hetR and *patS*, two genes necessary for heterocyst pattern formation, are widespread in filamentous nonheterocyst-forming cyanobacteria

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Heterocysts, cells specialized in N₂ fixation in cyanobacteria, appeared at near to 2.1 Ga. They constitute one of the oldest forms of differentiated cells in evolution, and are thus an interesting model for studies on evolutionary-developmental biology. How heterocysts arose during evolution remains unknown. In *Anabaena* PCC 7120, heterocyst development requires, among other genes, *hetR* for the initiation of heterocyst differentiation, and *patS*, encoding a diffusible inhibitor of heterocyst formation. In this study, we report that both *hetR* and *patS* are widespread among filamentous cyanobacteria that do not form heterocysts or fix N₂. *hetR* and *patS* are found in proximity on the chromosome in several cases, such as *Arthrospira platensis*, in which the level of HetR increased following nitrogen deprivation. The *hetR* gene of *A. platensis* could complement a *hetR* mutant of *Anabaena* PCC 7120, and *patS* of *A. platensis* could suppress heterocyst differentiation in *Anabaena* PCC 7120. Thus, key regulatory genes, including *hetR* and *patS*, involved in heterocyst development may have evolved before heterocysts appeared, suggesting that their function was not limited to heterocyst differentiation.

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INTRODUCTION

Many cyanobacteria can fix N₂, a process catalysed by nitrogenase, an enzyme complex highly sensitive to oxygen, whereas oxygen is produced by cyanobacteria as byproduct of photosynthesis. Cyanobacteria adopt two major strategies to resolve the problem of incompatibility between oxygenic photosynthesis and oxygen-sensitive nitrogen fixation, by separation of these two activities either in time or in space, or a combination of both strategies (Berman-Frank *et al.*, 2003). In the model organism *Anabaena* sp. strain PCC 7120 (hereafter *Anabaena* 7120), the spatial separation of these two activities is achieved by the differentiation of 5–10% of the cells in each filament into heterocysts, cells that specialize in nitrogen fixation (Meeks & Elhai, 2002; Zhang *et al.*, 2006a). A micro-oxic environment within heterocysts protects nitrogenase against inactivation by oxygen (Wolk *et al.*, 1994).

Heterocyst differentiation requires a number of genes involved in the different steps of the developmental process (Meeks & Elhai, 2002; Zhang *et al.*, 2006a). At least in *Anabaena* 7120, heterocyst differentiation is triggered by

the accumulation of 2-oxoglutarate, which acts as a signal of deprivation of combined nitrogen (Chen *et al.*, 2006; Laurent *et al.*, 2005; Li *et al.*, 2003). One of the receptors of the 2-oxoglutarate signal could be NtcA, a transcription factor highly conserved in cyanobacteria (Herrero *et al.*, 2004; Laurent *et al.*, 2005). Another critical factor for the initiation of heterocyst differentiation is HetR, a protease with DNA-binding activity (Buikema & Haselkorn, 1991; Huang *et al.*, 2004; Zhou *et al.*, 1998b). HetR has been reported so far in heterocyst-forming strains, in filamentous strains that fix N₂ and in *Arthrospira platensis*, a nondiazotrophic filamentous strain (Zhou *et al.*, 1998a). At the maturation stages of heterocyst differentiation, two envelope layers, composed of polysaccharides and glycolipids, are formed; genes encoding nitrogenase are expressed, and heterocysts become functional (Meeks & Elhai, 2002). In many heterocyst-forming strains, heterocysts are spaced semi-regularly along each filament, corresponding to a one-dimensional pattern (Meeks & Elhai, 2002; Zhang *et al.*, 2006a). The formation of the heterocyst pattern possibly requires interaction between HetR and PatS (Huang *et al.*, 2004). PatS, or a pentapeptide of PatS (PatS-5), has been proposed to be a diffusible molecule acting as an inhibitor of heterocyst differentiation (Yoon & Golden, 1998). According to the current model, HetR activates the expression of *patS*, and PatS or a derivative of

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PatS diffuses laterally to inhibit the differentiation of the neighbouring cells by acting negatively on the DNA-binding activity of HetR (Zhang *et al.*, 2006a). Another gene, *hetN*, is required for the maintenance of heterocyst pattern (Callahan & Buikema, 2001). Both *patS* and *hetN* are necessary to prevent heterocyst differentiation in the presence of a combined nitrogen source (Borthakur *et al.*, 2005).

How different forms of cells and organisms emerged during evolution remains a challenging question in biology. Evolutionary developmental biology (known as evo-devo biology) explores the molecular basis of the emergence and evolution of the form and shape of living organisms (Goodman & Coughlin, 2000). Heterocysts are interesting for evo-devo studies because they may have appeared in evolution between 2450 and 2100 Ma based on indirect fossil record and phylogenetic analysis (Tomitani *et al.*, 2006), making them one of the oldest forms of differentiated cells to have appeared on the Earth. Although we have an idea about when heterocysts appeared during evolution (Tomitani *et al.*, 2006), it remains unknown how they appeared. In this study, we show that *hetR* and *patS* are found not only in nitrogen-fixing filamentous cyanobacteria, but also in filamentous strains that do not fix N₂. The implication of these findings is discussed.

METHODS

Sequence analysis. Genome data of cyanobacteria were collected from the Integrated Microbial Genomes (IMG) website (<http://img.jgi.doe.gov>). Genomic data of *Arthrospira platensis* were kindly made available by Beijing Genomic Institute. The 16S rRNA sequences and HetR sequences used for phylogenetic analysis were obtained from GenBank. Phylogenetic analyses were performed with MEGA 4.0 (Tamura *et al.*, 2007) by using the minimum evolution (ME) method (Rzhetsky & Nei, 1992) together with the close-neighbour-interchange (CNI) searching algorithm (Nei & Kumar, 2000). The 16S rRNA tree was inferred with the Kimura two-parameter substitution model (Kimura, 1980), while the HetR tree was inferred with the Poisson correction substitution model (Zuckerkanndl & Pauling, 1965).

Bacterial strains and culture conditions. All cyanobacterial strains related to this study are listed in Table 1. *Schizothrix calcicola*, *Oscillatoria lutea*, *Lyngbya* sp. and *Phormidium mucicola* were obtained from the culture collection of the Institute of Hydrobiology, Chinese Academy of Sciences; *Arthrospira platensis* was obtained from the culture collection of the Institute of Botany, Chinese Academy of Sciences and was grown in modified SAG medium as described by Zhang *et al.* (2006b). *Anabaena* 7120 was grown in BG11 with nitrate as source of combined nitrogen. For cultures under conditions of combined-nitrogen starvation, nitrate was omitted from the BG11 medium (BG11₀) or SAG medium. All cyanobacterial strains were grown at 28 °C.

PCR for *hetR* and immunodetection of the HetR protein. Two degenerate PCR primers, PDhetR-up and PDhetR-dw (Table 2), were deduced from conserved motifs of HetR proteins and were used to amplify *hetR* sequences. The procedure for immunodetection was as described previously (Kuhn *et al.*, 2000), using a polyclonal antibody prepared against HetR of *Anabaena* 7120.

Plasmid constructions. We first constructed pRL25Z, a modified version of the replicative vector pRL25c in *Anabaena* 7120 (Wolk *et al.*, 1988). For this purpose, pRL25c was digested with *Bam*HI and *Eco*RI. The larger DNA fragment was purified and ligated with an artificial fragment, which was generated by annealing two oligonucleotides (GATCCCCGGG and AATCCCCGGG), and thus had a *Bam*HI site at one end and an *Eco*RI site at the other end and a *Sma*I site in the middle. The resulting vector pRL25Z has a size of 9507 bp and a linker with unique cloning sites for *Bam*HI, *Sma*I and *Eco*RI. All PCR primers are listed in Table 2. The *hetR* gene of *Arthrospira platensis* (*hetR_{ar}*) was obtained by PCR with the primers ParhetR498m and ParhetR1285, and the *patS_{ar}-hetR_{ar}* gene cluster of *Arthrospira platensis* was obtained with primers ParhetR979m and ParhetR1285. The PCR products were digested with *Bam*HI and inserted into the *Bam*HI site of pRL25Z, leading to pRar and pSRar, respectively (see Fig. 3). To obtain the construct pSar (Fig. 3), the *hetR_{ar}* corresponding sequence in pSRar was removed by digestion with *Eco*RI. The promoter region of *hetR_{ar}* was amplified with primers ParhetR498m and ParhetR9 and cloned at the *Sma*I site of pGFPTX (derived from pGFPuv by replacing the region of the *lac* promoter on pGFPuv with the T4 transcriptional terminator) to produce pGFPparhetR. pGFPparhetR was linearized with *Xmn*I and inserted into the *Sma*I site of pRL25Z, resulting in pRarGfp (Fig. 3). The promoter region of *patS_{ar}* together with the first 100 bp of its coding region was amplified with primers ParpatS434m and ParpatS100, digested with *Hind*III and *Kpn*I, and ligated into the same sites of pGFPTX; the resulting plasmid was then linearized with *Eco*RI and inserted into the *Eco*RI site of pRL25Z, leading to pSarGfp (Fig. 3). All pRL25Z-derived plasmids were introduced into *Anabaena* 7120 by conjugation (Wolk *et al.*, 1988).

Microscopy. A Nikon DXM 1200 digital camera mounted on a Nikon Eclipse E800 microscope was used to capture images as described before (Sakr *et al.*, 2006). The same microscopic setting was used to acquire series of images. For fluorescent images, areas under the microscope were chosen under bright-field and were excited at 465 nm only during the time of image acquisition to avoid unnecessary bleaching.

RESULTS

HetR orthologues are widespread among both diazotrophic and nondiazotrophic filamentous cyanobacteria

The *hetR* gene was first identified as a key regulator of heterocyst differentiation and found also in filamentous cyanobacteria that are able to fix molecular nitrogen without heterocyst formation (Buikema & Haselkorn, 1991). It was used as a marker for phylogenetic analysis of nitrogen-fixing filamentous cyanobacteria (Mes & Stal, 2005). Similar genes are absent in all unicellular cyanobacteria whose genomes have been sequenced. One report also mentioned the existence of a protein in *Arthrospira platensis* that could react with antibody raised against the HetR of *Anabaena* PCC 7120 (Zhou *et al.*, 1998a), but it remained unknown whether it represented an isolated case or a general observation in filamentous cyanobacteria. In this study, we used a polyclonal antibody raised against HetR of *Anabaena* 7120 to assess the presence of HetR in five nondiazotrophic filamentous cyanobacteria (Table 1), *Schizothrix calcicola*, *Oscillatoria lutea*, *Lyngbya* sp., *Phormidium mucicola* and *Arthrospira platensis*. All five

Table 1. Relevant features of cyanobacterial strains related to this study

Strain	Morphology	N ₂ fixation	Heterocyst formation	Habitat	Reference
<i>Anabaena</i> PCC 7120	Filamentous	Yes	Yes	Freshwater	Rippka <i>et al.</i> (1979)
<i>Anabaena variabilis</i> ATCC 29413	Filamentous	Yes	Yes	Freshwater	Wolk <i>et al.</i> (1994)
<i>Nostoc punctiforme</i> PCC 3102	Filamentous	Yes	Yes	Symbiotic	Rippka <i>et al.</i> (1979)
<i>Microcoleus chthonoplastes</i> PCC 7420	Filamentous	No	No	Marine	Rippka <i>et al.</i> (1979)
<i>Trichodesmium erythraeum</i> IMS 101	Filamentous	Yes	No	Marine	Zehr <i>et al.</i> (2000)
<i>Lyngbya</i> PCC 8106	Filamentous	Yes	No	Marine	Stal & Krumbein (1981)
<i>Lyngbya</i> FACHB 722	Filamentous	No	No	Soil	Maintained at the Institute of Hydrobiology, Wuhan, China
<i>Phormidium mucicola</i> FACHB 723	Filamentous	No	No	Soil	Maintained at the Institute of Hydrobiology, Wuhan, China
<i>Oscillatoria lutea</i> FACHB 406	Filamentous	No	No	Freshwater	Maintained at the Institute of Hydrobiology, Wuhan, China
<i>Schizothrix calcicola</i> FACHB 404	Filamentous	No	No	Freshwater	Maintained at the Institute of Hydrobiology, Wuhan, China
<i>Arthrospira platensis</i>	Filamentous	No	No	Salt lake	Maintained at the Institute of Botany, Beijing, China
<i>Synechocystis</i> PCC 6803	Unicellular	No	No	Freshwater	Rippka <i>et al.</i> (1979)
<i>Synechococcus</i> PCC 6301	Unicellular	No	No	Freshwater	Rippka <i>et al.</i> (1979)
<i>Synechococcus</i> PCC 7942	Unicellular	No	No	Freshwater	Rippka <i>et al.</i> (1979)
<i>Synechococcus</i> PCC 7335*	?	Yes	No	Marine	Rippka <i>et al.</i> (1979)
<i>Synechococcus</i> CC 9605	Unicellular	No	No	Seawater	Palenik (2001)
<i>Synechococcus</i> CC 9902	Unicellular	No	No	Seawater	Palenik (2001)
<i>Crocospaera watsonii</i> WH8501	Unicellular	Yes	No	Marine	Webb <i>et al.</i> (2001)
<i>Synechococcus</i> WH8102	Unicellular	No	No	Marine	Scanlan & West (2002)
<i>Prochlorococcus marinus</i> MED4	Unicellular	No	No	Marine	Scanlan & West (2002)
<i>Prochlorococcus marinus</i> MIT 9312	Unicellular	No	No	Marine	Scanlan & West (2002)
<i>Prochlorococcus marinus</i> MIT 9313	Unicellular	No	No	Marine	Scanlan & West (2002)
<i>Prochlorococcus marinus</i> NATL2A	Unicellular	No	No	Marine	Scanlan & West (2002)
<i>Prochlorococcus marinus</i> SS120	Unicellular	No	No	Marine	Scanlan & West (2002)
<i>Thermosynechococcus elongatus</i> BP-1	Unicellular	No	No	Hot spring	Yamaoka <i>et al.</i> (1978)
<i>Gloeobacter violaceus</i> PCC 7421	Unicellular	No	No	Rock surface	Rippka <i>et al.</i> (1979)

*The morphology and the classification of *Synechococcus* PCC 7335 are a matter of debate, as discussed in the text.

strains displayed a cross-reactivity with the antibody (Fig. 1). To further confirm these data, partial sequence of *hetR* was successfully amplified from *S. calcicola*, using degenerate oligonucleotide primers designed according to conserved regions of known *HetR* sequences. A *HetR*-encoding sequence was also identified from the genome of *A.*

platensis. These data clearly indicate that *HetR* homologues are widespread in both diazotrophic and nondiazotrophic filamentous cyanobacteria.

In *Anabaena* 7120, the level of the *HetR* protein is increased following limitation of combined nitrogen (Buikema &

Table 2. DNA primers used in this study

Primer	Sequence (5'–3')	Cloning site
PDhetR-up	CCTCGACCYAARCGRGT	–
PDhetR-dw	CCTCTCATGYTCTGYCA	–
ParhetR979m	CGTA GGATCC TATGACTGGGGGGTTGAC	<i>Bam</i> HI
ParhetR498m	CGTA GGATCC TCAATCAGAATCACTGTT	<i>Bam</i> HI
ParhetR9	G AAGCTT GATCAGATCTGTATC	<i>Hind</i> III
ParhetR1285	CGTA GGATCC GGCTATGGTAATGGTTTC	<i>Bam</i> HI
ParpatS434m	CTCT AAGCTT AGGTCTGGAGTCTCGTCA	<i>Hind</i> III
ParpatS100	TCTA GGTACC TCTAATTGCTGTGTGGTTG	<i>Kpn</i> I

Fig. 1. Immunoblot analysis using antibodies against HetR of *Anabaena* 7120. (a) Detection of HetR from several cyanobacteria. Lanes 1 to 4: protein samples extracted from *Schizothrix calcicola*, *Oscillatoria lutea*, *Lyngbya* sp. and *Phormidium mucicola*, respectively; lane 5, protein extract from *Anabaena* 7120. (b) Increase in HetR level in *Arthrospira platensis* following nitrogen starvation at 0 h, 18 h, and 42 h respectively. A similar amount of total protein was loaded on the first three lanes. rHetR, recombinant HetR of *Anabaena* 7120 as control.

Haselkorn, 1991; Zhou *et al.*, 1998a). Using immunoblotting, we found that the levels of HetR_{ar} (HetR of *Arthrospira platensis*) increased in cells of *A. platensis* incubated in the

absence of a combined nitrogen source for 18 or 42 h (Fig. 1b). The level of HetR_{ar} was found to be highest in filaments at 48 h of incubation in the absence of combined nitrogen, and then decreased (data not shown). These results show that although *A. platensis* is not able to fix N₂ or to form heterocysts, the level of HetR_{ar} is upregulated similarly as in heterocyst-forming cyanobacteria.

The HetR sequences identified here, together with some representative HetR sequences previously published, were used to perform a phylogenetic analysis (Fig. 2). This analysis shows that HetR from heterocyst-forming cyanobacteria forms a monophyletic clade distinct from that of non-heterocyst-forming cyanobacteria, suggesting that *hetR* genes found in the latter strains are unlikely to be the results of horizontal gene transfer from heterocyst-forming cyanobacteria.

***hetR_{ar}* is adjacent to a putative *patS* sequence in *Arthrospira platensis* and the corresponding genomic region is functional in regulating heterocyst development in *Anabaena* 7120**

The amino acid sequence of HetR_{ar} shares 76 % identity with HetR of *Anabaena* 7120. Since no reliable genetic

Fig. 2. Phylogenetic relationship of HetR sequences analysed by the minimal evolution (ME) method. The corresponding gi numbers of sequences are labelled on the left of the genus names. The numbers in the major branches are the bootstrap values calculated from 1000 replicate trees.

Fig. 3. (a) The genomic region including *patS_{ar}-hetR_{ar}* in *Arthrosira platensis* (top) and various plasmids carrying different regions. Green colour represents the *gfp* gene fused to the promoter region of *hetR_{ar}* or *patS_{ar}* (see Fig. 4 for GFP fluorescence). (b) Phenotypes of the *Anabaena* strains expressing *hetR_{ar}* and/or *patS_{ar}* after 24 h of combined-nitrogen deprivation. Three constructs were transferred by conjugation into various strains as shown: pSRar (b1, b2), pSar (b3, b4) and pRar (b5, b6). The wild-type (b1, b3, b5), a *hetR* mutant (b2, b6), and a *patS* mutant (b4) were used in this analysis. Arrowheads indicate heterocysts. wt, wild-type.

system exists for filamentous nondiazotrophic cyanobacteria such as *Arthrosira platensis*, we used *Anabaena* 7120 as a heterologous system to get insight into the possible function of *hetR_{ar}*. A genomic region including *hetR_{ar}* was cloned in pRL25Z, a replicative vector in *Anabaena* 7120. The construct, pSRar (Fig. 3), was then transferred into both the wild-type and the *hetR* mutant of *Anabaena* 7120 by conjugation. Surprisingly, the presence of this plasmid suppressed heterocyst differentiation in the wild-type and was unable to complement the *hetR* mutation (Fig. 3). As controls, a plasmid bearing a copy of *hetR* of *Anabaena* 7120 could complement the defect of the *hetR* mutant in heterocyst differentiation, whereas the same plasmid in the wild-type strain resulted in Mch (multiple contiguous heterocysts) phenotypes as reported previously (Buikema & Haselkorn, 1991).

Further examination of the DNA fragment in pSRar revealed that the ORF upstream of *hetR_{ar}* could encode a small protein of 84 amino acid residues with the sequence of the

last five residues identical to that of the PatS pentapeptide of *Anabaena* 7120 (Fig. 3). To test whether this ORF could confer a PatS-like function, two constructs were made, one with *hetR_{ar}* alone (pRar, Fig. 3), and another with the ORF corresponding to the putative *patS* gene (pSar). In the presence of pSar, neither the wild-type nor the *patS* mutant of *Anabaena* 7120 formed heterocysts in the presence or absence of a combined-nitrogen source, whereas the *patS* mutant gave rise to Mch phenotypes as expected (Fig. 3). Thus the presence of the upstream ORF had a heterocyst-suppressing activity. We therefore designated this ORF *patS_{ar}*. Similarly, the construct with *hetR_{ar}* alone led to a Mch phenotype when present in the wild-type of *Anabaena* 7120, and could complement the *hetR* mutant of the same organism (Fig. 3). Based on these results, we concluded that *hetR_{ar}* and *patS_{ar}* from *Arthrosira platensis* could regulate heterocyst differentiation in *Anabaena* 7120.

Although the products of *hetR_{ar}* and *patS_{ar}* could function in *Anabaena* 7120, their promoter regions show no obvious

similarity to those of *hetR* and *patS* in *Anabaena* 7120, raising the question of whether the transcriptional control could operate on *hetR_{ar}* and *patS_{ar}* in *Anabaena* 7120. Therefore, the putative promoter region of *hetR_{ar}* and that of *patS_{ar}* were fused to a *gfp* reporter. The two corresponding constructs, pRarGfp and pSarGfp (Fig. 3), were transferred into *Anabaena* 7120 by conjugation. After induction by nitrogen starvation, green fluorescence emitted by GFP could be detected more strongly in heterocysts than in vegetative cells for *Anabaena* 7120 bearing pSarGfp (Fig. 4). GFP fluorescence observed with the strain bearing pRarGfp displayed also a strong upregulation after combined-nitrogen stepdown; this upregulation was not confined specifically to heterocysts, and became less strong after 8 h of incubation under conditions of combined nitrogen starvation (Fig. 4). These results showed that both *patS_{ar}* and *hetR_{ar}* displayed activation by combined-nitrogen deprivation in *Anabaena* 7120, but only *patS_{ar}* displayed a cell-type-specific upregulation. This result is consistent with the observation obtained with *Anabaena* 7120 that cell-type-

specific expression of *hetR* is not necessary for heterocyst formation (Black *et al.*, 1993; Buikema & Haselkorn, 2001).

Putative *patS*-like sequences are widespread among filamentous cyanobacteria

Potential PatS-like peptides were scanned bioinformatically in 18 cyanobacterial strains whose genomes have been completely sequenced. These strains include (Table 1): *Anabaena variabilis* ATCC 29413, *Nostoc punctiforme* PCC 73102, *Trichodesmium erythraeum* IMS 101, *Arthrospira platensis*, *Crocospaera watsonii* WH8501, *Synechocystis* PCC 6803, *Gloeobacter violaceus* PCC 7421 and *Thermosynechococcus elongatus* BP-1, *Synechococcus* PCC 6301, *Synechococcus* PCC 7942 and 8 *Prochlorococcus* strains (*Prochlorococcus marinus* SS120, *Prochlorococcus marinus* MED4, *Prochlorococcus marinus* NATL2A, *Prochlorococcus marinus* MIT 9313, *Prochlorococcus marinus* MIT 9312, *Synechococcus* WH8102, *Synechococcus* CC9902 and *Synechococcus* CC9605). Those sequences with an RGSGR motif embedded within the protein sequence were

Fig. 4. Analysis of promoter regions of *patS_{ar}* and *hetR_{ar}* fused to *gfp* reporter in *Anabaena* 7120. The corresponding constructs are depicted in Fig. 3. (a, b) pSarGfp in a *patS* mutant at 24 h after nitrogen stepdown. (c–h) pRarGfp in the wild-type at time 0 h (c), 3 h (d), 8 h (e), 24 h (f) or 48 h (g, h) after combined-nitrogen stepdown. (a) Filament pictured under bright-field; (b, c, d, e, f, h) green epifluorescence emitted by GFP; (g) red epifluorescence emitted from photosynthetic pigments of the same filaments as in (h), to highlight heterocysts that display a dimmer red fluorescence as compared to vegetative cells. Arrowheads indicate heterocysts.

excluded. Several possible *patS*-like genes were identified. Such putative *patS*-like genes were not detected in the unicellular cyanobacterial genomes, but were present in all filamentous cyanobacteria whose genome sequences were available (Fig. 5). While, in the heterocyst-forming cyanobacteria such as *Anabaena* 7120, *Anabaena variabilis* or *N. punctiforme*, the *patS* gene could encode a peptide of 17 or 13 amino acids depending on the initiation codon that would actually be used, those deduced from non-heterocyst-forming cyanobacteria could encode a much larger peptide (Fig. 5). The *patS*-like gene in *Arthrospira platensis* could encode a peptide of 84 residues, and that in *T. erythraeum* could encode a peptide of 90 residues. The latter one was annotated as hypothetical (GenBank accession no. ABG51177). Fig. 5 shows the position of the *hetR* locus relative to that of *patS* on different genomes. In *A. platensis* and *T. erythraeum*, the *patS* gene and *hetR* genes form a cluster and are separated by 459 bp and 1284 bp, respectively, with no other ORF detected in the intergenic regions. In *Anabaena* 7120 and *A. variabilis*, these two genes are located on opposite DNA strands and are separated by about 50 kb. In *N. punctiforme*, they are located very far away from each other, at a distance of about 4.5 Mb.

While this paper was in preparation, several partially sequenced cyanobacterial genomes became available. In

three of them, namely *Lyngbya* sp. PCC 8106, *Microcoleus chthonoplastes* PCC 7420 and *Synechococcus* sp. PCC 7335, ORFs similar to both *hetR* and *patS*, clustered on the chromosome similarly as in *Arthrospira platensis*, could also be identified. The putative *hetR* gene and the putative *patS* gene are separated by 435 bp, 277 bp and 401 bp in *Lyngbya* sp. PCC 8106, *Microcoleus chthonoplastes* PCC 7420 and *Synechococcus* sp. PCC 7335, respectively (data not shown). While both *Lyngbya* sp. PCC 8106 and *Microcoleus chthonoplastes* PCC 7420 are filamentous, non-diazotrophic strains, the position of *Synechococcus* sp. PCC 7335 in the classification of cyanobacteria is still ambiguous (Table 1). Even though *Synechococcus* sp. PCC 7335 is considered as a unicellular strain, its 16S rRNA sequence belongs to a lineage of filamentous strains (Honda *et al.*, 1999; Wilmotte & Herdmann, 2001).

DISCUSSION

The *hetR* gene has been extensively studied in *Anabaena* 7120 as a key regulator of heterocyst development. The presence of *hetR* in all filamentous strains examined in this study gives the possibility that it might have been laterally transferred from heterocyst-forming cyanobacteria. However, this is not supported by phylogenetic analysis and the fact that its presence among filamentous strains

Fig. 5. (a) PatS or PatS-like sequences deduced from the genomes of several filamentous cyanobacteria (*Np*, *Nostoc punctiforme*; *A7120*, *Anabaena* 7120; *Av*, *Anabaena variabilis*; *Ap*, *Arthrospira platensis*; *Te*, *Trichodesmium erythraeum*). The last five residues conserved among the sequences are in bold. (b) Relative location of *patS* and *hetR* on the chromosomes of different strains.

appears to be general rather than exceptional. Consistently, the monophyletic clade of heterocyst-forming cyanobacteria as based on HetR sequences also suggests that the *hetR* gene originated before the divergence of heterocyst-forming and non-heterocyst-forming cyanobacteria but at or after the time of the appearance of filamentous cyanobacteria, because *hetR* is not found in any of the unicellular cyanobacteria so far examined. The only exception is *Synechococcus* PCC 7335, which appears to be more related to filamentous strains than unicellular strains based the phylogenetic trees obtained with 16S rRNA sequences (Honda *et al.*, 1999; Wilmotte & Herdmann, 2001). A recent study suggests that heterocyst-forming cyanobacteria probably emerged between 2450 and 2100 Ma (Tomitani *et al.*, 2006), so the *hetR* gene may have originated at least before this lower time limit. Having experienced such a long period of evolutionary history, the HetR proteins remain highly conserved throughout the whole sequence, in particular those residues critical for various HetR functions (Risser & Callahan, 2007), implying that HetR must play a very critical role, not limited to heterocyst differentiation as earlier studies suggested (Buikema & Haselkorn, 1991).

We found that in several filamentous cyanobacteria the last five residues of a peptide encoded by a gene adjacent to *hetR* were identical to the functional pentapeptide of PatS. Three arguments support the idea that these genes correspond to *patS*. First, similar sequences were found in several filamentous cyanobacteria, often with similar genetic organization relative to *hetR*. Second, the *patS*-like sequence of *Arthrospira platensis* has heterocyst-suppressing activity when cloned in *Anabaena* 7120. Finally, *patS_{ar}* displayed a cell-type-specific expression in heterocysts in *Anabaena* 7120. These results suggest that the two alien genes, *hetR_{ar}* and *patS_{ar}*, can interact with their native partners in the control of heterocyst development.

In *Anabaena* 7120, the expression of *hetR* was upregulated after combined-nitrogen deprivation. In two non-heterocyst-forming diazotrophic strains *Trichodesmium* and *Symploca*, the expression of *hetR* also seemed to be affected by nitrogen status (El-Shehawey *et al.*, 2003; Janson *et al.*, 1998). We found that the level of HetR increased in *Arthrospira platensis* following combined-nitrogen removal. Unlike unicellular strains, filamentous cyanobacteria may need a certain degree of intercellular cooperation for a better control of nitrogen distribution along filaments under nitrogen-limiting conditions. The semi-regular distribution of nitrogen-fixing heterocysts along filaments in some modern cyanobacteria such as *Anabaena* 7120 may be considered as the best example of such intercellular interactions. One observation in support of such a hypothesis is that *Arthrospira platensis* could survive nitrogen limitation for at least a month, with most cells showing weak red fluorescence indicative of extensive pigment degradation, while a few single cells along the filament retained a high level of pigment fluorescence (data not shown). This result could imply that a filament may

have evolved a mechanism to ensure the survival of at least some of the cells under adverse conditions. Since genetic manipulation of filamentous nondiazotrophic cyanobacteria remains difficult, whether *hetR* is involved in such a mechanism remains unknown.

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