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Physiological characterization and molecular profiling of toxic and non-toxic isolates of Cyanobacterium *Microcystis*

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Abstract

Our investigation aimed to utilize physiological attributes and molecular tools for distinguishing the toxic strain of *Microcystis* from other non toxic strains, belonging to the same genus. Physiological characterization of five *Microcystis* isolates indicated that the toxic strain (M1) exhibited significantly higher pigment accumulation (phycocyanin: 54.20 µg ml^{-1}; allophycocyanin: 18.2 µg ml^{-1}) and sugar content (74.25 µg ml^{-1}), which may be providing a competitive advantage for successful colonization and proliferation. Profiling using repeat sequence primers (STRR, Hip) was helpful in distinguishing different strains (M1-M5) and HIP TG profile was unique to M1. SDS-PAGE profile of the five strains indicated the presence of a unique band (25kDa) in M1. The combined use of SDS-PAGE and HipTG profiles can help in providing distinct fingerprint for the toxic strain, which can be useful in its identification.

Key words

Algal blooms, *Microcystis*, Molecular polymorphism, Repetitive sequences, SDS-PAGE profile

Introduction

Cyanobacteria are one of the most diverse groups of Gram-negative photosynthetic prokaryotes. They show remarkable ecological diversity, which can be attributed to their unique metabolic characteristics and high adaptive ability under a wide range of environmental conditions. Under suitable circumstances they are capable of growing into big biomass, often dominating other aquatic biota producing scums (Jaiswal *et al*., 2008). Cyanobacterial blooms are hazardous due to the production of a wide range of toxic secondary metabolites (Carmichael, 1994), which can adversely affect the flora and fauna at various trophic levels, besides resulting in deterioration of water quality. Some of the common blooms forming cyanobacteria are *Microcystis, Anabaena, Nodularia, Oscillatoria, Aphanizomenon* etc. Among them, *Microcystis* is the most notorious bloom former and effective producer of microcystin (Carmichael, 1994). *Microcystis* strains produce a family of related cyclic hepatopeptides that pose considerable threat to aquatic ecosystem. More than 65 microcystin variants have been isolated to date (Rantala *et al*., 2004). Toxin production by these strains is regulated by various physical and environmental factors (Jaiswal *et al*., 2008). Recent studies have brought to light, the significant genetic variation within and across *Microcystis* populations (Janse *et al*., 2004; Wilson *et al*., 2005). Among the various molecular markers used in cyanobacterial diversity analyses, the utility of repeat sequences/palindromes is well established (Rasmussen and Svenning, 1998). Another interspersed repeated sequence known to be commonly found in many but not all cyanobacterial species, is an 8bp highly iterated palindromic sequence known as Hip1 (Gupta *et al*., 1993). Robinson *et al*. (1995) showed that although use of Hip1 primer gave PCR fingerprinting characteristic to the species, the banding patterns were very complex. Smith *et al*. (1998) constructed PCR primer by extending the Hip1 sequence at the 3' end by two bases and they found it

useful in distinguishing several cyanobacterial species. PCR based identification of cyanobacterial strains using different sets of primers is well established (Prasanna *et al*., 2006; Pedro *et al*., 2011). Therefore, the present investigation was undertaken to study the physiological diversity and polymorphism among toxic and non-toxic *Microcystis* isolates.

Materials and Methods

Five *Microcystis* strains isolated from different water and soil samples collected from various sites in India and maintained in laboratory in Parker's medium. Growth was measured by determining the protein content of cultures (Herbert *et al*., 1971), thereafter Specific growth rates were calculated Kratz and Myers (1955).

Photosynthetic pigments and sugars : The exponentially growing cyanobacterial samples (10 ml) were centrifuged (8000g for 5 min) and the pellets were used for estimation of chlorophyll and phycobiliproteins spectrophotometrically following the methods of MacKinney (1941) and Bennett and Bogorad (1973), respectively. Sugars were estimated by following the method of Spiro *et al*. (1966).

SDS PAGE : Eight-day-old cultures of five *Microcystis* strains grown under controlled environmental conditions were pelleted, washed with sterile water and homogenized in a ratio of 1:2 (g fresh weight: ml of cold sample buffer) in sample buffer containing 125mM Tris-HCl (pH 6.8), 2% SDS, 20% glycerol (v/v), 1.0 mM phenyl methyl sulfonyl fluoride, 5% β-mercaptoethanol, 0.5% sodium azide, 20mM EGTA and 0.5% bromophenol blue. Gel electrophoresis was carried out following the procedure of Laemmli (1970) using a resolving gel of 12% and a stacking gel of 5%. 30 µl of each sample containing 150- 200µg ml^{-1} proteins was loaded into each well. The gels were scanned using Alpha Imager 3400 and the number of bands counted. The bands were calibrated by comparing with molecular weight marker standard (PWM) (Bangalore Genei Pvt. Ltd., India). The patterns were compared by using the information on apparent molecular masses of bands and their intensity. The analyses were repeated at least three times independently to verify the reproducibility of the patterns.

Molecular marker analyses : Cyanobacterial strains were pelleted by centrifugation and washed twice in sterile MilliQ water. The pellet was dissolved in 5µl of sterile water, and used directly as a template for PCR. The sequence of the oligonucleotide primers (Bangalore Genei Pvt Ltd., India) is given in Table 1. The PCR cycling conditions, primarily based on the procedure of Rasmussen and Svenning (1998), with the following cycler conditions: 1 cycle at 95°C for 6 min; 35 cycles of 94°C for 1 min, 56°C for 1 min and 65°C for 5 min, 1 cycle at 65°C for 16 min and a final step at 4 °C. Each PCR was carried out in a 25µl volume containing 50 pmol of each primer, 1.25mM dNTPs and 1U of *Taq* polymerase (Banglore Genei Pvt Ltd., India). The amplification was performed in a DNA Engine DYAD™ cycler (MJ Research Inc., USA).

The amplified products were resolved on 1.5% agorose gel in TAE buffer (pH 8.0). Molecular size standards of 100bp and 1Kb were run along with the amplified products to determine the size of resolved band. The gels were photographed using a CCD camera (Sony XC-75 CE). The amplification reaction and electrophoretic resolution were repeated at least thrice for each primer.

For each molecular marker, a binary matrix of all bands present in each strain was generated, using '1' for the presence of amplicon and '0' for its absence. Efficiency of discrimination was assessed in terms of the number of polymorphic markers generated and the ability to generate unique banding profiles, the latter represented as 'genotype index' (Mcgregor *et al*., 2000). Genotype index (GI), calculated as the number of genotypes with unique banding profiles, expressed as a fraction of total genotypes fingerprinted, has a range of 0-1. In case of the genetically dominant STRR marker PIC is synonymous with the term 'genetic diversity' as described by Weir (1990), and was calculated using the formula: $2[(1-q)^2 x ("Q^2]$, where Q is the proportion of genotype showing the absence of an amplicon and q represent the frequency of recessive allele.

Statistical analyses : The data recorded in triplicate for all the parameters in various strains and SE was calculated according to Snedecor and Cochran (1967).

Results and Discussion

Cyanobacteria are the dominant phytoplankton in eutrophic freshwater bodies and can grow to form thick scums that colour the water. Most blooms disappear in a few days, but the cells can release toxins lethal to animals and humans (Weier *et al*., 1982). Among various bloom forming genera, *Microcystis* is known to be of greatest concern due to its widespread prevalence in water bodies and its toxicity to aquatic and terrestrial organisms (Carmichael, 1992; Xu *et al*., 2010). Traditionally, microscopic identification of cyanobacteria alone or in combination with direct analysis of toxin has been used for detection of toxic cyanobacteria. Now DNA fingerprinting technologies e.g RAPD, AP-PCR, RFLP, AFLP are being employed for studying the genetic polymorphism among toxic and non-toxic strains (Nishihara *et al*., 1997; Hisbergues *et al*., 2003; Oberholster *et al*., 2005; Ouellette *et al*., 2006). However, there is a need to characterize toxic and non-toxic strains on the basis of simpler techniques. This study focuses on the use of physiological and biochemical attributes and fingerprints obtained through amplification of repeat

sequence primers as a tool for differentiating toxic and non-toxic strains. During the current study, five *Microcystis* strains isolated from different localities were compared for their growth attributes (Table 2). No significant differences were observed in specific growth rate of these strains, which was in the range of .044 to .050. M1 and M5 showed higher growth rate among the five strains followed by M2 and M4 and lowest values was recorded for M3. However, comparison of the five strains in terms of doubling time showed that this parameter did not reveal a significant variation and ranged between 20-22. Similar growth rates have been reported in previous studies (Roberts and Zohary, 1987; Long *et al*., 2001). However, recently Wilson *et al*. (2006) reported a doubling time in the range of 5.2 to 1.5d, which was unrelated to toxin production. Comparison of the strains on the basis of pigment accumulation and sugars revealed a considerable diversity (Table 2). Strain M1 showed highest values for chlorophyll content followed by M5. Phycocyanin (PC) content in the five *Microcystis* strains showed tremendous variation. M5 showed lowest PC content, while it was found highest (almost 5 folds higher than M5) in M1. However, not much variation was observed in phycoerythrin content of the strains, which varied from 2.09 - 2.58 µg ml^{-1}. Allophycocyanin content of the five strains also showed a wide variation with M1 showing the highest value of 18.2 µg ml^{-1} followed by M2. Sugar content of five *Microcystis* strains did not show much variation (Table 2), but again M1 recorded highest values for this parameter.

It is well known that bloom formers have to be highly competitive, so as to proliferate in a community consisting of diverse floral and faunal members (Klemer and Konopka, 1989). Allelochemicals produced by cyanobacteria, which include biocidal compounds, are also known to provide a competitive advantage. In the study, toxic *Microcystis* strain exhibited all the properties enabling its dominance in present the environment where it grows.

SDS PAGE of whole cell proteins of five *Microcystis* strains showed approximately 23 bands, which included 14 polymorphic bands (Fig. 1). Although the use of proteins as reliable markers has been controversial because they represent expressed part of genome, which are influenced by growth and environmental conditions, and toxin production in cyanobacteria is known to be influenced by changes in physical and environmental factors (Rapala *et al*., 1997; Jaiswal *et al.,* 2006). In the present study, utmost care was been taken to maintain uniform conditions during growth and reproducibility of the profiles was checked. The banding pattern of M2 and M3 strain were almost similar except, that M3 showed the presence of a 50 KDa band, which was absent in M2. M5 showed a very distinct banding pattern. Most of the protein bands in the range of 97 to 66 KDa were present in other four strains but absent in M5. The banding pattern of M1 included 18 bands and a unique band of approximately 25KDa was observed in its profile, which was not visible in any other strains; this might be related with the toxic nature of M1. Chan *et al*. (1994)

Table 1 : Molecular polymorphism in the *Microcystis* strains revealed by primers based on repetitive sequences

Primer	Sequence(5' - 3')	N_f	N_p	N_{bp}	GI	PIC
STRR1A	CCAATCCCCAATCCCC	10	10	5	1	0.384
$STRR_{mod}$	GCGCCCCAATCC	5	5	4	0.8	0.384
HipTG	GCGATCACTG	8	7	5	1	0.35

N_f: Number of amplicons; N_p: Number of polymorphic amplicons; N_{bp}: Number of banding profiles revealed across the strains; GI: Genotypic index: PIC: Polymorphism information content

Table 2 : Pigment and sugar content of five *Microcystis* isolates

Strains	Specific growth rate (k)	Chlorophyll	Phycocyanin	Phycoerythrin	Allophycocyanin	Sugar
M1	0.05	7.51±0.17	54.20±1.905	2.09±0.05	18.2±0.06	74.25±1.75
M2	0.048	5.17±0.075	52.08±0.72	2.58±0.038	7.8±0.20	70.56±1.87
M3	0.044	5.88±0.083	32.10±0.60	2.14±0.023	3.7±0.10	61.20±1.80
M4	0.048	5.91±0.022	31.18±1.03	2.50±0.064	3.8±0.08	72.27±2.03
M5	0.05	6.23±0.047	10.4±0.26	2.40±0.036	7.2±0.09	69.48±3.83
CD (0.05)		0.047	2.749	0.054	0.137	3.675

All the values are in µg ml^{-1}; Values in parentheses represent SE

used two dimensional gel electrophoresis to discriminate toxic and non-toxic strains of *Alexandrium minutum* found in harmful algal blooms. They found most notable differences in the range of 17.5 to 21.5KDa. They reported that a group of proteins were consistently present in non-toxic strains while two other proteins were prominent in the toxic strain. Strains M2 and M3 isolated from Varanasi were non-toxic, shared an almost common profile, with only one dissimilar band, indicative of their common geographical origin. Lyra *et al*. (1997) also demonstrated the use of SDS-PAGE of whole cell protein for grouping of cyanobacterial genera.

All the five strains were profiled using three specific primers: STRRmod, STRR1A and HipTG based on repetitive sequences. Results demonstrated that PCR fingerprinting generated by a group of distinct repetitive sequences present in cyanobacterial genome can be used to evaluate the diversity among *Microcystis* strains. PCR amplification of the strains using STRR1A primer revealed 10 polymorphic amplicons and 5 distinct banding profiles (Fig. 2). The primer was able to differentiate all the five strains. The profile of M1 included 5 bands in the range of 600- 250bp, which were common to M2 and M3. The banding patterns of M2 and M3 were very similar, with only two bands (1000 and 200bp) being absent in M3. M5 showed a very different pattern and most of the bands of higher molecular weight, present in other three strains, were missing in M5. Only two bands of 450 and 300 bp were present in the profile of M5. Amplification with $STRR_{mod}$ resulted in 5 bands with 100% polymorphism (Fig 3). Four different banding patterns were observed, three of which were unique to M1, M4 and M5, while strains M2 and M3 shared a common banding pattern. STRR primers have been used to study the diversity among 45 cyanobacterial isolates from 11 different *Gunnera* species originating from different geographical areas (Nilsson *et al*., 2000).

HipTG generated 8 amplicons, 7 of which were polymorphic (Fig. 4). Five different banding patterns were generated by the use of this primer and M5 showed the presence of only two low molecular weight bands (200-400bp). The profile of M1 strain was distinct with only two bands of 700 and 350bp were present. The use of extended Hip1 primers like HipTG proved useful in discriminating among the five *Microcystis* strains isolated from different regions in our study. The profile of toxic M1 obtained through amplification with this primer can prove useful as a fingerprint for identification of this strain.

It was observed that HipTG and STRR1A primers were able to discriminate among the five *Microcystis* strains. The GI of STR1A and HipTG were found to be higher (1.0) than that of $STRR_{mod}$ (0.8). However, the PIC values did not show

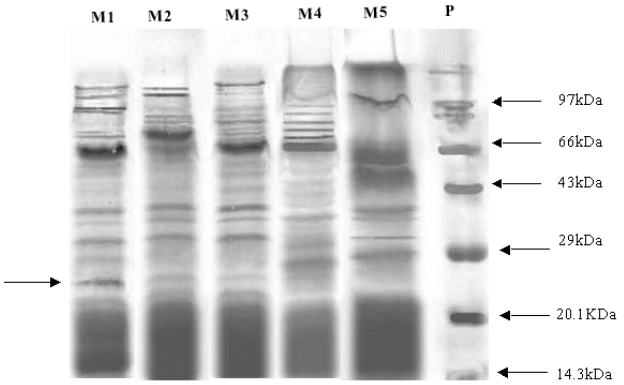

Fig. 1 : SDS-PAGE profiles of the *Microcystis* strains. P represents molecular marker and arrow represent the unique band in profile of M1.

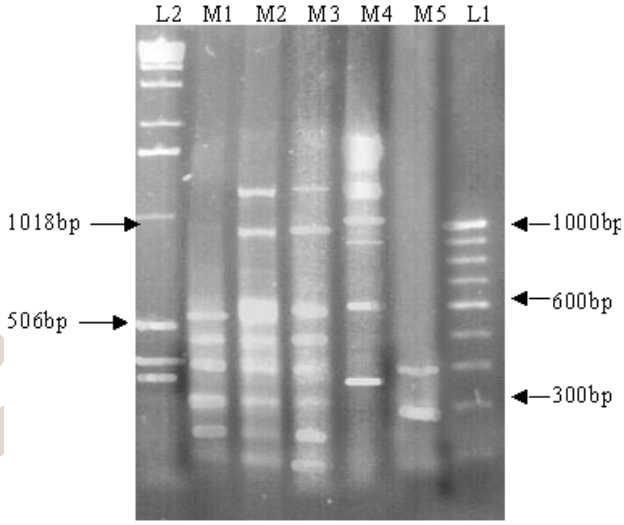

Fig. 2 : PCR-based DNA amplification profiles of the *Microcystis* strains revealed by STRR1A primer. L1 and L2 represent 100bp and 1kb molecular size standards, respectively.

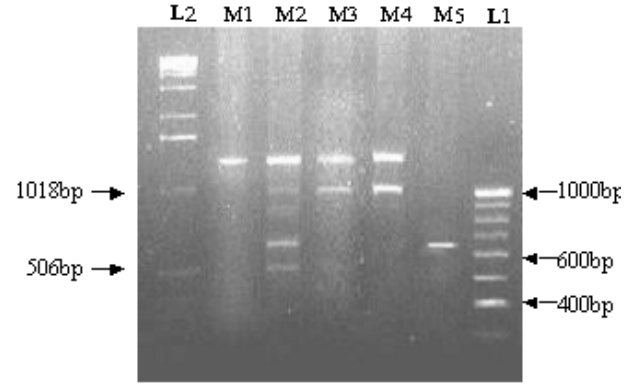

Fig. 3 : PCR-based DNA amplification profiles of the *Microcystis* strains revealed by STRRmod primer. L1 and L2 represent 100bp and 1kb molecular size standards, respectively.

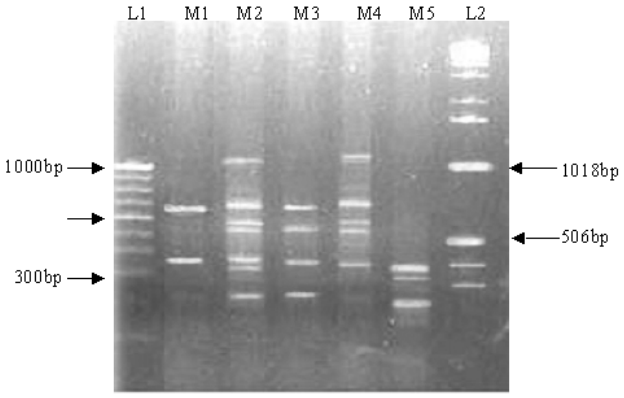

Fig. 4 : PCR-based DNA amplification profiles of the *Microcystis* strains revealed by HipTG primer. L1 and L2 represent 100bp and 1kb molecular size standards, respectively.

much variation. It was 0.38 for STRR1A and $STRR_{mod}$ and 0.35 for HipTG (Table 1).

In conclusion, the toxic *Microcystis* strain in the present study can be identified on the basis of the unique band (25Kda) in the SDS-PAGE profile and the HipTG based PCR fingerprint. Further, studies are being undertaken to evaluate its utility in detection of this strain in mixture/ aquatic samples.

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