Identification of a gene regulated by HetR, a master regulator of heterocyst differentiation, in the non-heterocyst-forming filamentous cyanobacterium Arthrospira platensis NIES-39

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Cyanobacteria are a morphologically and physiologically diverse group of bacteria, which contains unicellular and multicellular filamentous strains. Some filamentous cyanobacteria, such as Anabaena sp. strain PCC 7120, form a differentiated cell called a heterocyst. The heterocyst is a specialized cell for nitrogen fixation and is differentiated from a vegetative cell in response to depletion of combined nitrogen in the medium. In Anabaena PCC 7120, it has been demonstrated that hetR, which encodes a transcriptional regulator, is necessary and sufficient for heterocyst differentiation. However, comprehensive genomic analysis of cyanobacteria has shown that hetR is present in non-heterocyst-forming cyanobacteria. Almost all filamentous cyanobacteria have hetR, but unicellular cyanobacteria do not. In this study, we conducted genetic and biochemical analyses of hetR (NIES39_C03480) of the non-heterocyst-forming cyanobacterium Arthrospira platensis NIES-39. HetR of A. platensis was able to complement the hetR mutation in Anabena PCC 7120 and recognized the same DNA sequence as Anabaena HetR. A search of the A. platensis genome revealed the HetR-recognition sequence within the promoter region of NIES39_O04230, which encodes a protein of unknown function. Expression from the NIES39_O04230 promoter could be suppressed by HetR in Anabaena PCC 7120. These data support the conclusion that NIES39_O04230 is regulated by HetR in A. platensis NIES-39.
the sequence GG(N3)CC, which is necessary for the DNA binding of HetR. The HetR-binding affinity to the hetP promoter, which contains the expanded inverted repeat sequence GAGGGGT(N3)ACCCCTC, is the highest of all known HetR-binding sites (Videau et al., 2014).

The patS gene was discovered from the observation of multicopy suppression of heterocyst differentiation in Anabaena (Yoon and Golden, 1998). The patS mutant shows an aberrant heterocyst pattern with shorter heterocyst spacing and multiple contiguous heterocysts (Mch). PatS is a small protein of no more than 17 amino acids, and exogenous addition of the C-terminal five amino acids of PatS (PatS-5 peptide; RGSGR) prevents heterocyst formation. The PatS-5 peptide directly binds to HetR to inhibit its DNA binding (Feldmann et al., 2011; Huang et al., 2014). These observations support the idea that HetR function is related to the multicellular lifestyle of filamentous cyanobacteria; this is obvious in the case of heterocyst-forming cyanobacteria. Hence, understanding the functions of HetR in non-heterocyst-forming filamentous cyanobacteria would lead to identification of the underlying mechanism of the multicellular phenotype in cyanobacteria. In this study, we identified a gene regulated by HetR (NIES39_03480) in A. platensis NIES-39. HetR of A. platensis (HetR<sub>ana</sub>) recognized the same inverted repeat sequence as HetR of Anabaena (HetR<sub>ana</sub>), and its DNA binding was prevented by PatS-5. The HetR-recognition sequence was found upstream of NIES39_004230 (hereafter 004230) in the A. platensis genome, and the expression of 004230 was changed in a HetR-dependent manner, suggesting that HetR regulates the expression of 004230 in A. platensis NIES-39.

Materials and Methods

Bacterial strains and culture conditions. Anabaena sp. strain PCC 7120 and its derivatives were grown in BG-11 medium as described previously (Ehira and Ohmori, 2006). The strain DRhetRS (Ehira and Ohmori, 2012a) was used as the hetR mutant in this study. Spectinomycin and neomycin were added to the medium at 10 and 30 µg/ml, when required.

Mutant construction. Primers used in this study are listed in Table 1. A DNA fragment containing the hetR<sub>ana</sub> promoter and coding regions was amplified by PCR using the primer pair hetR-uF and hetR-RB and cloned into the EcoRV site of pBluescript II KS+ to express hetR<sub>ana</sub> in non-heterocyst-forming filamentous cyanobacteria. The cloned fragment was excised by digestion with BamHI and inserted into the BamHI site of pSU101 (Ehira et al., 2017) to construct pSU101-hetR<sub>ana</sub>. A plasmid for the HetR<sub>ana</sub> expression in Anabaena was constructed as follows: the hetR<sub>ana</sub> promoter region was amplified by PCR using the primer pair InFhetR-5F and InFhetR-5R, and the hetR<sub>ana</sub> coding region was amplified by PCR using the primer pair InFarphetR-F and InFarphetR-R. These two fragments were cloned into the EcoRV site of pBluescript II KS+ to express hetR<sub>ana</sub> from the hetR<sub>ana</sub> promoter. The assembled gene was transferred into the Anabaena strain PCC 7120 and its derivatives.

Table 1. Primers used in this study.

| Primer   | Sequence (5’-3’)            |
|----------|-----------------------------|
| hetR-uF  | TGGCAATGCGAAGGTTAAA         |
| hetR-RB  | TAGGATCCTGACCTGAAAGATGTTTCTC |
| InFhetR-5F| CTCGAGGAATTCGATGGCCAAATGCGAAAGGTTAAA |
| InFhetR-5R| ATTAAAGTTAGTGAATGTTGAAATTC |
| InFarphetR-F| TCAACTATTGTAATGTAATGGAAGATGACAGATCTG |
| InFarphetR-R| ATCGATAAAGCTTGGCAGACATGACAGATCTG |
| PO04230-F2  | AAGGATCCCTATCGAATCTTGGGTAAATCC     |
| PO04230-R2  | AAGGATCCCTATCGAATCTTGGGTAAATCC     |
| HetR-F    | GGCATATGAGTAAAGACATGACATGTTG |
| HetR-R    | CAGATTTGCGGATAGTACAGATCTG |
| arpHetR-F | GCCATATGAAAAAAGTTAGTACAGATCTG |
| arpHetR-R | GCCATATGCCCAACTCCAGAAGCTTTC |
| PhetP-F    | TTTGAGGATTTATGTTGCAAC     |
| PhetP-R    | TTTGAGGATTTTCTACTAGG     |
| PO04230-F | TCGTGTCTTGGTATGAGTTCGCTCA |
| PO04230-R | TGGTTTATTATGTTGCGCTTAGT |
| Cs3-M13-F  | TTGTAAAACGACGGCCAGTG |
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Fig. 1. Heterocyst differentiation in the *Anabaena* strain expressing hetR<sub>arp</sub>. Heterocyst differentiation was induced by transferring filaments grown with nitrate to a nitrogen-free medium, and then filaments were incubated for 24 h with or without PatS-5. The upper panels are the *Anabaena* strain expressing hetR<sub>arp</sub> (SRhetR<sub>arp</sub>) and the lower panels are the strain complemented by hetR<sub>ana</sub> (SRhetR<sub>ana</sub>). PatS was added to the medium in the amount indicated under each photo. Arrow heads indicate heterocysts.

Complementation of the hetR mutation in *Anabaena* by hetR from *A. platensis* NIES-39

We examined whether hetR of *A. platensis* NIES-39 (hetR<sub>arp</sub>) was able to complement the hetR mutation of *Anabaena*. The hetR<sub>arp</sub> gene that was expressed from the promoter of *Anabaena* hetR (hetR<sub>ana</sub>) was inserted into the hetR locus on the chromosome of the hetR mutant to construct strain SRhetR<sub>arp</sub>. Because hetR<sub>arp</sub> was inserted by single homologous recombination, the entire plasmid sequence was also integrated into the chromosome. Hence, a control strain SRhetR<sub>ana</sub> was generated by returning hetR<sub>ana</sub> to the hetR mutant. SRhetR<sub>arp</sub> formed heterocysts after 24 h of nitrogen deprivation, but it showed an increased frequency of heterocysts and an abnormal pattern (Fig. 1). Heterocyst frequency was higher in SRhetR<sub>arp</sub> (13.6%) than SRhetR<sub>ana</sub> (5.6%), and 19.9% of heterocysts in SRhetR<sub>arp</sub> were Mch, while no Mch was observed in SRhetR<sub>ana</sub>. The patS mutant gives rise to Mch phenotypes, and heterocyst formation is inhibited by exogenously added PatS-5, the C-terminal pentapeptide of PatS (Yoon and Golden, 1998). In the wild-type *Anabaena* strain, heterocysts were not observed in the presence of 0.1 µM PatS-5. The C-terminal pentapeptide of PatS (Yoon and Golden, 1998). In the wild-type *Anabaena* strain, heterocysts were not observed in the presence of 0.1 µM PatS-5.
The addition of 0.1 µM PatS-5 inhibited heterocyst formation in SRhetRana, but not in SRhetRarp (Fig. 1). A ten-fold higher concentration of PatS-5 (1 µM) was required for inhibition of heterocyst formation in SRhetRarp (Fig. 1). These results indicate that hetRarp can regulate heterocyst differentiation in Anabaena and that HetRarp is less sensitive to inhibition by PatS.

Identification of a HetR-regulated gene in A. platensis
HetRana recognizes the inverted repeat sequence GAGGGGT(N3)ACCCCTC, which is present within the hetP promoter of Anabaena (Higa and Callahan, 2010). We analyzed the interaction between HetRarp and the hetP promoter. Gel mobility shift assays were conducted using purified His-HetRarp and a DNA probe of the hetP promoter region. HetRarp bound to the hetP promoter in the same way as HetRana (Fig. 2A). Moreover, interaction between HetRarp and the hetP promoter was prevented by the addition of the PatS-5 peptide, indicating that this peptide inhibited the DNA-binding activity of HetRarp (Fig. 2B). These results suggest that HetRarp also binds to the inverted repeat sequence recognized by HetRana. Because PatS-5 (data not shown). The addition of 0.1 µM PatS-5 inhibited heterocyst formation in SRhetRarp, but not in SRhetRana (Fig. 1). A ten-fold higher concentration of PatS-5 (1 µM) was required for inhibition of heterocyst formation in SRhetRarp (Fig. 1). These results indicate that hetRarp can regulate heterocyst differentiation in Anabaena and that HetRarp is less sensitive to inhibition by PatS.

Fig. 2. Interaction between HetRarp and the hetP promoter region of Anabaena.
A. DNA-binding assays of HetR protein. A Cy3-labeled probe (3 nM) including the hetP promoter region was mixed with HetRarp or HetRana in the amount indicated above each lane, and the mixtures were subjected to electrophoresis. B. PatS-5 inhibition of the HetR-DNA interaction. PatS-5 was added to the mixtures of HetR and the probe in the amount indicated above each lane. Open arrows, probe alone; closed arrows, complex with HetR and the probe.

Fig. 3. Interaction between HetRarp and the 004230 promoter region of A. platensis.
A. Schematic representation of the 004230 upstream region. The HetR-recognition sequence is located from -170 to -186 with respect to the translation start site of 004230. A black line (-66 to -345) indicates a DNA probe used in gel mobility shift assay. B. A Cy3-labeled probe (3 nM) including the 004230 promoter region was mixed with HetRarp or HetRana in the amount indicated above each lane, and the mixtures were subjected to electrophoresis. Open and closed arrowheads indicate the positions of free probes and the complexes of HetR and DNA probes, respectively.

Fig. 4. Expression of the 004230-lacZ transcriptional fusion gene in Anabaena.
Filaments of the wild-type strain (A) and the hetR mutant (B) of Anabaena containing a plasmid harbouring the 004230-lacZ fusion were grown with nitrate (0 h), and were subjected to nitrogen deficiency for 24 h and 48 h. The upper panels show light micrographs and the lower panels show corresponding fluorescence micrographs.
A. platensis does not have the hetP gene, we searched the genomic sequence of A. platensis NIES-39 for the HetR-recognition sequence using an ultrafast search engine for nucleotide sequences, GGGenome (http://gggenome.dbcls.jp/en/). We found the sequence GAGGGGT(N3)ACCCCTC in the A. platensis genome located at 170 bp upstream of the O04230 gene (Fig. 3A). Both HetRana and HetRarp bound to the upstream region of O04230 (Fig. 3B). Two forms of the HetRarp-DNA complex (C1 and C2) were detected, while HetRana only formed the C1 complex. These data indicate the presence of two HetRarp-binding sites in the upstream region of O04230, one of which includes the consensus sequence and is recognized by both HetRarp and HetRana. HetRarp could bind to a sequence other than the consensus sequence, because we found no sequence similar to the consensus sequence within the upstream region of O04230.

To reveal whether the expression of O04230 was regulated by HetR, a transcriptional fusion gene between lacZ and the O04230 promoter was produced. A plasmid harboring this fusion gene was introduced into the wild-type strain and the hetR mutant of Anabaena. Expression from the O04230 promoter was determined by observing the fluorescence from the β-galactosidase-cleaved product of the substrate C12-FDG. When the Anabaena strains were grown with nitrate, β-galactosidase activity was hardly detected (Fig. 4). In the hetR mutant, however, increased fluorescence was observed by transferring filaments to a nitrogen-free medium, while fluorescence intensity was not changed in the wild-type strain, suggesting that HetR can repress the expression from the O04230 promoter.

Discussion

The hetR gene of A. platensis HZ01, which encodes a protein 99% identical with HetRarp, is capable of complementing the hetR mutation of Anabaena (Zhang et al., 2009), but HetR of non-heterocyst-forming cyanobacteria has not been biochemically characterized. Here, in vitro biochemical analyses show that HetRarp recognizes the inverted repeat sequence GAGGGGT(N3)ACCCCTC and that PatS-5 inhibits the DNA binding of HetRarp as well as that of HetRana (Figs. 2 and 3). Biochemical characteristics of HetR appear to be conserved in both heterocyst-forming and non-heterocyst-forming cyanobacteria, as evident from the results of complementation studies of the hetR mutant by hetRarp (Fig. 1). However, a few notable differences in phenotypes between SRhetRarp and SRhetRana were observed. SRhetRarp formed more heterocysts and Mch than SRhetRana, and exogenous PatS-5 was less effective in inhibiting heterocyst formation in SRhetRarp. These data suggest that HetRarp is less sensitive to PatS-5 than HetRana, patX (NIES39_C03490), which is referred to as patS in Zhang et al. (2009), is located upstream of hetR on the genome of A. platensis and inhibited the HetR function in Anabaena, suggesting that HetR would be regulated by PatX in A. platensis. The amino acid sequences identity between HetRarp and HetRana is 76%. Six amino acid residues (L252, E253, E254, D256, D270, and D278) of HetRana are involved in binding to PatS-6 peptide (ERGSGR) (Hu et al., 2015). All of these amino acid residues are conserved in HetRarp, and inhibition of the DNA-binding activity of HetRarp by PatS-5 was more sensitive than HetRana (Fig. 2). Hence, the lower sensitivity of SRhetRarp to PatS-5 might result from tolerance to degradation by hitherto unidentified proteases that are promoted by PatS-5 (Risser and Callahan, 2009). Alternatively, HetRarp might affect the expression of genes that were not regulated by endogenous HetR in Anabaena. Within the O04230 promoter, there was an additional HetRarp-binding site, other than the consensus sequence, in which HetRana did not bind (Fig. 3). Hence, HetRarp may bind to extra sites on the Anabaena genome, resulting in the enhancement of heterocyst formation. Amino acid residues that participate in binding to the consensus sequence were identified in HetR of Fischerella sp. MV11 (Kim et al., 2013) and all of them are conserved in HetRarp. Identification of the additional HetRarp-binding sequence would further understanding of DNA-binding mode of HetR.

We identified a gene, O04230, that had the HetR-binding site within its promoter region in A. platensis NIES-39. Expression from the O04230 promoter in Anabaena was affected by the presence of HetR (Fig. 4), suggesting HetR-dependent regulation of O04230. In A. platensis HZ01, the HetR level is highest 48 h after nitrogen deprivation (Zhang et al., 2009) and expression from the O04230 promoter increased after nitrogen deprivation in the hetR mutant of Anabaena. Thus, in A. platensis, the O04230 expression would repress by HetR under nitrogen-deprived conditions. The function of the protein encoded by O04230 is unknown. The O04230 protein is composed of 303 amino acids and is annotated as a hypothetical protein having an alpha/beta hydrolase family domain. O04230 orthologues are widely distributed in cyanobacteria, including heterocyst-forming and unicellular strains. An orthologue in Anabaena, alr1709, does not respond to nitrogen deprivation (Ehira and Ohmori, 2006), and the HetR-recognition sequence has not been found within its promoter region by genome-wide analysis of the HetR-binding site (Videau et al., 2014). In addition, in the unicellular cyanobacterium Synechocystis sp. PCC 6803, which does not have hetR, expression of sil0553, an O04230 orthologue, is not changed after nitrogen depletion (Osanai et al., 2006). Regulation of the O04230 expression by HetR under nitrogen-deprived conditions might be specific to non-heterocyst-forming filamentous cyanobacteria. We are now trying to identify genes regulated by HetR in other non-heterocyst-forming filamentous cyanobacteria.

Considering that hetP is absent in the A. platensis genome, the HetR regulon is likely to be quite different between non-heterocyst-forming and heterocyst-forming cyanobacteria. To reveal the physiological functions of HetR in non-heterocyst-forming cyanobacteria, including A. platensis, more research is needed to expand the HetR regulon by further identifying HetR-binding sites in the genome. Moreover, development of a genetic system for A. platensis would facilitate such a physiological study.
Acknowledgments

This work was supported by the Japan Society for the Promotion of Science (JSPS) [Challenging Research Exploratory 19K22290] and the Nagase Science and Technology Foundation to S.E. The authors would like to thank Enago (www.enago.jp) for the English language review.

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