Transcriptional dynamics of developmental genes assessed with an FMN-dependent fluorophore in mature heterocysts of *Anabaena* sp. strain PCC 7120

Patrick Videau, Reid T. Oshiro, Loralyn M. Cozy and Sean M. Callahan

Department of Microbiology, University of Hawaii, Honolulu, HI 96822, USA

*Anabaena* sp. strain PCC 7120 is a filamentous cyanobacterium that differentiates nitrogen-fixing heterocysts when available combined nitrogen is limiting. Growth under diazotrophic conditions results in a mixture of ‘new’ (recently differentiated) and ‘old’ (mature) heterocysts. The microoxic environment present in heterocysts makes the interpretation of gene expression using oxygen-dependent fluorophores, including GFP, difficult. The work presented here evaluates the transcriptional dynamics of three developmental genes in mature heterocysts utilizing EcFbFP, a flavin mononucleotide-dependent fluorophore, as the reporter. Expression of both GFP and EcFbFP from the heterologous petE promoter showed that, although GFP and EcFbFP floresced in both vegetative cells and new heterocysts, only EcFbFP floresced in old heterocysts. A transcriptional fusion of EcFbFP to the late-stage heterocyst-specific nifB promoter displayed continued expression beyond the cessation of GFP fluorescence in heterocysts. Promoter fusions of the master regulator of differentiation, hetR, and its inhibitors, patS and hetN, to GFP and EcFbFP were visualized to determine their role(s) in heterocyst function after morphogenesis. The expression of hetR and hetN was found to persist beyond the completion of development in most heterocysts, whereas patS expression ceased. These data are consistent with a model of heterocyst patterning in which patS is involved in *de novo* pattern formation, hetN is required for pattern maintenance, and hetR is needed for all stages of development.

INTRODUCTION

GFP and its derivatives have allowed for the study of gene expression at both the population and single-cell level. However, the oxygen dependence of GFP has stymied efforts to visualize transcriptional dynamics in anoxia (Tsien, 1998). An alternative to GFP is the FbFP [flavin mononucleotide (FMN)-based fluorescent protein] family of fluorophores that require FMN for maturation, a common metabolic oxidizing agent, rather than oxygen (Drepper *et al.*, 2007). One member of this family, EcFbFP, derived from the light oxygen voltage (LOV) domain of the YtvA protein from *Bacillus subtilis*, was codon-optimized for expression in *Escherichia coli*, and floresces in the blue spectrum in both aerobic and anaerobic conditions.

Studies have revealed several characteristics of FbFPs that make them appealing as fluorescent reporters. First, FbFPs are smaller proteins (~100–140 aa) than GFP-based fluorophores (~240 aa) (Mukherjee *et al.*, 2013). Second, EcFbFP was found to floresce within minutes of expression commensurate to cell density, whereas GFP and its derivatives displayed a 10–30 min delay in fluorescence that was not proportional to cell density (Drepper *et al.*, 2010). This delay could confound the interpretation of the timing of gene expression in certain experiments. Third, in work conducted on *E. coli* (Drepper *et al.*, 2010), *Saccharomyces cerevisiae* (Tielker *et al.*, 2009), *Candida albicans*, *Rhodobacter capsulatus* (Drepper *et al.*, 2007), *Porphyromonas gingivalis* (Choi *et al.*, 2011), *Bacteroides fragilis* (Lobo *et al.*, 2011), *Arabidopsis thaliana* (Chapman *et al.*, 2008) and mammalian cells (Walter *et al.*, 2012), FbFPs have proven to be suitable fluorophores in anoxia. To date, studies utilizing FbFPs have focused on either aerobic or anaerobic conditions. Here, we examine EcFbFP in an organism that consists of a heterogeneous population of both aerobic and anaerobic cell types simultaneously.

*Anabaena* sp. strain PCC 7120 (herein *Anabaena*) is a filamentous cyanobacterium that differentiates a periodic pattern of nitrogen-fixing heterocyst cells when starved for combined nitrogen (N−) (reviewed by Golden & Yoon, 2003; Wolk *et al.*, 1994). Heterocysts create a microoxic environment necessary for the oxygen-labile nitrogenase complex, encoded by the *nif* genes, to fix atmospheric...
dinitrogen. The formation of heterocysts takes about 24 h and is controlled by a cascade of regulators including hetR, the master regulator of differentiation, and its inhibitors, patS and hetN; the interactions of these three gene products define and maintain the pattern of vegetative cells destined to become heterocysts (Muro-Pastor & Hess, 2012). Placement of these regulators in spatial and temporal order is important for interpreting their role(s) during development. The hetR, patS and hetN genes are upregulated 0.5 (Black et al., 1993), 3 (Yoon & Golden, 1998) and 12 h (Bauer et al., 1997) after induction of differentiation, respectively, and expression studies with transcriptional fusions to GFP have shown localization to developing heterocysts after 6 (Black et al., 1993), 8 (Yoon & Golden, 2001) and 17 h N− growth (Callahan & Buikema, 2001), respectively. In contrast to the body of work elucidating transcriptional profiles of hetR, patS and hetN during development, the expression status of these genes following maturation has been difficult to study due to the low-oxygen environment within the heterocyst.

Here, we present evidence for the use of the EcFbFP fluorescent reporter in monitoring gene expression in the aerobic and anaerobic cell types produced by Anabaena. When the expression of EcFbFP was driven by the heterologous petE promoter, similar levels of fluorescence were observed in both vegetative and heterocyst cell types. A transcriptional fusion of the heterocyst-specific nifB promoter to EcFbFP persisted in heterocysts after the cessation of GFP fluorescence. Finally, whereas transcriptional fusions to the promoters of hetR and hetN displayed continued fluorescence in heterocysts after morphogenesis, the patS promoter was downregulated. This work demonstrates sustained expression of heterocyst-specific genes beyond development.

**METHODS**

**Bacterial strains and culture conditions.** Growth of E. coli and wild-type Anabaena sp. strain PCC 7120, induction of heterocysts in medium lacking combined nitrogen (N−), and the concentrations of antibiotics used in this study were as previously described (Borthakur et al., 2005; Higa & Callahan, 2010). Plasmids were conjugated from E. coli into Anabaena as previously described (Elhai & Wolk, 1988).

**Plasmid construction.** The strains and plasmids used in this study are listed in Table 1 and the primers used are listed Table 2. The integrity of all PCR-derived products was verified by DNA sequencing. Plasmid pPJAV213 is a mobilizable shuttle vector based on pAM504 (Wei et al., 1994) utilized for making transcriptional fusions to the copper-inducible petE promoter. The petE promoter region was amplified by PCR from chromosomal DNA with the primers PpetE-SacI-F and PpetE-EcoRI-Smal-BamHI-R. The product was cloned as a SacI–BamHI fragment into the same sites of pSMC187 (Higa et al., 2012).

Plasmid pPJAV216 is a source of the coding region of EcFbFP in the multiple cloning site of pBlueScript SK− (Stratagene). The coding region of EcFbFP was excised from pGLOW-KXXN-B1 (Evocaical) as an XbaI–BamHI fragment, blunt-ended and cloned into the Smal site of pBlueScript SK−.

**Table 1. Strains and plasmids utilized in this study**

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<th>Strain or plasmid</th>
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<td><strong>Anabaena sp. strains</strong></td>
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<td>PCC 7120</td>
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<td><strong>Plasmids</strong></td>
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<td>pBlueScript SK+</td>
<td>Cloning vector, AmR</td>
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<td>pGLOW-KXXN-B1</td>
<td>Source of EcFbFP, KmR CmR</td>
<td>Evocatal</td>
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<td>pAM504</td>
<td>Shuttle vector for replication in E. coli and Anabaena; KmR NmR</td>
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*AmR, Ampicillin; KmR, kanamycin; CmR, chloramphenicol; NmR, neomycin.*
Plasmid pPJAV222 is a mobilizable shuttle vector based on pAM504 to create transcriptional fusions to EcFbFP. The coding region of EcFbFP was amplified by PCR from pPJAV216 with the primers M13R and FbFP-BglII-R. The product was cloned as a BamHI–BglII fragment into the BamHI sites of pAM1956 (Yoon & Golden, 1998), thus replacing the coding region of GFP with the coding region of EcFbFP, and directionality was verified by PCR.

Plasmid pPJAV226 is a mobilizable shuttle vector based on pAM504 carrying EcFbFP transcriptionally fused to the petE promoter. The coding region of EcFbFP was amplified by PCR with the primers FbFP-Munl-F and FbFP-BglII-R. The product was cloned as a Munl–BglII fragment into the EcoRI–BamHI sites of pPJAV213.

Plasmid pPJAV294 is a mobilizable shuttle vector based on pAM504 carrying the nifB promoter transcriptionally fused to gfp. The nifB promoter was amplified by PCR from chromosomal DNA with the primers PnifB-I-F and PnifB-XhoI-R. The product was cloned into the Smal site of pAM1956, and directionality was verified by PCR.

Plasmid pPJAV295 is a mobilizable shuttle vector based on pAM504 carrying the nifB promoter transcriptionally fused to EcFbFP. The nifB promoter was amplified by PCR from chromosomal DNA with the primers PnifB-Xhol-F and PnifB-EcorV-R. The product was cloned into the Smal site of pPJAV222 and directionality was verified by PCR.

Plasmids pPJAV340, pPJAV341 and pPJAV343 are mobilizable shuttle vectors based on pAM504 carrying the hetR, hetN and patS promoters, respectively, transcriptionally fused to EcFbFP. The hetR, hetN and patS promoters were amplified by PCR from chromosomal DNA with the primer sets PhetR-BamHF and hetR-gfpR2, PhetN-BamHF and hetN-gfpR2, and PpatS-Munl-F and PpatS-OEX-R, respectively. The products were cloned into the Smal site of pPJAV222 and directionality was verified by PCR.

**RESULTS**

Fluorescence from EcFbFP is detectable in both vegetative and heterocyst cells

As a result of intrinsic fluorescence, the experimental design of studies in *Anabaena* and other photosynthetic organisms is often restricted to fluorophores and fluorescent dyes with peak emission spectra outside the range of the photosystem components. GFP is commonly utilized in gene expression studies in *Anabaena* as a transcriptional reporter. However, the microoxic environment within heterocysts is low in molecular oxygen that is necessary for proper folding of GFP, which can impair the interpretation of expression studies of genes involved in heterocyst function (Tsien, 1998). To assess the feasibility of a new tool for gene expression studies in both cell types produced by *Anabaena*, the gene for the FMN-dependent EcFbFP fluorophore was transcribed from the copper-inducible petE promoter (Buikema & Haselkorn, 2001) and blue fluorescence was visualized. Expression of EcFbFP from the petE promoter in the presence (N+) or absence (N−) of a source of combined nitrogen resulted in fluorescent vegetative cells and heterocysts; fluorescence was absent from cells harbouring a promoterless EcFbFP (Fig. 1a and data not shown). As with EcFbFP, GFP expressed from the petE promoter resulted in fluorescent vegetative cells, but, in contrast, many of the heterocysts fluoresced at a level no greater than cells harbouring a promoterless GFP after 168 h N− growth (Fig. 1a, b). Comparison of data from three independent trials of 100 heterocysts each showed a significant difference between GFP and EcFbFP reporters after 168 h N− growth (P=0.012; Fig. 1b). EcFbFP fluoresced in both *Anabaena* cell types irrespective of oxygen status, whereas fluorescence from GFP was limited to a subpopulation of heterocysts.
**Temporal transcription dynamics of developmental genes in mature heterocysts**

The *hetR*, *patS* and *hetN* genes play a central role in the cascade of events leading to cellular differentiation in *Anabaena*. The transcriptional profiles of all three genes have been extensively studied during the process of heterocyst formation but comparatively little is known about their regulation following heterocyst maturation. To determine whether expression of *hetR*, *patS* and *hetN* is maintained in heterocysts following morphogenesis, the promoter regions of these three genes were transcriptionally fused to EcFbFP and patterns of fluorescence were visualized. Transcription of *hetR*, *patS* and *hetN* localized to developing heterocysts roughly 8–18 h following induction of differentiation (Black *et al.*, 1993; Callahan & Buikema, 2001; Yoon & Golden, 1998). Transcriptional fusions of the *hetR*, *patS* and *hetN* promoters (P*hetR*, P*patS* and P*hetN*, respectively) to gfp and EcFbFP resulted in patterns of fluorescence localization similar to published accounts at 16–18 h following induction (Fig. 3) (Black *et al.*, 1993; Callahan & Buikema, 2001; Yoon & Golden, 2001). After 24 h N− growth, fluorescence from both the gfp and EcFbFP fusions to P*hetR*, P*patS* and P*hetN* was primarily localized to heterocysts and nearly all heterocysts counted were fluorescent (Fig. 4; P = 0.6595 for P*hetR*; P = 0.8020 for P*patS*; P = 0.6056 for P*hetN*). After 96 h N− growth, the percentage of fluorescing heterocysts dropped to half the levels of that observed after 24 h N− growth in cultures containing P*hetR*, P*patS* and P*hetN* fusions to gfp. The majority of heterocysts in strains containing P*hetR* and P*hetN* fusions to EcFbFP were fluorescent (Fig. 4a, c, d; P = 0.0085 for P*hetR*; P = 0.0087 for P*hetN*) but only half of the heterocysts fluoresced in the strain containing a P*patS* fusion to EcFbFP by 96 h of N− growth (Fig. 4b, d; P = 0.4444). The pattern of fluorescence for all reporter fusions at 16 h of N− growth was comparable to the pattern of fluorescence at the 96 h time point (data fusion localized to heterocyst cells (Wang & Xu, 2005). Nearly all heterocysts containing either a P*nifB–gfp* or –EcFbFP fusion were fluorescent after 24 h N− growth (Fig. 2c). By 168 h of N− growth, all of the heterocysts containing a P*nifB–EcfbFP* fusion fluoresced but only half of the heterocysts containing a P*nifB–gfp* fusion were fluorescent (P = 0.007). As *Anabaena* cells divide during N− growth, new heterocysts arise when the interval between older heterocysts is too great to effectively export combined nitrogen to all vegetative cells in the filament. GFP expressed in developing heterocysts is exposed to molecular oxygen before the microoxic environment is established. Therefore, we infer that the absence of GFP fluorescence in half of the heterocysts at 168 h N− growth was due to the lack of molecular oxygen required for folding rather than cessation of *nifB* transcription in old heterocysts. We conclude that *nifB* is transcribed in both new and old mature heterocysts and this transcription is accurately visualized from an EcfbFP promoter fusion.

**nifB is expressed in all mature heterocysts**

The *nifB* gene product is involved in the synthesis of the Fe–Mo cofactor required for functional nitrogenase (reviewed by Wolk *et al.*, 1994). Transcription of *nifB* is upregulated late in the differentiation process and a *nifB* promoter (P*nifB*) fusion to gfp showed that transcription localized exclusively to heterocysts (Flaherty *et al.*, 2011; Mulligan & Haselkorn, 1989; Wang & Xu, 2005). To assess late-stage heterocyst-specific gene expression, P*nifB* was transcriptionally fused to EcFbFP in a plasmid containing a promoterless EcFbFP (Fig. 2a), and heterocyst-specific fluorescence was visualized (Fig. 2b). Similar to published results from a gfp fusion, transcription from a P*nifB–EcfbFP*...
not shown). This suggests that patS is downregulated in mature heterocysts whereas transcription of hetR and hetN continues.

**DISCUSSION**

During growth in medium lacking a combined nitrogen source, Anabaena differentiates a periodic pattern of microoxic nitrogen-fixing heterocysts to provide nitrogen to vegetative cells in the interval between heterocysts (Fig. 5). This interval, however, is not of fixed length and increases as the vegetative cells divide. Cells at the midpoint between two functional heterocysts are starved of nitrogen; these cells are also the farthest away from sources of the PatS and HetN inhibitors, which allows an increase in HetR and, thus, the differentiation of a new heterocyst. This method of pattern maintenance ensures that sufficient combined nitrogen is available for continued growth. Therefore, the heterocysts that initially form in a filament have recently completed the differentiation process and can be regarded as ‘new’ heterocysts, but 24 h later after a second round of differentiation and thereafter, half of the heterocysts are 24 h or more ‘old’, while the other half, those that formed in the second round of differentiation are ‘new’. In newly formed heterocysts, GFP, or any oxygen-dependent fluorophore, produced before the establishment of the microoxic environment will continue to fluoresce for the lifetime of that protein molecule. Utilizing the P\textit{nifB–gfp} transcriptional fusion and taking into account doubling time during diazotrophic growth, the half life of GFP was determined to be roughly 18 h in heterocysts. This result is similar to data from Pseudomonas putida and E. coli, where the half life of GFP was estimated to be greater than 24 h (Andersen et al., 1998). Expression after the establishment of a microoxic environment will result in improperly
folded GFP and non-fluorescent heterocysts. The distinction between ‘new’ (recently differentiated) and ‘old’ heterocysts (>24 h old) is paramount in interpreting the results of GFP and EcFbFP in this study.

Characterization studies have shown that FbFPs are robust and versatile fluorophores capable of functioning in a wide range of conditions (Mukherjee et al., 2013). In Anabaena, EcFbFP fluoresced in aerobic vegetative cells and microoxic heterocysts with similar intensity when expressed from a heterologous promoter. When driven by the native nifB promoter, EcFbFP fluoresced in heterocysts and more accurately reflected PnifB transcription in mature heterocysts than an equivalent fusion with gfp. In a previous study, utility of the FbFP iLOV was demonstrated and used to characterize molecular transport in the periplasmic space of Anabaena (Zhang et al., 2013). These results, in addition to the work reported here, support the utility of FbFPs as fluorescent reporters for studies in Anabaena.

The hetR gene encodes the HetR protein, which is the master regulator of differentiation in Anabaena (Buikema & Haselkorn, 1991, 2001). HetR is known to interact with DNA, and has been proposed to bind three categories of binding site, one of which is suggested to be involved in diazotrophic metabolism (Videau et al., 2014). Mutation of one gene with a HetR binding site in the category related to diazotrophic metabolism in its promoter, alr1000, resulted in the development of morphologically distinct heterocysts that were only capable of fixing nitrogen in anoxic conditions. Similarly, ectopic expression of hetR in a ΔhetR mutant, which is normally incapable of differentiation, bypassed the mutation to form heterocysts only capable of anoxic nitrogen fixation (Higa & Callahan, 2010). These results suggest that, while morphogenesis can be forced in a ΔhetR mutant, HetR is necessary for heterocyst function and predicts that persistent expression of hetR is required in heterocysts for proper function.

The hetR gene is upregulated within 0.5 h after the removal of combined nitrogen, and transcripts of hetR localize to a pattern of single cells by 6 to 8 h of N− growth (Black et al., 1993). Furthermore, in Anabaena variabilis, a closely related heterocystous strain, hetR transcripts were abundant among developmental genes in mature heterocysts (Park et al., 2013). In the work presented here, transcriptional fusions of the hetR promoter with EcFbFP showed that fluorescence persisted in several generations of heterocysts, but GFP fluorescence driven by the same promoter was absent in roughly half of the heterocysts at 96 h N− growth (Fig. 4). This evidence is consistent with a requirement for maintained hetR expression in heterocysts to facilitate proper functioning following morphogenesis.

The inhibitors of heterocyst differentiation, HetN and PatS, are thought to be involved in the creation and maintenance of pattern formation, respectively. Mutation of patS results in a multiple contiguous heterocyst (Mch) phenotype

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**Fig. 4.** Transcriptional dynamics of hetR, hetN and patS post-development. (a–c) Bright-field (BF, greyscale), autofluorescence (AF, false colour red), and GFP or EcFbFP (FP, false colour green or blue, respectively) microscopy of wild-type Anabaena at either 24 or 96 h after removal of combined nitrogen. (a) Strains carry PhetR−gfp (pSMC127) or PhetR−EcFbFP (pPJAV340). (b) Strains carry PpatS−gfp (pAM1951) or PpatS−EcFbFP (pPJAV343). (c) Strains carry PhetN−gfp (pSMC126) or PhetN−EcFbFP (pPJAV341). Carets indicate heterocysts. Bars, 10 μm. (d) Percentage of heterocysts with fluorescence 24 or 96 h after removal of combined nitrogen. Indicated promoters drive expression of either GFP (light grey bars) or EcFbFP (dark grey bars). Percentage was calculated as the number of heterocysts with fluorescence out of 100. Each bar represents the mean of three independent trials. Error bars represent one SD. *P-value less than 0.01. †P-value greater than 0.4.
at 24 h N− growth that resolves to a normal pattern by roughly 96 h N− growth (Corrales-Guerrero et al., 2013; Yoon & Golden, 2001). In contrast, a ΔhetN mutant produces a normal pattern of heterocysts at 24 h N− growth and Mch arises by 48 h N− growth (Callahan & Buikema, 2001). The production of HetN from heterocysts is thought to resolve the early Mch phenotype of a ΔpatS mutant, whereas the terminal Mch phenotype displayed by a ΔhetN mutant results from the absence of the inhibitory signal involved in pattern maintenance. Transcription of patS is upregulated 3 h after the removal of combined nitrogen and was shown to return to pre-induction levels by 27 h N− growth (Yoon & Golden, 1998; Yoon & Golden, 2001). A transpositional fusion of the patS promoter to gfp displayed GFP localization to single cells by 8 h N− growth and heterocyst-specific fluorescence began to diminish after 24 h N− growth. Unlike patS, transcription of hetN is upregulated 12 h after the removal of combined nitrogen and localizes to single cells after commitment to differentiation. Work in Anabaena variabilis showed that hetN transcripts were abundant in mature heterocysts while patS transcripts were less so (Park et al., 2013). In the work presented here, transcriptional fusions of the patS and hetN promoters with EcFPs displayed diminished or continued fluorescence in heterocysts, respectively (Fig. 4). The transcriptional dynamics shown here, as well as phenotype and expression data, provides support for the role of PatS in pattern formation and HetN in pattern maintenance.

In Anabaena, the formation of a pattern of cells that will differentiate into heterocysts is regulated in a manner that resembles the activator–inhibitor model of biological pattern formation. Originally proposed by Geirer and Meinhardt, the activator–inhibitor model posits that a biological pattern is formed from a homogeneous population of cells through the interaction of a self-promoting activator of differentiation that enhances the production of a diffusible inhibitor (Geirer & Meinhardt, 1972). The inhibitor is capable of diffusing from source cells (cells actively producing the inhibitor) and inhibits autocatalysis or degradation of the activator. In the Anabaena system, HetR is an autocatalytic activator of differentiation that binds DNA (Black et al., 1993; Huang et al., 2004; Kim et al., 2013). PatS and HetN are inhibitors of differentiation that both contain a pentapeptide motif that likely interacts with HetR directly to signal it for degradation (Feldmann et al., 2012; Higa et al., 2012; Risser & Callahan, 2009; Yoon & Golden, 1998). The interplay between HetR, PatS and HetN is required for periodic pattern formation and heterocyst development. Here we show the utility of FbFPs in the study of the maintenance of a periodic pattern of microoxic heterocysts.

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