IDENTIFICATION OF NOVEL REGULATORY MECHANISMS CONTROLLING HETEROCYST DEVELOPMENT IN *Anabaena* SP. STRAIN PCC 7120

A Dissertation

by

MARIA RAMONA ALDEA

Submitted to the Office of Graduate Studies of
Texas A&M University
in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

August 2008

Major Subject: Microbiology

IDENTIFICATION OF NOVEL REGULATORY MECHANISMS CONTROLLING HETEROCYST DEVELOPMENT IN *Anabaena* SP. STRAIN PCC 7120

A Dissertation

by

MARIA RAMONA ALDEA

Submitted to the Office of Graduate Studies of
Texas A&M University
in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

Approved by:

Chair of Committee,	James W. Golden
Committee Members,	Deborah Bell-Pedersen
	Michael Polymenis
	Deborah Siegele
Head of Department,	Vincent M. Cassone

August 2008

Major Subject: Microbiology

ABSTRACT

Identification of Novel Regulatory Mechanisms Controlling Heterocyst Development in *Anabaena* Sp. Strain PCC 7120. (August 2008)

Maria Ramona Aldea, B.S., Babes-Bolyai University; M.S., Babes-Bolyai University

Chair of Advisory Committee: Dr. James W. Golden

The regulatory mechanisms that govern heterocyst development in *Anabaena* sp. strain PCC 7120 have been continuously refined over the last two decades. In this work, we show that three of the sigma factor genes present in the *Anabaena* sp. strain PCC 7120 genome are developmentally regulated. Time-lapse microscopy of *gfp* reporter strains indicated that expression of *sigC*, *sigG*, and *sigE* is upregulated specifically in differentiating cells at 4 h, 9 h and 16 h, respectively, after induction of heterocyst development. We proposed that the sigma factors encoded by these genes are involved in regulation of heterocyst-specific genes whose expression is relatively coincident with that of *sigC*, *sigG*, or *sigE*. Indeed, inactivation of the *sigC* gene caused delayed and reduced expression of genes required for the early stages of heterocyst development, and caused delayed development. Inactivation of the *sigE* gene caused a considerable drop in expression of *nifH*, a late gene required for nitrogen fixation.

We also provide evidence that c-di-GMP, a novel bacterial second messenger, is involved in regulating heterocyst development. The all2874 gene encodes a bona fide diguanylate cyclase, which synthesizes c-di-GMP, and the gene's inactivation resulted in

a decreased tendency to form heterocysts; this phenotype was exacerbated by high light intensity. We hypothesize that the putative operon all2875-all2874 senses and relays information about light conditions and this information is integrated into the decision to form heterocysts.

Finally, we identified the all0187 gene, which is expressed at 9 h, a time when cells that have initiated differentiation commit to complete the process. In nitrogen-free medium, all0187 mutant filaments formed abnormally long heterocysts and were unable to grow diazotrophically. Septum formation between heterocysts and their flanking vegetative cells was incomplete, leaving one or both poles of the heterocysts more opened and potentially more permeable to oxygen. Despite having nitrogenase activity, the all0187 mutant was unable to grow diazotrophically. We hypothesize that the diazotrophic growth defect is caused by the inability of the heterocysts to transport fixed nitrogen to the neighboring vegetative cells.

ACKNOWLEDGMENTS

I would like to extend my deepest gratitude to my advisor, Dr. James Golden, for his guidance, support, and undoubted faith. With his careful mentoring, Jim has gently pushed me to exceed my highest expectations.

I would also like to thank current and former members of my committee: Drs. Deborah Bell-Pedersen, Suma Datta, Andy LiWang, Deborah Siegele, and Michael Polymenis, for their support and advice over these years.

A special thank you to Dr. Nicolae Dragos, my mentor at Babes-Bolyai University. I owe the opportunity to start my graduate career at Texas A&M to his guidance, support, and faith in my potential as a scientist.

A heart felt thank you to all my friends and colleagues for their support and for making my experience at Texas A&M enjoyable and unforgettable. I would like to especially thank Lindsay Bennett and Rodrigo Mella for their loyal friendship and immeasurable help.

I would like to thank my fiancé, Josh Neunuebel, for his endless love, support, and encouragement. Thank you to my sister, Delia and my parents, Mia and Marius Aldea for their love, pride, and joy for all my accomplishments.

TABLE OF CONTENTS

CHAPTER Page

CHAPTER Page

Page

LIST OF FIGURES

FIGURE Page

LIST OF TABLES

CHAPTER I

INTRODUCTION*

Multicellularity has arisen several times during evolution and although common to eukaryotes, it is also found in prokaryotes (79). The generation of multicellularity and cell-type diversity enables an organism to acquire specialized functions and advantages in feeding, dispersion, and protection (79). One example of prokaryotic multicellularity that clearly exhibits cellular differentiation and the formation of a multicellular pattern is found in filamentous nitrogen-fixing cyanobacteria, e.g., the genera *Anabaena* and *Nostoc*. Cyanobacteria comprise a diverse group of gram-negative prokaryotes that perform oxygenic photosynthesis. Some are also able to "fix" nitrogen by reducing atmospheric dinitrogen to ammonium. Nitrogen fixation and oxygenic photosynthesis are incompatible processes because the nitrogenase enzyme complex is very oxygen-sensitive. Many filamentous cyanobacteria that fix nitrogen overcome this incompatibility by undergoing cellular differentiation, which spatially separates the two processes; nitrogen fixation takes place in terminally differentiated cells called heterocysts and photosynthesis operates in vegetative cells that continue to grow and divide (162). For *Anabaena*, nitrogen-limiting conditions induce about 5 to 10% of cells along a filament to differentiate, resulting in a one-dimensional developmental pattern of

---

This dissertation follows the style of Journal of Bacteriology.

single heterocysts separated by an average of 10-20 vegetative cells (Fig. 1.1B); this regulated pattern suggests the existence of cell-to-cell communication within filaments (57). Filaments grown in the presence of a combined nitrogen source consist of vegetative cells only (Fig. 1.1A). Differentiating cells pass through an intermediary stage, called the proheterocyst stage, when if a source of combined nitrogen becomes available differentiation can be reversed. Proheterocysts become committed to complete heterocyst differentiation between 9 to 14 hours after nitrogen step-down (173), and differentiation is completed by 18 to 24 hours.

Heterocysts undergo specific changes in physiology and morphology to generate the microoxic environment needed to accommodate nitrogen fixation (164). The O_2-producing photosystem II, which is part of the photosynthetic electron transport chain, is dismantled during heterocyst differentiation. An envelope that consists of two layers encircles the heterocyst and constrains the influx of environmental oxygen. The inner layer is composed of a hydroxylated glycolipid and the outer layer is composed of polysaccharide. Traces of oxygen that cross the envelope are consumed by increased respiration in heterocysts. Heterocysts are thought to become dependent on neighboring vegetative cells for reduced carbon, and in return, the heterocysts provide fixed nitrogen as amino acids that probably move through the periplasm (103) to the vegetative cells (Fig. 1.2). Thus, these two functionally distinct cell types must collaborate to support diazotrophic growth and communicate to regulate the developmental pattern for efficiency. Heterocystous cyanobacteria provide a relatively simple model of prokaryotic

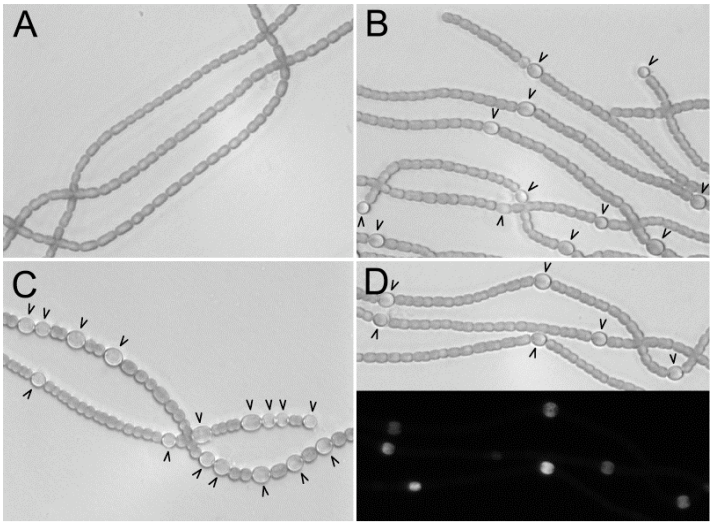

FIG. 1.1. Wild-type and mutant filaments of *Anabaena* sp. strain PCC 7120. (A) Wild-type filaments grown on medium containing nitrate consist of vegetative cells only. (B) Wild-type filaments grown on medium without a source of combined nitrogen showing a pattern of single heterocysts spaced along filaments. (C) A *patS* deletion mutant (strain AMC451) grown on medium without a source of combined nitrogen displaying multiple-contiguous-heterocysts phenotype. (D) GFP fluorescence of a *patS-gfp* reporter strain (AMC484) grown on medium without a source of combined nitrogen showing bright heterocysts (lower panel). Arrowheads indicate heterocysts.

multicellular development in which two cell types must continuously interact to support growth of the organism.

Regulation of heterocyst development

Most of the information about heterocyst development to date is based on the study of three species of heterocyst-forming filamentous cyanobacteria: *Anabaena* (also *Nostoc*) sp. strain PCC 7120, *Anabaena variabilis* ATCC 29413, and *Nostoc punctiforme* ATCC 29133. All three of these genomes have been sequenced as well as those of over two dozen other cyanobacterial species, which facilitates bioinformatic analyses. Numerous genes have been identified that are involved in heterocyst development and/or function. Here, we will focus on those genes involved in signaling and regulation.

Sensing nitrogen limitation

Ammonium is usually the preferred source of nitrogen for bacteria including heterocyst-forming cyanobacteria. Its presence exerts an inhibitory effect on assimilatory pathways of alternative nitrogen sources and also strongly inhibits heterocyst development; nitrate has a lesser inhibitory effect (164). In *E. coli*, the intracellular concentration of glutamine serves as the signal for nitrogen status and 2-oxoglutarate (2-OG) serves as the signal for carbon status (72, 109). Intracellular nitrogen status is sensed by PII (encoded by *glnB* or *glnK*), a protein that plays a central role in interpreting and responding to changes in the key carbon and nitrogen metabolites (111). A UTase/UR enzyme (encoded by *glnD*) controls the activity of PII by uridylylation and deuridylylation in response to levels of

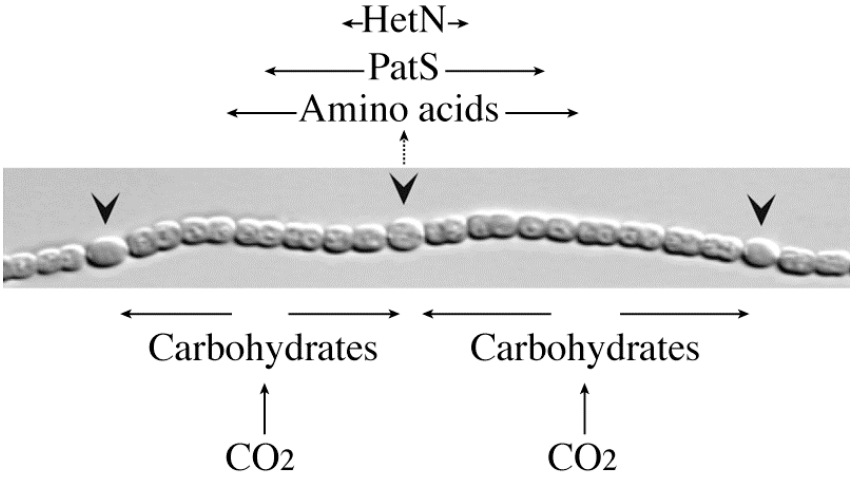

FIG. 1.2. Signal and metabolite flow between heterocysts and vegetative cells. Wild-type filament with a normal pattern of heterocysts (marked by the arrowheads) grown on medium without a source of combined nitrogen. Vegetative cells are thought to provide carbohydrates produced by photosynthesis to heterocysts, which in turn provide fixed nitrogen as amino acids to the reproductive vegetative cells. Heterocyst pattern is controlled by PatS and HetN-dependent signals and by the supply of nitrogen from heterocysts. The transfer and movement of small molecules along filaments is thought to occur via the periplasm. Horizontal arrows indicate the apparent effective range of each signal or metabolite.

glutamine (109). The uridylylated form of PII can interact with adenyltransferase (encoded by *glnE*) to activate glutamine synthase (GS). Native PII protein interacts with NtrB to modify the phosphorylation status of NtrC, and in turn, NtrC~P acts as a transcriptional regulator of various genes involved in nitrogen metabolism including *glnA*, which encodes GS (35, 109).

The cyanobacterial nitrogen sensory system diverges from that of *E. coli*, but is similar between unicellular and filamentous cyanobacteria. In the unicellular cyanobacterium *Synechococcus elongatus* PCC 7942, PII is not modified by uridylylation, as is the case in *E. coli*, but is instead phosphorylated (47). In heterocyst-forming filamentous cyanobacteria, PII is modified but the nature of the modification is not yet clear; phosphorylation of recombinant PII was achieved *in vitro* but not *in vivo* (46, 58). Functional analysis of PII in *N. punctiforme* has been impeded by the fact that *glnB* seems to be essential (62). In contrast to proteobacteria where PII activity is modified in response to glutamine levels, in cyanobacteria, PII is modified by phosphorylation in response to 2-OG levels (46).

The 2-OG molecule is an intermediate in the Krebs cycle, and is considered to be the metabolic junction between the carbon and nitrogen balance in bacteria. In cyanobacteria, the Krebs cycle is incomplete because they lack 2-OH dehydrogenase (164); as a result, the main function of 2-OG in cyanobacteria is to serve as the primary carbon skeleton for the incorporation of ammonium. Therefore, 2-OG can serve as a

measure of nitrogen status. Variations in the nitrogen supply are inversely correlated with levels of 2-OG, thus, nitrogen-limiting conditions produce an increase in 2-OG (119). The hypothesis that 2-OG controls heterocyst development has been confirmed by use of a non-metabolizable analog of 2-OG to mimic nitrogen-limiting conditions and provoke heterocyst development (89).

Another major difference in the mechanism of nitrogen control in cyanobacteria is the lack of the RpoN (sigma 54) sigma factor and RpoN-dependent transcription factors including NtrC. In cyanobacteria, nitrogen control is mediated by NtcA, a transcription factor in the CRP (cAMP receptor protein) family of DNA-binding proteins. NtcA regulates various genes important for nitrogen and carbon metabolism (67, 144). In *Anabaena* sp. strain PCC 7120, *ntcA* mutants are not able to use nitrate as a sole nitrogen source and require ammonium for growth; moreover, they show no signs of heterocyst differentiation indicating an essential role for NtcA in the initiation of differentiation (Fig. 1.3) (49, 67, 160). Under nitrogen-limiting conditions, *ntcA* is induced most strongly in differentiating cells (125). The *ntcA* gene is induced after nitrogen deprivation and is positively autoregulated (67, 133). In *S. elongatus*, NtcA DNA-binding activity is enhanced by the presence of 2-OG and, moreover, transcriptional activation by NtcA requires 2-OG (147, 156). DFPA (2,2-difluoropentanedioic acid), a synthetic analogue of 2-OG, was also able to stimulate DNA-binding of NtcA (89). Taken together, these data indicate that NtcA is a sensor of 2-OG that upregulates expression of genes for permeases and enzymes of the nitrogen assimilatory pathways

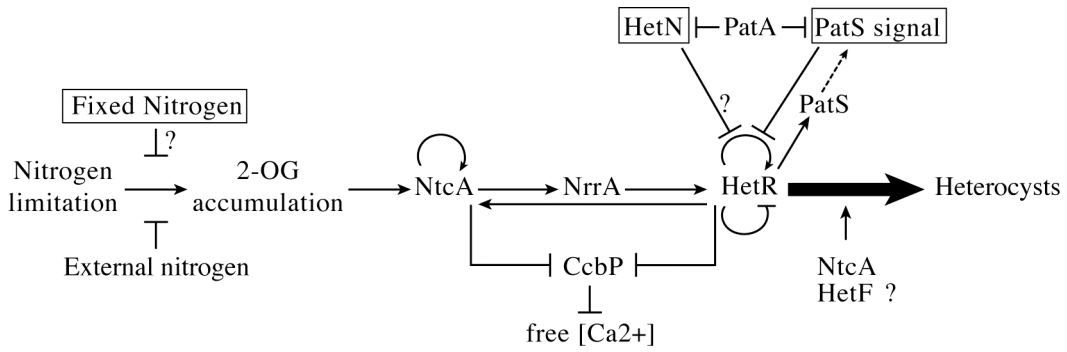

FIG. 1.3. Model showing the influence of cell-to-cell signals on heterocyst development and pattern formation. Nitrogen starvation results in accumulation of 2-oxoglutarate (2-OG), which leads to increased NtcA activity. Initiation of heterocyst development is controlled mainly by NtcA and HetR, which are positively autoregulated and mutually dependent on each other for upregulation; HetR also has autoproteolytic activity. The DNA-binding protein NrrA serves as a regulatory link between NtcA and HetR. It is directly activated by NtcA and then causes the initial induction of *hetR*; further induction of *hetR* relies on autoregulatory positive feedback. HetR is considered the master regulator of heterocyst development. NtcA and HetR collaborate to reduce the levels of CcbP in the differentiating cell, the former by directly inhibiting transcription and the latter by proteolysis, resulting in release of free calcium. NtcA is required for early as well as late stages of heterocyst development. HetF positively influences heterocyst development by an unknown mechanism. Three factors produced by the differentiating cell are proposed to influence heterocyst development and pattern formation by acting as cell-to-cell signals (enclosed in boxes). Fixed nitrogen originating from the differentiating cells and mature heterocysts in the form of amino acids presumably inhibit differentiation of neighboring cells and establish the ultimate spacing between heterocysts. Production of PatS is directly activated by HetR in differentiating cells and it is thought that PatS, or a posttranslationally processed peptide, is secreted and then enters neighboring cells where it inhibits HetR activity to block differentiation. HetN is also thought to generate an inhibitory signal from mature heterocysts that inhibits differentiation of adjacent cells, possibly by interfering with HetR activity. PatA may partially relieve the inhibitory effect of the HetN- and PatS-dependent signals. The dashed arrow indicates putative posttranslational processing of PatS, and the large arrow represents other developmental steps that take place between the activation of HetR and completion of the differentiation process.

needed for utilizing alternative sources of nitrogen and triggers heterocyst differentiation in heterocystous strains (67). In most cases, genes that are directly controlled by NtcA have the signature binding site TGTA-(N_8)-TACA centered at -41.5 with respect to the transcriptional start site (67, 77). In the unicellular cyanobacterium *S. elongatus*, full activation of some NtcA-dependent genes requires PII, and when nitrate is present, PII has an inhibitory effect on NtcA activity (6). Additionally, NtcA regulates PII at the transcriptional and post-translational level (41, 130). Recently, a search for interacting partners of nitrogen regulators found that the previously identified PipX protein, which is present only in cyanobacteria, acts as a link between the key nitrogen regulators PII and NtcA. PipX swaps between these two partners in a 2-OG-dependent manner (40).

HetR is a master regulator of heterocyst development

HetR plays a central role in heterocyst development and pattern formation (Fig. 1.3) (13, 21, 83, 175). HetR is a positive regulatory factor that is essential for heterocyst development. The *hetR* gene is expressed early in differentiating cells after nitrogen step-down. Transcription of *hetR* is positively autoregulated and begins to increase 30 minutes after nitrogen deprivation and by 3.5 h P_{hetR}-*luxAB* expression is localized to a subset of cells, well before morphological differentiation is observed (13, 20). HetR has two known activities, autoproteolysis, and specific DNA-binding that requires formation of a homodimer (69, 177). Mutations that interfere with either of these activities block heterocyst development at an early stage. HetR is an unusual serine-type protease. Two serine residues, Ser-152 and Ser-179, are required for autoproteolysis and heterocyst

differentiation, with Ser-152 thought to be in the active site of the protease (34). In *hetR* S179N and S152A mutants, HetR over-accumulates in filaments, but these mutants are heterocyst defective (34). The autoproteolytic activity may be important for regulating the accumulation of HetR in only differentiating cells because *hetR* is, at least initially, transcribed in undifferentiated cells.

Increased HetR levels or activity is sufficient to force heterocyst differentiation. Overexpression of *hetR* on a multicopy plasmid either from its native promoter or from the copper-regulated *petE* promoter, leads to increased heterocyst frequency regardless of the presence of nitrate or ammonium (20). Ectopic overexpression of a mutant allele of *hetR* ($hetR_{R223W}$) is able to bypass the main inhibitory signals of heterocyst pattern formation and results in a conditionally lethal phenotype caused by complete differentiation of nearly all cells under nitrogen-limiting conditions (83).

During heterocyst development, *ntcA* and *hetR* exhibit a mutual dependency (Fig. 1.3) (116). Activation of *hetR* expression in the early stage of heterocyst development precedes that of the *ntcA* gene, suggesting that the NtcA-dependent boost in *hetR* expression is mediated by preexisting NtcA protein (67). Expression of some genes involved in heterocyst development, such as *devH* and the *devBCA* operon is dependent upon both *ntcA* and *hetR* (44, 64). In some cases, this regulation may result from the HetR-dependent increase of NtcA levels (67, 124). Other genes such as *hetC*, which is also required for the early stage of heterocyst differentiation, exhibit only NtcA

dependence (116). NrrA, a response regulator, has recently been identified as the regulatory link between NtcA and HetR. The *nrrA* gene is directly dependent on NtcA and is transcribed in differentiating cells within 3 h after nitrogen deprivation (Fig. 1.3) (37, 115). In turn, NrrA binds specifically to the *hetR* promoter region and is required for increased *hetR* expression (36). In the absence of *nrrA*, HetR accumulation and, thus, heterocyst development are delayed (37), and extra copies of *nrrA* result in increased expression of *hetR* and heterocyst frequency (36).

Ca^{2+} has a regulatory role in heterocyst development

In bacteria, calcium ions play important roles in various cellular processes such as pathogenesis, sporulation in *Bacillus*, chemotaxis in *E. coli*, and heterocyst development in cyanobacteria (66, 141, 142, 152). The first accounts of Ca^{2+} involvement in heterocyst development originated from a study that manipulated the extracellular concentration of calcium showing that it influences heterocyst frequency and nitrogenase activity (141). A recent study used the Ca^{2+}-binding luminescent protein aequorin as a reporter to track intracellular calcium changes in cultures during heterocyst development (153). A transient increase primarily due to mobilization of internally stored Ca^{2+} occurred at about 1 h after nitrogen step-down. In a *hetR* mutant, the Ca^{2+} concentration increase was not disrupted suggesting that the Ca^{2+} signal acts earlier than *hetR* during heterocyst development (153). Subsequently, the use of obelin, a different Ca^{2+} reporter, allowed monitoring of intracellular calcium levels in individual cells (176). Heterocysts had a 10-fold higher calcium concentration than vegetative cells and the increase was

detected 4 h after removal of combined nitrogen. The free calcium levels were found to be inversely correlated with the expression of *ccbP*, which encodes a calcium-sequestering protein in *Anabaena* sp. strain PCC 7120 and other heterocyst-forming cyanobacteria (176). In the absence of combined nitrogen, inactivation of *ccbP* results in a multiple-contiguous-heterocyst phenotype, whereas overexpression has an inhibitory effect on *hetR* induction and heterocyst development. Expression of *ccbP* is down regulated in heterocysts and CcbP is not present in mature heterocysts (176). A regulatory pathway comprising HetR, CcbP, and NtcA controls intracellular free calcium during heterocyst development (Fig. 1.3) (140). HetR specifically degrades CcbP in a Ca^{2+}-dependent manner, and *ccbP* down-regulation in differentiating cells requires 2-OG-dependent binding of NtcA to its promoter region. The increase in free calcium in differentiating cells may regulate the Ca^{2+}-dependent serine protease activity of HetR and other Ca^{2+}-dependent proteases (100). It has also been suggested that PII modification is inhibited by calcium (101), which correlates with the observation that PII is unmodified in heterocysts, which is a requirement for normal nitrogen metabolism in heterocysts (90).

Other genes involved in the regulation of heterocyst development

The *hetF* gene is present in all heterocystous cyanobacteria, but the predicted HetF protein has no similarity to proteins of known function (165). In *N. punctiforme*, *hetF* mutants do not initiate heterocyst development and HetR accumulates non-specifically in all cells, while *hetF* overexpression produces a multiple-contiguous-heterocyst

phenotype but only in the absence of combined nitrogen. HetF is thought to have a role in constraining the accumulation of active HetR protein and increased *hetR* expression to differentiating cells (165).

PatA is a response regulator that contains a CheY-like phosphoacceptor domain at its C-terminus and a newly identified domain, PATAN, at its N-terminus, which may be involved in protein-protein interactions (97, 102). Inactivation of *patA* causes heterocysts to form almost exclusively at the terminal positions of filaments regardless of their length (97). This phenotype is maintained even when *hetR* or $hetR_{R223W}$ is overexpressed, suggesting that *patA* acts downstream *hetR* in the regulatory pathway controlling heterocyst development (20, 97). PatA may influence heterocyst development by attenuating the negative effects of the main inhibitory signals of heterocyst pattern formation, PatS and HetN (126). This could result if PatA modifies or interacts with HetR to make it less sensitive to the inhibitory signals.

The *patB* gene was originally thought to be involved in heterocyst pattern formation because the original mutant had an Mch (multiple contiguous heterocysts) phenotype (98). PatB contains an N-terminal domain with two putative 4Fe-4S centers and a C-terminal domain with a DNA-binding motif. It is now clear that *patB* is required for growth and survival in the absence of combined nitrogen (78). A P_{patB}-*gfp* reporter is expressed in heterocysts 16 h after nitrogen step-down (78). A *patB* frameshift mutant has a delayed Mch phenotype in the absence of combined nitrogen, whereas a deletion

mutant impairs growth and nitrogen fixation within 24 h of combined nitrogen step-down. The pattern formation defect of *patB* mutants is apparently a result of defective heterocyst function because the phenotype is similar to other mutants defective for nitrogen fixation, which have a mild Mch phenotype and decreased numbers of vegetative cells in the intervals between heterocysts (J. Golden, unpublished data).

The *hetC* gene encodes an ABC-type exporter and is required for heterocyst differentiation (82). A P_{hetC}-*gfp* reporter shows increased expression in differentiating cells (82) and *hetC* expression is NtcA-dependent (117). Inactivation of the *hetC* gene blocks heterocyst development at an early stage, resulting in a pattern of weakly fluorescent cells that express a P_{hetR}-*gfp* reporter (170); these cells are still able to divide under certain conditions (170). It is possible that HetC is required for normal morphogenesis of the heterocyst envelope and the absence of HetC results in a regulatory checkpoint that blocks further differentiation.

Overexpression of the *hetL* gene strongly stimulates heterocyst development (99). The predicted HetL protein is composed almost entirely of pentapeptide repeats with a consensus of A(D/N)L*X, where * is a polar amino acid. Pentapeptide repeat proteins may resemble DNA in structure and some members of the family bind to and inhibit DNA gyrase (157). *Anabaena* sp. strain PCC 7120 contains 30 genes encoding proteins that contain this motif. The *hetL* gene was identified because its overexpression suppresses the inhibition of heterocyst differentiation caused by extra copies of PatS.

Overexpression of *hetL* stimulates heterocyst development even in an *ntcA* mutant background; however, the differentiation of the *ntcA* mutant is blocked at an early stage, indicating that NtcA is required for later stages of differentiation (67, 99). Heterocyst development and diazotrophic growth appear to be normal in a *hetL* knockout mutant, showing that HetL is not essential for normal heterocyst development. Further study is needed to determine if HetL is normally involved in heterocyst development or if the overexpression phenotype is caused indirectly, possibly through interactions with other proteins that contain the pentapeptide repeat motif. Based on the characteristics of the pentapeptide repeat family of proteins, HetL might interact with a DNA-binding protein or transcription factor involved in regulating heterocyst development.

The *Anabaena* genome harbors twelve genes that encode putative sigma factors: nine on the chromosome, *sigA* (all5263), *sigB2* (alr3800, previously *sigE*), *sigC* (all1692), *sigD* (alr3810), *sigE* (alr4249, previously *sigF*), *sigF* (all3853), *sigG* (alr3280, previously sigma-E), *sigI* (all2193), and *sigJ* (alr0277, previously sigma-37); and three on plasmids, *sigB* (all7615), *sigB3* (all7608, previously *sigH*), and *sigB4* (all7179, previously *sigG*) (18, 19, 84, 85, 174). The sigma factor nomenclature in *Anabaena* has recently been modified by Yoshimura *et al.* and our own phylogenetic grouping of cyanobacterial sigma factors is in agreement; therefore, we have adopted the suggested nomenclature changes.

In *Anabaena* sp. strain PCC 7120, numerous genes are expressed or upregulated only in differentiating cells at specific times during the course of heterocyst development (106). The ordered sequence of events during heterocyst development may be the result of a hierarchy of transcriptional regulators, possibly including sigma factors, that control expression of different sets of genes at particular times (22, 84). The principal sigma factor encoded by *sigA* is expressed in all cells in the presence or absence of combined nitrogen and it is essential for viability (19, 139). We showed that some combinations of double mutants, such as *sigD sigB2* and *sigB2 sigE*, had deficiencies in establishing diazotrophic growth, but none of the tested alternative sigma factor genes, inactivated individually or in pairs (*sigB*, *sigC*, *sigD*, *sigB2*, and *sigE*), were found to be essential for development (18, 84). However, our recent studies using a *gfp* reporter to follow the expression of eight sigma factor genes found that three, *sigC*, *sigE*, and *sigG*, were upregulated in differentiating cells at different times during heterocyst development.

Later stages of heterocyst differentiation and morphogenesis

Synthesis of heterocyst envelope

Late stages of heterocyst development are characterized by structural changes that include the deposition of three cell layers – an outermost fibrous layer, an envelope polysaccharide layer, and an innermost glycolipid layer. The heterocyst envelope is thought to limit the entry of oxygen into the heterocyst to provide it with a microoxic environment, which is vital for the function of nitrogenase (164). Mutants that lack the envelope polysaccharide or the glycolipid layer are unable to grow aerobically (68, 163).

A cluster of *hep* genes is required for the deposition of the polysaccharide layer (68, 179). The heterocyst-specific glycolipids are composed of fatty alcohols glycosidically linked to sugar residues (42). The *hgl* genes are required for the glycolipid layer and are expressed during the middle stage of differentiation around the time that cells become committed to form heterocysts (11, 12, 25, 39, 43, 68, 92). The *devH* gene encodes a trans-acting regulatory protein required for formation of the glycolipid layer (64, 134).

Metabolic changes and nitrogen fixation

Heterocyst development culminates in the synthesis of nitrogenase and the supply of fixed nitrogen to vegetative cells. During nitrogen fixation, nitrogenase reduces atmospheric nitrogen to ammonia, which is then assimilated into amino acids (33). In addition to synthesizing the nitrogenase enzyme complex, heterocyst differentiation requires changes in the photosynthetic apparatus to stop oxygen production and increased production of enzymes for carbon metabolism to provide ATP and low-potential reductant for nitrogen fixation (164). Compounds from the photosynthetic vegetative cells are transported into heterocysts, probably in the form of sucrose (32), and heterocysts deliver nitrogenous compounds to the vegetative cells, probably in the form of amino acids such as glutamine (45, 164). Large polar cyanophycin granules, which store nitrogen, form near the intercellular junctions of mature heterocysts but their production is not essential for diazotrophic growth (180).

The nitrogen-fixation (*nif*) genes are organized into several operons in *Anabaena* sp. strain PCC 7120. The *nifHDK* operon encodes the structural components of nitrogenase (63) and the *nifB-fdxN-nifS-nifU* operon (112) and other genes (15) are required for assembly of the nitrogenase enzyme complex. Interestingly, a closely related cyanobacterium, *A. variabilis*, contains three different nitrogenase gene clusters, one expressed in heterocysts, one expressed in vegetative cells, and a third that produces a vanadium-dependent nitrogenase (149, 150). Little is known about the mechanisms controlling expression of cyanobacterial *nif* genes. The *nif* genes are expressed late during heterocyst development between 18 and 24 hours after nitrogen step down (54, 57, 63). In *Anabaena* sp. strain PCC 7120, two *nif* operons and the *hupSL* operon are each interrupted by DNA elements whose developmentally regulated site-specific excision is required to recreate the intact operons before the genes can be correctly expressed (28, 56). All three DNA rearrangements occur late during heterocyst development, between 18 and 24 hours after nitrogen step-down (26, 28, 52-54).

Pattern formation and maintenance

The *patS* gene is required for *de novo* pattern formation

Heterocystous cyanobacteria provide an excellent prokaryotic model for studying pattern formation in a multicellular organism because they form a one-dimensional developmental pattern composed of only two cell types, heterocysts and vegetative cells. Regulation of heterocyst frequency and spacing is necessary to ensure an efficient exchange of fixed nitrogen and fixed carbon between vegetative cells and heterocysts

(Fig. 1.2). A long-standing model proposes that heterocyst pattern formation is regulated by lateral inhibition by a diffusible inhibitor originating from differentiating cells that would inhibit differentiation of neighboring cells (106). The PatS peptide fulfills the role this diffusible inhibitor. In *Anabaena* sp. strain PCC 7120, the *patS* gene is predicted to encode a 13- or 17-amino acid-peptide and depending on the start codon chosen *in vivo* (172, 173). The *patS* ortholog in *N. punctiforme* contains only 13 codons (107). Overexpression of *patS* blocks heterocyst development, whereas a *patS* null mutant forms heterocysts even in the presence of nitrate, and forms multiple contiguous heterocysts and short vegetative-cell intervals between heterocysts resulting in about 30% heterocysts after nitrogen step-down (Fig. 1.1C).

The last five carboxy-terminal amino acid residues (RGSGR) of PatS are necessary and sufficient for inhibiting heterocyst development. Mutations in *patS* that affect these residues result in a loss of heterocyst-inhibition activity (172). Minigenes and heterologous genes that encode only these residues inhibit development (169). The corresponding synthetic pentapeptide (PatS-5) inhibits heterocyst development at submicromolar concentrations (172). Addition of the PatS-5 peptide to the growth medium of the *patS* null mutant reduced the frequency of heterocysts but did not restore a normal pattern. However, ectopic expression of *patS* from the heterocyst-specific *hepA* promoter restored normal spacing in a *patS* null mutant. These data suggest that PatS acts as a diffusible inhibitor in a cell nonautonomous manner and that a gradient of the

PatS signal, possibly a processed C-terminal peptide originating from differentiating cells is required to establish a normal pattern (57).

RNA blot analysis and P_{patS}-*lacZ* reporter strains show that *patS* is upregulated early during heterocyst development (172). A P_{patS}-*gfp* reporter strain showed that *patS* expression is localized to individual cells or small groups of cells by 8 to 10 h after nitrogen step-down (173). By 12 to 14 h, fluorescence is confined mostly to individual cells arranged in a pattern resembling that of mature heterocysts, and by 18 h the bright cells are almost exclusively proheterocysts (Fig. 1.1D). These data support a lateral-inhibition model in which a PatS product acts as an intercellular signal generated by differentiating cells to inhibit differentiation of neighboring cells. A normal pattern is not restored in a *patS* mutant when a *patS5* minigene is expressed in differentiating cells, suggesting that the pentapeptide produced by the minigene is confined to the cytoplasm and cannot be exported and function in cell-to-cell signaling (169). One hypothesis is that the full-length or processed PatS is exported from differentiated cells into the periplasmic space, and then taken up by neighboring vegetative cells where it inhibits differentiation. It has been shown that the periplasmic space is continuous and it is believed that it serves as a conduit along cyanobacteria filaments for metabolites and regulatory molecules including a PatS signal (103). The uptake of the PatS signal presumably requires oligopeptide permeases that consist of multisubunit ABC-transporters and periplasmic oligopeptide-binding proteins. Alternatively, metabolite exchange could take place through intercellular connections. Recent findings showed

cytoplasm-to-cytoplasm movement of a small fluorophore in *Anabaena*, which indicates the existence of non-specific intercellular channels that allow metabolite exchange between cells of the filament (113).

Our work with artificial minigenes indicate that the PatS receptor must be cytoplasmic (169). Huang *et al.* have now provided strong evidence that HetR is the PatS receptor (69). They discovered that HetR homodimer is a DNA-binding protein that binds specifically to the promoter regions of the heterocyst-specific genes *hepA*, *patS* and *hetR*, and that the DNA-binding activity is inhibited *in vitro* by the PatS-5 pentapeptide in a dose-dependent manner (69). The heterocyst-pattern defect and insensitivity to PatS produced by the $hetR_{R223W}$ allele provides genetic evidence for HetR being the PatS receptor (83). The observation that simultaneous overexpression of *patS* and *hetR* in a synthetic operon indicates that *patS* acts downstream of *hetR* transcription, which is also consistent with HetR being the PatS receptor (126). These data suggest that the PatS to HetR ratio may be a critical factor in developmental decisions; a high PatS to HetR ratio, enhanced by HetR autodegradation, would be characteristic of vegetative cells where differentiation is inhibited, but it is less clear how HetR remains active in differentiating cells (69).

An important unanswered question is what confers immunity to the differentiating cells against the inhibitory PatS signal? One explanation is that *hetR* upregulation in differentiating cells precedes and controls transcription of *patS*, which results in a high

HetR to PatS ratio in differentiating cells (69). A second possibility is that the full-length PatS peptide is not active immediately upon translation in differentiating cells, and that it requires cleavage or modification before or after export to become active (175). However, the ability of different polypeptides containing the RGSGR motif to inhibit heterocyst development suggests that PatS maturation can occur in these alternative forms or that maturation is not an absolute requirement for activity (169). However, inhibition activity and the ability to establish a normal pattern are not the same, so firm conclusions cannot be made based on the available data. Third, the active PatS concentration may be reduced in differentiating cells by mechanisms related to export or potential modification of PatS in proheterocyst. Fourth, in differentiating cells, the HetR receptor may become insensitive to the PatS signal due to posttranslational modifications, or a heterocyst-specific factor or protein that could interact with HetR to relieve PatS-dependent inhibition.

A recent epistasis analysis of four genes involved in pattern formation in *Anabaena* sp. strain PCC 7120 suggests that PatA has two distinct activities, to promote differentiation as well as to attenuate the negative effects of PatS and HetN on differentiation (126). PatA may be involved in the immunity of differentiation cells to the inhibitory signals by directly interfering with the inhibitory signals themselves or by interacting with HetR to render it insensitive to inhibition.

The *hetN* gene is required for maintenance of the heterocyst pattern

An additional inhibitory signal requires the *hetN* gene, which encodes a protein similar to ketoacyl reductases (14). HetN appears to play a role in the maintenance of heterocyst patterning, as opposed to the role of *patS* in establishing the initial pattern (23). When *hetN* is overexpressed from a copper-inducible promoter, heterocyst development is completely blocked. In the absence of *hetN* expression, filaments develop a normal pattern in the first 24 h after nitrogen step-down, but by 48 h excessive differentiation produces a multiple-contiguous-heterocyst phenotype (23). Immunoblot assays showed a low level of HetN in vegetative cells under non-inducing conditions, and after nitrogen step-down, HetN levels first dropped and then increased, with HetN localized to heterocysts (96). Overexpression of *hetN* blocks the accumulation of HetR protein (96), prevents the patterned expression of a *hetR-gfp* reporter, and also suppresses the multiple-contiguous-heterocyst phenotype caused by overexpression of *hetR* (23). Thus, it is proposed that a putative *hetN*-dependent signal blocks heterocyst development upstream and downstream of *hetR* transcription, possibly by blocking *hetR* positive autoregulation (126). Inactivation of both *patS* and *hetN* results in almost complete differentiation of filaments in the absence of combined nitrogen (16). A nitrogen source of ammonium, but not nitrate, inhibits heterocyst development in the double mutant. After nitrogen step-down from ammonium, the double mutant strain produces a multiple-contiguous-heterocyst phenotype similar to a *patS* mutant followed by further differentiation of nearly all cells. Thus, *patS*- and *hetN*-dependent suppression pathways are the major mechanisms that prevent heterocyst differentiation and influence

patterning (16, 126). However, this conclusion does not rule out a contributing role for the products of nitrogen fixation or other diffusible signals, or internal controls of differentiation related to metabolism or the cell cycle.

In two mutants that undergo almost complete differentiation under nitrogen-limiting conditions, *hetR*$_{R223W}$ and the *patS hetN* double mutant, the process of differentiation is asynchronous and it takes several days for nearly all cells to differentiate (16, 83). This seems to support previous ideas that, at any particular time, not all cells in a filament are equally competent to differentiate into heterocysts. This effect may be due to a regulation based on cell lineage, specific signals that create a preexisting pattern, or differential stores of nitrogen reserves (16, 83, 173). However, it is not yet known what factors influence developmental competence of individual cells within a filament.

Other predicted cell-to-cell communication in cyanobacteria

In addition to the ability to form heterocysts, some cyanobacteria have other developmental alternatives. For example, *N. punctiforme* vegetative cells have three possible developmental alternatives: heterocysts, akinetes, and hormogonia (105, 107). Akinetes are spore-like cells structurally equipped to endure cold and desiccation, and can remain viable for hundreds of years prior to germination (4). Akinetes usually develop when a culture approaches stationary-phase but they can be induce synchronously in a *zwf* mutant strain of *N. punctiforme* following dark incubation in the presence of fructose (9). Some genes that are required for heterocyst development are

also involved in akinete formation such as *hetR* and *hepA* (94, 162). Akinetes form along filaments in different developmental patterns in different cyanobacterial strains (4). They can be found adjacent to heterocysts, at intercalary positions between heterocysts, and in some cases all cells differentiate into akinetes. In the absence of heterocysts, the akinetes form at random positions along the filament, whereas the presence of heterocysts influences akinete placing implying the existence of cell-to-cell communication (1). These developmental patterns may involve signaling mechanisms similar to those found in heterocyst development, but nothing is currently known about the control of akinete developmental pattern formation.

Hormogonia are short differentiated filaments released after fragmentation of the parent filament that often produce gas vesicles and possess gliding motility. These filaments consist of small cells that result from rapid cell division in the absence of cell growth or DNA replication, which is possible because cyanobacteria have multiple genome copies in each cell (106). The role of hormogonia is to disseminate and colonize new portions of a habitat. Eventually, hormogonial filaments return to vegetative growth and can then produce heterocysts (105). The factors inducing hormogonia development seem to be related to changes in nutrient or light parameters under free-living conditions but can be influenced by the plant partner in symbiotic associations (105).

The ability to form symbiotic associations with plants and fungi speaks to the versatility of heterocystous cyanobacteria. *N. punctiforme* can establish symbiosis with the

bryophyte *Anthoceros punctatus*, the angiosperm *Gunnera* spp., and as an intracellular symbiont of the mycorrhizal fungus *Geosiphon piriforme* (105). The symbiotic interaction with the plant partners has a profound effect on the physiology and development of cyanobacteria. Heterocyst frequencies increase several fold, growth and photosynthesis are diminished, and hormogonium development is influenced by the action of extracellular factors produced by the plant (105, 106). It is clear that multicellular prokaryotic organisms such as heterocystous cyanobacteria must be able to integrate extracellular and intracellular signals to display the appropriate response to the environmental changes.

CHAPTER II

SIGMA FACTOR GENES *sigC*, *sigE*, AND *sigG* ARE UPREGULATED IN HETEROCYSTS OF THE CYANOBACTERIUM *Anabaena* SP. STRAIN PCC 7120∗

Introduction

The bacterial RNA polymerase holoenzyme consists of a core enzyme that is competent for transcription and a sigma factor, which is a dissociable subunit required for promoter-specific transcription initiation. Bacteria often possess multiple sigma factors, each with different promoter-recognition specificities. In many cases, a cell can respond to environmental and intracellular signals by selecting specific sigma factors from its repertoire to alter the transcriptional program. Sigma factors are important for transcriptional regulation during development in a number of bacterial genera such as *Bacillus* (10, 87), *Myxococcus* (158), *Streptomyces* (51), and *Caulobacter* (167).

The filamentous cyanobacterium *Anabaena* sp. strain PCC 7120 (hereafter *Anabaena* PCC 7120) responds to nitrogen deprivation by differentiating heterocysts, nitrogen-fixing cells that are semiregularly spaced along filaments of photosynthetic vegetative cells. Some genes are active in either vegetative cells or heterocysts, but others are active in both cell types (162). We and others have hypothesized that sigma factors may play a significant role in directing transcriptional control during heterocyst development in

∗Reprinted with permission from "Sigma Factor Genes *sigC*, *sigE*, and *sigG* are Upregulated in Heterocysts of the Cyanobacterium *Anabaena* sp. strain PCC 7120" by Aldea *et al*., 2007. Journal of Bacteriology, **189:**8392-8396, Copyright [2007] by American Society of Microbiology.

Anabaena PCC 7120; however, clear evidence supporting this hypothesis is still lacking (84).

Sigma factors are classified into two families based on sequence similarity: the σ^{70} and σ^{54} families (61). The σ^{70} family has been divided into four major groups based on phylogeny (61). Group 1 includes sigma factors responsible for the transcription of housekeeping genes, and, thus, they are essential for viability. Group 2 sigma factors are closely related to group 1 based on sequence similarity, but are dispensable under laboratory growth conditions. Group 3 sigma factors are more divergent from group 1 and can often be divided into groups of similar function such as heat shock, general stress responses, motility, and sporulation. Group 4 sigma factors, which were initially considered part of group 3 and called the extracytoplasmic function (ECF) family, are distantly related to the other representatives of the σ^{70} family (65).

The evolutionarily distinct σ^{54} family requires activator proteins and ATP to initiate transcription and includes sigma factors that regulate transcription of genes involved in various processes such as nitrogen fixation, flagellar synthesis, chemotaxis, and development (166). There are no obvious σ^{54} homologs in cyanobacteria or in high-GC, Gram-positive bacteria (143). Another feature that cyanobacteria share with some Gram-positive bacteria such as *Streptomyces* spp. is a relatively large number of group 2 sigma factors (166).

The cyanobacterial sigma factors investigated so far are involved in various processes such as the response to nutrient starvation, post-exponential phase growth, circadian rhythm, and symbiosis (29, 60, 61, 74, 114, 120, 128).

A total of twelve sigma factors, all belonging to the σ^{70} family, have been identified in the genome of *Anabaena* PCC 7120 (85). Here, we have adopted the revised nomenclature proposed by Yoshimura *et al.* (174). Although several sigma factor genes have been studied by reverse genetics in *Anabaena* PCC 7120 (*sigB*, *sigB2*, *sigC*, *sigD*, and *sigE*), their functions still remain obscure and none have been shown to be specifically involved in heterocyst development (18, 57, 84). A recent study has shown that the *Anabaena* PCC 7120 group 3 sigma factor encoded by *sigJ* regulates extracellular polysaccharide biosynthesis and is important for desiccation tolerance (174). The group 1 sigma factor gene, *sigA*, is expressed from two promoters: one is expressed constitutively in both nitrogen-replete and nitrogen-limiting growth conditions, and the other is expressed only during diazotrophic growth (19). The transcription of *sigB* is induced by nitrogen-limiting conditions, whereas that of *sigC* is induced by nitrogen or sulfur limitation (18). However, single and double mutants of *sigB* and *sigC* showed that they are dispensable for diazotrophic growth (18). Inactivation mutants of *sigB2*, *sigD*, or *sigE* can grow on nitrate or diazotrophically, but the *sigB2* and *sigD* mutants were slow to establish diazotrophic growth (84). A *sigB2 sigD* double mutant was able to form proheterocysts but was unable to grow diazotrophically, probably due to extensive fragmentation of filaments upon nitrogen

deprivation; the double mutant was complemented by either *sigB2* or *sigD* (84). The lack of a specific phenotype in any of the single sigma factor mutants suggests that there is some level of functional redundancy among these sigma factors but also that at least some group 2 sigma factors may be partially involved in heterocyst development. Therefore, we decided to study the expression patterns of the different sigma factors in *Anabaena* PCC 7120 filaments after nitrogen step-down as an important step toward elucidating how they might contribute to transcriptional control of developmentally regulated genes.

In the present study, we used *gfp* transcriptional fusions to investigate the developmental regulation of eight *Anabaena* PCC 7120 genes predicted to encode sigma factors: *sigB2*, *sigC*, *sigD*, *sigE*, *sigF*, *sigG*, *sigI*, and *sigJ*. Northern blot experiments showed that *sigC* inactivation caused delayed expression of genes known to be upregulated early during heterocyst differentiation. In a *sigE* mutant strain, *nifH* expression was not present at 24 h, suggesting that SigE regulates *nifH* expression.

Materials and methods

Strains and culture conditions

The strains and plasmids used in this study are presented in Table 2.1. *Anabaena* sp. strain PCC 7120 and its derivatives were grown in BG-11 (containing sodium nitrate) or BG-11_0 (BG-11 lacking sodium nitrate) at 30°C as previously described (54, 135). For derivatives of *Anabaena* PCC 7120 carrying shuttle plasmids, neomycin (25 μg/ml) or

TABLE 2.1. Bacterial strains and plasmids for the sigma factor gene study

Strain/plasmid	Relevant characteristics	Source reference
Anabaena		
PCC 7120	Wild type	R. Haselkorn
AMC1449	PCC 7120 P_{sigC} carried on pAM3648	This study
AMC1450	PCC 7120 P_{sigD} carried on pAM3650	This study
AMC1451	PCC 7120 P_{sigB2} carried on pAM3651	This study
AMC1452	PCC 7120 P_{sigE} carried on pAM3652	This study
AMC1454	PCC 7120 P_{sigF} carried on pAM3653	This study
AMC1455	PCC 7120 P_{sigJ} carried on pAM3654	This study
AMC1456	PCC 7120 P_{sigI} carried on pAM3656	This study
AMC1457	PCC 7120 P_{sigG} carried on pAM3657	This study
AMC645	*sigC* insertional inactivation mutant	(84)
AMC649	*sigE* insertional inactivation mutant	(84)
Plasmids		
pAM1956	Shuttle vector pAM505 containing promoterless *gfpmut2*	(172)
pAM2178	Conjugal suicide vector	(84)
pAM3648	pAM1956 containing 520-bp fragment upstream of *sigC* (all1692)	This study
pAM3650	pAM1956 containing 880-bp fragment upstream of *sigD* (alr3810)	This study
pAM3651	pAM1956 containing 554-bp fragment upstream of *sigB2* (alr3800)	This study
pAM3652	pAM1956 containing 800-bp fragment upstream of *sigE* (alr4249)	This study
pAM3653	pAM1956 containing 888-bp fragment upstream of *sigF* (all3853)	This study
pAM3654	pAM1956 containing 474-bp fragment upstream of *sigJ* (alr0277)	This study
pAM3655	pBluescript II KS+ containing 206-bp fragment upstream of *sigI* (all2193)	This study
pAM3656	pAM1956 containing *sigI* insert from pAM3655	This study
pAM3657	pAM1956 containing 815-bp fragment upstream of *sigG* (alr3280)	This study
pAM3751	pBluescript II KS+ containing 900-bp fragment upstream of *sigF* (all3853)	This study
pAM3952	pAM2178 containing 450-bp fragment internal to *sigG*	This study
pAM4191	pAM2770 containing the *sigC* gene expressed from the *petE* promoter	This study

streptomycin and spectinomycin (2 μg/ml each) were used when strains were grown on BG-11 or BG-11_0 agar plates. For liquid media, the antibiotic concentrations were reduced by half. For heterocyst induction by nitrogen step-down, filaments from an actively growing culture with an optical density at 750 nm of 0.1 to 0.3 were collected by centrifugation and washed three times with sterile water before being transferred to BG-11_0. For strains carrying the copper-inducible *petE* promoter, cells were washed twice by centrifugation and resuspension of the pellet with copper-deficient BG-11 medium before being resuspended in the final growth medium. Activity of the *petE* promoter was induced by transferring the cells to media supplemented with 0.6 μM $CuSO_4$.Plasmids were transferred to *Anabaena* by conjugation from *E. coli* following published protocols (38, 55) with some minor modifications (99).

DNA manipulations

Standard protocols were used for cloning, *E. coli* transformation, and PCR. Blue-white screening of *E. coli* strains was performed on LB (Lennox L broth) plates with 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal). Total DNA from cyanobacterial strains was isolated as previously described (54). Sequencing of plasmid DNA was performed by the Gene Technologies Laboratory (Texas A&M University, College Station, TX) following the Big Dye sequencing protocol (Applied Biosystems) with synthetic oligonucleotide primers (Table 2.2) that were used to amplify the PCR fragments for construction of the reporter plasmids.

TABLE 2.2. PCR primer sequences for the sigma factor gene study

Gene	Sense primer	Sequence (5'→3')	Antisense primer	Sequence (5'→3')
P_{sigC}	AMO -970	CGACCCGGGATTG GTAAGGGGTAATT GG	AMO -971	TCCGGATCCCAATA GATTGCGGTCAGC
P_{sigD}	AMO -1175	TAGGTCGACGCCC GAACTTCTAGAAC ACCAATTCAG	AMO -1176	TAGGAGCTCTAACT GAATAGATGCTCTTT GCCGTTG
P_{sigB2}	AMO -1177	GGAGTCGACTGTG AGAAGTAGGGAGC AGGA	AMO -1178	AGTGAGCTCTGGTG CGTTGTCCTCTACTC CG
P_{sigE}	AMO -1179	AATGTCGACAGTTC TTGAATTTTAAAGA TAAATATTCTTATT GCC	AMO -1180	TTGGAGCTCAATTTC ATGACAACTGTTACT ACTGAGATGG
P_{sigF}	AMO -1149	CTTGAGCTCCAAAG CTGTCTGGCAC	AMO -1150	GTAGTCGACGCACC GCATTTATTAAG
P_{sigJ}	AMO -1386	ACAGTCGACAGTA GCAGTATTACTATA GC	AMO -1387	TGCGAGCTCATAAA AATTCCCTTGTGGTA AC
P_{sigI}	AMO -1151	TGGAGCTCACACC GCCTGACGAATTAT ACCTC	AMO -1152	GGGTCGACGCGATT TTTAACCCATATC
P_{sigG}	AMO -1382	TTGGTCGACTATCC GGAAAAATG	AMO -1383	CGATTGGAGCTCAG ATCGTATGGCG
hepA	AMO -1970	ATTCCCCGATTTTA CGATCC	AMO -1971	GATTCGTGTTGCATC TGGTG
hetR	AMO -1137	CTGCTCGAGGGCA AACTGCTCAAG	AMO -646	TAAGTCCGCTCTTGG TCGTCTG
rnpB	AMO -1972	CTTGCTGGATAACG TCCAGT	AMO -1973	TGTTACCAAACGCCT CTAGC
sigC	AMO -1145	GATCATATGCCAGC AACATCTTTTTACG CAG	AMO -1146	CTACCCGGGATCTA ACTCAGAGATTCCA AATAGT
sigG	AMO -1491	CTGGTCGACGGTTG ATGCGAGG	AMO -1492	TCAGAGCTCGCCTCT CGCAGTTG

Plasmid constructions

Plasmids used in this study are listed in Table 2.1. Plasmids containing transcriptional reporters were constructed by cloning a DNA fragment, PCR-amplified from genomic DNA, containing the upstream-untranslated region of the gene of interest into the shuttle vector pAM1956, which carries a promoterless *gfpmut2* gene (172). The P_{sigC}-*gfp* transcriptional reporter was constructed by first cloning a fragment containing the PCR-amplified upstream region of *sigC* (using forward and reverse primers containing restriction sites SmaI and BamHI, respectively, at their 5' ends) into pBluescript II SK+. Subsequently, a SalI-SacI fragment containing the insert was subcloned into the corresponding sites of pAM1956 to generate pAM3648. For the construction of P_{sigI}-*gfp* and P_{sigF}-*gfp* transcriptional reporters, a fragment containing each upstream region was amplified by PCR 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 moved from pBluescript II KS+ to pAM1956. The P_{sigB2}-*gfp*, P_{sigD}-*gfp*, P_{sigE}-*gfp*, and P_{sigJ}-*gfp* transcriptional fusions were constructed by PCR amplification of the entire non-coding region immediately upstream of each gene using primers containing SalI and SacI restriction sites at their 5' ends and cloning this fragment into pAM1956. For the construction of the suicide vector pAM3952, a fragment containing 450 bp of the *sigG* open reading frame (+33 to +483) was amplified by PCR using forward and reverse primers that contained SacI and SalI sites,

respectively, at their 5' ends; the PCR-generated fragment was cloned into the suicide vector pAM2178. Plasmid constructs were verified by DNA sequencing.

Light and fluorescence microscopy

Fluorescence and bright-field images were captured using an Olympus IX70 inverted microscope (Olympus) with a Hamamatsu OrcaER C4742-95 CCD camera (Hamamatsu) and Simple PCI software version 6.1 (Compix Inc.). The Simple PCI software controlled the camera, and the microscope's light path, ProScan shutters (Prior Scientific), and automated focus. A Piston GFP band-pass filter set (# 41025; Chroma Technology Corp.) was used for fluorescence images.

Time-lapse microscopy

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* PCC 7120 sigma factor-reporter strains were grown in nitrate-containing medium to an OD_{750} 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-11_0. Induced *Anabaena* filaments in 5 to 10 μl BG-11_0 were applied to a BG-11_0 1% agarose pad in a single-chambered coverglass (Lab-Tek chamber slide system; Nalge Nunc International) were prepared as follows. A thin 150-μl pad of BG-11_0 1% agarose was made by placing a slightly trimmed coverglass, with a piece of toothpick glued to the top to serve as a handle, on the molten medium in the chambered

coverglass, and then removing the trimmed coverglass after the agarose cooled. The agarose pad was then carefully surrounded on all four sides by a total of about 2 ml of BG-11_0 1% agarose to maintain moisture in the thin agarose pad. Temperature around the microscope stage was maintained at approximately 30°C. A time-lapse sequence of images with a 10-minute time delay was acquired at 600 × magnification for 26 to 40 h using automated switching between light sources and autofocus. The bright-field illumination 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 hours 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 (Fig. 2.3).

Alcian blue staining

Alcian blue staining was used to detect the heterocyst-specific polysaccharide layer of the proheterocyst envelope. Briefly, an equal volume of *Anabaena* PCC 7120 culture

was mixed with a solution of 0.5% alcian blue (Sigma) in 50% ethanol-water and incubated for 10 min at room temperature before microscopic examination.

RNA isolation

Total RNA was extracted using RiboPure (Ambion) according to the manufacturer's instructions. *Anabaena* PCC 7120 filaments from an exponentially growing culture were inoculated into 100 ml of BG-11 nitrate-containing medium to obtain an OD_{750} of about 0.2. The cells were grown overnight, after which they were washed twice with water and then transferred to 100 ml of BG-11_0. The cells poured over 100 g of ice and collected by centrifugation at 4°C at 4, 8, 12, 18, 24, 36, and 48 h after nitrogen deprivation and the pellet was immediately frozen at -80°C until RNA isolation.

Northern RNA blot analysis

A total of 15 μg of RNA for each sample was denatured and run on a 1.5% denaturing formaldehyde gel in MOPS buffer and transferred to a Magna charge nylon membrane (GE Osmonics) using 10× SSPE (1.8 M NaCl, 100 mM sodium phosphate, pH 7.7, 10 mM EDTA). The blots were hybridized with radioactively labeled DNA probes prepared by random primer labeling and purified on Micro Bio-Spin P-30 columns (Bio-Rad). For probes, fragments amplified by PCR using the corresponding primers (Table 2.2) were labeled with ^{32}P-dCTP using random primer labeling. The hybridization solution contained 5× SSPE, 50% formamide, 0.5% SDS, and 5× Denhardt's solution (0.1% Ficoll, 0.1% polyvinylpyrolidone, 0.1% BSA fraction V) and hybridization was

performed overnight at 42°C. Blots were washed twice for 5 min with 2× SSC (0.3 M NaCl, 30 mM sodium citrate, pH 7.0)-0.1% SDS at room temperature, once for 10 min with 1× SSC-0.1% SDS at 65°C, and once for 10 min with 0.5× SSC-0.1% SDS at 65°C. Blots were exposed to a phosphorimager plate, which was scanned with a Fuji BAS 5000 Phosphorimager (Fuji Photo Co.).

Reverse transcription (RT)-PCR

Total RNA was subjected to DNaseI treatment using RiboPure (Ambion) according to the manufacturer's instructions. Two-microgram aliquots of each RNA sample were reverse-transcribed using the SuperScript II™ reverse transcriptase (Invitrogen) and primers specific for the *hepA* or the *rnpB* genes (Table 2.2) in a reaction volume of 20 μl according to manufacturer's instructions. The *rnpB* gene was used as an internal control. It encodes the subunit of ribonuclease P and is known to be constitutively expressed in *Anabaena* PCC 7120. The absence of DNA was checked by PCR. Two microliters from each cDNA pool were used as PCR template. The linear range of RT-PCR for both primer sets was determined by measuring the amount of PCR product obtained at different cycles (16-30 cycles). Thermal cycling conditions were as follows: 94ºC for 2 min, followed by 22 cycles of 94ºC for 30 s, 52ºC for 30 s, and 72ºC for 45 s, and additional 6 min at 72ºC.

Results

Classification of putative sigma factor genes in *Anabaena* sp. strain PCC 7120

The *Anabaena* PCC 7120 genome harbors twelve genes that encode putative sigma factors, nine on the chromosome: *sigA* (all5263), *sigB2* (alr3800, previously *sigE*), *sigC* (all1692), *sigD* (alr3810), *sigE* (alr4249, previously *sigF*), *sigF* (all3853), *sigG* (alr3280, previously sigma-E), *sigI* (all2193), and *sigJ* (alr0277, previously sigma-37), and three on plasmids: *sigB* (all7615), *sigB3* (all7608, previously *sigH*), and *sigB4* (all7179, previously *sigG*) (84, 174). The sigma factor nomenclature in *Anabaena* PCC 7120 has recently been modified by Yoshimura *et al.* based on phylogenetic analysis of sigma factors in *Anabaena* sp. strain PCC 7120, *A. variabilis* ATCC 29413, *Synechocystis* sp. strain PCC 6803, and *Synechococcus elongatus* PCC 7942 (174). These nomenclature changes were assigned based on analysis of phylogenetic clusters, such that sigma factors falling in one group were given the same name. We had independently constructed a phylogenetic tree from predicted amino acid sequences corresponding to regions 2 and 4 of sigma factors from several cyanobacterial strains: *Anabaena* PCC 7120, *Nostoc punctiforme* PCC 73102, *Synechocystis* sp. strain PCC 6803, *Synechococcus elongatus* PCC 7942, and *Synechococcus* sp. strain PCC 7002 (Fig. 2.1). Our own phylogenetic grouping of cyanobacterial sigma factors agrees with that of Yoshimura *et al.*; therefore, we have adopted the suggested nomenclature changes.

Sequence comparisons and phylogenetic relatedness were used to classify the products of the *sigF* and *sigJ* genes as group 3 sigma factors, which form one cluster. SigF and

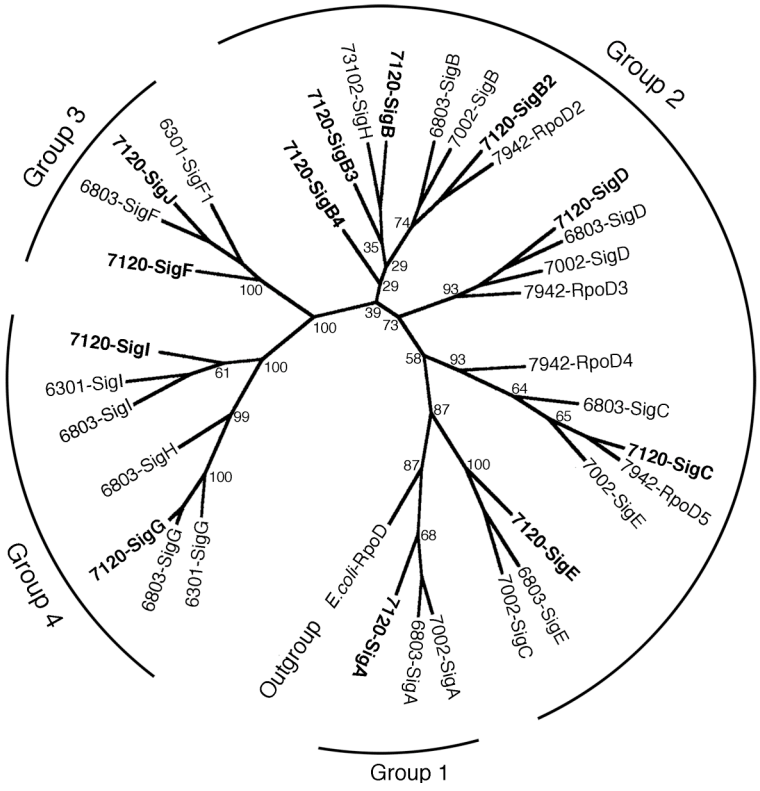

FIG. 2.1. Phylogenetic relationship of group 1, 2, 3, and 4 sigma factors. Sequences originated from *Anabaena* PCC 7120, *N. punctiforme* PCC 73102, *Synechocystis* sp. strain PCC 6803, *Synechococcus elongatus* PCC 7942, and *Synechococcus* sp. strain PCC 7002. The unrooted tree was generated with the PHYLIP software package version 3.65 using the neighbor-joining program to calculate distance matrices from a multiple sequence alignment of amino acid positions corresponding to regions 2 and 4 of the sigma factors produced by ClustalW software. Bootstrap values were obtained with 100 replications. The *E. coli* principal sigma factor, RpoD, was included as outgroup.

SigJ have close homologs in all sequenced cyanobacterial genomes and a SigF homolog has been implicated in high light and salt stress responses, motility, and pilus formation (71). Insertion mutants of these two genes in *Anabaena* PCC 7120 were constructed but their phenotypes did not differ noticeably from the wild-type strain under laboratory growth conditions in BG-11 and BG-11_0 medium, which indicates that they are dispensable for diazotrophic growth and heterocyst development (I. Khudyakov, personal communication).

Cyanobacterial group 4 sigma factors seem to form two clusters and *Anabaena* PCC 7120 has one representative in each cluster: SigG and SigI. These two putative sigma factors have homologs in the non-diazotrophic strain *Synechocystis* PCC 6803 (Fig. 2.1). The *Synechocystis sigG* gene appears to be essential for viability (71).

Attempted insertional inactivation of *sigG*

We used the suicide vector pAM3952, which carries an internal fragment of *sigG*, to attempt to inactivate *sigG* by single-crossover homologous recombination. The suicide vector was introduced into *Anabaena* sp. strain PCC 7120 by conjugation, but an extended period of time (30 days) was necessary for small exconjugants to appear. Conjugation was repeated twice, and each time exconjugants appeared only after 30 days of incubation. From our experience, the average time required for exconjugants (single recombinants) to grow following conjugation is 11-14 days. Thus, it is possible that the *sigG* single recombinant mutants are not able to grow without second-site suppressor

mutations or that, after 30 days, the antibiotic concentration had decreased and allowed growth of partially segregated mutants. After transfer to nitrate-containing medium, the potential single recombinants were not able to grow with streptomycin and spectinomycin concentrations in excess of 0.5 μg/ml (each). These low concentrations of antibiotic provide weak selection for the integrated suicide plasmid and, thus, presumably allowed growth of these strains due to incomplete segregation. This was tested with PCR assays, which easily detected the presence of the wild-type *sigG* gene and confirmed incomplete segregation. As mentioned above, the *sigG* homolog in *Synechocystis* PCC 6803 (*sigG*) was found to be essential for viability and our results suggest that *sigG* is essential for viability in *Anabaena* PCC 7120.

Reporter strains containing P_{sigB2}-*gfp*, P_{sigD}-*gfp*, P_{sigI}-*gfp*, and P_{sigJ}-*gfp* showed GFP reporter fluorescence in vegetative cells and heterocysts

The shuttle vector pAM1956, which carries a promoterless *gfpmut2* gene, was used to construct transcriptional fusions to the upstream regions for each of the sigma factor genes, except *sigA*, located on the chromosome of *Anabaena* PCC 7120: *sigB2*, *sigC*, *sigD*, *sigE*, *sigF*, *sigG*, *sigI*, and *sigJ*. The plasmid pAM1956 is based on a low- to medium-copy-number shuttle vector containing the cyanobacterial origin of replication from the *Nostoc* sp. strain PCC 7524 plasmid pDU1 (93, 173). 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. 2.2). In nitrate-grown cultures, the GFP fluorescence intensity varied in the vegetative cells along filaments such that groups of cells that showed lower expression tended to alternate with groups of cells producing stronger intensity (Fig. 2.2). 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. 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 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 75% of the heterocysts displayed higher fluorescence intensity compared to 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. In all four strains mentioned above, GFP fluorescence decreased in older heterocysts. The P_{sigI}-*gfp* reporter strain underwent extensive fragmentation when maintained in medium without a source of combined nitrogen for more than a month. This phenotype may be the result of altered expression of the endogenous *sigI* gene caused by the extra-copies of the promoter region present on the shuttle vector, which, for example, could be titrating out a regulatory factor.

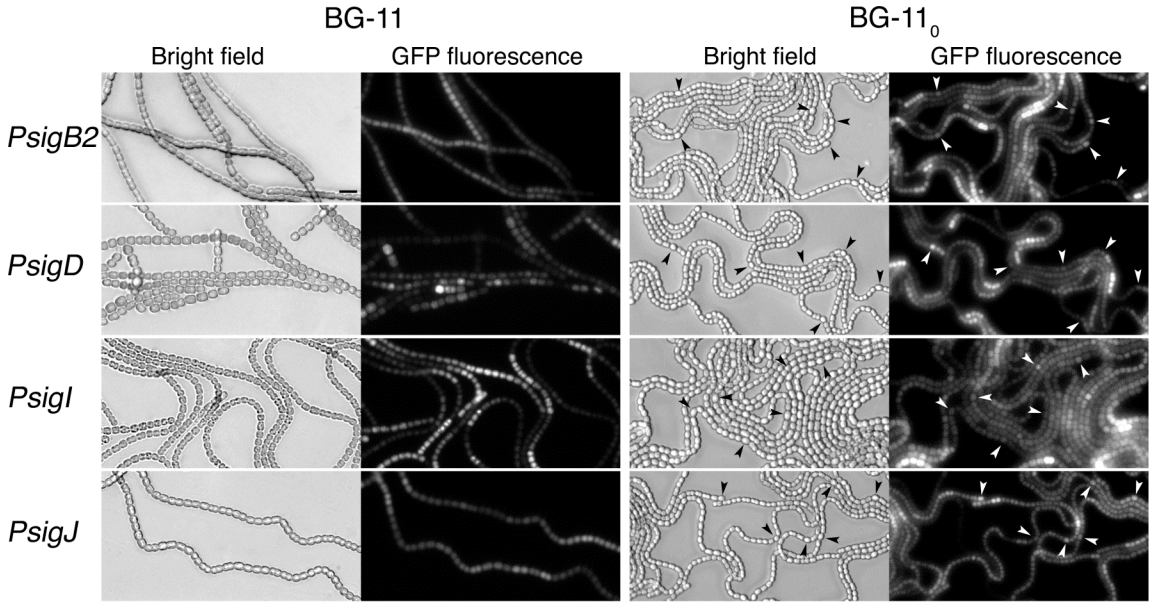

FIG. 2.2. GFP reporter fluorescence from strains containing *gfpmut2* expressed from promoters of the *sigB2*, *sigD*, *sigJ*, and *sigI* sigma factor genes in *Anabaena* sp. strain PCC 7120. Filaments grown in nitrate-containing BG-11 medium or at 24 h after nitrogen step-down to BG-11_0 medium to induce heterocyst development are shown. Arrowheads indicate mature heterocysts. (Left panels) Bright-field images. (Right panels) Corresponding GFP fluorescence images. Scale bar, 10 μm.

The P_{sigF}-*gfp* reporter strain did not show detectable GFP fluorescence when grown in medium with or without a source of combined nitrogen.

Reporter strains containing P_{sigC}-*gfp*, P_{sigE}-*gfp*, and P_{sigG}-*gfp* showed increased GFP fluorescence in heterocysts after nitrogen step-down

Of the eight sigma factor genes included in this study, the *sigC*, *sigE*, and *sigG* reporter strains showed GFP reporter expression primarily in differentiating cells 24 h after nitrogen step-down, which suggests that these three sigma factors may be important for normal heterocyst development. When grown diazotrophically for an extended time in medium lacking combined nitrogen, filaments of these three reporter strains maintained a heterocyst-specific GFP fluorescence pattern similar to the initial pattern produced 24 h after nitrogen step-down.

The relative levels and pattern of GFP fluorescence in vegetative cells of nitrate-grown cultures varied among these three reporter strains. The P_{sigC}-*gfp* reporter strain often had a small number of bright cells along filaments of mostly very dim cells (Fig. 2.3A). The P_{sigE}-*gfp* reporter strain displayed a more uniform low level of fluorescence in vegetative-cell filaments (Fig. 2.3B). The P_{sigG}-*gfp* reporter strain showed a higher and less uniform level of fluorescence in vegetative cells of nitrate-grown filaments, and immediately following nitrogen step-down (Fig. 2.3C).

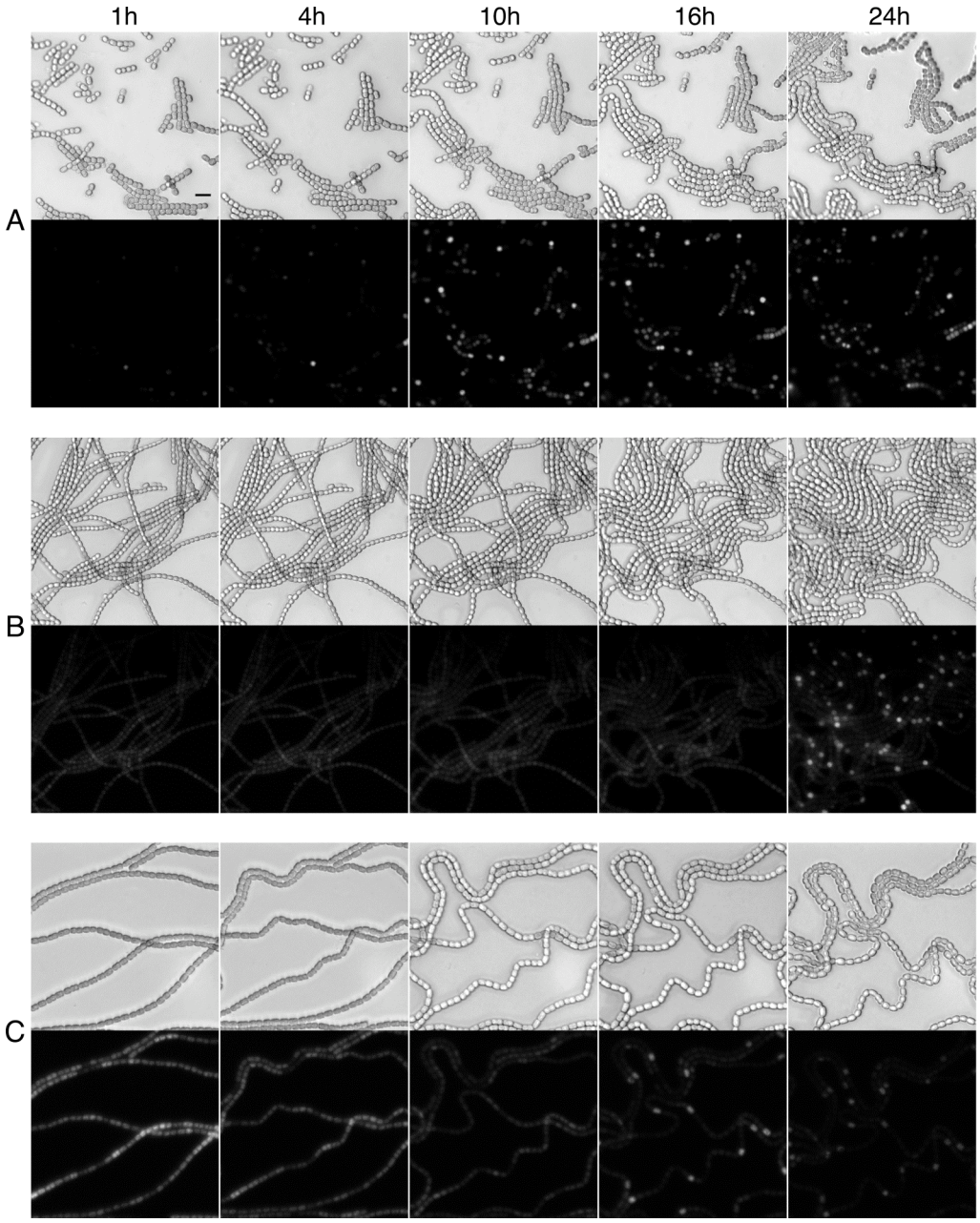

FIG. 2.3. Temporal and spatial GFP reporter fluorescence from promoters of sigma factor genes *sigC* (A), *sigE* (B), and *sigG* (C). (Upper panels) Bright field images. (Lower panels) Corresponding GFP fluorescence images. The strains were grown in nitrate-containing BG-11 medium and then transferred to BG-11_0 medium to induce heterocyst development. Selected time-lapse images are shown for the indicated times post induction. The *sigC* reporter strain filaments 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 μm.

We used time-lapse fluorescence microscopy to investigate the spatial and temporal patterns of GFP reporter expression for *sigC*, *sigE*, and *sigG*. For time-lapse microscopy, nitrate-grown filaments were washed and transferred to BG-11_0 medium without a source of combined nitrogen to induce heterocyst development, and images were acquired every 10 minutes for at least 24 h. Figure 2 contains images corresponding to the indicated time-points that were extracted from the time-lapse series.

At 1 h following nitrogen step-down, P_{sigC}-*gfp* expression was relatively unchanged compared to nitrate-grown filaments (Fig. 2.3A). At 4 h, individual cells, but also pairs of cells and dividing cells, showed increased GFP fluorescence. 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 (171), about 12% of the cells showed increased GFP fluorescence, of which about half were arranged in a pattern similar to that of mature heterocysts. Dividing bright cells maintained the original fluorescence levels, but after division only one daughter cell increased its fluorescence. After 10 h, other individual cells but also pairs and small clusters of about 4 cells began to fluoresce. By 24 h, most individual cells that showed the strongest early expression became heterocysts, and, subsequently, mature heterocysts became dimmer. Previous observations showed that *sigC* mRNA is synthesized by 6 h after nitrogen step-down, then peaks at 12 h followed by a drop to lower levels (18), which correlates with the dimming GFP reporter fluorescence in mature heterocysts. Also, the P_{sigC}-*gfp* reporter strain maintained in media containing nitrate often showed increased GFP

fluorescence in cells at the end of filaments. This phenomenon was observed in actively growing cells, but it was more evident in filaments from older cultures.

Time-lapse microscopy of the P_{sigE}-*gfp* strain showed no change in fluorescence levels until around 16 h after nitrogen step-down, when individual differentiating cells displayed increased fluorescence. In the following two 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. By 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 heterocyst-specific genes such as the *nif* genes that encode nitrogenase.

For the P_{sigG}-*gfp* reporter strain, after nitrogen step-down, vegetative cells were initially brighter than for the *sigC* and *sigE* reporter strains, but by 4 h after induction there was a marked decrease in GFP fluorescence. This drop continued in most of the cells along filaments, but by 10 h some individual cells showed slightly increased GFP fluorescence. At 11 h, the GFP fluorescence was localized almost exclusively to presumptive differentiating cells, although proheterocysts were not morphologically distinguishable at this time. By 16 h, proheterocyst showing GFP fluorescence were present. At 24 h, the 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 complete the differentiation process (171), suggesting that SigG could be involved in the mechanism of commitment.

Phenotype of the *sigC* mutant strain

The *sigC* gene was previously inactivated by our laboratory using single-crossover homologous recombination, but the *sigC* mutant strain (AMC645) was not fully characterized. Although inactivation of *sigC* was not essential for diazotrophic growth, we found a significant delay in heterocyst development. No morphological signs of differentiation were visible until around 40-48 h following nitrogen deprivation, whereas the wild-type strain formed heterocysts at 24 h. The heterocyst pattern in filaments of the *sigC* mutant was similar to the wild-type pattern. To determine if the mutant phenotype is indeed caused by inactivation of *sigC*, and not a second site mutation, we introduced a shuttle plasmid carrying the *sigC* gene expressed from the *petE* promoter into the mutant strain. The mutant phenotype was fully complemented when the s*igC* mutant strain containing the overexpression plasmid was grown in media containing 0.6 μM copper sulfate.

Expression of early genes required for heterocyst development is delayed and reduced in the absence of the *sigC* gene

Northern blot hybridization experiments showed that in the wild-type strain the *sigC* gene is expressed at 4 h after nitrogen step-down, 2 h earlier than it was initially shown to be upregulated (Fig. 2.4) (18). The GFP fluorescence pattern of the P_{sigC}-*gfp* reporter

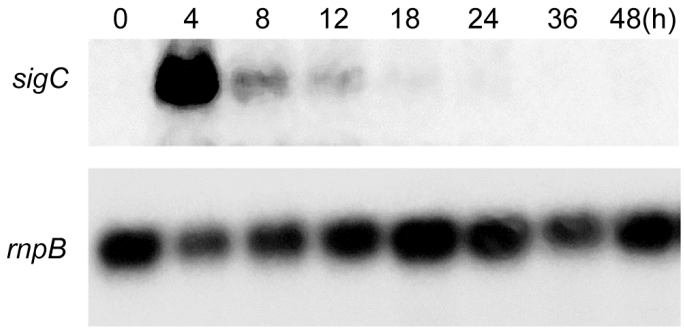

FIG. 2.4. Expression of the *sigC* gene increased at 4 h after nitrogen step-down and then gradually decreased. Total RNA was isolated from the wild type and the *sigC* mutant strain at 4, 8, 12, 18, 24, 36, and 48 h after nitrogen step-down. For the 0 h time-point, RNA was isolated from nitrate-grown cultures prior to nitrogen step-down. Northern RNA blots were hybridized with a labeled *sigC* probe, and an *rnpB* probe was used as a control for sample loading and transfer. These data are representative of three independent experiments.

strain also suggested an early increase of *sigC* expression in differentiating cells. Due to the timing of *sigC* upregulation after nitrogen step-down, we hypothesized that the SigC sigma factor associates with RNA polymerase to transcribe genes during the early stage of heterocyst development. The delayed heterocyst development observed in the *sigC* mutant strain supports this hypothesis. We used northern blot hybridization experiments or RT-PCR to examine expression of *hetR*, which is the earliest gene known to be involved in heterocyst development, as well as expression of *hepA* and *hepC*, which are involved in synthesis of the polysaccharide layer and known to be upregulated at 5-9 h after nitrogen step-down (Fig. 2.5). Two major transcripts are known to be produced for the *hetR* gene with a length of 1.4 kb and 1.9 kb. We detected both of these transcripts in the wild-type and the *sigC* mutant strain, but upregulation of *hetR* expression in the mutant strain showed a 20-h delay (Fig. 2.5). The *hepC* gene was upregulated at 8 h after nitrogen deprivation in the wild type.

Two transcripts were detected for *hepC* at 1.3 kb and 1 kb, although a previous study mentioned the presence of only one transcript of an undetermined size {Koksharova, 2002 #118}. Expression of the 1-kb transcript began to increase at 4 h after nitrogen step-down and peaked at 12 h, after which it gradually decreased (Fig. 2.5). The second transcript was also present at 4 h although not as abundantly as the larger transcript and its expression peaked at 12 h as well. In the mutant strain, the two transcripts began to increase only at 18 h following nitrogen step-down. Abundance of the 1.3-kb transcript peaked at 24 h, but was almost four times less abundant than the wild-type levels (Fig.

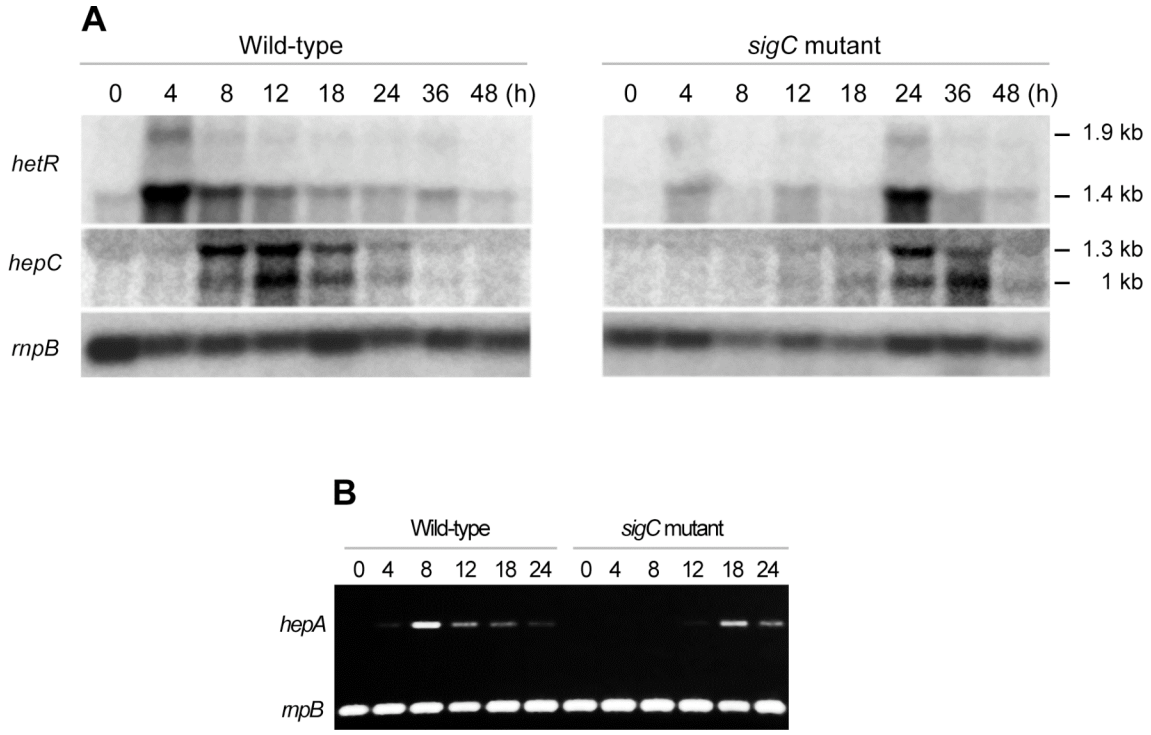

FIG. 2.5. The *sigC* mutant strain showed delayed and reduced amounts of *hetR*, *hepC*, and *hepA* transcripts. (A) Total RNA was isolated from the wild type and the *sigC* mutant strain at 4, 8, 12, 18, 24, 36, and 48 h after nitrogen step-down. For the 0 h time-point, RNA was isolated from nitrate-grown cultures prior to nitrogen step-down. Northern RNA blots were hybridized with labeled probes for *hetR* or *hepC*, and an *rnpB* probe was used as a control for sample loading and transfer. These data are representative of three independent experiments. (B) *hepA* transcript levels for the wild type and the *sigC* mutant strain at 4, 8, 12, 18, and 24 h after nitrogen step-down as measured by semiquantitative RT-PCR. For the 0 h time-point, RNA was isolated from nitrate-grown cultures. The *rnpB* transcript level was measured as a control for equal loading of the agarose gel wells. These data are representative of two independent experiments.

2.6). The 1-kb transcript levels peaked at 36 h, but were reduced to half of the wild type levels. Expression of *hepA* was under detection level by northern blot hybridization, thus we used a more sensitive approach for *hepA* transcript detection, RT-PCR.

The *hepA* gene showed a pattern of expression similar to that of *hep C* (Fig. 2.5). In the wild-type background, *hepA* was present at 4 h, and showed peak abundance at 8 h following nitrogen deprivation, after which it gradually decreased. In the *sigC* mutant background, *hepA* mRNA was not detectable until 12 h after nitrogen step-down and peak abundance was at 18 h, although not as great as in the wild-type background. Because we detected delayed and reduced expression of *hepA* and *hepC*, two genes that are required for synthesis of the polysaccharide layer, we sought to determine whether the first round of heterocysts formed by the *sigC* mutant strain were able to synthesize the polysaccharide layer. Alcian blue staining showed that the polysaccharide layer was present. Overexpression of *sigC* from the copper-inducible promoter *petE* did not alter the pattern of heterocysts, heterocyst morphology, or diazotrophic growth (data not shown).

Expression of *nifH* is delayed in the *sigE* mutant

Time-lapse microscopy of the P_{sigE}-*gfp* strain suggests that *sigE* is expressed around 16 h following heterocyst development. At this late stage during heterocyst development the heterocysts increase expression of *nifH* in preparation for nitrogen fixation. Using

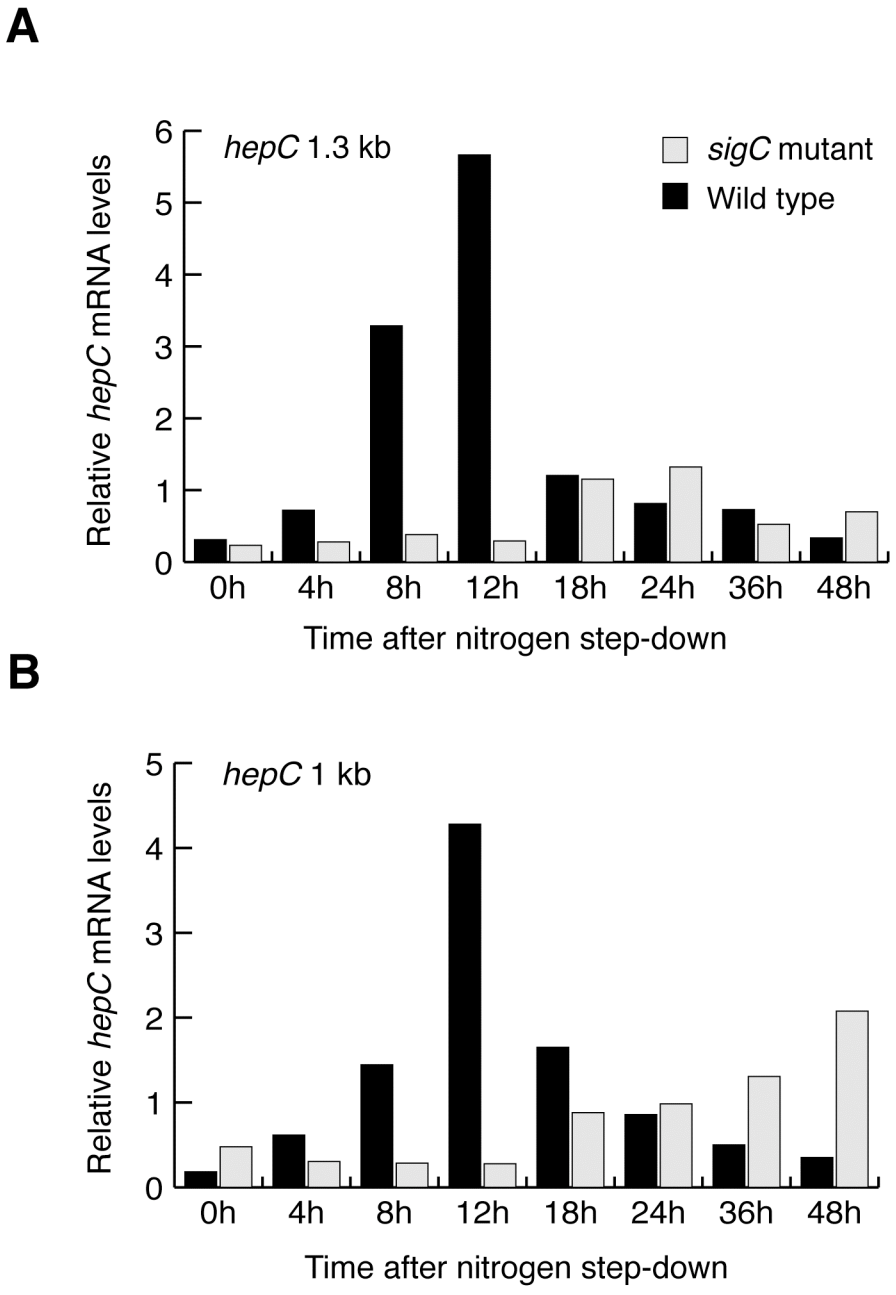

FIG. 2.6. Abundance of *hepC* transcript levels was reduced in the *sigC* mutant compared to the wild type. Densitometry of *hepC* mRNA levels in the wild-type strain and the *sigC* mutant strain at several time points after nitrogen step-down. The graph values are an average of two independent experiments. (A) Abundance for the 1.3-kb *hepC* transcript. (B) Abundance for the 1-kb *hepC* transcript.

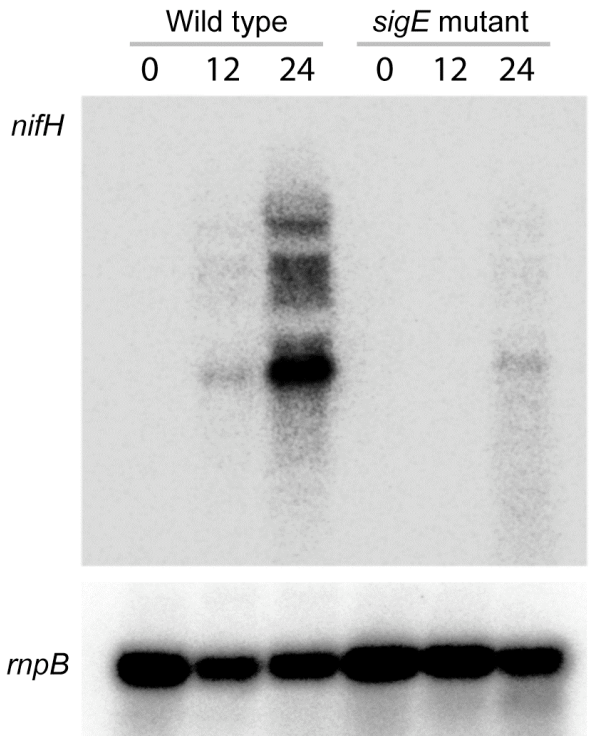

FIG. 2.7. The amount of *nifH* mRNA is considerably reduced in the *sigE* mutant strain compared to wild type. Total RNA was isolated from the wild type and the *sigE* mutant strain at 12 and 24 h after nitrogen step-down. For the 0 h time-point, RNA was isolated from nitrate-grown cultures prior to nitrogen step-down. Northern RNA blots were hybridized with labeled *nifH* probe, and an *rnpB* probe was used as a control for sample loading and transfer. These data are representative of two independent experiments.

northern blot hybridization experiments we found that *nifH* expression is delayed and greatly reduced in the *sigE* mutant compared to wild type (Fig. 2.7).

Discussion

Regulation of transcription initiation is one of the major forms of gene control in bacteria. Sigma factors associate with RNA polymerase to initiate transcription at specific promoters. The use of alternative sigma factors is important for the regulation of bacterial development by activating particular sets of genes in a specific spatial and temporal fashion. There are twelve putative sigma factor genes in the genome of *Anabaena* PCC 7120. It would be surprising if heterocystous cyanobacteria did not use this regulatory strategy during heterocyst differentiation, but reverse genetics experiments have failed to produce unambiguous results (18, 84). These previous studies found that none of the group 2 sigma factors *sigB*, *sigB2*, *sigC*, *sigD*, or *sigE* was individually required for heterocyst development. These results were interpreted to suggest that there may be functional overlap between the sigma factors, whereby two or more sigma factors recognize and initiate transcription from the same promoter (84).

Even though individual sigma factors may not be essential for heterocyst development, we hypothesized that certain sigma factors could be specifically involved in some aspects of the process. Furthermore, we hypothesized that the expression of the genes encoding sigma factors involved in heterocyst development would be developmentally regulated. In order to test this hypothesis, we used *gfp* transcriptional fusions to analyze

the spatial and temporal pattern of expression for all putative sigma factor genes located on the chromosome except *sigA*, which encodes the group 1 housekeeping sigma factor. The genes examined were: *sigB2*, *sigC*, *sigD*, *sigE*, *sigF*, *sigG*, *sigI*, and *sigJ*. For each gene, the upstream noncoding region was fused to a promoterless *gfpmut2* gene on the shuttle vector pAM1956. For these studies aimed at determining the temporal and special patterns of expression, we chose to place the reporter constructs on a low- to medium-copy-number shuttle plasmid to improve our chance of obtaining detectable levels of GFP fluorescence from all of the promoters. Overall, we found that three sigma factor genes had heterocyst-specific expression 24 h after induction of heterocyst development: two group 2 sigma factors, *sigC* and *sigE*, and one representative of group 4, *sigG*.

The vegetative cells of the P_{sigB2}-*gfp*, P_{sigD}-*gfp*, P_{sigI}-*gfp*, and P_{sigJ}-*gfp* reporter strains showed an irregular pattern of GFP fluorescence with clusters of brighter cells alternating with dimmer cells; this patchy distribution of fluorescence was observed regardless of the presence or absence of a source of combined nitrogen. We do not know what underlies this phenomenon, but we could speculate that the clusters of cells showing similar GFP fluorescence 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 possibly affect the copy number of the plasmid carrying the reporter fusion. All four of these reporter strains also showed at least some GFP fluorescence in heterocysts as well. Previous studies showed that *sigB2* and *sigD*

mutants had an abnormally slow transition to diazotrophic growth (84). The reporter-strain results together with the fragmentation phenotype and the transient impairment in establishing diazotrophic growth caused by inactivation of both of these sigma factors suggest that they may function in vegetative cells and heterocysts.

After nitrogen step-down, the P_{sigC}-*gfp*, P_{sigE}-*gfp*, and P_{sigG}-*gfp* reporter strains showed GFP fluorescence localized to heterocysts. Using time-lapse fluorescence microscopy, we determined the developmental regulation of the sigma-factor promoters. Based on their expression patterns during the course of cell differentiation, the *sigC*, *sigG*, and *sigE* sigma factor genes could be classified as early, middle, and late stage, respectively.

The time of expression of *sigC* indicates its involvement in the early stages of heterocyst development. Inactivation of *sigC* delays heterocyst development by at least 24 hours (84); however, *sigC* inactivation does not block heterocyst development or nitrogen fixation (18). 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* (4 h), *devH* (6 h), *patS* (6 h), *patA* (3-6 h), *patB* (3-6 h), and genes involved in the formation of the polysaccharide (*hepA*, *hepB*, *hepC*, *hepK*) and glycolipid (*hglC*, *hglD*, *hglE*, *hglK*, *hglB*, *hetN*, *hetI*) envelope layers, which are expressed within the 4 to 7 h interval after nitrogen step-down (2, 57). We examined expression of *hetR*, *hepA*, and *hepC* in the *sigC* mutant background and found that these early genes are indeed delayed and reduced in the absence of SigC, suggesting that these

early genes are indeed regulated by SigC. It remains to be determined whether SigC directly regulates expression of these genes. Overexpression of *sigC* did not affect heterocyst development. It is possible that the RNA polymerase that associates with SigC works in concert with a transcriptional activator to initiate expression, and that absence of this activator prevents expression of the target genes even if the sigma factor is present at a high concentration.

Interestingly, patterned expression of *sigC* was sometimes observed in nitrate-grown filaments before nitrogen step-down (unpublished results). This phenomenon is not always evident and further study is needed to determine the growth conditions that are favorable for patterned expression of *sigC* in nitrate-grown filaments. The individual cells that showed preexisting GFP fluorescence at the time of nitrogen step-down often differentiated into heterocysts. Thus, our observations suggest the presence of a partial pre-pattern under conditions that suppress heterocyst development.

The P_{sigG}-*gfp* reporter strain showed increased GFP fluorescence in developing cells around 9 h after nitrogen step-down (Fig. 2.3C), which suggests an involvement of SigG in the expression of middle stage genes. This is a crucial time at which differentiating cells commit to becoming heterocysts (171). During this stage, 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 (155).

The P_{sigE}-*gfp* reporter strain displayed a basal level of fluorescence until about 16 h after nitrogen step-down, when increased fluorescence localized exclusively to proheterocyst cells that later differentiated into heterocysts (Fig. 2.3B). Thus, the *sigE* reporter is expressed during the late stage of heterocyst differentiation when the cells are become micro-oxic, suggesting that the expression of *sigE* could be related to the reduced oxygen tension and that SigE could play an important role in transcriptional regulation of late genes. The main potential target genes for SigE during the late stages of differentiation are the nitrogen-fixation (*nif*) genes, which are expressed between 18 h and 24 h after nitrogen deprivation (57). We found that expression of the *nifH* gene in the *sigE* mutant strain was indeed delayed, which suggests that SigE is involved in adequate expression of genes known to be upregulated late during heterocyst differentiation. Although insertional inactivation of *sigE* did not block heterocyst differentiation or diazotrophic growth (84), this could be due to overlapping function among sigma factors, as previously mentioned.

Our results raise the question of what mechanisms are involved in the regulation of the sigma factor genes. For example, how are *sigC* and *sigE* upregulated during heterocyst differentiation, and what regulates the change in *sigG* expression from being strong in vegetative cells of nitrate-grown filaments to being expressed exclusively in heterocysts

after nitrogen step-down? A comparative genomics study of NtcA regulons in cyanobacteria found that six *Anabaena* PCC 7120 sigma factor genes, *sigA*, *sigB2*, *sigD*, *sigE*, *sigF*, and *sigG*, may be regulated by NtcA because their promoter regions bear an NtcA-binding sequence (144). Lemeille *et al.* have explored the regulatory relationships between sigma factors in the unicellular cyanobacterium *Synechocystis* PCC 6803 and they found interdependent regulation of the sigma factor genes (95). In *Synechocystis* PCC 6803, group 2 sigma factors SigC and SigB (SigB2 in *Anabaena* PCC 7120) contribute to growth phase-dependent expression of NtcA-dependent genes including *sigE* (*sigE* in *Anabaena* PCC 7120) (95). We expect that multiple mechanisms are involved in the regulation of sigma factor genes during heterocyst development.

Our results indicate that three sigma factors are specifically upregulated in the heterocysts of *Anabaena* 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 the reason group 2 sigma factors are not essential for heterocyst differentiation and nitrogen fixation is because they have at least partial overlapping promoter specificity, such that, in the absence of a sigma factor that normally controls the expression of a particular set of genes, other sigma factors can provide at least partial transcriptional control of those genes. An example of overlapping promoter specificity is found in *S. elongatus* PCC 7942, where group 1 and group 2 sigma factors can bind to and regulate transcription from the same promoter regions, although they do show preference for particular promoters.

Furthermore, these different sigma factors are also able to recognize canonical *E. coli* promoters (59). Goto-Seki *et al.* postulated that promoter-specificity crosstalk is a common characteristic among bacterial group 1 and group 2 sigma factors. Similarly, overlapping promoter specificity has also been described for *Synechocystis* sp. strain PCC 6803 (73, 74).

Summary

The genome of the heterocyst-forming cyanobacterium *Anabaena* sp. strain PCC 7120 contains twelve genes predicted to encode sigma factors. The temporal and spatial patterns of expression from all chromosomal sigma factor genes except *sigA*, which encodes the primary sigma factor, were studied by using shuttle vectors carrying transcriptional fusions of each gene's upstream region driving expression of a *gfp* reporter gene. On nitrate-containing medium, which suppresses heterocyst development, the P_{sigB2}-*gfp*, P_{sigC} -*gfp*, P_{sigD}-*gfp*, P_{sigE}-*gfp*, P_{sigG}-*gfp*, P_{sigI}-*gfp*, and P_{sigJ}-*gfp* reporter strains showed various levels of GFP fluorescence. The P_{sigF}-*gfp* reporter strain did not have detectable activity in cells grown either with or without nitrate. After inducing heterocyst development by shifting filaments to medium lacking combined nitrogen (nitrogen step-down), the P_{sigC}-*gfp*, P_{sigE}-*gfp*, and P_{sigG}-*gfp* reporter strains showed an increase in GFP fluorescence that was localized to heterocysts, and decreased fluorescence in vegetative cells. The remaining sigma factor genes did not show heterocyst-specific upregulation. Time-lapse microscopy of the P_{sigC}-*gfp* reporter strain showed that expression from the *sigC* promoter increased at 3-4 h after nitrogen step-down in individual cells.

Fluorescence of the P_{sigG}-*gfp* reporter strain was strong in vegetative cells immediately following nitrogen step-down, but within 4 h, the fluorescence in vegetative cells decreased to a low level and by 9 h, cells in a pattern resembling that of mature heterocysts showed strong fluorescence. The P_{sigE}-*gfp* reporter strain showed an overall low level of fluorescence until about 16 h after nitrogen step-down, followed by increased fluorescence localized to differentiating cells.

CHAPTER III

Anabaena SP. STRAIN PCC 7120 GENE all2874 ENCODES A DIGUANYLATE CYCLASE AND IS REQUIRED FOR NORMAL HETEROCYST DEVELOPMENT UNDER HIGH LIGHT GROWTH CONDITIONS

Introduction

Cyclic nucleotides are commonly used as second messengers in signal transduction networks of both prokaryotes and eukaryotes. Cyclic-di-GMP is a bacterial second messenger that has been recognized as an important regulatory molecule in diverse bacteria (76). It was discovered 20 years ago in *Acetobacter xylinum*, where it functions as a positive allosteric regulator of cellulose synthase (138). Recent studies have shown that c-di-GMP is involved in the regulation of a variety of complex physiological processes including production of exopolysaccharides, biofilm formation, motility, virulence, and cellular differentiation (76, 146). The intracellular concentration of c-di-GMP is controlled by two enzymes with opposing activity: diguanylate cyclase and phosphodiesterase, which catalyze c-di-GMP synthesis and breakdown, respectively. Diguanylate cyclase activity is associated with the conserved GGDEF domain, whereas phosphodiesterase activity is associated with the conserved EAL or HD-GYP domains, named after the conserved sequence motifs that are present in the active sites (146). The GGDEF and EAL domains are two of the largest superfamilies of output domains found in bacterial one- and two-component signal transduction systems (137). These two domains are often found on the same protein, which could then potentially act as a

bifunctional enzyme with opposing activities, although some studies suggest that only one of the domains may be active in some proteins.

Analysis of the domain architecture of proteins containing the GGDEF and/or EAL domains in sequenced bacterial genomes show that these domains are typically coupled with a variety of sensory input domains, suggesting that their activities are regulated by diverse environmental or intracellular signals (136). Some of the signals perceived by c-di-GMP signaling pathways include oxygen, blue light, red light, nutrient starvation, antibiotics, bile salts, intercellular signaling molecules, and mucin (76).

In cyanobacteria, the GGDEF domain is coupled with various signaling domains including light sensing domains such as the BLUF domain or those belonging to the PAS and GAF families. Certain photosynthetic bacteria possess photoreceptors known as bacteriophytochromes that sense red/far red light using the biliverdin chromophore (148). Most bacteriophytochromes work as photoactivated protein kinases, but several examples of atypical bacteriophytochromes harboring GGDEF and EAL domains have recently emerged. BphG1, a bacteriophytochrome from the anoxygenic phototrophic bacterium *Rhodobacter sphaeroides* 2.4.1 that contains GGDEF and EAL domains, has been shown to act as a bifunctional enzyme in which the diguanylate cyclase activity, but not the phosphodiesterase activity, is light-dependent (148). The cyanobacterial phytochrome Cph2 from *Synechocystis* sp. strain PCC 6803 contains GGDEF and EAL output domains and was shown to covalently attach bilin; its absence stimulated

movement towards blue light (161) and altered the light-dependent expression of numerous downstream genes (70). In *Anabaena* sp. strain PCC 7120, proteins encoded by all2875 and alr3170 were found to function as blue-light sensors; the latter gene is predicted to encode both GGDEF and EAL domains, but their activity has not been studied (122). In *Synechococcus elongatus* (PCC 7942) the PsfR protein, which contains a GGDEF domain, regulates the activity of the *psbAI* gene, which encodes the D1 protein of the photosystem II reaction center (151).

It is not yet well understood how c-di-GMP mediates its regulatory effects on downstream physiological processes. The recent *in silico* identification of the putative c-di-GMP-binding PilZ domain reported by Amikam and Galperin (8) has been followed by experimental evidence demonstrating c-di-GMP binding by PilZ-domain proteins from several bacteria. These c-di-GMP sensing proteins have been shown to regulate biofilm formation, motility, and virulence (146). Bioinformatic analysis of currently available genomes revealed that the majority of cyanobacteria do not harbor the PilZ domain (8). There are no PilZ domains in *Anabaena* sp. strain PCC 7120.

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 usually positioned every 10 to 15 cells along filaments of photosynthetic vegetative cells. Heterocysts provide a microoxic environment for nitrogenase through changes in their physiology and morphology, including production

of a thickened envelope consisting of inner glycolipid and outer polysaccharide layers (162). The O_2-producing photosystem II is dismantled during heterocyst differentiation and respiration is increased to consume oxygen that enters the cell. Numerous genes have been identified that are involved in heterocyst development and/or function. HetR is a positive regulatory factor that is essential for heterocyst development (13, 20, 83). The *hetR* gene is expressed early in differentiating cells after nitrogen step-down. HetR has two known activities, autoproteolysis and specific DNA-binding that requires formation of a homodimer (69). Increased HetR levels or activity is sufficient to force heterocyst differentiation. Overexpression of *hetR* on a multicopy plasmid either from its native promoter or from the copper-regulated *petE* promoter leads to increased heterocyst frequency regardless of the presence of nitrate or ammonium (20). Overexpression of a mutant allele of *hetR* (*hetR*$_{R223W}$) is able to bypass the main inhibitory signals of heterocyst pattern formation and results in a conditionally lethal phenotype caused by complete differentiation of nearly all cells under nitrogen-limiting conditions (83).

The initial heterocyst pattern and its maintenance are controlled by two negative regulators, PatS and HetN. The *patS* gene is predicted to encode a small peptide involved in establishing the initial heterocyst pattern by lateral inhibition (172). The last five carboxy-terminal amino acid residues (RGSGR) of PatS are necessary and sufficient for inhibiting heterocyst development. A P$_{patS}$-*gfp* reporter strain showed that *patS* expression is localized to individual cells or small groups of cells by 8 to 10 h after

nitrogen step-down (173). Overexpression of *patS* blocks heterocyst development, whereas a *patS* null mutant forms heterocysts even in the presence of nitrate, and forms multiple contiguous heterocysts and short vegetative-cell intervals between heterocysts, which results in about 30% heterocysts after nitrogen step-down (172). The PatS receptor is thought to be cytoplasmic (169) and current evidence suggests that HetR directly interacts with PatS because the DNA-binding activity of HetR is inhibited *in vitro* by a PatS-5 pentapeptide in a dose-dependent manner (69).

The *hetN* gene encodes a protein similar to ketoacyl reductases and plays a role in the maintenance of heterocyst pattern (14). When *hetN* is overexpressed from a copper-inducible promoter, heterocyst development is completely blocked. In the absence of *hetN* expression, filaments develop a normal heterocyst pattern in the first 24 h after nitrogen step-down, but by 48 h, excessive differentiation produces a multiple-contiguous-heterocyst phenotype (23). HetN is thought to function cell non-autonomously by cell-to-cell signaling to prevent differentiation of new heterocysts adjacent to pre-existing heterocysts, but the signaling mechanism is unknown.

The role of c-di-GMP in heterocyst development has not been explored. Because c-di-GMP is an important signaling molecule involved in cellular functions such as cellular differentiation, exopolysaccharide synthesis, and light sensing, we hypothesized that c-di-GMP may be important for some aspects of heterocyst development in *Anabaena* sp. strain PCC 7120. There are 14 genes containing the GGDEF domain in *Anabaena* sp.

strain PCC 7120 (154). In an effort to elucidate the role of c-di-GMP in heterocyst development, we have inactivated each of these 14 genes and screened for heterocyst-related phenotypes. Under our conditions, only one of the 14 mutant strains produced altered heterocyst development. Inactivation of all2874 caused a pronounced decrease in heterocyst frequency as well as a reduction in vegetative-cell size. The heterocyst-related defects were accentuated under high light growth conditions. We also showed that All2874 has diguanylate cyclase activity *in vitro*.

Materials and methods

Strains and culture conditions

The strains used in this study are presented in Table 3.1. Genetic loci designations for *Anabaena* sp. strain PCC 7120 are from CyanoBase (bacteria.kazusa.or.jp/cyanobase/). *Anabaena* sp. strain PCC 7120 and its derivatives were grown in BG-11 (containing sodium nitrate) or BG-11_0 (BG-11 lacking sodium nitrate) medium at 30°C as previously described (54). Cultures were grown under fluorescent white-light illumination at 25 μM photons m^{-2} s^{-1} (low), 50 μM photons m^{-2} s^{-1} (medium, normal), and 150 μM photons m^{-2} s^{-1} (high), as measured with a Biospherical Instruments Model QSL100 sensor, which collects light from all directions. For derivatives of *Anabaena* sp. strain PCC 7120 carrying plasmids, neomycin (Nm) (25 μg/ml) and/or streptomycin (Sm) and spectinomycin (Sp) (2 μg/ml each) were used when strains were grown on BG-11 or BG-11_0 agar plates. For liquid media, the antibiotic concentration was reduced by half. Heterocyst development was induced by nitrogen step-down; filaments from an actively

TABLE 3.1. Bacterial strains and plasmids for the diguanylate cyclase study

Strain/plasmid	Relevant characteristics	Source reference
Anabaena		
PCC 7120	Wild type	R. Haselkorn
AMC484	PCC 7120 carrying pAM1951; Nm^r	(172)
AMC1289	PCC 7120 carrying pAM3318; Nm^r	This study
AMC1572	Insertional inactivation of all2874 by single homologous recombination of pAM4108; Sp^r Sm^r	This study
AMC1574	PCC 7120 carrying pAM4114; Nm^r	This study
AMC1575	AMC1572 carrying pAM4114; Nm^r Sp^r Sm^r	This study
AMC1577	AMC1572 carrying pAM3318; Nm^r Sp^r Sm^r	This study
AMC1578	AMC1572 carrying pAM1951; Nm^r Sp^r Sm^r	This study
Plasmids		
pRL277	Conjugal suicide plasmid; Sp^r Sm^r	(13)
pRP89	pET11 containing C-terminal His-tagged *pleD**; Ap^r Cm^r	(129)
pAM1951	Shuttle plasmid pAM505 carrying a P_{patS}-*gfp* transcriptional fusion; Km^r Nm^r	(172)
pAM2770	Shuttle plasmid containing XhoI–P_{petE}–NdeI–lacZ-alpha SapI(Cys)-6His-(stop)-ClaI; Km^r Nm^r	(93)
pAM3318	Shuttle plasmid pAM504 carrying P_{petE}-$hetR_{R223W}$-Ω	(83)
pAM4097	pET30b+ expression plasmid containing the C-terminal 6His-tagged all2874 ORF; Km^r	This study
pAM4108	pRL277 containing 630-bp internal fragment of all2874; Sp^r Sm^r	This study
pAM4114	pAM2770 containing the entire all2874 ORF; Nm^r	This study

growing BG-11 culture with an optical density at 750 nm of 0.1 to 0.3 were collected by centrifugation and washed three times with sterile water before being transferred to the original volume of BG-11_0. For inducing multiple samples, cultures were grown under different illumination intensities in 2 ml of BG-11_0 in 16 × 150 mm culture tubes. Strains carrying the copper-inducible *petE* promoter were initially grown in medium lacking copper; filaments from standard medium were washed twice by centrifugation and resuspension of the pellet in copper-deficient BG-11 medium before being resuspended in the final growth medium. Activity of the *petE* promoter was induced by transferring filaments to medium supplemented with 0.6 μM $CuSO_4$. Copper-deficient media was prepared in plasticware and filter sterilized as previously described (20).

Plasmids were transferred to *Anabaena* by conjugation from *Escherichia coli* following published protocols (38, 55) with some minor modifications (99). *E. coli* strains were maintained in LB (Lennox L) liquid or agar-solidified media supplemented with appropriate antibiotics.

Growth rate determination

Cultures were grown on a shaking platform at 30°C and at different light intensities of 25, 50, and 150 μM photons m^{-2} s^{-1}. Growth was monitored spectrophotometrically as optical density at 750 nm. At 12-h intervals, 1-ml samples were removed from 100-ml cultures for optical density measurements. To avoid sampling errors caused by filament clumping in BG-11_0 medium, the filaments were dispersed by repeated pipetting before

optical density measurements. Antibiotics were not added to media during growth rate experiments.

DNA manipulations and plasmid constructions

Standard protocols were used for cloning, *E. coli* transformation, and PCR. Total DNA from cyanobacterial strains was isolated as previously described (54). DNA sequencing of plasmid inserts was performed by the Gene Technologies Laboratory (Texas A&M University) following the Big Dye sequencing protocol (Applied Biosystems).

Plasmids used in this study are listed in Table 3.1. The suicide plasmids used to inactivate the 13 GGDEF-domain genes other than all2874 are described in Table 3.3. For the construction of each suicide plasmid, an internal fragment of the open reading frame extending into the GGDEF motif (ranging from 580 to 1277 bp) was amplified from genomic DNA by PCR using forward and reverse primers that contained XhoI and SacI sites, respectively, at their 5' ends (Table 3.2); and the PCR-generated fragments were cloned into the conjugal suicide plasmid pRL277. For all2874, which is the focus of this study, the suicide plasmid pAM4108 was constructed by cloning a 630-bp internal fragment of all2874, PCR-amplified with primers AMO-1069 and AMO-1070, into pRL277. Plasmid pAM4114 carrying P_{petE}-all2874 was made by subcloning a 993-bp NdeI–XmaI fragment containing the all2874 ORF into the respective sites of plasmid pAM2770. All plasmid constructions were verified by DNA sequence analysis.

TABLE 3.2. PCR primer sequences for the diguanylate cyclase study

Gene	Sense primer	Sequence (5'→3')	Antisense primer	Sequence (5'→3')
All0219	AMO-1063	GCCTCGAGGGCACTGCGTGAAAGTG	AMO-1064	GATGAGCTCATCTCCGCCAAAACGC
All1012	AMO-1065	GAGTCTCGAGCTTATCTGTTAAAGCC	AMO-1066	GGCGAGCTCATCACCACCAAATCG
All1175	AMO-1067	GGCTCGAGGAACTACATCAGCGCGA	AMO-1068	TGTGAGCTCATCACCTCCCCAACGAG
All1219	AMO-995	GTTTTTCCTCGAGTTAAAAAAAATG	AMO-996	TAAAGAGCTCGCCACCTAAG
Alr2306	AMO-1053	GATCTCGAGGCAGACAATCTGCGTG	AMO-1054	TAAGAGCTCCGCCTAGTCTTGC
All2416	AMO-991	TCCTCGAGCCGCATCGCAG	AMO-992	AAGAGCTCACCACCATAACG
All2874	AMO-1069	ATGCTCGAGTCATCCGAGCGCAGTTAC	AMO-1070	CAGGAGCTCCTTCGCCACCATAACGAG
Alr3170	AMO-1071	TTCTCGAGACGTCGGCAAGGCATTC	AMO-1072	TGTGAGCTCACCCCCACCCAAACG
Alr3504	AMO-993	GTTACTCGAGGGAATATGCG	AMO-994	CAAAGAGCTCACCACCAA
Alr3599	AMO-1055	ACCTCGAGTAATTTTGGTGGCTG	AMO-1056	ATTGAGCTCCACCATAACGGGCAAC
All4225	AMO-1405	TTACTCGAGAAAAATTACACCGCCAA	AMO-1406	ATTGAGCTCCACTTAAATGGGCTAAAATATC
All4896	AMO-1073	GGCTCGAGTCGCATCGGAGGCATTAG	AMO-1074	CAGAGCTCCGTCACCACCCCATCTGC
All4897	AMO-1075	TACCTCGAGGCGACAGTGCTTATCG	AMO-1076	GTGGCTCATCTCCTCCTATACGGG
All5174	AMO-1077	ATCCTCGAGCCGGTCGAGAATGAGG	AMO-1078	ATGAGCTCTGTGCCTCCCCAGCGTC
All2874 ORF	AMO-1394	GGACATATGATGAAAAATACAGTTCCAG	AMO-1395	CTTCCCGGGTTAAGCTAGTCGATG
All2874 ORF	AMO-1927	GACCATATGAAAAATACAGTTCCAGAGAG	AMO-1928	CTTCTCGAGAGCTAGTCGATGGTGAG

TABLE 3.3. Suicide plasmids used to inactivate predicted diguanylate cyclase genes other than all2874

Plasmids	Relevant characteristics	Source reference
pAM4103	Conjugal suicide plasmid pRL277 containing a 643-bp internal fragment of all0219; Sp^r Sm^r	This study
pAM4168	Conjugal suicide plasmid pRL277 containing a 737-bp internal fragment of all1012; Sp^r Sm^r	This study
pAM4104	Conjugal suicide plasmid pRL277 containing a 792-bp internal fragment of all1175; Sp^r Sm^r	This study
pAM4105	Conjugal suicide plasmid pRL277 containing a 1277-bp internal fragment of all1219; Sp^r Sm^r	This study
pAM4106	Conjugal suicide plasmid pRL277 containing a 724-bp internal fragment of alr2306; Sp^r Sm^r	This study
pAM4107	Conjugal suicide plasmid pRL277 containing a 596-bp internal fragment of all2416; Sp^r Sm^r	This study
pAM4109	Conjugal suicide plasmid pRL277 containing a 835-bp internal fragment of alr3170; Sp^r Sm^r	This study
pAM4110	Conjugal suicide plasmid pRL277 containing a 602-bp internal fragment of alr3504; Sp^r Sm^r	This study
pAM3954	Conjugal suicide plasmid pAM2178 containing a 723-bp internal fragment of alr3599; Sp^r Sm^r	This study
pAM4116	Conjugal suicide plasmid pAM2178 containing a 580-bp internal fragment of all4225; Sp^r Sm^r	This study
pAM4111	Conjugal suicide plasmid pRL277 containing a 830-bp internal fragment of all4896; Sp^r Sm^r	This study
pAM4112	Conjugal suicide plasmid pRL277 containing a 883-bp internal fragment of all4897; Sp^r Sm^r	This study
pAM4113	Conjugal suicide plasmid pRL277 containing a 831-bp internal fragment of all5174; Sp^r Sm^r	This study

For each targeted gene, six exconjugants were picked and subcultured repeatedly to ensure complete segregation of the mutant phenotype. For the all2874 mutant strain, insertion of the pAM4108 plasmid at the desired locus and segregation of the mutation was confirmed by PCR. The pAM4114 plasmid carrying the entire all2874 ORF under control of the *petE* promoter was able to complement the mutant when grown in standard BG-11_0 media, which contains 0.3 μM $CuSO_4$ and induces half-maximal expression from the *petE* promoter (20).

To express and purify the All2874 protein, we constructed pAM4097. The all2874 ORF was amplified by PCR with primers AMO-1927 and AMO-1928, which contained NdeI and XhoI restriction sites at their 5' ends, respectively. The PCR product was then cloned into the pET-30b(+) (Novagen) expression plasmid at its NdeI and XhoI restriction sites, which generated plasmid pAM4097 carrying an in-frame fusion of all2874 with a carboxy-terminal 6×His tag (All2874-6His). DNA sequencing was used to verify the insert and tag region of pAM4097.

Microscopy

Fluorescence and bright-field images were captured using an Olympus IX70 inverted microscope (Olympus) with a CoolSNAP HQ2 camera (Photometrics) and Simple PCI software version 6.0 (Compix Inc.). The Simple PCI software controlled the camera, light-path shutters (ProScan, Prior Scientific), and automated focus. A Piston GFP band-pass filter set (# 41025; Chroma Technology Corp.) was used for fluorescence images.

Photomicrographs were minimally processed with Adobe Photoshop 8.0 to improve brightness and contrast.

Scoring of heterocyst pattern along the filaments was performed as previously described (173). Detached single heterocysts and aggregates of heterocysts were not scored. At least three biological replicate samples were scored for each experimental condition. Statistical analyses were performed using StatView software. Vegetative-cell size measurements were obtained using Image J software and micrographs of wild-type and all2874 mutant filaments at 96 h after nitrogen step-down under high light growth conditions; a total of 200 cells were measured for each of the two strains. Alcian blue staining was used to detect the heterocyst-specific polysaccharide layer of the proheterocyst envelope (99). Briefly, a solution of 0.5% alcian blue (Sigma) in 50% ethanol-water was mixed with an equal volume of an *Anabaena* culture and incubated for 10 minutes before microscopic examination.

Expression and purification of All2874

The pAM4097 plasmid carrying the all2874-6His fusion was transformed into *E. coli* strain BL21(DE3) producing strain AM4097. An overnight culture of AM4097 grown at 37°C in LB (Lennox L) medium supplemented with kanamycin (50 μg/ml) was diluted 1:100 into fresh media and grown at 37°C with vigorous shaking. At an OD_{600} of 0.5, expression of the fusion protein was induced by addition of isopropyl-beta-D-thiogalactopyranoside (IPTG) to a final concentration of 0.5 mM and incubation at 28°C

for 8 h. Cells were then harvested by centrifugation at 4000×g for 10 min, resuspended in binding buffer (50 mM Tris-HCl pH 8.0, 500 mM NaCl, 5 mM β-mercaptoethanol, and 20 mM imidazole) containing PMSF (0.1 mg/ml), and lysed by passage through a French press twice (18,000 psi). The lysate was cleared by centrifugation at 35,000×g for 25 min at 4°C. To reduce viscosity, the supernatant was briefly sonicated with a microprobe tip to shear genomic DNA and the centrifugation was repeated for 10 min. The supernatant was loaded onto a 1 ml HisTrap HP column (GE Healthcare) with a syringe pump and washed with 10 ml of binding buffer. A linear gradient of imidazole was used to elute the fusion protein using an Äkta FPLC system (GE Healthcare). Fractions containing All2874-6His were pooled and then dialyzed against 25 mM Tris-HCl pH 8.0, 250 mM NaCl, and 2.5 mM β-mercaptoethanol for 12 h at 4°C. Fractions containing >95% purified protein, as determined by SDS-PAGE (Fig. 3.9A), were used for enzyme activity assays. Protein concentrations were determined using the Bradford assay (Bio-Rad). The strain carrying plasmid pRP89, which expresses a C-terminal His-tagged fusion of PleD*, was generously provided by Dr. Urs Jenal (University of Basel). PleD* purification was performed as previously described (129).

Diguanylate cyclase assays

The protocol used for enzyme assays is based on methods described by Paul et al. (129) and modified by Kazmierczak et al. (80). Briefly, purified proteins were added to reaction buffer (25 mM Tris-HCl pH 8.0, 250 mM NaCl, 2.5 mM β-mercaptoethanol, and 10 mM $MgCl_2$) in a total reaction volume of 9 µl. Reactions were started by addition

of 1 μl of α-labeled [^{32}P]-GTP (3.33 pmol, 3000 Ci mmol^{-1}, PerkinElmer). Reactions were incubated at 25°C for 60 min before termination by addition of 2 μl of 0.5 M EDTA pH 8.0. The product of the PleD* reaction served as a control for the production of c-di-GMP. HetL, a protein from *Anabaena* sp. strain PCC 7120 that does not contain the GGDEF domain, was assayed as a negative control (data not shown).

Reaction products from diguanylate cyclase assays were separated by thin layer chromatography (TLC) on 20×20 cm PEI-cellulose plates (Macherey-Nagel), using 1:1.5 v/v saturated $(NH_4)_2SO_4$ and 1.5 M KH_2PO_4, pH 3.6, as the running buffer. From each reaction, a 2-μl volume was spotted and dried onto the PEI-cellulose TLC plates. Plates were developed in running buffer, air-dried for 3 h under a fume hood, and visualized by autoradiography.

Results

Identification of putative GGDEF- and EAL-proteins in *Anabaena* sp. strain PCC 7120

The *Anabaena* sp. strain PCC 7120 genome harbors 8 genes encoding a GGDEF domain but not the EAL domain, 1 gene encoding only the EAL domain, and 6 genes encoding both domains; all these genes are present on the chromosome. A schematic representation of the domain architecture of all the proteins predicted to have GGDEF domains is shown in Fig. 3.1A. The majority of these proteins contain additional

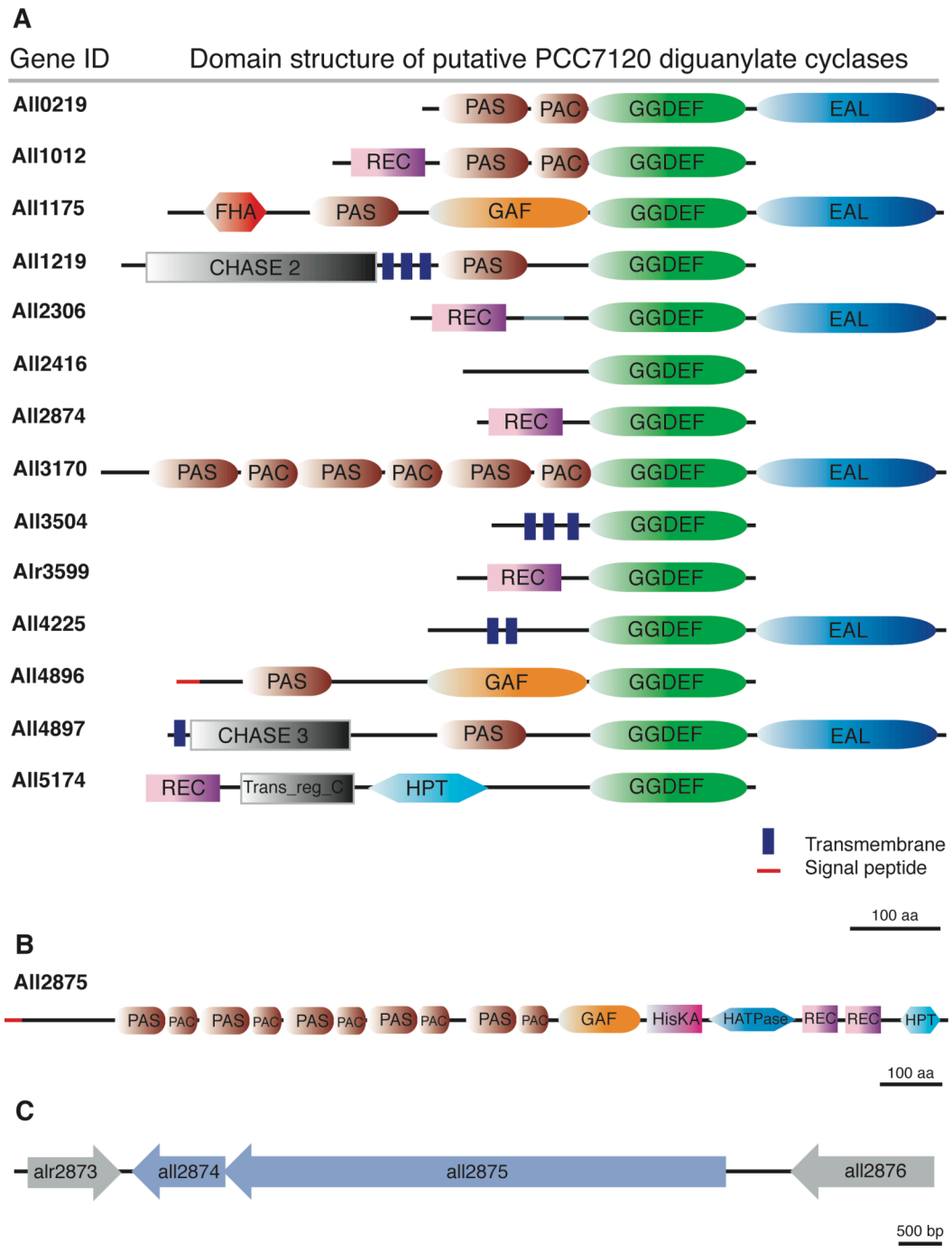

FIG. 3.1. (A) Domain organization of the 14 genes predicted to encode diguanylate cyclases in *Anabaena* sp. strain PCC 7120 determined by the SMART program (http://smart.embl-heidelberg.de). (B) Predicted domain organization of the All2875 protein. (C) Diagram of the chromosomal context of all2874.

domains predicted to be involved in signal transduction, including PAS, GAF, CBS, HAMP, CHASE, FHA, and receptor domains.

To determine the evolutionary relationship between the GGDEF domains, we constructed a phylogenetic tree based on the alignment of the predicted amino acid sequences of all the GGDEF domains present in *Anabaena* sp. strain PCC 7120 (Fig. 3.2); in the alignment we included the GGDEF domain from the PleD protein of *Caulobacter crescentus* whose diguanylate cyclase activity has been previously confirmed (145). The phylogenetic tree shows that the GGDEF domains can be grouped in two clusters. Cluster I contains almost exclusively GGDEF domains that are not coupled with EAL domains, and the majority display the variant GGEEF motif. All domains in cluster II have a conserved GGDEF motif and most of them are coupled with the EAL domain.

Inactivation of all2874 caused a decrease in heterocyst frequency

To determine if c-di-GMP signaling may play a role in heterocyst development, we inactivated all 14 genes predicted to encode diguanylate cyclases by single homologous recombination with suicide plasmids carrying an internal fragment of the targeted gene. We screened all the mutants for heterocyst defects, including: heterocyst differentiation and morphology, pattern formation, heterocyst frequency, and diazotrophic growth.

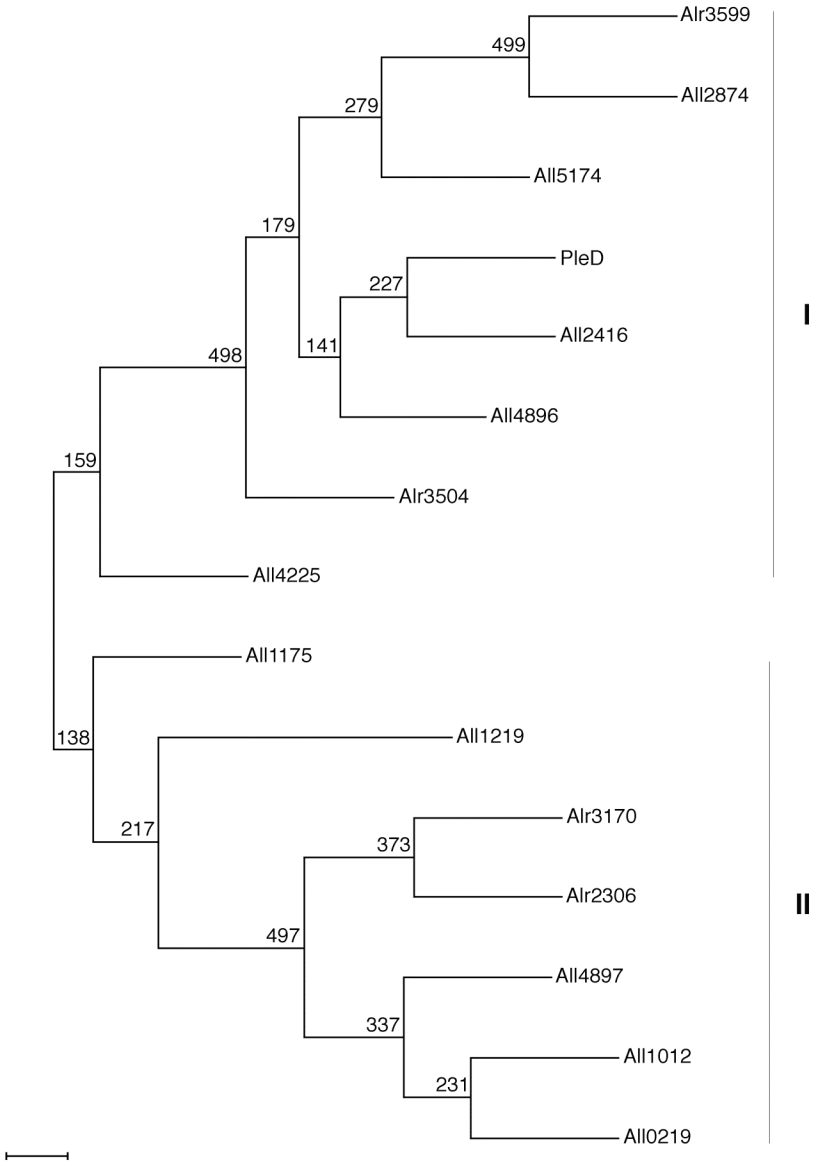

FIG. 3.2. Phylogenetic relationship of GGDEF domains from predicted diguanylate cyclases in the genome of *Anabaena* sp. strain PCC 7120. Genetic loci designations for *Anabaena* sp. strain PCC 7120 are from CyanoBase (bacteria.kazusa.or.jp/cyanobase/). The GGDEF domain from the PleD protein of *Caulobacter crescentus* was also included in the analysis. The unrooted tree was generated with the PHYLIP software package version 3.65 available through the BioBike system using the neighbor-joining program to calculate distance matrices from a multiple sequence alignment of amino acid positions corresponding to each GGDEF domain produced by ClustalW software. Bootstrap values were obtained with 500 replications. Two distinct phylogenetic groups are indicated. The scale bar represents 0.02 amino acid substitutions per residue.

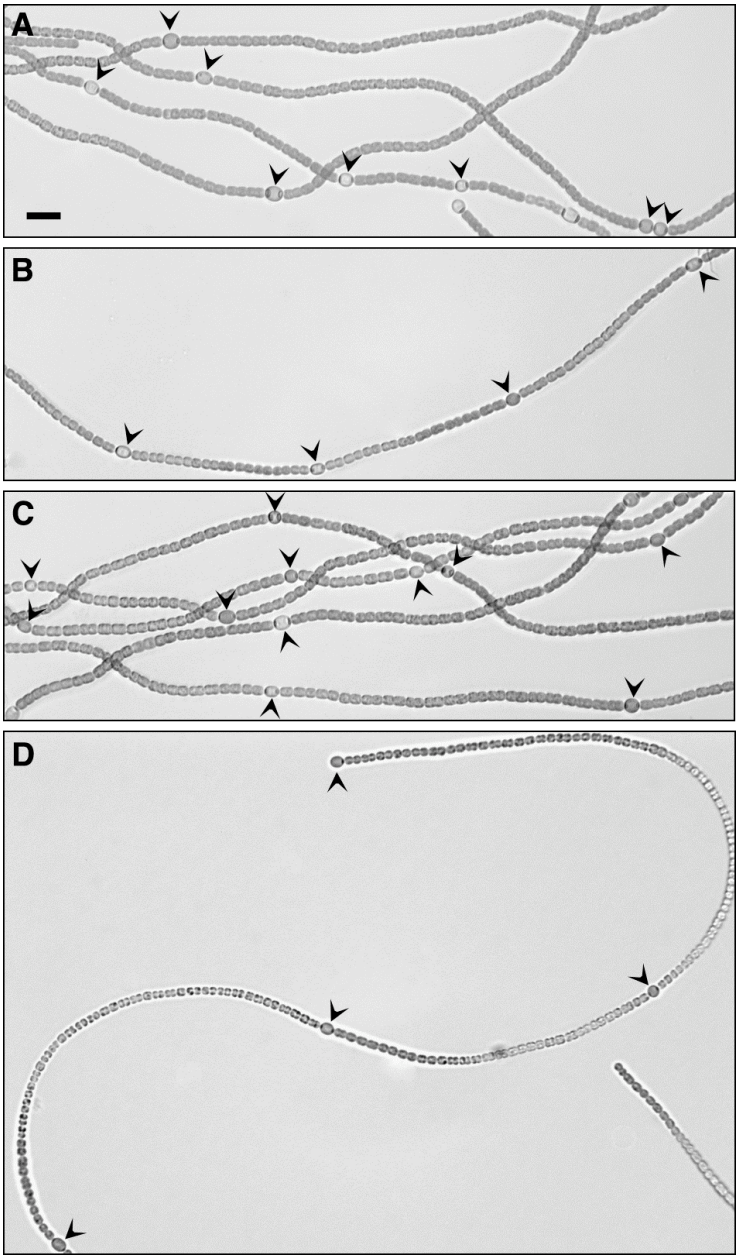

FIG. 3.3. Inactivation of all2874 caused a reduced heterocyst frequency. Filaments grown in BG-11 liquid medium under normal light intensity were transferred to BG-11_0 medium to induce heterocyst development and grown at high light. At 24 h after nitrogen step-down, the all2874 mutant (B) formed heterocysts with a frequency similar to that of the wild type (A), but by 96 h the heterocyst frequency of the all2874 mutant (D) was considerably lower than that of the wild type (C). Arrowheads indicate heterocysts. Scale bar, 10 μm.

Only one of the mutants, strain AMC1572 in which gene all2874 was disrupted, displayed a heterocyst-related phenotype (Fig. 3.3). Under our normal growth conditions on medium lacking a source of combined nitrogen, the initial heterocyst frequency was essentially wild type, but over the next four days, the mutant strain showed a pronounced decrease in heterocyst frequency (Fig. 3.5). In addition, vegetative-cell size of the mutant was diminished compared to that of the wild-type strain; at 96 h after nitrogen step-down under high light growth conditions, wild-type vegetative-cell length was 3.6±0.56 μm, whereas vegetative-cell length for the all2874 mutant was 1.89±0.33 μm.

Heterocyst frequency phenotype of the all2874 mutant is light- and/or growth rate-dependent

We noticed that older (denser) nitrogen-deprived cultures of the mutant strain showed a less severe phenotype. This prompted us to investigate if light intensity plays a role in the mutant phenotype because all2874 is potentially co-transcribed with the upstream gene all2875, which contains several PAS domains, including one that has been shown to be involved in sensing light (122). To determine whether light intensity affects heterocyst frequency of the mutant strain, filaments of the wild-type and mutant strains grown in nitrate-containing medium under normal light conditions were washed and transferred to medium lacking fixed nitrogen and grown at three different light intensities: low, medium, and high. At 24 h after nitrogen step-down, the heterocysts frequency of the all2874 mutant was similar to that of the wild-type strain under all three light conditions (Fig. 3.4 and Fig. 3.5). When grown at medium and high light intensity,

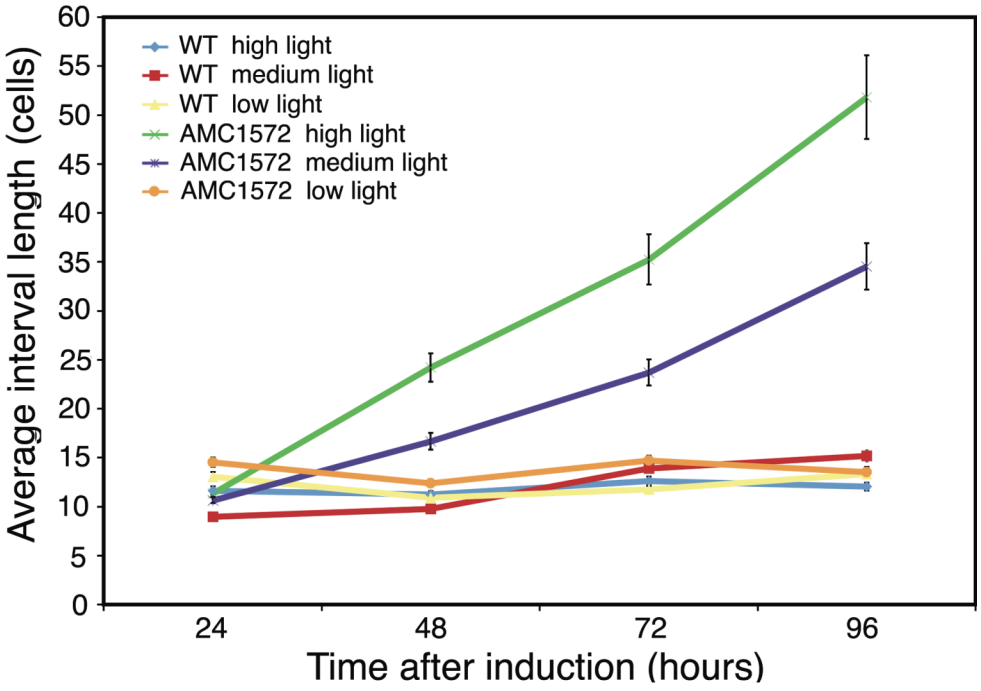

FIG. 3.4. The all2874 mutant showed a light intensity-dependent defect in heterocyst frequency. Vegetative-cell intervals between heterocysts of the wild type and all2874 mutant (AMC1572) grown at three different light intensities (low, medium, and high) for 4 days following nitrogen step-down. The average number of vegetative cells in the interval between heterocysts remained similar in the wild type grown under all three light conditions, whereas the all2874 mutant showed increased interval lengths over time in a light intensity-dependent manner. Error bars indicate standard error.

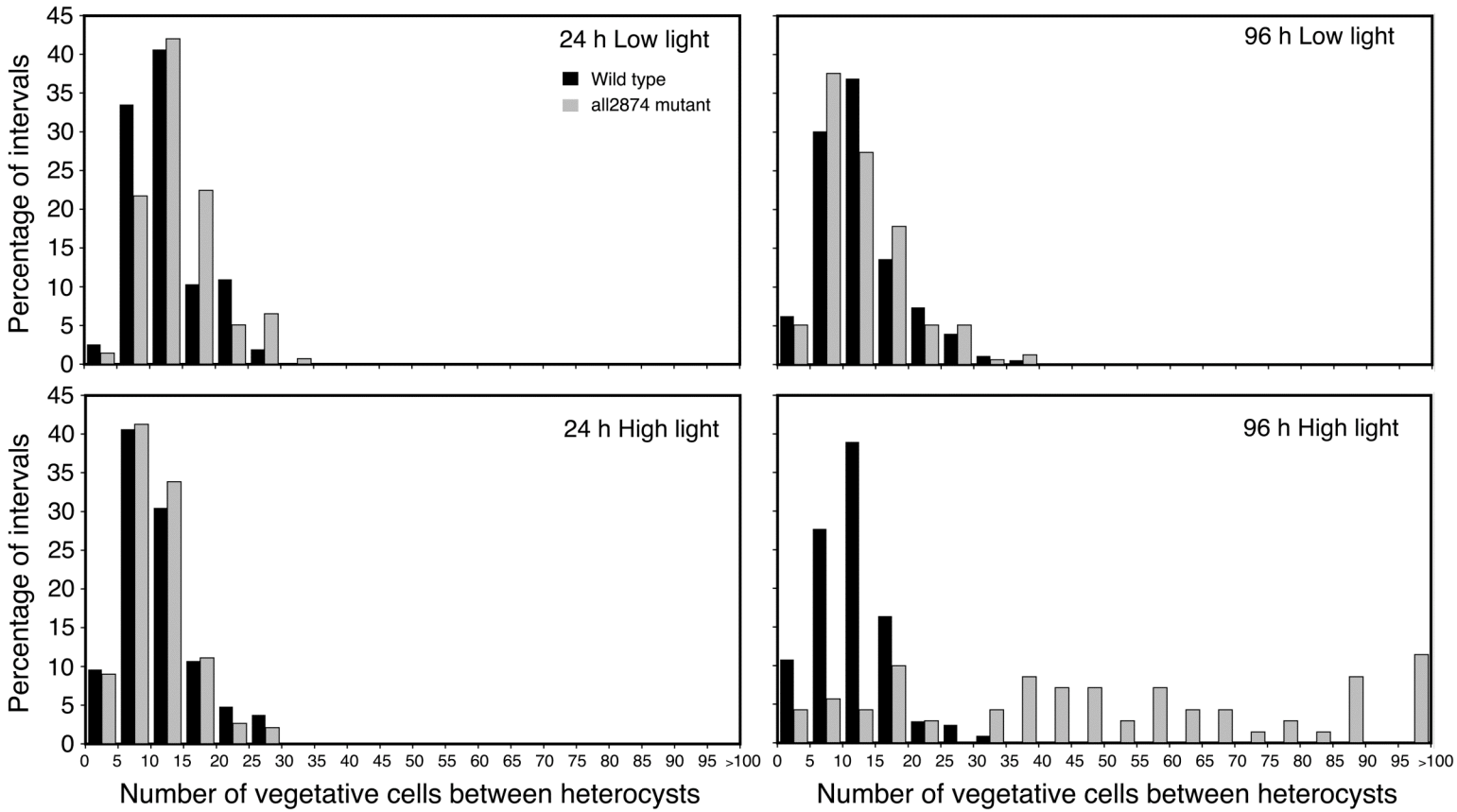

FIG. 3.5. The all2874 mutant strain was deficient in maintaining a normal heterocyst frequency when grown at high light intensity. The bar graph shows the distribution of the number of vegetative cells in the intervals between heterocysts at 24 h and 96 h after nitrogen step-down of the wild type and the all2874 mutant grown at low light or high light.

the average number of vegetative cells between heterocysts (vegetative-cell interval) exceeded that of the wild type and continued to increase over time. At 96 h after nitrogen step-down, the last time-point recorded, the average vegetative-cell interval increased to 34.5±24.8 when filaments were grown under medium light and 51.8±35.5 under high light (Fig. 3.4 and Fig. 3.5). In addition, the interval between heterocysts became more variable such that normal vegetative-cell intervals between heterocysts were interspersed with abnormally long intervals of up to 200 cells. The variability of heterocyst spacing is reflected in the large standard deviation of the average vegetative-cell intervals of the mutant filaments grown at high light. No significant changes in heterocyst frequency and spacing occurred in the wild-type strain grown under the different light conditions (Fig. 3.4 and Fig. 3.5).

We also compared the growth rate of the all2874 mutant and wild-type strains grown at low, medium, and high light intensity in the presence or absence of a source of combined nitrogen. In the presence of nitrate, growth of the mutant strain was similar to that of wild type under all three light conditions (data not shown). In the absence of a source of combined nitrogen, growth of the mutant strain was slower than that of the wild type at medium and high light intensity, but remained similar to wild type at low light, suggesting that the growth defect of the mutant is caused by the decreased heterocyst frequency at higher light levels (Fig. 3.6).

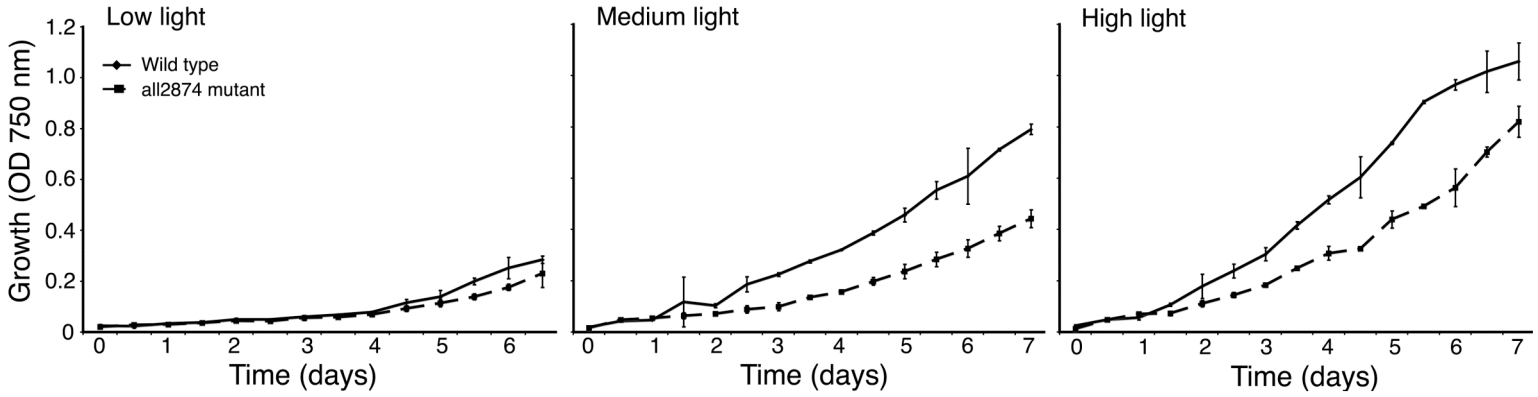

FIG. 3.6. Growth of the all2874 mutant strain (AMC1572) is slower than that of the wild type after nitrogen step-down at medium and high light intensities. Growth under low light conditions was similar for the two strains. Wild-type and all2874 mutant filaments from nitrate-containing BG-11 cultures were washed and then transferred to BG-11_0 at time zero to induce heterocyst development. Three independent cultures of the wild type and the all2874 mutant were used for each light condition. Diazotrophic growth of each culture was followed for 7 days by measuring the optical density at 750 nm (OD). Error bars indicate standard deviation.

Heterocyst development is blocked before upregulation of P_{patS}-*gfp* in the all2874 mutant

To determine if some cells within the long vegetative-cell intervals between heterocysts of the all2874 mutant strain had initiated differentiation but were blocked before morphogenesis, the shuttle plasmid pAM1951 (93, 173), which carries a *gfp* transcriptional fusion to the *patS* promoter, was introduced into the all2874 mutant, producing strain AMC1578. Filaments were then induced to form heterocysts by nitrogen step-down, and incubated at low or high light intensity. The results for low and high light were similar; Fig. 3.7 shows the high-light data. Surprisingly, we found that heterocyst differentiation was blocked for the first 3 to 4 days following nitrogen step-down in the all2874 mutant carrying the P_{patS}-*gfp* transcriptional fusion. During this period of time, the GFP fluorescence intensity from the *patS* reporter remained at a low level in all vegetative cells and there was no apparent pattern of GFP fluorescent cells. This is in contrast to the wild-type reporter strain, which showed an initial pattern of GFP-bright cells at 6 to 8 h after nitrogen step-down. After 4 days, some AMC1578 filaments differentiated a low frequency (2.5±1.56% at high light) of heterocysts. At high light, some filaments did not differentiate any heterocysts even after 7 days following nitrogen step-down.

Once the first round of heterocysts was formed, we wanted to know whether subsequent rounds of differentiation were occurring normally. We examined more than 100 vegetative-cell intervals containing 30 to 200 cells between heterocysts. In the majority

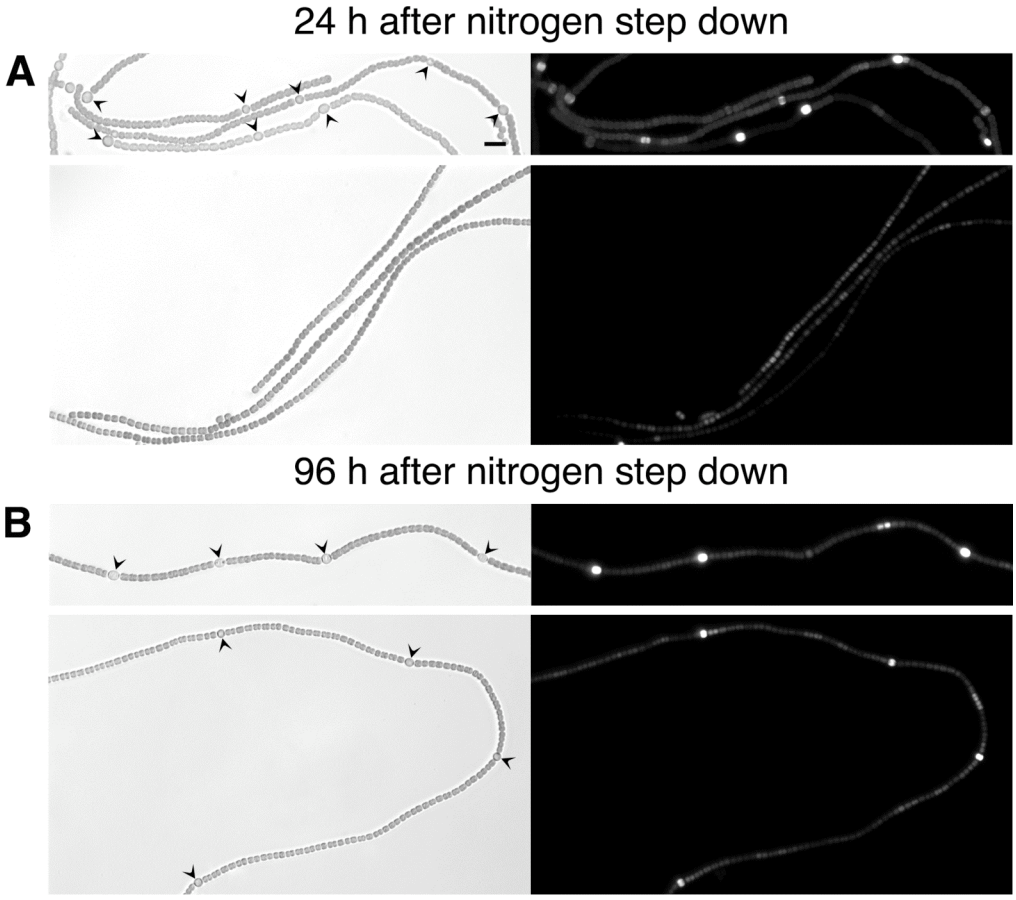

FIG. 3.7. A P_{patS}-*gfp* reporter is upregulated in only a low frequency of differentiating cells in the all2874 mutant background, and the presence of the P_{patS}-*gfp* reporter plasmid delayed heterocyst development. Images show GFP reporter fluorescence produced by the wild type and all2874 mutant containing a P_{patS}-*gfp* reporter on plasmid pAM1951 at 24 h (A) and 96 h (B) after nitrogen step-down under high light growth conditions. In both (A) and (B) the top two panels show wild type carrying the P_{patS}-*gfp* reporter (AMC484) and the bottom two panels show the all2874 mutant carrying the P_{patS}-*gfp* reporter (AMC1578). (Left panels) Bright-field images. (Right panels) Corresponding GFP fluorescence images. Arrowheads indicate heterocysts. Scale bar, 10 μm.

of filaments, intense GFP fluorescence was limited to only proheterocysts and heterocysts, indicating that the abnormally low frequency of heterocysts was due to an early block in differentiation before the upregulation of the *patS* gene. A few filaments contained some cells that were GFP bright, but these occurred at a low frequency with no apparent pattern. Similar results were obtained with wild-type and all2874 mutant strains carrying a P_{hetR}-*gfp* transcriptional fusion on a shuttle plasmid (data not shown); heterocyst development was delayed and the *hetR* reporter was upregulated in only the low percentage of cells that differentiated into heterocysts. These results indicate that the low frequency of heterocysts in the all2874 mutant is due to a decreased tendency to initiate heterocyst differentiation. In addition, the delayed development caused by the presence of the *patS* and *hetR* promoters on a multicopy plasmid suggests that the all2874 mutant is deficient for active transcription factors required to trigger differentiation.

All2874 is upstream of HetR in the heterocyst differentiation pathway

To determine whether All2874 is upstream or downstream of HetR in the regulatory cascade controlling heterocyst differentiation, we overexpressed the mutant allele $hetR_{R223W}$ in wild-type and the all2874 mutant background and compared heterocyst frequency over time at low light and high light. The shuttle plasmid pAM3318 carrying the $hetR_{R223W}$ allele under the copper-inducible *petE* promoter was used for overexpression. The wild-type and the all2874 mutant strain carrying pAM3318 were grown in copper-free medium containing nitrate at low light to minimize the tendency to

form heterocysts prior to induction. Heterocyst differentiation was induced by transferring the two strains to medium lacking combined nitrogen and containing 0.6 μM $CuSO_4$. At 24 h after nitrogen step-down, both strains displayed multiple contiguous heterocysts and a similar heterocyst frequency under both light conditions (Fig. 3.8). Over the following days, heterocyst frequency remained abnormally elevated in both strains at both light intensities (Fig. 3.8). Even after several days of growth at high light, the all2874 mutant overexpressing the $hetR_{R223W}$ allele never showed a reduction in heterocyst frequency. Therefore, the $hetR_{R223W}$ allele is epistatic to the all2874 mutation, which suggests that All2874 is upstream of HetR in the heterocyst differentiation pathway.

All2874 has diguanylate cyclase activity *in vitro*

To directly evaluate the ability of All2874 to synthesize c-di-GMP from α-^{32}P-GTP, we performed an *in vitro* diguanylate cyclase assay (80, 129). As a positive control, we included the constitutively active allele of the PleD protein (PleD*), a well-characterized diguanylate cyclase. All2874-6His produced c-di-GMP in a concentration-dependent manner (Fig. 3.9B). Purified HetL protein was used in the assay as a negative control and it produced no c-di-GMP product. These data show that purified All2874-6His functions as a diguanylate cyclase *in vitro*.

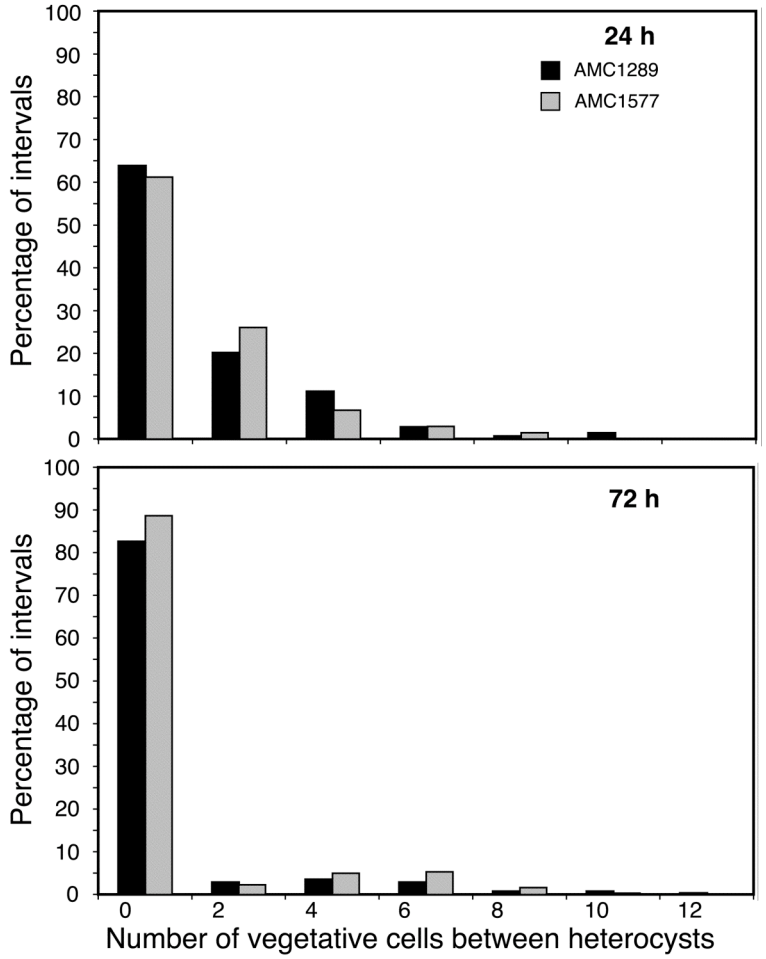

FIG. 3.8. Overexpression of the *hetRR223W* mutant allele is epistatic to the all2874 mutation. The bar graph shows the percentage of intervals that contain different numbers of vegetative cells in the interval between heterocysts at 24 h and 72 h after nitrogen step-down under high light growth conditions for a wild-type strain containing the pAM3318 plasmid carrying the *hetRR223W* allele expressed from the copper-inducible *petE* promoter (AMC1289), and the all2874 mutant containing the same plasmid (AMC1577).

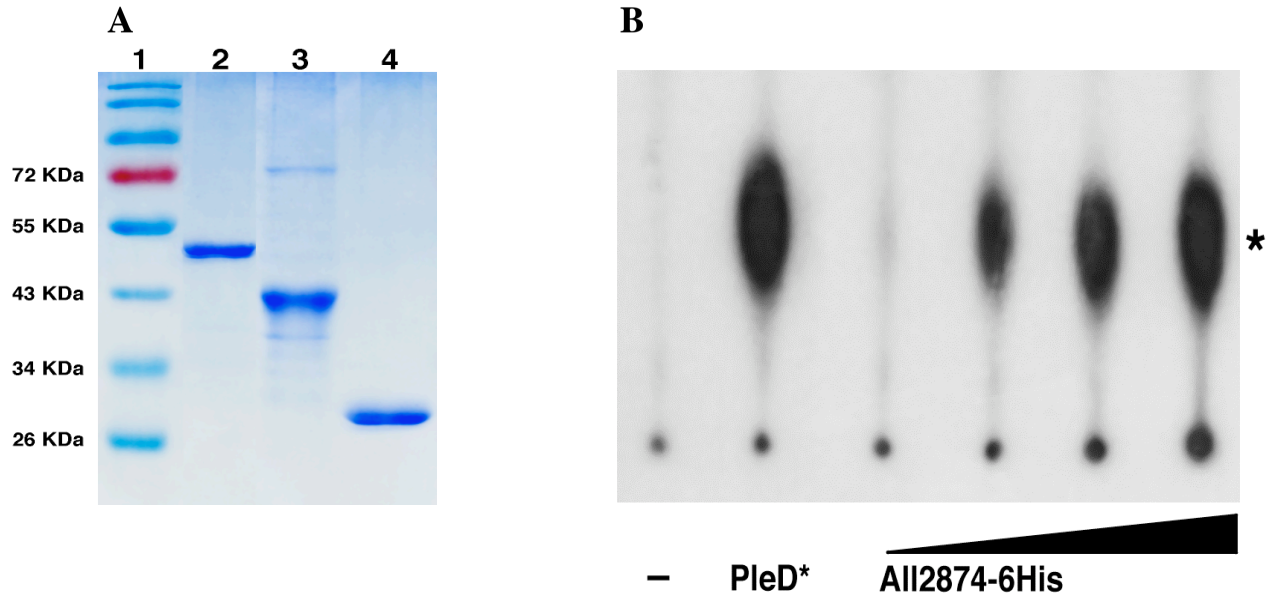

FIG. 3.9. Purification and enzymatic activity of All2875-6His. (A) SDS-PAGE gel stained with Coomassie brilliant blue showing purified proteins: lane 1, prestained molecular weight markers; lane 2, purified PleD* (~49.5 kDa); lane 3, purified All2874-6His (~38.2 kDa); and lane 4, purified HetL-6His (~ 27 kDa). (B) Purified All2874-6His exhibited diguanylate cyclase activity in a concentration-dependent manner. *In vitro* synthesis of c-di-GMP by All2874-6His was determined by thin layer chromatography. PleD* (3 μM) served as a positive control for the synthesis of c-di-GMP. Reactions were performed with increasing concentrations of All2874-6His (2 μM, 6.3 μM, 12.6 μM, and 18.3 μM). No protein was added to the reaction spotted in the left-most lane. The position of c-di-GMP is marked with an asterisk.

Discussion

The recently recognized second messenger c-di-GMP has been shown to be involved in signal transduction pathways that regulate a variety of prokaryotic cellular processes (76). The level of c-di-GMP signal is controlled by the reciprocal activities of diguanylate cyclase and phosphodiesterase. In many cases, cellular levels of c-di-GMP lead to changes in cell surface properties, which, in turn, affect motility and biofilm formation (75). Thus far, there is only one example in which inactivation of a gene encoding a diguanylate cyclase had an effect on cellular differentiation; PleD, from *Caulobacter crescentus*, was shown to be required for polar development (7). In this paper, we have shown that a diguanylate cyclase encoded by the all2874 gene is required for normal heterocyst development.

The chromosome of *Anabaena* sp. strain PCC 7120 harbors 14 genes containing the GGDEF domain (154), usually coupled with various sensor and/or response modules. The most prevalent of all these signal transduction domains is the PAS domain. The tendency to associate with PAS domains could be due to the functional versatility of these domains; PAS domains are important for monitoring changes in light, redox potential, overall energy level of a cell, oxygen, and small ligands (121).

A phylogenetic tree of the GGDEF domains from *Anabaena* sp. strain PCC 7120 shows the presence of two clusters that correlate with whether or not the GGDEF domain is coupled with an EAL domain. This phylogenetic clustering suggests that proteins

containing only the GGDEF domain have evolved separately from those containing both GGDEF and EAL domains. A recent study showed a similar phylogenetic division of the putative diguanylate cyclases in *Pseudomonas aeruginosa* strain PA14, and the same study found that several proteins belonging to either cluster were enzymatically active (88). We tested the *in vitro* diguanylate cyclase activity of All2874, which belongs to cluster I and harbors the GGEEF motif, and found that this protein was indeed able to synthesize c-di-GMP *in vitro*.

We have inactivated all of the genes containing GGDEF domains in *Anabaena* sp. strain PCC 7120 to determine the effect on heterocyst development. Only the all2874 mutant showed a distinct mutant phenotype. However, mutation of some genes may not produce an altered phenotype if there is functional redundancy with other genes or if they produce a phenotype that was not tested for.

After nitrogen step-down, the all2874 mutant initially formed heterocysts with a normal pattern and frequency at 24 h, but over the next few days, the frequency was severely diminished. We hypothesize that this phenotype is caused by a decreased tendency to trigger heterocyst differentiation that is clearly manifested during steady-state diazotrophic growth, but is masked during the first round of heterocyst differentiation, which is initiated by a sudden and extreme nitrogen step down. We envision that this later condition results in a more extensive modulation of regulatory factors and that it is less likely to represent conditions experienced under natural growth conditions.

We used a reporter strain containing a P_{patS}-*gfp* transcriptional fusion in the mutant background to examine whether some cells within the long vegetative-cell interval between heterocysts had initiated differentiation but were blocked before morphogenesis. Our results showed that the all2874 mutant failed to upregulate the *patS* reporter and therefore is affected in early regulatory events required to initiate cellular differentiation. Surprisingly, the reporter strain showed delayed heterocyst formation. The first round of heterocysts did not differentiate until about 4 days after nitrogen step-down, and then at only a reduced frequency. Since a shuttle plasmid carrying the *patS* promoter in the all2874 mutant background was sufficient to cause a long delay in heterocyst differentiation, we speculate that at least one protein that is important for heterocyst development and that binds to the *patS* promoter, such as HetR, is present at a lower effective concentration in the all2874 mutant compared to the wild type. Therefore, the *patS* promoter region carried on a multicopy plasmid would further reduce the pool of the transcription factor by titrating out the protein, resulting in a failure to trigger normal levels of heterocyst differentiation in the all2874 mutant.

In *Anabaena cylindrica*, high light was shown to stimulate heterocyst differentiation (3), but in the all2874 mutant strain, high light intensity caused the opposite effect. The all2874 gene is predicted to be co-transcribed with all2875, which encodes a two-component hybrid sensor response regulator that possesses multiple domains including several PAS domains and a GAF domain. The second PAS domain belongs to a subfamily that function as blue-light sensors due to the presence of the LOV (light,

oxygen, and voltage) domain that binds flavin mononucleotide (FMN) (110, 122). Many GAF domains in cyanobacteria can sense red light; however, the GAF domain present in all2875 is not predicted to have this property because of the absence of the conserved residues required for bilin-binding (110). Thus, we speculate that all2875 and all2874 are involved in sensing and conveying information about light intensity. However, it is possible that light levels may only indirectly impact heterocyst development through an effect on growth rate. Inactivation of all2874 could reduce the tendency to differentiate heterocysts equally at different light intensities, but the increased growth rate under high light growth conditions would accentuate the heterocyst frequency phenotype because differentiation would lag behind the rate of vegetative-cell doubling and result in longer vegetative-cell intervals. However, this latter hypothesis is not consistent with the very long 200 vegetative-cell intervals produced by the all2874 mutant.

To determine the epistatic relationship between *hetR* and all2874, we overexpressed $hetR_{R223W}$, which fails to respond to inhibitory signals, from the copper-inducible *petE* promoter in the all2874 mutant strain and analyzed the heterocyst frequency in filaments grown at high or low light intensity. Under both light conditions, the $hetR_{R223W}$ phenotype was produced; heterocyst frequency was abnormally high after 24 h, and after several days of growth, the frequency did not decrease in the all2874 mutant background. On the contrary, the multiple contiguous heterocyst phenotype produced by the $hetR_{R223W}$ allele in the all2874 mutant was slightly more pronounced than in the wild-type background. Therefore, the $hetR_{R223W}$ allele is epistatic to the all2874 mutation,

which suggests that All2874 functions upstream of HetR activity in the regulatory pathway controlling heterocyst development.

Summary

The genome of the heterocyst-forming cyanobacterium *Anabaena* sp. strain PCC 7120 harbors fourteen genes containing a GGDEF diguanylate cyclase domain. We found that inactivation of one of these genes, all2874, caused abnormal heterocyst development. The all2874 mutant showed a pronounced reduction in heterocyst frequency during diazotrophic growth and reduced vegetative-cell size compared to the wild type. The severity of the mutant phenotype varied with light intensity; at high light intensity, the mutant phenotype was accentuated, whereas at low light intensity the phenotype was similar to wild type. Under high light growth conditions, the initial heterocyst frequency and pattern for the all2874 mutant were normal, but within four days following nitrogen step-down, many intervals between heterocysts increased to as many as 200 vegetative cells. Filaments containing these unusually long vegetative-cell intervals between heterocysts also contained intervals of normal length. An all2874 mutant strain carrying a P_{patS}-*gfp* transcriptional reporter fusion failed to show normal upregulation of the reporter, which indicates that the decrease in heterocyst frequency is due to an early block in differentiation before induction of the *patS* gene. Genetic epistasis experiments suggest that All2874 acts upstream of HetR activity in differentiating cells. We also showed that purified All2874 functions as a diguanylate cyclase *in vitro*. We hypothesize

that All2874 is required for the normal regulation of heterocyst frequency under high light growth conditions.

CHAPTER IV

Anabaena SP. STRAIN PCC 7120 GENE all0187 IS DEVELOPMENTALLY REGULATED AND ESSENTIAL FOR DIAZOTROPHIC GROWTH AND HETEROCYST MORPHOGENESIS

Introduction

The filamentous cyanobacterium *Anabaena* (*Nostoc*) sp. strain PCC 7120 is capable of fixing CO_2 by oxygenic photosynthesis, while also maintaining the ability to assimilate molecular nitrogen when a combined nitrogen source such as ammonium or nitrate is not available (57). These two processes are seemingly incompatible because nitrogenase, the enzyme involved in reducing atmospheric dinitrogen to ammonium, is oxygen-sensitive. In unicellular nitrogen-fixing cyanobacteria, these two processes are temporally separated, with photosynthesis occurring during the day and nitrogen fixation at night (31). Some filamentous cyanobacteria, including *Anabaena* sp. strain PCC 7120, undergo development in which approximately 1 out of 10 vegetative cells differentiates into a specialized cell called a heterocyst that no longer carries out photosynthesis and instead fixes nitrogen (5).

Heterocyst differentiation involves morphological and physiological changes that produce the micro-oxic environment necessary for nitrogen fixation (5). This micro-oxic environment is generated by the inactivation of the O_2-producing photosystem II, an increase in respiration, and the deposition of a multilayered cell envelope consisting of

an inner layer of glycolipids and an outer layer of polysaccharides that diminish O_2 entry into the cell. The heterocyst pattern is thought to be regulated by cell-cell signaling involving the products of the *patS* and *hetN* genes, as well as fixed nitrogen produced by heterocysts (16). The two types of cells are nutritionally dependent upon each other. Heterocysts supply the vegetative cells with fixed nitrogen and, in turn, vegetative cells supply fixed carbon to the heterocysts (5, 131). It is not yet clear how metabolites are exchanged between vegetative cells and heterocysts. Recent evidence has shown that the periplasm is continuous along each filament of cells, but also that cytoplasm-to-cytoplasm movement of small molecules can occur in *Anabaena* (113).

The early stages of heterocyst differentiation require increased levels of NtcA and HetR, which are mutually dependent on each other for upregulation. NtcA is an autoregulatory transcription factor that belongs to the cAMP receptor protein (CRP) family (50, 159), whereas HetR is an autoregulatory serine-type protease with DNA-binding activity that is inhibited *in vitro* by a synthetic pentapeptide corresponding to the C-terminal region of the PatS product (69). Cells that have initiated heterocyst differentiation, but have not yet committed to differentiate are termed proheterocysts. During maturation, a polysaccharide layer is deposited around the proheterocyst followed by the deposition of a glycolipid layer between the cell wall and the polysaccharide layer. Synthesis and deposition of the polysaccharide layer require a cluster of genes that constitute the HEP island, as well as *hepA*, *hepB*, alr3699, and all4160; expression of these genes is upregulated early during heterocyst differentiation (5 to 8 h) (68). Several genes with

regulatory roles have also been shown to be required, such as *hepK* and *devR*, which form a two-component system, *henR* (response regulator), *hepN* (histidine kinase) and *hepS* (serine/threonine kinase). The glycolipid layer synthesis requires *hglB*, *hglC*, and *hglD*, and *hglE*, *hglK*, and *devBCA* have a role in transport and/or assembly (42). Mutations that compromise the integrity of the polysaccharide or the glycolipid layer cause a Fox^- phenotype characterized by the inability of the heterocysts to perform nitrogen fixation in the presence of oxygen.

Fan *et al.* (2006) previously identified gene all0187, the subject of this paper, in a transposon mutagenesis screen for mutants with a Fox^- phenotype. The all0187 mutant was unable to grow diazotrophically although the polysaccharide and glycolipid layers were present. However, the polar junctions connecting heterocysts to vegetative cells were incomplete or widely open, which they hypothesized might have allowed oxygen to enter the heterocyst and inactivate the nitrogenase (43).

We independently identified the *Anabaena* sp. strain PCC 7120 all0187 gene because its expression is upregulated after nitrogen step-down. We show that site-directed inactivation of all0187 caused a defect in vegetative cell division in medium that contained ammonium or nitrate. The mutant strain was unable to grow diazotrophically, although it was able to form partially functional heterocysts with nitrogenase activity. In addition, observed altered morphogenesis, in which heterocyst cells of the mutant

filaments exceeded wild-type length. Increased expression of all0187 after nitrogen step-down was localized to proheterocysts and heterocysts, as shown by the $P_{all0187}$-*gfp* reporter strain.

Material and methods

Strains and culture conditions

Strains and plasmids used in this study are listed in Table 4.1. *Anabaena* (*Nostoc*) sp. strain PCC 7120 and its derivatives were grown in 100 ml of BG-11 or BG-11_0 (lacking sodium nitrate) medium in 250 ml flasks at 30°C with white-light illumination of approximately 75 μM photons m^{-2} s^{-1} as previously described (168). Plates contained 40 ml of medium solidified with 1.5% agar; for BG-11_0 plates, the agar was washed to remove trace nitrogen compounds. For medium containing ammonium, ammonium chloride (2.5 mM) and MOPS buffer (5 mM, pH 8.0) were added to BG-11_0. For *Anabaena* sp. strain PCC 7120 strains containing shuttle or suicide plasmids grown on solid medium, antibiotics were used at the following concentrations: neomycin (Nm) 25 μg ml^{-1}, or spectinomycin (Sp) and streptomycin (Sm) 2 μg ml^{-1} each; when grown in liquid medium these concentrations were reduced by half. *E. coli* strains were grown in LB (Lennox L) medium containing appropriate antibiotics at 37˚C. *E. coli* strain DH10B was used for all cloning experiments. Suicide and shuttle plasmids were transferred into *E. coli* donor strain AM1359 by electroporation and transferred to *Anabaena* sp. strain PCC 7120 strains by conjugation using standard protocols (38) with some modifications (84). To limit the possibility of working with clones containing second-site mutations in

TABLE 4.1. Bacterial strains and plasmids for the all0187 gene study

Strains/plasmids	Relevant characteristic	Reference
Anabaena		
PCC 7120	Wild-type *Anabaena* sp. strain PCC 7120	R. Haselkorn
AMC1369	PCC 7120 carrying suicide plasmid pAM3438 with an internal fragment of all0187; Sm^r Sp^r	This study
AMC1370	PCC 7120 carrying shuttle plasmid pAM3450; Nm^r	This study
Plasmids		
pAM504	Shuttle plasmid pAM502 without the Sp/Sm cartridge containing promoterless *gfpmut2* with unique cloning sites XbaI, BamHI, XmaI, KpnI, SstI and AccI; Km^r Nm^r	(27)
pRL277	Conjugal suicide plasmid; Sm^r Sp^r	(38)
pAM1011	Contains an uncharacterized mutation resulting in a copy number in *Anabaena* PCC 7120 higher than that of the original pDU1-based shuttle plasmids; Km^r Nm^r	(168)
pAM1824	Shuttle vector containing the *Anabaena* PCC 7120 *rbcL* promoter on an EcoRI-SalI fragment from pAM496; Km^r Nm^r	(99)
pAM1956	Shuttle plasmid pAM505 carrying the *gfpmut2* gene; Km^r Nm^r	(172)
pAM3438	pRL277 containing 676-bp internal all0187 fragment; Sm^r Sp^r	This study
pAM3461	Shuttle plasmid pAM504 without the Spectinomycin cartridge containing promoterless *gfpmut2* with unique cloning sites XbaI, BamHI, XmaI, KpnI, SstI and AccI; Ap^r Sm^r Sp^r	(172)
pAM3448	pAM1824 containing the entire ORF all0187 including 76 bp upstream the translational start site under control of the *rbcL* promoter; Km^r Nm^r	This study
pAM3451	pBluescript-KS+ containing a -472 bp to +86 region relative to the translation start site of *all0187*; Ap^r	This study
pAM3450	pAM1956 carrying a SalI-SacI fragment from pAM3450 containing the -472 bp to +86 region; Km^r Nm^r	This study

genetic experiments, the phenotype of several independent exconjugant clones were determined to check for consistency.

DNA manipulations and plasmid constructions

Standard protocols were used for cloning, *E. coli* transformation, and PCR. Primers used in this study are listed in Table 4.2. DNA sequencing of plasmid inserts was performed by the Gene Technologies Laboratory (Texas A&M University, College Station, TX) following the Big Dye sequencing protocol (Applied Biosystems).

Plasmids used in this study are listed in Table 4.1. All plasmid constructions were verified by DNA sequence analysis. The open reading frame all0187 was inactivated by single homologous recombination. To construct the suicide plasmid pAM3438 an internal fragment of all0187 was amplified by PCR using oligonucleotides AMO-903 and AMO-904. The amplified fragment was digested with XhoI and SacI and inserted into the corresponding sites of the suicide plasmid pRL277. Six exconjugants were picked and streaked repeatedly to obtain complete segregation of the mutation. Recombination of the suicide plasmid into the all0187 gene and the absence of wild-type copies of the gene were confirmed by PCR and Southern blot analysis for three exconjugants (data not shown).

Plasmid pAM3348 was used for overexpression of all0187. This plasmid contains the all0187 gene (including 37 bp upstream and 2 bp downstream of the ORF) on a PCR

TABLE 4.2. PCR primer sequences for the all0187 gene study

Gene	Sense Primer	Sequence (5'→3')	Antisense primer	Sequence (5'→3')
all0187-internal	AMO-903	CTCGAGTGTTTCCCGTAACGTGGTTG	AMO-904	GAGCTCAAACTCACCCTAGAGCGACG
all0187	AMO-955	GCTTGATCGATCATAAATTTTAGGGAG	AMO-956	CCACTGTCGACTAACTATTTCCAATC
$P_{all0187}$-GFP	AMO-959	GGCCCCGGGCGTGCTTCTACCCTG	AMO-960	CACCTCTAGATTGAGATCCGGTATTTG
all0185	AMO-1032	GCCCAGATTTTCTTGGCAG	AMO-1033	CATGGGCTGCATTGACAC
all0186	AMO-1005	GTTATGCTGGCGTGG	AMO-1006	CAATACCCGTAACTC
alr0188	AMO-1007	GACTGGTTAAGTTCG	AMO-1008	GACTGGTTAAGTTCG
rnpB	AMO-489	GTACTGCGGGTAACTAAAG	AMO-490	AATGACTATTGACTAAAAG
nifH (all1445)	AMO-622	TTCACGGTCAACCTTACGG	AMO-1038	CGGTAAAGGCGG
hcwA (alr0093)	AMO-1549	CAGTGCTGCACCCGG	AMO-1550	GTTCCTGGTTGGGGT

fragment generated with primers AMO-955 containing an engineered ClaI site and AMO-956 containing an engineered *Sal*I restriction site. The PCR product was digested with ClaI and SalI and cloned into the same sites of pAM1824, which is a shuttle plasmid containing the *Anabaena* sp. strain PCC 7120 *rbcL* promoter on an EcoRI-SalI fragment from pAM496 cloned into pAM1011 in the same sites that contain a transcriptional terminator after de SalI site. pAM1011 contains an uncharacterized mutation resulting in a copy number in *Anabaena* sp. strain PCC 7120 higher than that of the original pDU1-based shuttle plasmids (168).

To construct a shuttle plasmid carrying a $P_{all0187}$-*gfp* transcriptional fusion, a fragment extending from 402 bp upstream to 63 bp downstream of the all0187 translational start site was amplified by PCR with primers AMO-959 and AMO-960. The fragment was digested with SmaI and XbaI and cloned into pBluescript-SK+ digested with EcoRV and XbaI generating pAM3351. A SalI-SacI fragment containing the insert was excised from pAM3351 and ligated into the same sites of pAM1956, a shuttle plasmid containing the promoterless *gfpmut2* gene, generating pAM3350.

RNA isolation

Total RNA was extracted using the RiboPure-Bacteria kit (Ambion) according to the manufacturer's instructions. *Anabaena* sp. strain PCC 7120 filaments from an exponentially growing culture were inoculated into 100 ml of BG-11_0 supplemented with 2.5 mM ammonium chloride and 5 mM MOPS [3-(N-morpholino) propanesulfonic acid]

(pH 8.0) to obtain an OD_{750} of 0.025-0.035. The cells were grown overnight until the OD_{750} reached 0.05 - 0.075, after which they were washed twice with water and then transferred to 100 ml of $BG\text{-}11_0$. The cells were collected by centrifugation at 4°C with 100 g of ice at 0, 4, 8, 12, 16, 20, and 24 h after nitrogen deprivation and the pellet was immediately frozen at -80°C until RNA isolation.

Northern RNA blot analysis

A total of 5 μg of RNA for each sample was denatured and run on a 1% denaturing formaldehyde gel in MOPS buffer and transferred to a Magna charge nylon membrane (GE Osmonics) with 10× SSPE. The blots were hybridized with radioactively labeled DNA probes prepared by random primer labeling and purified on Micro Bio-Spin P-30 columns (Bio-Rad). For probes, fragments amplified by PCR using the corresponding primers (Table 4.2) were labeled with ^{32}P-dCTP using random primer labeling. The hybridization solution contained 5× SSPE, 50% formamide, 0.5% SDS, and 5× Denhardt's solution and hybridization was performed overnight at 42°C. Blots were washed twice for 5 min with 2× SSC-0.1% SDS at room temperature, once for 10 min with 1× SSC-0.1% SDS at 65°C, and once for 10 min with 0.5× SSC-0.1% SDS at 65°C. Blots were exposed to a phosphorimager plate and scanned with a Phosphorimager BAS-5000 apparatus (Fujifilm).

Electron microscopy

Cultures were prepared for electron microscopy by collecting the cyanobacterial filaments by centrifugation and resuspending the pellet in 2 × BG-11, 2.5% acrolin, 2.5% glutaraldehyde, and 0.1% DMSO. Cells were fixed in a microwave for 2 minutes at 21˚C and 23 mmHg. The cells were washed three times in BG-11 medium and postfixed in 3% $K_3F(CN)_6$ over night at 4˚C. Fixed material was washed with distillated water four times at room temperature. Samples were embedded in 1% SeaKem low-gelling-temperature agarose. The samples were then dehydrated through a graded methanol series (two changes of 5 min each in 10, 20, 30, 40, 50, 60, 70, 80 and 90% methanol and three changes of 10 min each in 100% methanol) and two 10-min incubations in propylene oxide. The samples were infiltrated with a 1:1 mixture of propylene oxide-Epon 812 for 4 h and then with a 1:3 mixture of propylene oxide-Epon 812 overnight with gentle agitation. The samples were incubated in Epon 812 under a vacuum at room temperature for 4 h and then embedded in BEEM capsules with fresh degassed Epon 812 overnight at 55˚C. Thin sections were collected on Parlodion-carbon-coated copper grids and stained with 3% uranyl acetate for 30 min in a moist chamber. The sections were examined in a Siemens 101 electron microscope operated at 80 kV.

Light and fluorescence microscopy

Fluorescence microscopy was performed on a Zeiss Axioplan II microscope with a 40× objective and green fluorescent protein (GFP)-specific emission (518 ± 13 nm) filter

sets. The images were captured with a Hamamatsu 3 charge-coupled device (CCD) camera (C5810) attached to the microscope via an HR coupler (Diagnostic Instruments, Inc.). Some bright-field images were captured using an Olympus IX70 inverted microscope with a 100× objective (Olympus), connected to a Hamamatsu OrcaER C4742-95 CCD camera (Hamamatsu) and Simple PCI software version 6.0 (Compix Inc). A Piston GFP band-pass filter set (# 41025; Chroma Technology Corp.) was used for fluorescence images. The images were processed with Adobe Photoshop CS version 8.0 (Adobe System Incorporated).

Acetylene reduction assay

Wild-type *Anabaena* sp. strain PCC 7120 and the all0187 mutant strain AMC1369 were grown in 100 ml of BG-11 in 250 ml flasks under standard conditions to OD_{750} 0.4. The cells were collected by centrifugation in 250 ml conical bottles at room temperature, washed twice with 50 ml of water, resuspended in 3 ml of $BG\text{-}11_0$. Cells were then transferred to 10 ml tubes that were sealed with a rubber serum stopper. Acetylene replaced 1 ml of air and the tube was mixed by inversion for 1 min. The cells were incubated at 30^0C with standard illumination for 16 h and then 0.1 ml samples of gas were analyzed by gas chromatography with a 6-ft Porapak N column at 50°C.

Results

Identification and bioinformatic analysis of all0187

To identify genes that are involved in the early stages of heterocyst development in *Anabaena* sp. strain PCC 7120, we performed microarray experiments of the wild type comparing gene expression in the presence of ammonia and at 8 h after nitrogen step-down (data not shown). To assess their role in heterocyst development, we inactivated candidate genes obtained from our microarray experiments that showed a pattern of expression similar to *hetC*. The *hetC* gene is upregulated at 5-9 h after the onset of heterocyst differentiation and it is predicted to encode a bacterial ABC protein exporter (81, 118).

The all0187 gene product is predicted to contain an N-terminal transmembrane region (residues 83-105) but lacks a signal sequence. It is also predicted to contain a LytR/CpsA/Psr domain (residues 174-323), which is found in a group of proteins that have putative cell-wall maintenance properties (30, 86, 91). However, the function of the LytR domain is not yet known. This domain is not to be confused with the LytTR domain (previously also named LytR) that is found in a group of transcriptional regulators predicted to have DNA-binding properties (123). The all0187 gene is incorrectly annotated in several databases and by Fan *et al*. (2006) as being a transcriptional regulator because of this confusion in names. The LytR domain is located in the extracellular region of a number of membrane-bound proteins. The proteins in which this domain was originally described have unrelated functions: Psr is a penicillin

binding protein 5 (PDP-5) synthesis repressor, LytR influences transcription of its own gene and the *lytABC* operon by an unknown mechanism (91), and CpsA is involved in exocellular polysaccharide biosynthesis (86). All of these proteins have a short N-terminal cytoplasmic domain and a transmembrane domain that together form a signal-anchor, which can be inserted into the membrane. The promoter region of all0187 contains a putative NtcA binding site at position -60 relative to the start codon, which was identified by Su and collaborators in a bioinformatic screen for NtcA regulated genes (144).

Phenotype of the all0187 mutant

The all0187 gene was insertionally inactivated by single homologous recombination with a suicide plasmid that contained an internal fragment of the gene. Southern blot and PCR analysis showed that no copies of the wild-type gene could be detected in the mutant strain grown under antibiotic selection (data not shown). The phenotype of the resulting mutant strain, AMC1369, was analyzed in medium with or without a source of combined nitrogen. In medium with nitrate, vegetative cells of the mutant strain were unable to complete cell division and, consequently, appeared much longer than normal (Fig. 4.1B). In medium without a source of combined nitrogen, the mutant filaments were able to form heterocysts with a normal pattern along the filament, but were unable to grow diazotrophically. Curiously, vegetative cells began to show normal cell division within 8 to 48 h following nitrogen step-down (Fig. 4.1B). Time-lapse microscopy of mutant filaments after nitrogen step-down revealed that differentiating cells elongated

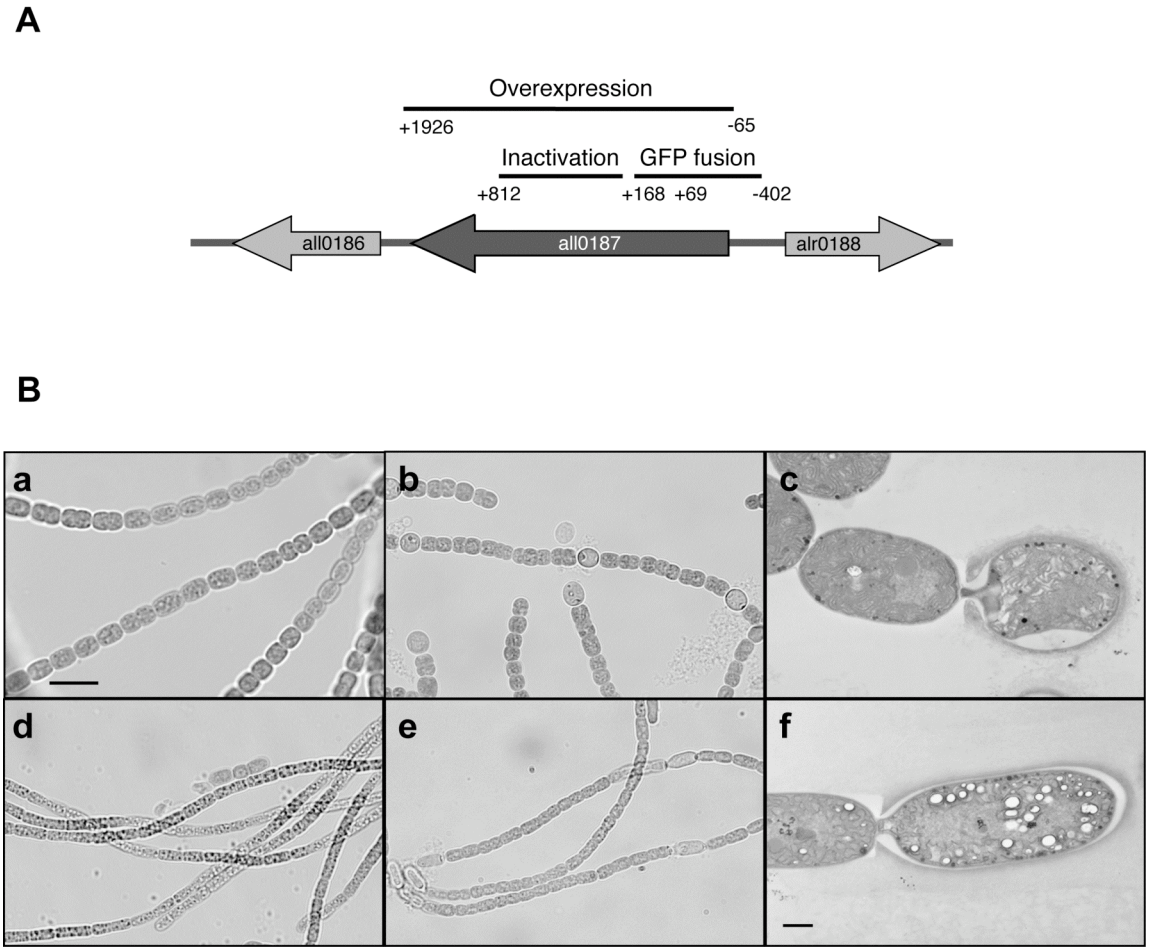

FIG. 4.1 Construction and characterization of the all0187 mutant strain.
(A) Representation of the of all0187 locus on the *Anabaena* sp. strain PCC 7120 chromosome and the DNA fragments used in our study for genetic experiments. Suicide plasmid pAM3438 contains an internal fragment of all0187 and was used to inactivate all0187 by single homologous recombination. Plasmid pAM3448 contains the entire all0187 ORF under control of the *rbcL* promoter and was used to overexpress all0187. Plasmid pAM3450 contains the promoter region of all0187 fused to the *gfpmut2* reporter gene. Numbering is relative to the start codon.
(B)The all0187 mutant strain (AMC1369) is showed defective vegetative cell division in nitrate-containing medium and abnormal heterocyst morphogenesis after nitrogen step-down. *Anabaena* s. strain PCC 7120 wild type (a, b, e) and all0187 mutant (c, d, f) strains grown in nitrate-containing BG-11 medium (a, c) or nitrate-free BG-11_0 medium (b, d, e, f). Size bars are 10 μm in the bright-field micrographs in panels a, b, c, and d, and 500 nm in the electron micrographs in panels e and f.

and did not complete septum formation with their neighboring vegetative cell(s) resulting in abnormally long heterocysts that were open-ended (data not shown). Differentiating cells visibly exceeded normal length (and continued to elongate) at around 9 h after nitrogen step-down, a time when proheterocysts are not yet morphologically distinguishable in the wild-type strain (Fig. 4.3). After nitrogen step-down, if vegetative cells did not undergo division prior to differentiating into a heterocyst, then the septum formation defect was not obvious.

To confirm that the observed phenotypes were due to the inactivation of all0187 and not to potential second-site mutations, the all0187 mutant was complemented with shuttle plasmid pAM3448, which carries the all0187 gene expressed from the *rbcL* promoter. The complemented strain had a wild-type phenotype, which shows that the inactivation of all0187 was responsible for all of the observed phenotypes. Overexpression of all0187 from pAM3448 in a wild-type background did not produce an abnormal phenotype in either nitrogen-containing or nitrogen-free medium (data not shown).

The all0187 mutant strain was unable to grow diazotrophically even though heterocysts were present. Therefore, we used electron microscopy to determine whether the heterocyst envelope layers were absent or altered (Fig. 4.1B). The electron micrographs showed that the glycolipid, polysaccharide, peptidoglycan, and outer membrane layers of the heterocyst envelope in AMC1369 were all intact, but that the cells exhibited zones of low electron density in the cytoplasm, that were not present in the wild type. Thin layer

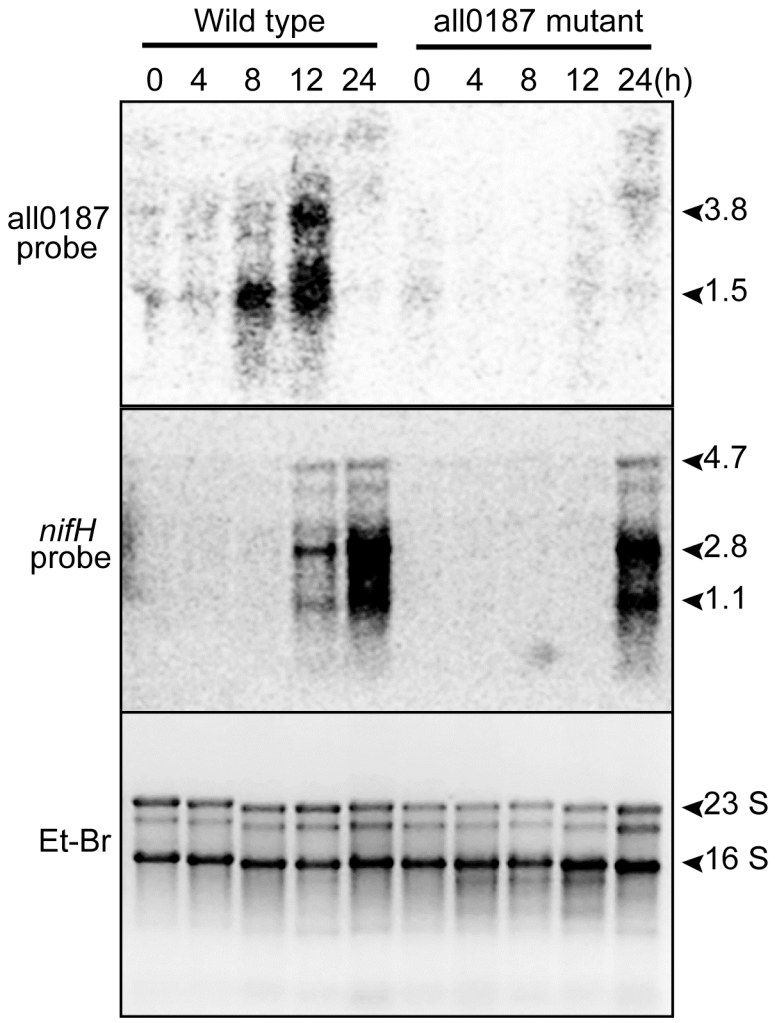

FIG. 4.2. Northern RNA blot analysis of *Anabaena* sp. strain PCC 7120 and the all0187 mutant strain during heterocyst development. The upper panel shows ethidium bromide staining as a loading control. The middle and lower panels show Northern RNA blots probed with a strand-specific radioactive probe for all0187 or *nifH* genes, respectively. For all0187 a 3.8-kb and a 1.5-kb transcript were detected as indicated by the arrows, and for *nifH*, three transcripts were detected at 4.7 kb, 2.8 kb, and 1.1 kb as indicated by the arrows.

chromatography experiments showed that heterocyst glycolipids were present in the mutant strain (data not shown). Similar results were obtained by Fan and collaborators (43).

Expression analysis of all0187

To assay whether expression of all0187 increases after nitrogen step-down, we performed northern RNA blot analysis. Total RNA was extracted from wild-type *Anabaena* sp. strain PCC 7120 and from the AMC1369 strain at several times after nitrogen step-down. Figure 4.2 shows that all0187 transcripts were detectable by 8 h after nitrogen step-down and continued to increase until 12 h, but were no longer detectable at 24 h following nitrogen depletion. To determine whether or not the increase in expression of all0187 takes place specifically in differentiating cells we used a $P_{all0187}$-*gfp* reporter strain. We found that after nitrogen step-down, GFP fluorescence increased in differentiating cells at around 9 h and continued to rise until around 16 h. GFP fluorescence began to decrease at 28 h following nitrogen depletion (data not shown). GFP fluorescence from vegetative cells remained at a low level following nitrogen step-down.

Because the all0187 mutant strain failed to growth diazotrophically even though it had an apparently normal heterocyst envelop, we determined if the *nifH* gene was expressed at normal levels. Previous studies have shown that the *nifHDK* operon is expressed around 12-18 h after heterocyst induction in the wild type (15, 159). Under our

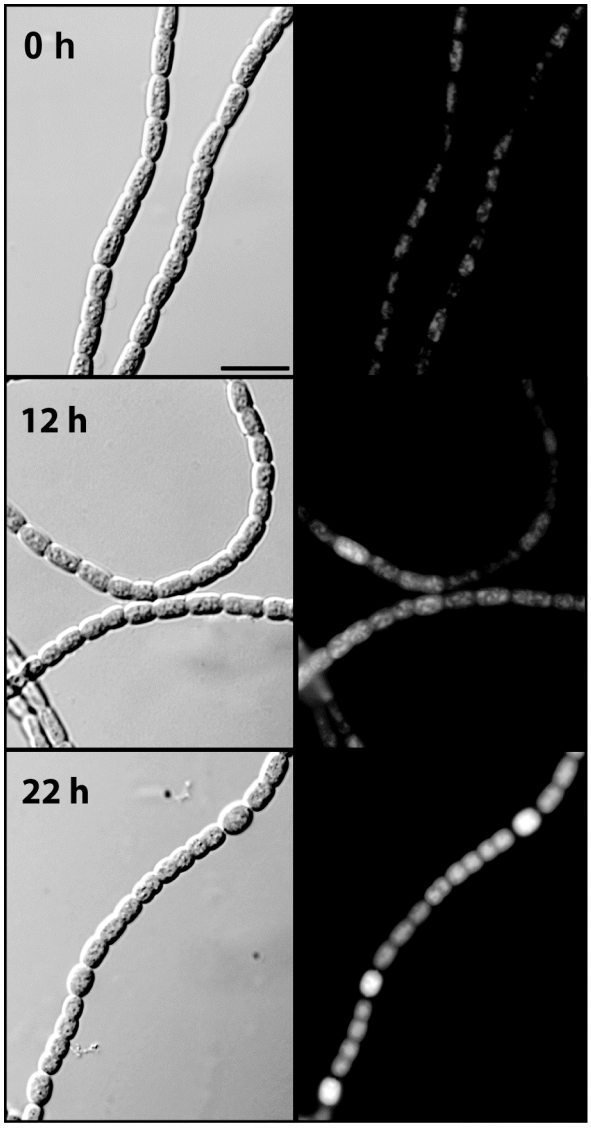

FIG. 4.3. A $P_{all0187}$-*gfp* reporter strain (AMC1370) showed increasing GFP fluorescence in differentiating cells of filaments at different times after nitrogen step-down. Differential interference contrast microscopy images of AMC1370 filaments (Left panels). Corresponding GFP fluorescence images (Right panels). Size bar 10 μm.

conditions, *nifH* expression in the wild type began to increase by 12 h after nitrogen step-down (Figure 4.2). However, in the all0187 mutant strain, *nifH* expression began to increase only by 24 h after nitrogen step-down. However, overt morphological heterocyst development was not delayed in this experiment.

An acetylene reduction assay was used to determine if functional nitrogenase activity was being produced in the all0187 mutant strain. Nitrogenase activity was only 30% lower in the all0187 mutant strain compared to wild type (data not shown). These results suggest that the diazotrophic growth defect caused by the inactivation of all0187 is not due to the inability of the cell to reduce molecular dinitrogen but must instead be due to an inability to supply fixed nitrogen to vegetative cells.

Discussion

The all0187 gene of *Anabaena* sp. strain PCC 7120 has previously been identified by Fan and colleagues in a transposon mutagenesis screen for mutants with a Fox^- phenotype (43). We independently identified this gene with microarray analysis of gene expression in wild type at different times after nitrogen step-down. The all0187 gene was found to be strongly upregulated at 8 h (data not shown). Using a $P_{all0187}$-*gfp* reporter strain, we determined that all0187 was expressed at a low level in vegetative cells grown on nitrate, but at around 9 h after nitrogen step-down all0187 was specifically upregulated in differentiating cells.

We inactivated the all0187 gene by single homologous recombination and obtained a phenotype similar to that originally described by Fan et al. 2006. The all0187 mutant was unable to grow diazotrophically and septum formation between heterocysts and vegetative cells was incomplete. The constriction defect was observed when vegetative cells divided after nitrogen step-down to give rise to two daughter cells, one of which differentiated into a heterocyst. In addition, we observed that mutant filaments grown in nitrate-containing media showed defective cell division characterized by improper septum placement and invagination. Interestingly, vegetative cell division returned to normal after nitrogen step-down and during diazotrophic growth, and defects were apparent only at the heterocyst-vegetative cell boundary. These results suggest that the all0187 gene is required for normal morphogenesis of the differentiating cells by controlling proper formation of the septum during cell division.

The all0187 gene is predicted to encode a protein containing an N-terminal transmembrane domain and a periplasmic LytR/CpsA/Psr domain. In *Bacillus subtilis* a *lytR* mutant shows upregulation of the *lytABC* operon, which is involved in synthesis and maintenance of the cell wall; LytC is an N-acetyl-muramoyl-L-alanine amidase (autolysin) involved in cleaving the peptidoglycan cell wall (91). The mutant phenotype is presumably due to defective feedback regulation related to cell wall defects. In many bacteria, the peptidoglycan cell wall is cleaved by multiple hydrolases, including amidases, which remove peptide side chains from the carbohydrate polymer. Peptidoglycan hydrolases are thought to be essential for separating daughter cells after

division is concluded, and their absence causes incomplete septal invagination (132). Based on the all0187 mutant phenotype, we can envision that the set of peptidoglycan hydrolases involved in separating vegetative cells are different than those involved in separating differentiating cells from vegetative cells. In *Anabaena* sp. strain PCC 7120, HcwA encodes an autolysin, which has been shown to be required for heterocyst maturation (178). We hypothesized that the abnormal cell division in the all0187 mutant might be caused by altered expression of *hcwA*. However, we did not detect a change in expression of *hcwA* in the absence of all0187 (data not shown). Alternatively, the enzymatic activity of HcwA may be altered in the all0187 mutant. It is also possible that the effect of all0187 inactivation is mediated through other enzymes involved in peptidoglycan layer formation or remodeling during septation.

In many cases, inability of an *Anabaena* sp. strain PCC 7120 mutant to grow diazotrophically is caused by a block in heterocyst differentiation or by a defect that compromises the integrity of the envelope layers, allowing more oxygen to enter the heterocysts and inactivate the nitrogenase. However, the all0187 mutant was able to form heterocysts with seemingly normal polysaccharide and glycolipid layers. We then asked whether the incomplete constriction of the septum between heterocyst and vegetative cells exposes the intracellular environment of the heterocyst to oxygen and, thus, inactivates the nitrogenase. Northern blot analysis showed that *nifH* expression in the all0187 mutant was upregulated after nitrogen step-down, but with a 12 h delay. Although morphological heterocyst development observed by light microscopy was not

delayed in the mutant strain, the delay in *nifH* expression may have been caused by late establishment of the micro-oxic environment due to the septum formation defect or possibly delayed deposition of the envelope layers.

The all0187 mutant retained seventy percent of the wild-type nitrogenase activity, and this amount of activity should have been sufficient to sustain diazotrophic growth, although at a slower rate. Inactivation of the heterocyst-specific gene *fdxH* reduced nitrogenase activity to less then 50%, which led to slow diazotrophic growth (104).

However, all0187 mutant filaments stop growing after a few days in medium without a source of combined nitrogen. These results suggest that after nitrogen step-down, heterocysts of the all0187 mutant are still partially functional in spite of the obvious morphological defects, and that the diazotrophic growth defect is caused by the inability of the heterocysts to deliver fixed nitrogen to neighboring vegetative cells. This explanation is plausible in the context of recent findings showing cytoplasm-to-cytoplasm movement of a small fluorophore in *Anabaena*, which indicates the existence of non-specific intercellular channels that allow metabolite exchange between cells of the filament (113). In the all0187 mutant, defective septum closure could interfere with the formation of these channels at the interface between heterocysts and vegetative cells and, thus, prevent normal metabolite exchange from taking place.

Summary

The *all0187* gene of the filamentous cyanobacterium *Anabaena* sp. strain PCC 7120 is predicted to encode a protein containing the LytR domain associated with regulation of cell wall maintenance. Northern RNA blot analysis showed that *all0187* expression was upregulated at 8 h after nitrogen step-down. Fluorescence microscopy of a $P_{all0187}$-*gfp* reporter strain revealed increasing GFP fluorescence in proheterocysts and heterocysts at around 9 h after nitrogen step-down, whereas vegetative cells maintained a low level of fluorescence. Insertional inactivation of *all0187* caused a partial cell division defect of vegetative cells grown in nitrate-containing medium. In nitrogen-free medium, mutant filaments formed abnormally long heterocysts and were unable to grow diazotrophically. Septum formation between heterocysts and their flanking vegetative cells was incomplete, leaving one or both poles of the heterocysts partially opened. We investigated whether these morphological defects lead to inactivation of nitrogenase due to lessened protection against oxygen. Acetylene reduction assays for nitrogenase activity showed that the mutant strain retained approximately seventy percent of the wild-type activity, indicating that heterocysts of the *all0187* mutant strain are partially functional. We hypothesize that the diazotrophic growth defect is caused by the inability of the heterocysts to transport fixed nitrogen to the neighboring vegetative cells. The contributions to the data included in this chapter are as follows: Rodrigo A. Mella-Herrera, M. Ramona Aldea, and James W. Golden.

CHAPTER V

CONCLUSIONS

Heterocyst development in the filamentous cyanobacterium *Anabaena* sp. strain PCC 7120 is one of the simplest models of cellular differentiation, where two cell types, the vegetative and heterocyst cells, rely on mutual nutritional support to survive under nitrogen deprived conditions. The heterocysts are arranged in a semiregular pattern along filaments of vegetative cells, which presumably facilitates efficient metabolite exchange. Distinct sets of genes become active at different times during the ~ 24 h required for a differentiating cell to reach morphological and functional maturity. Central to the initiation and progress of heterocyst development are interweaving transcriptional feedback loops in which some transcriptional factors regulate gene expression at a specific stage of the development and others play a regulatory role throughout development. A great deal is known about how initiation of heterocyst development is regulated, but it is not very clear what are the regulatory factors required for progression through development.

It has been proposed that different sigma factors act sequentially during heterocyst development directing RNA polymerase to transcribe the sets of genes that encode products needed at the early, middle, and late stages of development in *Anabaena* sp. strain PCC 7120. This hypothesis was based on the sigma factor cascade model shown to control sporulation in *Bacillus subtilis* where sigma factors become active at different

times in a cell-type specific manner and inactivation of one sigma factor causes a developmental block (87). It subsequently became evident that this model does not apply to heterocyst development in *Anabaena* sp. strain PCC 7120 because inactivation of sigma factor genes individually and in pairs failed to halt development (84).

Developmentally regulated sigma factors

Time-lapse fluorescence microscopy of GFP reporter strains proved to be a more fruitful approach to discern which of the eight chromosomally encoded sigma factors are potentially important for cellular differentiation in *Anabaena* sp. strain PCC 7120 (Chapter II). Sigma factors specifically expressed in heterocysts are very likely to be involved in development. The results indicated that the *sigC*, *sigG*, and *sigE* genes are upregulated specifically in differentiating cells at 4 h, 9 h, and 16 h, respectively, following nitrogen deprivation. Our study also found that promoter regions of four other sigma factor genes *sigB2*, *sigD*, *sigI*, and *sigJ* were constitutively active in both cell types regardless of the presence or absence of a source of combined nitrogen (Chapter II). This brings the sum of sigma factor genes likely active in the heterocyst cells up to seven. We can envision that multiple sigma factors with overlapping promoter specificities are present in the differentiating cells at any given time during development in *Anabaena* sp. strain PCC 7120, but the identity and relative amounts of the sigma factors present changes throughout development. Under these circumstances, promoter recognition properties and association with other transcription factors would determine which sigma factor is primarily used for transcription of a particular gene.

Regulatory targets of heterocyst-specific sigma factors

Timing of *sigC* expression seems to coincide with a role in transcribing genes required for early stages of heterocyst development. Expression of early genes such as *hepA*, *hepC*, and *hetR* is delayed and reduced in a *sigC* mutant. Inactivation of *sigE* caused delayed expression of the *nifH* gene, which indicates that SigE is important for expression of late genes. Lack of a more severe phenotype for the *sigC* or the *sigE* mutant is most likely due to partial functional redundancy among sigma factors, which has been observed in other cyanobacteria (127). The delayed heterocyst development in the *sigC* mutant suggests that the developmental genes that make up the SigC regulon are not transcribed as efficiently by alternate sigma factors, or maybe that sigma factors that recognize and transcribe SigC-dependent promoters are not available until later during heterocyst development. Microarray experiments for *sigC* and *sigE* that compare expression profiles of the sigma factor mutant and the wild type after nitrogen deprivation will provide a more complete insight into the sets of genes regulated by each of these sigma factors. To investigate *sigG* regulatory targets, a conditional mutant is needed because insertional inactivation proved to be lethal. One of the caveats of the microarray technique is its inability to detect small changes in gene expression. Functional redundancy among sigma factors will likely minimize the effect of the absence of one sigma factor on target gene expression, and the smaller amplitude changes in gene expression may be missed by the microarray. Real time RT-PCR will need to be used to more accurately quantify changes in expression of specific target genes.

The c-di-GMP bacterial second messenger is involved in heterocyst development

Second messengers are small molecules that integrate a multitude of sensory inputs to trigger the appropriate response of a cell or a population of cells to changes in the intra- and extracellular environments. During the last decade, the novel bacterial second messenger, c-di-GMP (3',5'-cyclic diguanylic acid), has emerged as a molecule important for diverse cellular processes such as biofilm formation, pathogenesis, virulence, exopolysaccharide formation, and development (137). Environmental stimuli such as photons, oxygen, amino acids, and electrons, are thought to regulate the activity of the enzymes that synthesize and hydrolyze c-di-GMP, diguanylate cyclase and phosphodiesterase, respectively (137). The *Anabaena* sp. strain PCC 7120 genome carries fourteen genes that have the GGDEF domain known to be associated with diguanylate cyclase activity. One of these genes, all2874, encodes a bona fide diguanylate cyclase (Chapter III). The all2874 gene is predicted to form an operon with the upstream all2875 gene, which encodes a multidomain protein including a PAS domain that senses blue light (122). Inactivation of all2874 caused a decreased tendency to form heterocysts and this phenotype was exacerbated by light intensity. Genetic epistasis experiments suggest that all2874 acts upstream of *hetR* (or HetR activity), the earliest known heterocyst-specific gene.

Our results lend support to the hypothesis that the putative operon all2875-all2874 senses and relays information about light conditions. This information feeds into the pathway of heterocyst differentiation at a very early stage. Cyanobacteria are

photosynthetic organisms, which means their growth is directly dependent upon light. It stands to reason that heterocyst development would be coordinated with the growth rate; in response to information about light intensity and implicitly the rate of growth, filaments would modulate heterocyst development to keep up with their growth rate. In *Anabaena cylindrica* high light leads to increased frequency of heterocysts (3). A disconnect between these two processes would cause heterocyst development to lag behind the growth rate resulting in a gradually decreasing percentage of heterocysts. The all2874 mutant phenotype illustrates this disconnect; the tendency to form heterocysts decreases with increasing light intensity, but at low light, when filaments grow more slowly, the heterocyst pattern is essentially wild type.

Regulation of diguanylate cyclase activity

Diguanylate cyclase activity can be regulated by phosphorylation (24). In one of the best studied examples, PleD, a diguanylate cyclase from *Caulobacter crescentus*, phosphorylation promotes dimerization, which is required for the catalytic activity of PleD (24). We speculate that a similar mechanism regulates the All2874 diguanylate cyclase in *Anabaena* sp. strain PCC 7120. The all2875 gene encodes a multidomain protein predicted to act as a two-component hybrid sensor and regulator, and it includes a sensor kinase, a response regulator, and a phosphorelay domain. In two-component hybrid proteins the signal is conveyed through a four-step phosphorelay. An environmental signal triggers autophosphorylation of the sensor kinase module and then the phosphoryl group is transferred through the rest of the modules and finally to its

cognate response regulator (48). Presumably, All2875 senses light and then conveys the information through a similar phosphorelay mechanism. Phosphorylation of the receiver domain in the All2874 response regulator protein would activate the GGDEF domain to catalyze c-di-GMP synthesis from two GTP molecules.

Despite rapid advances in the understanding of c-di-GMP as a signaling molecule, we have yet to discover the molecular mechanism underlying its regulatory effects. One regulatory function that is clearly mediated by c-di-GMP is the control of gene expression. DNA binding proteins may be influenced by c-di-GMP and thus directly affect gene expression, but c-di-GMP could also act on structural proteins and enzymes to direct cell physiology through posttranscriptional mechanisms (24). Few c-di-GMP receptors proteins are known and cyanobacteria do not have homologues for these proteins. It remains to be determined how production of c-di-GMP relates to the regulation of heterocyst development.

More detailed studies of sigma factors in organisms such as *Escherichia coli* and *Bacillus subtilis* have revealed that the activity of each sigma factor is regulated by multiple mechanisms. Post-translational modification, proteolytic degradation, or the presence of anti-sigma factors ensure that genes are expressed at appropriate levels in each cell type at the proper time (87). We show here that in *Anabaena* sp. strain PCC 7120, sigma factor genes are controlled at the transcriptional level, but it remains to be

determined what other regulatory strategies are employed to control activity and turnover of sigma factors required for heterocyst development.

Peptidoglycan remodeling during heterocyst formation

The cells that have committed to differentiate into heterocysts undergo morphological and biochemical changes to produce a propitious intracellular environment for nitrogenase activity. A polysaccharide layer is first deposited around the enlarged cell and beneath it a glycolipid layer is synthesized to minimize entry of oxygen, which would otherwise inactivate the nitrogenase. Once synthesized, the heterocyst envelope glycolipids and polysaccharides must traverse the cell wall before they are deposited at the outside surface of differentiating heterocysts. Therefore, it has been proposed that rearrangement, perforation, or partial degradation of the peptidoglycan layer is required for assembly of the glycolipid or polysaccharide layer of the heterocyst envelope and that this process leads to the expansion and rounding of differentiating cells during heterocyst formation (178). HcwA, a predicted N-acetylmuramoyl-L-alanine amidase (a peptidoglycan hydrolase), has been proposed to increase the permeability of the peptidoglycan layer of the cell wall, to facilitate access of glycolipids and polysaccharides (178). When deposition and assembly of the two-layered envelope is disrupted, heterocysts are unable to fix atmospheric nitrogen in the presence of oxygen (Fox^- phenotype) and, thus, filaments cannot survive in nitrogen-deprived medium. In other bacteria, peptidoglycan hydrolases are thought to be important for inserting new

material into the cell wall during cell growth, as well as splitting the septum that separates daughter cells at the end of cell division (132).

Involvement of All0187 in heterocyst-specific morphogenetic changes

Inactivation of the all0187 gene caused a Fox$^-$ phenotype and the heterocysts presented abnormal morphology (Chapter IV). The all0187 gene is annotated as a transcriptional regulator due to the presence of the LytR domain that in other bacteria has been associated with transcriptional attenuation of genes involved in cell wall maintenance; how this domain affects expression of other genes is not yet clear (30). The all0187 gene was strongly upregulated in our microarray experiments at 8 h after nitrogen step-down and a reporter strain carrying a *gfp* fusion with the promoter region of all0187 showed that upregulation of all0187 takes place specifically in differentiating heterocysts. The all0187 gene was recently identified in a transposon mutagenesis study that screened for Fox$^-$ mutants, but the authors did not explore the temporal and spatial patterns of this gene's expression (43). The study remarked on the incomplete constriction of the septum between heterocysts and flanking vegetative cells in the mutant strain, but did not investigate the impact of all0187 inactivation on the regulation of other genes. In addition, we noticed that vegetative cells are defective in cell division when grown in the presence of nitrate, but more normal division is recovered after nitrogen step-down. The incomplete septal constriction at the heterocyst-to-vegetative cell boundary resulted in one or both ends of the heterocyst appearing more open and potentially more permeable to oxygen. However, the mutant still retained seventy percent of its wild-type

nitrogenase activity, which should be sufficient to sustain growth although probably at a slower rate. Despite forming seemingly functional heterocysts, the all0187 mutant was unable to grow diazotrophically.

The all0187 mutant phenotype and the presence of the LytR domain indicate a role in cell wall maintenance. The elongated heterocyst and the defective septal constriction are consistent with abnormal activity of peptidoglycan hydrolases or penicillin binding proteins (peptidoglycan synthesis), which result in failure to constrict the septum between two daughter cells if not present in adequate levels (132). The production and turnover of the cell wall material could be affected such that cell growth continues beyond the wild-type size. In *E. coli*, the absence of FtsI, a penicillin binding protein, abolishes peptidoglycan synthesis at the septum, but still supports cell elongation (17). Time-lapse microscopy showed that at around 9 h following nitrogen step-down, the size of differentiating cells begins to surpass that of the wild type. In a wild-type strain, differentiating cells commit to forming heterocysts at around 9 h but do not show obvious signs of differentiation until about 12-16 h after nitrogen step-down. So these results suggest that remodeling of the peptidoglycan begins immediately after the cells have committed to differentiate into heterocysts. Microarray experiments should aid in determining what genes have altered expression in the all0187 mutant. Alternatively, the absence of all0187 could affect activity of peptidoglycan related enzymes.

Heterocysts-to-vegetative cell communication may be disrupted in the all0187 mutant

We propose that inability of the all0187 mutant to grow diazotrophically is an indirect result of impaired septum formation at the division site between heterocysts and flanking vegetative cells. Recent findings showed that intercellular movement of molecules takes place directly between the cytoplasms of adjacent cells in *Anabaena* sp. strain PCC 7120, which indicates the existence of non-specific intercellular channels that allow metabolite exchange between cells of the filament (113). SepJ is a membrane bound protein that was shown to localize at the cell-cell interface and was proposed to compose these channels. Mullineaux et al (2008) hypothesized that the intercellular channels would need to be formed as the cell divides, before cell wall formation inhibits lateral diffusion. In the all0187 mutant, defective septum closure could interfere with the formation of these channels at the interface between heterocysts and vegetative cells and, thus, prevent normal metabolite exchange. It would be interesting to know whether SepJ localization is disrupted in the all0187 mutant at the heterocyst to vegetative cell boundary.

Closing comments

Our view of the fundamental genetic components that govern heterocyst development has been continuously refined over the last two decades. The search for genes that are required for heterocyst development has received a boost in the last few years due the successful application of DNA microarray expression profiling for detection of genes that are upregulated during heterocyst development in *Anabaena* sp. strain PCC 7120. A

more detailed picture is available for the regulatory factors that trigger heterocyst development than those involved throughout its progression (Chapter I). One aspect that has not been given enough attention is how environmental signals, other then nitrogen deprivation, affect the decision to form heterocysts. For example, how does light intensity affect heterocyst development? How is heterocyst development regulated on a bright sunny day as opposed to a cloudy day? Not surprisingly, proteins containing light sensing domains such as GAF and PAS domains are abundant in cyanobacteria, including *Anabaena*. One such protein encoded by the all2875 gene was found to be capable of sensing light by virtue of one of its PAS domains. Our results indicate that the response regulator encoded by the all2874 gene may work in conjunction with All2875 to convey information about light intensity by synthesizing the c-di-GMP second messenger (Chapter III). This information might be integrated into the decision to form heterocysts by affecting expression of regulatory genes necessary for development.

We do not yet have a clear picture of how the heterocyst pattern is formed in *Anabaena*. The main inhibitory signals of heterocyst development, PatS and HetN, are believed to control pattern formation by lateral inhibition, but it is not known how these signals reach the neighboring cells (16). One theory is that these signals are exported out of the heterocyst and travel along the filament through the continuous periplasmic space. Recent research opens the possibility that intercellular connections may facilitate movement of various signals between cells (113). Nitrogen fixation products are also thought to influence pattern formation. Our *sigC* reporter strain results, which show that

some cells with preexisting fluorescence prior to nitrogen step-down formed heterocysts, point to another aspect of pattern formation that has not been explored: the existence of a pattern under nitrogen-replete conditions, even before induction of heterocyst development by nitrogen deprivation (Chapter II). We can envision that a pre-pattern would poise cells to form heterocysts, although mathematical models show that a pre-pattern is not a prerequisite for pattern formation. Meinhardt and Gierer (2000) propose that random fluctuation in gene expression coupled with autoregulation and long-range inhibition can generate a pattern in several organisms including *Anabaena* (108). The partial pattern of GFP fluorescence in the *sigC* reporter strain when nitrate is available suggests that SigC is involved in pre-pattern formation. But this hypothesis gives rise to the proverbial "chicken and egg" question. Does SigC regulate early genes required for heterocyst development, such as *hetR* or is it HetR that regulates SigC in the first place? Future research will hopefully resolve this issue.

Heterocyst formation is reversible until about 9-12 h when cells become committed to differentiate. We do not yet have a full understanding of the factors involved in commitment. How do heterocysts "seal the deal"? To determine what genes are involved in this stage, we would need to look for genes that are expressed around the same time frame. Few genes are known to be upregulated at this time. We have identified a sigma factor, *sigG* that is upregulated at 9 h after nitrogen step-down. Based on its timing of expression SigG may play a role in commitment (Chapter II). Data suggest that peptidoglycan remodeling takes place during differentiation. The question is, does

peptidoglycan remodeling occur before or after commitment? The all0187 mutant phenotype may suggest that this process occurs after commitment, since functional heterocysts were still able to form in the mutant despite an apparent impairment in peptidoglycan formation (Chapter IV).

The functional maturity of a heterocyst is reflected by its ability to fix nitrogen. One of the major gaps in our knowledge of heterocyst development is the regulation of the *nif* genes that encode the nitrogenase complex. Our reporter strain results showed *sigE* expression around 16 h after nitrogen step-down, when *nifH* is known to be upregulated (Chapter II). Furthermore, genetic data also suggests a role for SigE in regulation of *nifH* expression (Chapter II). *In vitro* transcription studies will help clarify the roles played by the developmentally regulated sigma factors during heterocyst formation and whether sigma factors collaborate with transcriptional activators such as NtcA and HetR to transcribe heterocyst-specific genes.

REFERENCES

1. **Adams, D. G.** 1997. Cyanobacteria, p. 109-148. *In* J. A. Shapiro and M. Dworkin (ed.), Bacteria as Multicellular Organisms. Oxford University Press, New York.

2. **Adams, D. G.** 2000. Heterocyst formation in cyanobacteria. Curr. Opin. Microbiol. **3:**618-624.

3. **Adams, D. G., and N. G. Carr.** 1981. Heterocyst differentiation and cell division in the cyanobacterium Anabaena cylindrica: effect of high light intensity. J Cell Sci **49:**341-352.

4. **Adams, D. G., and N. G. Carr.** 1981. The developmental biology of heterocyst and akinete formation in cyanobacteria. Crit. Rev. Microbiol. **9:**45-100.

5. **Aldea, M. R., K. Kumar, and J. W. Golden.** 2008. Heterocyst development and pattern formation. ASM Press, Washington, DC.

6. **Aldehni, M. F., and K. Forchhammer.** 2006. Analysis of a non-canonical NtcA-dependent promoter in *Synechococcus elongatus* and its regulation by NtcA and PII. Arch. Microbiol. **184:**378-386.

7. **Aldridge, P., R. Paul, P. Goymer, P. Rainey, and U. Jenal.** 2003. Role of the GGDEF regulator PleD in polar development of *Caulobacter crescentus*. Mol. Microbiol. **47:**1695-1708.

8. **Amikam, D., and M. Y. Galperin.** 2006. PilZ domain is part of the bacterial c-di-GMP binding protein. Bioinformatics **22:**3-6.

9. **Argueta, C., K. Yuksek, R. Patel, and M. L. Summers.** 2006. Identification of *Nostoc punctiforme* akinete-expressed genes using differential display. Mol. Microbiol. **61:**748-757.

10. **Barak, I., and A. J. Wilkinson.** 2005. Where asymmetry in gene expression originates. Mol. Microbiol. **57:**611-620.

11. **Bauer, C. C., K. S. Ramaswamy, S. Endley, L. A. Scappino, J. W. Golden, and R. Haselkorn.** 1997. Suppression of heterocyst differentiation in *Anabaena* PCC 7120 by a cosmid carrying wild-type genes encoding enzymes for fatty acid synthesis. FEMS Microbiol. Lett. **151:**23-30.

12. **Black, K., W. J. Buikema, and R. Haselkorn.** 1995. The *hglK* gene is required for localization of heterocyst-specific glycolipids in the cyanobacterium *Anabaena* sp. strain PCC 7120. J. Bacteriol. **177:**6440-6448.

13. **Black, T. A., Y. Cai, and C. P. Wolk.** 1993. Spatial expression and autoregulation of *hetR*, a gene involved in the control of heterocyst development in *Anabaena*. Mol. Microbiol. **9:**77-84.

14. **Black, T. A., and C. P. Wolk.** 1994. Analysis of a Het- mutation in *Anabaena* sp. strain PCC 7120 implicates a secondary metabolite in the regulation of heterocyst spacing. J. Bacteriol. **176:**2282-2292.

15. **Borthakur, D., M. Basche, W. J. Buikema, P. B. Borthakur, and R. Haselkorn.** 1990. Expression, nucleotide sequence and mutational analysis of two open reading frames in the *nif* gene region of *Anabaena* sp. strain PCC7120. Mol. Gen. Genet. **221:**227-234.

16. **Borthakur, P. B., C. C. Orozco, S. S. Young-Robbins, R. Haselkorn, and S. M. Callahan.** 2005. Inactivation of *patS* and *hetN* causes lethal levels of heterocyst differentiation in the filamentous cyanobacterium *Anabaena* sp. PCC 7120. Mol. Microbiol. **57:**111-123.

17. **Botta, G. A., and J. T. Park.** 1981. Evidence for involvement of penicillin-binding protein 3 in murein synthesis during septation but not during cell elongation. J. Bacteriol. **145:**333-340.

18. **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.

19. **Brahamsha, B., and R. Haselkorn.** 1991. Isolation and characterization of the gene encoding the principal sigma factor of the vegetative cell RNA polymerase

from the cyanobacterium *Anabaena* sp. strain PCC 7120. J. Bacteriol. **173:**2442-2450.

20. **Buikema, W. J., and R. Haselkorn.** 2001. Expression of the *Anabaena hetR* gene from a copper-regulated promoter leads to heterocyst differentiation under repressing conditions. Proc. Natl. Acad. Sci. USA **98:**2729-2734.

21. **Buikema, W. J., and R. Haselkorn.** 1991. Isolation and complementation of nitrogen fixation mutants of the cyanobacterium *Anabaena* sp. strain PCC 7120. J. Bacteriol. **173:**1879-1885.

22. **Cai, Y., and C. P. Wolk.** 1997. *Anabaena* sp. strain PCC 7120 responds to nitrogen deprivation with a cascade-like sequence of transcriptional activations. J. Bacteriol. **179:**267-271.

23. **Callahan, S. M., and W. J. Buikema.** 2001. The role of HetN in maintenance of the heterocyst pattern in *Anabaena* sp. PCC 7120. Mol. Microbiol. **40:**941-950.

24. **Camilli, A., and B. L. Bassler.** 2006. Bacterial small-molecule signaling pathways. Science **311:**1113-1116.

25. **Campbell, E. L., M. F. Cohen, and J. C. Meeks.** 1997. A polyketide-synthase-like gene is involved in the synthesis of heterocyst glycolipids in *Nostoc punctiforme* strain ATCC 29133. Arch. Microbiol. **167:**251-258.

26. **Carrasco, C. D., J. A. Buettner, and J. W. Golden.** 1995. Programmed DNA rearrangement of a cyanobacterial *hupL* gene in heterocysts. Proc. Natl. Acad. Sci. USA **92:**791-795.

27. **Carrasco, C. D., and J. W. Golden.** 1995. Two heterocyst-specific DNA rearrangements of nif operons in *Anabaena cylindrica* and *Nostoc* sp. strain Mac. Microbiology **141 (Pt 10):**2479-2487.

28. **Carrasco, C. D., S. D. Holliday, A. Hansel, P. Lindblad, and J. W. Golden.** 2005. Heterocyst-specific excision of the *Anabaena* sp. strain PCC 7120 *hupL* element requires *xisC*. J. Bacteriol. **187:**6031-6038.

29. **Caslake, L. F., T. M. Gruber, and D. A. Bryant.** 1997. Expression of two alternative sigma factors of *Synechococcus* sp. strain PCC 7002 is modulated by carbon and nitrogen stress. Microbiology **143:**3807-3818.

30. **Chatfield, C. H., H. Koo, and R. G. Quivey, Jr.** 2005. The putative autolysin regulator LytR in *Streptococcus mutans* plays a role in cell division and is growth-phase regulated. Microbiology **151:**625-631.

31. **Colon-Lopez, M. S., D. M. Sherman, and L. A. Sherman.** 1997. Transcriptional and translational regulation of nitrogenase in light-dark- and continuous-light-grown cultures of the unicellular cyanobacterium *Cyanothece* sp. strain ATCC 51142. J. Bacteriol. **179:**4319-4327.

32. **Curatti, L., E. Flores, and G. Salerno.** 2002. Sucrose is involved in the diazotrophic metabolism of the heterocyst- forming cyanobacterium *Anabaena* sp. FEBS Lett. **513:**175-178.

33. **Dixon, R., and D. Kahn.** 2004. Genetic regulation of biological nitrogen fixation. Nat. Rev. Microbiol. **2:**621-631.

34. **Dong, Y., X. Huang, X. Y. Wu, and J. Zhao.** 2000. Identification of the active site of HetR protease and its requirement for heterocyst differentiation in the cyanobacterium *Anabaena* sp. strain PCC 7120. J. Bacteriol. **182:**1575-1579.

35. **Edwards, R., and M. Merrick.** 1995. The role of uridylyltransferase in the control of *Klebsiella pneumoniae nif* gene regulation. Mol. Gen. Genet. **247:**189-198.

36. **Ehira, S., and M. Ohmori.** 2006. NrrA directly regulates the expression of *hetR* during heterocyst differentiation in the cyanobacterium *Anabaena* sp. strain PCC 7120. J. Bacteriol. **188:** 8520-8525.

37. **Ehira, S., and M. Ohmori.** 2006. NrrA, a nitrogen-responsive response regulator facilitates heterocyst development in the cyanobacterium *Anabaena* sp. strain PCC 7120. Mol. Microbiol. **59:**1692-1703.

38. **Elhai, J., A. Vepritskiy, A. M. Muro-Pastor, E. Flores, and C. P. Wolk.** 1997. Reduction of conjugal transfer efficiency by three restriction activities of *Anabaena* sp. strain PCC 7120. J. Bacteriol. **179:**1998-2005.

39. **Ernst, A., T. Black, Y. Cai, J. M. Panoff, D. N. Tiwari, and C. P. Wolk.** 1992. Synthesis of nitrogenase in mutants of the cyanobacterium *Anabaena* sp. strain PCC 7120 affected in heterocyst development or metabolism. J. Bacteriol. **174:**6025-6032.

40. **Espinosa, J., K. Forchhammer, S. Burillo, and A. Contreras.** 2006. Interaction network in cyanobacterial nitrogen regulation: PipX, a protein that interacts in a 2-oxoglutarate dependent manner with PII and NtcA. Mol. Microbiol. **61:**457-469.

41. **Fadi Aldehni, M., J. Sauer, C. Spielhaupter, R. Schmid, and K. Forchhammer.** 2003. Signal transduction protein P(II) is required for NtcA-regulated gene expression during nitrogen deprivation in the cyanobacterium Synechococcus elongatus strain PCC 7942. J Bacteriol **185:**2582-2591.

42. **Fan, Q., G. Huang, S. Lechno-Yossef, C. P. Wolk, T. Kaneko, and S. Tabata.** 2005. Clustered genes required for synthesis and deposition of envelope glycolipids in *Anabaena* sp. strain PCC 7120. Mol. Microbiol. **58:**227-243.

43. **Fan, Q., S. Lechno-Yossef, S. Ehira, T. Kaneko, M. Ohmori, N. Sato, S. Tabata, and C. P. Wolk.** 2006. Signal transduction genes required for heterocyst maturation in *Anabaena* sp. strain PCC 7120. J. Bacteriol. **188:**6688-6693.

44. **Fiedler, G., A. M. Muro-Pastor, E. Flores, and I. Maldener.** 2001. NtcA-dependent expression of the *devBCA* operon, encoding a heterocyst-specific ATP-binding cassette transporter in *Anabaena* spp. J. Bacteriol. **183:**3795-3799.

45. **Flores, E., A. Herrero, C. P. Wolk, and I. Maldener.** 2006. Is the periplasm continuous in filamentous multicellular cyanobacteria? Trends Microbiol. **14:**439-443.

46. **Forchhammer, K.** 2004. Global carbon/nitrogen control by PII signal transduction in cyanobacteria: from signals to targets. FEMS Microbiol. Rev. **28:**319-333.

47. **Forchhammer, K., and N. Tandeau de Marsac.** 1995. Phosphorylation of the PII protein (*glnB* gene product) in the cyanobacterium *Synechococcus* sp. strain PCC 7942: analysis of *in vitro* kinase activity. J. Bacteriol. **177:**5812-5817.

48. **Freeman, J. A., B. N. Lilley, and B. L. Bassler.** 2000. A genetic analysis of the functions of LuxN: a two-component hybrid sensor kinase that regulates quorum sensing in *Vibrio harveyi*. Mol. Microbiol. **35:**139-149.

49. **Frias, J. E., E. Flores, and A. Herrero.** 1994. Requirement of the regulatory protein NtcA for the expression of nitrogen assimilation and heterocyst development genes in the cyanobacterium *Anabaena* sp. PCC 7120. Mol. Microbiol. **14:**823-832.

50. **Frías, J. E., E. Flores, and A. Herrero.** 1994. Requirement of the regulatory protein NtcA for the expression of nitrogen assimilation and heterocyst development genes in the cyanobacterium *Anabaena* sp. PCC 7120. Mol. Microbiol. **14:**823-832.

51. **Genay, M., S. Catakli, A. Kleinclauss, A. Andrieux, B. Decaris, and A. Dary.** 2006. Genetic instability of *whiG* gene during the aerial mycelium development of *Streptomyces ambofaciens* ATCC23877 under different conditions of nitrogen limitations. Mutat. Res. **595:**80-90.

52. **Golden, J. W., C. D. Carrasco, M. E. Mulligan, G. J. Schneider, and R. Haselkorn.** 1988. Deletion of a 55-kilobase-pair DNA element from the chromosome during heterocyst differentiation of *Anabaena* sp. strain PCC 7120. J. Bacteriol. **170:**5034-5041.

53. **Golden, J. W., S. J. Robinson, and R. Haselkorn.** 1985. Rearrangement of nitrogen fixation genes during heterocyst differentiation in the cyanobacterium *Anabaena*. Nature (London) **314:**419-423.

54. **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.

55. **Golden, J. W., and D. R. Wiest.** 1988. Genome rearrangement and nitrogen fixation in Anabaena blocked by inactivation of *xisA* gene. Science **242:**1421-1423.

56. **Golden, J. W., and H.-S. Yoon.** 1998. Heterocyst formation in *Anabaena*. Curr. Opin. Microbiol. **1:**623-629.

57. **Golden, J. W., and H. S. Yoon.** 2003. Heterocyst development in *Anabaena*. Curr. Opin. Microbiol. **6:**557-563.

58. **Gonzalez, L., V. Phalip, and C. C. Zhang.** 2001. Characterization of PknC, a Ser/Thr kinase with broad substrate specificity from the cyanobacterium *Anabaena* sp. strain PCC 7120. Eur. J. Biochem. **268:**1869-1875.

59. **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.

60. **Gruber, T. M., and D. A. Bryant.** 1998. Characterization of the alternative sigma-factors SigD and SigE in *Synechococcus* sp. strain PCC 7002. SigE is implicated in transcription of post-exponential-phase-specific genes. Arch. Microbiol. **169:**211-219.

61. **Gruber, T. M., and C. A. Gross.** 2003. Multiple sigma subunits and the partitioning of bacterial transcription space. Annu. Rev. Microbiol. **57:**441-466.

62. **Hanson, T. E., K. Forchhammer, N. Tandeau de Marsac, and J. C. Meeks.** 1998. Characterization of the *glnB* gene product of *Nostoc punctiforme* strain ATCC 29133: *glnB* or the PII protein may be essential. Microbiology **144 (Pt 6):**1537-1547.

63. **Haselkorn, R., J. W. Golden, P. J. Lammers, and M. E. Mulligan.** 1986. Developmental rearrangement of cyanobacterial nitrogen-fixation genes. Trends Genet. **2:**255-259.

64. **Hebbar, P. B., and S. E. Curtis.** 2000. Characterization of *devH*, a gene encoding a putative DNA binding protein required for heterocyst function in *Anabaena* sp. strain PCC 7120. J. Bacteriol. **182:**3572-3581.

65. **Helmann, J. D.** 2002. The extracytoplasmic function (ECF) sigma factors. Adv. Microb. Physiol. **46:**47-110.

66. **Herbaud, M. L., A. Guiseppi, F. Denizot, J. Haiech, and M. C. Kilhoffer.** 1998. Calcium signalling in *Bacillus subtilis*. Biochim. Biophys. Acta **1448:**212-226.

67. **Herrero, A., A. M. Muro-Pastor, A. Valladares, and E. Flores.** 2004. Cellular differentiation and the NtcA transcription factor in filamentous cyanobacteria. FEMS Microbiol. Rev. **28:**469-487.

68. **Huang, G., Q. Fan, S. Lechno-Yossef, E. Wojciuch, C. P. Wolk, T. Kaneko, and S. Tabata.** 2005. Clustered genes required for the synthesis of heterocyst envelope polysaccharide in *Anabaena* sp. strain PCC 7120. J. Bacteriol. **187:**1114-1123.

69. **Huang, X., Y. Dong, and J. Zhao.** 2004. HetR homodimer is a DNA-binding protein required for heterocyst differentiation, and the DNA-binding activity is inhibited by PatS. Proc. Natl. Acad. Sci. USA **101:**4848-4853.

70. **Hubschmann, T., H. Yamamoto, T. Gieler, N. Murata, and T. Borner.** 2005. Red and far-red light alter the transcript profile in the cyanobacterium *Synechocystis* sp. PCC 6803: impact of cyanobacterial phytochromes. FEBS Lett. **579:**1613-1618.

71. **Huckauf, J., C. Nomura, K. Forchhammer, and M. Hagemann.** 2000. Stress responses of *Synechocystis* sp. strain PCC 6803 mutants impaired in genes encoding putative alternative sigma factors. Microbiology **146:**2877-2889.

72. **Ikeda, T. P., A. E. Shauger, and S. Kustu.** 1996. *Salmonella typhimurium* apparently perceives external nitrogen limitation as internal glutamine limitation. J. Mol. Biol. **259:**589-607.

73. **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.

74. **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.

75. **Jenal, U.** 2004. Cyclic di-guanosine-monophosphate comes of age: a novel secondary messenger involved in modulating cell surface structures in bacteria? Curr. Opin. Microbiol. **7:**185-191.

76. **Jenal, U., and J. Malone.** 2006. Mechanisms of cyclic-di-GMP signaling in bacteria. Annu. Rev. Genet. **40:**385-407.

77. **Jiang, F., S. Wisen, M. Widersten, B. Bergman, and B. Mannervik.** 2000. Examination of the transcription factor NtcA-binding motif by *in vitro* selection of DNA sequences from a random library. J. Mol. Biol. **301:**783-793.

78. **Jones, K. M., W. J. Buikema, and R. Haselkorn.** 2003. Heterocyst-specific expression of *patB*, a gene required for nitrogen fixation in *Anabaena* sp. strain PCC 7120. J. Bacteriol. **185:**2306-2314.

79. **Kaiser, D.** 2001. Building a multicellular organism. Annu. Rev. Genet. **35:**103-123.

80. **Kazmierczak, B. I., M. B. Lebron, and T. S. Murray.** 2006. Analysis of FimX, a phosphodiesterase that governs twitching motility in *Pseudomonas aeruginosa*. Mol. Microbiol. **60:**1026-1043.

81. **Khudyakov, I., and C. P. Wolk.** 1997. *hetC*, a gene coding for a protein similar to bacterial ABC protein exporters, is involved in early regulation of heterocyst differentiation in *Anabaena* sp. strain PCC 7120. J. Bacteriol. **179:**6971-6978.

82. **Khudyakov, I., and C. P. Wolk.** 1997. *hetC*, a gene coding for a protein similar to bacterial ABC protein exporters, is involved in early regulation of heterocyst differentiation in *Anabaena* sp. strain PCC 7120. J. Bacteriol. **179:**6971-6978.

83. **Khudyakov, I. Y., and J. W. Golden.** 2004. Different functions of HetR, a master regulator of heterocyst differentiation in *Anabaena* sp. PCC 7120, can be separated by mutation. Proc. Natl. Acad. Sci. USA **101:**16040-16045.

84. **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.

85. **Kill, K., T. T. Binnewies, T. Sicheritz-Ponten, H. Willenbrock, P. F. Hallin, T. M. Wassenaar, and D. W. Ussery.** 2005. Genome update: sigma factors in 240 bacterial genomes. Microbiology **151:**3147-3150.

86. **Koskiniemi, S., M. Sellin, and M. Norgren.** 1998. Identification of two genes, *cpsX* and *cpsY*, with putative regulatory function on capsule expression in group B streptococci. FEMS Immunol. Med. Microbiol. **21:**159-168.

87. **Kroos, L., B. Zhang, H. Ichikawa, and Y. T. Yu.** 1999. Control of sigma factor activity during *Bacillus subtilis* sporulation. Mol. Microbiol. **31:**1285-1294.

88. **Kulasakara, H., V. Lee, A. Brencic, N. Liberati, J. Urbach, S. Miyata, D. G. Lee, A. N. Neely, M. Hyodo, Y. Hayakawa, F. M. Ausubel, and S. Lory.** 2006. Analysis of *Pseudomonas aeruginosa* diguanylate cyclases and phosphodiesterases reveals a role for bis-(3'-5')-cyclic-GMP in virulence. Proc. Natl. Acad. Sci. USA **103:**2839-2844.

89. **Laurent, S., H. Chen, S. Bedu, F. Ziarelli, L. Peng, and C. C. Zhang.** 2005. Nonmetabolizable analogue of 2-oxoglutarate elicits heterocyst differentiation under repressive conditions in *Anabaena* sp. PCC 7120. Proc. Natl. Acad. Sci. USA **102:**9907-9912.

90. **Laurent, S., K. Forchhammer, L. Gonzalez, T. Heulin, C. C. Zhang, and S. Bedu.** 2004. Cell-type specific modification of PII is involved in the regulation of nitrogen metabolism in the cyanobacterium *Anabaena* PCC 7120. FEBS Lett. **576:**261-265.

91. **Lazarevic, V., P. Margot, B. Soldo, and D. Karamata.** 1992. Sequencing and analysis of the *Bacillus subtilis lytRABC* divergon: a regulatory unit encompassing the structural genes of the N-acetylmuramoyl-L-alanine amidase and its modifier. J. Gen. Microbiol. **138:**1949-1961.

92. **Lechno-Yossef, S., Q. Fan, S. Ehira, N. Sato, and C. P. Wolk.** 2006. Mutations in four regulatory genes have interrelated effects on heterocyst maturation in *Anabaena* sp. strain PCC 7120. J. Bacteriol. **188:**7387-7395.

93. **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.

94. **Leganes, F., F. Fernandez-Pinas, and C. P. Wolk.** 1994. Two mutations that block heterocyst differentiation have different effects on akinete differentiation in *Nostoc ellipsosporum*. Mol. Microbiol. **12:**679-684.

95. **Lemeille, S., J. Geiselmann, and A. Latifi.** 2005. Crosstalk regulation among group 2-sigma factors in *Synechocystis* PCC6803. BMC Microbiol. **5:**18.

96. **Li, B., X. Huang, and J. Zhao.** 2002. Expression of *hetN* during heterocyst differentiation and its inhibition of *hetR* up-regulation in the cyanobacterium *Anabaena* sp. PCC 7120. FEBS Lett. **517:**87-91.

97. **Liang, J., L. Scappino, and R. Haselkorn.** 1992. The *patA* gene product, which contains a region similar to CheY o*f Escherichia coli*, controls heterocyst pattern formation in the cyanobacterium *Anabaena* 7120. Proc. Natl. Acad. Sci. USA **89:**5655-5659.

98. **Liang, J., L. Scappino, and R. Haselkorn.** 1993. The *patB* gene product, required for growth of the cyanobacterium *Anabaena* sp. strain PCC 7120 under nitrogen-limiting conditions, contains ferredoxin and helix-turn-helix domains. J. Bacteriol. **175:**1697-1704.

99. **Liu, D., and J. W. Golden.** 2002. *hetL* overexpression stimulates heterocyst formation in *Anabaena* sp. strain PCC 7120. J. Bacteriol. **184:**6873-6881.

100. **Lockau, W., B. Massalsky, and A. Dirmeier.** 1988. Purification and partial characterization of a calcium-stimulated protease from the cyanobacterium, *Anabaena variabilis*. Eur. J. Biochem. **172:**433-438.

101. **Maheswaran, M., C. Urbanke, and K. Forchhammer.** 2004. Complex formation and catalytic activation by the PII signaling protein of N-acetyl-L-glutamate kinase from *Synechococcus elongatus* strain PCC 7942. J. Biol. Chem. **279:**55202-55210.

102. **Makarova, K. S., E. V. Koonin, R. Haselkorn, and M. Y. Galperin.** 2006. Cyanobacterial response regulator PatA contains a conserved N-terminal domain (PATAN) with an alpha-helical insertion. Bioinformatics **22:**1297-1301.

103. **Mariscal, V., A. Herrero, and E. Flores.** 2007. Continuous periplasm in a filamentous, heterocyst-forming cyanobacterium. Mol Microbiol **65:**1139-1145.

104. **Masepohl, B., K. Scholisch, K. Gorlitz, C. Kutzki, and H. Bohme.** 1997. The heterocyst-specific *fdxH* gene product of the cyanobacterium *Anabaena* sp. PCC 7120 is important but not essential for nitrogen fixation. Mol. Gen. Genet. **253:**770-776.

105. **Meeks, J. C., E. L. Campbell, M. L. Summers, and F. C. Wong.** 2002. Cellular differentiation in the cyanobacterium *Nostoc punctiforme*. Arch. Microbiol. **178:**395-403.

106. **Meeks, J. C., and J. Elhai.** 2002. Regulation of cellular differentiation in filamentous cyanobacteria in free-living and plant-associated symbiotic growth states. Microbiol. Mol. Biol. Rev. **66:**94-121; table of contents.

107. **Meeks, J. C., J. Elhai, T. Thiel, M. Potts, F. Larimer, J. Lamerdin, P. Predki, and R. Atlas.** 2001. An overview of the genome of *Nostoc punctiforme*, a multicellular, symbiotic cyanobacterium. Photosynth. Res. **70:**85-106.

108. **Meinhardt, H., and A. Gierer.** 2000. Pattern formation by local self-activation and lateral inhibition. Bioessays **22:**753-760.

109. **Merrick, M. J., and R. A. Edwards.** 1995. Nitrogen control in bacteria. Microbiol. Rev. **59:**604-622.

110. **Montgomery, B. L.** 2007. Sensing the light: photoreceptive systems and signal transduction in cyanobacteria. Mol. Microbiol. **64:**16-27.

111. **Moorhead, G. B., and C. S. Smith.** 2003. Interpreting the plastid carbon, nitrogen, and energy status. A role for PII? Plant Physiol. **133:**492-498.

112. **Mulligan, M. E., and R. Haselkorn.** 1989. Nitrogen fixation (*nif*) genes of the cyanobacterium *Anabaena* species strain PCC 7120. The *nifB-fdxN-nifS-nifU* operon. J. Biol. Chem. **264:**19200-19207.

113. **Mullineaux, C. W., V. Mariscal, A. Nenninger, H. Khanum, A. Herrero, E. Flores, and D. G. Adams.** 2008. Mechanism of intercellular molecular exchange in heterocyst-forming cyanobacteria. EMBO J. **27:**1299-1308.

114. **Muro-Pastor, A. M., A. Herrero, and E. Flores.** 2001. Nitrogen-regulated group 2 sigma factor from *Synechocystis* sp. strain PCC 6803 involved in survival under nitrogen stress. J. Bacteriol. **183:**1090-1095.

115. **Muro-Pastor, A. M., E. Olmedo-Verd, and E. Flores.** 2006. All4312, an NtcA-regulated two-component response regulator in *Anabaena* sp. strain PCC 7120. FEMS Microbiol. Lett. **256:**171-177.

116. **Muro-Pastor, A. M., A. Valladares, E. Flores, and A. Herrero.** 2002. Mutual dependence of the expression of the cell differentiation regulatory protein HetR and the global nitrogen regulator NtcA during heterocyst development. Mol. Microbiol. **44:**1377-1385.

117. **Muro-Pastor, A. M., A. Valladares, E. Flores, and A. Herrero.** 1999. The *hetC* gene is a direct target of the NtcA transcriptional regulator in cyanobacterial heterocyst development. J. Bacteriol. **181:**6664-6669.

118. **Muro-Pastor, A. M., A. Valladares, E. Flores, and A. Herrero.** 1999. The *hetC* gene is a direct target of the NtcA transcriptional regulator in cyanobacterial heterocyst development. J. Bacteriol. **181:**6664-6669.

119. **Muro-Pastor, M. I., J. C. Reyes, and F. J. Florencio.** 2005. Ammonium assimilation in cyanobacteria. Photosynth. Res. **83:**135-150.

120. **Nair, U., J. L. Ditty, H. Min, and S. S. Golden.** 2002. Roles for sigma factors in global circadian regulation of the cyanobacterial genome. J. Bacteriol. **184:**3530-3538.

121. **Narikawa, R., S. Okamoto, M. Ikeuchi, and M. Ohmori.** 2004. Molecular evolution of PAS domain-containing proteins of filamentous cyanobacteria through domain shuffling and domain duplication. DNA Res. **11:**69-81.

122. **Narikawa, R., K. Zikihara, K. Okajima, Y. Ochiai, M. Katayama, Y. Shichida, S. Tokutomi, and M. Ikeuchi.** 2006. Three putative photosensory light, oxygen or voltage (LOV) domains with distinct biochemical properties from the filamentous cyanobacterium *Anabaena* sp. PCC 7120. Photochem. Photobiol. **82:**1627-1633.

123. **Nikolskaya, A. N., and M. Y. Galperin.** 2002. A novel type of conserved DNA-binding domain in the transcriptional regulators of the AlgR/AgrA/LytR family. Nucleic Acids Res. **30:**2453-2459.

124. **Olmedo-Verd, E., E. Flores, A. Herrero, and A. M. Muro-Pastor.** 2005. HetR-dependent and -independent expression of heterocyst-related genes in an *Anabaena* strain overproducing the NtcA transcription factor. J. Bacteriol. **187:**1985-1991.

125. **Olmedo-Verd, E., A. M. Muro-Pastor, E. Flores, and A. Herrero.** 2006. Localized induction of the *ntcA* regulatory gene in developing heterocysts of *Anabaena* sp. strain PCC 7120. J. Bacteriol. **188:**6694-6699.

126. **Orozco, C. C., D. D. Risser, and S. M. Callahan.** 2006. Epistasis analysis of four genes from *Anabaena* sp. strain PCC 7120 suggests a connection between PatA and PatS in heterocyst pattern formation. J. Bacteriol. **188:**1808-1816.

127. **Osanai, T., M. Ikeuchi, and K. Tanaka.** 2008. Group 2 sigma factors in cyanobacteria. Physiol. Plant. **133:** 490-506.

128. **Osanai, T., Y. Kanesaki, T. Nakano, H. Takahashi, M. Asayama, M. Shirai, M. Kanehisa, I. Suzuki, N. Murata, and K. Tanaka.** 2005. Positive regulation of sugar catabolic pathways in the cyanobacterium *Synechocystis* sp. PCC 6803 by the group 2 sigma factor *sigE*. J. Biol. Chem. **280:**30653-30659.

129. **Paul, R., S. Weiser, N. C. Amiot, C. Chan, T. Schirmer, B. Giese, and U. Jenal.** 2004. Cell cycle-dependent dynamic localization of a bacterial response regulator with a novel di-guanylate cyclase output domain. Genes Dev. **18:**715-727.

130. **Paz-Yepes, J., E. Flores, and A. Herrero.** 2003. Transcriptional effects of the signal transduction protein P(II) (*glnB* gene product) on NtcA-dependent genes in *Synechococcus* sp. PCC 7942. FEBS Lett. **543:**42-46.

131. **Popa, R., P. K. Weber, J. Pett-Ridge, J. A. Finzi, S. J. Fallon, I. D. Hutcheon, K. H. Nealson, and D. G. Capone.** 2007. Carbon and nitrogen fixation and metabolite exchange in and between individual cells of *Anabaena oscillarioides*. Isme J. **1:**354-360.

132. **Priyadarshini, R., M. A. de Pedro, and K. D. Young.** 2007. Role of peptidoglycan amidases in the development and morphology of the division septum in *Escherichia coli*. J. Bacteriol. **189:**5334-5347.

133. **Ramasubramanian, T. S., T. F. Wei, A. K. Oldham, and J. W. Golden.** 1996. Transcription of the *Anabaena* sp. strain PCC 7120 *ntcA* gene: multiple transcripts and NtcA binding. J. Bacteriol. **178:**922-926.

134. **Ramirez, M. E., P. B. Hebbar, R. Zhou, C. P. Wolk, and S. E. Curtis.** 2005. *Anabaena* sp. strain PCC 7120 gene *devH* is required for synthesis of the heterocyst glycolipid layer. J. Bacteriol. **187:**2326-2331.

135. **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.

136. **Romling, U.** 2005. Characterization of the rdar morphotype, a multicellular behaviour in *Enterobacteriaceae*. Cell. Mol. Life Sci. **62:**1234-1246.

137. **Romling, U., and D. Amikam.** 2006. Cyclic di-GMP as a second messenger. Curr. Opin. Microbiol. **9:**218-228.

138. **Ross, P., H. Weinhouse, Y. Aloni, D. Michaeli, P. Weinberger-Ohana, R. Mayer, S. Braun, E. De Vroom, G. A. Van der Marel, J. H. Van Boom, and M.**

Benziman. 1987. Regulation of cellulose synthesis in *Acetobacter xylinum* by cyclic diguanylic acid. Nature **325:**279-281.

139. **Schneider, G. J., and R. Haselkorn.** 1988. RNA polymerase subunit homology among cyanobacteria, other eubacteria and archaebacteria. J. Bacteriol. **170:**4136-4140.

140. **Shi, Y., W. Zhao, W. Zhang, Z. Ye, and J. Zhao.** 2006. Regulation of intracellular free calcium concentration during heterocyst differentiation by HetR and NtcA in *Anabaena* sp. PCC 7120. Proc. Natl. Acad. Sci. USA **103:**11334-11339.

141. **Smith, R. J., S. Hobson, and I. R. Ellis.** 1987. Evidence for calcium-mediated regulation of heterocyst frequency and nitrogenase activity in *Nostoc* 6720. New Phytologist **105:**531-541.

142. **Straley, S. C., G. V. Plano, E. Skrzypek, P. L. Haddix, and K. A. Fields.** 1993. Regulation by Ca2+ in the *Yersinia* low-Ca2+ response. Mol. Microbiol. **8:**1005-1010.

143. **Studholme, D. J., and M. Buck.** 2000. The biology of enhancer-dependent transcriptional regulation in bacteria: insights from genome sequences. FEMS Microbiol. Lett. **186:**1-9.

144. **Su, Z., V. Olman, F. Mao, and Y. Xu.** 2005. Comparative genomics analysis of NtcA regulons in cyanobacteria: regulation of nitrogen assimilation and its coupling to photosynthesis. Nucleic Acids Res. **33:**5156-5171.

145. **Tal, R., H. C. Wong, R. Calhoon, D. Gelfand, A. L. Fear, G. Volman, R. Mayer, P. Ross, D. Amikam, H. Weinhouse, A. Cohen, S. Sapir, P. Ohana, and M. Benziman.** 1998. Three *cdg* operons control cellular turnover of cyclic di-GMP in *Acetobacter xylinum*: genetic organization and occurrence of conserved domains in isoenzymes. J. Bacteriol. **180:**4416-4425.

146. **Tamayo, R., J. T. Pratt, and A. Camilli.** 2007. Roles of cyclic diguanylate in the regulation of bacterial pathogenesis. Annu. Rev. Microbiol. **61:**131-148.

147. **Tanigawa, R., M. Shirokane, S. Maeda Si, T. Omata, K. Tanaka, and H. Takahashi.** 2002. Transcriptional activation of NtcA-dependent promoters of *Synechococcus* sp. PCC 7942 by 2-oxoglutarate *in vitro*. Proc. Natl. Acad. Sci. USA **99:**4251-4255.

148. **Tarutina, M., D. A. Ryjenkov, and M. Gomelsky.** 2006. An unorthodox bacteriophytochrome from *Rhodobacter sphaeroides* involved in turnover of the second messenger c-di-GMP. J. Biol. Chem. **281:**34751-34758.

149. **Thiel, T.** 1996. Isolation and characterization of the VnfEN genes of the cyanobacterium *Anabaena variabilis*. J. Bacteriol. **178:**4493-4499.

150. **Thiel, T., E. M. Lyons, and J. C. Erker.** 1997. Characterization of genes for a second Mo-dependent nitrogenase in the cyanobacterium *Anabaena variabilis*. J. Bacteriol. **179:**5222-5225.

151. **Thomas, C., C. R. Andersson, S. R. Canales, and S. S. Golden.** 2004. PsfR, a factor that stimulates *psbAI* expression in the cyanobacterium *Synechococcus elongatus* PCC 7942. Microbiology **150:**1031-1040.

152. **Tisa, L. S., B. M. Olivera, and J. Adler.** 1993. Inhibition of *Escherichia col*i chemotaxis by omega-conotoxin, a calcium ion channel blocker. J. Bacteriol. **175:**1235-1238.

153. **Torrecilla, I., F. Leganes, I. Bonilla, and F. Fernandez-Pinas.** 2004. A calcium signal is involved in heterocyst differentiation in the cyanobacterium *Anabaena* sp. PCC7120. Microbiology **150:**3731-3739.

154. **Ulrich, L. E., and I. B. Zhulin.** 2007. MiST: a microbial signal transduction database. Nucleic Acids Res **35:**D386-390.

155. **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.

156. **Vazquez-Bermudez, M. F., A. Herrero, and E. Flores.** 2002. 2-Oxoglutarate increases the binding affinity of the NtcA (nitrogen control) transcription factor for the *Synechococcus glnA* promoter. FEBS Lett. **512:**71-74.

157. **Vetting, M. W., S. S. Hegde, J. E. Fajardo, A. Fiser, S. L. Roderick, H. E. Takiff, and J. S. Blanchard.** 2006. Pentapeptide repeat proteins. Biochemistry **45:**1-10.

158. **Viswanathan, P., M. Singer, and L. Kroos.** 2006. Role of sigmaD in regulating genes and signals during *Myxococcus xanthus* development. J. Bacteriol. **188:**3246-3256.

159. **Wei, T.-F., T. S. Ramasubramanian, and J. W. Golden.** 1994. *Anabaena* sp. strain PCC 7120 *ntcA* gene required for growth on nitrate and heterocyst development. J. Bacteriol. **176:**4473-4482.

160. **Wei, T. F., T. S. Ramasubramanian, and J. W. Golden.** 1994. *Anabaena* sp. strain PCC 7120 *ntcA* gene required for growth on nitrate and heterocyst development. J. Bacteriol. **176:**4473-4482.

161. **Wilde, A., B. Fiedler, and T. Borner.** 2002. The cyanobacterial phytochrome Cph2 inhibits phototaxis towards blue light. Mol. Microbiol. **44:**981-988.

162. **Wolk, C. P.** 1996. Heterocyst formation. Annu. Rev. Genet. **30:**59-78.

163. **Wolk, C. P., Y. Cai, L. Cardemil, E. Flores, B. Hohn, M. Murry, G. Schmetterer, B. Schrautemeier, and R. Wilson.** 1988. Isolation and complementation of mutants of *Anabaena* sp. strain PCC 7120 unable to grow aerobically on dinitrogen. J. Bacteriol. **170:**1239-1244.

164. **Wolk, C. P., A. Ernst, and J. Elhai.** 1994. Heterocyst metabolism and development, p. 769-823. *In* D. A. Bryant (ed.), The molecular biology of cyanobacteria, vol. 1. Kluwer Academic Publishers, Dordrecht.

165. **Wong, F. C., and J. C. Meeks.** 2001. The *hetF* gene product is essential to heterocyst differentiation and affects HetR function in the cyanobacterium *Nostoc punctiforme*. J. Bacteriol. **183:**2654-2661.

166. **Wosten, M. M.** 1998. Eubacterial sigma-factors. FEMS Microbiol. Rev. **22:**127-150.

167. **Wu, J., and A. Newton.** 1997. Regulation of the *Caulobacter* flagellar gene hierarchy; not just for motility. Mol. Microbiol. **24:**233-239.

168. **Wu, X., D. W. Lee, R. A. Mella, and J. W. Golden.** 2007. The *Anabaena* sp. strain PCC 7120 *asr1734* gene encodes a negative regulator of heterocyst development. Mol. Microbiol. **64:**782-794.

169. **Wu, X., D. Liu, M. H. Lee, and J. W. Golden.** 2004. *patS* minigenes inhibit heterocyst development of *Anabaena* sp. strain PCC 7120. J. Bacteriol. **186:**6422-6429.

170. **Xu, X., and C. P. Wolk.** 2001. Role for *hetC* in the transition to a nondividing state during heterocyst differentiation in *Anabaena* sp. J. Bacteriol. **183:**393-396.

171. **Yoon, H.-S., and J. W. Golden.** 2001. PatS and products of nitrogen fixation control heterocyst pattern. J. Bacteriol. **183:**2605-2613.

172. **Yoon, H. S., and J. W. Golden.** 1998. Heterocyst pattern formation controlled by a diffusible peptide. Science **282:**935-938.

173. **Yoon, H. S., and J. W. Golden.** 2001. PatS and products of nitrogen fixation control heterocyst pattern. J. Bacteriol. **183:**2605-2613.

174. **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.

175. **Zhang, C. C., S. Laurent, S. Sakr, L. Peng, and S. Bedu.** 2006. Heterocyst differentiation and pattern formation in cyanobacteria: a chorus of signals. Mol. Microbiol. **59:**367-375.

176. **Zhao, Y., Y. Shi, W. Zhao, X. Huang, D. Wang, N. Brown, J. Brand, and J. Zhao.** 2005. CcbP, a calcium-binding protein from *Anabaena* sp. PCC 7120, provides evidence that calcium ions regulate heterocyst differentiation. Proc. Natl. Acad. Sci. USA **102:**5744-5748.

177. **Zhou, R., X. Wei, N. Jiang, H. Li, Y. Dong, K. L. Hsi, and J. Zhao.** 1998. Evidence that HetR protein is an unusual serine-type protease. Proc. Natl. Acad. Sci. USA **95:**4959-4963.

178. **Zhu, J., K. Jager, T. Black, K. Zarka, O. Koksharova, and C. P. Wolk.** 2001. HcwA, an autolysin, is required for heterocyst maturation in *Anabaena* sp. strain PCC 7120. J. Bacteriol. **183:**6841-6851.

179. **Zhu, J., R. Kong, and C. P. Wolk.** 1998. Regulation of *hepA* of *Anabaena* sp. strain PCC 7120 by elements 5' from the gene and by *hepK*. J. Bacteriol. **180:**4233-4242.

180. **Ziegler, K., D. P. Stephan, E. K. Pistorius, H. G. Ruppel, and W. Lockau.** 2001. A mutant of the cyanobacterium *Anabaena variabilis* ATCC 29413 lacking cyanophycin synthetase: growth properties and ultrastructural aspects. FEMS Microbiol. Lett. **196:**13-18.

VITA

Name: Maria Ramona Aldea

Address: Texas A&M University, Department of Biology, 3258 TAMU, College Station, TX 77843-3258

Email: maldea@mail.bio.tamu.edu

Education: B.S., Biology, Babes-Bolyai University, 2001

M.S., Cell Biology and Molecular Biotechnology, Babes-Bolyai University, 2003

Ph.D., Microbiology, Texas A&M University, 2008