HETEROCYST MORPHOGENESIS AND GENE EXPRESSION IN *Anabaena* SP. PCC 7120

A Dissertation

by

RODRIGO ANDRES MELLA HERRERA

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 2010

Major Subject: Microbiology

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Approved by:

Co-Chairs of Committees,	James W. Golden
	Michael Benedik
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	Michael Polymenis
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ABSTRACT

Heterocyst Morphogenesis and Gene Expression in *Anabaena* sp. PCC 7120. (August 2010)

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Co-Chairs of Advisors Committee: Dr. James W. Golden
Dr. Michael Benedik

Many multicellular cyanobacteria produce specialized nitrogen-fixing heterocysts. During diazotrophic growth of the model organism *Anabaena* (*Nostoc*) sp. strain PCC 7120, a regulated developmental pattern of single heterocysts separated by about 10 to 20 photosynthetic vegetative cells is maintained along filaments. Heterocyst structure and metabolic activity function to accommodate the oxygen-sensitive process of nitrogen fixation. This dissertation focuses on my research on heterocyst development, including morphogenesis, transport of molecules between cells in a filament, differential gene expression, and pattern formation.

We using microarray experiments we found that *conR* (all0187) gene is necessary for normal septum-formation of vegetative cells, diazotrophic grow, and heterocyst morphogenesis. In our studies we characterized the expression of sigma factors genes in *Anabaena* PCC 7120 during heterocyst differentiation, and we found that the expression of *sigC*, *sigG* and *sigE* is localized primarily in heterocysts. Expression studies using *sigE* mutant showed that *nifH* is under the control of this specific sigma factor.

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In Chapter IV, the construction of P_{nifHD}-*gfp* was conducted by Krithika Kumar.

Finally, I would like to thank my country, Chile, and her beautiful people.

NOMENCLATURE

µg	micro grams
µl	microliter
6xHis	six adjacent histidine residues
Ap	ampicillin
ATP	adenosine triphosphate
BSA	bovine serum albumin
CCD	camera charging device
cDNA	complementary deoxrribonucleic acid
Cm	chloramphenicol
CT	threshold cycle
Cy3	cyaninefluorochrome
Cy5	indodicarbocyanine
dATP	deoxyadenosine triphosphate
dCTP	deoxycytidine triphosphate
DIC	differential interference contrast
DMSO	dimethylsulfoxide
DNA	deoxyribonucleic acid
EDTA	ethylenediaminetetraacetic acid
EMSA	electrophoretic mobility shift assay
g	grams

GFP	green fluorescent protein
GTP	guanosine triphosphate
h	hours
IPTG	isopropyl-beta-D-thiogalactopyranoside
kb	kilobase
kDa	kiloDalton
Km	kanamycin
kV	kilo voltts
l	liter
M	molar
ml	milliliter
mM	millimolar
MOPS	3-(N-morpholino)propanesulfonic acid
mRNA	messenger ribonucleic acid
MW	molecular weight
N-6xHis	six adjacent amino-terminal histidine residues
Ni-NTA	nickel-nitriloacetic acid
Nm	neomycin
N-terminus	amino terminus
OD	optical density
ORF	open reading frame
PCR	polymerase chain reaction

pmol	pico molar
PSII	photosystem II
qRT-PCR	quantitative real-time reverse transcription PCR
RACE	rapid amplification of 5' cDNA ends
RT	reverse transcription
SDS	sodium dodecgl sulfate
Sm	streptomycin
Sp	spectinomycin
TRITC	tetramethyl rhodamine iso-thiocyanate
WT	wild type
X-Gal	5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside

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CHAPTER I

INTRODUCTION

Summary

Many nitrogen-fixing cyanobacteria grow as multicellular organisms composed of photosynthetic vegetative cells and heterocysts in a regulated developmental pattern similar to beads on a string. Heterocysts are terminally differentiated cells that are specialized morphologically and physiologically for supplying fixed nitrogen to the filament. Many multicellular cyanobacteria produce specialized nitrogen-fixing heterocysts. During diazotrophic growth of the model organism *Anabaena* (*Nostoc*) sp. strain PCC 7120, a regulated developmental pattern of single heterocysts separated by about 10 to 20 photosynthetic vegetative cells is maintained along filaments. Heterocyst structure and metabolic activity function to accommodate the oxygen-sensitive process of nitrogen fixation. This introduction focuses on recent research on heterocyst development, including morphogenesis, transport of molecules between cells in a filament, differential gene expression, and pattern formation.

This dissertation follows the style of Journal of Bacteriology.

Introduction

Organisms composed of multiple differentiated cell types can possess structures, functions, and behaviors that are more diverse and efficient than those of unicellular organisms. Among multicellular prokaryotes, heterocyst-forming cyanobacteria offer an excellent model for the study of cellular differentiation and multicellular pattern formation. Cyanobacteria are a large group of Gram-negative prokaryotes that perform oxygenic photosynthesis. They have evolved multiple specialized cell types, including nitrogen-fixing heterocysts, spore-like akinetes, and the cells of motile hormogonia filaments. Of these, the development of heterocysts in the filamentous cyanobacterium *Anabaena* (also *Nostoc*) sp. strain PCC 7120 (hereafter *Anabaena* PCC 7120) has been the best studied (Figure 1.1). Heterocyst development offers a striking example of cellular differentiation and developmental biology in a very simple form: filaments are composed of only two cell types and these are arrayed in a one-dimensional pattern similar to beads on a string.

Many cyanobacterial species are capable of nitrogen fixation. However, oxygenic photosynthesis and nitrogen fixation are incompatible processes because nitrogenase is inactivated by oxygen. Cyanobacteria mainly use two mechanisms to separate these activities: a biological circadian clock to separate them temporally, and multicellularity and cellular differentiation to separate them spatially. Heterocyst-forming cyanobacteria differentiate highly specialized cells to provide fixed nitrogen to the vegetative cells in a filament.

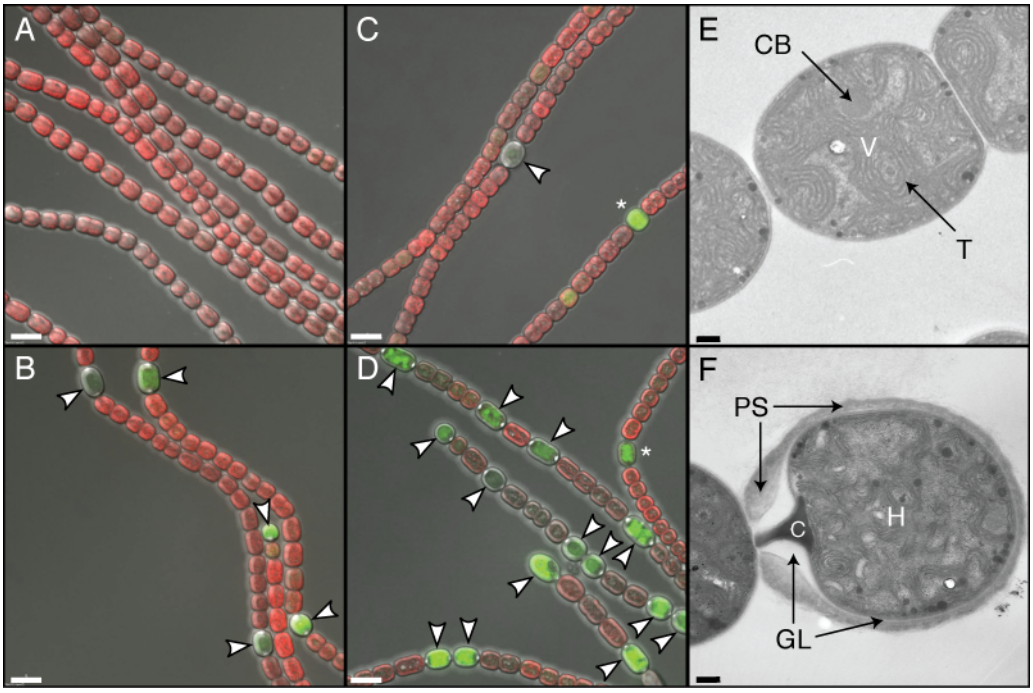

Figure 1.1. Heterocyst development in *Anabaena* PCC 7120. Filaments of the wild type carrying a *patS-gfp* reporter grown in medium containing nitrate are composed of vegetative cells (A), and have undergone heterocyst development one day after transfer to medium without combined nitrogen (B). A *patS* mutant strain carrying the same *patS-gfp* reporter grown in media containing nitrate contains a small number of heterocysts (C), and one day after transfer to medium without combined nitrogen shows a higher than normal frequency of heterocysts and an abnormal developmental pattern (D). (A, B, C, D) Merged DIC (grayscale), autofluorescence (red), and *patS-gfp* reporter fluorescence (green) images; arrowheads indicate heterocysts; asterisks indicate proheterocysts; size bar, 5 µm. (E, F) Transmission electron micrographs of wild-type vegetative cells (V) and a heterocyst (H) at the end of a filament; T, thylakoid membranes; PS, polysaccharide layer; GL, glycolipid layer; C, cyanophycin polar granule; size bar, 0.2 µm.

In the presence of a source of combined nitrogen such as nitrate or ammonium, *Anabaena* PCC 7120 grows as long filaments containing hundreds of photosynthetic vegetative cells. In the absence of combined nitrogen, it produces heterocysts, which are terminally differentiated nitrogen-fixing cells that form at semi-regular intervals between stretches of vegetative cells to produce a multicellular pattern of single heterocysts every ten to twenty vegetative cells along filaments (Figure 1.1). Some heterocyst-forming cyanobacteria produce heterocysts constitutively in their natural environment or display different developmental patterns. Heterocyst development involves integration of multiple external and internal signals, communication between the cells in a filament, and temporal and spatial regulation of genes and cellular processes. The study of heterocyst development in *Anabaena* PCC 7120 has proven to be an excellent model for the study of cell fate determination, pattern formation, and differential gene expression during prokaryotic multicellular development. Various aspects of heterocyst development, signaling, and regulation have been the subject of several recent reviews (3, 51, 71, 109, 177, 179).

Cellular differentiation, multicellularity, and transport allow specialized functions

Cyanobacteria have a Gram-negative cell wall that includes two distinct membranes, the plasma membrane and an outer membrane, and a peptidoglycan layer, which is thicker than in other Gram-negative bacteria, sandwiched between these two membranes (72). External to the cell wall is a carbohydrate-enriched glycocalyx that can

have different relative amounts of three recognizable layers: a closely associated sheath, a defined capsule, and loosely associated slime.

Heterocysts are typically distinguishable from vegetative cells by their somewhat larger and rounder shape, diminished pigmentation, thicker cell envelopes, and usually prominent cyanophycin granules at poles adjacent to vegetative cells (Figure 1.1). The additional envelope layers surrounding heterocysts help to protect the enzyme nitrogenase from oxygen (46). For details of the heterocyst cell wall and envelope, readers are referred to recent reviews (5, 123, 127). Mature heterocysts provide the microoxic environment required for nitrogen fixation, spatially separating oxygen-evolving photosynthesis in vegetative cells from nitrogen fixation. Differentiating cells undergo many metabolic and morphological changes (62). Oxygen-producing photosystem PSII is dismantled during differentiation and heterocysts show an increased rate of respiration (167). Morphological changes include the deposition of two additional envelope layers around the heterocyst: an inner "laminated" layer composed of two heterocyst-specific glycolipids (HGL) and an outer polysaccharide layer (HEP) (26, 27, 123). The heterocyst envelope polysaccharide layer is sometimes subdivided into a well-defined homogeneous inner layer and an external fibrous layer.

Heterocysts and vegetative cells are mutually interdependent. Because they lack photosystem II and carbon fixation, heterocysts are dependent on vegetative cells for a source of reductant and carbon, which is probably partially supplied as sucrose (36, 103). In *Anabaena* PCC 7120, vegetative cells must also supply glutamate to

heterocysts. (106). In return, vegetative cells obtain fixed nitrogen in the form of amino acids from the heterocysts (110).

The exchange of metabolites and intercellular signals that control the regulated spacing of the heterocysts require movement of molecules between cells along a filament, possibly through a continuous periplasm (49). According to this model, molecules exported from one cell would diffuse through the periplasm and then be taken up by other cells along a filament. It was recently shown that GFP expressed from the *patS* promoter, which is expressed at a low basal level in vegetative cells and strongly upregulated in differentiating heterocysts, and targeted to the periplasm by a cleavable twin-arginine signal peptide, could diffuse through the heterocyst periplasm to nearby vegetative cells (104). GFP attached to the cytoplasmic membrane was only seen in heterocysts and not in adjacent vegetative cells. Electron micrographs of *Anabaena* PCC 7120 intercellular junctions appear to show an intact peptidoglycan layer around each cell and sometimes a distinct "junctional space" between these peptidoglycan layers (Figure 1.2) is observed, which could be a barrier for secreted large molecules. Recent data support the exchange of molecules through intercellular junctions or channels directly connecting the cytoplasm of adjacent cells (114). Calcein, a small fluorescent molecule loaded into the cytoplasm of cells was found to quickly diffuse between cells. Electron micrographs suggest the existence of connections between adjacent cells named microplasmodesmata (Figure 1.2) (54, 55).

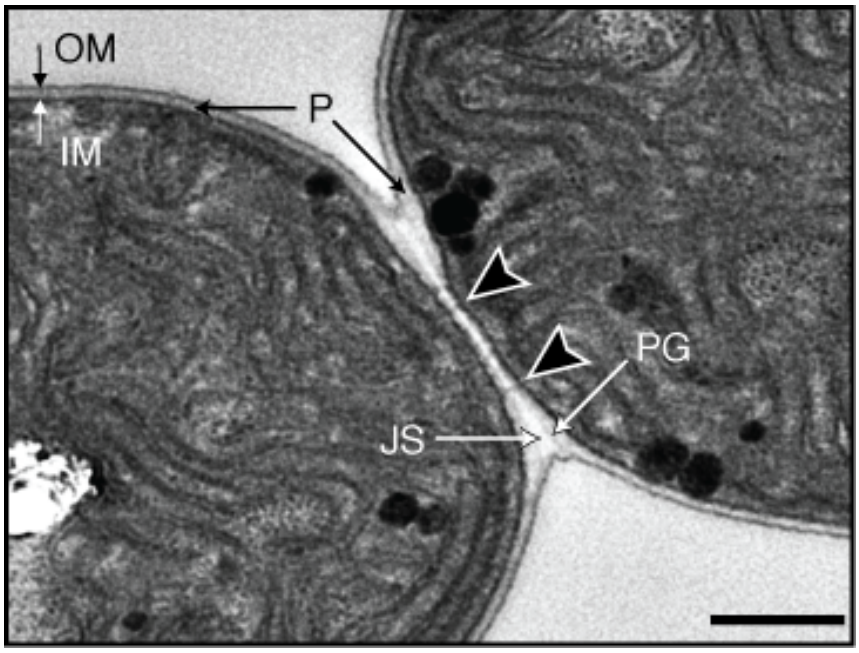

Figure 1.2. Transmission electron micrograph of the junction between two vegetative cells. Arrowheads indicate microplasmodesmata, which are potential cell-to-cell channels. Note the "junctional space" between the cell wall peptidoglycan layers of the two cells. IM, inner membrane; OM, outer membrane; P, periplasm; PG, peptidoglycan; JS, junctional space. Size bar, 0.5 μm.

The microplasmodesmata may be channels formed by protein oligomers, and SepJ (FraG) has been suggested as a candidate channel forming protein (50, 122). SepJ has a large extracytoplasmic domain that may be involved in spanning the cell wall and bridging the gap between adjacent cells (50). Localization studies with a SepJ-GFP reporter showed that it is expressed in both cell types and that it is localized at intercellular septa (50). *sepJ* mRNA levels increase after nitrogen depletion and *sepJ* null mutants are unable to differentiate heterocysts completely (50, 122). The diffusion of calcein required SepJ, suggesting that SepJ could be the channel forming protein connecting the cytoplasm of two adjacent cells (114).

Gene expression and morphogenesis during heterocyst development

The timeline of heterocyst development begins with sensing combined-nitrogen limitation and culminates with nitrogen fixation in the mature heterocyst. Heterocyst development is complete in about 20 hours and involves cellular differentiation of selected vegetative cells into heterocysts and less obvious changes in gene expression and metabolism in the remaining vegetative cells. Several studies employing DNA microarray methods show global changes in gene expression after nitrogen step-down (25, 39, 40, 142). The process of differentiation is reversible if a source of combined nitrogen is added within 9-12 hours after nitrogen deprivation, after which point the cells are “committed” to forming heterocysts (149, 175).

Initiation and early stages of heterocyst development

The presence of a source of combined nitrogen such as ammonium or nitrate inhibits the differentiation of heterocysts. In cyanobacteria, 2-oxoglutarate, an intermediate in the Krebs cycle, constitutes the signal for nitrogen deprivation (96, 177). The Krebs cycle in cyanobacteria is incomplete because of the lack of 2-oxoglutarate dehydrogenase. As a result, 2-oxoglutarate's main function is to serve as a precursor in a variety of biosynthetic reactions. It is the primary carbon skeleton for incorporation of ammonium and is considered the metabolic junction between carbon and nitrogen balance in cyanobacteria (119, 120, 156). Nitrogen-limiting conditions result in an increase in the levels of 2-oxoglutarate. An artificial analog of 2-oxoglutarate, 2,2-difluoropentanoic acid, DFPA, added to medium resulted in heterocyst development even in the presence of ammonium, showing that 2-oxoglutarate plays a key role in controlling heterocyst development (96).

NtcA, a transcriptional regulator belonging to the CRP (cyclic AMP receptor protein) family of proteins, senses 2-oxoglutarate levels. The protein is conserved in all cyanobacteria and regulates a number of genes involved in carbon and nitrogen metabolism (71, 103). In *Anabaena* PCC 7120, NtcA is required for the expression of the genes in pathways for ammonium and nitrate assimilation, as well as heterocyst development (131, 163). The *ntcA* gene is induced soon after nitrogen deprivation and is autoregulated (119, 131, 132). *ntcA* mutants are unable to use nitrate as the sole source of nitrogen and are blocked from initiating heterocyst development (52, 162). Genes that are activated by NtcA typically have the consensus binding site TGTA-(N_8)-TACA

centered at -41.5 nucleotides upstream of the transcription start point (TSP) (71). The DNA binding activity of NtcA is enhanced in the presence of 2-oxoglutarate and 2-oxoglutarate is necessary for transcriptional activation by NtcA (147, 157). Additionally, DFPA, the synthetic analogue of 2-oxoglutarate, stimulates DNA binding activity of NtcA *in vitro* (34, 96).

HetR is a master regulator of heterocyst development and plays a key role in differentiation and pattern formation (20). *hetR* is one of the earliest genes induced in differentiating cells and is positively autoregulated. Transcription of *hetR* increases as early as 30 minutes after nitrogen deprivation and by 3.5 hours expression is confined to spaced foci arranged in a pattern similar to that of differentiating cells (8, 19). Null mutants of *hetR* fail to produce heterocysts, and overexpression of *hetR* (18, 19) and particular point mutants (88) result in increased heterocyst frequency.

HetR protein is a serine type protease that has autoprotease activity and DNA binding activity that requires formation of a HetR homodimer (75, 181). Mutations that affect these activities block heterocyst development at an early stage, however the role of particular amino acid residues is unclear (37, 137). The heterocyst inhibitory peptide PatS interferes with HetR DNA-binding activity *in vitro* (75). A $hetR_{R223W}$ point mutant is insensitive to the main inhibitory signals of pattern formation, PatS and HetN, and produces a conditionally lethal phenotype due to complete differentiation under nitrogen limiting conditions (88).

Expression of *ntcA* and *hetR* show a mutual dependency during heterocyst development (118). *hetR* is not induced in an *ntcA* mutant and *ntcA* expression is

transiently induced in a HetR-dependent manner (52, 118). Expression of some genes involved in the developmental process is dependent on both *ntcA* and *hetR*; however, in some cases the dependency is probably due to the interdependent upregulation of *hetR* and *ntcA* (48, 67, 155). NrrA, a response regulator, has been identified as the regulatory link between NtcA and HetR (38). *nrrA* is transcribed in differentiating cells within 3 h after nitrogen deprivation and is directly dependent on NtcA (39, 116). Earlier work on the transposon reporter strain TLN14, now known to be a fusion to the *nrrA* gene, showed rapid induction one hour after nitrogen stepdown (21). An *nrrA* mutant strain shows a delay in heterocyst development caused by a delay in accumulation of HetR, and extra copies of *nrrA* result in increased expression of *hetR* and thus, increased heterocyst frequency (38, 39).

The *hetC* gene, which encodes a member of the family of ATP-binding cassette type exporters, is required for an early step in the differentiation of heterocysts as observed by a P_{hetC}-*gfp* reporter, which showed an increase in expression in proheterocysts and heterocysts (87, 118). A *hetC* mutant carrying a P_{hetR}-*gfp* reporter shows a pattern of weakly fluorescent cells that are blocked from further stages of development (160, 173). It is possible that the *hetC* mutant fails to complete an early step in morphogenesis of the envelope that then triggers a developmental checkpoint that prevents further differentiation.

Heterocyst cellular differentiation produces an environment for nitrogen fixation

The middle and later stages of heterocyst development are distinguished by structural and physiological changes. These changes begin with morphogenesis of the heterocyst envelope by the deposition of an outer polysaccharide layer and an inner glycolipid layer, which decrease the entry of oxygen into the heterocyst (Figure 1) (46). Mutants that lack the envelope polysaccharide or the glycolipid layer are unable to grow diazotrophically in the presence of air (44, 74, 123, 165, 166).

Deposition of the external polysaccharide layer is one of the earliest morphological changes during heterocyst differentiation. DevR and HepK, which comprise a two component regulatory system, are involved in biosynthesis of the polysaccharide layer (182). The *hep* genes *hepA*, *hepB*, and *hepC*, and a cluster of genes present around *hepA* are required for the deposition of the polysaccharide layer (74, 159, 184).

The *hglB*, *hglC*, *hglD*, and *hglE* genes along with a cluster of nearby genes are required for the synthesis of heterocyst glycolipids (24, 44). DevH, a trans-acting regulatory protein, is required for the formation of the glycolipid layer, either by directly regulating the expression of the genes or indirectly through other gene products (134). The *hglK* gene is required for the localization of the glycolipids and may be directly involved in their deposition (7).

Differentiation and maturation of heterocysts is dependent on DevBCA and HgdD, which are thought to be a glycolipid exporter and outer membrane efflux tunnel, respectively (47, 112), and autolysin HcwA, which is presumably involved in cell wall

remodeling (183). The regulatory genes *hepK*, *hepN*, *henR*, and *hepS* are required for normal heterocyst maturation (98).

Nitrogen fixation in heterocysts

Heterocyst development culminates with nitrogen fixation, which is the ATP-dependent process of reducing atmospheric nitrogen to ammonia by the enzyme nitrogenase. In many heterocyst-forming cyanobacteria, including *Anabaena* PCC 7120, nitrogenase is synthesized only in heterocysts, where it is protected from irreversible inactivation by oxygen (46). However, some heterocyst-forming cyanobacteria contain an additional set of *nif* genes that allows nitrogen fixation in vegetative cells under anoxic conditions (148). Nitrogenase is a well-conserved enzyme in all nitrogen-fixing organisms and consists of two components: dinitrogenase (Mo-Fe protein) and dinitrogenase reductase (Fe protein) (139). Dinitrogenase reductase serves to transfer electrons from electron donors such as ferredoxin or flavodoxin to dinitrogenase. In *Anabaena* sp. PCC 7120, the *fdxH* gene codes for a heterocyst-specific ferredoxin (108). In addition to the common molybdenum nitrogenase, some cyanobacteria contain an alternative nitrogenase that utilizes a vanadium cofactor (129).

In *Anabaena* PCC 7120, the nitrogen-fixation (*nif*) genes are expressed specifically in heterocysts late during development, between 18 and 24 hours after nitrogen deprivation (43, 59). The *nifHDK* operon encodes the molybdenum-containing nitrogenase enzyme complex. Upstream of the *nifHDK* operon is another *nif* operon, the *nifB-fdxN-nifS-nifU* (113). The *nifVZT* genes form a *nif* gene cluster separated from the

main *nif* gene cluster (143). Other cyanobacterial *nif* genes include *nifE*, *nifN*, *nifX*, and *nifW* (148, 167). The transcriptional regulators that control the expression of the *nif* genes in cyanobacteria have not been identified.

Heterocyst-specific uptake hydrogenase, encoded by the *hupSL* operon, recovers reductant from H_2 produced by nitrogenase (146). In *Nostoc punctiforme* the upstream region of the *hupSL* operon contains a putative NtcA binding site (73). Mobility shift assays showed that NtcA binds to the *hupSL* promoter. However, deletion of the NtcA binding site did not affect the expression of *hupSL* significantly. Study of cis-acting regulatory regions in the *hupSL* promoter using truncated sequences of the *hupSL* promoter fused to either *gfp* or *luxAB* reporter genes indicates that sequences required for heterocyst-specific expression of the *hupSL* genes are located in a region starting 57 base pairs upstream of the transcription start point and extending 258 base pairs downstream.

Heterocyst development is accompanied by changes in the photosynthetic apparatus and carbon metabolism to provide ATP and low potential reductant for nitrogen fixation (110, 167). Respiration by cytochrome c oxidase generates ATP for nitrogen fixation and helps create the microoxic environment. Whereas *cox1* encoding mitochondrial type cytochrome c oxidase is expressed in vegetative cells, *cox2* and *cox3* are expressed only in heterocysts and are required for growth during nitrogen fixation (85, 152).

In *Anabaena* PCC 7120, three DNA rearrangements take place during the late stages of heterocyst development that affect nitrogen fixation and uptake hydrogenase

operons (28-30, 56-58). The rearrangements are the result of developmentally programmed site-specific recombination between direct repeats that flank the DNA elements that are deleted from the chromosome (56). One rearrangement excises an 11-kb element from the *nifD* gene (16, 58). The excision is catalyzed by *xisA* located on the 11-kb element (15, 60, 69, 95). A *xisA* null mutant forms heterocysts but is unable to excise the element or grow on media lacking a source of combined nitrogen (59, 60). A second rearrangement excises a 55-kb element from the *fdxN* gene (57) and requires the *xisF*, *xisH*, and *xisI* genes (31, 133). A third programmed DNA arrangement deletes a 10.5-kb element from the *hupL* gene and requires the *xisC* recombinase gene (28, 30). All three DNA elements appear to be parasitic DNA sequences that are passed to daughter vegetative cells as they are carried innocuously in genes that are only required in heterocysts. And, all three DNA elements have acquired the ability to excise from the chromosome in terminally differentiated heterocysts so that the genes in which they reside can be expressed. This developmental regulation has apparently evolved independently for each element but the regulatory mechanism is not yet known.

RNA polymerase sigma factors are developmentally regulated

The control of gene expression in bacteria is primarily regulated at the level of transcription initiation. Bacteria contain an RNA polymerase (RNAP) enzyme that requires an additional σ factor for promoter recognition and transcription initiation. In many cases a bacterium utilizes specific sigma (σ) factors to activate a set of genes in response to environmental and intracellular signals (23). For example, in *Bacillus*

subtilis, temporally and spatially regulated σ factors modulate the expression of genes during starvation-induced sporulation (23, 93). It was hypothesized that global changes in gene expression during heterocyst development in *Anabaena* PCC 7120 are regulated by σ factors (4, 89). The σ 70 family of sigma factors has been divided in four major groups: group 1 is responsible for transcription of housekeeping genes, group 2 is similar to group 1 but not essential under laboratory growth conditions, group 3 is involved in the expression of genes associated with sporulation, motility, stress response, and heat shock, and group 4 participates in multiple biological processes (23). *Anabaena* PCC 7120 has twelve putative sigma factors identified by sequence similarity (4, 176). Genetic analysis has not identified any sigma factor genes that are essential for heterocyst differentiation or nitrogen fixation, suggesting that there is a level of functional redundancy between the sigma factors (89).

Genetic analysis using *gfp* as a reporter identified three sigma factor genes, *sigC*, *sigG*, and *sigE*, that are developmentally upregulated after nitrogen deprivation (4). The expression of *sigC* is upregulated 4 hours after nitrogen step-down and by 10 hours the expression is restricted mostly to single cells in a heterocyst-like pattern. SigC may be involved in regulating early heterocyst-specific genes (4). The expression of *sigE* (alr4249) is low in vegetative cells grown in nitrate-containing media and is upregulated in individual cells at about 16 hours after nitrogen depletion. At 24 hours after nitrogen step-down, a *sigE* reporter is expressed in mature heterocysts suggesting that SigE may be involved in the expression of late heterocyst specific genes such as the *nif* and *hup* genes. Expression of a *sigG* reporter is seen in cells in media containing nitrate but

decreases in all cells soon after nitrogen depletion. At around 9 hours after nitrogen step-down, increased expression is localized to single cells and by 16 hours, expression is localized to morphologically distinguishable proheterocyst cells; at 24 hours, the heterocysts show decreased reporter expression. These data suggest that SigG is involved in the expression of genes during the middle stages of differentiation such as those involved in morphogenesis and the creation of a microoxic environment, and possibly genes that are necessary for commitment to complete differentiation (4).

Heterocyst pattern formation requires dynamic signaling

Pattern formation has been observed in many prokaryotic systems. For example, *Myxococcus xanthus* forms a complex three-dimensional pattern when starving bacteria self-organize to form fruiting bodies (83), and *Pseudomonas aeruginosa* produces biofilms during infection (91). Temporary patterns have been studied in *Escherichia coli* and *Salmonella typhimurium* when these organisms swim in gradients of nutritional chemoattractant (9, 17). The developmentally regulated pattern of heterocysts in multicellular cyanobacteria offers a model to understand biological pattern formation, signals that control pattern formation, and downstream regulatory pathways.

A longstanding model of heterocyst pattern formation is that differentiating cells produce an inhibitory signal that diffuses along a filament to form a gradient that inhibits differentiation of neighboring cells in a concentration dependent manner (164, 165). The *patS* gene in *Anabaena* PCC 7120 is essential for normal pattern formation and is predicted to encode a 13- to 17- amino-acid peptide depending on the start codon used *in*

vivo (174, 175). All filamentous cyanobacteria for which a genome sequence is available contain *hetR* and *patS* genes, and although the *patS* genes encode peptides from 13 to 90 amino acids, they all have an identical five amino acid sequence, RGSGR, at their C-terminal ends (178). This PatS pentapeptide sequence may be the essential element of the predicted diffusible inhibitor controlling, at least in part, heterocyst pattern.

The *patS* gene is transcribed early during heterocyst development. Expression analysis using northern RNA blots and a *patS-lacZ* reporter showed that *patS* is upregulated by 6 hours after nitrogen deprivation (174). A *patS-gfp* reporter strain in nitrate-containing media showed low levels of expression in all cells. At 8 to 10 hours after nitrogen step-down increased fluorescence was localized to small groups of cells or individual cells (175). By 12 to 14 hours after nitrogen step-down, most of the GFP signal was localized in individual cells that were arranged in a pattern similar to that of mature heterocysts, and by 18 hours the fluorescence was localized almost entirely to heterocysts (also see Figure 1.1).

A *patS* null mutant produces heterocysts even in the presence of nitrate, and forms multiple contiguous heterocysts after nitrogen deprivation with up to 30% of the cells differentiating to form heterocysts, while extra copies or overexpression of *patS* results in a complete suppression of heterocysts (174, 175). Mutations in the last five carboxy-terminal residues of PatS result in a loss-of-function phenotype. A synthetic RGSGR pentapeptide, PatS-5, blocks heterocyst differentiation at nanomolar concentrations while a four amino acid synthetic peptide did not inhibit differentiation (174). Addition of PatS-5 to growth medium of a *patS* null mutant at a concentration that

decreases the frequency of heterocysts to the wild-type level does not restore a wild-type heterocyst pattern, but ectopic expression of *patS* from a heterocyst-specific promoter, *hepA*, in a *patS* null mutant background does restore the normal pattern. These data are consistent with PatS functioning as a diffusible inhibitor and that a gradient of PatS is required for the establishment of a normal pattern (61). However, other somewhat more complicated mechanisms are possible and direct support for this hypothesis would require, for example, the development of methods to detect gradients of small molecules in cyanobacterial filaments.

The PatS signal must move from differentiating cells to neighboring cells to inhibit HetR, however, a *patS5* minigene expressed in heterocysts in a *patS* null background does not produce a normal pattern, indicating that PatS-5 produced by the minigene cannot function as a cell-to-cell signal and may remain in the cytoplasm of the differentiated cell, possibly because of the lack of a domain that is needed for transport to adjacent cells or export to the periplasmic space (172). The ability of large peptides containing an internal RGSGR sequence motif to inhibit heterocyst development supports the idea that the receptor for PatS is localized in the cytoplasm (172). *In vitro* studies show that the DNA-binding activity of HetR is inhibited by PatS-5 pentapeptide in a dose-dependent manner, implying that the HetR-PatS ratio is important for controlling heterocyst differentiation and indicating that HetR is a PatS receptor (75). Upregulation of *hetR* expression is abolished when PatS-5 is added to the growth medium (75). *hetR* mutant $hetR_{R223W}$ is insensitive to the PatS inhibitory signal and overexpression of the $hetR_{R223W}$ allele results in a conditionally lethal phenotype after

nitrogen deprivation because nearly all cells differentiate (88). Overexpression of *patS* and *hetR* in a synthetic operon inhibits heterocyst formation indicating that *patS* acts downstream of *hetR*, which is consistent with PatS inhibiting the activity of HetR (125).

The *hetN* gene encodes a protein similar to ketoacyl reductase. Like *patS*, overexpression of *hetN* gene results in complete suppression of heterocyst development. A *hetN* null mutant shows a wild-type pattern at 24 hours after nitrogen depletion but forms multiple contiguous heterocysts by 48 hours, suggesting that *hetN* is not necessary for *de novo* heterocyst pattern, and instead is required for the maintenance of the preexisting heterocyst pattern (22). A *patS-hetN* double mutant shows complete differentiation of heterocysts in the absence of combined nitrogen, producing a lethal phenotype. These data indicate that the two inhibitory signal pathways may be independent (11). Together, *patS*- and *hetN*-mediated inhibitory pathways are the primary mechanism for establishing heterocyst pattern. A model of the regulatory pathway for heterocyst development is shown in figure 1.3.

However, other factors can influence pattern formation such as the cell cycle, the physiology of individual cells, signals or nutrients from vegetative cells, and the products of nitrogen fixation (3, 61, 177). It is known that single cells from fragmented

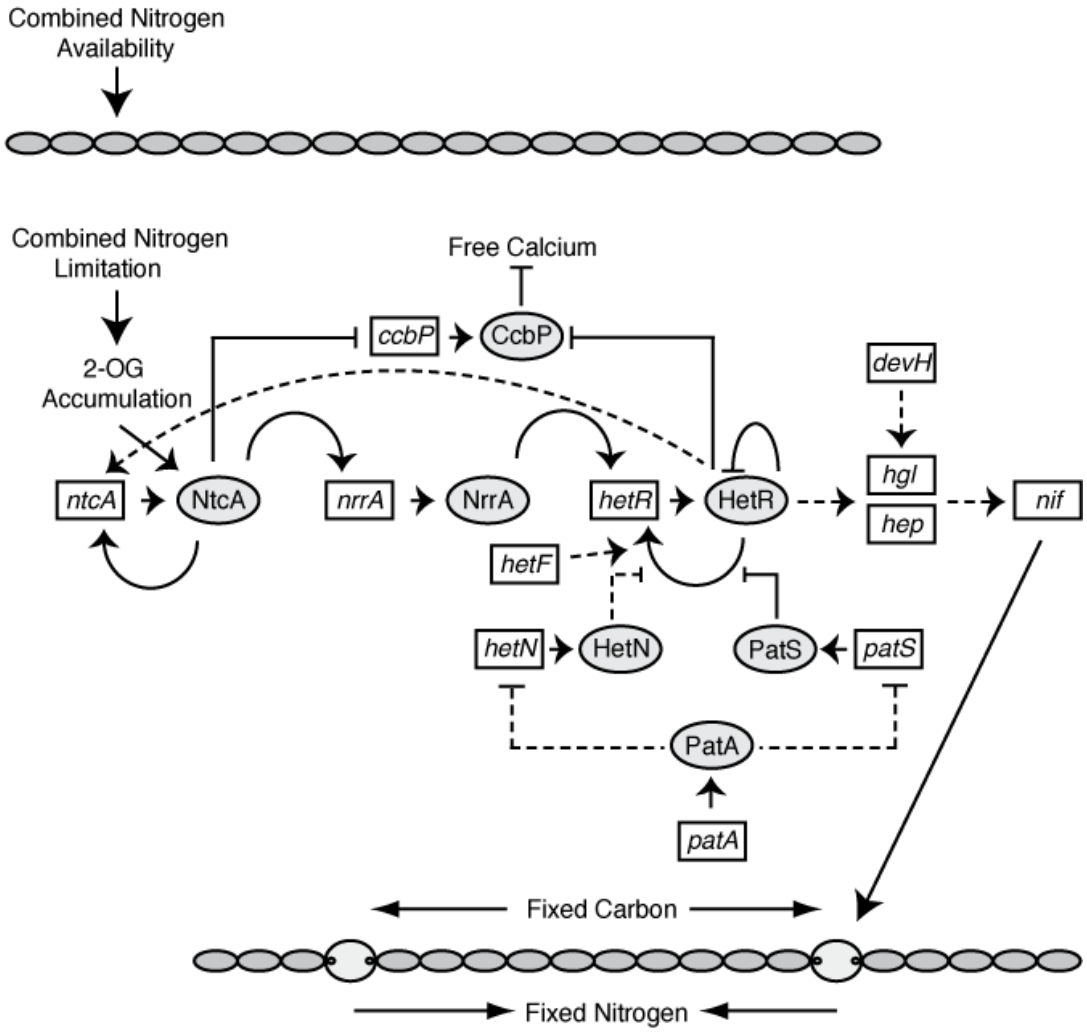

Figure 1.3. Model of the regulatory pathway for heterocyst development. For clarity, the figure shows only selected genes, proteins, and events. Open boxes represent genes and gray ovals represent proteins. Lines ending in arrows and bars indicate positive and negative interactions, respectively. Dashed lines represent indirect and/or unknown interactions or missing steps. Short arrows are between genes and their products. See the text for details.

filaments will not differentiate but instead always divide first followed by differentiation of one of the daughter cells, and it has been suggested that an activator, possibly 2-oxoglutarate, of differentiation is produced by vegetative cells and serves as a regulator of pattern formation (179). There is some evidence for the products of nitrogen fixation supplied from heterocysts contributing to the average spacing between heterocysts along filaments (3, 175). However, data from *A. variabilis*, which can fix nitrogen in vegetative cells, have been interpreted as an argument against the products of nitrogen fixation having a significant effect on heterocyst pattern (149). This seemingly simple question has been difficult to answer because mutants in which heterocysts cannot supply nitrogen to the filament cannot grow diazotrophically, which makes determining the maintenance of heterocyst pattern impossible.

CHAPTER II

Anabaena SP. STRAIN PCC 7120 GENE *conR* (ALL0187) CONTAINS A LytR-CpsA-Psr DOMAIN, IS DEVELOPMENTALLY REGULATED, AND IS ESSENTIAL FOR DIAZOTROPHIC GROWTH AND HETEROCYST MORPHOGENESIS

Summary

The *conR* (all0187) gene of the filamentous cyanobacterium *Anabaena* sp. strain PCC 7120 is predicted to be part of a family of proteins that containing the LytR-CpsA-Psr domain associated with septum formation and cell wall maintenance. Northern RNA blot analysis showed that *conR* expression was upregulated at 8 h after nitrogen step-down. Fluorescence microscopy of a P_{conR}-*gfp* reporter strain revealed increased GFP fluorescence in proheterocysts and heterocysts beginning at 9 h after nitrogen step-down, whereas vegetative cells maintained a lower level of fluorescence. Insertional inactivation of *conR* caused a septum-formation defect of vegetative cells grown in nitrate-containing medium. In nitrate-free medium, mutant filaments formed abnormally long heterocysts and were unable to grow diazotrophically. Septum formation between heterocysts and adjacent vegetative cells was abnormal, often with one or both poles of the heterocysts appearing partially open. In a *conR* mutant (AMC1369), expression of *nifH* was delayed after nitrogen step-down. 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 *conR* mutant strain are partially functional. We hypothesize that the diazotrophic growth defect is caused by an inability of the heterocysts to transport fixed nitrogen to the neighboring vegetative cells.

Introduction

The filamentous cyanobacterium *Anabaena* (also *Nostoc*) sp. strain PCC 7120 (hereafter *Anabaena* 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 (61). These two processes are seemingly incompatible because nitrogenase, the enzyme involved in reducing atmospheric dinitrogen to ammonium, is oxygen-sensitive. In many unicellular nitrogen-fixing cyanobacteria, these two processes are temporally separated, with photosynthesis occurring during the day and nitrogen fixation occurring at night (35). Some filamentous cyanobacteria, including *Anabaena* PCC 7120, undergo development in which five to ten percent of vegetative cells differentiate into a specialized cell called a heterocyst that no longer carries out photosynthesis and instead fixes nitrogen (3).

Heterocyst differentiation involves morphological and physiological changes that produce the microoxic environment necessary for nitrogen fixation (3). This microoxic 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 diminishes O_2 entry into the cell. The heterocyst pattern of single heterocysts separated by ten to fifteen vegetative cells 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 (3, 11, 136, 177). Heterocysts supply vegetative cells with fixed nitrogen, and vegetative cells supply fixed carbon to the heterocysts (3, 128). It is not yet completely clear how metabolites are exchanged between vegetative cells and heterocysts. Recent evidence has shown that the

periplasm is continuous along each filament of cells and could provide a conduit for exchange (104), but also that cytoplasm to cytoplasm movement of small molecules can occur in *Anabaena* PCC 7120 (114).

The early stages of heterocyst differentiation require increased levels of the regulators NtcA and HetR, which are mutually dependent on each other for upregulation (117). NtcA is an autoregulatory transcription factor that belongs to the cAMP receptor protein (CRP) family (71). 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 (75). 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 outer membrane 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 after induction (74). Several genes with regulatory roles have also been shown to be required for regulation of the *hep* genes, including *hepK* and *devR*, which form a two-component system, *henR* (response regulator), *hepN* (histidine kinase), and *hepS* (serine/threonine kinase). Synthesis of the glycolipid layer requires *hglB*, *hglC*, and *hglD*, and *hglE*, *hglK*, and *devBCA* have a role in transport and/or assembly (44). 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, which they named *conR*, the subject of this paper, in a transposon mutagenesis screen for mutants with a Fox$^-$ phenotype. Their *conR* mutant was unable to grow diazotrophically although the polysaccharide and glycolipid envelope 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 (45).

ConR it is predicted to contain a LytR/CpsA/Psr domain, which is located in the extracellular region of a number of membrane-bound proteins (76). The proteins containing this domain have been described to be involved in a variety of processes, with most related to the cell wall, but biochemical functions are not known (76). 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 (92).

We independently identified the *Anabaena* PCC 7120 *conR* gene in expression microarray experiments and confirmed that its expression is upregulated after nitrogen step-down. A P_{conR}-*gfp* reporter showed that increased expression was localized to proheterocysts and heterocysts. Site-directed inactivation of *conR* caused a defect in vegetative cell division in addition to defects in heterocyst morphogenesis that resulted in abnormally long heterocyst cells. Although the *conR* mutant strain was unable to grow diazotrophically, it was able to form partially functional heterocysts with significant levels of nitrogenase activity.

Materials and methods

Strains and culture conditions

Strains and plasmids used in this study are listed in Table 2.1 *Anabaena* 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 μmol photons m^{-2} s^{-1} as previously described (171). Plates contained 40 ml of medium solidified with 1.5% agar; for BG-11_0 plates, the agar was washed (161) to remove trace nitrogen compounds. All primers used in this study are listed in Table 2.2. 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* 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; for liquid medium these concentrations were reduced by half. *Escherichia 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* PCC 7120 strains by conjugation using standard protocols (42) with minor modifications (89). For all cyanobacterial genetics experiments, to limit the possibility of working with clones containing second-site mutations in genetic experiments, the phenotype of several independent exconjugant clones were determined to check for consistency.

Table 2.1. Bacterial strains and plasmids for *conR*

Strains	Relevant characteristic	Reference
Anabaena		
PCC 7120	Wild-type *Anabaena* sp. strain PCC 7120	(7)
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 unique cloning sites *Xba*I, *Bam*HI, *Xma*I, *Kpn*I, *Sst*I and *AccI*; Km^r Nm^r	(162)
pRL277	Conjugal suicide plasmid; Sm^r Sp^r	(42)
pAM1011	Higher-copy-number version of pCCB110 shuttle plasmid; Km^r Nm^r	(171)
pAM1824	Shuttle plasmid containing the *Anabaena* PCC 7120 *rbcL* promoter on an *Eco*RI-*Sal*I fragment from pAM496; Km^r Nm^r	(101)
pAM1956	Shuttle plasmid pAM505 containing the *gfpmut2* gene; Km^r Nm^r	(174)
pAM2178	pARO180 with Omega Sp^r Sm^r cassette inserted into unique SspI site; Ap^r Sp^r Sm^r	(89)
pAM3438	pRL277 containing internal fragment of all0187; Sm^r Sp^r	This study
pAM3448	pAM1824 containing the entire all0187 ORF, including 76 bp upstream from the translational start site, under control of the *rbcL* promoter; Km^r Nm^r	This study
pAM3450	pAM1956 carrying a *Sal*I-*Sac*I fragment from pAM3351 containing the -472 bp to +86 region of all0187; Km^r Nm^r	This study
pAM3451	pBluescript-KS+ containing a -472 to +86 bp region relative to the translation start site of all0187; Ap^r	This study
pAM2981	pRL277 containing internal fragment of all3290; Sm^r Sp^r	This study
pAM4403	pRL277 containing internal fragment of alr0295; Sm^r Sp^r	This study
pAM4404	pRL277 containing internal fragment of alr4884; Sm^r Sp^r	This study

Table 2.1 continued

pAM4405	pRL277 containing internal fragment of alr0996; Sm^r Sp^r	This study
pAM4406	pRL277 containing internal fragment of all1738; Sm^r Sp^r	This study
pAM4407	pRL277 containing internal fragment of all3169; Sm^r Sp^r	This study
pAM4408	pRL277 containing internal fragment of alr4808; Sm^r Sp^r	This study
pAM4409	pRL277 containing internal fragment of alr0141; Sm^r Sp^r	This study
pAM4410	pRL277 containing internal fragment of all4738; Sm^r Sp^r	This study
pAM4411	pRL2178 containing internal fragment of alr3871; Ap^r Sm^r Sp^r	This study
pAM3986	pRL2178 containing internal fragment of all1682; Ap^r Sm^r Sp^r	This study
pAM3988	pRL2178 containing internal fragment of all4160; Ap^r Sm^r Sp^r	This study
pAM4062	pRL2178 containing internal fragment of alr3924; Ap^r Sm^r Sp^r	This study
pAM4065	pRL2178 containing internal fragment of all1688; Ap^r Sm^r Sp^r	This study

Table 2.2. DNA primers used for ***conR***

Gene	Forward Primer	Sequence (5'→3')	Reverse primer	Sequence (5'→3')
all0187-internal	AMO-903	CTCGAGTGTTTCCCGTAACGTGGTTG	AMO-904	GAGCTCAAACTCACCCTAGAGCGACG
all0187-ORF	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
all3290	AMO-659	AACCAACATATG GTGATTCGTCCTTGTAAACC	AMO-660	AACCAACCCGGGTCATGGCTTGACTACCTCC
alr0295	AMO-886	CAAGGCTCGAGAGAAACTGCTGTAG	AMO-887	GACCTGAGCTCAAAAACAGCAGAGTATC
alr4882	AMO-888	CTTCAGCTCGAGCAGGATAAAATGTCTGC	AMO-889	ACAATAGAGCTCTGAGCGATCGCTAACCC
alr0996	AMO-892	GACCTCGAGCAAAGAAATTGCTGGTGGTAATG	AMO-893	GTTGAGCTCAGAGTCTGTTTTGGGTGTAG
all1738	AMO-895	GTAGACCAAGTATGG	AMO-896	GAATTCGACCACCAATTACTAC
all3169	AMO-897	AAGCTTGGGGGTGACCTGCATCC	AMO-898	TCTGAATTCACTGGCGCTGGCTAGAATC
alr4808	AMO-899	GACTTGTGAAACCATTGC	AMO-900	CCGAATTCAAACCAAACCCACAG
alr0141	AMO-901	TAGAAGCTTCTTTGACAAGTCGGGG	AMO-902	ACAGAATTCCACAGAGCGAAGAGC

Table 2.2 continued

all4738	AMO-927	GTGACTCGAGGTTATGAGTTATTGC	AMO-928	CACCGAGCTCTTGTGCGCCCAAGCC
alr387	AMO-937	AGAGAGCTCCAGTTGGCACACTTG	AMO-938	ACAAGGATCCGGCTGTAGTTGAAAGG
all1682	1794	CAGAAGCTTGTACAAATACTAGTG	1795	TTCCAATCCCCAATATAAAACAAC
all4160	1800	TGAAAGCTTGGAATGTCACTCCCC	1801	ACTGAGCTCCTGCCATTCACTTGC
alr3924	1802	TAAGTCTAGAGCTTACGACTTGGGGC	1803	GCGAGAATTCCACCAATTACTGTTAG
all1688	1820	CTCTCGTCGACCAGATTTCCCAAGAG	1821	ATACGAATTCCTGTATCTTCTACTTG

DNA manipulations and plasmid constructions

Standard protocols were used for cloning, *E. coli* transformation. Total DNA from cyanobacterial strains was isolated as previously described (89). DNA sequencing of plasmid inserts was performed by the Gene Technologies Laboratory (Texas A&M University) following the Big Dye sequencing protocol (Applied Biosystems).

All plasmid constructions were verified by DNA sequence analysis. The open reading frame *conR* was inactivated by single homologous recombination. To construct the suicide plasmid pAM3438, an internal fragment of *conR* was amplified by PCR using oligonucleotides AMO-903 and AMO-904. The amplified fragment was digested with *Xho*I and *Sac*I 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 *conR* 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 *conR*. This plasmid contains the *conR* gene (including 37 bp upstream and 2 bp downstream of the ORF) on a PCR fragment generated with primers AMO-955, containing an engineered *Cla*I site, and AMO-956, containing an engineered *Sal*I restriction site. The PCR product was digested with *Cla*I and *Sal*I and cloned into the same sites of pAM1824, which is a shuttle plasmid containing the *Anabaena* PCC 7120 *rbcL* promoter on an *Eco*RI-*Sal*I fragment from pAM496 cloned into the same sites on pAM1011, which contains a transcriptional terminator downstream of the *Sal*I site (133).

To construct a shuttle plasmid carrying a P_{conR}-*gfp* transcriptional fusion, a fragment extending from 402 bp upstream to 63 bp downstream of the *conR* translational start site was amplified by PCR with primers AMO-959 and AMO-960. The fragment was digested with *Sma*I and *Xba*I and cloned into pBluescript-SK+ digested with *Eco*RV and *Xba*I to produce pAM3451. A *Sal*I-*Sac*I fragment containing the insert was excised from pAM3451 and ligated into the same sites of pAM1956, a shuttle plasmid containing the promoterless *gfpmut2* gene, producing pAM3450.

RNA isolation

Total RNA was extracted using the RiboPure-Bacteria kit (Ambion) according to the manufacturer's instructions. *Anabaena* PCC 7120 filaments from a dark-green BG-11 culture were inoculated into 100 ml of BG-11_0 supplemented with 2.5 mM ammonium chloride and 5 mM MOPS (pH 8.0) to obtain a starting OD_{750} of 0.025-0.035. The cells were grown overnight until the OD_{750} reached 0.05 to 0.075, after which they were collected by centrifugation, washed twice with water, and then transferred to 100 ml of BG-11_0. At 0, 4, 8, 12, 16, 20, and 24 h after nitrogen deprivation, 100 ml cultures were poured into a conical 250 ml centrifuge tube containing 100 g of ice to rapidly stop mRNA synthesis and degradation, and the filaments were collected by centrifugation at 5,000 × g for 10 minutes at 4°C. The cell pellet was immediately frozen at -80°C for storage until RNA extraction was performed.

Northern RNA blot analysis

Five μg of total RNA for each sample was denatured and run on a 1% agarose denaturing formaldehyde gel in MOPS buffer and transferred to a MAGNACHARGE nylon membrane (GE Water & Process Technologies) with 10× SSPE. 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) were labeled with ^{32}P-dCTP by 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 (Fujifilm).

Microarray experiments

Labeled *Anabaena* PCC 7120 RNA samples were hybridized to a custom microarray slide manufactured by Integrated Genomics. Each slide contained probes for 768 open reading frames (ORFs) from the *Anabaena* PCC 7120 genome, including heterocyst-related genes, regulatory genes, photosynthesis genes, clock genes, and selected additional genes. The array was distributed in 24 blocks; each block contains 16 × 15 DNA probe spots. The array included 6 duplicates of each gene, 96 negative controls (repeated 6 times each), and 11 positive controls (repeated 48 to 196 times). In total, each slide contained 5760 spots; 50 probes were 70-base oligonucleotides and the

remainder were PCR products. Oligonucleotides and PCR products were UV cross-linked onto Corning GAPS II coated slides (Invitrogen). Each spot had a diameter of approximately 150 μm and spots were aligned in an area of 1.5 × 2.8 cm.

For hybridization, total RNA was converted to an amino-allyl single-stranded cDNA and post-labeled with Cy5 or Cy3 fluorescent dyes (Amersham Biosciences) using the Superscript-II reverse transcriptase system and random primers (Invitrogen). Slides were pre-hybridized in 25% formamide, 5× SSC, 0.1% SDS, and 2% BSA for 45 min at 42°C, then rinsed twice in distilled water and dried by centrifugation at 500 × g for 3 minutes. Hybridization was performed in a dark humid chamber at 42°C for 16 h using a 4× hybridization solution (Amersham Biosciences). Slides were then washed with a pre-warmed solutions of 2× SSC-0.2% SDS at 55°C followed by 1× SSC-0.2% SDS at 37°C and finally with 0.1× SSC at room temperature for 10 minutes. Slides were then air-dried and subsequently scanned using an Affymetrix 428 Array scanner (Affymetrix). Array images were generated in GPX and TIFF formats using GenePix-Pro software.

Normalization and statistical analysis of the raw data were performed with GeneSpring Analysis Platform (Silicon Genetics) software. Specific labeled RNA samples were normalized using *rnpB* as an internal control and then all samples were normalized against the median of the control sample grown on ammonium. The measurement for each gene in a sample was divided by the median of that gene's measurements in the corresponding control samples. Finally, the expression values for all genes were clustered using 90% correlation with *hetC* values to identify genes upregulated after nitrogen step-down.

Electron microscopy

Cultures were prepared for electron microscopy by collecting 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% $K_3F(CN)_6$ overnight at 4˚C. Fixed material was washed with distilled 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 with 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). Differential interference contrast (DIC) images were captured using an

Olympus IX70 inverted microscope with a 100× objective (Olympus) and Hamamatsu OrcaER C4742-95 CCD camera 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 with the IX70 to minimize background from autofluorescence. The images were minimally processed to improve contrast and brightness with Adobe Photoshop CS version 8.0.

Acetylene reduction assay

Wild-type *Anabaena* PCC 7120 and the *conR* 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 $BG\text{-}11_0$, and transferred to 10 ml tubes that were sealed with a rubber serum stopper. One ml of air was replaced with acetylene and the tube was mixed by inversion for 1 min. The cells were incubated at 30°C 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 *conR*

To identify genes that are potentially involved in the early stages of heterocyst development in *Anabaena* PCC 7120, we performed DNA microarray transcription profiling experiments of the wild type to examine gene expression in the presence of ammonium, nitrate, and at different times after nitrogen step-down. From these data, we

identified genes that showed a pattern of expression similar to *hetC* (Figure 2.1). The *hetC* gene is upregulated at 5 to 9 h after the onset of heterocyst differentiation and is predicted to encode a bacterial ABC protein exporter (87, 154).

Candidate genes that were regulated similarly to *hetC* were inactivated to assess their role in heterocyst development. To inactivate all3290, alr0295, alr4882, alr0996, all1738, alr4808, all4738, alr3871, all1682, all4160, alr3924, and all1688, an internal fragment of each gene were amplified by PCR and cloned into a suicide plasmid (Table 2.2). After conjugation and isolation of segregated clones, none of the mutants exhibited a diazotrophic growth defect or defects in heterocyst differentiation or pattern formation. For the all3169 and alr0141 loci, no exconjugants were obtained (data not shown).

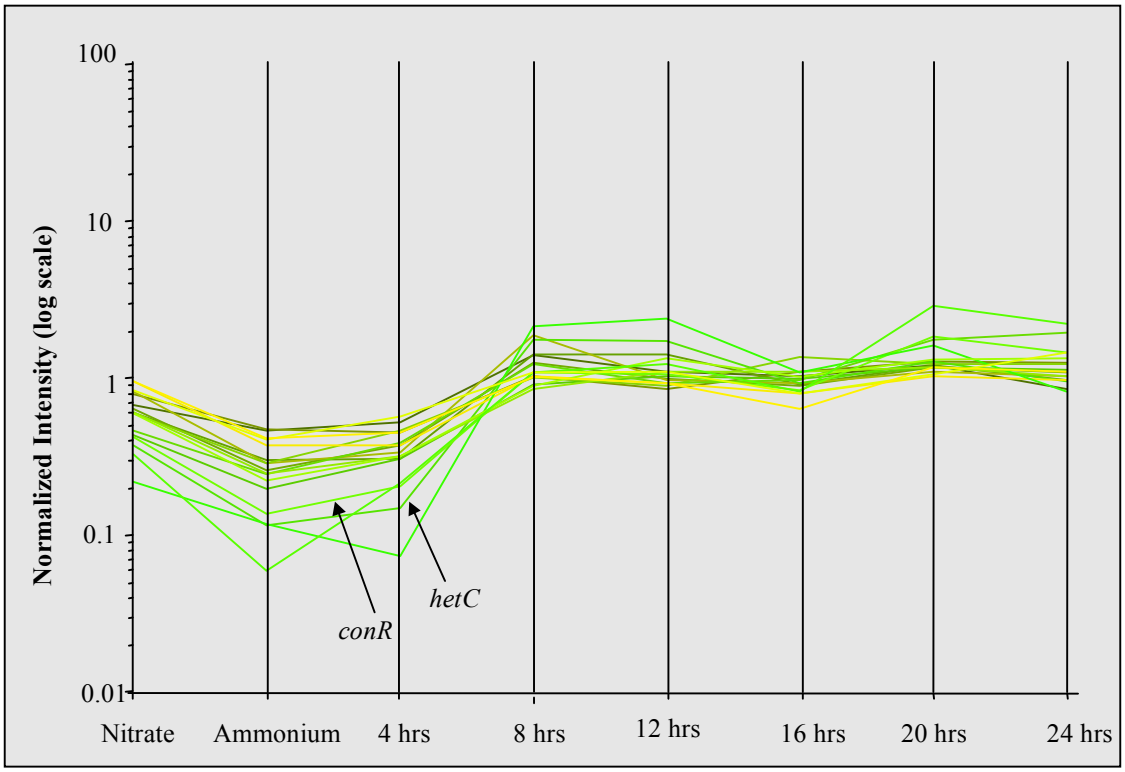

Figure 2.1. Graph of genes that have at least 90% correlation to the signal values of the *hetC* gene. Samples were grown in BG-11 medium containing nitrate, ammonium, or after nitrogen stepdown for the times shown. All genes in this set have lower expression in nitrate- and ammonium-containing medium and are upregulated after 8 hrs of nitrogen deprivation.

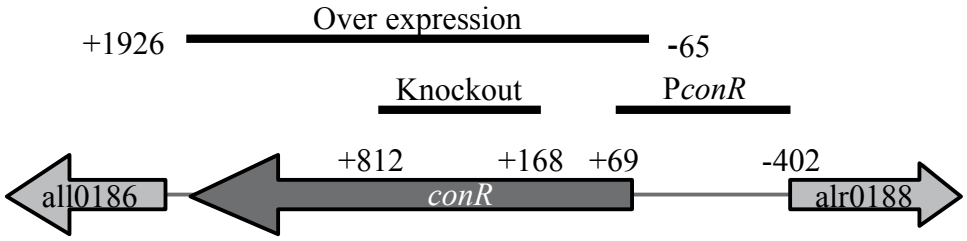

Figure 2.2. Map of *conR* on the *Anabaena* PCC 7120 chromosome and the DNA fragments used in our study for genetic experiments. Suicide plasmid pAM3438 contains an internal fragment of *conR* (Knockout) and was used to inactivate *conR* by single homologous recombination into the chromosome. Plasmid pAM3448 contains the entire *conR* ORF under control of the *rbcL* promoter and was used to overexpress *conR*. Plasmid pAM3450 contains the promoter region of *conR* fused to the *gfpmut2* reporter gene. Numbering is relative to the first nucleotide of the *conR* ORF.

Inactivation of all0187 (Figure 2.2) produced defective diazotrophic growth among other defects described below. Concurrent with our work, a single transposon mutant of all0187 was briefly described by Fan *et al*., who named the gene *conR* (45).

The *conR* gene product is predicted to contain an N-terminal transmembrane region (residues 83-105) but lacks a predicted signal sequence. It is predicted to contain a LytR/CpsA/Psr domain (residues 174-323), which is found in a group of proteins that are involved in speptation and cell-wall maintenance (33, 76, 84). However, the biochemical 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 (124). The *conR* gene is incorrectly annotated in several databases and by Fan *et al*. (45) as being a transcriptional regulator because of this confusion. Our bioinformatic analysis of the *Anabaena* PCC 7120 genome identified two genes predicted to contain a LytR/CpsA/Psr domain, *conR* (all0187) and alr2479, and both genes are incorrectly annotated as transcriptional regulators. Our microarray experiments showed that only *conR*, and not alr2479, was developmental regulated after nitrogen step-down.

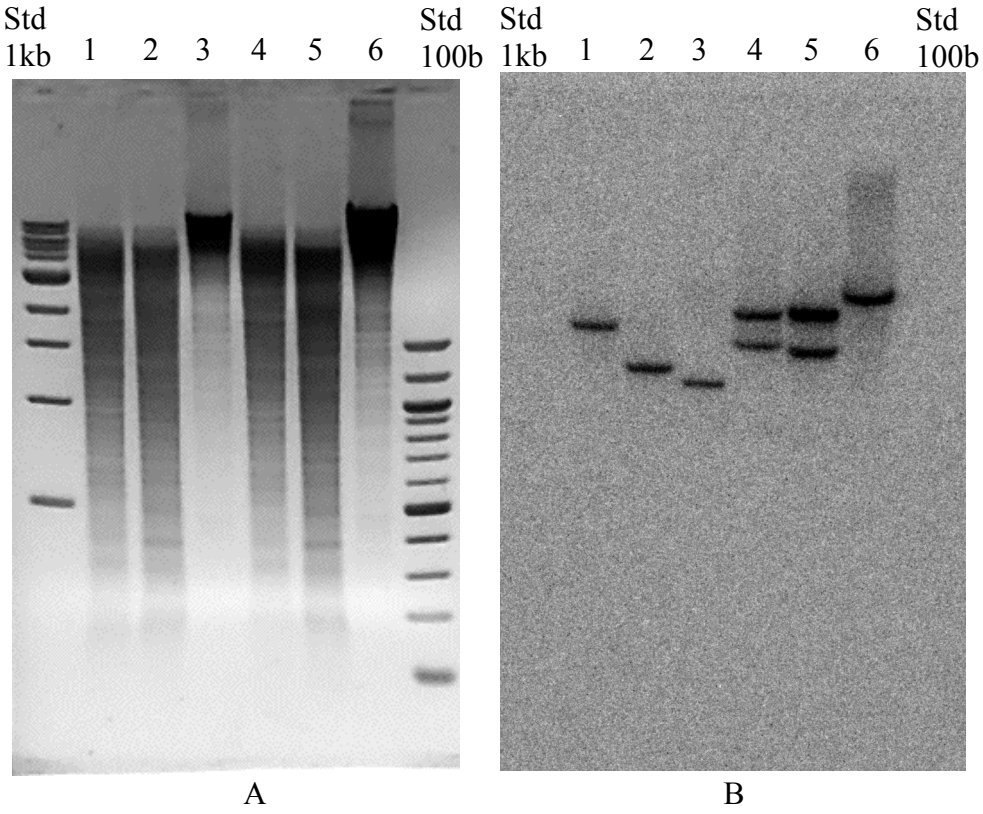

Figure 2.3. Confirmation of single-recombination mutant of *conR* by Southern blot analysis. Chromosomal DNA was isolated from *Anabaena* PCC 7120 wild type or the *conR* mutant strain AMC1369, and 10 µg of chromosomal DNA was digested and the fragments were separated in 1% agarose gels and analyzed by Southern blot using a specific PCR fragment probe for *conR*. (A) Ethidium bromide staining. (B) Radioactive label. Lanes 1, 2, and 3, *Anabaena* PCC 7120 wild type; lanes 4, 5, and 6, strain AMC1369. DNA samples were digested with HincII (1 and 4), SspI (2 and 5) and XbaI (3 and 6). Size standards are in the outermost lanes.

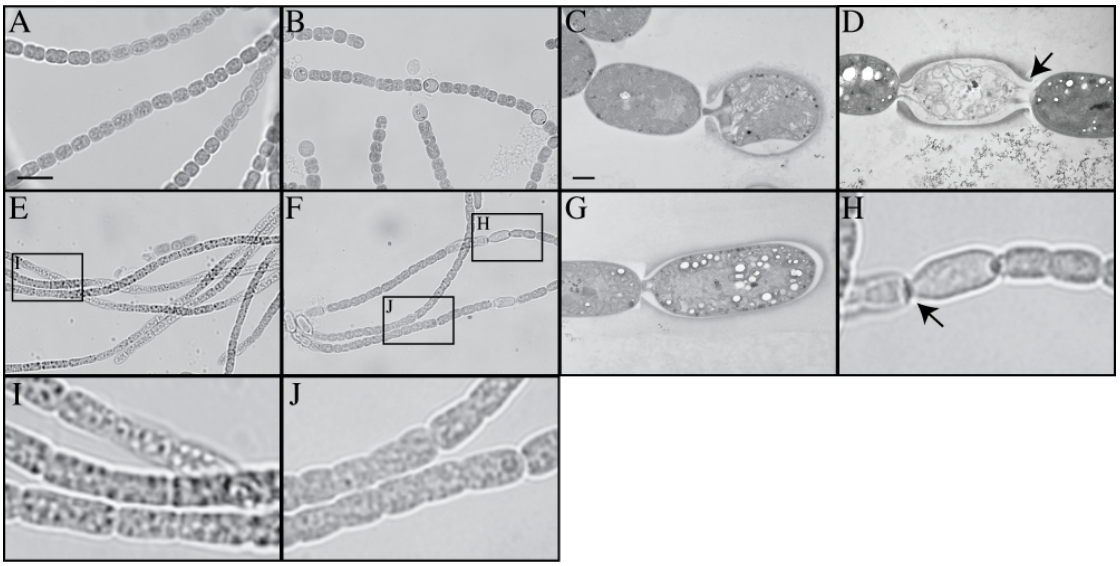

Figure 2.4. *conR* mutant phenotype. The strain AMC1369 is defective for normal vegetative cell division and heterocyst morphogenesis after nitrogen step-down. *Anabaena* PCC 7120 wild type (a, b, c) and *conR* mutant AMC1369 (D, E, F, G, H, I, J) strains grown in nitrate-containing BG-11 medium (A, E) or nitrate-free BG-11_0 medium (B, C, D, F, G, H, I, J). Size bars are 10 μm in the DIC micrographs in panel A and 500 nm in the electron micrographs in panel C. Panel H and J shows the boxed region in F, arrows indicate abnormal cell poles that appear open. Panel I shows the boxed region in E.

Characterization of the *conR* mutant

The *conR* gene was insertionally inactivated by single homologous recombination with a suicide plasmid that contained an internal fragment of the gene (Figure 2.2). Southern blot (Figure 2.3) and PCR analysis (data not shown) showed that no copies of the wild-type gene could be detected in the mutant strain grown under antibiotic selection. The phenotype of the resulting mutant strain, AMC1369, was analyzed in medium with or without a source of combined nitrogen. In medium containing nitrate, the mutant cells grew slower that the wild type.

Vegetative cells of the mutant strain appeared highly granular and were partially defective for septum formation and cell division and, consequently, some cells appeared longer than normal (Figure 2.4 E and I). In medium without a source of combined nitrogen, the AMC1369 mutant filaments were able to form heterocysts with a fairly normal pattern along the filament, but were unable to grow diazotrophically (Figure 2.4 F). The vegetative cells had a somewhat more normal appearance after nitrogen step-down but still showed defective septum formation (Figure 2.4 J). AMC1359 cells transferred from BG-11 to BG-11_0 liquid media yellowed and died after 2 to 3 days.

Time-lapse microscopy of mutant filaments after nitrogen step-down revealed that differentiating cells elongated and usually did not complete septum formation with neighboring vegetative cell(s) resulting in abnormally long heterocysts that appeared open at their ends (Figure 2.4 D and 2.4 H). Differentiating cells 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. 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.

The mutant phenotype described above was observed in several independent exconjugant clones. However, to confirm that the observed phenotypes were due to the inactivation of *conR* and not to second-site mutations, the *conR* mutant strain AMC1369 was complemented with shuttle plasmid pAM3448, which carries the *conR* gene expressed from the *rbcL* promoter. The complemented strain had a wild-type phenotype, which shows that the inactivation of *conR* was responsible for all of the observed phenotypes. Overexpression of *conR* 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 *conR* 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 (Figure 2.4 G). 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 often contained circular inclusions of low electron density in the cytoplasm, that were not present in the wild type. These inclusions are presumably poly-[beta]-hydroxybutyrate (PHB) granules that accumulate because the filaments are limited for fixed nitrogen (169). Thin layer chromatography experiments showed that heterocyst glycolipids were present in the mutant strain (data not shown). Fan et al. had previously observed that a mutant strain with a transposon insertion in the all0187 locus could not grow diazotrophically but contained heterocyst glycolipids similar to the wild type. They

also noticed that the junctions between vegetative cells and between vegetative cells and heterocysts were often partially defective, and therefore they named the gene *conR* (45).

Expression of *conR*

To validate the microarray data and confirm that expression of *conR* increases after nitrogen step-down, we performed northern RNA blot analysis on independently isolated RNA samples. Total RNA was extracted from wild-type *Anabaena* PCC 7120 and from the *conR* mutant strain AMC1369 at several times after nitrogen step-down. Figure 2.5 shows that *conR* transcripts increased from a basal level by 8 h after nitrogen step-down and continued to increase until 12 h, but were back down to basal levels by 24 h following nitrogen step-down.

To determine whether the increase in expression of *conR* takes place specifically in differentiating cells we used a P_{conR}-*gfp* reporter (Figure 2.6). Time-lapse microscopy of the wild type carrying the P_{conR}-*gfp* reporter on shuttle plasmid pAM3450 showed 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 step-down (data not shown). GFP fluorescence from vegetative cells remained at a lower level compared to heterocysts following nitrogen step-down (Figure 2.6). These time-course *gfp*-reporter experiments allow us to follow the temporal and spatial expression from reporters but cannot provide precise quantitative measurement of gene expression because of the lack of an internal standard.

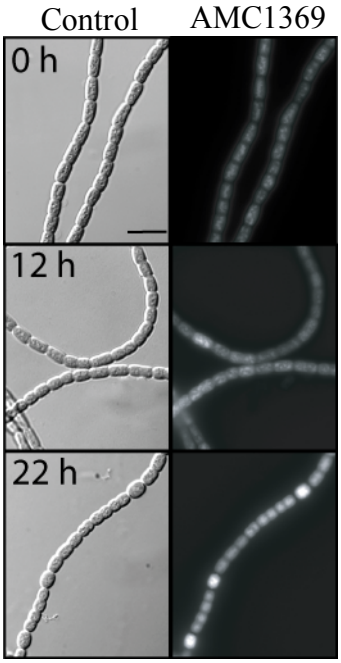

Figure 2.5. A P_{conR}-*gfp* reporter strain (AMC1370) showed increasing GFP fluorescence in differentiating cells after nitrogen step-down. Differential interference contrast microscopy (DIC) images of AMC1370 filaments (left) and corresponding GFP fluorescence images (right). Size bar, 10 μm.

Nitrogen fixation in the *conR* mutant

Because the *conR* mutant strain failed to growth diazotrophically even though it had an apparently normal heterocyst envelope, we determined if the *nifH* gene was expressed at normal levels. Previous studies have shown that the *nifHDK* operon is expressed 12-18 h after heterocyst induction in the wild type (10, 162). Under our conditions, *nifH* expression in the wild type began to increase by 12 h after nitrogen step-down (Figure 2.5). However, in the *conR* mutant strain, *nifH* expression was delayed, and began to increase only by 24 h after nitrogen step-down. Overt morphological heterocyst morphogenesis was not delayed.

An acetylene reduction assay was used to determine if functional nitrogenase activity was being produced in the *conR* mutant strain. Even though the *conR* mutant cannot grow diazotrophically, nitrogenase activity was only 30% lower in the *conR* mutant strain compared to wild type (Figure 2.7). These results suggest that the diazotrophic growth defect caused by the inactivation of *conR* is not due to the inability of the heterocysts to reduce molecular dinitrogen but is instead due to an inability to supply fixed nitrogen to vegetative cells.

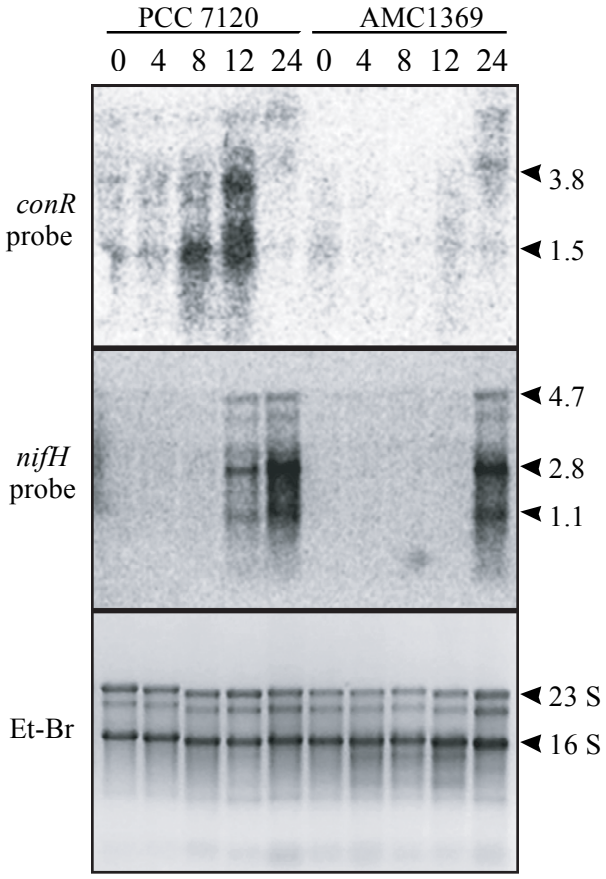

Figure 2.6. Northern RNA blot analysis of *Anabaena* PCC 7120 and the *conR* mutant strain AMC1369 during heterocyst development. Lanes are labeled with hours post nitrogen step-down. The lower panel is the ethidium bromide stained gel to show sample loading. The upper and middle panels show northern RNA blots probed with a strand-specific radioactive probe for *conR* or *nifH* genes, respectively. For *conR* 3.8-kb and 1.5-kb transcripts were detected, and for *nifH*, three transcripts were detected at 4.7 kb, 2.8 kb, and 1.1 kb.

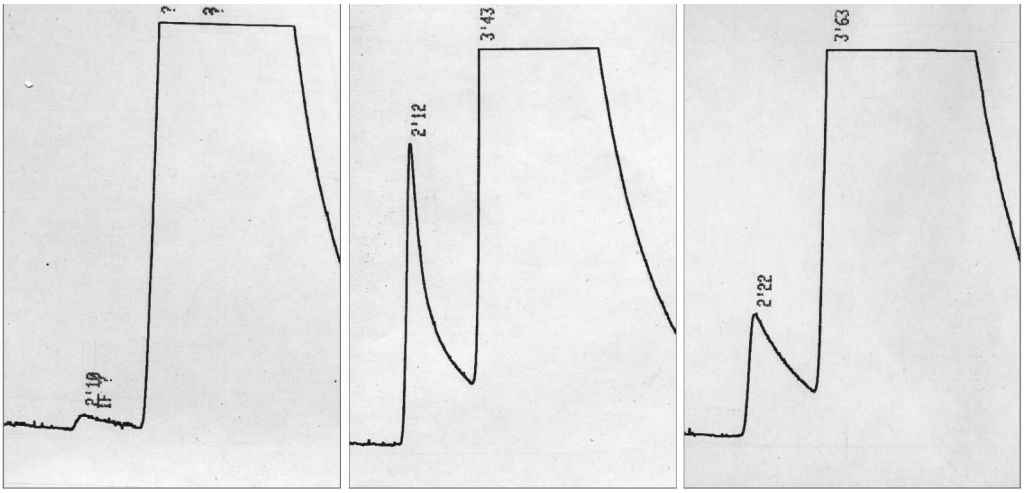

Figure 2.7. Acetylene reduction assay. *Anabaena* sp. PCC 7120 and *conR* mutant strain AMC1369 were incubated in BG-11_0 at 30ºC for 24 hrs in the presence of 10% ethylene. A 1 ml gas sample was analyzed by gas chromatography in a high pressure Hewlett Packard gas chromatograph (HWP) using a C18 column. The control culture was uninoculated BG-11_0.

Discussion

DNA microarray transcript profiling data showed that the *Anabaena* PCC 7120 *conR* gene was expressed similarly to genes involved in the early stages of heterocyst development. In work done concurrently with ours, the *conR* gene was briefly characterized by Fan and colleagues who had identified it in a transposon mutagenesis screen for mutants defective for diazotrophic growth (45). Our microarray experiments showed that the expression of *conR* was up-regulated at 8 h after nitrogen step-down. A heterocyst induction time course with a P_{conR}-*gfp* reporter strain showed upregulated expression by 9 h after induction and showed that the highest expression levels were present in differentiating cells. The *conR* gene was inactivated by single homologous recombination, and similar to the observations of a transposon mutant by Fan et al. (45), the mutant cannot grow diazotrophically, and produces heterocysts with abnormal morphology and defective junctions with vegetative cells. Filaments of the *conR* mutant strain AMC1369 grown in nitrate-containing media showed defects in cell division and septum formation; this phenotype was also observed by Fan et al. (45). Interestingly, after nitrogen step-down, the defect in septum formation between vegetative cells in the *conR* mutant was less severe, which could be a consequence of a nutritional stress response or slowed growth caused by nitrogen limitation.

A bioinformatics screen for NtcA-regulated genes identified a putative NtcA binding site at position -60 relative to the start codon in the promoter region of *conR* (145), which is consistent with the upregulation of *conR* that occurs in differentiating heterocysts.

The *conR* 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 up-regulation 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 peptidoglycan (97). Recently a LytR domain protein has been described in *Streptococcus pneumoniae*, in which the disruption of the *lytR* gene results in misplacement of the septum (84). 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, autolysins, are thought to be essential for separating daughter cells after division is concluded, and their absence causes incomplete septal invagination (130). Based on the *conR* mutant phenotype, it is possible that the set of peptidoglycan hydrolases involved in septation between vegetative cells, or their regulation, are at least partially different from those involved in septation between differentiating heterocysts and vegetative cells. Autolysins may also be required for modification of the heterocyst peptidoglycan to facilitate assembly of the outer envelope layers, and autolysin activity may explain the often larger and rounder shape of heterocysts. In *Anabaena* PCC 7120, the autolysin gene *hcwA* is required for heterocyst maturation (183).

We hypothesized that the abnormal cell division of the *conR* mutant might be caused by altered expression of *hcwA*. However, we did not detect a change in *hcwA* messenger RNA levels in the *conR* mutant (data not shown). Alternatively, the enzymatic activity or localization of HcwA or other autolysins may be altered in the *conR* mutant.

In many cases, the inability of an *Anabaena* PCC 7120 mutant to grow diazotrophically may be caused by a defect that compromises the integrity of the envelope layers, allowing more oxygen to enter the heterocysts and inactivate nitrogenase. However, the *conR* mutant formed heterocysts with outwardly normal polysaccharide and glycolipid layers. We asked if the incomplete constriction of the septum between heterocyst and vegetative cells exposes the intracellular environment of the heterocysts to oxygen and, thus, causes a decrease in the expression or activity of nitrogenase. Northern blot analysis showed that *nifH* expression in the *conR* mutant was up-regulated 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 delayed establishment of a microoxic environment due to delayed or partially defective septum formation or possibly delayed or partially defective deposition of the envelope layers.

Unexpectedly, the *conR* mutant retained seventy percent of wild-type levels of nitrogenase activity, and this amount of activity should be sufficient to sustain diazotrophic growth (153), although at a slower rate. Inactivation of the heterocyst-specific gene *fdxH* reduced nitrogenase activity to less then 50%, but still allowed slow diazotrophic growth (108). However, *conR* mutant filaments completely stopped growing after a few days in medium without a source of combined nitrogen and the cells died. Therefore, the vegetative cells are not receiving nitrogen even though at least some of the heterocysts of the *conR* mutant are capable of creating a microoxic environment that supports nitrogenase activity in spite of the obvious morphological defects. These data show that the diazotrophic growth defect is not caused simply by defective nitrogen

fixation, but may involve defective delivery of fixed nitrogen from heterocysts to neighboring vegetative cells. Recent findings show cytoplasm-to-cytoplasm movement of the small fluorophore calcein in *Anabaena* PCC 7120, which indicates the existence of non-specific intercellular channels that allow metabolite exchange between cells of the filament (114). In the *conR* mutant, defective septum formation and closure could interfere with the formation of these channels at the junction between heterocysts and vegetative cells and, thus, prevent normal metabolite exchange from taking place.

CHAPTER III

SIGMA FACTOR GENES *sigC*, *sigE*, AND *sigG* ARE UPREGULATED IN HETEROCYSTS OF THE CYANOBACTERIUM *Anabaena* PCC 7120[*]

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.

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[*] 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 MA., Mella-Herrera RA., Golden JW., 2007. Journal of Bacteriology, **189:**8392-8396, Copyright 2007 by American Society for Microbiology.

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.

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* (6, 94), *Myxococcus* (158), *Streptomyces* (53), and *Caulobacter* (170).

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 (165). We and others have hypothesized that sigma factors may play a significant role in directing transcriptional control during heterocyst

development in *Anabaena* PCC 7120; however, clear evidence supporting this hypothesis is still lacking (89).

Sigma factors are classified into two families based on sequence similarity: the σ^{70} and σ^{54} families (65). The σ^{70} family has been divided into four major groups based on phylogeny (65). 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 (68).

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 (168). There are no obvious σ^{54} homologs in cyanobacteria or in high-GC, Gram-positive bacteria (144). Another feature that cyanobacteria share with some Gram-positive bacteria such as *Streptomyces* spp. is a relatively large number of group 2 sigma factors (168).

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 (32, 64, 65, 81, 120, 121, 126).

A total of twelve sigma factor genes, all belonging to the σ^{70} family, have been identified in the genome of *Anabaena* PCC 7120 (90). Here, we have adopted the revised nomenclature proposed by Yoshimura *et al*. (176). 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 (13, 61, 89). 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 (176). 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 (14). The transcription of *sigB* is induced by nitrogen-limiting conditions, whereas that of *sigC* is induced by nitrogen or sulfur limitation (13). However, single and double mutants of *sigB* and *sigC* showed that they are dispensable for diazotrophic growth (13). 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 (89). A *sigB2 sigD* double mutant was able to form proheterocysts but was unable to grow diazotrophically, possibly due to extensive fragmentation of filaments upon nitrogen deprivation; the double mutant was complemented by either *sigB2* or *sigD* (89). 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 stepdown 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*. Additionally, northern blot experiments showed that *sigC* inactivation caused delayed expression of genes known to be upregulated early during heterocyst differentiation.

Materials and methods

Strains and culture conditions

The strains and plasmids used in this study are presented in Table 3.1. *Anabaena* 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 (59, 135). For derivatives of *Anabaena* PCC 7120 carrying shuttle plasmids, neomycin (25 μg/ml) or 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 stepdown, 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

Table 3.1. Bacterial strains and plasmids

Strain/plasmid	Relevant characteristics	Source reference
Anabaena		
PCC 7120	Wild type	(7)
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	(89)
AMC649	*sigE* insertional inactivation mutant	(89)
Plasmids		
pAM1956	Shuttle vector pAM505 containing promoterless *gfpmut2*	(175)
pAM2178	Conjugal suicide vector	(89)
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

Table 3.2. PCR primer sequences

Gene	Sense primer	Sequence (5'→3')	Antisense primer	Sequence (5'→3')
P_{sigC}	AMO-970	CGACCCGGGATTGGTAAGGGGTAATTGG	AMO-971	TCCGGATCCCAATAGATTGCGGTCAGC
P_{sigD}	AMO-1175	TAGGTCGACGCCCGAACTTCTAGAACACCAATTCAG	AMO-1176	TAGGAGCTCTAACTGAATAGATGCTCTTTGCCGTTG
P_{sigB2}	AMO-1177	GGAGTCGACTGTGAGAAGTAGGGAGCAGGA	AMO-1178	AGTGAGCTCTGGTGCGTTGTCCTCTACTCCG
P_{sigE}	AMO-1179	AATGTCGACAGTTCTTGAATTTTAAAGATAAATATTCTTATTGCC	AMO-1180	TTGGAGCTCAATTTCATGACAACTGTTACTACTGAGATGG
P_{sigF}	AMO-1149	CTTGAGCTCCAAAGCTGTCTGGCAC	AMO-1150	GTAGTCGACGCACCGCATTTATTAAG
P_{sigJ}	AMO-1386	ACAGTCGACAGTAGCAGTATTACTATAGC	AMO-1387	TGCGAGCTCATAAAAATTCCCTTGTGGTAAC
P_{sigI}	AMO-1151	TGGAGCTCACACCGCCTGACGAATTATACCTC	AMO-1152	GGGTCGACGCGATTTTTAACCCATATC
P_{sigG}	AMO-1382	TTGGTCGACTATCCGGAAAAATG	AMO-1383	CGATTGGAGCTCAGATCGTATGGCG
hepA	AMO-1970	ATTCCCCGATTTTACGATCC	AMO-1971	GATTCGTGTTGCATCTGGTG
hetR	AMO-1137	CTGCTCGAGGGCAAACTGCTCAAG	AMO-646	TAAGTCCGCTCTTGGTCGTCTG
rnpB	AMO-1972	CTTGCTGGATAACGTCCAGT	AMO-1973	TGTTACCAAACGCCTCTAGC
sigC	AMO-1145	GATCATATGCCAGCAACATCTTTTTACGCAG	AMO-1146	CTACCCGGGATCTAACTCAGAGATTCCAAATAGT
sigG	AMO-1491	CTGGTCGACGGTTGATGCGAGG	AMO-1492	TCAGAGCTCGCCTCTCGCAGTTG

twice by centrifugation and the pellet was resuspended 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* PCC 7120 or *Anabaena* mutant strains by conjugation from *E. coli* following published protocols (42, 57) with some minor modifications (101).

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). Strains used in this study are mentioned in Table 3.1. Total DNA from cyanobacterial strains was isolated as previously described (59). 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 the synthetic oligonucleotide primers (see Table 3.2 in page 60) that were used to amplify the PCR fragments for construction of the reporter plasmids.

Plasmid constructions

Plasmids used in this study are listed in Table 3.1 (see table in page 59). 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

(62). 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 for 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 GFP 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 then 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) 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 during the experiment; 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 to prepare figures (Figure 3.3).

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. At 4, 8, 12, 18, 24, 36, and 48 h after nitrogen deprivation, the cells were rapidly chilled by pouring the culture over 100 g of ice in a 250 ml conical centrifuge bottle, and then collected by centrifugation at 4°C. The filament pellets were 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 MAGNACHARGE nylon membrane (GE Water & Process Technologies) 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 3.2) were labeled with [α-32P]dCTP. The hybridization solution contained 5× SSE, 50% formamide, 0.5% SDS, and 5× Denhardt's solution (0.1% Ficoll, 0.1% polyvinylpyrrolidone, 0.1% BSA) 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 DNase I treatment using a RiboPure kit (Ambion) according to the manufacturer's instructions. Two-microgram portions of each RNA sample were reverse-transcribed using SuperScript II™ reverse transcriptase (Invitrogen) and primers specific for the *hepA* or the *rnpB* genes (Table 3.2) in a reaction volume of 20 μl according to manufacturer's instructions. The *rnpB* gene was used as a control. It encodes the RNA component of ribonuclease P. The absence of DNA in RNA samples was checked by PCR. Two microliters from each cDNA sample 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 finally an additional 6 min at 72ºC.

Results

Classification of putative sigma factor genes in *Anabaena* 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*) (13, 14, 89, 90, 176). 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* PCC 7120, *A. variabilis* ATCC 29413, *Synechocystis* sp. strain PCC 6803, and *Synechococcus elongatus* PCC 7942 (176). 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 (Figure 3.1). 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 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 (78). 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 (Figure 3.1). The *Synechocystis sigG* gene appears to be essential for viability (78).

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* PCC 7120 by conjugation, but an extended period of time (30 days) was necessary for small exconjugant colonies to appear. Conjugation was repeated twice, and each time exconjugants appeared only after about 30 days of incubation. From our experience, the average time required for exconjugants

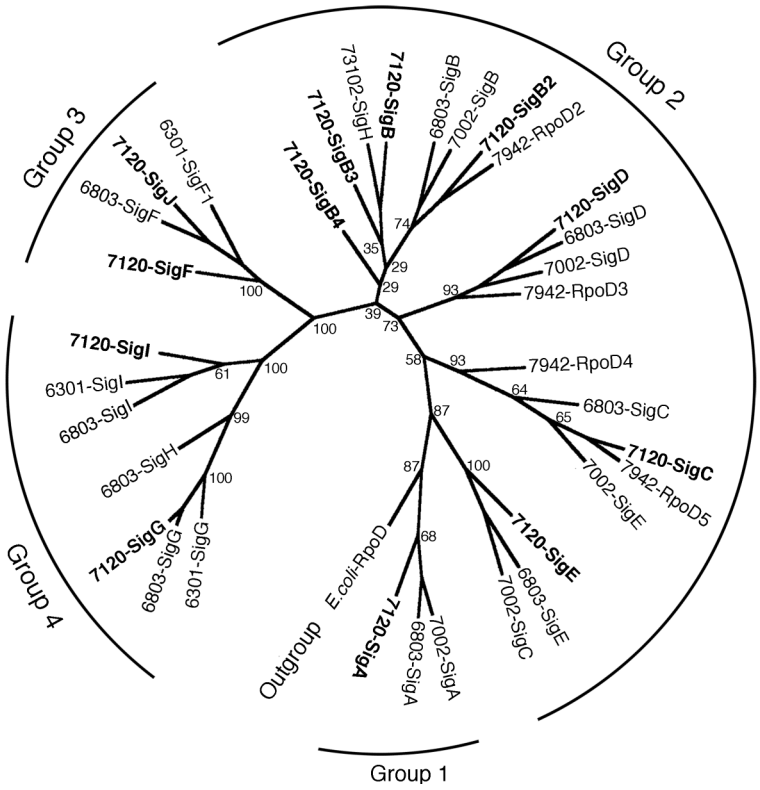

Figure 3.1. Phylogenetic relationship of group 1, 2, 3, and 4 sigma factors. Sequences originated from *Anabaena* PCC 7120, *N. punctiforme* PCC 73102, *Synechocystis* sp. strain PCC 6803, *Synechococcus elongatus* PCC 7942, and *Synechococcus* sp. strain PCC 7002. The unrooted tree was generated with 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.

(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 in the medium had decreased to a level that allowed growth of partially segregated mutants. After transfer to nitrate-containing medium, the potential single recombinants were not able to grow unless the streptomycin and spectinomycin concentrations were below 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. Segregation was tested by PCR, which showed 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 (99, 175). A control strain containing the pAM1956 vector alone produced no detectable GFP fluorescence.

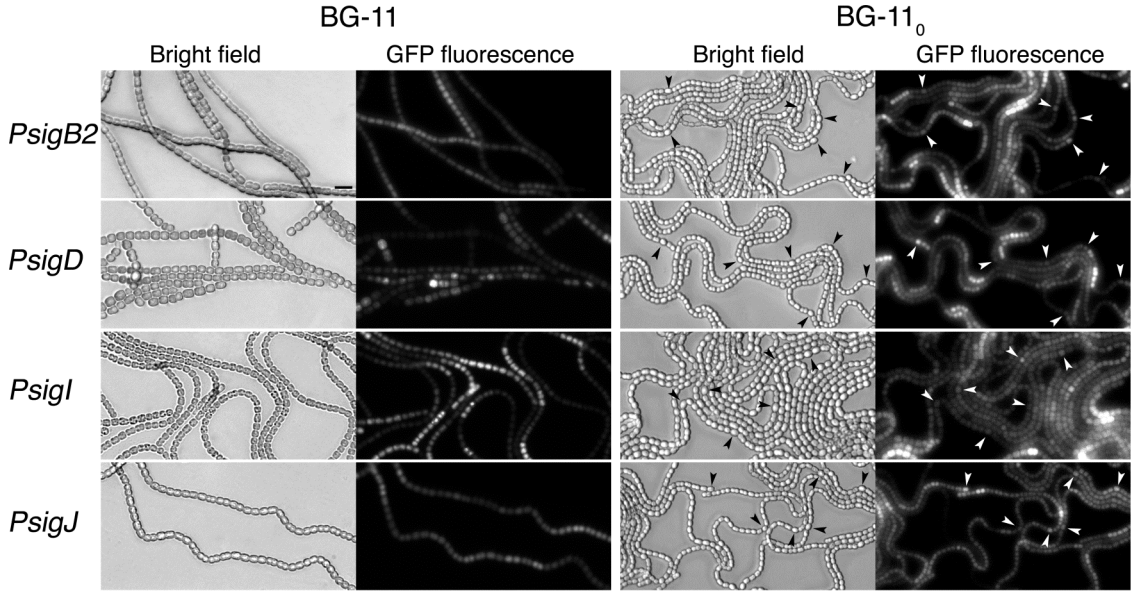

Figure 3.2. GFP reporter fluorescence from strains containing *gfpmut2* expressed from promoters of the *sigB2*, *sigD*, *sigI*, and *sigJ* sigma factor genes in *Anabaena* PCC 7120. Filaments grown in nitrate-containing BG-11 medium or at 24 h after nitrogen step-down to BG-11_0 medium to induce heterocyst development are shown. Arrowheads indicate mature heterocysts. (Left panels) Bright-field images. (Right panels) Corresponding GFP fluorescence images. Scale bar, 10 μm.

The *sigB2*, *sigD*, *sigI*, and *sigJ* promoter regions were all active in vegetative cells of nitrate-grown filaments (Figure 3.2). In nitrate-grown cultures, the GFP fluorescence intensity varied in the vegetative cells along filaments such that groups of cells that showed lower intensity tended to alternate with groups of cells producing stronger intensity (Figure 3.2). 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 stepdown. At 24 h after nitrogen stepdown, 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 stepdown. 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.

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 stepdown

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 stepdown, 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 stepdown.

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 (Figure 3.3A). The P_{sigE}-*gfp* reporter strain displayed a more uniform low level of fluorescence in vegetative-cell filaments (Figure 3.3B). 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 stepdown (Figure 3.3C).

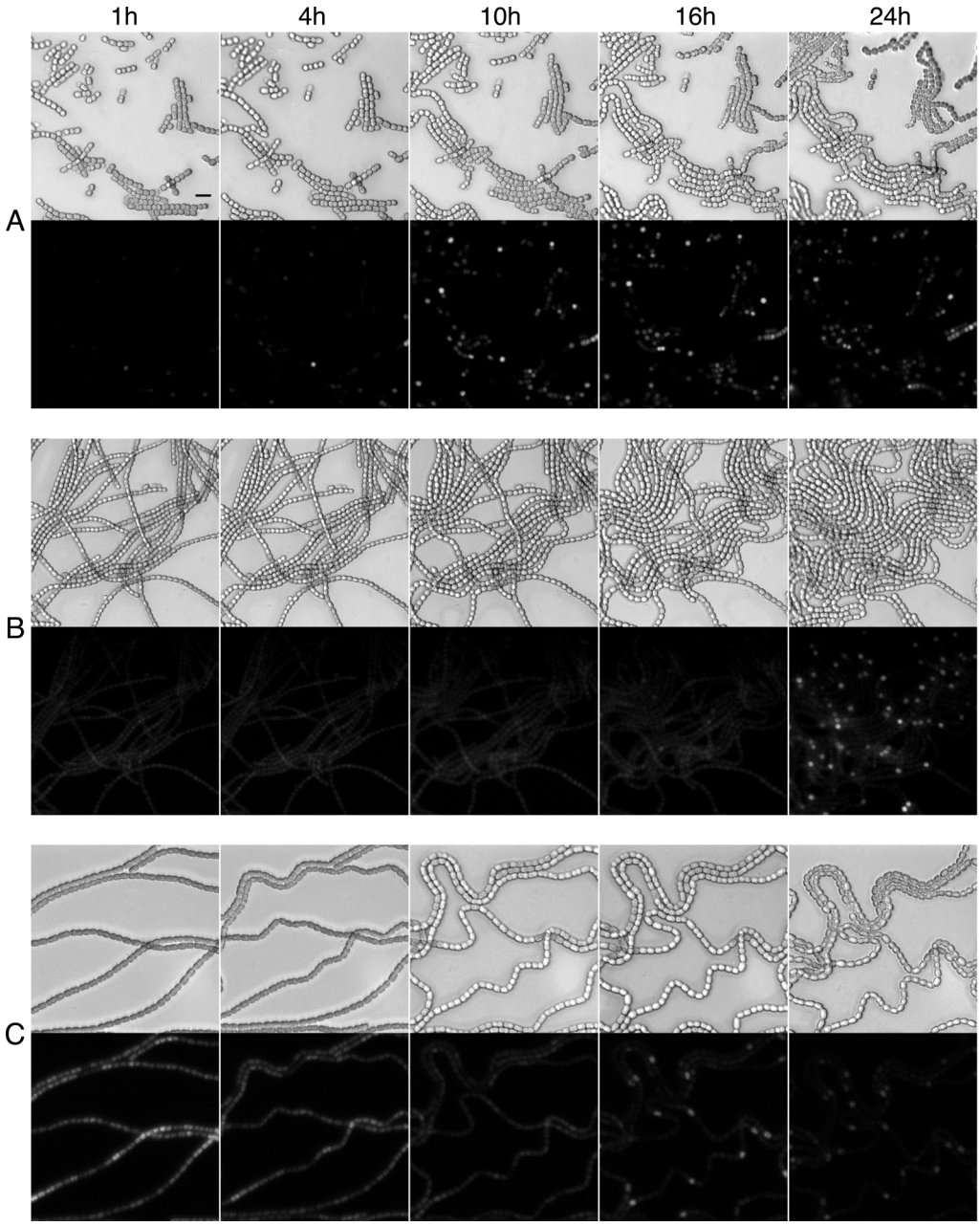

Figure 3.3. Temporal and spatial GFP reporter fluorescence from promoters of sigma factor genes *sigC* (A), *sigE* (B), and *sigG* (C). (Upper panels) Bright field images. (Lower panels) Corresponding GFP fluorescence images. The strains were grown in nitrate-containing BG-11 medium and then transferred 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* during heterocyst development. 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 3.3 contains images corresponding to the indicated time-points that were extracted from the time-lapse series.

At 1 h following nitrogen stepdown, P_{sigC}-*gfp* expression was relatively unchanged compared to nitrate-grown filaments (Figure 3.3A). 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 (175), 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. As mature heterocysts aged they became dimmer. Previous observations showed that *sigC* mRNA is synthesized by 6 h after nitrogen stepdown, and then peaks at 12 h followed by a drop to lower levels (13), 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 ends of filaments. This phenomenon was observed in actively growing cells, but it was most 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 stepdown, at which time 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 stepdown. 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 stepdown, 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, proheterocysts 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 stepdown when cells become committed to complete the differentiation process (175), 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. Our further analysis of the *sigC* mutant strain showed that although *sigC* was not essential for diazotrophic growth, the mutant did show 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 was delayed and reduced in the *sigC* mutant

Northern RNA blot hybridization experiments showed that in the wild-type strain the *sigC* gene was expressed at 4 h after nitrogen stepdown, 2 h earlier than it was previously shown to be upregulated (Figure 3.4) (13). The GFP fluorescence pattern of the P_{sigC}-*gfp* reporter strain also suggested an early increase of *sigC* expression in differentiating cells. Due to the timing of *sigC* upregulation after nitrogen stepdown, 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 and RT-PCR to examine expression of *hetR*, which is the a gene known to be specifically 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 stepdown (Figure 3.5).

Two major transcripts are known to be produced for the *hetR* gene with lengths 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 (Figure 3.5) compared to the wild type.

Two transcripts were detected for *hepC* at 1.0 kb and 1.3 kb, although a previous study mentioned the presence of only one transcript of an undetermined size (117).

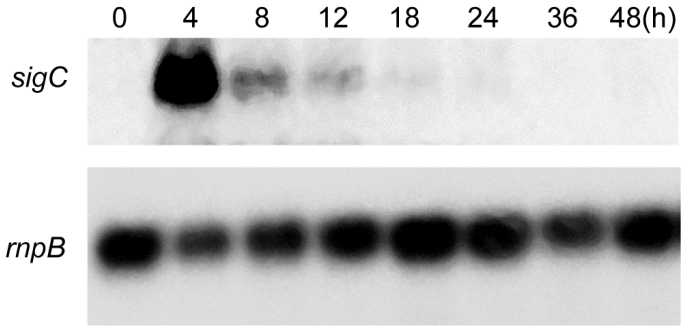

Figure 3.4. Expression of the *sigC* gene increased at 4 h after nitrogen step-down and then gradually decreased. Total RNA was isolated from the wild type and the *sigC* mutant strain at 4, 8, 12, 18, 24, 36, and 48 h after nitrogen step-down. For the 0 h time-point, RNA was isolated from nitrate-grown 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.

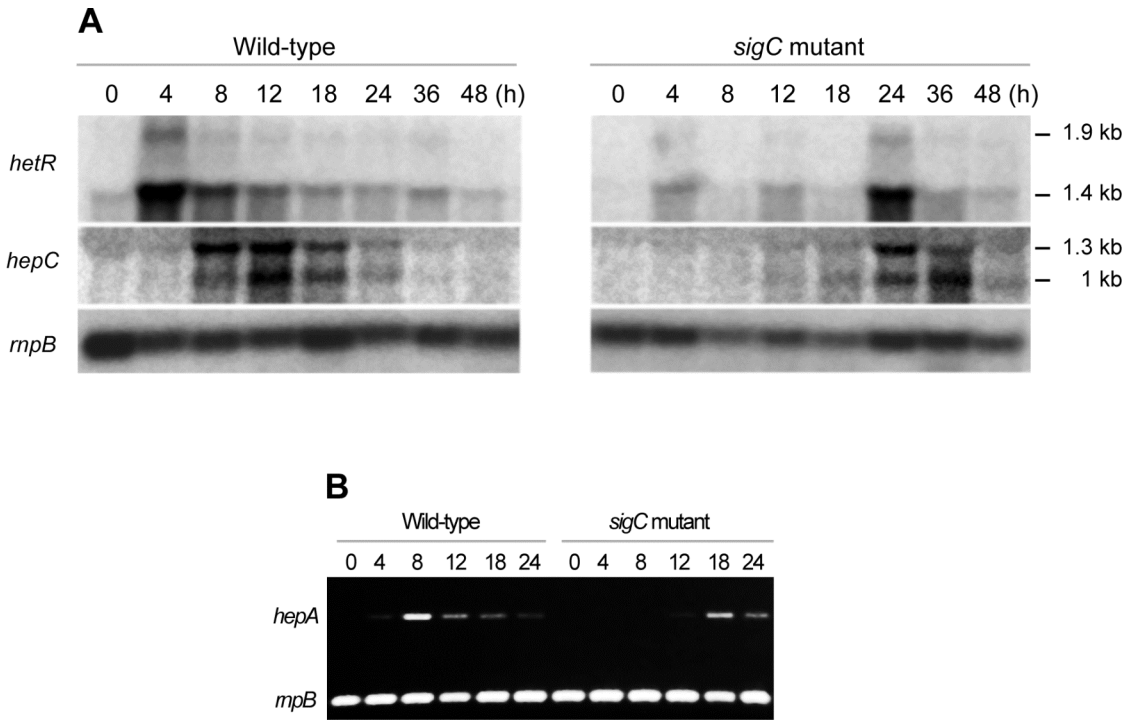

Figure 3.5. The *sigC* mutant strain showed delayed and reduced amounts of *hetR*, *hepC*, and *hepA* transcripts. (A) Total RNA was isolated from the wild type and the *sigC* mutant strain at 4, 8, 12, 18, 24, 36, and 48 h after nitrogen step-down. For the 0 h time-point, RNA was isolated from nitrate-grown 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 in separate reactions as a positive control for the RT-PCR reactions and loading of the agarose gel. These data are representative of two independent experiments.

Expression of both transcripts had increased by 8 h after nitrogen stepdown and peaked at 12 h, after which they gradually decreased (Figure 3.5). In the *sigC* mutant strain, the two transcripts began to increase at 12 h following nitrogen stepdown and did not peak until about 24 h. The *hepC* messages in the *sigC* mutant were two to four fold less abundant than the wild-type levels (Figure 3.6).

Expression of *hepA* was not detected by northern blot hybridization, therefore we used semiquantitative RT-PCR for measuring *hepA* transcript levels. The *hepA* gene showed a pattern of expression similar to that of *hep C* (Figure 3.5). Following nitrogen deprivation in the wild-type background, a low level of *hepA* was present at 4 h, and showed peak abundance at 8 h, after which it gradually decreased.

In the *sigC* mutant background, *hepA* mRNA was not detectable until 12 h after nitrogen stepdown and peak abundance was at 18 h, although not as high 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* in a wild-type background did not alter the pattern of heterocysts, heterocyst morphology, or diazotrophic growth (data not shown).

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. There are twelve putative sigma factor genes in the genome of *Anabaena* PCC 7120. 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. 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 showing a specific involvement of particular sigma factors in heterocyst development (13, 89). 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 (89).

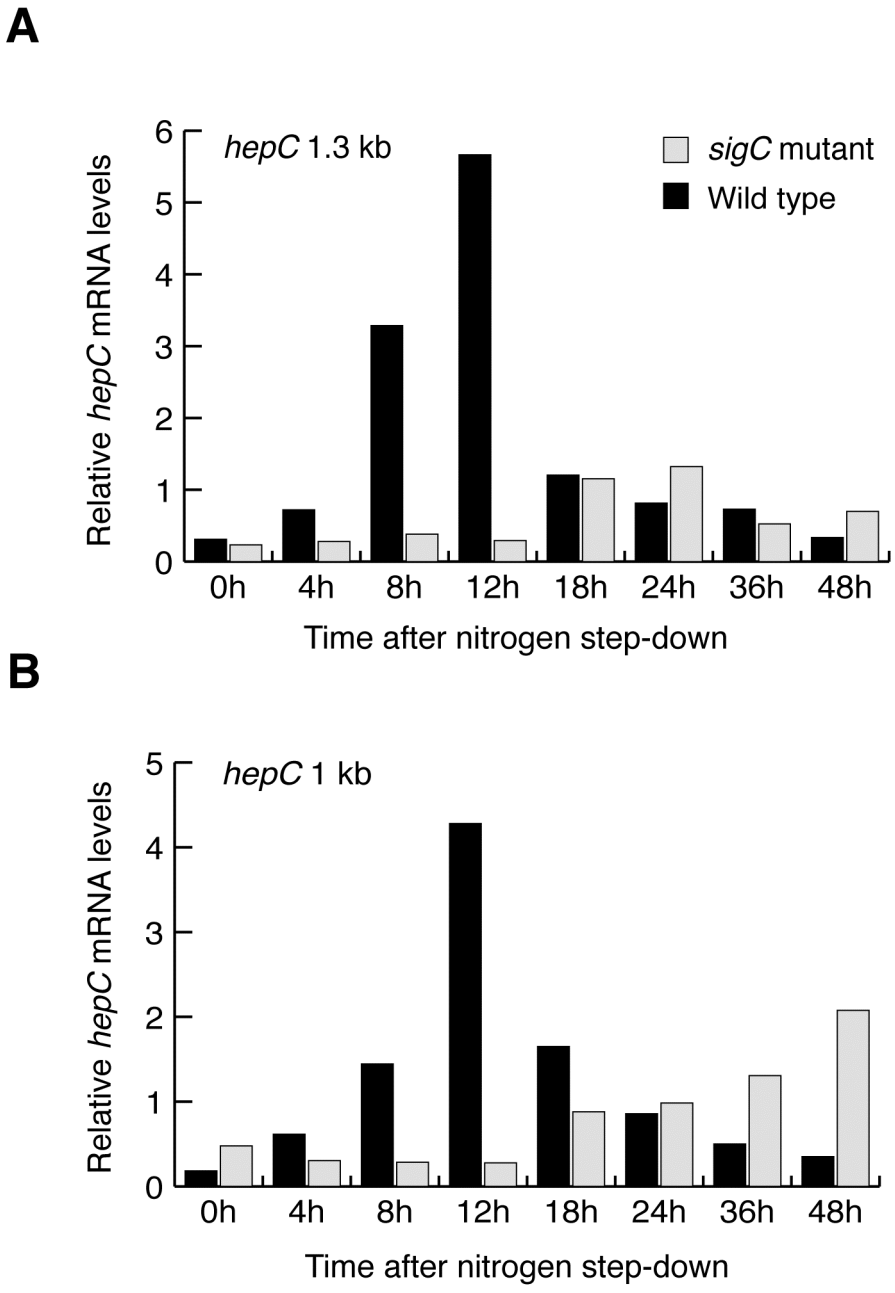

Figure 3.6. Abundance of *hepC* transcript levels was reduced in the *sigC* mutant compared to the wild type. Densitometry of *hepC* mRNA levels in the wild-type strain and the *sigC* mutant strain at several time points after nitrogen step-down. The graph values are an average of two independent experiments. (A) Abundance for the 1.3-kb *hepC* transcript. (B) Abundance for the 1-kb *hepC* transcript. All values were normalized again wild type at 24 h after nitrogen stepdown.

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 reporter 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 (89). 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 (89); however, *sigC* inactivation does not block heterocyst development or nitrogen fixation (13). 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, 61). 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 SigC works in concert with other transcription factors to regulate gene expression, and that increased levels of SigC is not sufficient to greatly change the expression of target genes. It is also possible that increase expression of the SigC regulon does not produce major changes in heterocyst development.

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 of cells poised to different into heterocysts under conditions that outwardly suppress heterocyst development.

The P_{sigG}-*gfp* reporter strain showed increased GFP fluorescence in developing cells around 9 h after nitrogen step-down (Figure 3.3C), which suggests that SigG is involved in the expression of middle stage heterocyst genes. This is a crucial time at which differentiating cells commit to becoming heterocysts (175). 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 (152). However, *sigG* was also expressed in vegetative cell grown on nitrate-containing medium. Therefore, its role in gene expression related to heterocyst development remains unclear.

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 (Figure 3.3B). Thus, the *sigE* reporter is expressed during the late stage of heterocyst differentiation when the cells are become microoxic, 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 most important 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 (61). Although insertional inactivation of *sigE* did not block heterocyst differentiation or diazotrophic growth (89), 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 cheifly 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 consensus sequence (6). 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 (100). 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) (100). We expect that similar 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 (63). Furthermore, these different sigma factors are also able to recognize canonical *E. coli* promoters (63). 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 (80, 81), and among *Bacillus subtilis* extracytoplasmic function sigma factors (107).

CHAPTER IV

SIGMA FACTOR E (σ^E) IN *ANABAENA* PCC 7120 IS REQUIRED FOR THE NORMAL EXPRESSION OF HETEROCYST-SPECIFIC GENES

Summary

The multicellular filamentous cyanobacterium *Anabaena* PCC 7120 produces specialized cells for nitrogen fixation called heterocysts. Previous work showed that the group two sigma factor *sigE* (alr4249) is heterocyst specific and that its expression is upregulated 16 h after nitrogen step-down. Mobility shift assays showed that the transcription factor NtcA binds to the upstream region of *sigE,* and that this binding is enhanced by 2-oxoglutarate (2-OG) in a dose dependent manner. However, site-directed mutagenesis of the NtcA binding site in the *sigE* promoter of a P_{sigE}-*gfp* reporter did not decrease the fluorescent signal *in vivo*. Northern blot and qRT-PCR analyses of the expression of *nifH* in wild-type and *sigE* mutant backgrounds revealed delayed and lower transcript levels during heterocyst differentiation. We developed a P_{nifHD}-*gfp* reporter construct that showed strong heterocyst-specific expression during the late stage of development. Time-lapse microscopy of the P_{nifHD}-*gfp* reporter in a *sigE* mutant background failed to show normal developmentally regulated GFP signal in heterocysts. In a *sigE* over-expression strain, the P_{nifHD}-*gfp* reporter showed early expression of GFP fluorescence. The *sigE* over-expression strain also showed more rapid heterocyst development and increased heterocyst frequency compared to the wild-type strain.

Introduction

Bacteria respond to environmental and intracellular cues by regulation of gene expression, frequently via changes in transcriptional initiation of particular genes. Transcriptional regulation in bacteria is associated with two major families of sigma factors: sigma 70 (σ^{70}) which is subdivided into 4 groups (65), and sigma 54 (σ^{54}) which has no clear homologs in cyanobacteria or in gram-positive bacteria with high-GC content (144). For example, in *Bacillus subtilis*, temporally and spatially regulated σ factors modulate the expression of genes during starvation-induced sporulation (23, 93), and in the gram-negative bacterium *Pseudomonas aeruginosa*, sigma factors control the expression of antibiotic- and phagocyte-resistance genes, specifically during biofilm formation (66, 105). In cyanobacteria, sigma factors have been shown to be involved in a variety of regulatory responses (79) For example, *Synechocystis* sp. PCC 6803 uses the sigma factor SigB for transcription of the *hspA* gene, which encodes a small heat-shock protein (150).

The multicellular cyanobacterium *Anabaena* PCC 7120 is a well-studied heterocyst-forming cyanobacterium with highly tractable genetics and a fully sequenced genome. Heterocysts are considered a good biological model for the study of bacterial differentiation, pattern formation, and nitrogen fixation. *Anabaena* PCC 7120 grows as long chains of photosynthetic vegetative cells in media containing fixed nitrogen (ammonium, nitrate, or urea, for example). After nitrogen step-down (transfer to media lacking fixed nitrogen), one out of about 10 to 20 cells in a filament differentiates to form a heterocyst. These heterocysts are capable of fixing nitrogen, which they supply to

vegetative cells in the form of amino acids, and the vegetative cells supply fixed carbon to the heterocysts.

Heterocysts are morphologically and physiologically differentiated from vegetative cells, which is the result of large scale changes in gene expression. One of the early steps in heterocyst differentiation is the increase expression of *ntcA*, which is an auto-regulatory transcription factor belonging to the cAMP receptor protein (CRP) family. NtcA together with HetR (a master regulator for heterocyst differentiation) regulates the expression of many genes involved in heterocyst differentiation and the assimilation of atmospheric nitrogen (75, 117, 180). NtcA activity is modulated by 2-oxoglutarate (2-OG), which is an intermediate in the Krebs cycle and its increase constitutes the signal for nitrogen starvation. Studies have shown that the addition of an artificial analog of 2-OG (DFPA) to the growth medium results in heterocyst formation even in the presence of ammonium (96). 2-OG interacts with NtcA to enhance DNA binding to the promoter regions of heterocyst development genes, such as *hetC*, which encodes a protein similar to bacterial ABC exporters, and *devB*, which encodes an ABC-type transporter that is necessary for the deposition of the heterocyst glycolipid layer (52, 96, 117, 120, 157, 162, 177). Both *hetC* and *devB* contain a canonical NtcA binding site ($TGTAN_8TACA$) in their promoter regions, and mutations in the NtcA binding site altered the normal developmental expression (115).

During heterocyst differentiation, a polysaccharide layer is deposited around the proheterocyst, followed by the deposition of a glycolipid layer between the outer membrane and the polysaccharide layer. Synthesis and deposition of the polysaccharide

layer requires the expression of a cluster of genes that constitute the HEP island (74). The expression of these genes is up-regulated during the early stages of heterocyst differentiation. For example, *hepB* is expressed between 5 and 8 h after nitrogen step-down (74). Synthesis of the glycolipid layer requires the expression of *hglB*, *hglC*, *hglD*, *hglE*, *hglK,* and *devBCA*, which are all up-regulated early during heterocyst differentiation (44). After deposition of the polysaccharide layer and glycolipid layer, the morphologically differentiated heterocysts become micro-oxic partly because these layers prevent oxygen diffusion into the heterocyst (46). In addition, heterocysts increase respiration in order to decrease oxygen levels (152). Two cytochrome c oxidase-type respiratory terminal oxidases (Cox2 and Cox3) have been shown to be essential for heterocyst function and for heterocyst respiration. The expression of *cox2* and *cox3* take place during the middle stage of heterocyst development, predominantly in heterocysts (152).

Complete maturation of heterocysts involves the expression of genes in the late stages of heterocyst development that are necessary for nitrogen fixation and nitrogen assimilation into amino acids. The *nifHDK* operon encodes nitrogenase polypeptides and is up-regulated around 18 h after nitrogen step-down in heterocysts (3, 59). Other late heterocyst-specific genes include *fdxH*, which encodes a [2Fe-2S] ferredoxin (108), and *hupL*, which encodes the large subunit of uptake hydrogenase (28, 30).

The identification of two sigma factors (*sigC* and *sigB*) in *Anabaena* PCC 7120 was archived by comparison of region similarity to the "rpoD" box sequence corresponding to the principal sigma factor in multiple organisms (13). Homologues of

sigma factor families share at least two of the four regions that are necessary for transcription initiation. Brahamsha and Haselkorn (11) found that *sigB* and *sigC* are highly conserved in the amino terminal 1b region of *sigA*, which is the primary sigma factor in *Anabaena* PCC 7120. *sigB* and *sigC* also are similar to the 2.4 and 4 regions, which are involved in recognition of the -10 and -35 sequences of promoters (13). The authors showed that *sigB* and *sigC* are expressed under nitrogen limiting conditions, specifically after 12 hours of nitrogen step-down. However, single and double mutants of *sigB* and *sigC* showed that they are not essential for heterocyst differentiation or diazotrophic growth. A similar result was obtained by Khudyakov I. and Golden J. (87), in which the insertional inactivation of *sigC*, *sigF*, *sigB2*, *sigD*, or *sigE*, can grow on nitrate or diazotrophically, but the *sigB2* and *sigD* mutants were slow to establish diazotrophic growth (89). A double mutant of *sigB2 sigD* can form proheterocysts but was unable to grow diazotrophically, possibly due to extensive fragmentation of filaments upon nitrogen deprivation (89).

Previous studies in *Anabaena* PCC 7120 used *gfp* transcriptional reporter fusions to analyze the spatial and temporal pattern of expression for all sigma factor genes located on the chromosome except *sigA* (4). These studies found that three sigma factor genes have heterocyst-specific expression after induction of heterocyst development: two group 2 sigma factors, *sigC* and *sigE*, and one representative of group 4, *sigG* (4). Reporter strains containing P_{sigC}-*gfp*, P_{sigE}-*gfp*, and P_{sigG}-*gfp* showed increased GFP fluorescence at least partially localized to differentiating cells and heterocysts. Expression of *sigC* starts at 4 h after nitrogen step-down suggesting that it may be

involved in the early stages of heterocyst development. The *sigG* gene is expressed in nitrogen containing media, and soon after nitrogen step-down *sigG* expression decreases in the vegetative-cell filaments. However, 10 h after nitrogen step-down some individual cells along the filaments show increased *sigG* expression, and at 24 h *sigG* expression is localized in heterocysts. These results suggest that SigG is involved in the expression of middle stage heterocyst genes as well as genes in vegetative cells grown on nitrate. The expression of *sigE* is localized in differentiating heterocysts around 16 h after nitrogen step-down, suggesting that SigE might be important for the regulation of genes that are expressed in the late stages of heterocyst development.

In this work, we further examined the expression of *sigE*, and determined if *sigE* is required for the expression of middle and late genes, such as the *nifHDK* operon or *fdxH, hupL, hepB,* and *hglE* during heterocyst differentiation. As we mentioned before, insertional inactivation of *sigC* and *sigE* did not change the capability of *Anabaena* PCC 7120 to grow under diazotrophic conditions or to establish a heterocyst pattern (89). However, *sigC* inactivation causes a 24 h delay in heterocyst differentiation, and a reduced expression of heterocyst specific genes *hepA* and *hepC* (Ramona Neunuebel, personal communication). We present evidence that *sigE* is required for the normal expression of the *nifH*, *fdxH*, and *hepB* genes, which are specific for heterocyst differentiation.

Material and methods

Strains and culture conditions

Strains and plasmids used in this study are listed in Table 4.1. *Anabaena* PCC 7120 and its derivatives were grown as previously described (59, 135). *Escherichia 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. Shuttle plasmids and suicide plasmids were transferred into *E. coli* donor strain AM1359 by electroporation and transferred to *Anabaena* PCC 7120 strains by conjugation using standard protocols (42) with some modifications (89).

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. Total DNA from *Anabaena* PCC 7120 was isolated as previously described (89). DNA sequencing of plasmid inserts was performed by the Gene Technologies Laboratory (Texas A&M University) following the Big Dye sequencing protocol (Applied Biosystems). The *sigE* over-expression plasmid was constructed by PCR amplification of *sigE* (alr4249) with primers containing an NdeI site at the 5' end and a XmaI site at the 3' end (AMO-2222 and AMO-2223) and then cloning the fragment into the same sites in pAM2770, which contains a copper inducible promoter (*petE*).

Site-directed mutagenesis of the NtcA binding site of the *sigE* promoter was conducted using standard protocols (86). Primers AMO-2217 and AMO-2218 were used to mutate the NtcA binding site of pAM3652 (P_{sigE}-*gfp)* (Table 4.2). Rapid amplification

of 5' cDNA ends (RACE) was performed using standard protocols and AMO-2266, AMO-2268, and AMO-2269 primers.

Electrophoretic mobility shift assay (EMSA)

A 6×His tagged NtcA protein was over-expressed from an IPTG-inducible plasmid pET28b+ and purified using a HisTrap HP 1 ml nickel column (GE Healthcare) using standard procedures. A DNA fragment upstream the *sigE* gene, containing a putative NtcA binding site, was then amplified by PCR using oligonucleotides RAM-Fw and RAM-Rv each containing an engineered EcoRI site at the 5' end. The PCR product

Table 4.1. Bacterial strains and plasmids for *sigE*

Strains	Relevant characteristic	Reference
Anabaena		
PCC 7120	Wild-type *Anabaena* sp. strain PCC 7120	(7)
AMC649	*Anabaena* PCC 7120 *sigE::luxAB*-Sp^r Sm^r cassette at ClaI site of *sigE*	(89)
AMC1452	*Anabaena* PCC 7120 carrying P_{sigE}-*gfp* on pAM3652; Nm^r	(4)
AMC1774	*Anabaena* PCC 7120 carrying pRL277-P_{nifHD}-*gfp*; Sm^r Sp^r	This study
AMC1773	*Anabaena* PCC 7120 carrying P_{petE}-*sigE* on pAM3961; Nm^r	This study
AMC1775	*Anabaena* PCC 7120 carrying pRL277-P_{nifHD}-*gfp* and P_{petE}-*sigE* on pAM3961; Sm^r Sp^r Nm^r	This study
AMC1776	*Anabaena* PCC 7120 carrying pRL278-P_{nifHD}-*gfp*; Nm^r	This study
AMC1777	AMC649 carrying pRL278-P_{nifHD}-*gfp*; Nm^r	This study
Plasmids		
pAM3929	*ntcA* was cloned into IPTG inducible promoter plasmid pET28a in EcoRI and XhoI sites	This study
pRL277	Conjugal suicide plasmid; Sm^r Sp^r	(42)
pRL278	Conjugal suicide plasmid; Nm^r	(42)
pAM2770	Shuttle vector containing XhoI-P_{petE}-NdeI-l*acZ*α-*Sap*I(Cys)-6His *(*stop)-ClaI; Km^r Nm^r	(99)
pAM3961	*sigE* (ORF alr4249) cloned into pAM2770 containing P_{petE} in NdeI and XmaI sites; Km^r Nm^r	This study
pAM3652	pAM1956 containing 800-bp fragment upstream of *sigE* (alr4249)	(4)

Table 4.2. DNA primers used for *sigE*

Gene	Forward Primer	Sequence (5'→3')	Reverse primer	Sequence (5'→3')
nifH probe	AMO-622	TTCACGGTCAACCTTACGG	AMO-1038	CGGTAAAGGCGG
ntcA	AMO-2026	CCGGAATTCATCGTG ACACAAGATAAG	AMO-2027	TAACCTCGAGTTAAGTGAACTGTCTGC
Site DirectedMutagenesis of P_{sigE}-*gfp*	AMO-2217	GTATTTTGAATGCAGATTTTATTC	AMO-2218	GAATAAAATCTGCATTCAAAATAC
nifH Real Time PCR	AMO-2185	GCACAAGAAATCTACATC	AMO-2186	TACGAAGTGAATCATTTG
hupL RT	AMO-2189	TGCTTCTCACTTAACCTCTG	AMO-2190	GTCAATGGCGAACAATCC
fdxH RT	AMO-2191	TACCAAGTTAGATTGATC	AMO-2192	AAGTAACACAAAGTAGAG
hepB RT	AMO-2193	GAGGTTTGGAAAGATATATTTATG	AMO-2194	TCTGGAGATGCTAAGTTAG
hglE RT	AMO-2197	TTGTTGAGGTGTATTGAG	AMO-2198	CAGGAACATCAGTGATAC
rpoA RT	AMO-2204	AGTTTGGACAAATGGTAG	AMO-2205	GCTTGAGACAGTTATAGG
ntcA Over Expression	AMO-2026	CCGGAATTCATCGTGACACAAGATAAG	AMO-2027	TAACCTCGAGTTAAGTGAACTGTCTGCTG
sigE probe	AMO-2222	CCTATTCCCGGGCTCAAATCAACCAACC	AMO-2223	AACAGTCATATGGAAATTATGTACCAAAC
Rapid amplification of 5' cDNA ends of *sigE*	AMO-2266	CAAGGAAACTATGAATATTGC	AMO-2267	ATTATTAAGCTTGTTCCCAATTCAGCAATATTCATAG
(dT)17 Adaptor RACE	AMO-2268	GACTCGAGTCGACATCGATTTTTTTTTTTTTTTT	AMO-2269	GACTCGAGTCGACATCG
Mobility Shift Assays	RAM-Fw	CTTGAATTCTAAAGATAAATATTCTTATTG CC	RAM-Rv	GTGGAATTCAAATATTTATTGCACGTAT

was digested with EcoRI and labeled with [α-32P]dATP (3000 Ci $mmol^{-1}$) by fill-in using the Klenow fragment. Binding assays were carried out in a final volume of 10 µl containing 10 mM Tris-HCl (pH 7.5), 50 mM KCl, 1 mM dithiothreitol, 5 ng bovine serum albumin μl^{-1}, 12.5% (v/v) glycerol, and 10 fmol of the DNA fragment; the reaction was supplemented with 2-oxoglutarate (0.6 mM) where indicated. The labeled probe was incubated with 1-4 pmol of purified 6×His-NtcA protein for 30 minutes at room temperature. DNA fragments were separated by electrophoresis on a non-denaturing 5% polyacrylamide gel, vacuum-dried, and visualized by phospho-imaging (Fuji Film, Tokyo, Japan).

RNA isolation

A RiboPure-Bacteria kit (Ambion) was used to isolate total RNA from *Anabaena* PCC 7120 and its derivates. Filaments from growing BG-11 cultures were inoculated into 250 ml flasks containing100 ml of $BG\text{-}11_0$ supplemented with 2.5 mM ammonium chloride and 5 mM MOPS (pH 8.0) and grown on an orbital shaker under standard conditions to obtain an OD_{750} of 0.025 - 0.035. One flask was grown for each time-point sample for each strain. The cultures were grown overnight to OD_{750} 0.05 - 0.075, and then the cells were collected by centrifugation in 50 ml conical tubes, washed twice with $BG\text{-}11_0$, and transferred to 100 ml of $BG\text{-}11_0$ in 250 ml flasks. The flasks were incubated as above for the appropriate time after nitrogen step-down. Then, each culture was poured into a conical 250 ml centrifuge tube containing 100 g of crushed ice and the

filaments were collected by centrifugation at 5,000 × g for 10 minutes at 4°C. The cell pellet was immediately frozen at -80°C until RNA isolation.

Northern RNA blot analysis

For each sample, ten μg of total RNA was separated on a 5% agarose denaturing formaldehyde gel in MOPS buffer, and then transferred by capillary action to a Magna charge nylon membrane (GE Water & Process Technologies) with 10× SSPE. DNA probes were prepared by random primer labeling and purified on Micro Bio-Spin P-30 columns (Bio-Rad). Blots were hybridized with radioactively labeled probes; fragments amplified by PCR using the corresponding primers (Table 4.2) were labeled with [α-32P]-dCTP by random primer labeling. Hybridizations and washes of the blots were conducted using standard protocols (141). Blots were exposed to a phosphorimager plate and scanned with a Phosphorimager BAS-5000 (Fujifilm).

Time-lapse microscopy

Time-lapse microscopy was used to record DIC, autofluorescence, and GFP-fluorescence images during heterocyst development. Filaments of each reporter strain to be studied were grown in nitrate-containing medium to an OD_{750} of 0.2-0.3, washed, and resuspended in a small volume of $BG\text{-}11_0$ for heterocyst induction. 10 µl of induced *Anabaena* PCC 7120 filaments were applied to a single-chambered coverglass (Lab-Tek chamber slide system; Nalge Nunc International) and covered with a thin agarose pad. The thin agarose pad was made by covering a single dialysis tube (1.5 cm by 2.5 cm

VWR Scientific, MW cut off = 3,500 kDa) that had been immersed in BG-11_0 with 300 µl of 0.7% low melting point agarose in filtered BG-11_0. After the agarose cooled and solidified, small sections of the agarose pad were sliced (around 0.4 to 0.4 mm square), and used to cover the induced filaments in the single-chambered coverglass. The excess media was removed and the agarose pad was then carefully surrounded on all four sides by a total of about 2 ml of BG-11_0 in 0.7% low melting agarose in order to maintain moisture in the thin agarose pad. Induced *Anabaena* PCC 7120 filaments in the chambered coverglass were placed in a DeltaVision Core system (Applied Precision) with a WeatherStation attached to an Olympus IX71 inverted microscope. The temperature was adjusted to 30ºC. SoftWoRx software was used to acquire time-lapse images for 24 to 42 h with a 15-minute time delay using a 40× objective. The induced *Anabaena* PCC 7120 filaments were illuminated with approximately 75 μmol photons m^{-2} s^{-2} by an LED white light source (Schott IFC 60825) attached to the DeltaVision Core system and automatically controlled by softWoRx software, which controlled switching between the external white light and the fluorescence excitation light for taking images. For all images, a polychroic beam splitter was used. DIC exposure time was 0.05 seconds, and for autofluorescence images, a TRITC filter set was used (EX555/EM617) with an exposure time of 0.05 seconds. For GFP images, a Chroma GFP filter set was used (HQ EX470/EM515) with a 0.5 second exposure time. Time-lapse images were processed using softWoRx software, and individual images from specific time-points were exported as required.

Scoring of heterocyst patterns along the filaments was performed as previously described (175). 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 the BioBike web interface (41).

Quantitative Real-Time RT–PCR Analysis (qRT-PCR)

qRT-PCR analysis, was carried out with a StepOnePlus 96 well qRT-PCR system (Applied Biosystems) and *Power* SYBR Green PCR Master Mix (Applied Biosystems) mixed with 100 pmol/µl of each primer and 50 ng of total cDNA. All primers used in this study are listed in Table 4.2. Primers for *nifH*, *fdxH*, *hupL*, *hepB*, and *rpoA* were designed with Vector NTI software (Invitrogen) and each primer was analyzed using the BioBike web interface for sequence-similarity searches against the *Anabaena* PCC 7120 genome. cDNA synthesis from total RNA samples (grown on nitrate or 12, 24, and 36 h after nitrogen step-down) was performed with an iScript cDNA kit (Bio-Rad). All samples were run in triplicate using the following amplification conditions: one cycle of 2 minutes at 52°C and 10 minutes at 95°C, and then 40 cycles at 95°C for 15 seconds and 60°C for 30 seconds. CT values for each gene were averaged and normalized against *rpoA* as an internal control.

Results

NtcA interacts with the promoter region of *sigE in vitro*

It has been shown that some heterocyst-specific promoters carry an NtcA-binding site with a consensus sequence of $TGTAN_8TACA$, which is located 22 nucleotides upstream from the -10 TAN_3T box (39, 70). Bioinformatics analysis of the *sigE* promoter region identified a putative canonical NtcA binding site twenty-two nucleotides upstream of a TAN_3T sequence, from position -683 to position -698 relative to the *sigE* translational start site (Figure 4.1). To determine if this site interacted with NtcA, we performed electrophoretic mobility shift assays (EMSA) using a radioactively labeled DNA PCR fragment containing the putative NtcA binding site, purified 6×His-tagged NtcA protein, and the effector 2-oxoglutarate (2-OG). Figure 4.2 shows a band-shift of the labeled DNA fragment in the presence of increasing amounts of NtcA, indicating NtcA binding. The affinity of NtcA for this promoter fragment increases in a dose dependent manner in the presence of 2-OG. This result suggests that *sigE* is developmentally regulated by NtcA and its effector 2-OG.

Site directed mutagenesis of the NtcA binding site in plasmid pAM3652, which carries P_{sigE}-*gfp* was performed. The NtcA binding site (5'-$TGTAN_8TAC$-3') in the *sigE* promoter region was substituted with 5'-GTGC N_8TAC-3', and the plasmid was introduced into wild-type *Anabaena* PCC 7120. The resulting strain, AMC1778, was analyzed by time-lapse microscopy after nitrogen step-down (data not shown). No differences in GFP fluorescence levels were detected between P_{sigE}-*gfp* wild type and the mutated P_{sigE}-*gfp*. This result suggests that the NtcA binding site is not required for the

expression of *sigE in vivo*, and it is possible that the NtcA binding site may be required for expression of the all4848 gene.

Northern blot analysis of *sigE* showed that is developmentally regulated and that transcript levels were highest at 12 and 24 h after nitrogen step-down. The *sigE* transcripts were present as a smear, suggesting a normal short half life, with an upper edge around 3 kb. At 30 and 48 h, *sigE* transcript levels were decreased compared with 12 and 24 h after nitrogen step-down (Figure 4.3). We cannot explain the results obtained for the 36 h time point sample; although the RNA sample showed slightly more degradation, the hybridization showed fewer small fragments and two large prominent bands. Attempts to map the *sigE* transcription start site using rapid amplification of 5' cDNA ends (RACE) were unsuccessful, presumably because the transcripts were partially degraded.

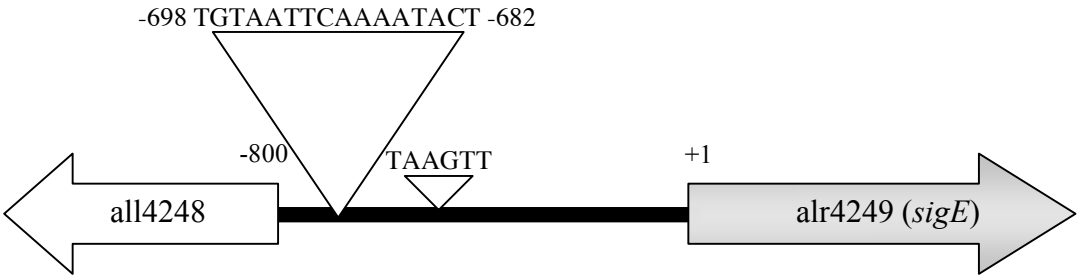

Figure 4.1. Map of the *sigE* region of *Anabaena* PCC 7120. The *sigE* gene (alr4249) is shown in light gray. The NtcA binding site is located upstream from position -682 to position -698 relative to the *sigE* translational start site (5'→3'). A putative -10 box is located 22 nucleotides down stream from the NtcA binding site.

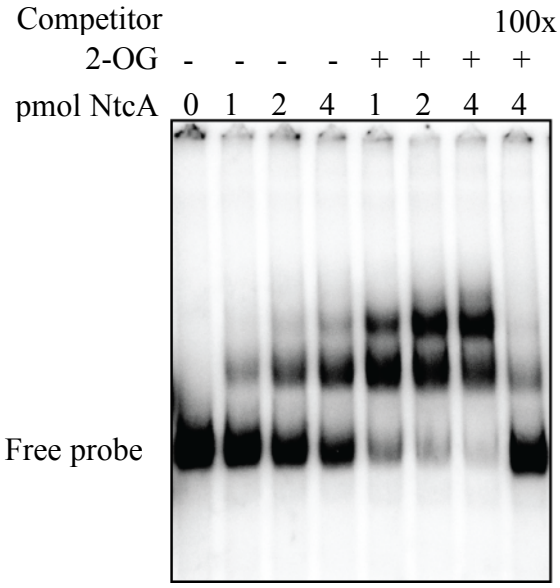

Figure 4.2. Electrophoretic mobility shift assay. Binding of purified 6×His-NtcA to the wild-type *sigE* promoter assayed by electrophoretic mobility shift assay (EMSA). The effect of 2-oxoglutarate (2-OG) on binding of NtcA to DNA fragments containing the *sigE* promoter region was assayed for reaction mixtures containing 0.1 fmol of labeled DNA fragment. The last lane contains a 100x excess of competitor (unlabeled probe).

nifH expression is delayed and reduced in the *sigE* mutant strain

sigE is expressed during the late stages of heterocyst development and the expression is localized to heterocysts. Therefore, it is possible that SigE participates in the expression of late heterocyst-specific genes (4). To determine if SigE is required for expression of genes in the late stages of heterocyst development, we analyzed the expression of the *nifH* gene, which encodes the nitrogenase iron protein. Total RNA from the wild type and the *sigE* mutant strain AMC649 was isolated at different time points after nitrogen step-down and then analyzed by northern blot. Figure 4.4 shows the expression of *nifH* from the wild type and *sigE* mutant when grown in media containing nitrate, and at 12, 24, 30, 36, and 48 h after nitrogen step-down. Transcripts from *nifH* in the wild-type background were detected at low levels at 12 h after nitrogen step-down. In the *sigE* mutant strain AMC649, *nifH* transcripts were detected only by 24 h after nitrogen step-down. The *sigE* mutant strain showed an increase of *nifH* transcript levels at 30 h after nitrogen step-down, but at lower levels compared to the wild-type strain. At 36 h after nitrogen step-down, *nifH* transcript levels for the *sigE* mutant were similar to that of the wild-type strain, but by 48 h after nitrogen step-down the *nifH* transcripts were significantly decreased compared with the wild type. These results show that expression of *nifH* is delayed and reduced in the *sigE* mutant, and suggest that SigE is required for normal full expression of *nifH*.

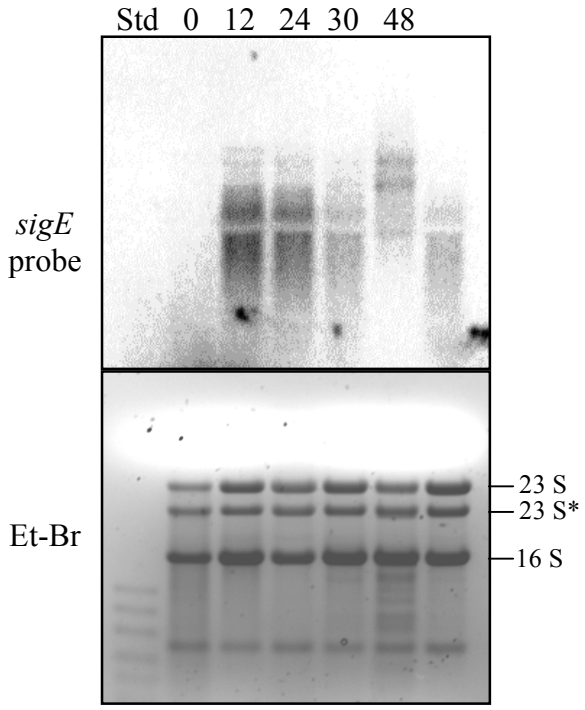

Figure 4.3 Northern RNA blot analysis of *Anabaena* PCC 7120 during heterocyst development. Lanes are labeled with hours post nitrogen step-down. The upper panel shows a northern RNA blot probed with a strand-specific radioactive probe for the *sigE* transcripts. The lower panel is the ethidium bromide stained gel to show sample loading.

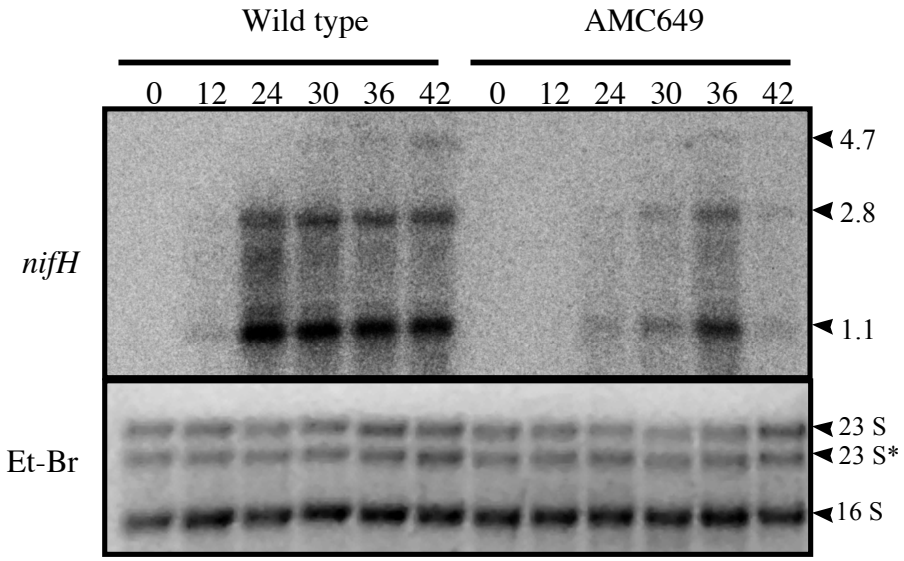

Figure 4.4. Northern blot analysis of *nifH* transcripts from wild-type *Anabaena* PCC 7120 and the *sigE* mutant strain AMC649 during heterocyst development. Lanes are labeled with hours post nitrogen step-down. The lower panel is an ethidium bromide stained gel to show sample loading. The upper panel shows Northern blots probed with a strand-specific radioactive probe for the *nifH* transcript. Three transcripts for *nifH* were detected at 4.7 kb, 2.8 kb, and 1.1 kb. The *sigE* mutant strain AMC649 had delayed and reduced levels of *nifH*

SigE regulates the expression of P_{nifHD}-*gfp* reporter during heterocyst development

Time-lapse microscopy was used to determine if SigE affects a P_{nifHD}-*gfp* reporter florescence signal and/or the heterocyst pattern *in vivo*. Strains containing a P_{nifHD}-*gfp* reporter integrated into the *Anabaena* PCC 7120 chromosome at the *nifH* locus were used for these studies; the P_{nifHD}-*gfp* reporter construct was constructed and tested by Krithika Kumar (personal communication). Three strains that contain the P_{nifHD}-*gfp* reporter were induced to undergo heterocyst development: the wild type, a *sigE* knockout mutant, and a *sigE* overexpression strain. For heterocyst induction, nitrate-grown filaments were washed and transferred to BG-11_0 medium without a source of combined nitrogen. Images were acquired every 15 minutes for 24 h for strains AMC1774 and AMC1775, and for 42 h for strain AMC1777. Figure 4.5 contains images corresponding to the indicated time-points that were extracted from the time-lapse series. The images on the left show strain AMC1774, which contains the pRL277-P_{nifHD}-*gfp* reporter in the chromosome in a wild-type background. The images in the center show strain AMC1775, which contains the pRL277-P_{nifHD}-*gfp* reporter in the chromosome and a shuttle vector that contains an extra copy of *sigE* expressed from the copper-inducible *petE* promoter. The images on the right show strain AMC1777, which is the *sigE* knockout mutant strain AMC649 engineered to also contain the pRL278-P_{nifHD}-*gfp* reporter in the chromosome.

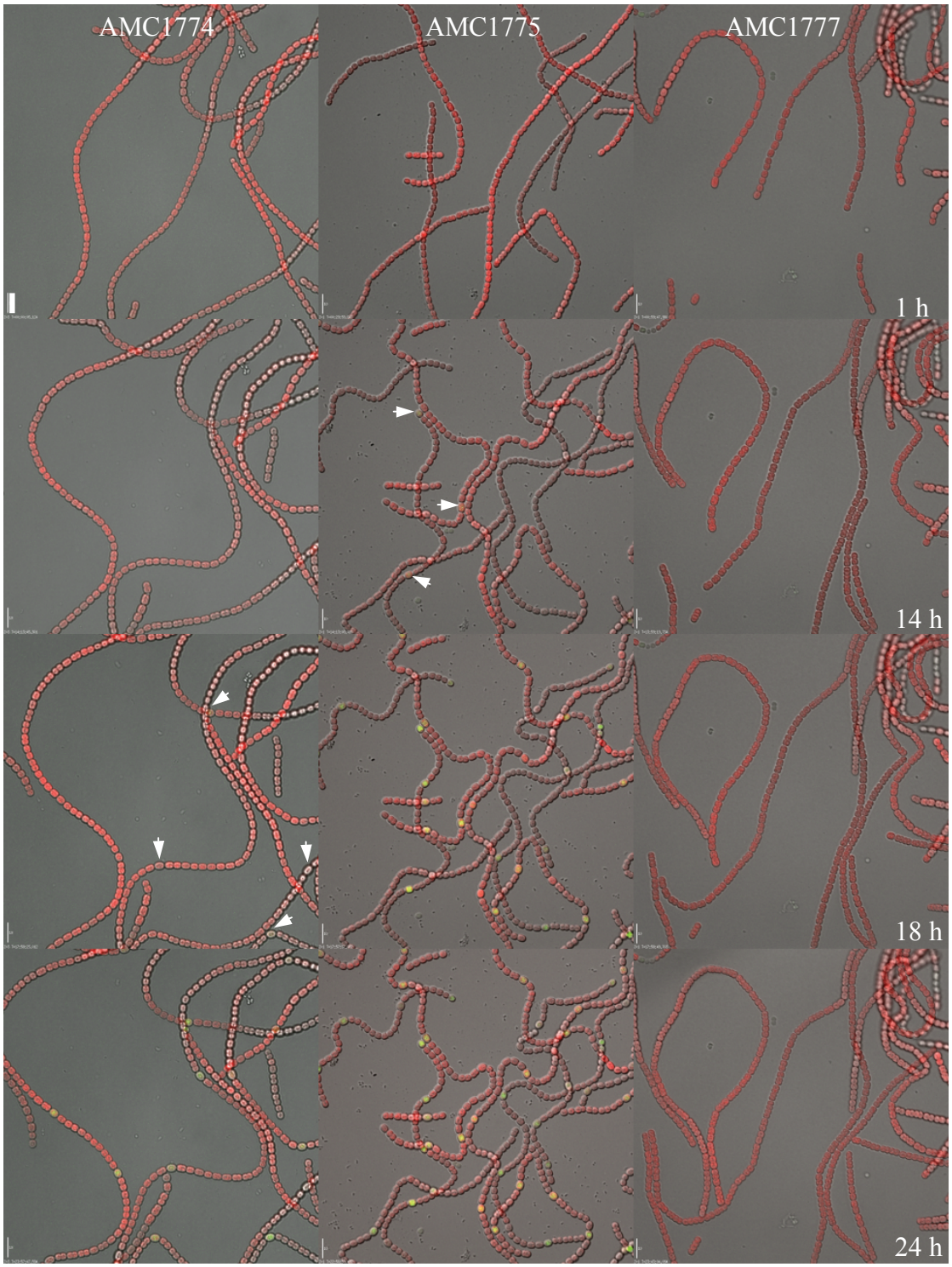

Figure 4.5. Time-lapse microscopy. *nifHD-gfp* reporter construct integrated into the *nifH* locus on the chromosome in a wild-type background (AMC1774), a *sigE* over-expression background (AMC1775), and a *sigE* mutant background (AMC1777). Images are merged DIC (grayscale), autofluorescence (red), and GFP reporter fluorescence (green). Arrows indicate weak GFP fluorescence from proheterocysts. Scale bar, 10 µm.

At 1 h after nitrogen step-down, no GFP fluorescence or heterocyst morphological characteristics were evident in any of the three strains (AMC1774, AMC1775, and AMC1777). By 14 h, when morphological differentiation of proheterocysts was not yet obvious in the wild type, the *sigE* over-expression strain (AMC1775) showed GFP fluorescence in individual cells that were morphologically distinguishable as proheterocysts. AMC1774 and AMC1777 filaments did not show any GFP fluorescence or proheterocysts after 14 h of heterocyst induction.

At 18 h after nitrogen step-down, the wild type strain (AMC1774) showed GFP fluorescence signal in some individual cells, which correlates with the expression of *nifH* shown by northern blot experiments (Figure 4.4). At this time, slight morphological changes in the cells that showed GFP fluorescence were observed. At 18 h after nitrogen step-down the percentage of heterocyst frequency in the wild-type strain was 26.6 ± 4.6 (Figure 4.6). The *sigE* over-expression strain showed strong GFP fluorescence at 18 h after heterocyst induction and showed clear heterocyst morphological differentiation with a heterocyst frequency percentage of 12.4 ± 4.7 (Figure 4.6). In the *sigE* mutant strain (AMC1777), no GFP fluorescence or morphological differentiation were detected after 18 h of heterocyst induction.

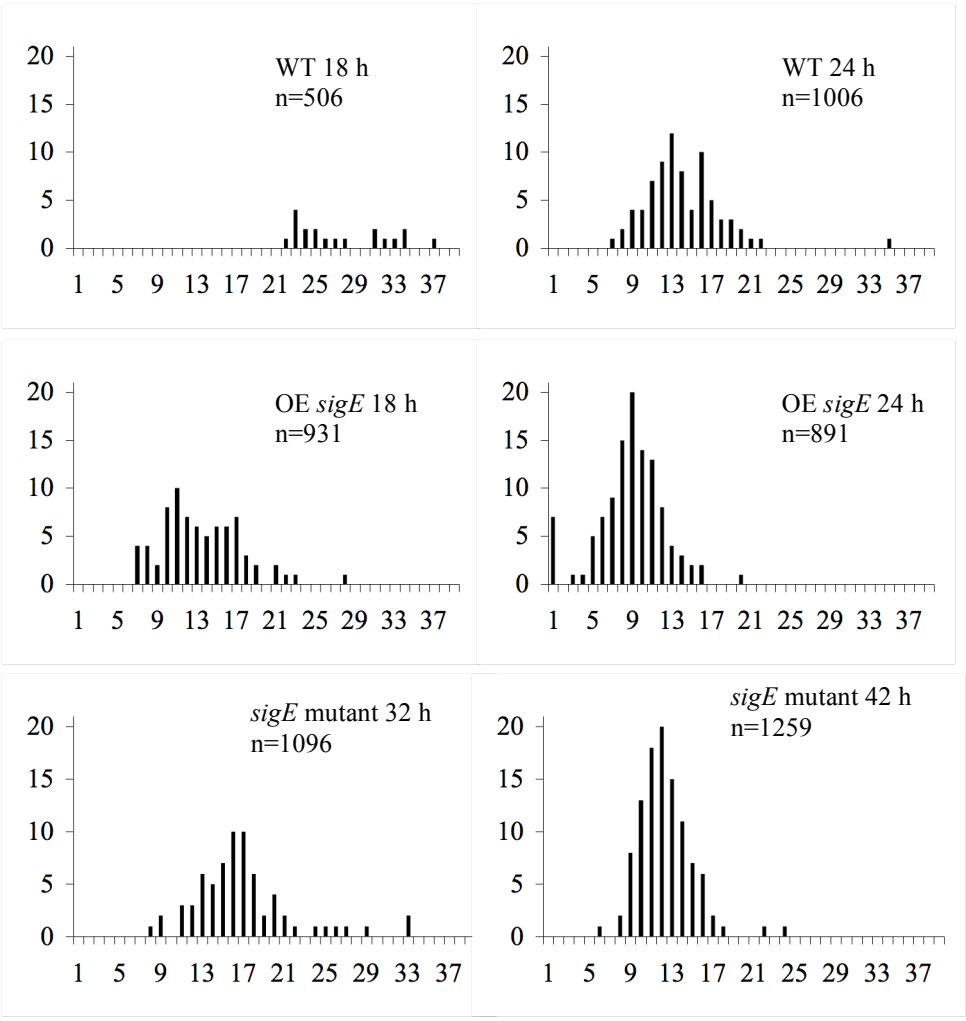

Figure 4.6. Heterocyst pattern of a *nifHD-gfp* reporter. P_{nifHD}-*gfp* construct was integrated into the *nifH* locus on the chromosome in a wild-type (WT) background (AMC1774) and in a *sigE* over-expression (OE) background (AMC1775) after nitrogen step-down for 18 and 24 h. For the *sigE* mutant strain, heterocyst pattern was determined by counting mature heterocyst after nitrogen step-down for 32 and 42 h. The number of vegetative cells between heterocysts was determined by microscopy at 18 h, 24 h, 32 h and 40 h after heterocyst induction. The average number of vegetative cells between heterocysts after nitrogen stepdown for wild type at 18 h was 26.6 ± 4.6 and at 24 h was 13.1 ± 4.0; for over-expression of *sigE* at 18 h was 12.4 ± 4.7, and at 24 h was 7.9 ± 3.2; and for *sigE* mutant at 32 h was 15.8 ± 4.891578, and at 42 h was 11.3 ± 2.6. The x-axis represents the percentage of intervals and in the y-axis the number of vegetative cells between heterocysts.

At 24 h after nitrogen step-down, the P_{nifHD}-*gfp* reporter in a wild type background (AMC1774) showed GFP fluorescence in individual differentiated cells (Figure 4.5) with a heterocyst frequency percentage of 13.1 ± 4.0 (Figure 4.6). The *sigE* over-expression strain (AMC1775) showed a higher frequency percentage of GFP-fluorescent heterocysts of 7.9 ± 3.2. These data show that extra copies of *sigE* lead to premature expression of *nifH* and heterocyst formation, and an increase in heterocyst frequency percentage. At 24 h after nitrogen step-down, the *sigE* knockout mutant strain (AMC1777) did not show any GFP fluorescence signal or heterocyst morphological changes. By 32 h after nitrogen step-down, heterocyst morphological changes in individual cells were observed in AMC1777 with a heterocyst frequency percentage of 15.8 ± 4.8; no GFP fluorescence was evident. At 42 h, AMC1777 had a pattern of mature heterocysts similar to the wild type with a frequency percentage of 11.3 ± 2.6, but no GFP fluorescence signal was evident.

SigE regulates the expression of heterocyst-specific genes

Previous observations in the *sigE* mutant strain (AMC649) found that *sigE* had little effect on the ability of the mutant strain to establish diazotrophic growth under standard laboratory conditions (89). However, our northern RNA blot hybridization experiments showed that in the wild-type strain, the *sigE* gene was expressed at 12 h after nitrogen step-down (Figure 4.3), which support an earlier study that showed GFP fluorescence produced by a P_{sigE}-*gfp* reporter increased at 16 h after heterocyst induction in differentiating cells. These results suggest that the SigE sigma factor may be required

for the transcription of genes that are expressed in the late stages of heterocyst differentiation. The overall delay in heterocyst development that was observed in the *sigE* mutant strain, as well as the delay in *nifH* expression, supports this hypothesis. To determine if SigE is required for the expression of heterocyst-specific genes, we used qRT-PCR to examine the expression levels of genes that have been described to be up-regulated during middle and late stages of heterocyst development. We selected *nifH*, *fdxH* (encoding a heterocyst-specific ferredoxin) and *hupL* (encoding the large subunit of uptake hydrogenase) as genes that are expressed during late stages of heterocyst development (28, 153). For genes expressed during the middle stage of heterocyst development, we selected *hglE* (encoding a typeI polyketide synthase involved in heterocyst glycolipid synthesis) and *hepB* (encoding a glycosyl transferase involved in heterocyst envelope polysaccharide synthesis) (24, 82, 159). The expression of *rpoA*, which encodes the RNA polymerase alpha subunit, was analyzed by qRT-PCR during heterocyst differentiation, and the values remained invariable and at relatively low levels (Figure 4.7); therefore, we used *rpoA* as an internal control for our qRT-PCR experiments.

Figure 4.7 shows the expression levels of *nifH*, *fdxH*, *hupL, hglE, and hepB* relative to *rpoA* in the wild-type background as well as in the *sigE* mutant strain during heterocyst induction. In media containing nitrogen (0 h), *nifH* transcripts were not detected in either the wild type or the *sigE* mutant. After 12 h of nitrogen step-down, small amounts of *nifH* transcript were detected, but only in the wild-type strain. At 24 h a peak of *nifH* transcript was observed in the wild-type strain, while in the *sigE* mutant

strain, only small amounts of *nifH* transcript were detected. By 36h, the levels of *nifH* transcript in the *sigE* mutant strain increased to about half of the wild-type transcript level. This result is similar to the observations obtained by northern blot anyalysis and with the P_{nifHD}-*gfp* reporter, which supports the hypothesis that SigE regulates the expression of *nifH*.

At 24 h after nitrogen step-down, the expression of the *fdxH* gene was detected only in wild-type filaments (Figure 4.7). By 36 h, *fdxH* expression increased in wild-type filaments but was detectable at only low levels in the *sigE* mutant strain, which resemble the same expression behave that *nifH* gene. These data suggest that SigE regulates the expression of *fdxH* during heterocyst development.

The expression levels of *hupL* and *hglE* were not significantly affected in the *sigE* mutant strain compared to the wild type control. Expression of *hupL* has been shown to occur during the late stages of heterocyst development (28). Our data showed a very low level of expression of *hupL* in nitrate-containing medium, increased expression at 12 and 24 h, and the highest level of expression at 36 h after nitrogen step-down.

Transcript levels of *hglE* were highest at 12 h and 36 h after heterocyst induction with a slight decrease at 24 h. At 36 h *hglE* transcript levels in the *sigE* mutant were half the wild-type levels (Figure 4.7).

The *hepB* gene showed expression in nitrate-containing medium and upregulation at 12 and 36 h after nitrogen step-down. In the *sigE* mutant strain the expression of *hepB* was significantly reduced at both 0 and 12 h, but was not reduced at 24 and 36 h. This result was unexpected because *sigE* is expressed at a low level in nitrate-containing medium and at early times after nitrogen step-down, and suggests that SigE may regulate gene expression at early and middle stages of heterocyst development. At 24 and 36 h after nitrogen step-down, *hepB* expression levels in the wild-type and *sigE* mutant strains were similar, which contrasts with the results for *nifH*, *fdxH*, and *hglE*. This shows that SigE may be involved with the regulation of genes at all stages of heterocyst development and that other factors must also be involved to produce different patterns of expression for different genes.

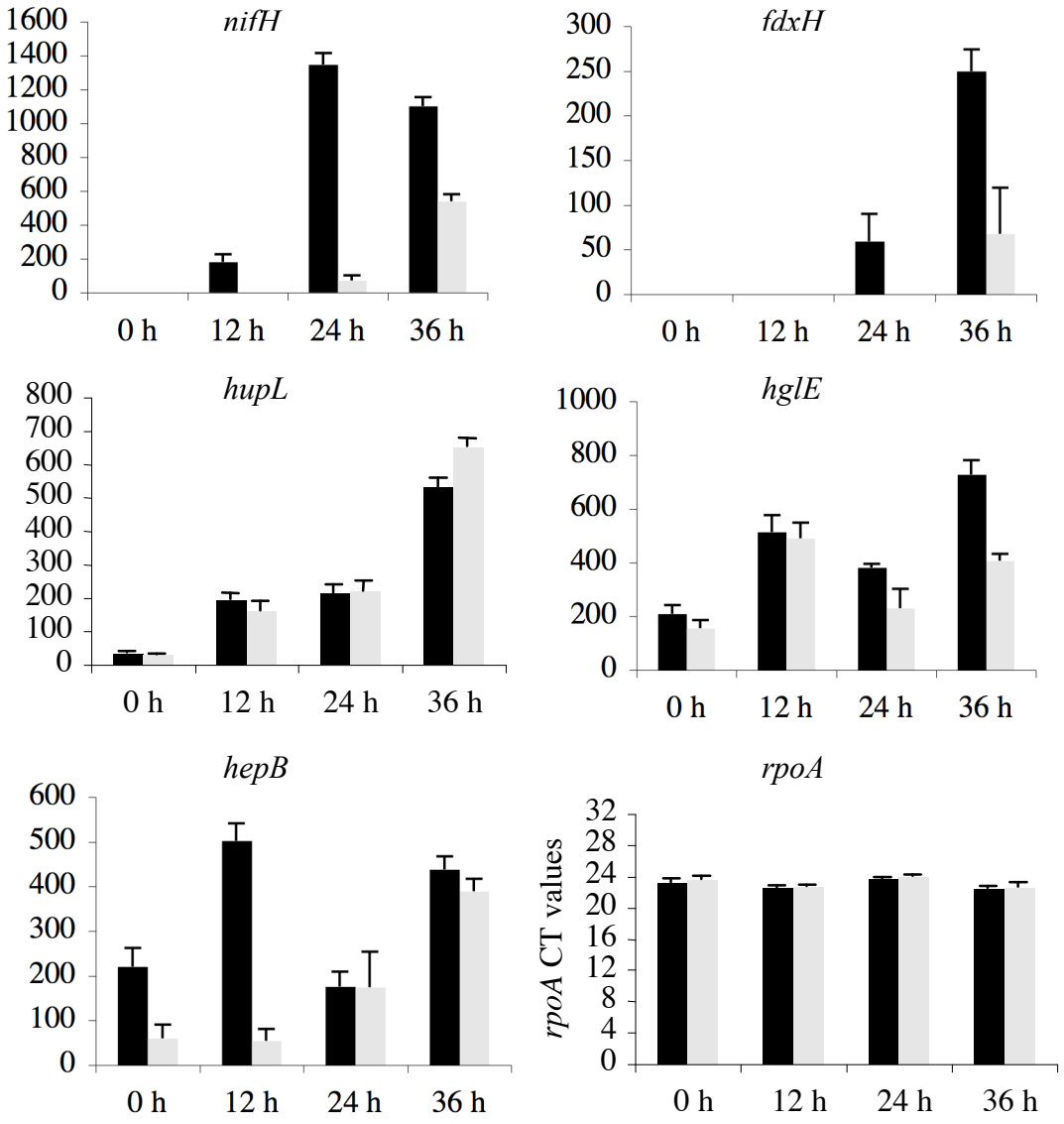

Figure 4.7. Quantitative reverse transcription PCR (qRT-PCR) measurements. Expression levels of *nifH, fdxH, hupL, hglE, and hepB* were determined using total RNA from wild-type *Anabaena* PCC 7120 (black bars) and the *sigE* mutant AMC649 (grey bars) at four time points after nitrogen stepdown. The data represent average values and standard error measurements from three technical replicates, normalized against the RNA polymerase alpha subunit (*rpoA*) gene. Average of the CT values for *rpoA* were determined and plotted for wild-type *Anabaena* PCC 7120 and the *sigE* mutant AMC649 at four time points.

Discussion

Transcriptional regulation in bacteria is dependent on sigma factors, which direct the initiation of transcription at specific promoters. Sigma factors associate with the RNA polymerase core enzyme and bind to specific sequences in the promoter region to start transcription. In *Anabaena* PCC 7120, there are twelve sigma factor genes in the genome. Previous studies have found that none of the group 2 sigma factors *sigB*, *sigB2*, *sigC*, *sigD*, or *sigE* are individually required for heterocyst development (13, 89). These results suggest that there might be functional overlap between the sigma factors in *Anabaena* PCC 7120, similar to other cyanobacteria (4, 89, 102, 176).

In *Anabaena* PCC 7120, the *sigE* gene, which belongs to the σ^{70} family, is up-regulated in heterocysts after nitrogen step-down (4). Our bioinformatic analysis of the *sigE* upstream region identified a putative NtcA binding site, which could explain the developmental regulation of *sigE*. However, the NtcA binding site is much closer to the upstream ORF all4248, which encodes the primosomal protein N', which is involved in DNA replication. Our studies using EMSA showed that NtcA binds to a DNA fragment containing this site, and that this binding is enhanced by the presence of 2-OG. However, site directed mutagenesis of the NtcA binding site on a P_{sigE}-*gfp* plasmid showed normal developmental expression *in vivo*. It could be possible that the expression of *sigE* is regulated by multiple transcription factors in addition to NtcA, such as NrrA or HetR, which have been shown to be heterocyst-specific transcriptional regulators (39, 115). Expression analysis of *sigE* by northern blot showed developmental up-regulation of transcripts beginning 12 h after nitrogen step-down, which is consistent with our

previous observations using P_{sigE}-*gfp* (4). However, the *sigE* transcripts were degraded, presumably because of a short half-life, and it was not possible to estimate the size of the full-length message. Attempts to identify the *sigE* transcriptional start site were performed by RACE analysis without success.

Because *sigE* is expressed during the late stages of heterocyst development, we hypothesized that *sigE* may be involved in the expression of the nitrogen-fixation (*nif*) genes. The regulation of the *nifHDK* operon has been studied for several years in *Anabaena* PCC 7120 (43, 59, 131, 133, 151, 154), however little is known about the transcription components that directly regulate the *nifHDK* operon. We examined the expression of the *nifH* gene in a *sigE* mutant strain using northern blot analysis, qRT-PCR, and a *gfp* reporter. Our experiments showed that the expression of *nifH* in the *sigE* mutant is reduced and delayed during heterocyst development, which, along with the fact that *sigE* is expressed late during heterocyst differentiation, indicates that *sigE* directly or indirectly regulates *nifH* expression at least partially. Establishing direct regulation of *nifH* expression by SigE will require *in vitro* transcription assays using purified components. In addition to the delayed expression of *nifH*, the *sigE* mutant showed delayed heterocyst morphological differentiation, which indicates that the SigE regulon includes additional genes required for normal heterocyst differentiation. In the *sigE* mutant, mature heterocysts became visible 32 h after nitrogen step-down (about 14 h longer than the wild-type strain), but by about 42 h, the heterocyst frequency was similar to the wild type. Time-lapse microscopy using a P_{nifHD}-*gfp* reporter in the *sigE* mutant background did not show any GFP signal even in mature heterocysts at 42 h.

However, the sensitivity for detecting GFP fluorescence in the time-lapse experiments is lower than for regular fluorescence microscopy, in which bleaching and damage to cells produced by higher excitation intensities is not a problem. Therefore, low levels of P_{nifHD}-*gfp* expression would not have been detected in these experiments.

Overexpression of *sigE* caused premature expression of P_{nifHD}-*gfp* and a high heterocyst frequency. The expression of the P_{nifHD}-*gfp* reporter in a wild-type background was visible at 18 h after nitrogen step down, which is about the time that *nifH* transcripts can been visualized by northern blot analysis (153, 162). The expression of P_{nifHD}-*gfp* in the *sigE* overexpression strain is first detectable 14 h after heterocyst induction (four hours earlier than the wild-type strain), which is consistent with our other data and suggests that *sigE* regulates the expression *nifH*. In addition, heterocyst morphogenesis and maturation occurred earlier and the final heterocyst frequency was higher in the *sigE* overexpression strain. At this time we do not know if *sigE* interacts directly with the promoter of the *nifHDK* operon to initiate transcription, but it could be possible that the earlier expression of P_{nifHD}-*gfp* is due to an indirect effect of *sigE*. SigE could regulate the expression of a set of genes necessary for heterocyst maturation, which then indirectly affect the expression of the *nif* genes. As mentioned earlier, *in vitro* transcription assays would be necessary to elucidate this question.

Because *sigE* influenced the expression of *nifH*, we hypothesized that *sigE* might also regulate the expression of other late stage heterocyst-specific genes such as *fdxH* and *hupL* (28, 153). Real-time quantitative RT-PCR revealed that the expression levels of *nifH* and *fdxH* were both reduced in the *sigE* mutant strain when compared with the

wild-type strain. In our experiments, expression of *fdxH* in the wild-type strain started to accumulate around 24 h after heterocyst induction and was strongly induced by 36 h after nitrogen step-down. In the *sigE* mutant strain, *fdxH* expression was not detectable at 24 h after nitrogen step-down, and at 36 h the transcriptional levels were about a third of the wild type. On the other hand, the expression of *hupL* was up-regulated normally after nitrogen step-down and was not affected by the inactivation of *sigE*. These results show that *sigE* regulates the expression of a subset of late stage heterocyst-specific genes, but not all late stage genes. These results suggest that at least some factors required for the activation of *hupL* expression are different from those that activate *nifH* and *fdxH* expression.

The *hepB* and *hglE* genes are necessary for the heterocyst polysaccharide layer and for the heterocyst glycolipid layer respectively, and they are developmentally up-regulated during the middle stage of heterocyst differentiation (2, 44, 159). Our qRT-PCR data showed that in the wild-type strain both genes are expressed at low levels in nitrogen-containing media and their expression is up-regulated 12 h after nitrogen step-down. In the *sigE* mutant strain the *hglE* expression levels at 12 h were similar to the wild-type strain, however 36 h after nitrogen step-down the transcript levels of *hglE* were half that of wild type levels, suggesting that SigE regulates the expression of genes in the middle to late stage of heterocyst development. Consistent with this idea, the transcript levels of *hepB* in the *sigE* mutant are significantly lower than the wild-type transcription levels at 12 h after nitrogen step-down, but the transcription is not affected at late stages of heterocyst differentiation. This result implies that SigE activates the

early up-regulation of *hepB* transcription after nitrogen step-down, but is not required for the expression at the later stage of heterocyst differentiation.

The results presented in this work indicate that SigE controls the expression of multiple heterocyst-specific genes that are regulated at middle and late stages during heterocyst differentiation.

CHAPTER V

CONCLUSIONS

Summary

This chapter will integrate and expand the discussion from previous chapters and will address the following topics: (1) The relationship between *conR* and septum formation in *Anabaena* PCC 7120. (2) How *conR* influences heterocyst morphogenesis. (3) Cell to cell transport in a *conR* mutant strain. (4) Expression of sigma factors during heterocyst differentiation. (5) The *sigC* and *sigG* regulons. (6) Transcription regulation of the *nifH* gene by *sigE*. (7) Expression analysis of heterocyst-specific genes in a *sigE* mutant strain.

Relationship between **conR** *and septum formation in* **Anabaena** *PCC 7120*

The *conR* gene identified in *Anabaena* PCC 7120 is predicted to be part of a family of proteins that contain the LytR-CpsA-Psr domain. Members of the LytR-CpsA-Psr family are relevant for beta-lactam resistance, biofilm formation, and stress tolerance, and are suggested to play a role in septum formation and cell wall maintenance (76, 77, 84). However, the biochemical function of the LytR-CpsA-Psr domain is still unknown. In Chapter II, we investigated the effect of inactivation of the *conR* gene on heterocyst formation in *Anabaena* PCC 7120. The *conR* mutant strain showed multiple phenotypes: (1) The *conR* mutant strain showed defective septum formation in vegetative cells grown with nitrate or grown diazotrophically. (2) The *conR*

mutant strain could not grow in media lacking fixed nitrogen and showed defective heterocyst morphogenesis. (3) Cell to cell transport between heterocysts and vegetative cells is likely to be disrupted in the *conR* mutant strain because heterocysts could fix nitrogen but could not support diazotrophic growth.

Like most prokaryotes, *Anabaena* PCC 7120 reproduces by binary fission, which is a relatively simple type of cell division. The cell elongates and the chromosomes replicate, separate, and finally a septum is formed mid-cell separating the two daughter cells. This process is divided into several steps: (1) selection of the site where the septum will be formed; (2) assembly of the tubulin-like protein FtsZ in the center of the cell; (3) recruitment of plasma membrane and cell wall components to the Z-ring; (4) assembly of the machinery for cell wall synthesis; and (5) constriction of the Z-ring and septum formation. Numerous studies have shown that in each of these steps of cell division many proteins play a specific role in a dynamic way (138). Homologues of cyanobacterial cell division genes have been identified in Gram-negative and Gram-positive bacteria by comparative analysis and mutational studies (111). In *Anabaena* PCC 7120, *ftsZ* polymerizes into a ring structure at the mid-cell position and presumably triggers the assembly of septum proteins, which drives the process of cell constriction (140). Inhibition of *ftsZ* polymerization produces elongated vegetative cells, poor septum formation, and suppresses heterocyst differentiation (140). At this time, we do not know the biochemical function of ConR. However, because ConR has a predicted membrane spanning segment and a periplasmic domain, and the inactivation of *conR* produces

defects in septum formation, we hypothesize that ConR may be involved in the assembly of septum proteins stimulated by the Z ring.

Recent studies in *Streptococcus pneumoniae* have shown that the disruption of the *lytR* gene, which encodes a LytR-CpsA-Psr domain protein, results in nonsymmetrical septum formation and cells with several septa distributed at multiple sites within the same cell (84). This is similar to our results with our conR mutant results in that the cells can form septa, but the septa are partially defective. It is likely that ConR in *Anabaena* PCC 7120 interacts with cell division machinery and the inactivation of *conR* affects the assembly of the machinery for cell wall synthesis and/or the localization of proteins involved in constriction. Alternatively, *conR* could affect the enzymatic activity or localization of the cell wall maintenance machinery such that inactivation of *conR* could cause a defect in peptidoglycan synthesis or deposition, which would result in a defective septum formation. Localization studies using *ftsZ-gfp* in the *conR* mutant strain, as well as localization of *conR-gfp,* would help elucidate the function of ConR in *Anabaena* PCC 7120.

Involvement of* conR *in heterocyst-specific morphogenetic changes

Inactivation of the *conR* gene caused abnormal morphology of heterocysts (Chapter II). Microarray experiments showed that *conR* is developmentally regulated in *Anabaena* PCC 7120 after nitrogen step-down, and a reporter strain carrying a *gfp* fusion with the promoter region of *conR* showed that up-regulation of *conR* takes place specifically in differentiating heterocysts. In the *conR* mutant, the heterocyst glycolipid

and polysaccharide layers are similar to a wild-type control. However, the size of the heterocysts was larger, which may be do to swelling that results from defects in the peptidoglycan cell wall.

The *conR* mutant strain frequently exhibited incomplete septal constriction at the heterocyst-to-vegetative cell boundary, which resulted in one or both ends of the heterocyst appearing more open and potentially more permeable to oxygen. However, the mutant retained seventy percent of its wild-type nitrogenase activity, which should be sufficient to sustain diazotrophic growth, although probably at a slower rate. Although the *conR* mutant formed seemingly functional heterocysts, it was unable to grow diazotrophically, probably because cell-cell transport or metabolite exchange between vegetative cells and heterocysts is interrupted in the *conR* mutant strain.

The *conR* mutant phenotype and the presence of the LytR-CpsA-Psr domain suggest that ConR plays a role in cell wall maintenance and septum formation. The elongated heterocysts and defective septal constriction seen in the *conR* mutant could be best explained by abnormal peptidoglycan synthesis, which results in a failure to constrict the septum between two daughter cells (130). The production and turnover of cell wall material could be affected such that cell growth continues beyond the wild-type size. For example, the absence of FtsI, a penicillin binding protein, in *E. coli* abolishes peptidoglycan synthesis at the septum but still allows cell elongation (1, 12). Time-lapse microscopy of the *conR* mutant showed that at around 9 h following nitrogen step-down, the size of differentiating cells begins to exceed the wild type size. 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. These results suggest that remodeling of the peptidoglycan begins immediately after the cells have committed to differentiate into heterocysts.

Heterocyst-to-vegetative cell transport may be disrupted in the* conR *mutant

The *conR* gene was previously identified in a transposon mutagenesis study that screened for Fox$^-$ mutants, but the authors did not explore the temporal and spatial patterns of *conR* expression (45). The previous study noted the incomplete constriction of the septum between heterocysts and flanking vegetative cells in the mutant strain, but did not investigate the impact of *conR* mutant on the expression of *nifH* or the capacity of the cells to fix nitrogen. We propose that the inability of the *conR* mutant to grow diazotrophically is a direct result of impaired septum formation at the division site between heterocysts and flanking vegetative cells. Recent findings have shown that intercellular movement of molecules takes place directly between the cytoplasms of adjacent cells in *Anabaena* PCC 7120, which indicates the existence of non-specific intercellular channels that allow metabolite exchange between cells of the filament (114). SepJ is a membrane-bound protein that localizes to the cell-cell interface between adjacent cells and is proposed to form the intercellular 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 of protein monomers that must oligomerize to form the channels (114). In the *conR* mutant, defective septum closure could interfere with the formation of these channels at the interface between heterocysts

and vegetative cells, thus preventing normal metabolite exchange. It have been though that SepJ in *Anabaena* PCC 7120 is part of the cell to cell transportation machinery, and it would be interesting to know whether SepJ localization is disrupted in the *conR* mutant at the heterocyst to vegetative cell boundary. It is possible that ConR interacts with proteins that are necessary to build the structures for cell-cell transport. This question could be addressed by finding proteins that interact with ConR by using protein pull-down or two-hybrid system experiments.

Regulation of sigma factors during heterocyst differentiation

As stated in Chapter III, the *Anabaena* PCC 7120 genome encodes twelve putative sigma factors, and it has been proposed that certain sigma factors act sequentially during heterocyst development to regulate different sets of genes that are necessary at the early, middle, and late stages of heterocyst development (4). Our 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 individual sigma factors cause a well-defined developmental block (94). However, it was made clear that this model does not directly apply to heterocyst development in *Anabaena* PCC 7120 because inactivation of sigma factor genes individually and in several different pairs failed to block heterocyst development (89).

As we described in Chapter III, the promoter regions of eight chromosomally encoded sigma factor genes were fused to a GFP reporter in order to analyze their

expression during heterocyst differentiation in *Anabaena* PCC 7120. Time-lapse fluorescence microscopy of GFP reporter strains indicated that the *sigC*, *sigG*, and *sigE* genes are up-regulated specifically in differentiating cells at 4 h, 9 h, and 16 h following deprivation of combined nitrogen. 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. From these results we can infer that the number of sigma factor genes active in the heterocyst cells is at least five. In this scenario, in the absence of one sigma factor, it is possible that other sigma factors overlap in promoter specificity to a degree that could activate gene expression in differentiating cells and allow development to proceed, even if the target genes were being expressed at incorrect stages during development. It is not known how the specificity and relative amounts of particular sigma factors change during heterocyst differentiation. In the wild type, the expression timing and level for each sigma factor, its promoter recognition properties, and its association with other transcription factors would determine which sigma factor is primarily used for transcription of a particular gene.

Regulatory targets of SigC

Using microarray experiments and time-lapse fluorescence microscopy we determined that the expression of *sigC* is developmentally regulated during the early stages of heterocyst differentiation, suggesting that *sigC* regulates the transcription of genes required for the early stages of heterocyst development. In the *sigC* mutant strain,

the expression of early genes such as *hepA*, *hepC*, and *hetR* is delayed and reduced. Lack of a more severe phenotype for the *sigC* mutant is possibly due to partial functional redundancy among sigma factors, which has been observed in other cyanobacteria (126). We found that in the *sigC* mutant strain, heterocyst development is delayed suggesting that the genes required for development that are part of the SigC regulon are not transcribed as efficiently by other sigma factors. An alternative hypothesis would be that a sigma factor(s) that can recognize and transcribe the SigC regulon is expressed later during heterocyst development.

A comparative expression analysis using microarray experiments of sigma-factor mutants and the wild type after nitrogen deprivation could provide a more complete picture of the sets of genes that are regulated by each sigma factor. One of the limitations 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 would help to determine more accurate quantitative changes in expression of specific target genes.

Regulatory targets of SigG

As we mentioned before, P_{sigG}-*gfp* is expressed in nitrogen-containing media but by 4 h after nitrogen step-down the P_{sigG}-*gfp* expression is no longer observed. By 9-10 hrs P_{sigG}-*gfp* expression is localized in single cells, which become heterocysts after 18 h

of nitrogen step-down. We attempted to inactivate *sigG* using different approaches without success. To investigate *sigG* regulatory targets, a depletion mutant is needed because insertional inactivation proved to be lethal. In order to crate a depletion mutant in *Anabaena* PCC 7120 we will need to clone *sigG* under the control of an inducible promoter, such as *petE*, which is a copper-inducible promoter. After this construction is introduced into the *Anabaena* PCC 7120 chromosome, we can then inactivate the wild-type copy of *sigG* in presence of copper. To analyze the effect of a *sigG* depletion mutant strain during heterocyst development, copper will need to be removed from the media for each experiment. A different approach would be to use an expression vector with temperature-dependent replication. The *sigG* gene would be cloned and expressed from the vector at the permissive temperature, which would allow the chromosomal *sigG* gene to be inactivated. Then the nonpermissive temperature would be used to produce the deplete conditions.

Regulation of* nifH *gene expression by SigE

Previous studies conducted by Khudyakov et al. have shown that inactivation of *sigE* is not required for heterocyst formation or nitrogen fixation, and these authors suggested that this could be a result of overlapping function between sigma factors (89). Our experiments showed that inactivation of *sigE* produced a delay in heterocyst maturation, but eventually the heterocyst pattern and heterocyst frequency were approximately normal. As described in Chapter III and IV, the expression of *sigE* is up-regulated during the middle to late stages of heterocyst development, and time-lapse

microscopy of the P_{sigE}-*gfp* reporter strain showed that the expression takes place specifically in differentiating cells. This implies that *sigE* regulates the expression of heterocyst-specific genes in the middle to late stages of heterocyst differentiation and maturation. The most important 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 heterocyst induction. Transcriptional regulation of the *nifHDK* operon has been poorly studied over the last two decades, and there is little knowledge about the mechanism and components that directly interact with the *nifHDK* operon to regulate its expression.

Our expression analysis of the *nifH* transcript in the *sigE* mutant strain showed that *nifH* expression levels are reduced and delayed when compared with the wild-type strain, indicating that SigE is important for the expression of *nif* genes. Time-lapse fluorescence microscopy of the P_{nifHD}-*gfp* reporter strain in the wild-type background showed developmental expression of *nifHD* after 18 h of heterocyst induction. However, the P_{nifHD}-*gfp* reporter in the *sigE* mutant background did not show any GFP signal even 42 h after nitrogen step-down, when mature heterocysts are morphologically evident. Overexpression of *sigE* causes premature expression of the P_{nifHD}-*gfp* reporter and heterocyst differentiation, as well as higher heterocyst frequency when compared with the wild type. We do not know how SigE is regulating the expression of *nifH*, or if this regulation of *nifH* expression is direct or indirect. We would like to know which genes are under control of SigE. Because *sigE* is developmentally regulated and its expression is primarily in heterocysts, we hypothesize that SigE regulates the expression of

heterocyst-specific genes. As a next step to defining the SigE regulation, we could use microarray or RNA SEQ experiments to compare the expression of all genes in the genome in wild-type and *sigE* mutant backgrounds.

In order to determine if SigE is directly responsible for *nifH* expression, *in vitro* transcription assays using purified RNA polymerase components from *Anabaena* PCC 7120 would be necessary. Another possibility would be to use our P_{nifHD}-*gfp* reporter to address this question. We have found that some *Anabaena* transcription factors will function in *E. coli* cells to control expression of *Anabaena* promoters. To determine if SigE can activate the expression of P_{nifHD}-*gfp*, we could transform *E. coli* with a P_{nifHD}-*gfp* reporter and with a plasmid that contains *sigE* expressed from an inducible promoter.

Expression analysis of heterocyst-specific genes in a* sigE *mutant strain

In Chapter IV we analyzed the expression of heterocyst-specific genes in the *sigE* mutant strain compared with the wild type during heterocyst development by qRT-PCR. The expression of *sigE* is detectable at 12 h after nitrogen step-down, and we hypothesize that SigE regulates transcription of genes during the middle and late stages of heterocyst development because our data show that SigE is required for the normal progression of heterocyst maturation. As we expected from our northern blot and reporter experiments, the transcript levels of *nifH* in the *sigE* mutant strain were significantly lower than in the wild-type strain, confirming our previous results. We also analyzed the expression of heterocyst-specific genes *fdxH*, *hupL*, *hepB*, and *hglE*, which are expressed during middle and late stages of heterocyst development. Our results

showed that the expression of the *fdxH* gene, which encodes a [2Fe-2S] ferredoxin, is affected in the *sigE* mutant, suggesting that *fdxH* is part of the SigE regulon. In the *sigE* mutant strain, the transcription levels of *hepB* were significantly diminished after 12 h of nitrogen step-down when compared with the wild-type strain, but by 24 h after nitrogen step-down, *hepB* expression levels are similar to the wild-type strain. The expression levels of *hupL* (a late gene) and *hglE* (a middle gene) were not significantly different in the *sigE* mutant when compared with the wild type. These results are difficult to fit into a consistent hypothesis and indicate that additional levels of regulation must be occurring. These data suggest that SigE regulates expression of a subset of heterocyst-specific genes in the middle and late stages of heterocyst development, but that there are different heterocyst regulatory pathways that do not involve SigE. As mentioned before, microarray or RNA SEQ experiments of the wild type and mutant strains during heterocyst development would help us to identify the genes that are regulated by SigE.

REFERENCES

1. **Adam, M., C. Fraipont, N. Rhazi, M. Nguyen-Disteche, B. Lakaye, J. M. Frere, B. Devreese, J. Van Beeumen, Y. van Heijenoort, J. van Heijenoort, and J. M. Ghuysen.** 1997. The bimodular G57-V577 polypeptide chain of the class B penicillin-binding protein 3 of *Escherichia coli* catalyzes peptide bond formation from thiolesters and does not catalyze glycan chain polymerization from the lipid II intermediate. J. Bacteriol. **179:**6005-6009.

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

3. **Aldea, M. R., K. Kumar, and J. W. Golden.** 2008. Heterocyst development and pattern formation, p. 75-90. *In* W. W. C. and B. L. Bassler (ed.), Chemical communication among bacteria. ASM Press, Washington, DC.

4. **Aldea, M. R., R. A. Mella-Herrera, and J. W. Golden.** 2007. Sigma factor genes *sigC*, *sigE*, and *sigG* are upregulated in heterocysts of the cyanobacterium *Anabaena* sp. strain PCC 7120. J. Bacteriol. **189:**8392-8396.

5. **Awai, K., S. Lechno-Yossef, and C. P. Wolk.** 2009. Heterocyst envelope glycolipids, p. 179-202. *In* H. Wada and N. Murata (ed.), Lipids in photosynthesis: essential and regulatory functions. Springer, New York.

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

7. **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.

8. **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.

9. **Blair, D. F.** 1995. How bacteria sense and swim. Annu. Rev. Microbiol. **49:**489-522.

10. **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.

11. **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.

12. **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.

13. **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.

14. **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.

15. **Brusca, J. S., C. J. Chastain, and J. W. Golden.** 1990. Expression of the *Anabaena* sp. strain PCC 7120 *xisA* gene from a heterologous promoter results in excision of the *nifD* element. J. Bacteriol. **172:**3925-3931.

16. **Brusca, J. S., M. A. Hale, C. D. Carrasco, and J. W. Golden.** 1989. Excision of an 11-kilobase-pair DNA element from within the *nifD* gene in *Anabaena variabilis* heterocysts. J. Bacteriol. **171:**4138-4145.

17. **Budrene, E. O., and H. C. Berg.** 1995. Dynamics of formation of symmetrical patterns by chemotactic bacteria. Nature **376:**49-53.

18. **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.

19. **Buikema, W. J., and R. Haselkorn.** 1991. Characterization of a gene controlling heterocyst differentiation in the cyanobacterium *Anabaena* 7120. Genes Dev. **5:**321-330.

20. **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.

21. **Cai, Y., and C. P. Wolk.** 1997. Nitrogen deprivation of *Anabaena* sp. strain PCC 7120 elicits rapid activation of a gene cluster that is essential for uptake and utilization of nitrate. J. Bacteriol. **179:**258-266.

22. **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.

23. **Campbell, E. A., L. F. Westblade, and S. A. Darst.** 2008. Regulation of bacterial RNA polymerase sigma factor activity: a structural perspective. Curr. Opin. Microbiol. **11:**121-127.

24. **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.

25. **Campbell, E. L., M. L. Summers, H. Christman, M. E. Martin, and J. C. Meeks.** 2007. Global gene expression patterns of *Nostoc punctiforme* in steady-state dinitrogen-grown heterocyst-containing cultures and at single time points during the differentiation of akinetes and hormogonia. J. Bacteriol. **189:**5247-5256.

26. **Cardemil, L., and C. P. Wolk.** 1981. Isolated heterocysts of *Anabaena variabilis* synthesize envelope polysaccharide. Biochim. Biophys. Acta. **674:**265-276.

27. **Cardemil, L., and C. P. Wolk.** 1979. The polysaccharides from heterocyst and spore envelopes of a blue-green alga. Structure of the basic repeating unit. J. Biol. Chem. **254:**736-741.

28. **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.

29. **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.

30. **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.

31. **Carrasco, C. D., K. S. Ramaswamy, T. S. Ramasubramanian, and J. W. Golden.** 1994. *Anabaena xisF* gene encodes a developmentally regulated site-specific recombinase. Genes Dev. **8:**74-83.

32. **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.

33. **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.

34. **Chen, H., S. Laurent, S. Bedu, F. Ziarelli, H. L. Chen, Y. Cheng, C. C. Zhang, and L. Peng.** 2006. Studying the signaling role of 2-oxoglutaric acid using analogs that mimic the ketone and ketal forms of 2-oxoglutaric acid. Chem. Biol. **13:**849-856.

35. **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.

36. **Cumino, A. C., C. Marcozzi, R. Barreiro, and G. L. Salerno.** 2007. Carbon cycling in *Anabaena* sp. PCC 7120. Sucrose synthesis in the heterocysts and possible role in nitrogen fixation. Plant Physiol. **143:**1385-1397.

37. **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.

38. **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.

39. **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.

40. **Ehira, S., M. Ohmori, and N. Sato.** 2003. Genome-wide expression analysis of the responses to nitrogen deprivation in the heterocyst-forming cyanobacterium *Anabaena* sp. strain PCC 7120. DNA Res. **10:**97-113.

41. **Elhai, J., A. Taton, J. P. Massar, J. K. Myers, M. Travers, J. Casey, M. Slupesky, and J. Shrager.** 2009. BioBIKE: a Web-based, programmable, integrated biological knowledge base. Nucleic Acids Res. **37:**W28-32.

42. **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.

43. **Elhai, J., and C. P. Wolk.** 1990. Developmental regulation and spatial pattern of expression of the structural genes for nitrogenase in the cyanobacterium *Anabaena*. EMBO J. **9:**3379-3388.

44. **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.

45. **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.

46. **Fay, P.** 1992. Oxygen relations of nitrogen fixation in cyanobacteria. Microbiol. Rev. **56:**340-373.

47. **Fiedler, G., M. Arnold, S. Hannus, and I. Maldener.** 1998. The DevBCA exporter is essential for envelope formation in heterocysts of the cyanobacterium *Anabaena* sp. strain PCC 7120. Mol. Microbiol. **27:**1193-1202.

48. **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:**3795-379.

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

50. **Flores, E., R. Pernil, A. M. Muro-Pastor, V. Mariscal, I. Maldener, S. Lechno-Yossef, Q. Fan, C. P. Wolk, and A. Herrero.** 2007. Septum-localized protein required for filament integrity and diazotrophy in the heterocyst-forming cyanobacterium *Anabaena* sp. strain PCC 7120. J. Bacteriol. **189:**3884-3890.

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

52. **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.

53. **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.

54. **Giddings, T. H., and L. A. Staehelin.** 1981. Observation of microplasmodesmata in both heterocyst-forming and non-heterocyst forming filamentous cyanobacteria by freeze-fracture electron microscopy. Arch. Microbiol. **129:**295-298.

55. **Giddings, T. H., and L. A. Staehelin.** 1978. Plasma membrane architecture of *Anabaena cylindrica*: occurrence of microplasmodesmata and changes associated with heterocyst development and the cell cycle. Eur. J. Cell. Biol. **16:**235-249.

56. **Golden, J. W.** 1997. Programmed DNA rearrangements in cyanobacteria, p. 162-173. *In* F. J. de Bruijn, J. R. Lupski, and G. Weinstock (ed.), Bacterial genomes: physical structure and analysis. Chapman & Hall, New York.

57. **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.

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

59. **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:**7098-70105.

60. **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.

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

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

63. **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.

64. **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.

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

66. **Hassett, D. J., M. D. Sutton, M. J. Schurr, A. B. Herr, C. C. Caldwell, and J. O. Matu.** 2009. *Pseudomonas aeruginosa* hypoxic or anaerobic biofilm infections within cystic fibrosis airways. Trends Microbiol. **17:**130-138.

67. **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.

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

69. **Henson, B. J., L. E. Pennington, L. E. Watson, and S. R. Barnum.** 2008. Excision of the *nifD* element in the heterocystous cyanobacteria. Arch. Microbiol. **189:**357-366.

70. **Herrero, A., A. M. Muro-Pastor, and E. Flores.** 2001. Nitrogen control in cyanobacteria. J. Bacteriol. **183:**411-425.

71. **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.

72. **Hoiczyk, E., and A. Hansel.** 2000. Cyanobacterial cell walls: news from an unusual prokaryotic envelope. J. Bacteriol. **182:**1191-1199.

73. **Holmqvist, M., K. Stensjo, P. Oliveira, P. Lindberg, and P. Lindblad.** 2009. Characterization of the *hupSL* promoter activity in *Nostoc punctiforme* ATCC 29133. BMC Microbiol. **9:**54-55.

74. **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.

75. **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.

76. **Hubscher, J., L. Luthy, B. Berger-Bachi, and P. Stutzmann Meier.** 2008. Phylogenetic distribution and membrane topology of the LytR-CpsA-Psr protein family. BMC Genomics **9:**617.

77. **Hubscher, J., N. McCallum, C. D. Sifri, P. A. Majcherczyk, J. M. Entenza, R. Heusser, B. Berger-Bachi, and P. Stutzmann Meier.** 2009. MsrR contributes to cell surface characteristics and virulence in *Staphylococcus aureus*. FEMS Microbiol. Lett. **295:**251-260.

78. **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.

79. **Imamura, S., and M. Asayama.** 2009. Sigma factors for cyanobacterial transcription. Gene Regul. Syst. Bio. **3:**65-87.

80. **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.

81. **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.

82. **Jang, J., L. Wang, R. Jeanjean, and C. C. Zhang.** 2007. PrpJ, a PP2C-type protein phosphatase located on the plasma membrane, is involved in heterocyst maturation in the cyanobacterium *Anabaena* sp. PCC 7120. Mol. Microbiol. **64:**347-358.

83. **Jelsbak, L., and L. Sogaard-Andersen.** 2002. Pattern formation by a cell surface-associated morphogen in *Myxococcus xanthus*. Proc. Natl. Acad. Sci. USA **99:**2032-2037.

84. **Johnsborg, O., and L. S. Havarstein.** 2009. Pneumococcal LytR, a protein from the LytR-CpsA-Psr family, is essential for normal septum formation in *Streptococcus pneumoniae*. J. Bacteriol. **191:**5859-5864.

85. **Jones, K. M., and R. Haselkorn.** 2002. Newly identified cytochrome c oxidase operon in the nitrogen-fixing cyanobacterium *Anabaena* sp. strain PCC 7120 specifically induced in heterocysts. J. Bacteriol. **184:**2491-2499.

86. **Ke, S. H., and E. L. Madison.** 1997. Rapid and efficient site-directed mutagenesis by single-tube 'megaprimer' PCR method. Nucleic Acids Res. **25:**3371-3372.

87. **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.

88. **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.

89. **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.

90. **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.

91. **Klausen, M., A. Heydorn, P. Ragas, L. Lambertsen, A. Aaes-Jorgensen, S. Molin, and T. Tolker-Nielsen.** 2003. Biofilm formation by *Pseudomonas aeruginosa* wild type, flagella and type IV pili mutants. Mol. Microbiol. **48:**1511-1524.

92. **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.

93. **Kroos, L.** 2007. The *Bacillus* and *Myxococcus* developmental networks and their transcriptional regulators. Annu. Rev. Genet. **41:**13-39.

94. **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.

95. **Lammers, P. J., S. McLaughlin, S. Papin, C. Trujillo-Provencio, and A. J. Ryncarz, 2nd.** 1990. Developmental rearrangement of cyanobacterial *nif* genes: nucleotide sequence, open reading frames, and cytochrome P-450 homology of the *Anabaena* sp. strain PCC 7120 *nifD* element. J. Bacteriol. **172:**6981-6990.

96. **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.

97. **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.

98. **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.

99. **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.

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

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

102. **Lopez-Gomollon, S., J. A. Hernandez, S. Pellicer, V. E. Angarica, M. L. Peleato, and M. F. Fillat.** 2007. Cross-talk between iron and nitrogen regulatory networks in *Anabaena* (*Nostoc*) sp. PCC 7120: identification of overlapping genes in FurA and NtcA regulons. J. Mol. Biol. **374:**267-281.

103. **Marcozzi, C., A. C. Cumino, and G. L. Salerno.** 2009. Role of NtcA, a cyanobacterial global nitrogen regulator, in the regulation of sucrose metabolism gene expression in *Anabaena* sp. PCC 7120. Arch. Microbiol. **191:**255-263.

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

105. **Martin, D. W., M. J. Schurr, H. Yu, and V. Deretic.** 1994. Analysis of promoters controlled by the putative sigma factor AlgU regulating conversion to

mucoidy in *Pseudomonas aeruginosa*: relationship to sigma E and stress response. J. Bacteriol. **176:**6688-6696.

106. **Martin-Figueroa, E., F. Navarro, and F. J. Florencio.** 2000. The GS-GOGAT pathway is not operative in the heterocysts. Cloning and expression of *glsF* gene from the cyanobacterium *Anabaena* sp. PCC 7120. FEBS Lett. **476:**282-286.

107. **Mascher, T., A. B. Hachmann, and J. D. Helmann.** 2007. Regulatory overlap and functional redundancy among *Bacillus subtilis* extracytoplasmic function sigma factors. J. Bacteriol. **189:**691969-27.

108. **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.

109. **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.

110. **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.

111. **Miyagishima, S. Y., C. P. Wolk, and K. W. Osteryoung.** 2005. Identification of cyanobacterial cell division genes by comparative and mutational analyses. Mol. Microbiol. **56:**126-143.

112. **Moslavac, S., K. Nicolaisen, O. Mirus, F. Al Dehni, R. Pernil, E. Flores, I. Maldener, and E. Schleiff.** 2007. A TolC-like protein is required for heterocyst development in *Anabaena* sp. strain PCC 7120. J. Bacteriol. **189:**7887-7895.

113. **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.

114. **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.

115. **Muro-Pastor, A. M., E. Flores, and A. Herrero.** 2009. NtcA-regulated heterocyst differentiation genes *hetC* and *devB* from *Anabaena* sp. strain PCC 7120 exhibit a similar tandem promoter arrangement. J. Bacteriol. **191:**5765-5774.

116. **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.

117. **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.

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. **Muro-Pastor, M. I., J. C. Reyes, and F. J. Florencio.** 2001. Cyanobacteria perceive nitrogen status by sensing intracellular 2-oxoglutarate levels. J. Biol. Chem. **276:**38320-38328.

121. **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.

122. **Nayar, A. S., H. Yamaura, R. Rajagopalan, D. D. Risser, and S. M. Callahan.** 2007. FraG is necessary for filament integrity and heterocyst maturation in the cyanobacterium *Anabaena* sp. strain PCC 7120. Microbiology **153:**601-607.

123. **Nicolaisen, K., A. Hahn, and E. Schleiff.** 2009. The cell wall in heterocyst formation by *Anabaena* sp. PCC 7120. J. Basic Microbiol. **49:**5-24.

124. **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:**2453-9245.

125. **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.

126. **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.

127. **Pereira, S., A. Zille, E. Micheletti, P. Moradas-Ferreira, R. De Philippis, and P. Tamagnini.** 2009. Complexity of cyanobacterial exopolysaccharides: composition, structures, inducing factors and putative genes involved in their biosynthesis and assembly. FEMS Microbiol. Rev. **33:**917-941.

128. **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.

129. **Pratte, B. S., K. Eplin, and T. Thiel.** 2006. Cross-functionality of nitrogenase components NifH1 and VnfH in *Anabaena variabilis*. J. Bacteriol. **188:**5806-5811.

130. **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.

131. **Ramasubramanian, T. S., T. F. Wei, and J. W. Golden.** 1994. Two *Anabaena* sp. strain PCC 7120 DNA-binding factors interact with vegetative cell- and heterocyst-specific genes. J. Bacteriol. **176:**1214-1223.

132. **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.

133. **Ramaswamy, K. S., C. D. Carrasco, T. Fatma, and J. W. Golden.** 1997. Cell-type specificity of the *Anabaena fdxN*-element rearrangement requires *xisH* and *xisI*. Mol. Microbiol. **23:**1241-1249.

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:**2326-3231.

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. **Risser, D. D., and S. M. Callahan.** 2009. Genetic and cytological evidence that heterocyst patterning is regulated by inhibitor gradients that promote activator decay. Proc. Natl. Acad. Sci. USA **106:**19884-19888.

137. **Risser, D. D., and S. M. Callahan.** 2007. Mutagenesis of *hetR* reveals amino acids necessary for HetR function in the heterocystous cyanobacterium *Anabaena* sp. strain PCC 7120. J. Bacteriol. **189:**2460-2467.

138. **Rothfield, L., A. Taghbalout, and Y. L. Shih.** 2005. Spatial control of bacterial division-site placement. Nat. Rev. Microbiol. **3:**959-968.

139. **Rubio, L. M., and P. W. Ludden.** 2008. Biosynthesis of the iron-molybdenum cofactor of nitrogenase. Annu. Rev. Microbiol. **62:**93-111.

140. **Sakr, S., R. Jeanjean, C. C. Zhang, and T. Arcondeguy.** 2006. Inhibition of cell division suppresses heterocyst development in *Anabaena* sp. strain PCC 7120. J. Bacteriol. **188:**1396-1404.

141. **Sambrook, J., and D. W. Russell.** 2006. Northern hybridization. Cold Spring Harbor Protocols**. 2:**3723-3725.

142. **Sato, N., M. Ohmori, M. Ikeuchi, K. Tashiro, C. P. Wolk, T. Kaneko, K. Okada, M. Tsuzuki, S. Ehira, H. Katoh, S. Okamoto, H. Yoshimura, T. Fujisawa, A. Kamei, S. Yoshihara, R. Narikawa, T. Hamano, S. Tabata, and S. Kuhara.** 2004. Use of segment-based microarray in the analysis of global gene expression in response to various environmental stresses in the cyanobacterium *Anabaena* sp. PCC 7120. J. Gen. Appl. Microbiol. **50:**1-8.

143. **Stricker, O., B. Masepohl, W. Klipp, and H. Bohme.** 1997. Identification and characterization of the *nifV-nifZ-nifT* gene region from the filamentous cyanobacterium *Anabaena* sp. strain PCC 7120. J. Bacteriol. **179:**2930-2937.

144. **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.

145. **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.

146. **Tamagnini, P., E. Leitao, P. Oliveira, D. Ferreira, F. Pinto, D. J. Harris, T. Heidorn, and P. Lindblad.** 2007. Cyanobacterial hydrogenases: diversity, regulation and applications. FEMS Microbiol. Rev. **31:**692-720.

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. **Thiel, T., E. M. Lyons, J. C. Erker, and A. Ernst.** 1995. A second nitrogenase in vegetative cells of a heterocyst-forming cyanobacterium. Proc. Natl. Acad. Sci. USA **92:**9358-9362.

149. **Thiel, T., and B. Pratte.** 2001. Effect on heterocyst differentiation of nitrogen fixation in vegetative cells of the cyanobacterium *Anabaena variabilis* ATCC 29413. J. Bacteriol. **183:**280-286.

150. **Tuominen, I., M. Pollari, E. Tyystjarvi, and T. Tyystjarvi.** 2006. The SigB sigma factor mediates high-temperature responses in the cyanobacterium *Synechocystis* sp. PCC6803. FEBS Lett. **580:**319-323.

151. **Ungerer, J., and T. Thiel.** 2007. Regulation of *nifH1* by cis-acting regulatory elements in the *nifH1* promoter region in the cyanobacterium *Anabaena variabilis* ATCC 29413, p. 50-51, 9th Cyanobacterial Molecular Biology Workshop, Delavan, WI.

152. **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:**1239-49.

153. **Valladares, A., I. Maldener, A. M. Muro-Pastor, E. Flores, and A. Herrero.** 2007. Heterocyst development and diazotrophic metabolism in terminal respiratory oxidases mutants of the cyanobacterium *Anabaena* sp. strain PCC 7120. J. Bacteriol.

154. **Valladares, A., A. M. Muro-Pastor, M. F. Fillat, A. Herrero, and E. Flores.** 1999. Constitutive and nitrogen-regulated promoters of the *petH* gene encoding ferredoxin:NADP+ reductase in the heterocyst-forming cyanobacterium *Anabaena* sp. FEBS Lett. **449:**159-164.

155. **Valladares, A., A. M. Muro-Pastor, A. Herrero, and E. Flores.** 2004. The NtcA-dependent P1 promoter is utilized for *glnA* expression in N_2-fixing heterocysts of *Anabaena* sp. strain PCC 7120. J. Bacteriol. **186:**7337-7343.

156. **Vazquez-Bermudez, M. F., A. Herrero, and E. Flores.** 2003. Carbon supply and 2-oxoglutarate effects on expression of nitrate reductase and nitrogen-regulated genes in *Synechococcus* sp. strain PCC 7942. FEMS Microbiol. Lett. **221:**155-159.

157. **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.

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. **Wang, Y., S. Lechno-Yossef, Y. Gong, Q. Fan, C. P. Wolk, and X. Xu.** 2007. Predicted glycosyl transferase genes located outside the HEP island are required for formation of heterocyst envelope polysaccharide in *Anabaena* sp. strain PCC 7120. J. Bacteriol. **189:**5372-5378.

160. **Wang, Y., and X. Xu.** 2005. Regulation by *hetC* of genes required for heterocyst differentiation and cell division in *Anabaena* sp. strain PCC 7120. J. Bacteriol. **187:**8489-8493.

161. **Waterbury, J. B., and J. M. Willey.** 1988. Isolation and growth of marine planktonic cyanobacteria. Methods Enzymol. **167:**100-105.

162. **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.

163. **Wei, T. F., T. S. Ramasubramanian, F. Pu, and J. W. Golden.** 1993. *Anabaena* sp. strain PCC 7120 *bifA* gene encoding a sequence-specific DNA-binding protein cloned by *in vivo* transcriptional interference selection. J. Bacteriol. **175:**4025-4035.

164. **Wilcox, M., G. J. Mitchison, and R. J. Smith.** 1973. Pattern formation in the blue-green alga, *Anabaena*. I. Basic mechanisms. J. Cell Sci. **12:**707-723.

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

166. **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.

167. **Wolk, C. P., A. Ernst, and J. Elhai.** 1994. Heterocyst metabolism and development, p. 769-863. *In* D. A. Bryant (ed.), The molecular biology of cyanobacteria, vol. 1. Kluwer Academic Publishers, Dordrecht, Netherlands.

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

169. **Wu, G. F., Q. Y. Wu, and Z. Y. Shen.** 2001. Accumulation of poly-[beta]-hydroxybutyrate in cyanobacterium *Synechocystis* sp. PCC6803. Bioresource Technology **76:**85-90.

170. **Wu, J., and A. Newton.** 1997. The Caulobacter heat shock sigma factor gene rpoH is positively autoregulated from a sigma32-dependent promoter. J Bacteriol **179:**514-521.

171. **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.

172. **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:**6422-4629.

173. **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.

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

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

176. **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.

177. **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.

178. **Zhang, J. Y., W. L. Chen, and C. C. Zhang.** 2009. *hetR* and *patS*, two genes necessary for heterocyst pattern formation, are widespread in filamentous nonheterocyst-forming cyanobacteria. Microbiology **155:**1418-1426.

179. **Zhao, J., and C. P. Wolk.** 2008. Developmental biology of heterocysts, p. 397-418. *In* D. E. Whitworth (ed.), Myxobacteria: multicellularity and differentiation. ASM Press, Washington, DC.

180. **Zhou, R., Z. Cao, and J. Zhao.** 1998. Characterization of HetR protein turnover in *Anabaena* sp. PCC 7120. Arch. Microbiol. **169:**417-423.

181. **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.

182. **Zhou, R., and C. P. Wolk.** 2003. A two-component system mediates developmental regulation of biosynthesis of a heterocyst polysaccharide. J. Biol. Chem. **278:**19939-19946.

183. **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.

184. **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.

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