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The Making of a Heterocyst in Cyanobacteria

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Abstract

Heterocyst differentiation that occurs in some filamentous cyanobacteria, such as *Anabaena* sp. PCC 7120, provides a unique model for prokaryotic developmental biology. Heterocyst cells are formed in response to combined-nitrogen deprivation and possess a microoxic environment suitable for nitrogen fixation following extensive morphological and physiological reorganization. A filament of *Anabaena* is a true multicellular organism, as nitrogen and carbon sources are exchanged among different cells and cell types through septal junctions to ensure filament growth. Because heterocysts are terminally differentiated cells and unable to divide, their activity is an altruistic behavior dedicated to providing fixed nitrogen for neighboring vegetative cells. Heterocyst development is also a process of one-dimensional pattern formation, as heterocysts are semiregularly intercalated among vegetative cells. Morphogens form gradients along the filament and interact with each other in a fashion that fits well into the Turing model, a mathematical framework to explain biological pattern formation.

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INTRODUCTION

Cyanobacteria are gram-negative, oxygen-evolving photosynthetic prokaryotes (98). The appearance of cyanobacteria with their oxygen-producing photosynthetic capacity was one of the greatest events on Earth, shaping the course of evolution with the accumulation of oxygen (90, 92). Diazotrophic cyanobacteria occur widely, free-living or symbiotically, and are major players in the nitrogen cycle (127). Nitrogen fixation is catalyzed by nitrogenase, which is oxygen labile (110). How, then, do nitrogen-fixing cyanobacteria reconcile the two incompatible biochemical reactions, oxygenic photosynthesis and nitrogen fixation? Cyanobacteria evolved two innovative strategies to separate the two activities: temporal and spatial separation. Most unicellular diazotrophic cyanobacteria, such as marine *Synechococcus* and *Crocosphaera* strains, use the circadian clock as the major mechanism and perform photosynthesis during the day and nitrogen fixation during the night (94). Filamentous cyanobacteria such as those from the orders of Nostocales and Stigonematales rely on morphologically and metabolically differentiated cells, called heterocysts, for nitrogen fixation (82, 110). Other filamentous cyanobacteria such as *Trichodesmium* use both strategies, although without morphological cell differentiation (9). The most frequently used genetic model for studying heterocyst development is *Anabaena* (*Nostoc*) sp. PCC 7120 (*Anabaena* hereafter) (**Figure 1**). A few other strains, especially *Anabaena variabilis* ATCC 29413 and *Nostoc punctiforme* ATCC 29133/PCC 73102 (*N. punctiforme* hereafter), are also used.

Heterocyst formation is unique in prokaryotic developmental biology, for two reasons. First, a filament has true multicellular and organismic behaviors. Different cells along the filaments not only exchange nutrients and signals (34, 36, 41) but also possess synchronized circadian clocks that tick along together for the expression of at least one reporter gene tested (1). In addition, these cells share a common outer membrane and a continuous periplasm along a filament (64). The function of such a periplasmic organization remains unknown. Second, although some strains form heterocysts only at the end of a filament (82), for *Anabaena*, individual heterocysts are intercalated among vegetative cells and have a Gaussian-type distribution pattern along the filament (110). Heterocysts are terminally differentiated, without cell-division capacity. With vegetative cell division, a new heterocyst forms, generally at mid-distance between the two existing heterocysts. Therefore, heterocyst distribution along *Anabaena* filaments constitutes a one-dimensional pattern. Heterocysts provide fixed nitrogen to surrounding vegetative cells, and in turn they receive fixed carbon from the latter as a reductant (34, 110).

Heterocyst formation allows *Anabaena* to grow diazotrophically under aerobic conditions. To do so, extensive morphological and metabolic changes take place during development. At the

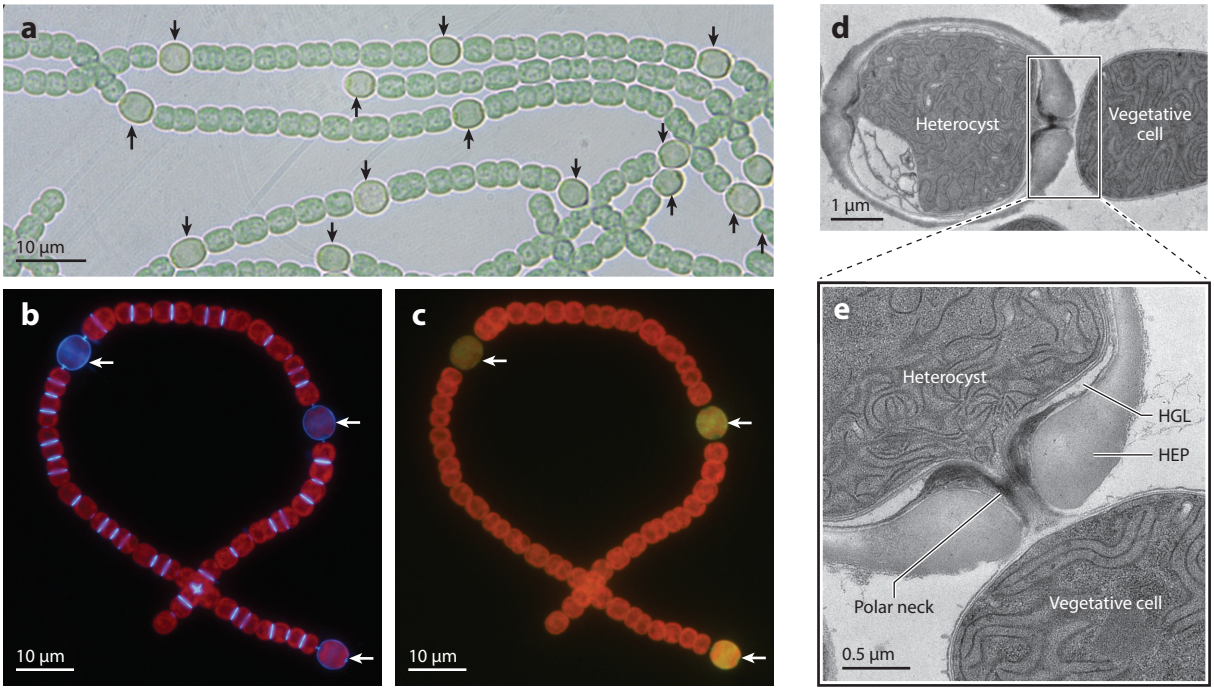

Figure 1

Anabaena sp. PCC 7120 filaments cultured under diazotrophic conditions. After deprivation of combined nitrogen (20–24 h), heterocysts are formed to perform nitrogen fixation. (*a*) Filaments imaged by light microscope. Heterocysts (*arrowheads*) are morphologically different from vegetative cells and are intercalated, in a semiregular manner, among vegetative cells. (*b*,*c*) Fluorescence microscopy of *Anabaena* filaments. Vegetative cells contain photosynthetic pigments that fluoresce red, whereas heterocysts (*arrowheads*) contain much less pigment and appear a dim fluorescent red. During differentiation, heterocysts form particular structures at the cell envelope to ensure a microoxic environment suitable for nitrogen fixation. (*b*) The peptidoglycan layers are labeled in bright blue by HADA in heterocysts, and vegetative cells are preferentially marked by HADA at the division sites. (*c*) Green fluorescent protein is expressed under the control of a heterocyst-specific promoter. Strongly differential gene expression occurs in heterocysts in comparison to vegetative cells. (*d*,*e*) Electron microscope images of *Anabaena* cells. A heterocyst and a vegetative cell are shown in panel *d*, and a part of the image is enlarged in panel *e*. Note the outer HEP layer, inner HGL layer, and protruding neck in connection with the neighboring vegetative cell. Abbreviations: HADA, 7-hydroxycoumarin-amino-D-alanine; HEP, heterocyst exopolysaccharide; HGL, heterocyst glycolipid.

morphological level, heterocysts form two extra layers deposited outside the outer membrane, a heterocyst glycolipid (HGL) layer and a heterocyst exopolysaccharide (HEP) layer (**Figure 1**). The HGL layer limits oxygen diffusion from outside, and the HEP layer protects the fragile HGL layer (110). Heterocysts also have strengthened peptidoglycan (PG) layers (74). As a consequence of the morphological changes, combined with metabolic reorganization as discussed in the section titled Morphogenesis and Maturation, heterocysts create an internal microoxic environment suitable for nitrogen fixation. Here, we concentrate on more recent advances. Readers may refer to other excellent reviews (34–37, 41, 54, 63, 66, 74, 110, 114).

MAJOR STEPS OF HETEROCYST DIFFERENTIATION

In this section, we review the molecular mechanisms driving different steps of heterocyst differentiation, which lasts about 20–24 h from initiation until maturation.

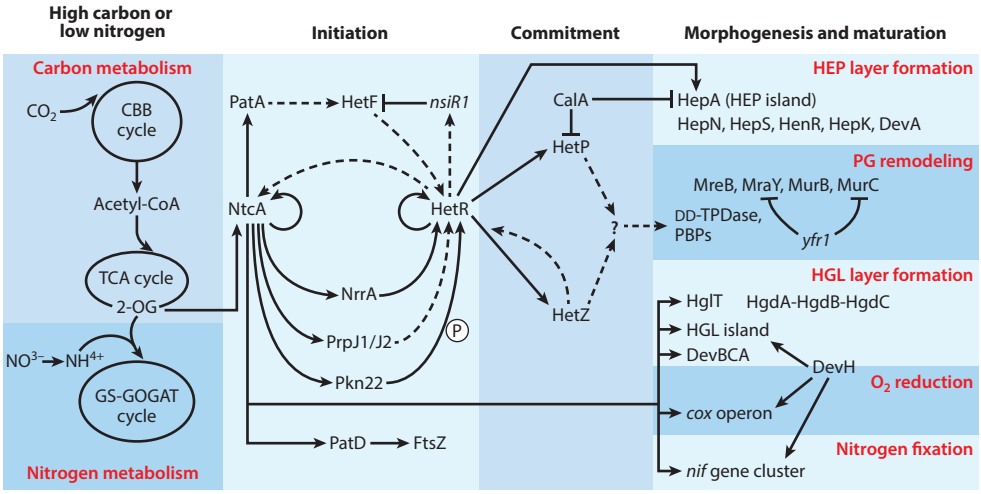

Figure 2

Regulation networks shaping major steps of heterocyst development in *Anabaena* sp. PCC 7120. Heterocyst formation depends on carbon and nitrogen regimes. Combined-nitrogen deprivation (low nitrogen) or oversupply of carbon leads to an accumulation of 2-OG, which acts on NtcA for the initiation of heterocyst differentiation. NtcA and HetR, two transcription factors, orchestrate the developmental program of heterocyst formation. Commitment occurs at the point when the developmental process becomes irreversible. During maturation, the heterocyst envelope is reorganized, with the deposition of the HEP layer and HGL layer and the strengthening of the PG layers. These morphological changes, together with physiological modifications such as increased respiration for O_2 reduction, create an internal microoxic environment suitable for nitrogen fixation. For simplicity, we do not distinguish genes and proteins for the networks, which involve regulation at multiple levels. For regulatory elements involved in heterocyst patterning, see **Figures 4** and **5**. Dashed arrows and question mark indicate steps still lacking experimental support. Note that cyanobacteria in general lack the 2-OG dehydrogenase, and the TCA cycle is an open one. Abbreviations: 2-OG, 2-oxoglutarate; CBB, Calvin-Benson-Bassham; DD-TPDase, DD-transpeptidase; GS-GOGAT, glutamine synthetase glutamine-oxoglutarate amidotransferase; HEP, heterocyst exopolysaccharide; HGL, heterocyst glycolipid; PBP, penicillin-binding protein; PG, peptidoglycan; TCA, tricarboxylic acid.

Initiation

In *Anabaena*, as both differentiation and nitrogen fixation are costly, heterocyst formation occurs only in the absence of a combined-nitrogen source, such as nitrate or ammonium. It is increasingly evident that it is the nitrogen-carbon balance that triggers this process, not just nitrogen deprivation as it is generally presented (**Figure 2**). The trigger for heterocyst differentiation is accumulation of 2-oxoglutarate (2-OG), one of the nitrogen-carbon balancing signals (119). Indeed, when supplied with a high level of carbon dioxide, *Anabaena* can also differentiate heterocysts even in the presence of a combined-nitrogen source (39, 50).

The 2-OG signal is perceived by NtcA, a highly conserved cyanobacterial transcription factor belonging to the CRP (cAMP receptor protein) family (56, 128). Genes under the control of NtcA are involved not only in nitrogen metabolism and heterocyst differentiation but also in other metabolic pathways, such as DNA metabolism, iron acquisition, carbon metabolism, photosynthesis, and stress responses (80). 2-OG enhances the DNA-binding efficiency of NtcA and allows transcriptional initiation of genes under the control of NtcA (56, 99). Structural studies indicate that NtcA forms a homodimer, and the binding of 2-OG at the effector-binding domain

of each monomer reduces the distance between the two DNA-recognizing F helices from 37 Å to 34 Å, for a perfect interaction with DNA targets (128).

NtcA activates, directly or indirectly, several key regulatory genes required for the initiation of heterocyst differentiation, including *hetR* (**Figure 2**). Both *ntcA* and *hetR* are autoregulated, and each positively regulates the expression of the other (11, 72). This regulation loop is key for heterocyst initiation, but the precise mechanism underlying the mutual regulation of *ntcA* and *hetR* remains poorly understood. NtcA activates *hetR* indirectly. The nitrogen response regulator NrrA is one of the links between NtcA and HetR (29, 30). Under nitrogen starvation, NtcA directly upregulates the expression of *nrrA*, and NrrA enhances *hetR* expression and heterocyst formation. However, NrrA is not the only intermediate between NtcA and HetR, because heterocyst differentiation and *hetR* upregulation are not abrogated in an *nrrA*-deficient mutant (29, 30). The protein kinase Pkn22 and two homologous phosphatases, PrpJ1 and PrpJ2, are also possible links between *ntcA* and *hetR* regulation (48, 85). It is not known how HetR regulates *ntcA* expression.

While NtcA is essential but not sufficient for heterocyst differentiation, HetR is both necessary and sufficient for the initiation of heterocyst differentiation. Indeed, inactivation of *hetR* leads to the failure of heterocyst differentiation, and its overexpression promotes heterocyst development even under repressive conditions (16, 17), suggesting that HetR is the master regulator. *hetR* is expressed at a basal level in vegetative cells and activated early in developing cells (11, 16). As a DNA-binding protein, HetR directly or indirectly upregulates hundreds of genes involved in heterocyst differentiation, as well as some targets unrelated to heterocysts (33, 47, 69, 103). This is consistent with the fact that HetR is conserved in filamentous cyanobacteria with or without heterocyst formation (31). In vitro, HetR binds to the upstream regions of several genes important for heterocyst differentiation, such as *hetZ*, *hetP*, *hepA*, and *hetR* itself (27, 42, 53, 87).

Structural analyses of the HetR dimer provided much insight into the molecular mechanism of HetR action, which is described below in the section titled Pattern Formation. Both dimeric and tetrameric forms of HetR can bind to DNA in vitro (53). HetR may be regulated by phosphorylation, although contradictory results have been published. One report found that purified HetR from *Escherichia coli* was autophosphorylated at the Ser179 residue, and the phosphorylation negatively regulated the formation of functional tetrameric HetR (101). Another study identified Pkn22 as a protein kinase for HetR and suggested that phosphorylation has a positive role on HetR activity (85). Pkn22 interacted with HetR and phosphorylates the latter at the Ser130 residue when both Pkn22 and HetR were coproduced in *E. coli* (85). More studies are needed to clarify the sites and roles of phosphorylation of HetR and the kinase(s) involved. Note that the available three-dimensional structures of HetR do not reveal an ATP-binding motif or a protein kinase fold (45, 52, 53).

NtcA and HetR are not the only players in the initiation of heterocyst differentiation. The second messenger ppGpp and the gene *hanA*, which encodes the histone-like DNA-binding protein HU, are also essential for the initiation of heterocyst differentiation (51, 125), but their mechanisms are unknown. Other components, such as PatA and HetF, have also been identified (60, 83, 111). HetF was predicted to be a caspase-type protease; inactivation of *hetF* results in no heterocyst formation, whereas overexpression of *hetF* leads to a multiple contiguous heterocyst (Mch) phenotype (83, 111). The *hetF* gene is expressed in vegetative cells but downregulated in developing cells at the late developmental stages (113). In addition to the failure to initiate heterocyst differentiation, the *hetF* deletion mutant has a marked cell-division phenotype, particularly when light levels are high (113). HetF acts at the step of septal PG synthesis as a component of the divisome (113). An array of 12 antisense RNAs, *nsiR1*, overlaps the promoter region of *hetF* and is expressed early in developing cells in an *ntcA*- and *hetR*-dependent manner (14). Although

high-level expression of *nsiR1* represses the expression of *hetF* and produces a *hetF*-deficient mutant phenotype (14), removal of all copies of *nsiR1* does not affect the expression of *hetF* or give a noticeable phenotype (113).

PatA is composed of an N-terminal PATAN domain and a C-terminal CheY-like domain. In *Anabaena*, *patA* is directly activated by NtcA (6). Inactivation of *patA* leads to heterocyst differentiation almost exclusively at the ends of the filaments, and *patA* overexpression increases the heterocyst frequency and causes aberrant vegetative cell morphology (83, 118). On the basis of genetic relationships, it was proposed that PatA antagonizes the inhibitory action of PatS and HetN, so that intercalary heterocysts can arise (79). The formation of terminal heterocysts in the *patA*-deficient mutant was explained by the fact that a cell at the end of the filaments receives inhibitory signals (see the section titled Pattern Formation) from only one side of a filament.

The mechanism of PatA and HetF in heterocyst differentiation remains obscure. These two regulatory elements have much in common, although they display opposite expression patterns, with HetF present in vegetative cells and PatA in developing cells (113, 118). First, in the absence of either HetF or PatA, a high level of HetR accumulates in the filaments (83, 111), which would be expected to cause a high level of differentiation; however, the *hetF*-deficient mutant lacks heterocysts, and the *patA*-deficient mutant forms heterocysts almost exclusively at the end of the filaments (60, 83, 111). Second, HetF works downstream of PatA in the same genetic pathway, as overexpression of *hetF* in the *patA*-deficient mutant restores a normal phenotype (83). Third, both PatA and HetF can interact with some components of the divisome (102, 113). However, because of the differential expression pattern of the two corresponding genes, PatA was proposed to interact with the divisome in developing cells in order to arrest cell division and promote heterocyst differentiation (102), whereas HetF participates directly in vegetative cell division (113). Functional cell division is necessary for the initiation of heterocyst differentiation (89); therefore, understanding the link between cell division and heterocyst differentiation may be key to determining the molecular mechanism of PatA and HetF in heterocyst differentiation. The regulation of cell division may play at least two important functions in heterocyst development: first, the management of cell-division arrest for developing heterocysts, and second, the coordination of late-stage cell division with the establishment of nanopores across the septal PG layer for cell-cell communication (see the section titled Cell-Cell Communication). Furthermore, the cell-division protein FtsZ is also suggested to play a role in both cell division and heterocyst differentiation, and PatD interacts with FtsZ in order to control the activity of FtsZ in developing cells (107).

Commitment

Following initiation, heterocyst differentiation advances to the commitment step, defined as the point of no return in the developmental process (**Figure 2**). Experimentally, it is the point, ~8 h after initiation, when addition of a combined-nitrogen source can no longer push developing cells back to a vegetative state (117). Several key genes involved in this step have been identified, including *hetZ*, *hetP*, and *hetP* homologs (104, 105, 120, 126). Both *hetZ* and *hetP* are directly under the transcriptional control of HetR (27, 33). Inactivation of *hetP* or *hetZ* delays heterocyst differentiation, and overexpression of *hetP* or *hetZ* results in the accumulation of supernumerary heterocysts both under nitrogen-deprivation and repressive conditions (104, 105, 120).

The relationship between *hetP* and *hetZ* reported by different studies is controversial. One view is that HetZ acts upstream of HetP, because the overexpression of *hetZ* cannot overcome *hetP* inactivation in heterocyst differentiation (105). However, another view is that HetP and HetZ functionally overlap each other, because overexpression of HetP can restore heterocyst differentiation in *hetZ*-deficient mutants and vice versa (120). In addition, overexpression of HetR can recover

the heterocyst differentiation in *hetZ*-deficient mutants and *hetP*-deficient mutants, but not in a *hetZ* and *hetP* double-deficient mutant. Functionally redundant and overlapping roles of these two genes are also consistent with results showing that ectopic expression of *hetP* and *hetZ* together partially bypass the requirement of *hetR* for heterocyst formation (120, 121). HetP and HetZ show no similarity to each other. HetP interacts with its homologs (Asl1930, Alr2902, and Alr3234), and these homologs play a role in delaying the commitment step or inhibiting the development of heterocysts (104). *hetP* is repressed by a transcriptional regulator, CalA/cyAbrB1, in the presence of combined-nitrogen sources (44). HetP has nonspecific DNA-binding activity in vitro, while HetZ can interact with HetR and was predicted to be a DNA-binding protein (120). The *hetZ* gene is found within a gene cluster including *patU3*, and *hetZ* and *patU3* display antagonistic actions in heterocyst differentiation and pattern formation (126); the mechanism is also unknown. HetZ has positive-feedback control of the expression of *hetR* and *patS*, a regulatory loop that may serve as a signal amplifier of HetR and PatS, so that these morphogens reach threshold concentrations critical for developmental commitment (28). Indeed, HetR levels may be a key determinant of the commitment step, as ectopic high-level expression of HetR can bypass the commitment step with heterocysts formed in the presence of a combined-nitrogen source (17). However, HetZ and HetP must have unknown downstream targets because *hetR* overexpression in the *hetZ* and *hetP* double-deficient mutant does not trigger heterocyst differentiation (120). Therefore, HetZ and HetP have a relay function between initiation and morphogenesis, but the molecular mechanism remains obscure.

Morphogenesis and Maturation

Once a cell is committed to differentiation, genes required for the synthesis and deposition of heterocyst-specific cell envelope layers are expressed, leading to the formation of the HGL layer and the HEP layer (110) (**Figure 2**). HEP is composed of β-1,3-linked mannosyl-glucosyl-glucosyl-glucose tetrasaccharide (the main chain) and mannose, glucose, glucosyl glucose, galactose, or xylose (side chains). Recently, it was found that HepP, a putative membrane protein that belongs to the major facilitator superfamily, is involved in the transport of glycoside(s) and participates in the formation of the HEP layer (75). The activation mechanism of HEP production is still poorly understood, and it seems to require several regulators. The *hepA* gene is activated by HetR under diazotrophic conditions and is repressed by CalA/cyAbrB1 in the presence of combined-nitrogen sources (44, 103). In addition, several signal transduction systems are essential for HEP deposition, including HepN (histidine kinase), HepS (serine/threonine kinase), HenR (response regulator), and the two-component system HepK-DevR$_A$ (for details, see reviews in References 36 and 74).

The HGL layer consists of long-chain polyhydroxy alcohols that are glycosidically linked to hexose. In *Anabaena*, two kinds of HGL have been identified: major and minor (38). A large number of proteins participate in HGL synthesis and localization (63). The gene cluster *alr5351–alr5357* (annotated as *hglE$_A$*, *hglF*, *hglG*, *hglD*, *hglC*, *hglA*, and *hglB*) in *Anabaena* encodes enzymes associated with the biosynthesis of HGL aglycones (63, 88). *hglT*, which encodes a heterocyst glycolipid synthase, catalyzes the final step of HGL biosynthesis that transfers glucose to the fatty alcohol (40). Four genes of a second gene cluster (*all5343–all5347*), encoding All5343-HgdA-HgdB-HgdC, are essential for synthesis and proper deposition of the HGL layer (96). Two export systems have been suggested for deposition of HGL in order to cover the entire heterocyst: one composed of HgdBCD for export at the lateral sides and another composed of DevBCA and HgdD for heterocyst polar necks, where a protruding and particularly thick layer of HGL is found (95) (**Figure 1**). HGL formation is regulated by different pathways. For example, a PP2C (protein

phosphatase 2C)-type membrane protein phosphatase is encoded by *prpJ1*, and in *prpJ1*-deficient mutants, only the minor HGL was present (48, 49). *pkn30* and *pkn44* encode protein kinases of the HstK family, and in the *pkn30pkn44* double-deficient mutant, only the major HGL was present (93). In an *hglK*-inactivated mutant, both HGLs are synthesized but not deposited outside of the cells (2, 10). Partial inactivation of *devH*, which encodes a CRP-family transcriptional regulator, results in the absence of both HGLs and affects the expression of several genes involved in the later stages of heterocyst differentiation, such as the *cox* operons, the *hgl* island, and the *nif* gene cluster (55). Therefore, DevH appears to be an important regulator for the coordination of heterocyst morphogenesis and maturation.

Active remodeling of the PG layers takes place at the end of heterocyst morphogenesis, as shown by HADA (7-hydroxycoumarin-amino-D-alanine, a fluorescent D-alanine analog) labeling (122) (**Figure 1**). Several mutations affecting PG synthesis lead to defective heterocyst differentiation. These mutations are related to PG-synthesis genes *murC* and *murB*; PG-elongation gene *alr5045* (encoding a DD-transpeptidase); PG-formation and -maintenance genes *pbpB*, *all2981*, *alr4579*, and *alr5051* (encoding penicillin-binding proteins); and PG precursor–synthesis gene *mraY* (19, 57, 62, 106). The expression of *mraY* and *murC* is negatively regulated by a conserved, cyanobacterial small noncoding RNA, Yfr1 (15). Overexpressing Yfr1 in *Anabaena* led to filament breakage at the connections between vegetative cells and heterocysts. This phenotype may be caused by incorrect PG synthesis at the cell wall, since the PG layers of neighboring cells are connected (15). PG synthesis in most bacteria is carried out by the divisome and elongasome complexes, under the control of FtsZ and MreB, respectively. Heterocyst PG synthesis is at least partly mediated by the interaction of PatD with FtsZ in developing cells (107).

At the metabolic level, mature heterocysts display extensive differences with vegetative cells (more details can be found in a review in Reference 110) (**Figure 2**). In essence, mature heterocysts express genes related to nitrogen fixation and supply fixed nitrogen to the filament. Photosystem II, which is responsible for oxygen production, is absent in heterocysts, and photosystem I produces ATP to support nitrogen fixation in these cells. This metabolic property, coupled to enhanced respiration, helps to create a microoxic environment within heterocysts that is suitable for nitrogen fixation. Heterocysts cannot fix carbon dioxide because of the absence of photosystem II; thus, the reductant necessary for nitrogen fixation is supplied by vegetative cells.

CELL-CELL COMMUNICATION

Intercellular communication is indispensable for the coordination of not only the developmental process but also filament growth. The continuous periplasmic space has been proposed as a possible channel for intercellular communication, but the little evidence that is available is contradictory (64, 123). By contrast, tremendous progress has been made on the channels connecting two neighboring cells for intercellular communication (**Figure 3**). The use of small fluorescent markers, the intercellular movement of which can be tracked and quantified by fluorescence recovery after photobleaching (FRAP), has been the key to unraveling the mechanism of such intercellular exchanges (70, 76).

Intercellular structures have long been observed with electron microscopes (36). The first solid evidence for the existence of cell-cell communication structures was the discovery of nanopores, present in isolated septal PG disks (58). These nanopores separating vegetative cells are ~20 nm in diameter (36). By electron tomography combined with potassium permanganate staining, proteinaceous structures perpendicular to the cytoplasmic membrane of the adjacent cell were observed in the junctions between vegetative cells (109). Later, cryopreservation combined with electron tomography revealed that the structures are channels that traverse the septal PG layers

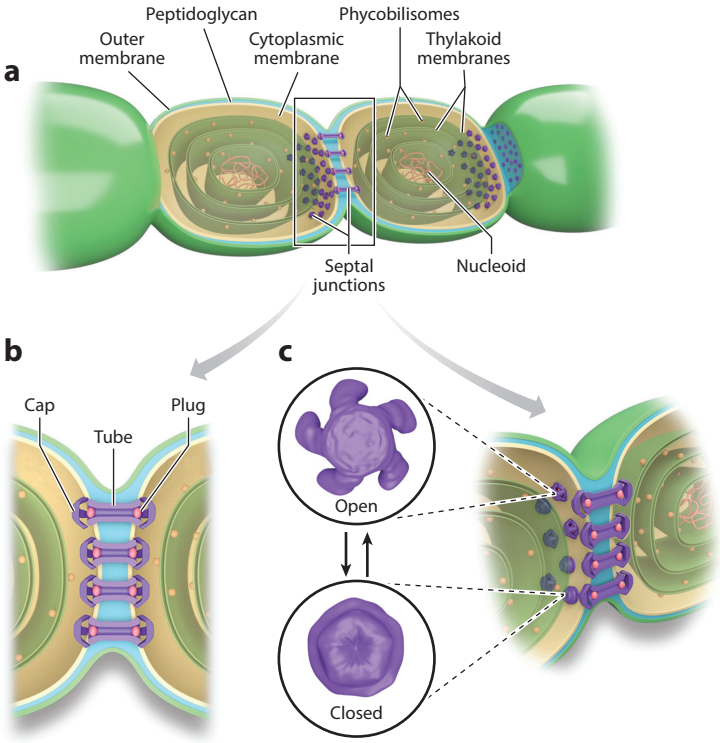

Figure 3

Models of cell-cell communication structures in *Anabaena* sp. PCC 7022. (*a*) Schematic view of SJs, which serve as cell-cell communication channels in *Anabaena* filaments. Note that the cells share a common outer membrane along the filament, whereas each cell is surrounded by a cytoplasmic membrane and PG layers. At the intercellular septal disks, adjacent cells have thick, connected PG layers. For *Anabaena*, 40–60 SJs per septal disk are found, and they are gated in response to environmental conditions. (*b*) Detailed view of the longitudinal section of SJ complexes enlarged from the region framed in panel *a*. An SJ complex has three modules: tube, plug, and cap. The length of an SJ depends on the thickness of the cell septum. SJs are not drawn in proportion to the size of the septal disk. (*c*) Schematic lateral view of the septal disk region framed in panel *a*. The conformation of the caps of the septal junctions switches between open and closed in response to environmental conditions. Abbreviations: PG, peptidoglycan; SJ, septal junction.

in *Anabaena* (77). These structures are similar to metazoan gap junctions and were given different names in the past, such as microplasmodesmata, septosomes, and channels. They are now called septal junctions (SJs) (35). The SJs are 12 nm in diameter, smaller than the nanopores described above. Therefore, they are proposed to be embedded in nanopores as a direct conduit between two adjacent cells. The length of SJs depends on the thickness of the cell septum, indicating a flexible assembling process (77, 108). The in situ and near-native architecture of the SJ complex was revealed by using electron cryotomography of cryo-focused ion beam–milled cyanobacterial filaments (108). An SJ consists of three parts: a tubelike channel traversing the septal PG, a plug at both ends of the tube, and a fivefold-symmetric cytoplasmic cap covering the plug (108) (**Figure 3**).

PG hydrolases are involved in the establishment of nanopores. In *N. punctiforme*, inactivation of the cell wall amidase AmiC2 leads to pleiotropic effects including absence of nanopore array and intercellular molecular exchange, irregular cell division planes, and impaired cell differentiation (20, 59). AmiC2 is involved in drilling the PG layer for the establishment of nanopores in

N. punctiforme. A homolog of AmiC2 from the same organism, AmiC1, seems essential for growth, and its roles in nanopore formation are unknown (20, 59). In *Anabaena*, three cell wall amidases have been identified: AmiC1, AmiC2, and AmiC3. Inactivation of any of the three corresponding genes results in a reduction of the intercellular-molecular exchange rate under nitrate regime (8, 12, 129). *amiC1*- or *amiC2*-deficient mutants display reduced nanopore numbers, while *amiC3*-deficient mutants have smaller nanopores than the wild type. *amiC1*-deficient mutants cannot grow under diazotrophic conditions because of reduced nitrogenase activity, and they take longer to form mature heterocysts than the wild type does (8, 12, 129). The LytM factor Alr3353 interacts with AmiC1 and enhances its hydrolytic activity (13). *amiC3* mutants were unable to grow diazotrophically, and no mature heterocysts were observed in the absence of combined nitrogen. By contrast, *amiC2* mutants can grow normally under nitrogen-fixing conditions. These studies suggest redundant functions of amidases (8, 12, 129).

Genetic studies have identified several proteins involved in SJ formation that are also necessary for filament integrity, as the corresponding mutants show often fragmented filaments (phenotype Fra). Inactivation of *sepJ* (previously known as *fraG*) impaired intercellular transfer of fluorescent markers and heterocyst differentiation (65, 70, 73). The number of nanopores at the septal PG disks was 50% lower in the *sepJ*-deficient mutant and increased following *sepJ* overexpression (65). Although SepJ was found at cell-cell junctions, it is not an SJ component, because observation by electron cryotomography showed the SJ structure was unaffected after *sepJ* deletion (108). SepJ has a PG-binding motif and may play a role in coordinating septum maturation at the late stages of cell division and precise amidase-dependent placement of nanopores traversing the septal PG layers (81). SepJ interacts with the cell-division proteins FtsQ and ZipN and the recently identified divisome protein SepI (22, 97). Similar to SepJ, SepI is required for filament integrity, cell morphology, proper septum size, and nanopore formation (97). Another PG-binding protein, SjcF1, may play a similar role as SepJ (86). The *sjcF1*-inactivated mutant displays similar phenotypes as the *sepJ*-deficient mutant in intercellular molecular transfer and SJ structure, and SjcF1 interacts with SepJ and FraC (discussed later in this section). Inactivation of *sjcF1* produces SJ channels with larger and more variable diameters compared with those of the wild type. Its role in septum maturation is to restrict the diameters of the SJ channels to a more uniform range (86). The interaction network of SepJ, SepI, and SjcF1 ensures coordination between SJ formation at the septal PG disks and cell division. In addition, the formation of nanopores and SJs seems to be coordinated with formation of the heterocyst envelope. HepP, which is necessary for HEP layer formation, is required for the proper localization of SepJ and nanopore formation (75). HglK, which is required for HGL layer formation, is found predominantly at the cell-cell junctions and participates in nanopore formation (2).

The membrane proteins FraC and FraD, which are encoded by the conserved *fraCDE* operon found in filamentous cyanobacteria, are also important for SJ formation (3, 7, 78). Inactivation of *fraC* or *fraD* in *Anabaena* produces a Fra phenotype, affects fluorescence marker transfer (especially under diazotrophic conditions), and reduces nanopore number (3, 78). These mutants form heterocysts but are unable to support diazotrophic growth (7, 68). The number of nanopores in the *fraCfraD* double-deficient mutant was dramatically lower, only 6% of that in the wild type (3). Visualized by electron cryotomography, the SJ structure of the *fraD*-deficient mutant lacks the plug module, that of the *fraCfraD* double-deficient mutant lacks the cap and the plug modules, and that of the *fraC* mutant presents a mixture of partially or completely assembled SJ structures (108). These results suggest that FraD is a structural component of SJs and FraC is involved in their assembly (108). FraE, however, is localized at heterocyst poles, and the *fraE*-deficient mutant cannot form a heterocyst neck (**Figure 1**), which normally has SJs located at its polar PG disks (3). Inactivation of *fraE* also reduced nanopore number on the heterocyst septal PG disks and the

transfer rate of a fluorescence marker from vegetative cells to heterocysts. FraE is involved mainly in heterocyst maturation, which is necessary for SJ formation (3).

Genetic studies on SepJ on the one hand and FraC and FraD on the other led to an initial suggestion that two types of SJ complexes existed, one related to SepJ and another related to FraCD, because the transfer rates of the fluorescent markers calcein and 5-CF were impaired differently in the *sepJ*- and *fraCfraD*-deficient mutants (76). However, this view is not supported by recent studies using electron cryotomography that revealed only a single type of SJ in *Anabaena* (108). The difference in cell-cell communication between the two mutants can be explained by the differences in SJ structure and nanopore number. Several other proteins are also involved in cell-cell communication machinery, although the mechanism is unclear. Inactivation *glsC*, *glsP*, or *hepP*, all of which encode the glucoside transporters involved in esculin uptake, influences the intercellular molecular exchange between vegetative cells of *Anabaena* (75). The number of nanopores per septum in *glsP*- and *hepP*-deficient mutants is similar to that in the wild type; however, the *glsC*-deficient mutant shows decreased nanopore numbers. GlsP and HepP interact with SepJ, and GlsC is required for subcellular localization of SepJ (75). Therefore, in addition to the role of nutrient uptake, glucoside transporters may also participate in SJ formation through interaction with SepJ. SjdR, a TonB-like protein, is proposed as a regulator for SJ disk formation, playing an essential role in septal nanopore distribution, formation of heterocyst polar necks, and vegetative cell–heterocyst septa (91).

Cell-cell communication is subject to regulation. It was observed early on that the transfer of fluorescent markers from vegetative cells to heterocyst is slower than that between vegetative cells (75, 76). It was also found that in mature diazotrophic filaments of *Anabaena*, the diameter of PG disks and that of the nanopores at heterocyst–vegetative cell junctions are larger than those between vegetative cells of the same filament (3). These observations suggest that the structure of nanopores between heterocysts and vegetative cells may be different from those present between vegetative cells. In addition to nitrogen-regime modulation of structures in cell-cell communication, individual cells on the same filament and the function of SJs respond to environmental signals (4). FRAP experiments allow a cell's capability for intercellular transfer of fluorescent markers to be tested, enabling communicating and noncommunicating cells on a filament to be distinguished. Noncommunicating cells coexist with communicating cells even within single filaments in *Anabaena*, and communicating cells are more abundant under nitrogen deprivation (4). Thus, SJs between cells seem to be coordinately regulated with the need for cell-cell communication in the filament. SJ function is dependent on membrane proton gradients, as SJs lost communication capacity after treatment of the *Anabaena* filaments with CCCP (carbonyl cyanide 3-chlorophenylhydrazone), a protonophore that dissipates the proton gradient across membranes (108). This no-communication behavior was caused by a conformational change of the SJs' cap module. In contrast to CCCP-treated cells, the cap in non-CCCP-treated cells can change reversibly from an open state, as arches in fivefold symmetry, to a closed state, with no individual arches visible (**Figure 3**). SJs can also adopt a closed state in response to stress, such as oxidative stress and light stress (108). Therefore, SJs gate cell-cell communication rapidly by dynamic structural changes in response to environmental conditions.

PATTERN FORMATION

Heterocyst distribution along the filaments of *Anabaena* has long been recognized as a semiregular pattern (54, 110). A mathematical model first proposed by Alan Turing in 1952 (100) and later redefined by Hans Meinhardt (67) led to a framework known as the Turing model or the reaction-diffusion model for our general understanding of biological pattern formation. On the

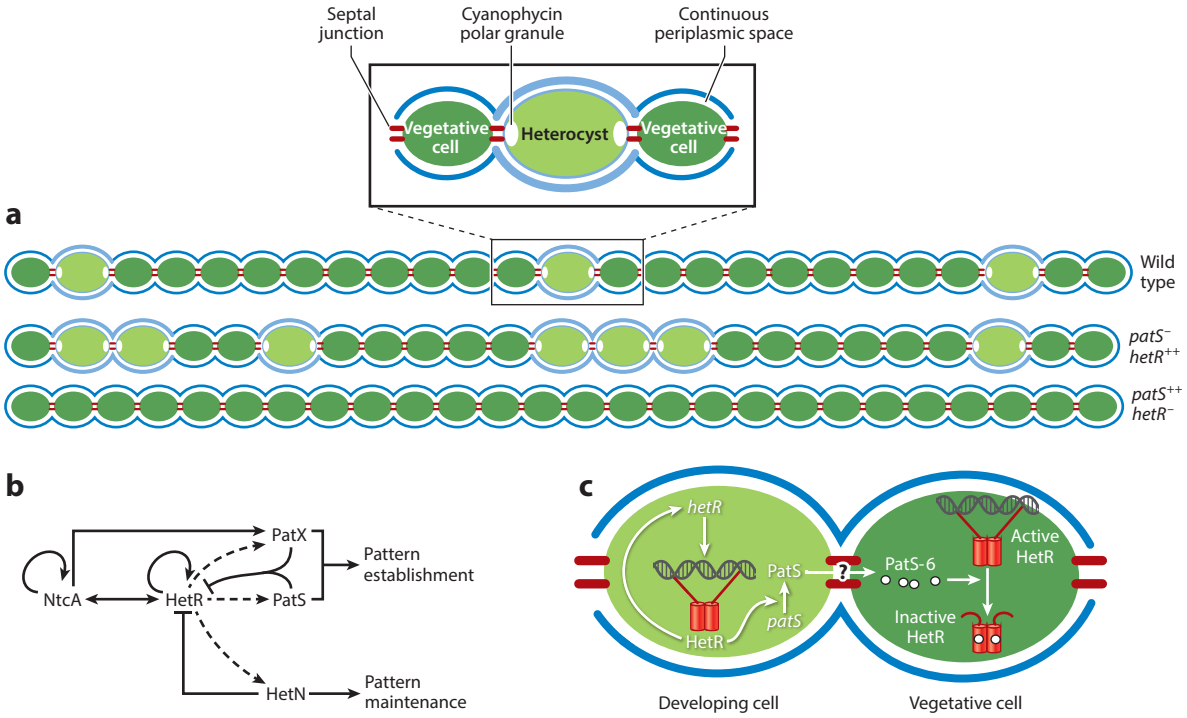

Figure 4

Molecular mechanism of heterocyst patterning. (*a*) In the wild type, heterocysts are intercalated, in a semiregular manner, among vegetative cells, and they account for 5% to 10% of the cells of each filament. One heterocyst with two immediate neighboring vegetative cells are enlarged above the wild-type filament. Mature heterocysts synthesize cyanophycin, a nitrogen reserve visible at the heterocyst polar regions. Heterocyst patterning can be disturbed by mutations. A strain with either overexpression of *hetR* (*hetR*$^{++}$) or inactivation of *patS* (*patS*$^{-}$) displays a phenotype of multiple contiguous heterocysts. A strain with either overexpression of *patS* (*patS*$^{++}$) or inactivation of *hetR* (*hetR*$^{-}$) prevents heterocyst differentiation. (*b*) Turing-type regulatory network involved in heterocyst patterning. After initiation by NtcA, the autoregulatory transcription factor HetR activates, possibly indirectly, the expression of genes encoding inhibitory signals for pattern establishment (PatS and PatX) or maintenance (HetN). The inhibitors exert feedback inhibition on HetR. The dotted arrows indicate mechanisms that remain to be elucidated. (*c*) Cell type–specific actions of the regulatory elements for heterocyst pattern formation, with PatS as an example. A cell that starts to increase *hetR* expression (developing cell) directs the production of PatS. PatS is processed to an active form (PatS-6) in the developing cell or during the transfer to neighboring vegetative cells through an unknown mechanism. PatS-6 binds to the hood domains of the HetR dimer and converts the active form of HetR to an inactive form. Therefore, the developmental potential of the neighboring vegetative cells is inhibited.

basis of this model, a pattern may arise from a relatively stable and uniform cell population through the action of two factors, an activator and its antagonist or inhibitor. The activator should have an autoactivation property and act locally, while its inhibitor, produced through the action of the activator, functions over a long range through diffusion to inhibit the activator. The activator and the inhibitor are morphogens, and their interaction and differential spatial distribution create a pattern (**Figures 4** and **5**). Establishment of heterocyst pattern in *Anabaena* fits well into the Turing model, and mathematical simulation can reproduce the basic characteristics of the heterocyst pattern (71).

HetR has all the properties of an activator in a Turing system. *hetR* is positively autoregulated and acts only in the cells that produce it (11, 47). Two types of diffusible inhibitors of heterocyst development have been partly demonstrated, although in both cases the exact nature remains to be revealed in vivo. The first type corresponds to a fixed-nitrogen product made by heterocysts

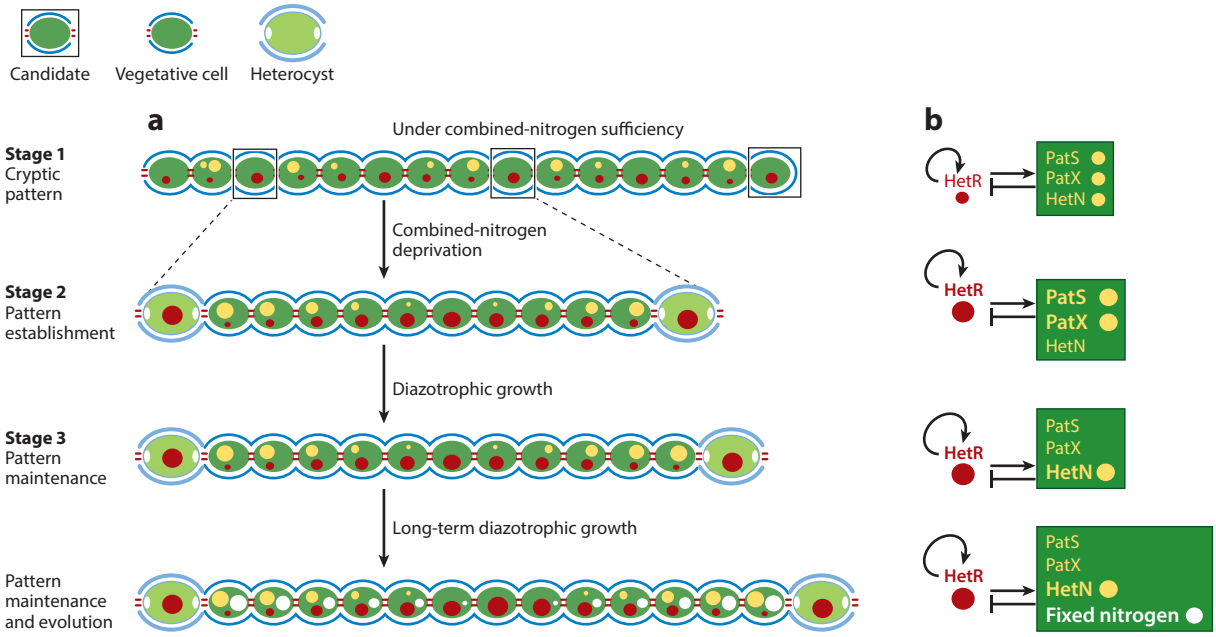

Figure 5

A model of heterocyst patterning with morphogen gradients. (*a*) Peptide inhibitory signals derived from PatS, PatX, or HetN (*yellow circles*) and an unknown fixed-nitrogen product (*white circles*) diffuse from the source cells along the filament and form a gradient along the filament. They directly or indirectly inhibit HetR (*red circles*) activity, thereby shaping a reverse gradient of HetR. The size of each circle is proportional to the amount of each morphogen. Stage 1 occurs before combined-nitrogen deprivation, and the genetic noise of genes encoding the morphogens creates a cryptic pattern. At the moment of combined-nitrogen deprivation, the candidate cell may be better prepared to differentiate into a heterocyst in stage 2. At this stage, peptidic inhibitors derived from PatS and PatX appear to be the major players for pattern establishment. At stage 3, HetN is activated for pattern maintenance and, together with a product of fixed nitrogen with a potential for longer-distance diffusion, accounts for a pattern with a lower percentage of heterocysts along the filament. At stages 2 and 3, a cell with the highest level of HetR and the lowest levels of inhibitors, at mid-distance between the two existing heterocysts, is a candidate for de novo differentiation. (*b*) Proposed Turing network operating at each step of the pattern formation process indicated in panel *a*.

(117), whose function is discussed below. The second type is a peptide signal (or signals) derived from PatS, PatX, and HetN, which share a common pentapeptide motif, RGSGR (21, 31, 116) (**Figures 4** and **5**). *patS* and *patX* participate in the establishment of the initial pattern and have redundant functions, and *hetN* is required for pattern maintenance. Although direct evidence is still lacking for the diffusion from cell to cell, several lines of evidence strongly indicate that RGSGR-type peptidic signals derived from PatS, PatX, and HetN act as long-range diffusible inhibitors for heterocyst development. First, the RGSGR pentapeptide can suppress heterocyst differentiation when added in the growth medium (23, 116). Second, mutations occurring in the RGSGR motif–coding region abolished the inhibitory function of *patS*, *patX*, and *hetN* (24, 43). Third, all three genes are activated specifically in developing cells, although at different stages of the developmental process. *patS* and *patX* are activated early and downregulated in mature heterocysts (31, 116), whereas *hetN* is activated at the late stage of heterocyst differentiation and remains activated in mature heterocysts (23). Finally, immunofluorescence assays using antibodies raised against the RGSGR motif detected the presence of such motif-containing signals in vegetative cells surrounding a heterocyst (24). Thus, the corresponding proteins are made in developing cells and/or

mature heterocysts to inhibit the differentiation of their neighbors, in a non-cell-autonomous manner (**Figures 4** and **5**).

The relationship between HetR and PatS/PatX/HetN meets the basic criteria of a Turing system. Peptide signals are dependent on HetR for their production and in turn inhibit HetR in the neighbors of the source cells (**Figure 4**). The three genes, *patS*, *patX*, and *hetN*, are unlikely to be controlled directly by HetR since their promoter regions lack a typical HetR-binding motif (27, 33). The promoter regions of *patS* and *patX* possess a DIF1 motif, a candidate HetZ-dependent regulatory element (28, 31). *patX* expression is also directly regulated by NtcA (31, 80). *hetN* upregulation in proheterocysts is not yet elucidated but is indirectly controlled by HetR, as the latter is required to initiate the developmental process.

Studies indicate that RGSGR-containing peptides act on HetR. The *patS* gene encodes a peptide of 17 amino acid residues (23, 116), and it is generally accepted that this peptide is processed into an RGSGR-containing oligopeptide, corresponding to the last five (PatS-5) to eight (PatS-8) PatS residues, for in vivo signaling (124). Depending on how they are experimentally tested, the same oligopeptides give contradictory results (23, 32, 112). When expressed by *patS* minigenes on a multicopy plasmid, and thus possibly overexpressed, PatS-5–PatS-8 displayed inhibitory effects on heterocyst differentiation (112). When similar minigenes were integrated into the chromosome, only PatS-8 could lead to patterned heterocyst formation (23). With the use of cell extracts, the form of PatS-7 was first identified, followed by PatS-6 and PatS-5 (124). In vitro, PatS-6 gives the highest affinity (K_d = 7 nM or 12 nM, depending on the study) for interaction with HetR; the binding affinity to HetR was dramatically decreased with PatS-5 (K_d = 227 nM) and PatS-7 (K_d = 9.2 mM) (32, 45). PatS-6, but not PatS-5, was also successfully crystallized in complex with the hood domain of HetR (45). Taken together, compelling evidence suggests that PatS-6 is the active form of PatS for in vivo signaling. PatS, PatX, and HetN might be processed by unknown proteases in order to generate the PatS-6 signal, so a peptide with a minimum length (for example in the form of PatS-8) would be necessary for substrate recognition and cleavage; this hypothesis conciliates in vitro and in vivo data. As for PatS-5, a high concentration of PatS-5 used in cell culture or expressed from a multicopy plasmid (23, 112) may compensate for its poor binding affinity to HetR (32, 33).

Structural analyses of HetR, the HetR-DNA complex, and the HetR hood domain in complex with PatS-6 give us much insight into the molecular mechanism of PatS action on HetR (45, 52, 53). HetR from *Anabaena* forms a highly intertwined homodimer, with four distinct domains: the N-terminal DNA-binding domain, the central flap domains that extend on each side of the whole structure, and the C-terminal hood domain. Two PatS-6 molecules bind to the dimerized hood domain, leading to a significant conformational change in the latter (45). In the HetR dimer, the backbone structure formed with the DNA-binding domain and the hood domain is stable, whereas the two flap domains on the two sides are flexible. The DNA-binding domain interacts with DNA, and this interaction is stabilized with the help of one α-10 helix from each flap domain, fixing the structure of the HetR dimer in a stable conformation with DNA. When PatS-6 binding leads to the conformational change of the hood domain, it destabilizes the interaction between the flap domain and DNA and finally results in the release of HetR from DNA (45) (**Figure 4**).

With all of the information available, a model can thus be proposed to explain heterocyst pattern formation (**Figure 5**). Meeks & Elhai (66) proposed a two-stage model for heterocyst pattern formation. At the initiation stage, they propose, a cell-cycle gating hypothesis whereby a group of cells at a certain stage of the cell cycle is competent to perceive the 2-OG signal, leading to a biased and crude pattern. In the second stage, the HetR/PatS interaction promotes pattern resolution. On the basis of current data, we revise the Meeks-Elhai model and propose a three-stage model (**Figure 5**). Although cell division constitutes a key element in heterocyst development, as

discussed in the subsection titled Initiation, no concrete evidence is available for the existence of a competence window for differentiation during the cell cycle (5). However, it is known that both *hetR* and *patS* are expressed at a low, basal level under nitrogen sufficiency (5, 11, 16, 112, 116). Such genetic noise can generate a low level of cell competition, with the HetR-PatS acting as a Turing system (26). Therefore, under nitrogen sufficiency, a cryptic pattern, or prepattern, exists with an interval of four to five vegetative cells (25, 26). This interval is shorter than the mature heterocyst pattern, which can be explained by the much lower amounts of HetR and PatS/PatX produced under such conditions. This can be considered the first stage of pattern formation. The second stage lasts from the initiation of heterocyst development until the formation of mature heterocysts. When deprived of combined nitrogen, a cell (predetermined by genetic noise at the time of nitrogen stepdown) that makes the highest amount of HetR will amplify its advantage through positive autoregulation and the synthesis of PatS and PatX. PatS- and PatX-derived signals diffuse to neighboring vegetative cells in which HetR is inhibited (**Figure 5**). The PatS signal likely forms a gradient, decreasing from source cells (23), and may be correlated with a reverse gradient of HetR (84). Vegetative cells continue to divide, increasing the distance of predetermined developing cells at the first stage, and this is possible because of the high inhibitory pressure due to the lateral diffusion of increasing amounts of PatS/PatX produced. At stage 3, HetN starts to replace PatS/PatX as a main source of inhibitory signal to maintain the heterocyst pattern (**Figure 5**). Following this stage, the heterocyst intervals increase in filaments over time under extended diazotrophic conditions. Under our laboratory conditions, the percentage of heterocysts decreases from 8% at stage 2 (about 20–24 h after initiation) to about 6.5% at 48 h, and then 6% at 72 h (46). This dynamic evolution is caused by a fixed-nitrogen product as an inhibitory signal that gives a longer range of lateral inhibition than the peptide signals (71, 117). Possible candidates include amino acids and ammonium transferred from heterocysts to vegetative cells (18).

Several questions still need to be addressed. The transfer route of PatS- and HetN-derived peptide signals from developing cells or mature heterocysts to vegetative cells is not yet fully understood, although it may depend on SJs (23). How HetN, PatX, and PatS are processed into peptide signals remains enigmatic, and this is especially true considering the inhibitory effect of several other proteins with RGSGR motifs under conditions of ectopic expression (112). As proposed (116), the maturation steps of PatS, PatX, or HetN may also be linked to the inhibition-immunity effect; that is, their unprocessed forms may be poorly active in source cells for HetR inhibition, and their maturation coupled to transport or diffusion toward vegetative cells for lateral inhibition. Another factor, HetL, also has an immunity function in the protection of HetR against PatS inhibition in developing cells (115). However, since the deletion of *hetL* gives a negligible phenotype difference (61), it remains to be confirmed that HetL and its homologs found in *Anabaena* have redundant inhibition-immunity functions in the protection of HetR in developing cells (115).

PERSPECTIVES

Although many players involved in heterocyst development have been identified, only a few have been studied in detail for their mechanisms of action. The relationship and hierarchy of different players and the biochemical basis of their action need to be elucidated. There are also several aspects of heterocyst differentiation that remain poorly understood. Among them, how does the developmental process advance beyond the commitment step? Are there inhibitors acting at the commitment step that abort the developmental process if combined nitrogen becomes available again? We also know little about the sequential events for the building of heterocyst-specific cell envelopes at the morphogenesis step. For example, how are different layers deposited at the

cell wall? Is there interdependency for the formation of different layers? High-resolution, real-time microscopic studies may provide insight into these questions. Finally, it is increasingly evident that some developmental components are involved in cell division and cell division components are involved in development. How is the choice of cell fate made, between reproductive division and terminal differentiation? With the recently developed genetic tools that make the manipulation of essentials genes easier, such as those involved in cell division, we may begin to dissect the relationship between cell division and differentiation.

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