Cyanobacterial Heterocysts

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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 together to accommodate the oxygen-sensitive process of nitrogen fixation. This article focuses on recent research on heterocyst development, including morphogenesis, transport of molecules between cells in a filament, differential gene expression, and pattern formation.

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. 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 (Figs. 1 and 2).

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. For example, the unicellular *Cyanothece* sp. strain ATCC 51142 stores glycogen during the day and fixes nitrogen at night (Toepel et al. 2008), whereas the filamentous *Trichodesmium erythraeum* IMS101 fixes nitrogen during the day in groups of specialized cells (Sandh et al. 2009). Heterocyst-forming cyanobacteria differentiate highly specialized cells

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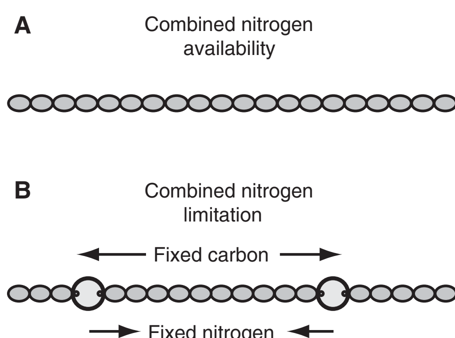

Figure 1. Heterocyst development in *Anabaena* PCC 7120. (*A*) *Anabaena* PCC 7120 grown in medium containing a source of combined nitrogen grows as filaments of photosynthetic vegetative cells. (*B*) In the absence of combined nitrogen, heterocysts differentiate at semiregular intervals, forming a developmental pattern of single heterocysts every 10 to 20 vegetative cells along filaments. Heterocysts are often larger than vegetative cells, have a thicker multilayered envelope, and usually contain cyanophycin granules at their poles adjacent to a vegetative cell.

to provide fixed nitrogen to the vegetative cells in a filament.

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 semiregular intervals between stretches of vegetative cells to produce a multicellular pattern of single heterocysts every ten to twenty vegetative cells along filaments (Figs. 1 and 2). Some heterocyst-forming cyanobacteria show different regulation or display different developmental patterns but these topics are beyond the scope of this article. 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 evelopment. Various aspects of heterocyst development, signaling, and regulation have been the subject of several recent reviews (Meeks and Elhai 2002; Forchhammer 2004; Herrero et al. 2004; Zhang et al. 2006; Aldea et al. 2008; Zhao and Wolk 2008).

Although beyond the scope of this article, it should be noted that cyanobacteria have recently attracted increased attention because of their important roles in environmental carbon and nitrogen fixation (Montoya et al. 2004), and their potential for providing renewable chemicals and biofuels (Dismukes et al. 2008).

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 (Hoiczyk and Hansel 2000). 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. These layers protect the cells from desiccation and presumably from phages and predators. Cyanobacteria contain extensive internal thylakoid membranes (Fig. 2), which are the site of photosynthetic reactions, but these will not be considered further here.

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 (Fig. 2). The additional envelope layers surrounding heterocysts help to protect the enzyme nitrogenase from oxygen (Fay 1992). For details of the heterocyst cell wall and envelope, readers are referred to recent reviews (Awai et al. 2009; Nicolaisen et al. 2009; Pereira et al. 2009). Mature heterocysts provide the microoxic environment required for nitrogen fixation, spatially separating oxygen-evolving photosynthesis in vegetative

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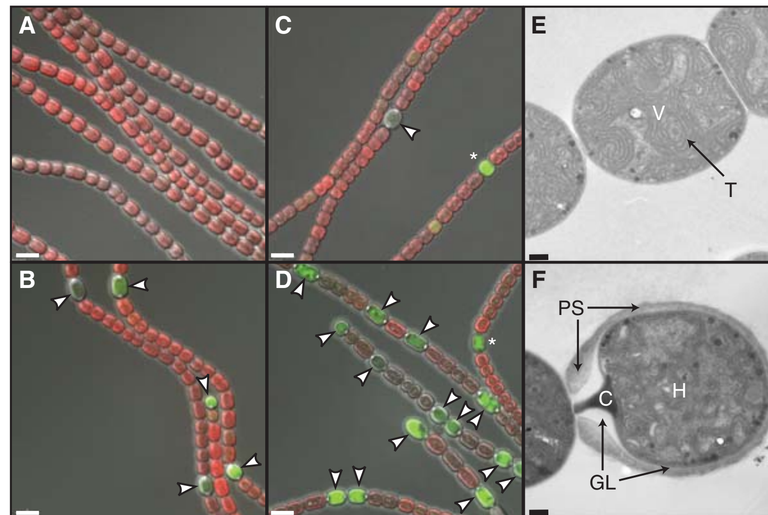

Figure 2. Heterocyst development in *Anabaena* PCC 7120. Filaments of the wild type carrying a *patS-gfp* reporter grown in medium containing nitrate are composed of vegetative cells (*A*), and have undergone heterocyst development 1 d after transfer to medium without combined nitrogen (*B*). A *patS* mutant strain carrying the same *patS-gfp* reporter grown in media containing nitrate contains a small number of heterocysts (*C*), and 1 d 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 of photosynthetic pigments (red), and *patS-gfp* reporter fluorescence (green) microscopic 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, polar cyanophycin granule; size bar, 0.2 μm.

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cells from nitrogen fixation. Differentiating cells undergo many metabolic and morphological changes (Golden and Yoon 1998). Oxygen-producing photosystem PSII is dismantled during differentiation and heterocysts show an increased rate of respiration (Wolk et al. 1994). 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) (Cardemil and Wolk 1979; Cardemil and Wolk 1981; Nicolaisen et al. 2009). 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 (Cumino et al. 2007; Marcozzi et al. 2009). In *Anabaena* PCC 7120, vegetative cells must also supply glutamate to heterocysts, which convert it to glutamine and other amino acids (Martin-Figueroa et al. 2000). In return, vegetative cells obtain fixed nitrogen in the form of amino acids from the heterocysts (Meeks and Elhai 2002). The temporal and spatial distributions of fixed carbon and nitrogen were studied using high-resolution nanometer-scale secondary ion mass spectrometry (NanoSIMS), in *Anabaena oscillarioides* (Popa et al. 2007). Newly fixed nitrogen is rapidly exported from heterocysts and distributed to nearby vegetative cells.

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 (Flores et al. 2006). 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 up-regulated 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 (Mariscal et al. 2007). GFP attached to the cytoplasmic membrane was only seen in heterocysts and not in adjacent vegetative cells. However, another group found that GFP targeted to the periplasm of vegetative cells or heterocysts using different cell-type specific promoters (P-*hepA*, P-*patB*, or P-*rbcL*) and the signal sequence of the *Escherichia coli* TorA protein attached to GFP showed no intercellular diffusion from one cell to the next (Zhang et al. 2008). The exported GFP diffused around individual cells but not beyond the cell borders. Although it is unclear why these two research groups came to different conclusions, it may be because of the different signal sequences that were used. The data obtained by Zhang et al. could be the result of a failure to cleave the TorA signal peptide from the GFP reporter, which could result in the GFP being anchored to the membrane or localized to the space on the inner side of the peptidoglycan layer. 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 (Fig. 3) 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 (Mullineaux et al. 2008). 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 (Fig. 3) (Giddings and Staehelin 1978; Giddings and Staehelin 1981). The microplasmodesmata may be channels formed by protein oligomers, and FraG (SepJ) has been suggested as a candidate channel forming protein (Flores et al. 2007; Nayar et al. 2007). FraG has a large extracytoplasmic domain that may be involved in spanning the cell wall and bridging the gap between adjacent cells (Flores et al. 2007). Localization studies with a FraG-GFP reporter showed that it is expressed in both cell types and that it is localized at intercellular septa (Flores et al. 2007). *fraG* mRNA levels increase after nitrogen depletion and *fraG* null mutants are unable to differentiate heterocysts completely (Flores et al. 2007; Nayar et al. 2007). The diffusion of calcein required FraG, suggesting that FraG could be the channel forming protein connecting the cytoplasm of two adjacent cells (Mullineaux et al. 2008).

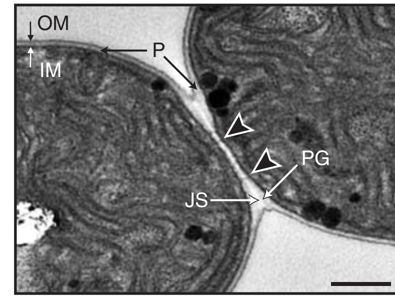

Figure 3. Transmission electron micrograph of the junction between two vegetative cells. Arrowheads indicate microplasmodesmata, which are potential cell-to-cell channels. Note the "junctional space" between the cell wall peptidoglycan layers of the two cells, which may indicate a partial barrier between the periplasmic compartments of adjacent cells. IM, inner membrane; OM, outer membrane; P, periplasm; PG, peptidoglycan cell wall; JS, junctional space. Size bar, 0.5 µm.

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 at 30°C 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 using DNA microarray methods show global changes

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in gene expression after nitrogen step-down (Ehira et al. 2003; Sato et al. 2004; Ehira and Ohmori 2006b; Campbell et al. 2007). The process of differentiation is reversible if a source of combined nitrogen or the inhibitory PatS pentapeptide is added within 9–12 hours after nitrogen deprivation, after which point the cells are committed to forming heterocysts (Fig. 4) (Thiel and Pratte 2001; Yoon and Golden 2001).

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 (Laurent et al. 2005; Zhang et al. 2006). 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 (Muro-Pastor et al. 2001; Vazquez-Bermúdez et al. 2003; Muro-Pastor et al. 2005). 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 (Laurent et al. 2005).

NtcA, a transcriptional regulator belonging to the CRP (cyclic AMP receptor protein) family of proteins, senses 2-oxoglutarate levels (Fig. 5). The protein is conserved in all cyanobacteria and regulates a number of genes involved in carbon and nitrogen metabolism (Herrero et al. 2004; Marcozzi et al. 2009). 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 (Wei et al. 1993; Ramasubramanian et al. 1994). The *ntcA* gene is induced soon after nitrogen deprivation and is autoregulated (Ramasubramanian et al. 1994; Ramasubramanian et al. 1996; Muro-Pastor et al. 2002). *ntcA* mutants are unable to use nitrate as the sole source of nitrogen and are blocked from initiating heterocyst development (Frias et al. 1994; Wei et al. 1994). 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) (Herrero et al. 2004). The DNA binding activity of NtcA is enhanced in the presence of 2-oxoglutarate, and 2-oxoglutarate is necessary for transcriptional activation by NtcA (Tanigawa et al. 2002; Vazquez-Bermúdez et al. 2002). Additionally, DFPA, the synthetic analogue of 2-oxoglutarate, stimulates DNA binding activity of NtcA in vitro (Laurent et al. 2005; Chen et al. 2006).

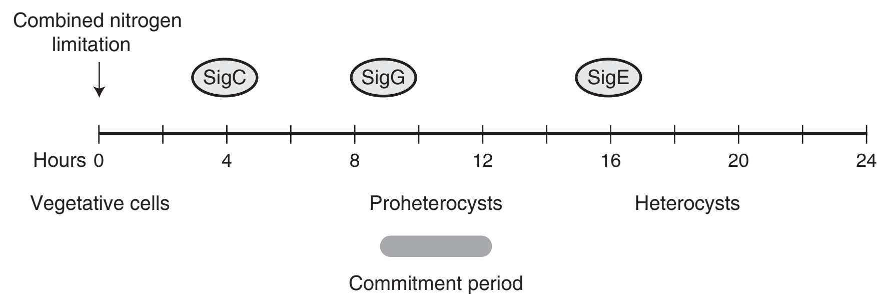

Figure 4. Developmentally regulated σ factors in *Anabaena* PCC 7120. The positions of the σ factor icons mark the times that GFP transcriptional reporters for the σ factor genes are up-regulated during heterocyst development. Filaments are composed of vegetative cells before nitrogen limitation. Immature proheterocysts are observed during approximately the same period of time when cells become committed to complete differentiation. Mature heterocysts are present by 20 h. See text for details.

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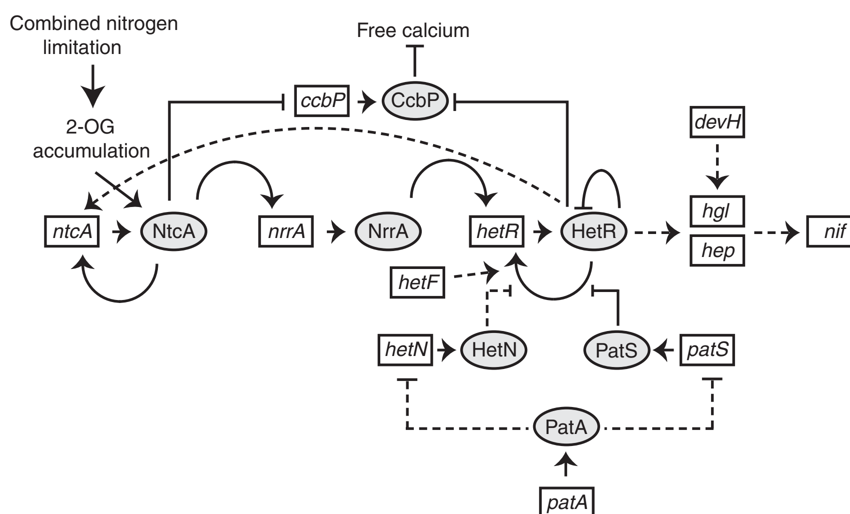

Figure 5. Model of regulatory interactions during heterocyst development. For clarity, the figure shows only selected genes, proteins, and events. Open boxes represent genes and gray ovals represent proteins. Lines ending in arrows and bars indicate positive and negative interactions, respectively. Dashed lines represent indirect and/or unknown interactions or missing steps. Short arrows are between genes and their products. See the text for details.

HetR is a master regulator of heterocyst development and plays a key role in differentiation and pattern formation (Buikema and Haselkorn 1991b). Null mutants of *hetR* fail to produce heterocysts, and overexpression of *hetR* (Buikema and Haselkorn 1991a; Buikema and Haselkorn 2001), and particular point mutants (Khudyakov and Golden 2004) result in increased heterocyst frequency. *hetR* is one of the earliest genes induced in differentiating cells and is positively autoregulated (Fig. 5) (Black et al. 1993; Buikema and Haselkorn 2001). 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.

HetR protein is a serine type protease that has autoprotease activity and DNA binding activity that requires formation of a HetR homodimer (Zhou et al. 1998; Huang et al. 2004). The heterocyst inhibitory peptide PatS interferes with HetR DNA-binding activity in vitro (Huang et al. 2004). Mutations that affect these activities block heterocyst development at an early stage; however, the role of particular amino acid residues is unclear (Dong et al. 2000; Risser and Callahan 2007). A *hetR*$_{R223W}$ point mutant is insensitive to the main inhibitory signals of pattern formation, PatS, and HetN (Fig. 5), and produces a conditionally lethal phenotype because of complete differentiation under nitrogen limiting conditions (Khudyakov and Golden 2004).

Expression of *ntcA* and *hetR* show a mutual dependency during heterocyst development (Muro-Pastor et al. 2002). *hetR* is not induced in an *ntcA* mutant and *ntcA* expression is transiently induced in a HetR-dependent manner (Frias et al. 1994; Muro-Pastor et al. 2002). Expression of some genes involved in the developmental process is dependent on both *ntcA* and *hetR*; however, in some cases, the dependency is probably because of the interdependent up-regulation of *hetR* and *ntcA* (Hebbar and Curtis 2000; Fiedler et al. 2001; Valladares et al. 2004). NrrA, a response regulator, has been identified as the regulatory link between NtcA and HetR (Fig. 5) (Ehira and Ohmori 2006a). *nrrA* is transcribed in differentiating cells within 3 hours after nitrogen deprivation and is directly dependent on NtcA (Ehira and Ohmori 2006b; Muro-Pastor et al. 2006). Earlier work on the transposon reporter strain TLN14, now known to be a fusion to the *nrrA* gene, showed

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rapid induction 1 hour after nitrogen stepdown (Cai and Wolk 1997). 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 (Ehira and Ohmori 2006a; Ehira and Ohmori 2006b).

In *Anabaena* PCC 7120, the calcium-binding luminescent protein aequorin was used to detect an increase in intracellular calcium levels after nitrogen deprivation (Torrecilla et al. 2004). The Ca^{++} reporter obelin was used to show a 10-fold higher Ca^{++} concentration in heterocysts compared with vegetative cells at 4 hours after nitrogen deprivation (Zhao et al. 2005). The increase in Ca^{++} concentrations is because of the decreased expression of *ccbP*, which encodes a calcium sequestering protein, CcbP (Fig. 5) (Zhao et al. 2005). *ccbP* message is down-regulated in differentiating cells and absent in mature heterocysts. Inactivation of *ccbP* causes a multiple-contiguous-heterocyst (Mch) phenotype and overexpression inhibits heterocyst development. It has been hypothesized that a regulatory pathway consisting of HetR, CcbP, and NtcA controls intracellular free calcium; HetR specifically degraded CcbP in a calcium-dependent manner, and *ccbP* down-regulation required 2-oxoglutarate-dependent binding of NtcA to its promoter (Shi et al. 2006). The increase in Ca^{++} in differentiating cells is thought to be important for HetR's Ca^{++}-dependent serine protease and/or other Ca^{++}-dependent proteolytic activities.

HetF influences heterocyst development by a positive effect on *hetR* expression (Fig. 5) (Wong and Meeks 2001; Risser and Callahan 2008). In mutants of *hetF*, expression of *hetR* was not localized to heterocysts and initiation of heterocyst development was not seen (Wong and Meeks 2001). Overexpression of *hetF* produces a multiple-contiguous-heterocyst phenotype, but only in the absence of combined nitrogen (Wong and Meeks 2001). Like HetR, HetF is likely to be a protease (Risser and Callahan 2008). An elegant mosaic analysis was used to show that HetF is required specifically in cells that differentiate (Risser and Callahan 2008). However, it is also thought that HetF plays a role in restricting *hetR* expression and the accumulation of HetR protein to differentiating cells (Wong and Meeks 2001).

Like *hetF*, *patA* also influences heterocyst development via a positive effect on *hetR* expression, but its effect is related to pattern formation (Fig. 5) (Liang et al. 1992; Risser and Callahan 2008). The *patA* gene encodes a response regulator similar to the CheY protein, which functions as a phosphorylation-activated switch (Liang et al. 1992). *patA* mutants form heterocysts almost exclusively at the ends of filaments. This mutant phenotype is maintained even when *hetR* is overexpressed in a *patA* mutant background, suggesting that *patA* acts downstream of *hetR* (Liang et al. 1992; Buikema and Haselkorn 2001). It is possible that PatA influences heterocyst development by attenuating the negative effects of the main inhibitory signals of heterocyst pattern formation, PatS and HetN (Orozco et al. 2006).

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 (Khudyakov and Wolk 1997; Muro-Pastor et al. 1999). A *hetC* mutant carrying a P_{hetR}-*gfp* reporter shows a pattern of weakly fluorescent cells that are blocked from further stages of development (Xu and Wolk 2001; Wang and Xu 2005). 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.

Two novel genes, *hetL* and *asr1734*, have been shown to be involved in regulating heterocyst development, but their exact roles and biochemical functions remain unclear. HetL is a pentapeptide-repeat protein composed almost entirely of 40 tandem pentapeptide repeats forming 10 complete coils (Ni et al. 2009). The *hetL* gene was isolated in a genetic screen designed to identify genes involved in PatS signaling (Liu and Golden 2002). Overexpression of *hetL* in a *patS*-overexpression strain allows heterocysts to form by bypassing the PatS

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inhibitory signal. Overexpression of *hetL* in the wild type produces a multiple-contiguous-heterocyst phenotype. *hetL* overexpression even induces partial heterocyst development in an *ntcA*-null mutant. A *hetL*-null mutant shows normal heterocyst development and diazotrophic growth, indicating that *hetL* plays a nonessential role in heterocyst development.

The *asr1734* gene is found in only heterocyst-forming cyanobacteria and a $P_{asr1734}$-*gfp* reporter showed localized expression in proheterocysts and heterocysts after nitrogen stepdown. An *asr1734* knockout mutant shows elevated levels of *ntcA* mRNA and forms 15% heterocysts and a weak Mch phenotype in media without fixed nitrogen. Overexpression of *asr1734* inhibits heterocyst development in the wild type and in two genetic backgrounds that stimulate heterocyst formation, a *patS* null and a $hetR_{R223W}$ mutant, suggesting that Asr1734 acts downstream of PatS and HetR (Wu et al. 2007).

Cyclic-di-GMP signaling appears to be involved in heterocyst development. The *all2874* gene, which encodes a diguanylate cyclase, is required for normal heterocyst development during growth under conditions of high light intensity (Neunuebel and Golden 2008). An *all2874* mutant shows a significant reduction in heterocyst frequency and reduced vegetative cell size. An *all2874* mutant strain carrying a P_{patS}-*gfp* transcriptional reporter did not show normal up-regulation of the reporter, indicating that the decrease in heterocyst frequency is because of an early block in 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 (Fig. 2) (Fay 1992). Mutants that lack the envelope polysaccharide or the glycolipid layer are unable to grow diazotrophically in the presence of air (Wolk et al. 1988; Wolk 1996; Fan et al. 2005; Huang et al. 2005; Nicolaisen et al. 2009).

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 (Zhou and Wolk 2003). The *hep* genes *hepA*, *hepB*, and *hepC*, and a cluster of genes present around *hepA* are required for the deposition of the polysaccharide layer (Zhu et al. 1998; Huang et al. 2005; Wang et al. 2007).

The heterocyst glycolipid layer is assembled beneath the polysaccharide layer and is composed of fatty alcohols glycosidically linked to sugar residues. The *hglB*, *hglC*, *hglD*, and *hglE* genes along with a cluster of nearby genes are required for the synthesis of these glycolipids (Campbell et al. 1997; Fan et al. 2005). 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 (Fig. 5) (Ramirez et al. 2005). The *hglK* gene is required for the localization of the glycolipids and may be directly involved in their deposition (Black et al. 1995). ORF *all5341*, named *hglT*, is predicted to encode a glycosyl transferase and is required for the formation of the glycolipid layer (Awai and Wolk 2007).

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 (Fiedler et al. 1998; Moslavac et al. 2007a). The autolysin HcwA, which is presumably involved in cell wall remodeling, is required for heterocyst maturation (Zhu et al. 2001). The combination of autolysin activity and turgor pressure is presumably responsible for the slightly larger and rounder shape of heterocysts. The regulatory genes *hepK*, *hepN*, *henR*, and *hepS* are also required for normal heterocyst maturation (Lechno-Yossef et al. 2006).

Recent proteomic studies of the heterocyst and vegetative cell wall have identified proteins in the inner and outer membranes as a step toward understanding the dynamics of the cell wall proteome during cell differentiation

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(Moslavac et al. 2005; Moslavac et al. 2007b). Analysis of proteins of the outer membrane of vegetative cells and of heterocysts revealed a high similarity, suggesting that the relative protein concentrations within the outer membrane may vary between the two cell types but not the composition (Nicolaisen et al. 2009).

The cell-wall-related proteins can be categorized as proteins involved in (1) signal transduction, (2) synthesis, and (3) transport. Among the signal transduction proteins in *Anabaena* PCC 7120, histidine kinases and protein phosphatases have recently been shown to act downstream of NtcA and have been suggested to be involved in heterocyst cell wall formation (Wang et al. 2002; Cheng et al. 2006). In *Anabaena* PCC 7120, 131 genes encode putative histidine kinases, 52 genes code for serine/threonine and tyrosine kinases, and 13 genes code for a group of histidine and Ser/Thr hybrid (HSTKI) proteins (Ohmori et al. 2001; Phalip et al. 2001). Two genes, *pkn30* and *pkn44*, that encode HSTKI proteins are involved in regulation of HGL2 synthesis (Shi et al. 2007). A single mutant of either kinase has no obvious phenotype, but a double mutant has an immature HEP layer and is defective in synthesis of the HGL2. PrpJ, a Ser/Thr phosphatase encoded by *all1731*, is involved in the regulation of the HGL1 (Jang et al. 2007).

Among the proteins that define cell wall structure, OmpA, encoded by *all3289* and *alr5049*, acts as a physical linker between the outer membrane and the peptidoglycan layer. The *alr1278* gene encoding OstA/Imp is thought to be involved in remodeling of the lipid content of the outer membrane during heterocyst development (Nicolaisen et al. 2009). OstA/Imp is involved in transport of lipid A to the cell surface (Bos et al. 2004). Expression analysis showed that *alr1278* was up-regulated early following nitrogen deprivation (Ehira and Ohmori 2006b).

The outer membrane of *Anabaena* PCC 7120 includes integral membrane proteins and lipoproteins attached to the outer membrane by amino-terminal lipids (Nicolaisen et al. 2009). These proteins comprise porin-type proteins, proteins that define the cell wall structure, and proteins involved in transport and assembly (Nicolaisen et al. 2009). In *Anabaena* PCC 7120, All4499 and All4550, which show homology with the outer membrane porin OprB of *Pseudomonas aeruginosa*, were identified in membranes of vegetative cells and were the most abundant outer membrane proteins in heterocysts (Moslavac et al. 2005; Moslavac et al. 2007b). Genome-wide expression analysis of genes after nitrogen deprivation showed an increase in expression of *all4499* 8 hours after nitrogen deprivation and a decrease in expression of *all4550* 24 hours after nitrogen depletion. In addition, ORF *alr0834*, classified as encoding a general porin, is up-regulated during the early stages of heterocyst differentiation (Ehira and Ohmori 2006b).

A large number of outer membrane proteins are involved in protein transport and assembly. *all0406* encodes a protein with high similarity to autotransporters, which are Gram-negative extracellular proteins that are characterized by the ability to translocate themselves across the outer membrane (Nicolaisen et al. 2009). HgdD, a TolC like protein, is an outer membrane efflux channel and functions in HGL-layer assembly. The *hgdD* gene is up-regulated during heterocyst development and the protein is essential for HGL-layer formation. A *hgdD* knockout mutant is defective in deposition of the HGL layer and the phenotype is similar to the defect observed for a *devBCA* knockout. These data suggest the formation of a DevBCA-HgdD secretion complex that is essential for the formation of the HGL layer (Moslavac et al. 2007a).

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 (Fay 1992). However, some heterocyst-forming cyanobacteria contain an additional set of *nif* genes that allows nitrogen fixation in vegetative cells under anoxic conditions (Thiel et al. 1995). 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) (Rubio and Ludden 2008). 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 (Masepohl et al. 1997). In addition to the common molybdenum nitrogenase, some cyanobacteria contain an alternative nitrogenase that uses a vanadium cofactor (Pratte et al. 2006).

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 (Elhai and Wolk 1990; Golden et al. 1991). The *nifHDK* operon encodes the molybdenum-containing nitrogenase enzyme complex. Upstream of the *nifHDK* operon is another *nif* operon, *nifB-fdxN-nifS-nifU* (Mulligan and Haselkorn 1989). The *nifVZT* genes form a *nif* gene cluster separated from the main *nif* gene cluster (Stricker et al. 1997). Other cyanobacterial *nif* genes include *nifE*, *nifN*, *nifX*, and *nifW* (Wolk et al. 1994; Thiel et al. 1995). The transcriptional regulators that control the expression of the *nif* genes in cyanobacteria have not been identified.

A heterocyst-specific uptake hydrogenase, encoded by the *hupSL* operon, recovers reductant from H_2 produced by nitrogenase (Tamagnini et al. 2007). In *Nostoc punctiforme,* the upstream region of the *hupSL* operon contains an NtcA binding site and NtcA binds to the *hupSL* promoter in vitro, but deletion of the binding site did not affect the expression of *hupSL* significantly (Holmqvist et al. 2009). This study showed 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. Whereas NtcA is not required for transcriptional regulation of *hupSL* in *N. punctiforme*, NtcA is required for the induction of *hupSL* in *Anabaena variabilis* after nitrogen step-down, as shown by reduced induction of *hupSL* in an *ntcA* mutant (Weyman et al. 2008). A consensus NtcA-binding site is upstream of the *A. variabilis hupSL* operon, and NtcA binds to this region (Weyman et al. 2008).

Heterocyst development is accompanied by changes in the photosynthetic apparatus and carbon metabolism to provide ATP and low potential reductant for nitrogen fixation (Wolk et al. 1994; Meeks and Elhai 2002). 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 (Jones and Haselkorn 2002; Valladares et al. 2003).

In *Anabaena* PCC 7120, three DNA rearrangements take place during the late stages of heterocyst development that affect nitrogen fixation and uptake hydrogenase operons (Golden et al. 1985; Golden et al. 1988; Carrasco et al. 1995; Carrasco and Golden 1995; Golden 1997; Carrasco et al. 2005). 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 (Golden 1997). One rearrangement excises an 11-kb element from the *nifD* gene (Golden et al. 1985; Brusca et al. 1989). The excision requires *xisA* located on the 11-kb element (Golden and Wiest 1988; Brusca et al. 1990; Lammers et al. 1990; Henson et al. 2008). A *xisA* null mutant forms heterocysts but is unable to excise the element or grow on media lacking a source of combined nitrogen (Golden and Wiest 1988; Golden et al. 1991). A second rearrangement excises a 55-kb element from the *fdxN* gene (Golden et al. 1988) and requires the *xisF, xisH*, and *xisI* genes (Carrasco et al. 1994; Ramaswamy et al. 1997). A third programmed DNA arrangement deletes a 10.5-kb element from the *hupL* gene and requires the *xisC* recombinase gene (Carrasco et al. 1995; Carrasco et al. 2005). 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

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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 σ 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 uses specific sigma (σ) factors to activate a set of genes in response to environmental and intracellular signals (Campbell et al. 2008). For example, in *Bacillus subtilis*, temporally and spatially regulated σ factors modulate the expression of genes during starvation-induced sporulation (Kroos 2007; Campbell et al. 2008). It was hypothesized that global changes in gene expression during heterocyst development in *Anabaena* PCC 7120 are regulated by σ factors (Khudyakov and Golden 2001; Aldea et al. 2007). The σ 70 family of σ factors has been divided into 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 (Campbell et al. 2008). *Anabaena* PCC 7120 has 12 putative σ factors identified by sequence similarity (Aldea et al. 2007; Yoshimura et al. 2007). Genetic analysis has not identified any σ factor genes that are essential for heterocyst differentiation or nitrogen fixation, suggesting that there is a level of functional redundancy between the σ factors (Khudyakov and Golden 2001).

Genetic analysis using *gfp* as a reporter identified three σ-factor genes, *sigC*, *sigG*, and *sigE*, that are developmentally up-regulated after nitrogen deprivation (Aldea et al. 2007). The expression of *sigC* is up-regulated 4 hours after nitrogen step-down, and by 10 hours, the expression is restricted mostly to single cells in a heterocyst-like pattern (Fig. 4). SigC may be involved in regulating early heterocyst-specific genes (Aldea et al. 2007). The expression of *sigE* (alr4249) is low in vegetative cells grown in nitrate-containing media and is up-regulated in individual cells at about 16 hours after nitrogen depletion, 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 vegetative cells in media containing nitrate but decreases in all cells soon after nitrogen depletion. At around 9 hours after nitrogen step-down, increased *sigG* expression is localized to single cells and by 16 hours, expression is localized to morphologically distinguishable proheterocyst cells; at 24 hours, heterocysts show somewhat 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 (Aldea et al. 2007).

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 (Jelsbak and Søgaard-Andersen 2002), and *Pseudomonas aeruginosa* produces biofilms during infection (Klausen et al. 2003). Temporary patterns have been studied in *Escherichia coli* and *Salmonella typhimurium* when these organisms swim in gradients of nutritional chemoattractant (Blair 1995; Budrene and Berg 1995). 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 (Wilcox et al. 1973; Wolk 1996). 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 (Fig. 5) (Yoon and Golden 1998; Yoon and Golden 2001). 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 carboxy-terminal ends (Zhang et al. 2009). 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 up-regulated by 6 hours after nitrogen deprivation (Yoon and Golden 1998). A *patS-gfp* reporter strain in nitrate-containing media showed low levels of expression in all cells. At 8–10 hours after nitrogen step-down, increased fluorescence was localized to small groups of cells or individual cells (Yoon and Golden 2001). By 12–14 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 Fig. 2).

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, whereas extra copies or overexpression of *patS* results in a complete suppression of heterocysts (Yoon and Golden 1998; Yoon and Golden 2001). 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, whereas a four amino acid synthetic peptide did not inhibit differentiation (Yoon and Golden 1998). 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 (Golden and Yoon 2003). 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 (Wu et al. 2004). 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 (Wu et al. 2004). 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 (Huang et al. 2004). Up-regulation of *hetR* expression is abolished when PatS-5 is added to the growth medium (Huang et al. 2004). *hetR* mutant *hetR*$_{\mathrm{R223W}}$ is insensitive to the PatS inhibitory signal and overexpression of the *hetR*$_{\mathrm{R223W}}$ allele results in a conditionally lethal phenotype after nitrogen deprivation because nearly all cells differentiate (Khudyakov and Golden 2004).

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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 (Orozco et al. 2006).

The *hetN* gene encodes a protein similar to ketoacyl reductase. Like *patS*, overexpression of *hetN* gene results in complete suppression of heterocyst development (Fig. 5). 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 (Callahan and Buikema 2001). 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 (Borthakur et al. 2005). Together, *patS*- and *hetN*-mediated inhibitory pathways are the primary mechanism for establishing heterocyst pattern.

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 (Golden and Yoon 2003; Sakr et al. 2006; Aldea et al. 2008). It is known that single cells from fragmented 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 (Zhao and Wolk 2008). There is some evidence for the products of nitrogen fixation supplied from heterocysts contributing to the average spacing between heterocysts along filaments (Yoon and Golden 2001; Aldea et al. 2008). 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 (Thiel and Pratte 2001). 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.

In *N. punctiforme*, the novel genes *patU* and *patN* are required to inhibit excess heterocyst differentiation (Meeks et al. 2002). In *Anabaena* PCC 7120, a cluster of genes that influence pattern formation and heterocyst differentiation, *hetZ*, *patU5*, and *patU3*, is conserved among heterocyst- and nonheterocyst-forming filamentous cyanobacteria (Zhang et al. 2007). All three genes are up-regulated after nitrogen deprivation and encode proteins with unknown function. A *hetZ* mutant does not differentiate heterocysts, unlike a *patU3* mutant, which forms multiple contiguous heterocysts. When *patU3* is inactivated in a *patA* mutant background, the *patA* mutant phenotype is abolished, restoring the formation of intercalary heterocysts (Zhang et al. 2007). Although these genes are clearly involved in the regulation of heterocyst development, their biochemical functions remain to be determined.

CONCLUDING REMARKS

Cyanobacteria have evolved several differentiated cell types in addition to heterocysts, including akinetes and the cells of hormogonia, to perform specialized functions. In *N. punctiforme*, vegetative cells have three developmental fates: heterocysts, akinetes, and hormogonial cells (Meeks et al. 2001; Meeks et al. 2002). Akinetes are perennating sporelike cells formed to endure cold and desiccation. Heterocysts and akinetes show pattern formation that must be regulated by cell-to-cell communication between cells within a filament. In the absence of heterocysts, akinetes form at random positions along a filament, whereas the presence of heterocysts influences akinete placing. In some organisms, akinetes differentiate from cells immediately adjacent to heterocysts, and in others chains of akinetes are produced in which the youngest are furthest away from the heterocysts. Hormogonia are typically short filaments of differentiated cells that lack heterocysts and often express gas vesicles and show gliding motility. One role of hormogonia is

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to disperse and inhabit new environments (Tandeau de Marsac 1994; Meeks and Elhai 2002). Although our knowledge of these additional forms of cyanobacterial development are limited compared with our understanding of heterocyst development, it is evident that cyanobacteria are capable of striking and complex multicellular development that involves the integration of environmental, extracellular, and intracellular signals to control cellular differentiation and morphogenesis. The study of multicellular developmental biology in cyanobacteria, as well as other bacterial developmental systems, has uprooted the common misconception of bacterial organisms as simple autonomous single cells.

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