Regulation by hetC of Genes Required for Heterocyst Differentiation and Cell Division in Anabaena sp. Strain PCC 7120

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Unlike those of the wild-type strain, proheterocysts of the Anabaena sp. strain PCC 7120 hetC strain keep dividing. ftsZ, the most critical cell division gene, is up-regulated in hetC proheterocysts. Heterocyst differentiation genes hglD, hglE, patB, nifB, and xisA are no longer expressed in the hetC mutant. hetC also regulates the expression of patA, a pattern formation gene.

Anabaena sp. strain PCC 7120, a filamentous nitrogen-fixing cyanobacterium, is one of the research models for prokaryotic cell differentiation and pattern formation. Its specialized nitrogen-fixing cells, called heterocysts, are semiregularly distributed along filaments. In the process of differentiation from a vegetative cell to a heterocyst, cell division is stopped, an envelope consisting of outer polysaccharide layer and inner glycolipid layer is formed, photosynthetic oxygen production no longer takes place, and respiration is remarkably intensified (37). Due to the degradation of phycobilisomes, the phycobiliprotein fluorescence is basically missing in heterocysts (1).

Many genes involved in heterocyst differentiation have been identified. The master regulatory gene hetR (5) encodes a DNA-binding protein that probably recognizes the promoter regions of itself and other heterocyst differentiation genes (19). hetC encodes an ABC peptide or protein transporter (22), whose expression is regulated by ntcA, another DNA-binding protein gene (29), and hetR (22). A hetC mutant is unable to stop cell division of proheterocysts (38). In wild-type mature heterocysts, the expression of essential cell division protein FtsZ is substantially reduced (23), suggesting that cell division genes could be regulated during the process of differentiation. A gene cluster containing hepA and hepC (18, 42), and hepB (37), a separately located gene, are required for the formation of the envelope polysaccharide layer. The expression of hepA has been shown to be dependent on a two-component signal transduction system, HepK/DevR (41), and a histidine kinase gene, alm0117 (30). htm, hglCD, and hglE (2, 4, 8, 30) and an operon, devBCA (14, 15), are involved in the formation of glycolipid layer. For cytoplasmic differentiation, coxBACI and coxBACII are the two operons encoding terminal oxidases of the respiratory chain (20, 32), and patB is a regulatory protein gene that affects the nitrogen-fixing capability of heterocysts (21, 25). In the nif region of Anabaena sp. strain PCC 7120, nifB-fdxN-nifS-nifU and nifHDRK are two independently transcribed operons (36). Three DNA rearrangements take place at a late stage of heterocyst development, including two within fdxN and nifD, respectively (16). xisA encodes an excisase required for the removal of an 11-kb DNA element from nifD (17), patA (24), patS (39), and hetN (4, 7) play important roles in heterocyst pattern formation or maintenance. Assisted by green fluorescent protein (GFP) or the luxAB reporter gene, hetR (3, 5), hetC (38), hepA (35), hepK (41), devBCA (26), nifH (12), coxBACII (20), patB (21), patS (39), and hetN (7) have been shown to be expressed exclusively or mainly in heterocysts and proheterocysts, while ccbP, a calcium-binding protein gene, is down-regulated in heterocysts (40).

Bacterial cell division is dependent on a complex macromolecular machinery assembled at the division site (13). FtsZ, the most critical component of the division machinery, is a tubulin-like GTPase and can polymerize to form a ring-like structure, to which other components are recruited sequentially. In Escherichia coli, a min system consisting of MinC, MinD, and MinE participates in selection of the division site. MinC interacts with FtsZ and prevents polymerization of FtsZ or interaction of other cell division proteins with the FtsZ ring. SulA, an SOS-induced inhibitor, halts cell division by binding to FtsZ on its GTP-hydrolyzing surface (9). Among the proteins that join the FtsZ ring are FtsA, ZipA (ZapA), FtsEX, FtsK, FtsQ, FtsL/FtsB, FtsW, FtsI, FtsN, AmiC, and EmV (33). The counterparts of many cell division genes in E. coli or Bacillus subtilis have been predicted to reside in Anabaena sp. strain PCC 7120 (28). Employing a shuttle vector carrying a GFP reporter gene, we systematically investigated the role of hetC in regulation of gene expression in heterocyst differentiation and cell division, directly showing its function in controlling cell division, envelope formation, and cytoplasmic differentiation.

Methods. Anabaena sp. strain PCC 7120 was obtained from the Freshwater Algae Culture Collection of Institute of Hydrobiology, Chinese Academy of Sciences. The wild-type and hetC mutant strains were grown, selected, and induced to differentiate as described elsewhere (30). Plasmids were conjugatively transferred into Anabaena sp. strain PCC 7120 or a...
hetC mutant strain (11). A hetC::C.CE2 mutant was generated by positive selection using sacB (6) and confirmed by PCR (data not shown). Fluorescence and bright-field images were captured through an Olympus BX41 microscope with a JVC3 charge-coupled device color video camera (TK-C1381) (Victor Company of Japan, Limited) assisted by the software 10moons-2000/PRO (version 4.5). The shutter speed was set at 1/50 s and automatic gain control gain at 18 dB. Images for GFP expression were visualized using a Sapphire GFP filter set (Exciter D395/40, dichroic 425DCLP, and emitter D510/40) (Chroma Technology Corp., Brattleboro, Vt.). A total of 20 to 30 heterocysts or proheterocysts were observed for each gene, all showing consistent results except where indicated otherwise.

In order to generate the hetC::C.CE2 mutant, plasmid pHB1281 was constructed as follows. A genomic region containing the hetC gene was amplified in vitro by PCR using primers hetC-11 and hetC-21 (Table 1) and cloned into pMD18-T (Takara) vector. A DNA fragment containing a C.CE2 (Emr/Cmr) cassette was excised from pRL598 (4) with BamHI, blunted with T4 DNA polymerase, and inserted into hetC, replacing a 1-kb EcoRV fragment. The hetC::C.CE2 fragment was excised with XbaI and PstI, blunted with T4 DNA polymerase, and subcloned into PstI, blunted with T4 DNA polymerase, site of pRL278 (3).

The GFP reporter plasmid pHB912 was constructed on the basis of the use of pRL25C (34) as follows: a promoterless gfp was amplified from pBADgfp (10) by PCR using primers gfpcyc3-3 and gfpcyc3-2 (Table 1) and cloned into pMD18-T, resulting in pHB775. A DraI fragment containing /H9024 cassette (Spr/Smr) from pRL57 (6) was inserted into the SmaI site of pHB775, resulting in pHB828. The /H9024-gfp fragment was excised with EcoRI-PstI from pHB828, blunted with T4 DNA polymerase, and cloned into the BglII site, blunted with T4 DNA polymerase, of pHB774, which is a derivative of pRL25C with BamHI-EcoRI fragments deleted, resulting in pHB912 (Fig. 1). The DNA fragments containing different promoters were generated with PCR using the primers listed in Table 1, cloned into pMD18-T, and confirmed by sequencing. In order to subclone the promoters directionally into pHB912, we introduced BamHI-cut pUC19 into the BglII site of pHB912.

![FIG. 1. The structure of the promoter-probing vector pHB912. The start codon of promoter-less gfp is in bold face. The underlined triad bases are the three stop codons in different potential reading frames to keep from a translational fusion with the gfp reporter gene. The stem-loop symbols at both ends of the Ω cassette indicate the short inverted repeats that terminate background transcription (31).](http://jb.asm.org/)}
producing two plasmids with pUC19 in different respective orientations, and then removed the pUC19 portion with SmaI and Sse8387I, resulting in linear DNA of pHB912 derivatives with SmaI and Sse8387I ends in different orientations. The cloned promoters were excised from pMD18-T vector with SmaI and Sse8387I and cloned into the corresponding derivatives of pHB912.

**Investigation of gene regulation during heterocyst differentiation with a replicative plasmid containing GFP reporter gene.** We cloned the promoter regions of *hetR*, *hetC*, *hepB*, *hepK*, *devB*, *hglD*, *hglE* (*alr5351*) (see reference 30), *hetN*, *patS*, *patA*, *patB*, *xisA*, *nifB*, *coxBII*, and *rbcL* into the shuttle vector pHB912, respectively, introduced the resulting plasmids into *Anabaena* sp. strain PCC 7120, and found that *rbcL* is down-regulated while the others are expressed exclusively in heterocysts or are up-regulated in heterocysts relative to vegetative cells (Fig. 2 and unpublished data). The results seen with *hetR*, *hetC*, *hepK*, *devB*, *hglE*, *hetN*, *patS*, *patB*, *coxBII*, and *rbcL* indicated that the detection system is reliable for the study of gene regulation during heterocyst differentiation. Among these genes, *hepB*, *hglD*, *xisA*, *nifB*, and *patA* were shown for the first time to be up-regulated in heterocysts. Detection of the pro-
moter of hep4 with this vector, however, produced a marginally visible GFP signal in heterocysts (data not shown). Using this system, we also detected the expression of predicted cell division genes ftsE (alr1706), ftsW (all0154), ftsX (all1757), ftsY (all1759), ftsZ (alr3858), minCDE (alr3455-alr3456-asr3457), sulA (all2390), all2033, and all2797, and found that after 24 h of nitrogen deprivation, ftsZ is down-regulated, sulA is up-regulated, and other genes are not regulated in heterocysts (Fig. 2 and unpublished data). Unlike the results seen with E. coli, ftsX is separately located with ftsY and ftsE in Anabaena sp. strain PCC 7120, and between ftsY and ftsE is all1758, a gene of unknown function. The Anabaena minC, minD, and minE form a cluster, as seen with E. coli. We tested ftsE, ftsX, and ftsY as separate genes but minCDE as an operon. all2033 and all2797 are the other two genes similar to minC. Our results suggested that the transition of cell division state of proheterocysts is controlled by two joint forces, decrease of FtsZ and increase of its inhibitor SulA, so as to ensure an immediate arrest of cell cycle. A previous detection of FtsZ expression with Western blotting (23) produced results consistent with ours. It would be interesting to know whether the Anabaena SulA is also SOS induced and, if so, the relations between heterocyst development and the SOS system.

**Regulation of heterocyst-specific genes by hetC.** A hetC mutant was constructed by replacing a 1-kb EcoRV fragment of hetC with the C.CE2 cassette from pRL598 (4). Plasmids containing promoters of heterocyst development genes were introduced into the hetC::C.CE2 strain, and expression of GFP from these promoters was observed after 24 and 48 h of nitrogen deprivation and, for the promoters of hepB and devB, also after 12 h. As seen with the wild-type strain, hepB, hepK, devB, patS, hetN, and coxBII were expressed in semiregularly distributed proheterocysts of the hetC mutant, while hglD, hglE, patB, nifB, and xisA were not expressed in those smaller dividing cells (Fig. 2). Expression of PnifB-gfp in Anabaena sp. strain PCC 7120 alr0117::Tn5-1087b (30), a mutant that lacks the polysaccharide layer of the heterocyst envelope, showed that expression of nifB was not regulated by alr0117 (Y. Wang and X. Xu, unpublished) or, by inference, oxygen. Therefore, it was considered unnecessary to observe the influence of hetC on nifB under anaerobic conditions. Among the genes responsible for the formation of the polysaccharide layer, hepA (38) depends on hetC for expression, and expression of hepB is delayed in a hetC mutant; and among the genes responsible for the formation of the glycolipid layer, hglD and hglE depend on hetC for expression, and expression of devB is delayed in a hetC mutant. Genes patB, xisA, nifB, and coxBII are required for cytoplasmic differentiation and up-regulated late in heterocyst differentiation. Again, patB, xisA, and nifB but not coxBII depend on hetC for expression. nblA, a gene required for degradation of phycobilisome (1), was evidently expressed in hetC proheterocysts, as shown with the loss of phycobiliprotein fluorescence (Fig. 2). The pattern formation or maintenance genes patS and hetN showed expression patterns in the hetC strain that were similar to those seen with the wild type. However, another pattern formation gene, patA, was not up-regulated in hetC proheterocysts but was apparently down-regulated in whole filaments of the mutant (Fig. 2). We found low-level expression of GFP in about 80% of proheterocysts and sporadically in vegetative cells of the hetC mutant. Slight differences in GFP expression from PpatA were also noticeable among vegetative cells of the wild type. Although essential to the cessation of cell division during heterocyst differentiation, hetC regulates only some of genes involved in heterocyst maturation. Those genes not regulated by hetC, like devBCA, may depend on NtcA (15) or other factors to be expressed specifically in heterocysts.

**Regulation of cell division genes by hetC during heterocyst differentiation.** Previous studies showed that hetC may play an important role in the transition of cell division state. Using the GFP detection system, we examined expression of cell division genes in a hetC background and found that unlike the results seen with the wild type, ftsZ is up-regulated in proheterocysts (Fig. 2). Consistent with such a reverse regulation, hetC proheterocysts continue to divide even more actively than vegetative cells. In the hetC mutant, however, about 70% of proheterocysts showed expression of sulA comparable to that in wild-type heterocysts. Therefore, the regulation of sulA may not be affected by hetC (Fig. 2). In addition to ftsZ, certain other components of the cell division machinery may also be regulated during the transition of cell division state. One can imagine that a higher level of certain counteracting factors for SulA, such as a protease, could be an additional element affecting the phenotype of a hetC mutant. We hypothesize that up-regulation of hetC at an early stage of heterocyst differentiation may indirectly affect the expression of ftsZ and possibly other cell division genes, enabling presumptive proheterocysts to develop into heterocysts.

As in most other processes of prokaryotic cell differentiation, a presumptive proheterocyst in Anabaena sp. strain PCC 7120 stops dividing before initiating heterocyst differentiation. In the previous report, a hetC mutant was deprived of nitrogen for 2 days and transferred to setting of weaker light for prolonged nitrogen step down, and differentiating cells were found to be dividing and less autofluorescent (38). The hetC phenotype was interpreted as loss of function in the transition of cell dividing state. However, such a phenomenon could also be explained as being dependent on the special conditions. In this study, we examined the expression of many heterocyst development genes in a hetC background without any special con-
ditions and demonstrated that some of the genes are up-regulated in differentiating cells. These results provide further evidence to confirm that the smaller dividing cells of a hetC mutant are indeed proheterocysts. We also examined the expression of cell division genes in a hetC background and directly showed the regulation of fsZ by hetC. Evidently, the transition of cell division state in proheterocysts depends on hetC. Because cessation of cell division is the first sign of the onset of cell differentiation, it could be a trigger for the morphogenetic process. The results of this study, however, present two alternative perspectives: (i) cessation of cell division parallels other morphogenesis processes; (ii) the cell division state controls some genes involved in heterocyst differentiation. We tentatively summarize the regulatory effects of hetC on heterocyst differentiation in Fig. 3. Last, we note that the continued division of proheterocysts in a hetC mutant is not necessarily contradictory to the hypothesis (27) that cells that are competent to initiate heterocyst differentiation are in a critical stage of the cell cycle.

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REFERENCES


