Sigma Factor Genes *sigC*, *sigE*, and *sigG* Are Upregulated in Heterocysts of the Cyanobacterium *Anabaena* sp. Strain PCC 7120[†]

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We used *gfp* transcriptional fusions to investigate the regulation of eight sigma factor genes during heterocyst development in the cyanobacterium *Anabaena* sp. strain PCC 7120. Reporter strains containing *gfp* fusions with the upstream regions of *sigB2*, *sigD*, *sigI*, and *sigJ* did not show developmental regulation. Time-lapse microscopy of *sigC*, *sigE*, and *sigG* reporter strains showed increased green fluorescent protein fluorescence in differentiating cells at 4 h, 16 h, and 9 h, respectively, after nitrogen step down.

Regulation of transcription initiation is a major mechanism of gene control in bacteria. Sigma factors associate with RNA polymerase to initiate transcription at specific promoters. In many cases, bacteria respond to environmental and intracellular signals by expressing specific sigma factors to activate particular sets of genes. The filamentous cyanobacterium *Anabaena* sp. strain PCC 7120 responds to deprivation of combined nitrogen by undergoing a developmental program to produce heterocysts, nitrogen-fixing cells that are positioned every 10 to 15 cells along filaments of photosynthetic vegetative cells. It has been hypothesized that sigma factors may play a role in directing transcriptional control during heterocyst development. The σ^{70} family of sigma factors has been divided into four major groups based on phylogeny: group 1 is involved in transcription of housekeeping genes; group 2 is closely related to group 1 but is dispensable under laboratory growth conditions; group 3 is more divergent from group 1 and can often be placed into groups with similar functions, such as heat shock, general stress responses, motility, and sporulation; and group 4 sigma factors are distantly related to the other representatives of the σ^{70} family (5).

There are 12 putative sigma factor genes in the genome of *Anabaena* sp. strain PCC 7120. The sigma factor nomenclature in *Anabaena* sp. strain PCC 7120 has recently been modified by Yoshimura et al. (14). Our own independent phylogenetic analysis of cyanobacterial sigma factors (see Fig. S1 in the supplemental material) agrees with that of Yoshimura et al.; therefore, we have adopted the revised nomenclature.

Although the group 2 sigma factor genes *sigB*, *sigB2*, *sigC*, *sigD*, and *sigE* in *Anabaena* sp. strain PCC 7120 have been studied by reverse genetics and *sigB* and *sigC* have been shown to be upregulated after nitrogen step down, their functions still remain obscure and none have been shown to be specifically involved in heterocyst development (1, 3, 8). The lack of a specific phenotype for any of the constructed sigma factor

mutants suggests that there is some level of functional redundancy among the sigma factors.

In the present study, we used *gfp* transcriptional fusions to investigate the temporal and spatial patterns of expression for eight *Anabaena* sp. strain PCC 7120 genes predicted to encode sigma factors: the group 2 sigma factor genes *sigB2* (alr3800; previously *sigE*), *sigC* (all1692), *sigD* (alr3810), and *sigE* (alr4249; previously *sigF*); the group 3 sigma factor genes *sigF* (all3853) and *sigJ* (alr0277; previously sigma-37); and the group 4 sigma factor genes *sigG* (alr3280; previously sigma-E) and *sigI* (all2193).

Methods. *Anabaena* sp. strain PCC 7120 and its derivatives were grown in BG-11 or BG-11₀ (BG-11 lacking sodium nitrate) medium at 30°C as previously described (2, 10). *Anabaena* genetics experiments were performed essentially as previously described (8, 9, 13). Antibiotics were omitted from BG-11₀ medium for synchronous heterocyst induction experiments. The plasmids used in this study are listed in Table 1, and the DNA primers are listed in Table S1 in the supplemental material. Plasmids containing transcriptional reporters were constructed by cloning the PCR-amplified upstream-untranslated region of each sigma factor gene into the shuttle vector pAM1956, which carries a promoterless *gfpmut2* reporter gene (12). The P_{*sigC*}-*gfp* transcriptional reporter was constructed by amplifying the upstream region of *sigC* with forward and reverse primers that contained SmaI and BamHI restriction sites, respectively, at their 5' ends into pBluescript II SK+. A SalI-SacI fragment containing the insert was subcloned into the corresponding sites of pAM1956 to generate pAM3648. For the construction of P_{*sigB2*}-*gfp* and P_{*sigF*}-*gfp* transcriptional reporters, a fragment containing each upstream region was amplified using forward and reverse primers containing SacI and SalI sites, respectively, at their 5' ends. These fragments were cloned into pBluescript II KS+ to generate pAM3655 and pAM3751, respectively. For each, a SacI-KpnI fragment containing the upstream region was subcloned into pAM1956. The P_{*sigB2*}-*gfp*, P_{*sigD*}-*gfp*, P_{*sigE*}-*gfp*, and P_{*sigJ*}-*gfp* transcriptional fusions were constructed by PCR amplification of the upstream region using primers containing SalI and SacI restriction sites at their 5' ends and then cloning the fragment into pAM1956. Plasmid constructs were verified by DNA sequencing.

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TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant characteristic(s)	Source or reference
<i>Anabaena</i> sp. strains		
PCC 7120	Wild type	R. Haselkorn
AMC1449	PCC 7120 carrying P _{sigC} -gfp on pAM3648	This study
AMC1450	PCC 7120 carrying P _{sigD} -gfp on pAM3650	This study
AMC1451	PCC 7120 carrying P _{sigB2} -gfp on pAM3651	This study
AMC1452	PCC 7120 carrying P _{sigE} -gfp on pAM3652	This study
AMC1454	PCC 7120 carrying P _{sigF} -gfp on pAM3653	This study
AMC1455	PCC 7120 carrying P _{sigG} -gfp on pAM3654	This study
AMC1456	PCC 7120 carrying P _{sigI} -gfp on pAM3656	This study
AMC1457	PCC 7120 carrying P _{sigG} -gfp on pAM3657	This study
Plasmids		
pAM1956	Shuttle vector pAM505 containing promoterless <i>gfpmut2</i> with unique upstream cloning sites SalI, SacI, KpnI, and SmaI	12
pAM2178	Conjugal suicide vector	8
pAM3648	pAM1956 containing 520-bp fragment upstream of <i>sigC</i> (all1692)	This study
pAM3650	pAM1956 containing 880-bp fragment upstream of <i>sigD</i> (alr3810)	This study
pAM3651	pAM1956 containing 554-bp fragment upstream of <i>sigB2</i> (alr3800)	This study
pAM3652	pAM1956 containing 800-bp fragment upstream of <i>sigE</i> (alr4249)	This study
pAM3653	pAM1956 containing 888-bp fragment upstream of <i>sigF</i> (all3853)	This study
pAM3654	pAM1956 containing 474-bp fragment upstream of <i>sigI</i> (alr0277)	This study
pAM3655	pBluescript II KS+ containing 206-bp fragment upstream of <i>sigI</i> (all2193)	This study
pAM3656	pAM1956 containing <i>sigI</i> insert from pAM3655	This study
pAM3657	pAM1956 containing 815-bp fragment upstream of <i>sigG</i> (alr3280)	This study
pAM3751	pBluescript II KS+ containing 900-bp fragment upstream of <i>sigF</i> (all3853)	This study

Fluorescence and bright-field images were captured using an Olympus IX70 inverted microscope (Olympus, Melville, NY) with a Hamamatsu OrcaER C4742-95 charge-coupled-device camera (Hamamatsu, Bridgewater, NJ) and Simple PCI software version 6.1 (Compix Inc., Cranberry Township, PA). The Simple PCI software controlled the camera, the microscope's light path through ProScan shutters (Prior Scientific, Rockland, MA), and automated focus. A Piston green fluorescent protein (GFP) band-pass filter set (catalog no. 41025; Chroma Technology Corp., Brattleboro, VT) was used for fluorescence images.

Time-lapse microscopy was used to record bright-field and GFP fluorescence images during synchronous heterocyst development of sigma factor-reporter strains. Filaments of *Anabaena* sp. strain PCC 7120 reporter strains were grown in nitrate-containing medium containing appropriate antibiotics to an optical density at 750 nm of approximately 0.2, and heterocyst development was induced by washing the filaments with purified water to remove nitrate and resuspending the filaments in BG-11₀ medium. Induced *Anabaena* filaments in 5 to 10 μ l BG-11₀ medium were applied to a BG-11₀ medium–1% agarose pad in a single-chambered cover glass (Lab-Tek chamber slide system) prepared as follows. A thin 150- μ l pad of BG-11₀ medium–1% agarose was made by placing a slightly trimmed cover glass, with a piece of toothpick glued to the top to serve as a handle, on the molten medium placed toward one end of the chambered cover glass and then removing the cover glass after the agarose cooled. The agarose pad was then surrounded on all four sides by a total of about 2 ml of BG-11₀ medium–1% agarose to maintain moisture in the thin agarose pad. The temperature around the microscope stage was maintained at approximately 30°C. A time-lapse sequence with 10-minute time delays of bright-field and fluorescence images was acquired at 600 $\times$ magnification for 26 to

40 h by using automated switching between light sources and autofocus before each bright-field image capture. During the delays, the cells received bright-field illumination for their growth; the intensity was adjusted to produce a maximum growth rate without killing the cells. The rate of cell division along filaments was variable and ranged from about 6 to 18 h or sometimes longer. To obtain synchronous induction of heterocyst development, it was necessary to open the bright-field light source iris diaphragms to their maximum setting to illuminate a larger patch of cells on the agarose pad. The fluorescence excitation light intensity was diminished with neutral-density filters to obtain the highest intensity that showed no GFP fluorescence bleaching; these lower excitation intensities caused no decrease in cell growth rate or viability but did reduce the level of GFP fluorescence. Time-lapse images were processed using Simple PCI software, and individual images from specific time points were exported as required (see Fig. 2).

Reporter strains for *sigB2*, *sigD*, *sigI*, and *sigJ* showed GFP reporter fluorescence in vegetative cells and heterocysts. The plasmid pAM1956 is based on a low- to medium-copy-number shuttle vector (9, 13). To determine the temporal and spatial patterns of expression, we chose to place the reporter constructs on a shuttle plasmid to obtain detectable levels of GFP fluorescence from all promoters being studied. However, it is possible that the use of reporter fusions on a replicative plasmid may not accurately reflect the normal levels of a gene's product and could potentially produce abnormal regulation of the promoter under investigation. A control strain containing the pAM1956 vector alone produced no detectable GFP fluorescence.

The *sigB2*, *sigD*, *sigI*, and *sigJ* promoter regions were all active in vegetative cells of nitrate-grown filaments (Fig. 1). In nitrate-grown cultures, the GFP fluorescence intensities varied in the vegetative cells along filaments such that groups of cells that showed lower expression levels tended to alternate with

FIG. 1. GFP reporter fluorescence from strains containing *gfpmut2* expressed from promoters of the *sigB2*, *sigD*, *sigI*, and *sigJ* sigma factor genes in *Anabaena* sp. strain PCC 7120 filaments grown in nitrate-containing BG-11 medium and 24 h after nitrogen step-down to BG-11₀ medium to induce heterocyst development. Arrowheads indicate mature heterocysts. The panels on the left for each medium are bright-field images. The panels on the right are corresponding GFP fluorescence images. Scale bar, 10 μ m.

groups of cells producing stronger intensities. We do not know the underlying mechanism responsible for this variation in GFP fluorescence along filaments, but it did not interfere with the ability to detect qualitative changes in fluorescence between vegetative cells and differentiating heterocysts after nitrogen step down. We could speculate that the clusters of cells showing similar GFP fluorescence intensities have comparable physiological states determined by their lineage or position in the cell cycle; the state of the cells might influence expression of the reporter gene or could possibly affect the copy number of the plasmid carrying the reporter fusion. At 24 h after nitrogen step down, filaments of the *P_{sigB2}-gfp*, *P_{sigD}-gfp*, *P_{sigI}-gfp*, and *P_{sigJ}-gfp* reporter strains showed GFP fluorescence in both vegetative cells and heterocysts, and none showed heterocyst-specific upregulation of GFP fluorescence. Time-lapse microscopy revealed that for the *P_{sigD}-gfp*, *P_{sigI}-gfp*, and *P_{sigJ}-gfp* reporter strains, the GFP fluorescence intensities from differentiating cells remained similar to that of the original vegetative cells prior to nitrogen step-down. For the *P_{sigB2}-gfp* reporter strain, approximately three-quarters of the heterocysts displayed higher fluorescence intensities than those displayed by the vegetative cells. However, time-lapse microscopy showed that this phenotype is apparently a consequence of decreased GFP fluorescence in vegetative cells and not upregulation in differentiating cells. The *P_{sigF}-gfp* reporter strain did not show detectable GFP fluorescence when grown in medium with or without a source of combined nitrogen (data not shown).

Previous studies showed that *sigB2* and *sigD* mutants have an abnormally slow transition to diazotrophic growth and that a double mutant exhibits extensive fragmentation of filaments upon nitrogen deprivation (8). Our *gfp* reporter results together with the fragmentation phenotype and transient impairment in establishing diazotrophic growth suggest that SigB2 and SigD may function in both vegetative cells and heterocysts.

Reporter strains for *sigC*, *sigE*, and *sigG* showed increased GFP reporter fluorescence in heterocysts after nitrogen step down. In nitrate-grown cultures, the *P_{sigC}-gfp* reporter strain often had a small number of bright cells along filaments of

mostly very dim cells, the *P_{sigE}-gfp* reporter strain displayed a more-uniform low level of fluorescence in vegetative cells, and the *P_{sigG}-gfp* reporter strain showed a higher and less-uniform level of fluorescence in vegetative cells.

Time-lapse microscopy of the *P_{sigC}-gfp* reporter strain showed that at 4 h following nitrogen step down, individual cells, but also pairs of cells and dividing cells, showed increased GFP fluorescence (Fig. 2A; and see Movie S1 in the supplemental material). By 10 h, when morphological differentiation of proheterocysts was not obvious but by which time about half of the differentiating cells are committed to form heterocysts (13), about 12% of the cells showed increased GFP fluorescence, of which about half were arranged in a pattern similar to that of heterocysts. Dividing brighter cells maintained the original fluorescence levels, but after division, usually only one daughter cell increased its fluorescence (see Movie S1 in the supplemental material). By 24 h, most individual cells that showed the strongest early *P_{sigC}-gfp* expression became heterocysts; however, only a portion of vegetative cells with moderate levels of fluorescence differentiated into heterocysts. For all *gfp* reporter strains, mature heterocysts gradually became dimmer after 24 h.

The *P_{sigC}-gfp* reporter strain maintained in medium containing nitrate often showed increased GFP fluorescence in cells at the ends of filaments. This phenomenon was observed in actively growing cells, but it was more evident in filaments from older cultures. Interestingly, patterned expression of *P_{sigC}-gfp* was sometimes observed in nitrate-grown filaments before nitrogen step down (unpublished results). The time of expression of *sigC* indicates its involvement in the early stages of heterocyst development. Previous studies have shown that *sigC* inactivation delays heterocyst development by at least 6 hours (8); however, *sigC* inactivation does not block heterocyst development or nitrogen fixation (1). SigC is potentially involved in the transcription of heterocyst-specific genes whose initial expression is coincident with that of the *sigC* gene. Examples of such genes include *hetC* and *hetP*, *devH*, *patS*, *patA*, *patB*, and genes involved in the formation of the polysaccharide (*hepA*, *hepB*,

FIG. 2. GFP reporter fluorescence from promoters of sigma factor genes *sigC* (A), *sigE* (B), and *sigG* (C). Upper panels show bright-field images; lower panels show the corresponding GFP fluorescence images. The strains were grown in nitrate-containing BG-11 medium and then transferred to BG-11₀ medium to induce heterocyst development. Time-lapse images were collected every 10 min for at least 24 h, and selected images are shown for the indicated time points after induction. Filaments of the *sigC* reporter strain were partially fragmented by mild sonication prior to nitrogen step down in the time series shown; similar results were obtained with unfragmented filaments. Scale bar, 10 μ m.

hepC, *hepK*) and glycolipid (*hglC*, *hglD*, *hglE*, *hglK*, *hglB*, *hetN*, *hetI*) envelope layers.

The *P_{sigE}-gfp* reporter strain showed no change in fluorescence levels until around 16 h after nitrogen step down, when individual differentiating cells displayed increased fluorescence (Fig. 2B; and see Movie S2 in the supplemental material). In the following 2 hours, the fluorescence levels rapidly increased in these cells. A pattern resembling that of mature heterocysts was distinguishable around 17 h after nitrogen step down. At 24 h, GFP fluorescence remained bright in mature heterocysts but decreased afterwards. These results suggest that SigE could be involved in the expression of late-stage heterocyst-specific genes. Potential target genes for SigE during the late stages of differentiation include the nitrogen fixation (*nif*) genes, which are expressed between 18 h and 24 h after nitrogen deprivation (3). Although insertional inactivation of *sigE* did not completely block heterocyst function or diazotrophic growth (8), this could be due to partial functional redundancy among sigma factors.

The *P_{sigG}-gfp* reporter strain initially had brighter fluorescence in vegetative cells than the *sigC* and *sigE* reporter strains,

but by 4 h after nitrogen step down, there was a clear decrease in GFP fluorescence (Fig. 2C; and see Movie S3 in the supplemental material). This drop continued in all cells along filaments. By 9 h, the strongest, but still fairly weak, GFP fluorescence was localized almost exclusively to presumptive differentiating cells, although proheterocysts were not morphologically distinguishable at this time. By 16 h, identifiable proheterocysts showed increased GFP fluorescence. At 24 h, GFP fluorescence was localized to heterocysts but was somewhat decreased in intensity. Interestingly, the localized rapid increase in GFP fluorescence in the *sigG* reporter strain occurred during the time between 9 and 13 h after nitrogen step down, when cells become committed to completing the differentiation process (13), suggesting that SigG could be involved in the mechanism of commitment. The timing of *P_{sigG}-gfp* expression suggests an involvement of SigG in the expression of “middle” genes. During this stage of development, differentiating cells undergo morphological and physiological changes to produce a micro-oxic environment necessary for nitrogenase to function, such as deposition of the heterocyst envelope layers and expression at 9 h of the *cox2* and *cox3* genes encoding cytochrome *c* oxidases (11).

Our results indicate that the sigma factor genes *sigC*, *sigE*, and *sigG* are specifically upregulated in differentiating heterocysts of *Anabaena* sp. strain PCC 7120. However, as mentioned above, reverse-genetics experiments have shown that *sigB*, *sigB2*, *sigC*, *sigD*, and *sigE* are not individually required for heterocyst development. It seems likely that group 1 and group 2 sigma factors may have at least partial overlapping promoter specificities. An example of overlapping promoter specificities is found in *Synechococcus elongatus* PCC 7942, where group 1 and group 2 sigma factors can bind to and regulate transcription from the same promoter regions (4). Similarly, overlapping promoter specificities have also been described for *Synechocystis* sp. strain PCC 6803 (6, 7). The regulons controlled by the *Anabaena* sp. strain PCC 7120 SigC, SigE, and SigG sigma factors remain to be determined.

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REFERENCES

1. Brahamsha, B., and R. Haselkorn. 1992. Identification of multiple RNA polymerase sigma factor homologs in the cyanobacterium *Anabaena* sp. strain PCC 7120: cloning, expression, and inactivation of the *sigB* and *sigC* genes. *J. Bacteriol.* **174**:7273–7282.
2. Golden, J. W., L. L. Whorff, and D. R. Wiest. 1991. Independent regulation of *nifHDK* operon transcription and DNA rearrangement during heterocyst differentiation in the cyanobacterium *Anabaena* sp. strain PCC 7120. *J. Bacteriol.* **173**:7098–7105.
3. Golden, J. W., and H. S. Yoon. 2003. Heterocyst development in *Anabaena*. *Curr. Opin. Microbiol.* **6**:557–563.
4. Goto-Seki, A., M. Shirokane, S. Masuda, K. Tanaka, and H. Takahashi. 1999. Specificity crosstalk among group 1 and group 2 sigma factors in the cyanobacterium *Synechococcus* sp. PCC7942: *In vitro* specificity and a phylogenetic analysis. *Mol. Microbiol.* **34**:473–484.
5. Gruber, T. M., and C. A. Gross. 2003. Multiple sigma subunits and the partitioning of bacterial transcription space. *Annu. Rev. Microbiol.* **57**:441–466.
6. Imamura, S., M. Asayama, H. Takahashi, K. Tanaka, H. Takahashi, and M. Shirai. 2003. Antagonistic dark/light-induced SigB/SigD, group 2 sigma factors, expression through redox potential and their roles in cyanobacteria. *FEBS Lett.* **554**:357–362.
7. Imamura, S., K. Tanaka, M. Shirai, and M. Asayama. 2006. Growth phase-

- dependent activation of nitrogen-related genes by a control network of group 1 and group 2 sigma factors in a cyanobacterium. *J. Biol. Chem.* **281**:2668–2675.
8. **Khudyakov, I. Y., and J. W. Golden.** 2001. Identification and inactivation of three group 2 sigma factor genes in *Anabaena* sp. strain PCC 7120. *J. Bacteriol.* **183**:6667–6675.
 9. **Lee, M. H., M. Scherer, S. Rigali, and J. W. Golden.** 2003. PlmA, a new member of the GntR family, has plasmid maintenance functions in *Anabaena* sp. strain PCC 7120. *J. Bacteriol.* **185**:4315–4325.
 10. **Rippka, R., J. Deruelles, J. B. Waterbury, M. Herdman, and R. Y. Stanier.** 1979. Generic assignments, strain histories and properties of pure cultures of cyanobacteria. *J. Gen. Microbiol.* **111**:1–61.
 11. **Valladares, A., A. Herrero, D. Pils, G. Schmetterer, and E. Flores.** 2003. Cytochrome *c* oxidase genes required for nitrogenase activity and diazotrophic growth in *Anabaena* sp. PCC 7120. *Mol. Microbiol.* **47**:1239–1249.
 12. **Yoon, H. S., and J. W. Golden.** 1998. Heterocyst pattern formation controlled by a diffusible peptide. *Science* **282**:935–938.
 13. **Yoon, H. S., and J. W. Golden.** 2001. PatS and products of nitrogen fixation control heterocyst pattern. *J. Bacteriol.* **183**:2605–2613.
 14. **Yoshimura, H., S. Okamoto, Y. Tsumuraya, and M. Ohmori.** 2007. Group 3 sigma factor gene, sigJ, a key regulator of desiccation tolerance, regulates the synthesis of extracellular polysaccharide in cyanobacterium *Anabaena* sp. strain PCC 7120. *DNA Res.* **14**:13–24.