

## Molecular characterization of *hetR* gene from *Cylindrospermum stagnale* A1345 isolated from arid soil

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### Abstract

*HetR* gene is a key regulator of heterocyst differentiation in filamentous cyanobacteria. It has been previously isolated and characterized from heterocystous cyanobacterium like *Anabaena* that has intercalary heterocysts and from non-heterocystous cyanobacteria like *Trichodesmium* and *Symploca* PCC 8002. This study reports the characterization of *hetR* gene from *Cylindrospermum stagnale* A1345, a cyanobacterium containing only terminal heterocysts. The expression profile of *hetR* gene and its correlation with the nitrogenase enzyme activity and heterocyst differentiation after nitrogen depletion has been investigated. Genomic analysis was carried out for copy number determination of the gene and northern blots for transcript profile. Immunoblot analysis of the Fe-protein subunit of nitrogenase enzyme complex at different time intervals revealed that the protein was detectable only around 24 hrs after nitrogen depletion and transcript levels of *hetR* gene correlated with the nitrogenase enzyme activity. Proheterocysts were observed at 24 hrs and fully developed heterocysts were found between 36 hrs and 48 hrs.

Key words: *hetR* gene, *Cylindrospermum* A1345, heterocysts, cyanobacteria, heterocyst differentiation, nitrogenase enzyme.

### Introduction

Cyanobacteria play a major role in sustenance of nitrogen levels in the soil and aquatic ecosystem (Stewart, 1980). Biological nitrogen fixation catalyzed by the enzyme nitrogenase is irreversibly inhibited by molecular oxygen and reactive oxygen species (Berman-Frank *et al.*, 2003). Cyanobacteria have developed a number of strategies to protect nitrogenase from oxygen such as temporal separation of oxygenic photosynthesis and nitrogen fixation in undifferentiated and unicellular forms (Stal and Krumbein, 1987; Gallon, 1989; Anand, 1998) or spatial separation of nitrogen fixation and oxygen evolving photosynthesis. Differentiation of cells into specialized heterocysts provides a suitable microanaerobic environment for the functioning of nitrogenase while the undifferentiated vegetative cells are used for photosynthesis (Adams and Duggan, 1999). Heterocysts are differentiated from vegetative cells under nitrogen deprived condition and they provide fixed nitrogen to its vegetative partner and in turn receive fixed carbon (Wolk *et al.*, 1994). It has been estimated that about 140 to 1000 genes are involved in the differentiation and function of the heterocysts (Lynn *et al.*, 1986; Wolk 2000).

Among the many genes involved in heterocyst formation, *hetR* plays a major role in heterocyst differentiation and pattern formation (Schiefer *et al.*, 2002). The *hetR* is an autoregulated gene, coding for 299aa protein and is required for heterocyst formation (Buikema and Haselkorn,

1991; 1993). The *hetR* is transcribed at low level in all vegetative cells in the presence of nitrate. Under nitrogen deprived condition, induction of the *hetR* begins within 2-3.5 hrs and enhanced transcription occur only in the cells which are initiated to differentiate into heterocyst. The induction increases at-least three fold in 10% of the cells by 6 hrs and 20 fold within the differentiating cells (Black *et al.*, 1993). Mutation studies of *hetR* have been revealed that the amino acid residues D17, G36 and H69 are essential for normal heterocyst differentiation and maintenance of HetR protein turnover. The residue D17 is required for DNA binding activity (Risser and Callahan, 2007) and the residues S152 and S179 are required for the normal function of HetR protein (Zhou *et al.*, 1998a; Dong *et al.*, 2000). Immunoblot analyses showed that HetR protein started accumulating within 3 hrs under nitrogen depleted conditions and the heterocysts had about 20 fold higher proteins than vegetative cells (Zhou *et al.*, 1998b).

Analysis of mutant of *Anabaena* sp PCC7120. has shown that *patA* and *hetF* genes are required for regulation of *hetR* gene and the increased expression of *hetR* in developing cells (Buikema and Haselkorn, 2001; Risser and Callahan, 2008). The expression of *hetR* and *ntcA* are mutually dependent (Muro-Pastor *et al.*, 2002). The NtcA is an autoregulatory protein required for the full expression of the genes involved in nitrogen and carbon metabolism and triggering heterocyst differentiation, development and function (Ramasubramanian *et al.*, 1996;

Adams, 2000; Herrero *et al.*, 2001; 2004; Golden and Yoon, 2003; Zhang *et al.*, 2006). The *hanA* gene codes for HU protein, required for *hetR* expression and is regulated by NtcA (Khudyakov and Wolk, 1996).

The HetR functions as a homodimer *in vivo* and has DNA-binding activity. The dimerization of HetR is required for its DNA-binding activity and heterocyst differentiation (Huang *et al.*, 2004).  $Ca^{2+}$  ions play very important role in *hetR* induction and heterocyst differentiation. CcbP, the  $Ca^{2+}$  binding protein regulates the concentration of  $Ca^{2+}$  in the heterocyst (Zhao *et al.*, 2005).

The gene *hetR* was also found in nitrogen-fixing non-heterocystous cyanobacteria (Buikema and Haselkorn, 1991). The *hetR* gene has been cloned and sequenced from several species of *Trichodesmium* and *Symploca* PCC 8002 which have the ability to fix the atmospheric nitrogen during daylight, without forming specialized cells (heterocysts) and from *Leptolyngbya* PCC73110 which fixes nitrogen anaerobically. Anti-HetR antibodies cross-reacted with a protein in *Spirulina platensis* which revealed that the *hetR* gene was also present in the non-heterocystous cyanobacteria that do not fix nitrogen (Zhou *et al.*, 1998b). No obvious function has been attributed to the *hetR* gene in the non-heterocystous nitrogen-fixing cyanobacteria (Janson *et al.*, 1998). Among the heterocystous cyanobacteria *Cylindrospermum* contains only two terminal heterocysts, unlike *Anabaena*, where many intercalary heterocysts are seen (Fogg *et al.*, 1973; Reddy and Talpasayi, 1974).

At the time of initiation of this study, only two partial sequences of *hetR* gene from the genus *Cylindrospermum* were reported. Very little information was available on the genomic organization and gene expression of this organism. Hence it would be interesting to study full length *hetR* gene of *Cylindrospermum* A1345 by isolating and characterizing it.

The current study has been aimed at understanding the genomic organization and the expression pattern of *hetR* gene in the terminal heterocystous cyanobacteria. The copy number of *hetR* gene and the correlation between expression of *hetR* gene with that of nitrogenase enzyme and heterocyst differentiation were determined from the present study.

## Materials and Method

### Cultures and Culture Conditions

*Cylindrospermum stagnale* (Kütz.) Born. et Flah. A1345 was isolated from uncultivated arid soil at P.M. Palayam, Namakkal District, Tamil Nadu, India. The strain was identified by morphological characters (Desikachary, 1959). The species was further characterised at molecular level by 16S rDNA sequence (GenBank accession no. DQ897365). The culture was grown in cyanobacterial medium BG11 (Rippka *et al.*, 1979) at 28°C and axenized

by Imipenem (Merck), a broad spectrum  $\beta$ -lactam antibiotic (Ferris and Hirsch, 1991). Growth studies on *Cylindrospermum* A1345 was carried out by measuring the Chlorophyll-*a* and total protein content (Tandeau de Marsac and Houmar, 1988).

*E. coli DH5 $\alpha$ '* was used for cloning and characterization of *hetR* gene. The *E. coli* was maintained on LB medium with appropriate antibiotics.

### Heterocyst induction

The cells were initially grown in nitrogen rich BG11 medium upon which the filaments develop without heterocysts and then were transferred to the nitrogen depleted BG11<sub>0</sub> medium and the morphological changes were observed under a light microscope at x450 magnification.

### Southern analysis

The isolation of 5' regulatory sequences and reconstruction of full-length *hetR* gene sequences from *Cylindrospermum* A1345 has been previously reported by the authors (GenBank accession no. DQ439538; Kuriakose *et al.*, 2009). Southern analysis of *Cylindrospermum* A1345 was carried out to determine the copy number of *hetR* gene (Southern, 1975; Sambrook *et al.*, 1989). Approximately 2  $\mu$ g of total DNA was restricted separately with the restriction enzymes EcoRV and TaqI, subjected to electrophoresis on 1.4% denaturing agarose gel and blotted onto Nylon N<sup>+</sup> membrane according to Sambrook *et al.* (1989). The blot was probed with *hetR* gene which obtained by digesting the plasmid (pCSHETR) that carried *hetR* gene of *Cylindrospermum* A1345 with appropriate restriction enzyme and radio-labeled using RediprimeII Random labeling kit (Amersham Biosciences). Hybridization was carried out at 65°C overnight with phosphate buffer and washed twice with 3X SSC and 0.5X SSC along with 0.1% SDS at 65°C for 15 minutes each. The blot was then exposed to X-ray film at -70°C for 1 hr and developed.

### Northern Analysis

Northern analysis of *hetR* transcripts in *Cylindrospermum* A1345 at different time intervals (0 hr, 1 hr, 4 hrs, 8 hrs, 12 hrs, 16 hrs, 24 hrs, 36 hrs, and 48 hrs) following N<sub>2</sub> step down were carried out. Initially *Cylindrospermum* A1345 was grown in BG11 medium and then transferred to BG11<sub>0</sub> medium (N<sub>2</sub> deprived). RNA was extracted from about 100 mg cells of *Cylindrospermum* A1345 by Chomczynski and Sacchi (2006) method. The quantity and purity of isolated RNA was estimated by spectrophotometric readings at wavelengths of 260 nm and 280 nm. The total RNA was electrophoresed at 50V for 4 hrs in 1X MOPS buffer. After electrophoresis, the RNA was transferred onto Nylon N<sup>+</sup> membrane by capillary

blotting. The blot was probed with radiolabelled *hetR* gene from the plasmid pCSHETR at 42°C using formaldehyde buffer for 16 hrs, washed twice with 2X SSC/0.1% SDS and 0.5X SSC/0.1% SDS for 15 minutes at 65°C and exposed to X-ray at RT for 10 minutes and developed. The same blot was stripped and re-probed with radiolabelled 16S rDNA of *Cylindrospermum* A1345

#### Western Blot Analysis

Western blot analysis of the Fe-protein of the nitrogenase of *Cylindrospermum* A1345 was done according to Zehr *et al.*, 1993. The universal Fe protein antiserum was gifted by Dr. Luis M. Rubio (University of California, Berkeley). The *Cylindrospermum* A1345 grown in BG11 medium were transferred to BG11<sub>0</sub> medium. The cells were harvested by centrifugation at different time points (0 hr, 12 hrs, 24 hrs, 36 hrs and 48 hrs) and cells were sonicated (UP200S Hielscher) in extraction buffer (50mM Tris (pH 6.8); 3% (w/v) SDS; 5% (v/v) β-Mercaptoethanol; 2mM Phenylmethylsulfonyl fluoride; 1mM EDTA) to break the cells. The lysate was centrifuged at 12000rpm for 10 minutes. The supernatant was precipitated with acetone and resuspended in extraction buffer without SDS. The total protein of the lysate was estimated by Bradford (1976) method. 10µg of protein from each sample was heated to 95°C for 5 minutes, cooled to room temperature and loaded onto a 12% SDS PAGE. The proteins were separated at 75V for 3 hrs and then transferred to PVDF membrane at 15V for overnight at 4°C. The membrane was blocked with 3% non-fat milk protein for 1 hr and incubated with 1:5000 dilution of primary antibody for 2 hrs (against universal Fe protein raised in rabbit) and then with secondary antibody for 2 hrs (anti-rabbit IgG) in TBSTT buffer followed by BCIP/NBT color development.

#### Assay of Nitrogenase Activity of *Cylindrospermum* A1345

Nitrogenase activity was assayed by acetylene reduction technique (Stewart *et al.*, 1967). The *Cylindrospermum* A1345 grown in BG11 medium were transferred to BG11<sub>0</sub> medium for N<sub>2</sub> step down. The assay was carried out at different time points (0 hr, 12 hrs, 24 hrs, 36 hrs and 48 hrs) in glass vials of 25 mL capacity fitted with rubber stoppers and sealed with aluminium caps. The amount of ethylene produced was measured with AIMIL – Nucon 5700 model Gas Chromatograph (GC) equipped with Poropak N

column and Flame Ionisation Detector (FID). Nitrogen was used as carrier gas and ethylene standard was used as reference.

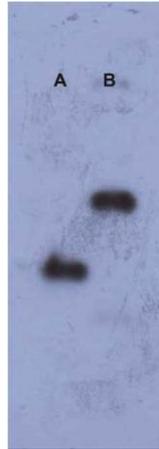
#### Sequence Analysis

The BLAST analysis of deduced amino acid sequence of *Cylindrospermum* A1345 HetR was carried out using NCBI protein blast (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>). The conserved domain search (CD search) was done online using <http://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi>. The HetR sequence was compared with other HetR sequences of peptidase family S48 (cl11616) protein sequences viz. *Anabaena cycadae* (ABE66460), *Anabaena variabilis* ATCC 29413 (AAG24634), *Nostoc sp.* PCC 7120 (NP\_486379), *Nostoc azollae* 0708 (ZP\_03764624), *Nostoc punctiforme* PCC 73102 (YP\_001865334), *Nodularia spumigena* CCY9414 (ZP\_01631106), *Trichodesmium erythraeum* IMS101 (YP\_721649), *Leptolyngbya boryana* (Q93CE8), *Lyngbya sp.* PCC 8106 (ZP\_01623413) and *Synechococcus sp.* PCC 7002 (YP\_001733689) from GenBank. The sequence alignment was done with ClustalW program in the BioEdit (Hall, 1999). The phylogenetic analyses performed with MEGA4 (Tamura *et al.*, 2007) and the evolutionary history of HetR protein was inferred using the Neighbor-Joining method (Saitou and Nei; 1987).

#### Results

##### *Copy number of hetR gene in Cylindrospermum A1345*

The cloned DNA fragment (1994bp) containing *hetR* gene sequence had two sites each for restriction enzyme EcoRV and TaqI. The EcoRV enzyme had one site in the cloned HetR coding sequence and another one in the promoter region giving rise to a 935bp fragment upon digestion. The TaqI enzyme had one site in the HetR coding region and one near the 5' end of the sequence giving give rise to a 1225 bp fragment. Hence the total DNA was restricted with these enzymes as they cut only once within the *hetR* gene. Southern analysis showed single band in the blot when restricted with each enzyme indicating single copy of *hetR* in *Cylindrospermum* A1345. (Fig.1).

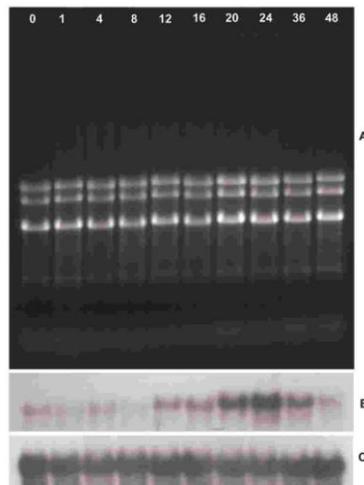


**Fig.1. Southern blot analysis of *Cylindrospermum* A1345 to determine the copy number of *hetR* gene showing single band for Eco RV (A) and Taq I (B) respectively**

*Expression pattern of hetR gene and differentiation of heterocyst in Cylindrospermum A1345*

Induction and expression of *hetR* transcripts during differentiation of heterocysts in *Cylindrospermum* A1345 were analysed and compared with previous reports. The blot carrying total RNA obtained at various time intervals after nitrogen depletion was probed with *hetR* gene. The blot showed an induction of the transcript levels

at 12 hrs peaking at 24 hrs followed by a gradual decrease (Fig.2). This correlated with the heterocyst differentiation and nitrogenase activity as shown in Fig. 3 & 5. The proheterocysts formation began at 12 hrs to 24 hrs and fully differentiated heterocysts were formed by 48 hrs. Most of the filaments were having fully developed heterocysts by 36 hrs



**Fig. 2. Northern blot analysis of expression of the *hetR* gene in *Cylindrospermum* A1345. Total RNA was isolated at different time points following nitrogen step-down. (A) Denaturing agarose gel (1.4%) electrophoresis of total RNA (B) Total RNA transferred membrane blot was hybridized with *hetR* gene specific probe. (C) The same blot was hybridized with 16S rDNA of *Cylindrospermum* A1345 specific probe to showing equal loading of total RNA.**

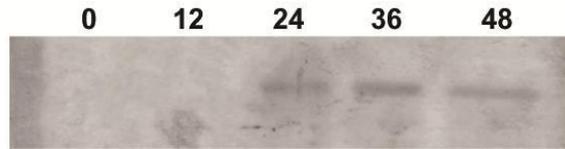


Fig. 3. Western immunoblot analysis of Fe protein of nitrogenase in *Cylindrospermum* A1345.

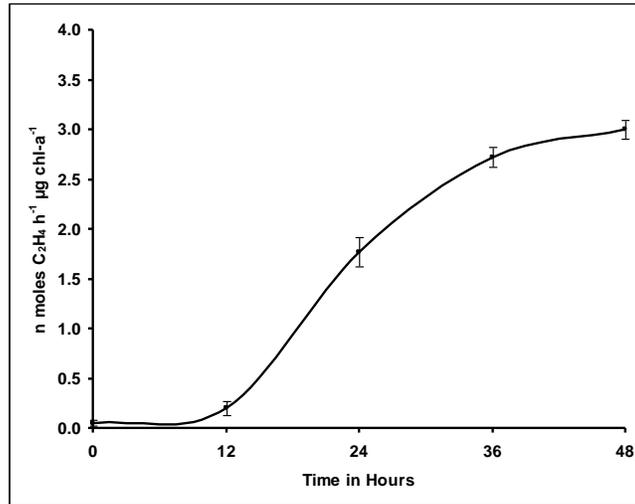


Fig.4. Nitrogenase activity of *Cylindrospermum* A1345 assayed by the acetylene reduction technique

#### Nitrogenase activity

Immunoblot analysis of the Fe-protein subunit of nitrogenase enzyme complex at different time intervals revealed that the protein was first detectable at around 24 hrs (Fig 3). The acetylene reduction assay of

*Cylindrospermum* A1345 showed that the nitrogenase activity started after 12 hrs and substantial activity was observed only around 36 hrs (Fig. 4) when most of the heterocysts were fully developed.

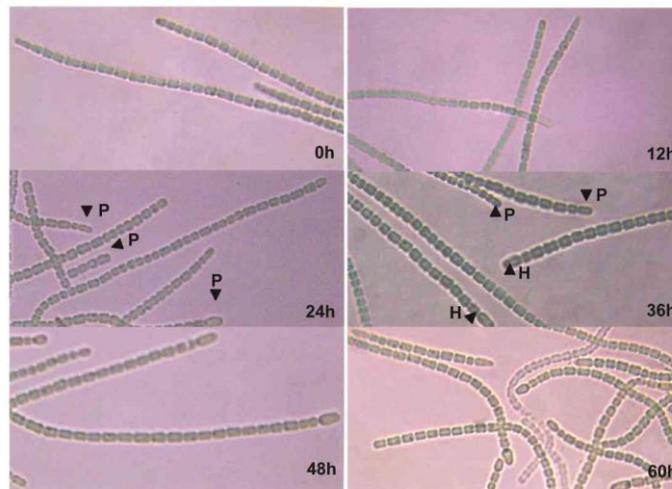
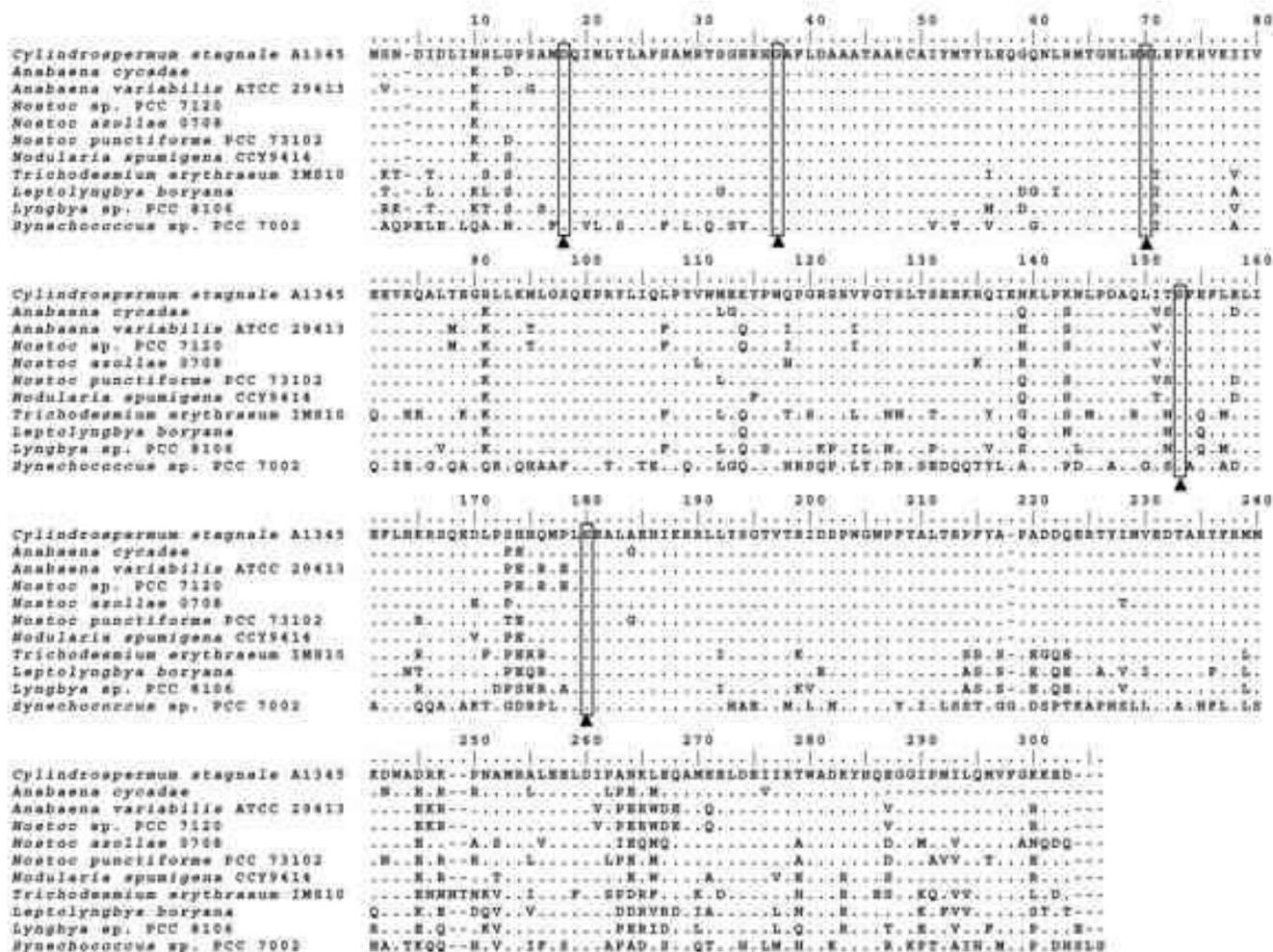


Fig. 5. Photomicrograph of *Cylindrospermum* A1345 (x450) shows the heterocyst differentiation after nitrogen step-down. P- Proheterocyst; H- Heterocyst.

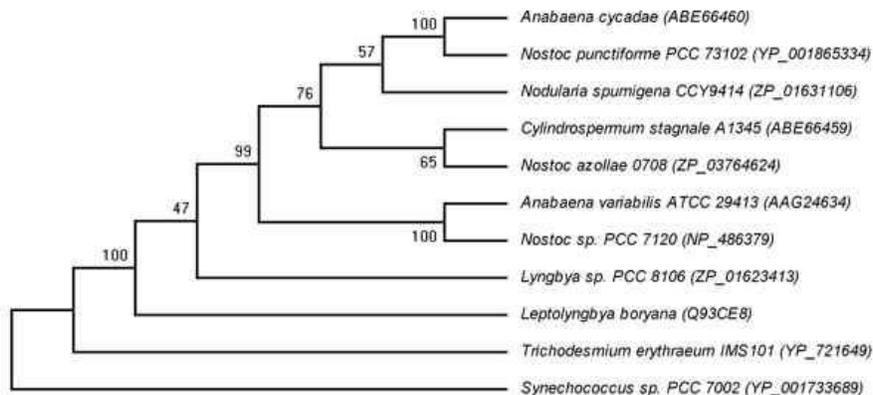
*Computational analysis of hetR gene of Cylindrospermum A1345*

The BLAST analysis of *hetR* gene of *Cylindrospermum* A1345 showed a sequence similarity of 80-84% at DNA level and 96% at protein level with other available *hetR* sequences in the public database. The conserved domain (CD) search of deduced amino acid sequences of HetR protein showed that the *hetR* had conserved domain belonging to peptidase\_S48 superfamily (Accession No. cl11616) and had best scoring hit with the family member PRK13245 (Bit Score: 563.40 and E-value: 2e-161). The HetR sequence analysis revealed that

all the previously reported amino acid residues viz. S152 and S179 (Zhou *et al.*, 1998a; Dong *et al.*, 2000), D17, G36 and H69 (Risser and Callahan, 2007) which are required for its normal function and protein turnover were conserved (Fig 6). These results indicated that the protein was highly conserved across the cyanobacterial genus though some changes in amino acid sequences at the C-terminus were also observed (Fig 6). The evolutionary tree of HetR protein was grouped this species along closely with *Nostoc azollae* 0708 (ZP\_03764624) and closely related to other nitrogen fixing cyanobacteria like *Nostoc* and *Anabaena* (Fig. 7)



**Fig. 6.** Multiple alignment of the deduced amino acid sequence of HetR from *Cylindrospermum* A1345 showing the sequence similarity to the deduced amino sequences of HetR from different cyanobacteria (Peptidase S48 family). Black arrowheads indicate that the amino acid residues are conserved in all organisms and thought to be essential for the normal functi



**Fig. 7. The evolutionary relationship of HetR sequences of different cyanobacteria (Peptidase S48 family) analysed by Neighbor-Joining method.**

### Discussion

The *hetR* gene is a key regulator of heterocyst differentiation and it has been extensively studied in *Anabaena* sp. PCC7120. Among the heterocystous cyanobacteria *Cylindrospermum* contains only two terminal heterocysts, unlike *Anabaena*, where many intercalary heterocysts are seen (Fogg *et al.*, 1973; Reddy and Talpasayi, 1974). We have previously isolated both 5' and 3' ends of *hetR* gene from *Cylindrospermum* A1345 organism using RAGE-PCR (Kuriakose *et al.*, 2009). The 1994 bp genomic region consisting the coding sequence, promoter and 3' region was submitted to GenBank (Accession number: DQ439538). Even though *hetR* gene has been reported from non-heterocyst forming cyanobacteria such as *Phormidium noryanum*, *Spirulina platensis*, *Trichodesmium* and *Symploca* PCC 8002 and unicellular cyanobacterium *Synechococcus* sp. PCC 7002 (YP\_001733689) in previous studies (Buikema and Haselkorn, 1991; Janson *et al.*, 1998; Zhou *et al.*, 1998a, Zhang *et al.*, 2009), we failed to amplify this gene by PCR using degenerate primers in the non-heterocyst forming cyanobacteria *Oscillatoria* spp (data not shown). In our studies, only one band was observed for each restriction enzyme used in Southern analysis and therefore indicates that *hetR* gene exist as single copy in *Cylindrospermum* A1345. Since the enzymes have only one site within the coding region of *hetR* gene and the second site would be on the genome, the number of bands observed directly indicates the number of copies of the gene. We had earlier observed only single bands in our efforts to clone the full length sequence by genome walking indicating single copy of the gene. This was further confirmed in this study by Southern analysis (Kuriakose *et al.*, 2009). The promoter sequence obtained by RAGE was approximately 430bp and it would be interesting to further characterize this promoter during nitrogen signaling based on the results obtained in this study.

Previous studies indicated that not all the cells differentiate into heterocysts (Buikema and Haselkorn, 2001); and there is a massive upregulation of the *hetR* gene in the heterocyst forming cells (Zhou *et al.*, 1998b). The present study was consistent with the previous studies as the induction of *hetR* gene in *Cylindrospermum* A1345 following nitrogen step-down began at 12 hrs. The levels peaked at 24 hrs followed by a gradual reduction reaching basal levels around 48 hrs as shown by northern analysis. The transcript levels of *hetR* gene also correlated with heterocyst formation in *Cylindrospermum* A1345. Proheterocysts were observed at 24 hrs and fully developed heterocysts were found between 36 hrs and 48 hrs. Previous studies in *Anabaena* showed that *hetR* gene was induced after 2 to 3.5 hrs of nitrogen step-down and peaked between 4.5 and 9 hrs (Black *et al.*, 1993; Muro-pastor *et al.*, 2002). In *Cylindrospermum licheniforme* the proheterocysts appeared 12 to 15 hrs after fragmentation, and 12 to 15% of the cells became heterocysts within 24 hrs (van De Water and Simon, 1982). Our studies were more consistent with the studies in *Cylindrospermum licheniforme* and differed with that of *Anabaena*. The difference in *hetR* gene expression and heterocyst differentiation in *Cylindrospermum* A1345 may be due to difference in signal perception among different nitrogen fixing cyanobacteria and retention of nitrogen within the cells at the time of transfer to nitrogen deprived medium (BG11<sub>0</sub>). The *hetR* is transcribed at low level in all vegetative cells at all the times and the transcription level was increased while heterocyst differentiation and gradually decreased subsequently. The increased transcription occurs only in the cells which are initiated to differentiate into heterocyst (Black *et al.*, 1993). Interestingly, in non-heterocystous cyanobacterium *Symploca* PCC 8002 the *hetR* transcription level decreased gradually after nitrogen step-down and reached minimum level (Janson *et al.*, 1998). The *hetR* gene has also been

reported from unicellular cyanobacterium *Synechococcus* sp. PCC 7002 (YP\_001733689). The function of *hetR* in non-heterocystous and unicellular cyanobacterium is not known and the HetR protein may have evolved before heterocysts appeared in nature (Zhang *et al.*, 2009). Our studies suggest that the function of HetR may not be limited to heterocyst differentiation and pattern formation. The widespread nature of HetR in non heterocystous and unicellular cyanobacteria suggested that there might be a larger role for the protein in the process.

The western blot analysis and nitrogenase assay showed that the nitrogenase activity started at 12 hrs when heterocysts formation had actually begun. The nitrogenase enzyme activity thus does not appear to be directly related to the HetR protein and heterocyst formation but a strong indirect role is indicated.

These results confirmed that the *hetR* gene is strictly regulated and the pattern of expression correlated with that observed in other heterocystous cyanobacteria like *Anabaena* though there was variation in the time intervals of induction. The BLAST results and sequence comparison of *hetR* from *Cylindrospermum* A1345 suggested that this gene was highly conserved among cyanobacterial genus. Further work like translational fusion of *hetR* gene under its own promoter to a reporter like GFP can help in understanding the spatial and temporal patterns of expression of the *hetR* and help in better understanding of the gene regulation during heterocyst formation in this organism.

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