9×10^{-5}
4	2.01×10^{-7}	2.1×10^{-7}	1.4×10^{-7}	0.9×10^{-7}	1.8×10^{-7}	0.6×10^{-7}	0.5×10^{-7}	0.6×10^{-7}	0.1×10^{-7}
5	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
DNase treated DNA (hr)	0.03×10^{-6}	0.02×10^{-6}	0.01×10^{-6}	0.04×10^{-6}	0.05×10^{-6}	0.03×10^{-6}	0.01×10^{-6}	0.02×10^{-6}	0.01×10^{-6}

*Transformation frequencies are given as the number of transformants obtained in relation to viable colony forming units (CFU). An average of 100 cells was taken as one CFU unit. The data presented here are an average of three independent experiments.

Permeaplasts of the three strains of different growth phases were incubated with donor DNA extracted from Ethionine resistant Nostoc muscorum ($1\ \mu g/2\times10^{-6}$ CFU of recipient cells) for 60 min. in the presence of photosynthetic light at a temperature of 28 ± 2°C and the process was terminated by addition of DNase I ($100\ \mu g\ ml^{-1}$) to the incubation mixture. Incubation of permeaplasts with DNase treated DNA was done in a similar way as described above.

Table 12

Effect of photosynthetic light and dark treatments on Transformation frequency of N. muscorum, N. calcicola and A . doliolum to Ethionine resistance.

Cyanobacterial strains	Transformation frequency*		
	Light +	Dark ‡	DNase treated DNA
Nostoc muscorum	15.14×10^{-4}	0.8×10^{-6}	0.03×10^{-6}
Nostoc calcicola	9.5×10^{-4}	0.42×10^{-6}	0.01×10^{-6}
Anabaena doliolum	7.2×10^{-4}	0.3×10^{-6}	0.02×10^{-6}

*Transformation frequencies are given as number of transformants obtained in relation to viable colony forming units (CFU) in the transformation mixture. An average of 100 cells was taken as a CFU unit. The data presented here are an average of three independent experiments.

\+ Transformation was carried out in the presence of light (50 μmol $m^{-2}s^{-1}$).

‡ Transformation was carried out in darkness for upto 60 min.

Permeaplasts of the three strains were incubated with donor DNA from Ethionine resistant N. muscorum (1 μg/2 x 10^6 CFU of recipient cells) for 60 mins in the presence and absence of photosynthetic light, at a temperature of 28 ± 2°C and the process was terminated by the addition of DNase I (100 μg ml^{-1}) to the incubation mixture. Incubation of permeaplasts with DNase treated DNA was carried out in the same manner as described above.

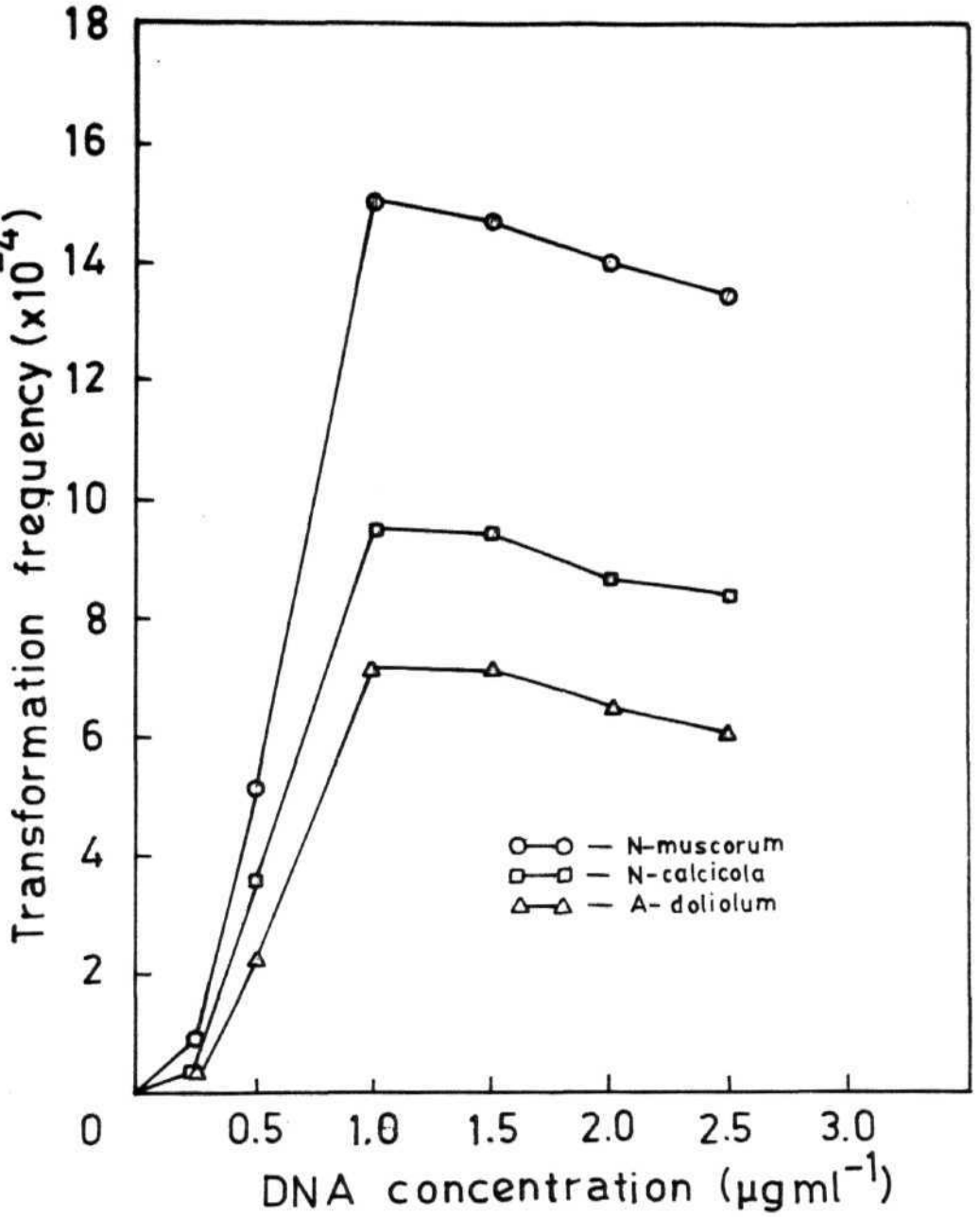


Fig. 8: DNA concentration dependence of the transformation.

DNA contact period on permeaplast transformation frequency under photosynthetic condition. **As shown in Fig. 8** the transformation frequency increased with DNA concentration and reached a saturation value at **a** DNA concentration of 1.0M g ml^{-1}. This concentration **of** DNA was **used** in all subsequent experiments in order to avoid complications in the results because of this factor. Transformation reaction mixture containing 1.0 ug ml^{-1} DNA incubated at a temperature of 28±2°C, under photosynthetic light, showed gradual rise in transformation frequency with the increase in DNA contact period and maximum transformation frequency in all the three heterocystous cyanobacteria was achieved with a contact period of about 60 mins as shown in Table 13.

To summarise it seems there is a lack of naturally occuring competent stage for transformation in heterocystous cyanobcateria. Permeaplast preparation in TE buffer and not in medium is the pre-requisite for raising transformable recipient cells, which require photo-synthetic light for undergoing efficient process of transformation. The three other factors for successful transformation in permeaplast systems are age of permeaplast, DNA concentration and DNA contact period.

Growth, heterocyst frequency and nitrogenase activity of parent, transformant and spontaneous mutant strains with respect to Ethionine resistance are compared and shown in Table 14. As expected parental strains of N. muscorum, N. calcicola or A. doliolum showed severe growth inhibition, heterocyst formation and nitrogenase activity by Ethionine. Ethionine resistant mutant of N. muscorum and transfor-mants of N. muscorum, N. calcicola and A. doliolum grew well, produced

Table 13

Effect of contact period on Transformation frequency to Ethionine resistance in permeaplasts of N. muscorum, N. calcicola and A. doliolum.

Contact period (Time)	Transformation frequency*		
	Nostoc muscorum	Nostoc calcicola	Anabaena doliolum
15 min	2.7×10^{-4}	1.32×10^{-4}	1.03×10^{-4}
30 min	7.31×10^{-4}	4.9×10^{-4}	3.5×10^{-4}
60 min	15.2×10^{-4}	9.5×10^{-4}	7.0×10^{-4}
75 min	4.13×10^{-4}	2.15×10^{-4}	1.8×10^{-4}
Dnase treated DNA	0.03×10^{-6}	0.01×10^{-6}	0.02×10^{-6}

*Transformation frequencies are given as number of transformants obtained in relation to viable colony forming units (CFU) in the transformation mixture. An average of 100 cells was taken as a CFU unit. The data presented here are an average of three independent experiments.

Permeaplasts of the three strains were incubated with donor DNA extracted from Ethionine resistant N. muscorum (1 μg/2×10^6 CFU of recipient cells) in the presence of photosynthetic light at a temperature of 28 ± 2 °C and samples were withdrawn at different time intervals and the process was terminated by addition of DNase I (100μg ml^{-1}) to the incubation mixture. Incubation of permeaplasts with DNase treated DNA for 60 mins. under similar conditions mentioned above was done.

Table 14

Growth, heterocyst frequency and nitrogenase activity (acetylene-reducing activity) of various cyanobacterial strains in N_2-medium containing (+) or lacking (-) L-Ethionine.

Cyanobacterial Strains	Growth in N_2-medium		Heterocyst frequency (%)		Nitrogenase activity (ARA)	
	(+)	(-)	(+)	(-)	(+)	(-)
Nostoc muscorum						
Wild-type	0.0	0.46	0.0	5-6	0.0	8.9
Mutant	0.48	0.50	5-6	5-6	7.8	7.72
Transformant	0.41	0.43	5-6	5-6	7.3	7.5
Nostoc calcicola						
Wild-type	0.0	0.47	0.0	5-6	0.0	7.79
Transformant	0.43	0.45	5-6	5-6	7.25	7.3
Anabaena doliolum						
Wild-type	0.0	0.40	0.0	5-6	0.0	6.87
Transformant	0.44	0.42	5-6	5-6	6.16	6.23

The source of inoculum was nitrate grown non-heterocystous, non-nitrogen fixing cultures. Growth was measured with 6 day old cultures (optical density at 665 nm), heterocyst frequency (number of heterocysts per hundred vegetative cells) and nitrogenase activity (n mol C_2H_4 formed mg chl$\underline{a}^{-1}$ hr^{-1}) were determined in cultures grown for 2 days (48 hr) of mutants, transformants and wild-type strains. L-Ethionine was added at a concentration of 130 µM wherever necessary. Each value is an average of three independent experiments.

heterocysts and showed nitrogenase activity in the presence as well as in the absence of Ethionine in the growth medium. However, though the mutant strain and transformants showed similar heterocyst frequency to that of parental strain both in the absence and presence of Ethionine, diazotrophic growth of mutant strain was in general found to be comparatively more resistant to Ethionine inhibition than that of their transformants. The transformants have been checked for their stability by subculturing them successively for 10 successive subcultures and then examined their resistance to Ethionine. The successive subculturings were found not to dilute or decrease the level of Ethionine resistance. It is therefore concluded that transformation for Ethionine resistance by the method of permeaplast system in N. muscorum, N. calcicola and A. doliolum is genetically as stable as the corresponding spontaneously occuring mutant Ethionine resistant strain.

DISCUSSION

Factors affecting and regulating genetic transformation in unicellular cyanobacteria have been studied extensively. The factors that have been taken into consideration in such studies include, purified DNA versus DNA-RNA complexes, competence, DNA concentration, DNA contact period, photosynthetic light and dark, homospecific and heterospecific nature of transformation process, selective conditions for optimum expression of selected markers and other miscellaneous factors. For precision and convenience the transformation systems so far studied in various unicellular cyanobacteria with regard to the process of transformation and the various factors influencing it are summarised in Table 15.

Table 15

GENES CLONED IN CYANOBACTERIA BY TRANSFORMATION.

Donor	Recipient	Function or gene product	Reference
Escherichia coli	Anacystis nidulans R2	Chloramphenicol resistance	Golden and Sherman (1984) Dzelkalns et al (1984)
		Ampicillin resistance	Williams and Szalay (1983) Golden and Sherman (1984) Lau and Straus (1985)
		Kananycin resistance	Lau and Straus (1985)
Escherichia coli	Fremyella diplosiphon	Chloramphenicol resistance	Cobley (1985)
Gloeocapsa alpicola	Gloeocapsa alpicola	Streptomycin resistance	Devilly and Houghton (1977)
Synechocystis 6803	Synechocystis 6803	Erythromycin resistance Ethionine resistance	Grigorieva and Shestakov (1982)
Escherichia coli	Synechocystis 6803	Kanamycin resistance	Chauvat et al. (1985)
		Ampicillin resistance	Williams and Mcintosh
		Chloramphenicol resistance	Chauvat et al. (1985)
Agmenellum quadruplicatum	Agmenellum quadruplicatum	Streptomycin resistance	Stevens and Porter (1980)
Escherichia coli	Agmenellum quadruplicatum	Amplicillin resistance	Buzby et al. (1983)
		Chloramphenicol resistance	" "
Escherichia coli	Anabaena 7120	Chloramphenicol resistance	Wolk et al. (1984)

Donor	Recipient	Function or gene product	Reference
Staphylococcus aureus	*Anabaena* 7120	Erythromycin resistance	Wolk *et al.* (1984)
Escherichia coli	"	Neomycin resistance	" "
"	"	Streptomycin resistance	" "
Anabaena 7120	*Anabaena* 7120	Nitrogen fixation	Jiawan *et al.* (1984)
Anacystis nidulans 602	*Anacystis nidulans* 602	Erythromycin resistance	Shestakov and Khyen (1970)
"	"	Streptomycin resistance	" "

Sensitivity of transformation process to DNases is strongly suggestive of purified DNA to be the agent of transformation process (Avery et al, 1944). In cyanobacteria **there are reports** showing involvement **of** DNA-RNA hybrid complex as one distinct mode of transformation which is different from the DNA mediated transformation process completely sensitive to DNase (Herdman and Carr, 1971; Devilly and Houghton, 1977). However subsequent studies in general have shown successful transformation in unicellular cyanobacteria with pure DNA preparation. The DNA-mediated transformation system for Ethionine resistance marker was found significantly sensitive to DNase present in the treatment mixture thus suggesting that the transformation process leading to inhibitor resistance is basically DNA dependent. Unlike transformation in unicellular cyanobacteria where it is found to be a natural process, that in hcterocystous forms seem to require artificial induction through the preparation of permeaplasts. The reason for success of transformation in such preparations could be due to unhindered and undamaged uptake of the transforming DNA by perrneaplasts. The extracellular DNase in TE buffer may be prevented from degrading donor DNA by the chelating agent. If DNA is the cause of transformation its concentration should influence the frequency of transformants. In Agmenellum quadruplicatum (Stevens and Porter, 1980) and Synechocystis 6803 (Grigorieva and Shestakov, 1982) the number of genomic: transformants were found to be a linear function of DNA concentration. The present result on transformation with DNA concentration is in agreement with this pattern and it is therefore, concluded that in heterocystous forms, frequency of transformants is a linear function of DNA concentration as reported for unicellular cyanobacteria.

The duration of DNA contact with the recipient population was studied in **order to find** out **the** optimum **time period for transformation to be** maximally effective. The transformation frequency rose with the increase in DNA contact period reaching a saturation value at 60 min, thereby suggesting that DNA contact period like DNA concentration is also important as a factor of significance in cyanobacterial transformation. Similar results have been found for transformation of azide resistance in heterocystous cyanobacteria (Singh et al 1987).

DNA binding and uptake has been shown to be an energy dependent process in heterotrophic transformable bacteria (Lacks, 1970). Cyanobacteria in general are photoautotrophs dependent on photosynthetic energy for all kinds oi cellular functions. This has lead to examining a role of photosynthesis in cyanobacterial transformation. Chauvat et al (1983) found uptake of transforming DNA to be more or less similar in photosynthetic light and in dark. They were showing no significant role of photosynthesis in the uptake ot transforming DNA. Golden and Sherman (1984) found more efficient process oi transformation taking place in the dark than in photosynthetic light. These two authors proposed that energy depleted state of cyanobacterial cells are more efficient in DNA uptake process leading to stable transformation. Williams and Szalay (1983) found greater than ten fold decrease in plasmid transformation frequency in A. nidulans R2 in dark. Singh et al (1987 a&b) showed requirement of photosynthetic light for efficient transformation to azide resistance and herbicide resistance in heterocystous cyanobacteria. Our results are in agreement with these reports.

The permeaplast based transformation system might in general be photosynthetic light dependent in view of their stability problem and primarily photosynthetic nature. It would be interesting to study the relative importance of photosynthetic light in transformable permeaplast preparations of **unicellular cyanobacteria.**

$CaCl_2$ **treatment has** been **reported** to **improve** DNA **uptake in** Gloeocapsa alpicola (Devilly and Houghton, 1977) and transformation in Agmenellum quadruplicatum (Stevens and Porter, 1980). Our preparation did not respond to $CaCl_2$ treatment with respect to transformation to Ethionine resistance.

Among the heterotrophic transformable bacteria the well characterised gram positive organisms showed no specificity with regard to the origin of DNA, that they will take up, but gram negative organisms did show that they will take up only DNA from the same or very closely related species (Smith et al, 1981; Goodgal, 1982). Golden and Sherman (1984) studied this problem in cyanobacterium A. nidulans R2 and conducted DNA-DNA competition experiments, where they showed that heterologous DNA from variety oi sources competes effectively with A. nidulans DNA during the process of uptake. Similar findings have been reported by Stevens and Porter (1980) for A. quadruplicatum thus the unicellular cyanobacteria, that has been examined ior specificity of DNA uptake do not show the specificity of gram negative heterotrophs despite their being themselves gram negative. It will be interesting to study similar DNA uptake specificity in heterocystous cyanobacteria.

Effectiveness of permeaplast system in transformation in heterocystous cyanobacteria has been previously reported (Singh et al, 1987). The present study confirms the suitability of this approach in genetic transformation in heterocystous forms. Recently electroporation has been used to induce heterocystous cyanobacteria to be transformable by plasmid DNA. It will be interesting to compare **the relative** efficacy **of permeaplast method and electroporation** method **in** chromosomal **and** plasmid **DNA** transformation in cyanobacteria in **general** and **in heterocystous cyanobacteria in particular.**

Overlaying method of addition of selective agent was used for scoring transformants and was found to be very effective. Incubation of the transformed cells on non-selective medium for 48 hrs is found to be very essential in order to get good transformation frequency, because uptake, integration and expression of the DNA fragment might be a time consuming process in cyanobacteria and it is therefore not surprising that transformed samples should be allowed to stabilise and grow under nonselective growth conditions before exposing them to selective agents.

B. GENETIC TRANSFORMATION OF ANTIBIOTIC RESISTANCE MARKERS, HERBICIDE RESISTANCE MARKERS AND *nif* AND *het* MARKERS

EXPERIMENTAL PROCEDURE

The plasmid pBR 322 (4.3 kbp) replicates freely in *E. coli* HB 101 and is an extremely versatile cloning vector with Ampicillin resistance and Tetracycline resistance as selective markers and the complete nucleotide sequence of this plasmid is known. Tetracycline

resistance marker is a unstable resistance marker in cyanobacteria growing photoautotrophically and in **the** studies earlier reported pBR 322 has been transferred to unicellular forms with the selectable marker Ampicillin resistance. The plasmid confers in E. coli a level of 572 µM of Ampicillin resistance. The cyanobacterial transformants show much lower level of Ampicillin resistance for reasons yet unknown (Kuck and Mulligan, 1984). In the present study effort was made to study comparatively pBR 322 transformation in unicellular cyanobacterium Synechococcus sp and heterocystous cyanobacteria A. doliolum and N. muscorum.

The broad outline of the procedure as described by Maniatis et al (1982) was followed in studying the pBR 322 mediated transformation to Ampicillin resistance phenotype in the three cyanobacteria.

Bulk preparation and purification of pBR322 from E. coli HB101

1. Inoculation:

A single bacterial colony from Ampicillin containing plate was inoculated into 10 ml of Luria Broth medium (Tryptone $10 g.l^{-1}$, Yeast extract $5 g.l^{-1}$ and NaCl $10 g.l^{-1}$, pH 7.5) containing 28.6uM of Ampicillin and incubated at 37°C overnight with vigorous shaking. The following morning, 0.1 ml of overnight grown culture was inoculated into 25 ml of LB medium in a 100 ml flask containing 28.6 uM of Ampicillin and incubated at 37°C with vigorous shaking until culture reached late log phase (OD_{600} 0.6).

II. Amplification:

The late Jog phase culture of 25 ml was then inoculated into 500 ml of LB medium prewarmed to 37°C with 28.6 µM Ampicilin in a 2 litre flask, incubated for 2.5 hrs at 37°C with vigorous shaking. The OD_{600} of the culture would approximately be 0.4. A stock of Chloramphenicol (105.23 mM) in ethanol was made and 2.5 ml of it was **added** to the culture so as to give a final concentration of 0.52 mM of Chloramphenicol The culture was further incubated for 12-16 hrs with vigorous shaking at 37°C.

III. Harvesting and Lysis of Bacteria:

Harvesting:

The bacterial cells were harvested by centrifugation at 4000 g for 10 mins at 4°C and supernatant discarded. Pellet was washed in 100 ml of ice-cold STE (0.1 M NaCl, 10 mM Tris. Cl [pH 7.8] and 1 mM EDTA).

Lysis by Sodium dodecyl sulphate (SDS):

The bacteria) pellet was resuspended in 10 ml of an ice-cold solution of 10% sucrose in 50 mM Tris. Cl [pH 8.0l. 2 ml of freshly **prepared** solution of Lysozyme (10 mg ml^{-1} in 0.2 5 M Tris.Cl, pH 8.0) was added and was left on ice for 15 mins. 4 ml o1 10% SDS was added and mixed quickly with a glass rod so as to disperse the SDS evenly through the bacterial suspension and gently so as not to shear the liberated bacterial DNA. 6.0 ml of 5 M NaCl was added immediately and mixed

gently and thoroughly and kept on ice for nearly 1 hr. The whole thing was centrifuged **to** remove high molecular weight DNA and the bacterial debris, for 30 mins at 30,000 rpm at 4°C. The pellet was discarded and the supernatant was saved. Supernatant was then extracted twice with phenol/chloroform and once with chloroform. After each extraction the acqueous layer was transferred to a clean tube. The acqueous phase was transferred to centrifuge bottles and two volumes of chilled ethanol was added, mixed well and allowed to stand for 1-2 hrs at -20°C and 15 mins at -70°C. The nucleic acids were recovered by centrifugation at 1500 g for 15 mins at 4°C. The supernatant was discarded and pellet was washed with 95% ethanol at room temperature and after as much as possible of ethanol was discarded, the pellet was dried briefly in a vacuum dessicator. DNA was dissolved in 8 ml of TE (pH 8.0).

Purification of plasmid DNA:

The plasmid DNA was purified by centrifugation to equillibrium in Cesium chloride - Ethidium bromide gradients as described previously. Two bands were visible, upper band consisting of linear bacterial DNA and nicked circular plasmid DNA and the lower band closed circular plasmid DNA. The lower band was collected into a glass tube and Ethidium bromide was removed from it as described previously and the sample dialysed against TE. The plasmid DNA was visualised after gel electrophoresis.

Transformation method:

The exponentially growing recipient cultures were harvested,

washed once with N_2-medium and then with TE buffer (Tris Cl 10 mM [pH 7.5] and 1 mM EDTA) and resuspended in 1 ml of TE buffer at a concentration of 2 **x** 10^6 colony forming units (CFU). Permeaplasts **were prepared** by the addition of 2 mg ml^{-1} of Lysozyme (Sigma) and incubated for 1 hr at 36°C in the presence of photosynthetic light. They were washed once with TE buffer after incubation with Lysozyme and resuspended in TE. Donor DNA, extract was added to a final concentration of 1µg ml^{-1}. The transformation mixture was incubated at 28±2°C under photosynthetic light (50 moles $urn^{-2}s^{-1}$) for upto 60 min. The uptake process was stopped by the addition of DNase I and spread on non-selective agar plates (at 2 x 10^3 CFU/plate). The required concentration of selective agents were overlayed after 48 hrs of incubation at 28±2°C, in the light.

Growth, heterocyst frequency and nitrogenase activity were measured according to the method described in Chapter II.

RESULTS

Antibiotic resistance markers : Ampicillin resistance and Streptomycin resistance

Ampicillin resistance

When tested before undertaking the transformation studies, Synechococcus sp and A. doliolum were found inherently sensitive to 0.572 MM and 1.0 µM of Ampicillin respectively, in contrast to this N. muscorum was found growing as effectively in Ampicillin as in antibiotic free medium. N. muscorum therefore carries natural resistance

to Ampicillin. Permeaplast preparations of A. doliolum did not show any evidence of transformation with pBR322 plasmid DNA under different range of DNA concentration starting from 0.1-2 ug different DNA contact periods and under conditions of photosynthetic light and dark. It is therefore, concluded that transformation with pBR322 in A. doliolum is not possible under the given set of conditions. In contrast the non-nitrogen fixing unicellular cyanobacterium underwent successful transformation with pBR322 with a frequency characteristic of such transformants in other unicellular cyanobacteria. However, the level of resistance of Ampicillin was nearly two fold higher in this cyanobacterium in comparison to that reported for other unicellular cyanobacteria. The comparison of transformation efficiency in this cyanobacterium was made with respect to the competence of whole cells and their permeaplasts. As shown in Table 16 the transformation frequency was nearly two fold higher in permeaplast preparation over that in normal whole cells. This point suggests that although unicellular cyanobacteria are naturally transformable with E. coli plasmid DNA, this frequency could be significantly increased through artificial manipulation of recipient cells (permeaplasts).

A comparison of the growth characteristics of parent Synechococcus sp and its plasmid transformant with and without Ampicillin are shown in Fig. 9. As expected parent did not grow in Ampicillin containing 5.72 μM of antibiotic. Transformant grew both in 5.72 μM and 14.3 μM of the antibiotic. However, growth of the transformant without antibiotic grew as well as its normal parent. Presence of antibiotic did slow the growth of transformants at both concentrations.

Table 16

pBR 322-mediated transformation to Ampicillin resistance in A. doliolum and Synechococcus sps.

Treatment	Transformation frequency*	
	Anabaena doliolum	Synechococcus sps.
Whole cells + no DNA	0.0	0.0
Permeaplasts + no DNA	0.0	0.0
Whole cells + DNA	0.0	3.23×10^{-4}
Permeaplasts + DNA	0.0	5.7×10^{-4}
Whole Cells + DNase treated DNA	0.0	2.5×10^{-6}
Permeaplasts + DNase treated DNA	0.0	3.2×10^{-6}

*Transformation frequencies are given as the number of transformants obtained in relation to viable colony forming units (CFU) in the transformation mixture. An average of 100 cells was taken as a CFU unit. The data presented here are an average of three independent experiments. Permeaplasts and whole cells of the two strains were incubated with pBR 322 plasmid (E. coli) (1 µg/2 x 10^6 CFU of recipient cells) for 60 mins in the presence of photosynthetic light, at a temperature of 28 ± 2°C and the process was terminated by the addition of DNase I (100 µg ml^{-1}) to the incubation mixture. Permeaplasts and whole cells were also incubated the same way as described above without DNA and with DNase treated DNA and transformants were scored on 5.72µM of Ampicillin.

Fig. 9 Growth pattern of parent Anabaena doliolum in N_2-medium and parent Synechococcus sp. and its Ampicillin reseistant transformant in NO_3^--medium in the absence and presence of Ampicillin

Parent A. doliolum grown without Ampicillin

Parent A. doliolum grown with Ampicillin (5.72 μM)

Parent Synechococcus sp. grown with Ampicillin (5.72μM)

Parent Synechococcus sp. grown without Ampicillin

Transformant grown with Ampicillin (5.72AM)

Transformant grown with Ampicillin (14.3μM)

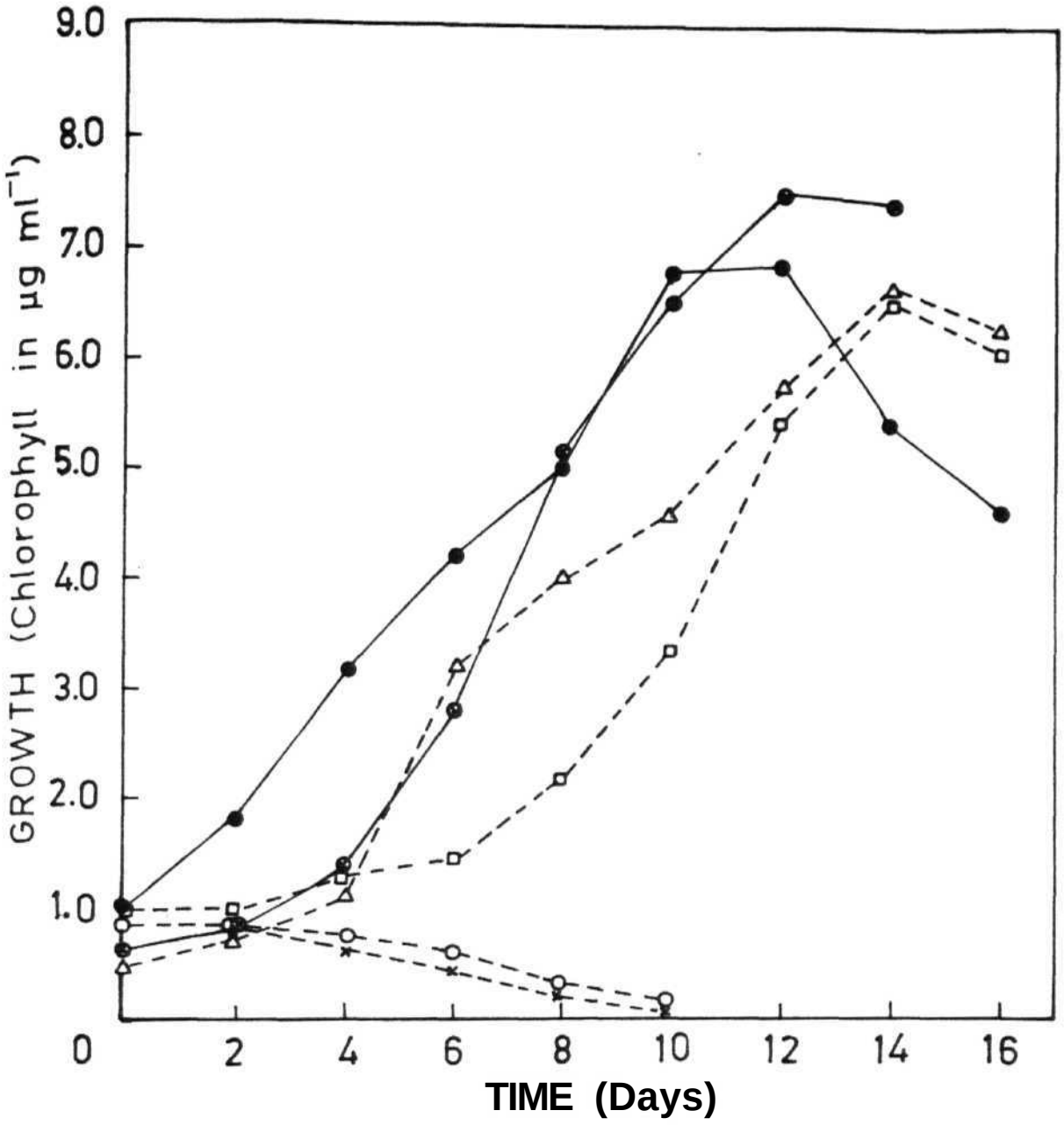

9.0
8.0
7.0
6.0
5.0
4.0
3.0
2.0
1.0
0
GROWTH (Chlorophyll in µg ml⁻¹)
2
4
6
8
10
12
14
16
TIME (Days)

Fig. **9**

Fig. 10 **Growth** pattern of parent Nostoc muscorum, A. doliolum Nostoc calcicola, Synechococcus sp. and Streptomycin resistant mutant of N. muscorum under diazotrophie conditions in the absence and presence of 0.6 µM Streptomycin


Parent N. muscorum without Streptomycin


Parent N. muscorum with Streptomycin


Parent Synechococcus sp. without Streptomycin


Parent Synechococcus sp. with Streptomycin


Parent Nostoc calcicola without Streptomycin


Parent Nostoc calcicola with Streptomycin


Parent Anabaena doliolum with Streptomycin


Parent A. doliolum without Streptomycin


Mutant N. muscorum with Streptomycin


Mutant N. muscorum without Streptomycin

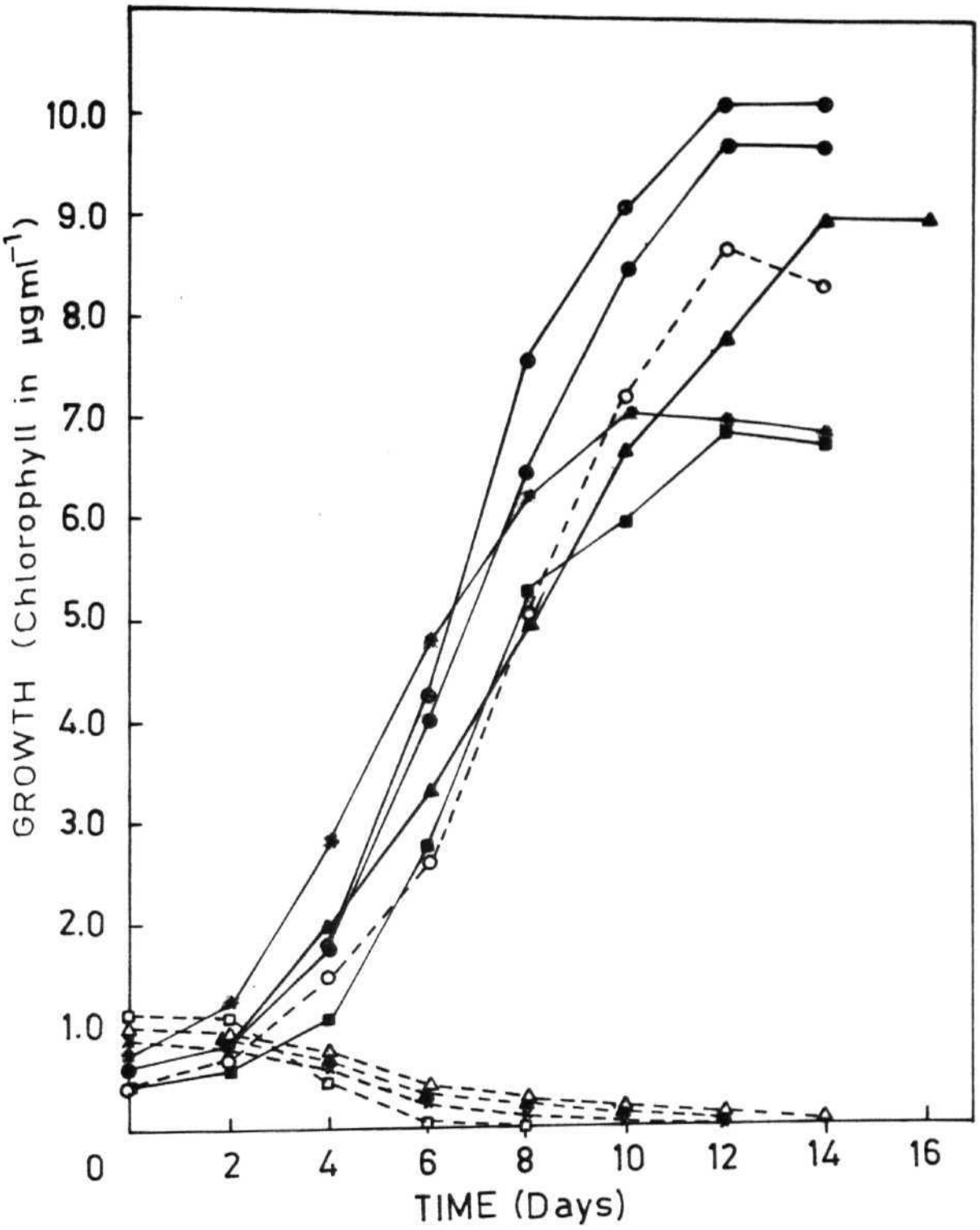

GROWTH (Chlorophyll in µgml⁻¹)
10.0
9.0
8.0
7.0
6.0
5.0
4.0
3.0
2.0
1.0
0
2
4
6
8
10
12
14
16
TIME (Days)

Fig.10

The transformant has been subcultured in antibiotic **free medium** for four successive subcultures but with no loss of Ampicillin resistance phenotype, thereby suggesting pBR322 mediated process of transformation in the unicellular cyanobacterium to be a stable feature.

Streptomycin resistance:

Nostoc muscorum undergoes spontaneous mutation with a frequency of 0.2-2.5 x 10^{-9} but all attempts to isolate spontaneous mutants for Streptomycin of A. doliolum failed. The reason for such a difference in spontaneous mutation characteristic to Streptomycin resistance is not known. This provided an opportunity to find out whether spontaneous Streptomycin resistance mutant phenotype could *be* transformed to A. doliolum. For comparison Synechococcus sp was also included in this study.

Streptomhycin was very toxic to parental strains of all the three cyanobacteria as none of them could grow in 0.6 µM of Streptomycin as shown in Fig. 10. The spontaneous Streptomycin resistant mutant of N. muscorum isolated from nutrient plates containing 3.5 µM of Streptomycin grew well upto 68 µM of Streptomycin. Since permeaplast preparations were found to be more effective as recipient cells lor transformation to Ethionine resistance and Ampicillin resistance in case of Synechococcus sp. The Streptomycin resistant donor DNA was used with permeaplasts from parent A. doliolum, parent N. muscorum and parent Synechococcus sp for transformation to Streptomycin resistance. $2x10^6$ CFU recipient cells were taken for permeaplast preparation and $2x10^3$ CFU were plated on each plate. Streptomycin was added

by overlaying method to a concentration of 0.6 μM and 1.2 μM after 48 hrs and the plates were incubated in the presence of light.

No transformants could be found in A. doliolum, N. muscorum and Synechococcus sp. It is therefore concluded that Streptomycin resistant marker of N. muscorum is not transformable to itself, to A. doliolum and to Synechococcus sp. The level of Streptomycin resistance in JN. muscorum is a clear indication that this phenotype is not plasmid determined, but whether plasmid or chromosomally determined, the fact remains that this marker is non- transiormable.

Herbicide resistance markers: DCMU and Atrazine

The experiments were also conducted to find out DCMU-resistance and Atrazine-resistance of N. muscorum whether they are transformable to A. doliolum, N. calcicola and to Synechococcus sp. The herbicide resistant mutant characteristic of N. muscorum have already been described in the previous chapter. Before starting the transiormation experiment lethal concentration of Atrazine to the three cyanobacterial strains were determined and it was found that 0.6 uM Atrazine was completely growth inhibitory in N. calcicola and A. doliolum. Synechococcus sp was found unable to grow in 0.4 uM Atrazine. Similar experiments were done for DCMU, 0.2 μ M was found lethal to Synechococcus sp and *0.1* μM lethal to A. doliolum and N. calcicola. The transformation experiments for the two herbicide resistant phenotypes were carried out as described in the method. The DCMU resistant donor DNA and Atrazine resistant donor DNA were separately isolated from their respective mutants and were used under optimum transiormation

condition with permeaplasts from A. doliolum, N. calcicola and Synechococcus sp. Such DNA treated permeaplast populations were plated on non-selective medium for 48 hrs. Such plates were then subsequently overlayed with afresh selective medium containing 1 µM Atrazine or DCMU as the case may be. The number of colonies growing in the presence of herbicides were scored for transformation frequency determination. They were tested for their stability to herbicide resistant phenotype after their isolation. One of the transformant colony was grown into bulk culture and tested for higher level of herbicide resistance. They were found to grow even in 4 µM DCMU or fcMM Atrazine as the case may be. It is important to point out here that all attempts to select transformants at more than 1 µM herbicide concentration failed. It therefore became necessary to go for selection oi transformants at lower level of the herbicide.

The growth characteristic of the parent, DCMU-resistant transformant (11a,b,c & d) and Atrazine-resistant strain (12a,b,c & d) were all compared. Transformants like their respective mutant strains grew with the herbicides and DCMU transformants showed cross resistance to Atrazine (13a) and Atrazine transformants showed cross-resistance to DCMU (13b). These transformants were as usual checked for their stability and found to be genetically stable. The tables 17 & 18 contain the data of transformation frequency in various cyanobacterial strains. It is important to mention here that DCMU and Atrazine resistance gene is transformable to A. doliolum, heterocystous N_2-fixing cyanobacterium closely related to N.muscorum and to unicellular non-N_2-fixing Synechococcus sp. a very distant relative of N. muscorum. The transformation to herbicide resistance phenotype was completely sensitive to DNase.

Fig. 11a Growth pattern of parent *N. muscorum* and its DCMU resistant transformant in N_2-medium lacking or containing 4 μM DCMU


Parent grown with DCMU


Parent grown without DCMU


Transformant grown with DCMU


Transformant grown without DCMU

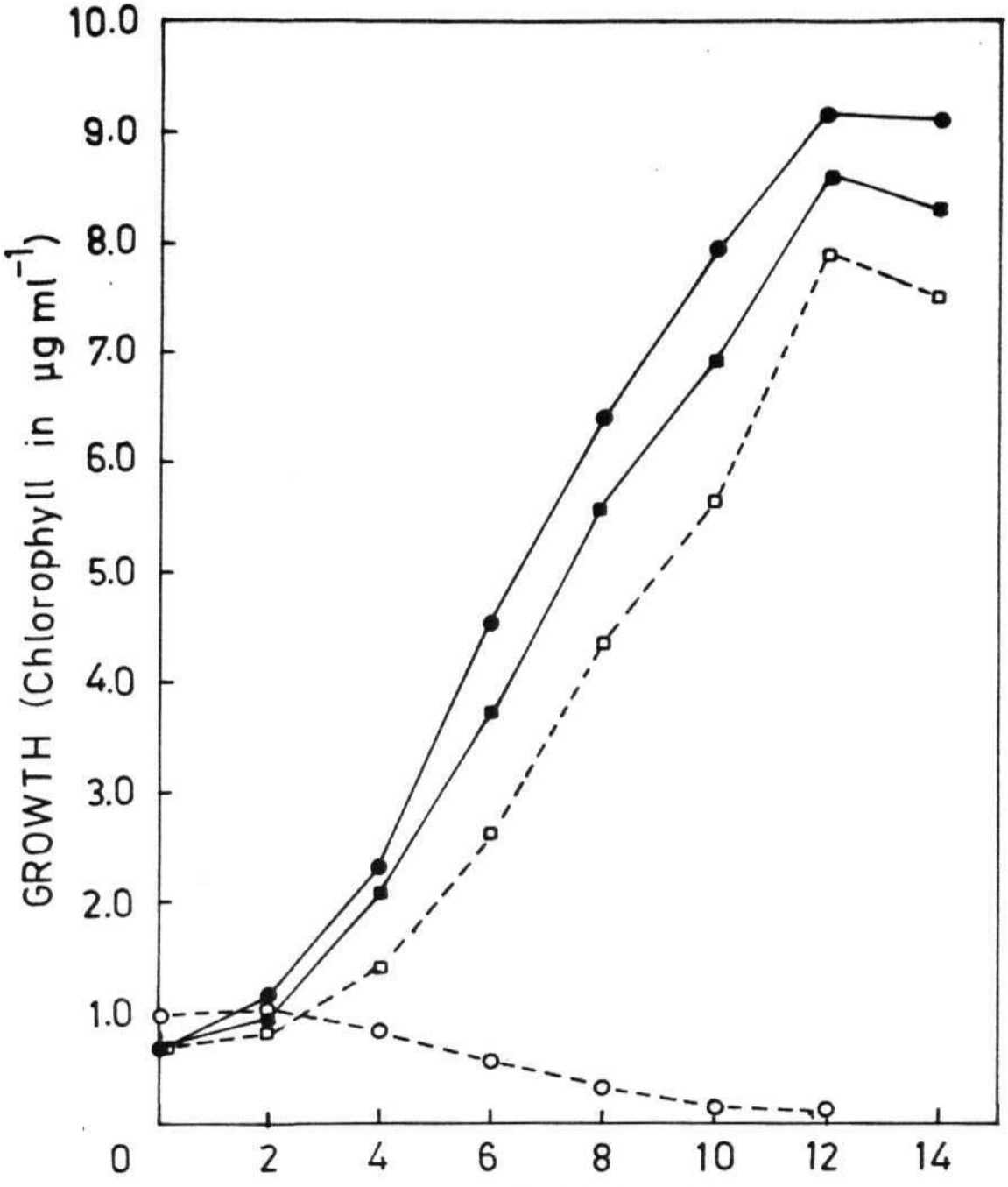

10.0
9.0
8.0
7.0
6.0
5.0
4.0
3.0
2.0
1.0
0
2
4
6
8
10
12
14
GROWTH (Chlorophyll in µg ml^{-1})
TIME (Days)

Fig. 11(a)

Fig. 11b Growth pattern of parent Nostoc calcicola and its DCMU resistant transformant under diazotrophic conditions lacking or containing 4μM DCMU

Parent grown with DCMU

Parent grown without DCMU

Transformant grown with DCMU

Transformant grown without DCMU

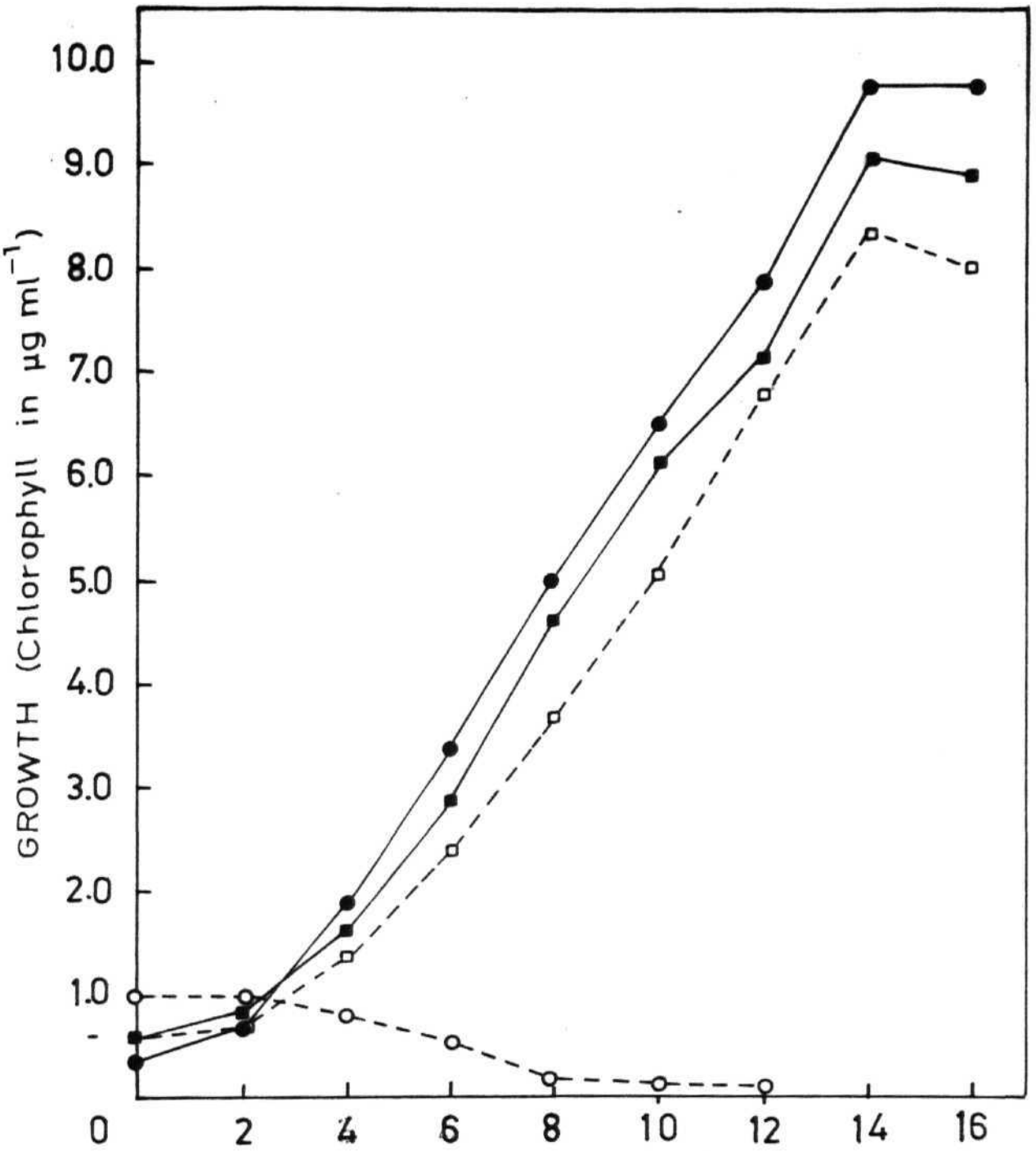

GROWTH (Chlorophyll in µg ml⁻¹)
10.0
9.0
8.0
7.0
6.0
5.0
4.0
3.0
2.0
1.0
0
2
4
6
8
10
12
14
16
TIME (Days)

Fig. 1Kb)

Fig. 11c Growth pattern of parent Anabaena doliolum and its DCMU resistant transformant under diazotrophic conditions lacking or containing 4 μM DCMU

Parent grown with DCMU

Parent grown without DCMU

Transformant grown with DCMU

Transformant grown without DCMU

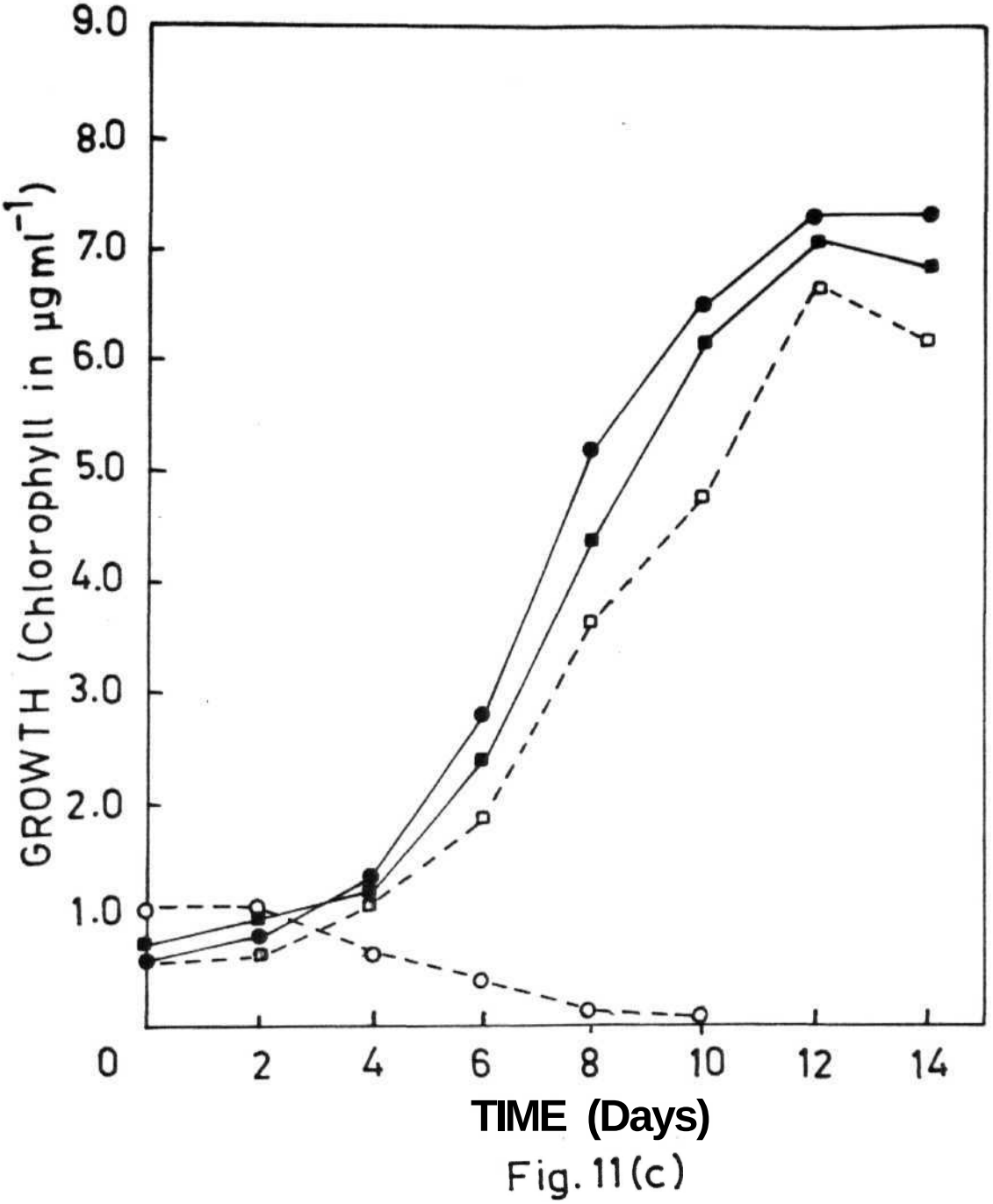

9.0
8.0
7.0
6.0
5.0
4.0
3.0
2.0
1.0
0
2
4
6
8
10
12
14
GROWTH (Chlorophyll in µg ml^{-1})
TIME (Days)

Fig. 11(c)

Fig. 11d Growth pattern of parent Synechococcus sp. and its DCMU resistant transformant in NO_3^--medium lacking or containing 4 M DCMU

Parent grown with DCMU

Parent grown without DCMU

Transformant grown with DCMU

Transformant grown without DCMU

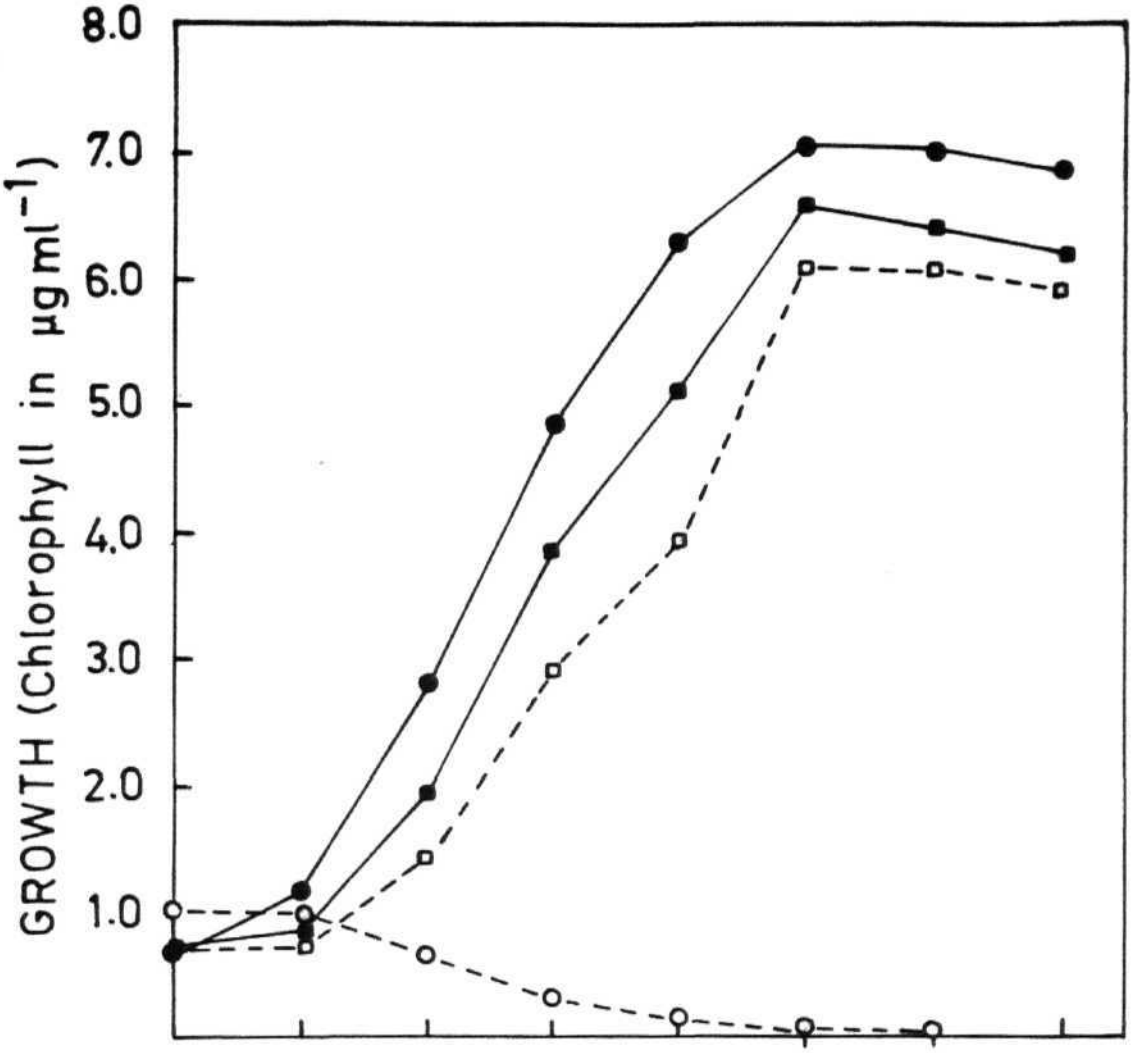
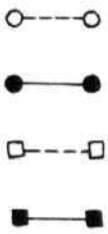

8.0
7.0
6.0
5.0
4.0
3.0
2.0
1.0
0
2
4
6
8
10
12
14
GROWTH (Chlorophyll in µg ml^{-1})
TIME (Days)

Fig.11(d)

Fig. 12a **Growth** pattern **of** parent Nostoc muscorum and its Atrazine resistant transformant under diazotrophic conditions in the absence and presence of 5μM Atrazine



Parent grown with Atrazine

Parent grown without Atrazine

Transformant grown with Atrazine

Transformant grown without Atrazine

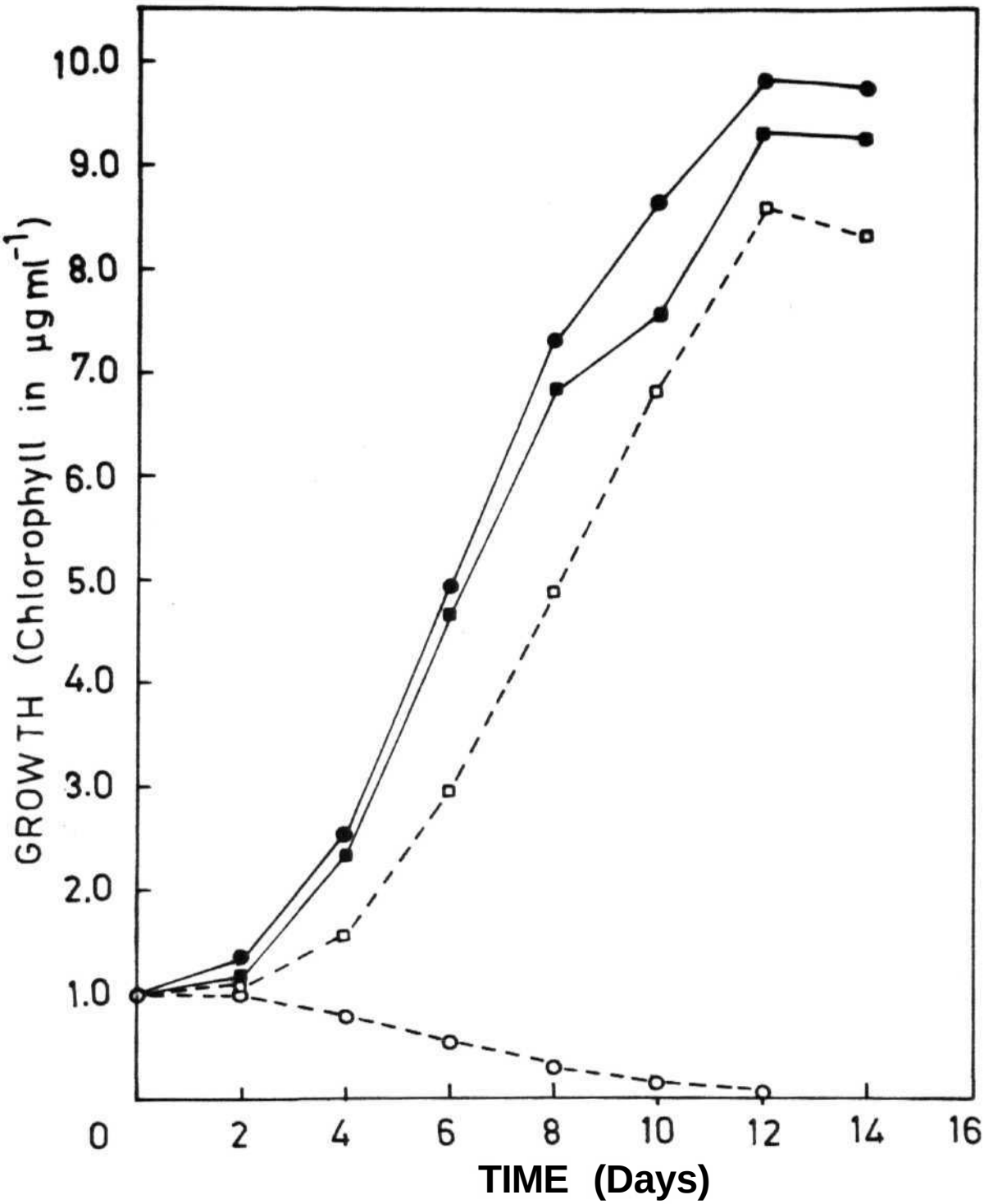

10.0
9.0
8.0
7.0
6.0
5.0
4.0
3.0
2.0
1.0
GROWTH (Chlorophyll in μg ml^{-1})
0
2
4
6
8
10
12
14
16
TIME (Days)

Fig.12 **(a)**

Fig. 12b Growth pattern of parent Nostoc calcicola and its Atrazine resistant transformant under diazotrophic conditions in the absence and presence of 5μ_M Atrazine

Parent grown with Atrazine

Parent grown without Atrazine

Transformant grown with Atrazine

Transformant grown without Atrazine

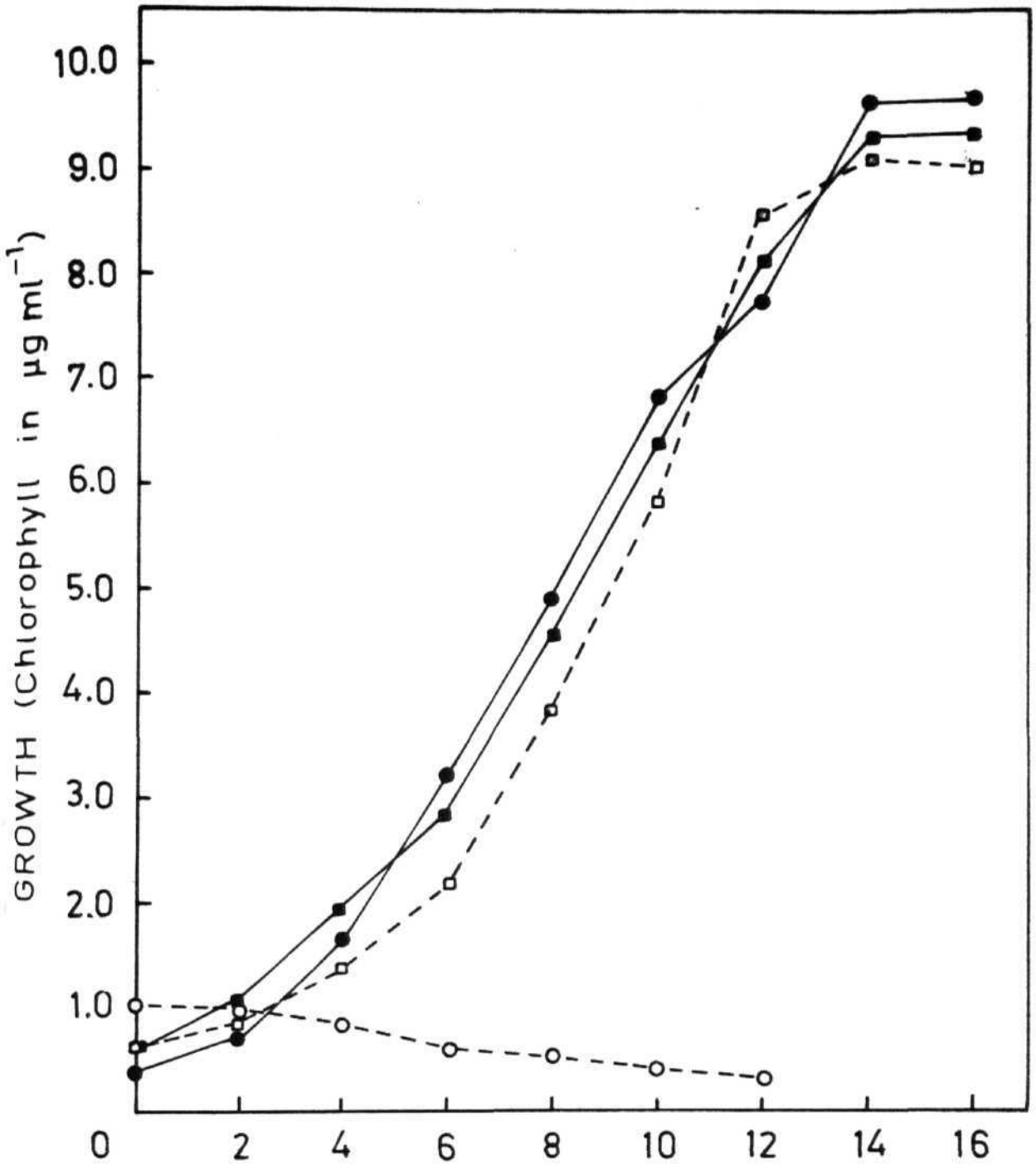
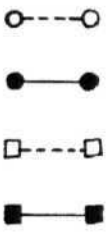

GROWTH (Chlorophyll in µg ml⁻¹)
10.0
9.0
8.0
7.0
6.0
5.0
4.0
3.0
2.0
1.0
0
2
4
6
8
10
12
14
16
TIME (Days)

Fig.12(b)

Fig. 12c Growth pattern of parent Anabaena doliolum and its Atrazine resistant transformant under diazotrophic conditions in the absence and presence of 5μM Atrazine



Parent grown with Atrazine

Parent grown without Atrazine

Transformant grown with Atrazine

Transformant grown without Atrazine

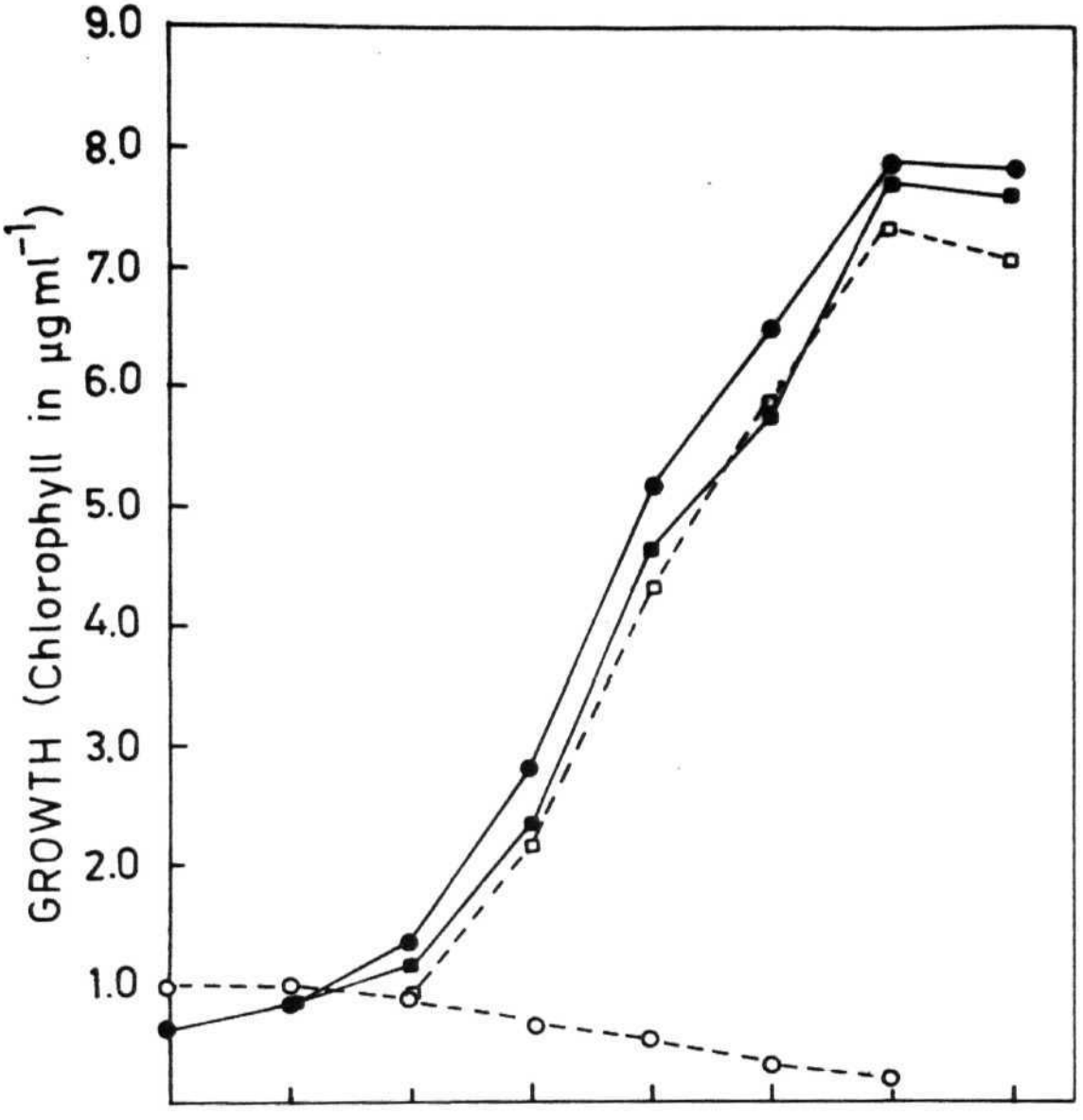
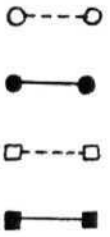

9.0
8.0
7.0
6.0
5.0
4.0
3.0
2.0
1.0
0
2
4
6
8
10
12
14
GROWTH (Chlorophyll in µg ml^{-1})
TIME (Days)

Fig. 12 (c)

Fig. 12d Growth pattern of parent Synechococcus sp and its Atrazine resistant transformant in NO_3^--medium in the absence and presence of 5μM Atrazine



Parent grown with Atrazine

Parent grown without Atrazine

Transformant grown with Atrazine

Transformant grown without Atrazine

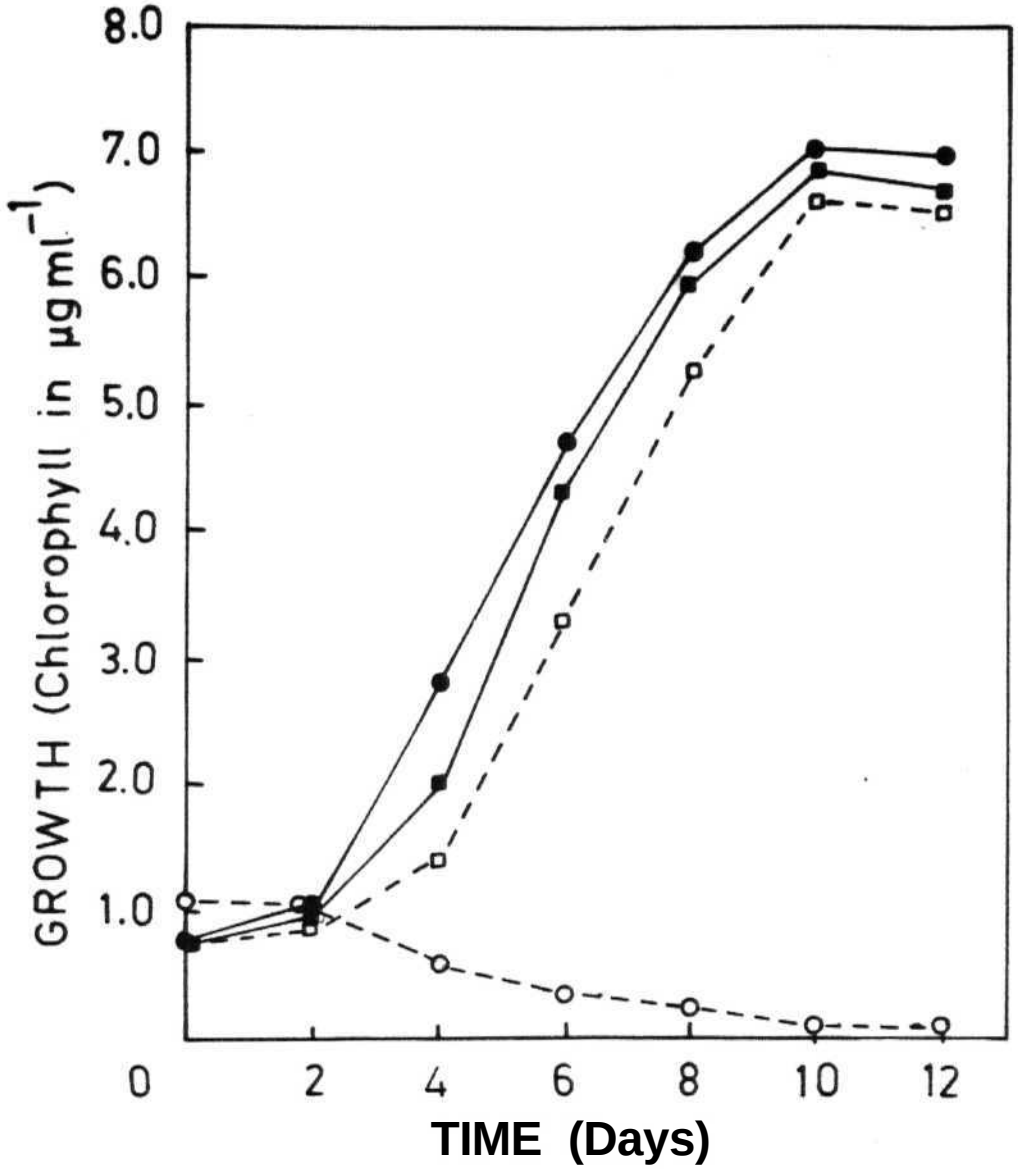
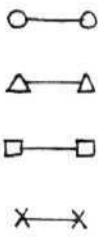

8.0
7.0
6.0
5.0
4.0
3.0
2.0
1.0
GROWTH (Chlorophyll in µg ml^{-1})
0
2
4
6
8
10
12
TIME (Days)

Fig. 12 (d)

Fig. 13a Growth pattern of Nostoc muscorum, Nostoc calcicola, Anabaena doliolum and Synechococcus sp. transformants resistant to DCMU under diazotrophic conditions, in the absence and in the combined presence of DCMU (4 μM) and Atrazine (5 μM)

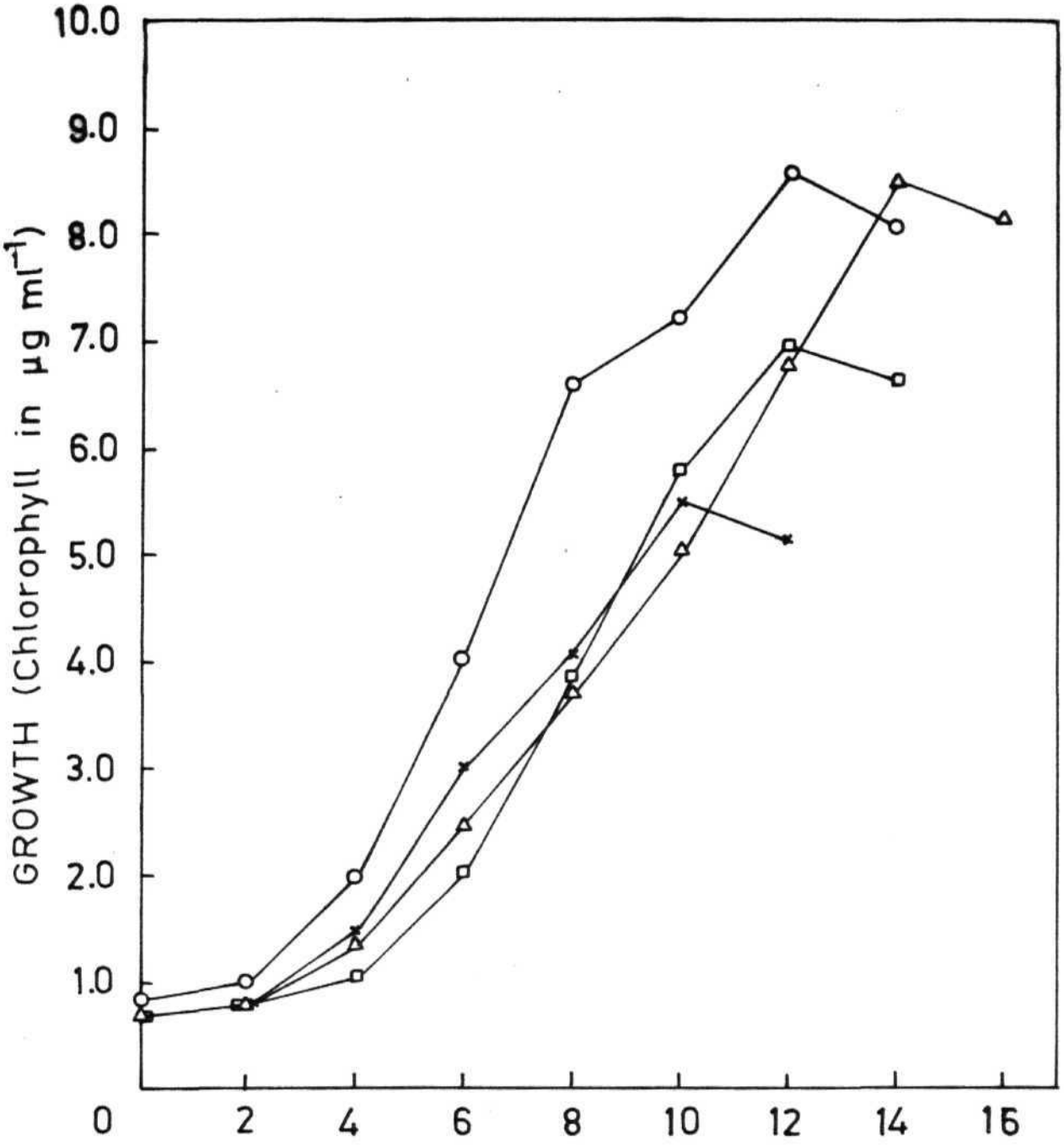


○—○ N. muscorum transformant

△—△ N. calcicola transformant

□—□ A. doliolum transformant

×—× Synechococcus transformant


10.0
9.0
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6.0
5.0
4.0
3.0
2.0
1.0
0
GROWTH (Chlorophyll in µg ml(-1))
2
4
6
8
10
12
14
16
TIME (Days)

Fig.13 (a)

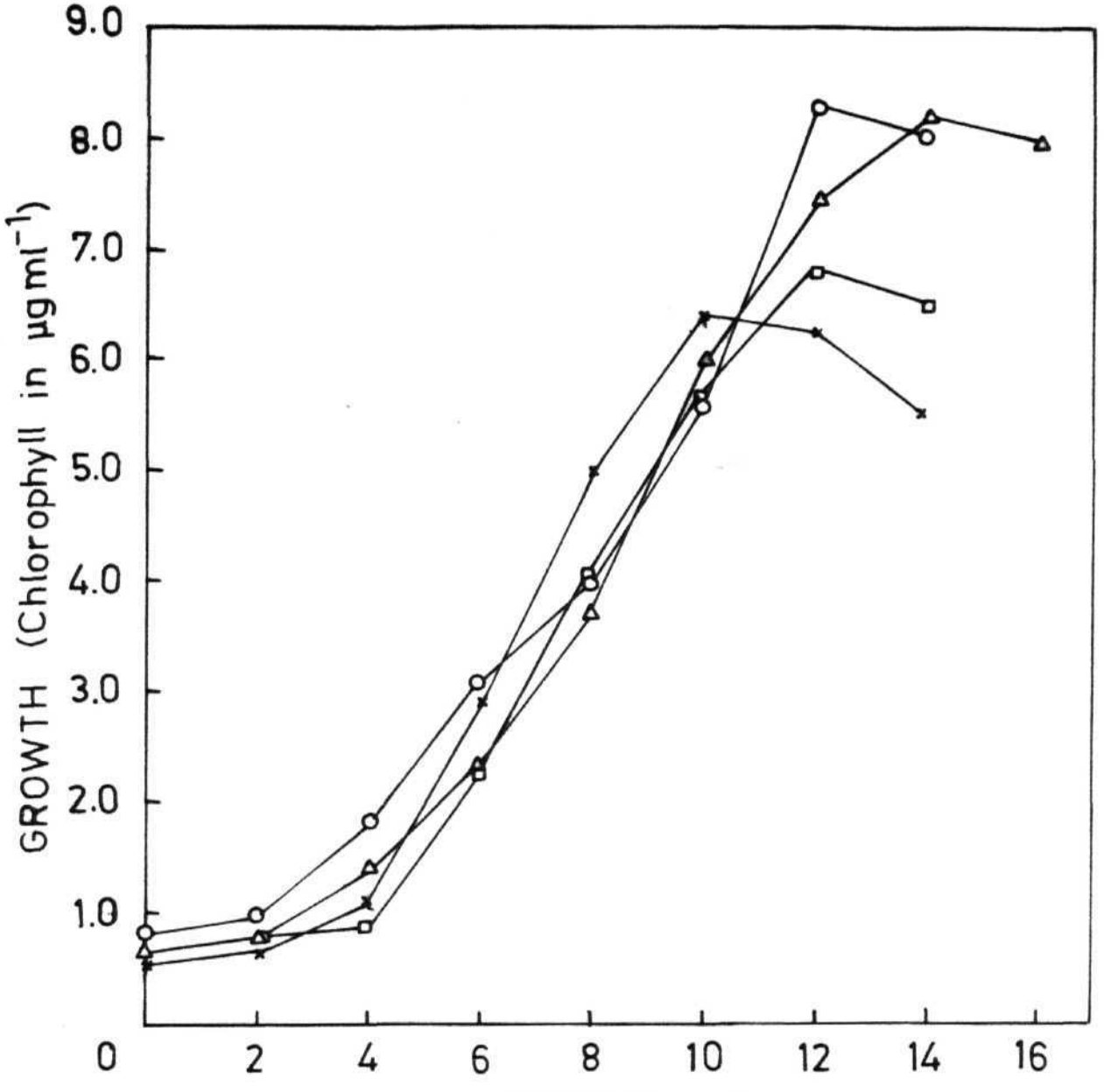
Fig. 13b Growth pattern of Nostoc muscorum, Nostoc calcicola. Anabaena doliolum and Synechococcus sp. transformants **resistant to Atrazine under** diazotrophic conditions, in the absence and the combined presence of DCMU (4μM) and Atrazine (5μM)


N. muscorum transformant


N. calcicola transformant


A. doliolum transformant


Synechococcus sp. transformant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5.0
4.0
3.0
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8
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12
14
16
GROWTH (Chlorophyll in µgml^{-1})
TIME (Days)

Fig.13(b)

Table 17

Transformation in N. muscorum, N. calcicola, A. doliolum, Synechococcus sps. with purified DNA from DCMU-resistant strain of N. muscorum.

Treatment	Transformation frequency*			
	Nostoc muscorum	Nostoc calcicola	Anabaena doliolum	Synechococcus sps.
Permeaplasts + No DNA	0.0	0.0	0.0	0.0
Permeaplasts + DNA	2.5×10^{-4}	7.4×10^{-5}	6.7×10^{-5}	6×10^{-5}
Permeaplasts + DNase treated DNA	0.0	0.0	0.0	0.0

*Transformation frequencies are given as number of transformants obtained in relation to viable colony forming units (CFU) in the transformation mixture. An average of 100 cells was taken as a CFU unit. The data presented here are an average of three independent experiments.

Permeaplasts of all the four strains were incubated with donor DNA from DCMU-resistant N. muscorum (1 μg/ 2×10^6 CFU of recipient cells) for 60 mins in the presence of photosynthetic light, at a temperature of 28 ± 2°C and the process was terminated by the addition of DNase I (100 μg ml^{-1}) to the incubation mixture. Permeaplasts were incubated without DNA and with DNase treated DNA as described above and transformants were scored on 1 μM DCMU.

Table 18

Transformation in N. muscorum, N. calcicola, A. doliolum and Synechococcus sps. with purified DNA from Atrazine-resistant strain of N. muscorum.

Treatment	Transformation frequency*			
	Nostoc muscorum	Nostoc calcicola	Anabaena doliolum	Synechococcus sps.
Permeaplast + no DNA	0.0	0.0	0.0	0.0
Permeaplast + DNA	3.26×10^{-4}	2.28×10^{-4}	1.56×10^{-4}	8.9×10^{-5}
Permeaplast + DNase treated DNA	0.0	0.0	0.0	0.0

*Transformation frequencies are given as number of transformants obtained in relation to viable colony forming units (CFU) in the transformation mixture. An average of 100 cells was taken as a CFU unit. The data presented here are an average of three independent experiments.

Permeaplasts of all four strains were incubated with donor DNA from Atrazine resistant N. muscorum (1 μg/ 2×10^6 CFU of recipient cells) for 60 mins, in the presence of photosynthetic light, at a temperature of 28 ± 2°C and the process was terminated by the addition of DNase I (100 μg ml^{-1})to the incubation mixture. Permeaplasts were also incubated without DNA and with DNase treated DNA as described above and transformants were scored on 1 μM Atrazine.

Het* Nif$^+$ marker:

The Het" Nif" mutant as described by Singh et al (1977) of Nostoc muscorum was used in transformation experiment to examine the ability of parent Het$^+$ Nif$^+$ donor DNA to transform the mutant strain to Nif^4 Het" phenotype or Nif$^+$ Het$^+$ phenotype. The permeaplast of the mutant strain were prepared as described previously and incubated with donor DNA 1 $\mu g\ ml^{-1}$ concentration for 60 mins. The control experiments involved use of permeaplast incubation without DNA and the other set of permeaplast were incubated with DNase treated DNA. The transformants were scored on N_2-medium at low light intensity. This was done to recover Nif^4 Het$^-$ transformants if possible, because of low light intensity, photosynthetic O_2 evolution could be very low and this low O_2 concentration may permit the Nif$^+$ Het$^-$ transformant to grow slowly at the expense of N_2 as nitrogen source. Under the same condition Het$^+$ Nif$^+$ transformants would grow faster. The transformation results suggest that Het$^-$ Nif$^-$ mutant strain is transformable with the Het$^+$ Nif$^-$ donor DNA to two kinds of phenotypes with differing frequencies, one the Het$^-$ Nif$^-$ phenotype and the other Het$^+$ Nif$^+$ phenotype (Table 19). The Het' Nif$^-$ mutant strain did revert spontaneously to Het^4 Nif$^+$ phenotype but never to Het$^-$ Nif$^+$ phenotype under the given growth conditions. The reversion frequency was nearly 100 fold lower than the transformation frequency to Het$^+$ Nif$^+$ phenotype. These results are very interesting and promising in view of the reported nif gene rearrangement associated with heterocyst differentiation and aerobic nitrogenase activity within heterocyst. The heterocyst frequency and nitrogenase activity of parent, mutant and transformants were compared

Table 19

Transformation in *Nostoc muscorum* (*Het*$^-$ *Nif*$^-$) mutant with purified DNA from parent *N. muscorum* to *Het*$^-$ *Nif*$^+$ and *Het*$^+$ *Nif*$^+$ phenotypes.

Treatment	Transformation frequency*	
	Het$^-$ *Nif*$^+$	*Het*$^+$ *Nif*$^+$
Permeaplasts + no DNA	0.0	0.0
Permeaplasts + DNA	1.2×10^{-5}	1.05×10^{-5}
Permeaplasts + DNase treated DNA	0.0	0.0

* Transformation frequencies are given as number of transformants obtained in relation to viable colony forming unit (CFU) in the transformation mixture. An average of 100 cells was taken as a CFU unit. The data presented here are an average of three independent experiments.

Permeaplasts of *N. muscorum* (*Het*$^-$ *Nif*$^-$) mutant are incubated with donor DNA from parent *N. muscorum* (1 μg/2 x 10^6 CFU of recipient cells) for 60 mins. in the presence of photosynthetic light, at a temperature of 28 ± 2°C and the process was terminated by the addition of DNAse I (100 μg ml^{-1}) to the incubation mixture. Permeaplasts were also incubated without DNA and with DNase treated DNA as described above and transformants were scored on N_2-medium.

Table 20

Heterocyst frequency and nitrogenase activity of parent *Nostoc muscorum* and its *Het*$^-$ *Nif*$^+$ and *Het*$^+$ *Nif*$^+$ transformants.

Cyanobacterial strains with phenotypic constitution	Heterocyst Frequency	Nitrogenase (ARA)
Parent		
Het$^+$ *Nif*$^+$	5-6	8.5
Mutant		
Het$^-$ *Nif*$^-$	0.0	0.0
Transformants		
Het$^-$ *Nif*$^+$	0.0	2.6
Het$^+$ *Nif*$^+$	5-6	7.92

Heterocyst frequency was calculated as number of heterocysts per 100 vegetative cells.

Nitrogenase activity measured in terms of acetylene reducing activity (ARA) expressed as n mol C_2H_4 formed mg Chl*a*$^{-1}$ hr^{-1}.

All the above results are an average of three independent experiments which do not vary by more than 10%.

(Table 20) and Het⁻ Nif⁺ transformant showed little nitrogenase activity and Het⁺ Nif⁺ transformant was similar to parent with **respect to hetero-**cyst frequency and **nitrogenase activity.**

DISCUSSION

Unicellular cyanobacteria grow in fine suspension and they can be easily quantified in terms of their growth and plating efficiency under different treatment conditions. A search for gene transfer system in cyanobacteria naturally looked towards these forms as suitable experimental system. This approach led to the discovery of DNA mediated, DNase sensitive transformation system for Erythromycin resistance or Streptomycin resistance in A. nidulans. During subsequent years explosive development and growth took place in the knowledge and application of recombinant DNA techniques which could be utilised as more precise and effective tool in probing the expression, structure and organisation of various alien or native genes in a common host like E. coli. With earlier demonstration of workable transformation system in A. nidulans was taken advantage of (Van den Hondel et al, 1980) to generate chimeric plasmids containing Ampicillin resistance transposon (Tn 901) of E. coli and capable of replication in A. nidulans. This technique for the first time showed the expression of bacterial gene in a cyanophycean host background. This approach was subsequently extended to generate plasmid shuttle vectors that can efficiently and effectively be transformable and replicable in cyanobacterial/E. coli host system. Success in this approach led to the development of this technique as a most powerful tool for cloning of bacterial genes in **their** alien host (Kuhlemeier et al, 1981; Shinozaki et al, 1982, Sherman

and Van de Putte, 1982; Buzby et al, 1985; Daniell et al, 1986 and Chauvat et al, 1986).

A plasmid shuttle vector pRL6 containing Neomycin resistance **and** Chloramphenicol resistance genes has recently been transformed in Anabaena sp. M 131 by the newly introduced technique of electroporation and further study of this transformation system showed a role of cyanobacterial restriction enzyme sensitive restriction site, in regulating the plasmid transformation frequency (Thiel and Poo, 1989). Previous studies have shown that heterocystous cyanobacteria are transformable with total DNA from azide resistance and herbicide resistance (Singh et al, 1987 a and b). Ampicillin resistance phenotype of pBR322 is transformable to Synechococcus but not to A. doliolum. This finding does suggest a natural barrier against pBR322 mediated transformation to Ampicillin resistance in A. doliolum. The previous reports of successful transformation with Ampicillin resistance marker of pBR322 have shown limitation of the expression efficiency of Ampicillin resistance gene in the unicellular cyanobacterial host to 1.0 µM-5.72 *MM* of the antibiotic. In our system this expression has doubled, thus indicating a role of cyanobacterial host in regulating the efficiency of expression of Ampicillin resistant gene of pBR322.

The single step spontaneous mutation to high level streptomycin resistance in N. muscorum indicates the phenotypic determinant to be of chromosomal nature. Our attempt to introduce this genetic marker in N. muscorum, N. calcicola, A. doliolum and in Synechococcus sp repeatedly failed. Evidently heterocystous diazotrophic cyanobacterial

forms like N. muscorum, N. calcicola and A. doliolum does not seem to be acceptable host for Streptomycin resistance marker. The inability of Synechococcus sps to undergo transformation with Streptomycin resistant marker of N. muscorum is intriguing in view of previously reported successful transformation of this marker in unicellular cyanobacteria (Shestakov and Khyen, 1970). In the latter case the source of Streptomycin resistance marker has been the unicellular cyanobacterium A. nidulans which has also been the recipient of this marker. The most simple conclusion, the present results lead to with respect to Streptomycin resistance marker of N. muscorum is that, it is not a stable acceptable transformable marker in either heterocystous, filamentous or unicellular cyanobacterial host. It is difficult to suggest any specific reason for this situation at the moment.

Crop protection chemicals find indispensable use in modern agriculture, in view of their selective inhibitory action on crop weeds and crop pests. There have been attempts to use those herbicides which are specifically inhibitory to unique metabolic aspects of plants such as photosynthesis. In addition, herbicides inhibitory to plant photosynthesis have been used as effective tools to understand the process of photosynthesis at molecular level. The most commonly used photosynthetic herbicides are DCMU and Atrazine, both of which inhibit the activity of reaction centre D1 protein of PS11. Recent comparative studies on molecular, organisational and functional aspect of photosynthesis in chloroplasts and cyanobacteria suggests them to be very similar. The genetics of Chloroplast photosynthesis is at the moment difficult to analyse, in view of the lack of viable gene transfer system. An alternative

to this approach in Chloroplast genetics, has been suggested to be manipulation of genetics of photosynthesis in cyanobacteria and its use in understanding the Chloroplast genetics (Golden et al, 1985). Among cyanobacteria, the facultatively heterotrophic strain PCC 6S03 of Synechocystis is being increasingly used as a model system for the study of oxygenic photosynthesis partly because mutants defective in photosynthetic apparatus are viable in this organism (Astier et al, 1984) and also because in vitro mutated genes can be substituted for the wild type allele by integrative transformation (Vermaas et al, 1988). Such and other studies have shown that PSI1 is a protein-pigment complex, the core of which consists of five proteins : CP-47, CP-43, D1 (32 kd protein), D2 (34 kd protein) and Cyt b_{559} (Lang and Haselkorn, 1989). The psb A gene coding for 32 kd protein from spinach Chloroplast DNA was used as a heterologous probe to isolate the homologous Anabaena psb A gene from a recombinant DNA library and to study in detail its organisation in cyanobacteria. This novel approach led to the discovery of three types of psb A gene namely psb Al, psb AII and psb AII1 occuring at different places in the cyanobacterial genome (Curtis and Haselkorn, 1984).

Molecular analysis of Atrazine resistant mutants associated with light sensitivity has shown Q_B binding region of Dl protein to be the cause of such light sensitivity leading to photoinhibition of photosynthesis and growth (Kirilovsky et al, 1989). Further, detailed study of dominance and sensitivity with respect to DCMU resistance have been studied, through the transformation based technique of specific gene inactivation and deletion. In Synechococcus sps. PCC 7942, by

selectively altering one member at a time of the three member psb A gene family, Brusslan and Haselkorn (1989) have demonstrated that Diuron resistance alleles are dominant to Diuron sensitive alleles.

Mutation to DCMU resistance and Atrazine resistance has been shown to occur in the previous chapter. These herbicide resistant genetic markers have been found to be effectively transformable to other heterocystous cyanobacteria like N. calcicola and A. doliolum and to unicellular non-N_2-fixing cyanobacterium like Synechococcus sp. These findings have serious implications in construction of diazotrophic cyanobacterial strains for use as biofertiliser in rice agriculture. Furthermore it seems that heterocystous cyanobacteria are transformable for certain markers like herbicide resistance but not to other markers like Ampicillin resistance or Streptomycin resistance. May be the process of transformation in heterocystous forms is marker specific, it would be interesting to analyse biochemical and physiological causes of such marker specificity of transformation processes.

nif gene and het gene organisation and number are not well studied. Heterologous nif probe of Klebsiella pneumoniae have been used to isolate and identify nif gene organisation in vegetative cells and heterocysts (Mazur et al, 1980). This approach led to identification of only four nif genes in Anabaena sp: nif D, nif H, nif K and nif S. Further studies have shown that in Anabaena vegetative cell DNA, the nif D gene coding the a subunit of nitrogenase is interrupted by an 11Kb pair excisable DNA element which gets excised during differentiation of vegetative cells into heterocysts. The excision results in restoration of the nif D coding sequence and of the entire nif H, D,

K transcription unit. A second developmentally regulated gene rearrangement has also been observed close to the nif S gene in Anabaena (Haselkorn et al, 1987). This nif gene rearrangement has suggested to be required for their **expression into** active nitrogenase (Haselkorn et al, **1987).**

Heterocyst differentiation from vegetative cells require laying down of new outer polysaccharide layer and inner glycolipid layer on the preexisting cell wall, destruction of proteins involved in O_2 evolution and CO_2 assimilation during photosynthesis by proteases and induction of nitrogenase enzyme, ATP production and reductant. It has been shown that heterocysts provide the anaerobic environment necessary ior N_2-fixation (Haselkorn, 1978; Wolk, 1982). Mutants defective in heterocyst specific glycolipid show oxygen sensitive nitrogenase activity, thereby implicating a direct role of heterocyst glycolipid layer in protection of heterocyst nitrogenase from exogenous O_2 (Murry and Wolk, 1989). Some heterocyst forming cyanobacteria have been" shown to fix N_2 without forming mature heterocysts (Rippka and Stanier, 1978). Anabaena sp strain 7118, a nonheterocyst forming mutant of A. variabilis has been found to show only anaerobic nitrogenase activity (Rippka and Stanier, 1978). Recently the expression of nif genes in terms of their mRNA and nitrogenase protein have been studied in Anabaena variablis under anaerobic conditions. Nitrogenase mRNA started appearing after 1.5 to 2 hrs of anaerobic incubation under nitrogen starvation growth condition and this was followed by appearance of nitrogenase activity 1 hr later. Such anaerobic cultures on exposure to O_2 stopped producing nitrogenase mRNA (Helber et al, 1988). These studies in

general suggests that vegetative cell nif genes can be expressed in the absence of O_2 much before the development of heterocysts under N_2-fixing condition. The most obvious implication of such a conclusion is that heterocyst is the exclusive site of N_2-fixation under aerobic growth condition and that vegetative cells become N_2 fixers only under anaerabic condition, if that be so, the nif gene rearrangement during heterocyst differentiation may not be a necessary condition for *nii* gene expression leading to nitrogenase activity. The available findings are still not very clear about the definite physiological role of developmentally regulated nif gene rearrangements in heterocystous forms.

Genetic recombinationa) analysis of het and nif genes in N. muscorum has been made where het^- nif^+ recombinants have been shown to possess, microaerobic vegetative cell nitrogenase activity (Singh and Singh, 1981).

The present transformation experiments, involving Het^- Nif^- permeaplasts and Het^+ Nif^+ donor DNA led to production of two classes of transiormants one Het^- Nif^+ phenotype showing microaerobic and anaerobic nitrogenase activity and the second class with Het^+ Nif^+ phenotype showing aerobic nitrogenase activity. These findings further confirmed that Het^- Nif^- mutants are regulatory mutants of positive nature and that Het class and Nif class of genes require functional regulatory genetic determinant for their coordinated developmental regulation. These results also suggest nif gene rearrangement associated with heterocyst differentiation not to be prerequisite for nif gene expression and N_2-fixation in N. muscorum.

CONCLUSIONS

CONCLUSIONS

1. Mutations leading to Chlorate-resistance in N. muscorum were used to identify possible number of genetic determinants in control of nitrate assimilation. Such Chlorate-resistant mutants were biochemically and physiologically analysed for defect in nitrate assimilation.

2. The results suggested involvement of three distinct genetic determinants controlling nitrate assimilation. One class of determinant controlled apoprotein component of nitrate reductase, the second class, Mo-cofactor component of nitrate reductase and the third class, transport component of nitrate assimilation process.

3. Spontaneously occuring mutants of N. muscorum resistant to Streptomycin, L-Ethionine, DCMU and Atrazine have been isolated with a mutation frequency characteristic of prokaryotic system.

4. Streptomycin resistant mutant did not show cross-resistance to L-Ethionine, DCMU or Atrazine.

5. L-Ethionine resistant mutant did not show cross-resistance to Streptomycin, DCMU or Atrazine.

6. Anabaena doliolum lack this ability to mutate spontaneously to Streptomycin resistance or Ethionine resistance.

7. DCMU resistant mutant of Nostoc muscorum showed cross-resistance to Atrazine and Atrazine resistant mutant of N. muscorum showed cross-resistance to DCMU.

8. L-Ethionine resistant marker of N. muscorum was used to study the process of transformation and the factors controlling competence and transformation frequency.

9. Nostoc muscorum, Nostoc calcicola and Anabaena doliolum showed lack of natural competence for stable transformation.

10. Artificial preparation of permeaplasts of heterocystous cyanobacteria made them competent for transformation.

11. Transformation efficiency of permeaplasts was maximum in 1 hour old preparations, but declined with the age of such preparations.

12. Preparation of permeaplasts in Tris EDTA (pH 7.5) buffer improved considerably transformation efficiency.

13. The transformation process was DNase sensitive.

14. Photosynthetic light was an essential condition for the transformation process.

15. Transformation frequency increased with DNA concentration reaching a saturation point at 1 ug ml^{-1} of DNA concentration.

16. Transformation frequency also increased with DNA contact period reaching maximum with a period of 60 mins.

17. Overlaying of the DNA treated population after a period of 48 hrs with the selective agent containing medium was necessary to score the viable transformants.

18. Transformation occured with homologous as well as heterologous system.

19. Transformants obtained were as stable as the mutant phenotypes.

20. The conditions standardised for transformation to Ethionine resistance were used for scoring transformants to Atrazine resistance, DCMU resistance, Streptomycin resistance and Ampicillin resistance.

21. DCMU-resistant phenotype of N. muscorum was readily transformable to N. muscorum, N. calcicola, A. doliolum and Synechococcus sp. thus showing that DCMU resistance gene is transformable in homologous as well as heterologous combination.

22. Atrazine-resistant gene of N. muscorum was similarly transformable to itself, A. doliolum, N. calcicola and Synechococcus sp.

23. The herbicide resistant transformants like herbicide resistant mutants showed herbicide cross-resistance relationship.

24. Streptomycin resistance gene of N. muscorum was not transformable to itself, to N. calcicola, to A. doliolum and to Synechococcus sp.

25. Ampicillin resistance gene of pBR 322 was transformable to Synechococcus sp but not to A. doliolum. N. muscorum and N. calcicola were found naturally resistant to Ampicillin. Hence pBR 322 transformation study was not conducted with them.

26. Successful transformation for herbicide resistance markers and amino acid analogue in homologous and heterologous condition but not for Streptomycin marker under similar condition, suggested marker specific nature of transformation in heterocystous cyanobacteria.

27. *Het*$^-$ *Nif*$^-$ mutant strain of *N. muscorum* was transformable with the DNA from its *Het*$^+$ *Nif*$^+$ parental strain giving rise to two phenotypic classes of transformants, the *Het*~ *Nif*$^+$ class and *Het*$^+$ *Nif*$^+$ class.

28. Expression of *nif* genes in vegetative cells of *Het*" *Nif*$^+$ class of transformants under microaerobic condition suggested that heterocyst is a site of N_2-fixation under aerobic growth condition and vegetative cell site of N_2-fixation under microaerobic or anaerobic growth condition.

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MGG

Genetic transfer of herbicide resistance gene(s) from *Gloeocapsa* spp. to *Nostoc muscorum*

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Summary. An aerobic diazotrophic *Gloeocapsa* strain contained genes conferring resistance to the growth toxic effects of rice field herbicides Machete and Basalin. The results of genetic crosses and of DNA-mediated genetic transformation experiments both suggested the absence of a heterospecific barrier for the transfer of herbicide resistance genes from a *Gloeocapsa* strain to *Nostoc muscorum* and their stable expression and maintenance in the latter. These findings will have considerable implications in cyanobacterial biofertilizer technology.

Key **words:** Herbicide resistance - Transformation - Recombination - Cyanobacteria

Diazotrophic cyanobacteria have been implicated in sustaining the cultivation of wetland rice for centuries without added nitrogen fertilizer because of their inherent ability to add fixed nitrogen to such habitats under aerobic photosynthetic conditions (Singh 1961; Roger and Kulasooriya 1980). In recent years the practice of using diazotrophic cyanobacteria as an efficient source of biofertilizer for rice crops has been advocated and adapted in many developing nations (Venkataraman 1972). The present cyanobacterial biofertilizer technology, however, suffers from a lack of herbicide-resistant cyanobacterial strains which can grow and add fixed nitrogen in herbicide-treated rice fields (Singh 1974; Singh et al. 1979). Accordingly a successful cyanobacterial biofertilizer technology will require the use of cyanobacterial strains which are resistant to rice field herbicides. This will be possible only after a source of herbicide resistance genes has been identified and methods developed for their genetic transfer to appropriate diazotrophic cyanobacterial strains, native to rice field ecosystems.

Recently we have reported the natural occurrence of two rice field herbicide resistance genes, controlling Machete resistance (Mat^r) and Basalin resistance (Bas^r), in an aerobic diazotrophic strain of *Gloeocapsa* (Singh et al. 1986). In this paper we describe the methods and present evidence to show that the herbicide resistance phenotypes Mat^r and Bas' of the *Gloeocapsa* strain can be genetically transferred to *Nostoc muscorum* in an efficient and stable manner, thereby demonstrating that there is no heterospecific barrier in *N. muscorum* for the stable expression and maintenance of the *Gloeocapsa* herbicide resistance genes.

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The two cyanobacterial strains used were the diazotrophic Mat' Bas' *Gloeocapsa* strain and *N. muscorum* sensitive to growth inhibition by Machete and Basalin (Singh et al. 1986). A streptomycin-resistant (Str^r) clone of *N. muscorum* occurring spontaneously was isolated and raised to form a stock culture for further use in the present study. The herbicide-resistant *Gloeocapsa* strain and streptomycin-resistant *N. muscorum* were both grown and maintained in axenic clonal culture in modified Chu No. 10 medium devoid of any fixed source of nitrogen under light and temperature conditions described previously (Singh and Singh 1981).

Since this growth medium contained N_2 as the only source of nitrogen we designated its use as diazotrophic growth conditions. The methods for testing streptomycin sensitivity, estimating the spontaneous mutation frequency, crossing two genetically distinct parents under conditions counter selective for one parent and estimating recombinant frequency were as described by Stewart and Singh (1975) and Singh and Singh (1981). DNA-mediated genetic transformation studies were also conducted in order to check the results obtained in the crosses. Donor DNA from the Mat', Bas' *Gloeocapsa* strain was used to transform Str' *N. muscorum* to Machete resistance. The transformation procedure of Daniell and McFadden (1986) was used, with appropriate modifications.

Recently we have shown the genetic basis of the Mat^r and Bas' phenotypes in the *Gloeocapsa* strain (Singh et al. 1986). The Mat', Bas' *Gloeocapsa* strain and the Mat^s, Bas^s *N. muscorum* strain were both nearly similar in their sensitivity to streptomycin: neither could grow in the presence of 2 µg/ml streptomycin. As shown in Table 1, while the *Gloeocapsa* strain failed to mutate spontaneously to the Str' phenotype, *N. muscorum* did so, but with a very low frequency. The Str^r *N. muscorum* thus obtained was used in the subsequent crosses and DNA-mediated transformation experiments. Neither wild-type *N. muscorum* nor its Str' derivative were found to mutate spontaneously to Mat' (Table 1). This finding is not surprising in view of our earlier observations (Singh and Vaishampayan 1978; Singh et al. 1979).

The crossing experiment involved incubating together the Mat', Bas', Str* *Gloeocapsa* strain and the Mat", Bas^s, Str^r *N. muscorum* strain in equal proportions (in terms of cfu) in diazotrophic growth medium containing 100 µg/ml

Table 1. Diazotrophic growth and specific characteristics of various cyanobacterial strains

Strains	Diazotrophic growth* (OD 663 nm)				Specific characteristics				
					Resistant phenotype			Frequency of spontaneous mutation ($\times 10^{-9}$) to	
	Control	+ streptomycin (100 µg/ml)	+ Machete (40 µg/ml)	+ Basalin (40 µg/ml)	Str^r	Mat^r	Bas^r	Str^r	Mat^r
Gloeocapsa strain	0.25	0.0	0.22	0.23	–	+	+	–	NR
Nostoc muscorum (parent)	0.84	0.78	0.0	0.0	+	–	–	1.8–2.5	0.0
N. muscorum (recombinant)	0.75	0.74	0.68	0.66	+	+	+	NA	NA

The dala presented are averages of 3 independent experiments. Crosses were made by mixing the *Gloeocapsa* and *N. muscorum* parents in equal proportions (approximately 10^6 cfu/ml) in diazotrophic growth medium containing 100 µg/ml streptomycin under growth conditions counter selective for the Mat^r, Bas^r *Gloeocapsa* spp parent. At the end of the incubation period 0.1 ml of mixed culture was spread on diazotrophic agar medium containing 40 µg/ml Machete. The spontaneous mutation frequency of these strains for different characteristics was calculated as described in the text. The recombinants appeared with a frequency of about 1.4×10^{-4}. NR. naturally resistant; NA, not applicable; —. sensitive; +. resistant

* Growth was measured as an increase in O.D. at 663 nm of a diazotrophic culture grown for 8 days

streptomycin. After % h the mixed culture was spread on diazotrophic agar medium containing 40 µg/ml Machete to score any possible/presumptive recombinants. Mat^r clones of Str^r *N. muscorum* appeared with a frequency of about 1.4×10^{-4}. All such Mat^r *N. muscorum* clones were found to have acquired the non-selected Bas^r phenotype as well. Accordingly such recombinants were designated as A', *muscorum* (Mat^r. Bas^r. Str^r) (Table 1). These herbicide-resistant clones of *N. muscorum* retained their properties even after several sub-culturings in the absence of herbicides, thus suggesting the stable maintenance and expression of the two herbicide resistance genes of the *Gloeocapsa* strain in A'. *muscorum*. Since *N. muscorum* did not show any spontaneous mutation to the Mat^r phenotype and since only mixed culturing of the *N. muscorum* and *Gloeocapsa* strains under conditions counter-selective for the *Gloeocapsa* strain produced Mat^r, Bas^r clones of *N. muscorum* it can be concluded that herbicide-resistant *N. muscorum* clones have arisen as a result of the transfer of herbicide resistance genes from the *Gloeocapsa* strain to *N. muscorum*. The present results have a considerable significance as they provide evidence for the tranfer of genes between two cyanobacterial strains belonging to different taxonomic orders.

The results obtained from the crosses prompted us to undertake DNA-mediated genetic transformation studies. In order to confirm the possibility of 'genetic' transfer of herbicide resistance genes from the *Gloeocapsa* strain to A. *muscorum*. as suggested by the crosses. total DNA was isolated and purified from the *Gloeocapsa* strain and used to transform A. *muscorum* to Machete resistance. The results of such DNA-mediaied transformation experiments are given in Table 2. The transformation frequency obtained was comparable to the observed recombination frequency in crosses and also that obtained with unicellular cyanobacteria (Stevens and Porter 1986). This transformation system was found to be extremely sensitive to DNAase treatment and was relatively unaffected by RNAase treatment (Table 2). Further characterization and improvement

Table 2. DNA-mediated transformation of *Nostoc muscorum* to Machete resistance

Treatment	No. of transformants per 2×10^6 cfu	Frequency
Control (no DNA)	0.0	0.0
DNA	520	2.6×10^{-4}
DNAase-treated DNA	5	2.5×10^{-6}
RNAase-treated DNA	368	1.84×10^{-4}

DNA was isolated from the *Gloeocapsa* strain following the method of Mazur et al (1980) with slight modifications. Permeaplasts of *N. muscorum* were prepared according to Daniell and McFadden (1986) with appropriate modifications. Permeaplasts of *N. muscorum* (Str^r) were incubated with the donor DNA (t µg/2 x 10^6 cfu of recipient cells) for 30 min in the presence of photosynthetic light (2500 Lx) and the transformation process was terminated by adding 100 µg/ml DNAase to the incubation mixture. DNAase treatment was essentially as in Daniell and McFadden (1986) The cells were then spread on selective plates containing 40 µg/ml Machete

of the efficiency of this transformation system is currently being worked out.

DNA-mediated genetic transfer has not been successfully demonstrated in any filamentous, diazotrophic, heterocystous cyanobacteria (Ruck and Mulligan 1984; Wolk et al. 1984). The reason for this failure has been attributed to the presence of a variety of restriction endonucleases which attack and degrade homo- and heterospecific DNA in these cyanobacteria (Duyvesteyn et al. 1983). Since, we found a heterospecific DNA-mediaied genetic transformation system functioning for herbicide resistance genes, in *N. muscorum*, it is obvious that a homospecific or heterospecific transformation system may operate, depending upon the nature of donor DNA and recipient host, in filamentous hcterocystous cyanobacteria. Both the crosses and DNA-mediated transformation experiments clearly established

that the herbicide resistance phenotype of the *Gloeocapsa* strain has a genetic basis and that the herbicide resistance genes can be transferred, expressed and maintained in A'. *muscorum*.

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