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MINI-REVIEW

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# Cellular differentiation in the cyanobacterium *Nostoc punctiforme*

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**Abstract** *Nostoc punctiforme* is a phenotypically complex, filamentous, nitrogen-fixing cyanobacterium, whose vegetative cells can mature in four developmental directions. The particular developmental direction is determined by environmental signals. The vegetative cell cycle is maintained when nutrients are sufficient. Limitation for combined nitrogen induces the terminal differentiation of heterocysts, cells specialized for nitrogen fixation in an oxic environment. A number of unique regulatory events and genes have been identified and integrated into a working model of heterocyst differentiation. Phosphate limitation induces the transient differentiation of akinetes, spore-like cells resistant to cold and desiccation. A variety of environmental changes, both positive and negative for growth, induce the transient differentiation of hormogonia, motile filaments that function in dispersal. Initiation of the differentiation of heterocysts, akinetes and hormogonia are hypothesized to depart from the vegetative cell cycle, following separate and distinct events. *N. punctiforme* also forms nitrogen-fixing symbiotic associations; its plant partners influence the differentiation and behavior of hormogonia and heterocysts. *N. punctiforme* is genetically tractable and its genome sequence is nearly complete. Thus, the regulatory circuits of three cellular differentiation events and symbiotic interactions of *N. punctiforme* can be experimentally analyzed by functional genomics.

**Keywords** Akinetes · Cellular differentiation · Cyanobacteria · Heterocysts · Hormogonia · *Nostoc punctiforme* · Symbiosis

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## Introduction

*Nostoc punctiforme* [Pasteur Culture Collection (PCC) 73102, synonym American Type Culture Collection (ATCC) 29133] is a filamentous nitrogen-fixing cyanobacterium with an extraordinarily wide range of ecological niches, physiological properties and vegetative cell developmental alternatives. The developmental biology of *N. punctiforme* is the topic of this review. The shotgun phase of sequencing the *N. punctiforme* genome has been completed (http://www.jgi.doe.gov) and a preliminary analysis was recently published (Meeks et al. 2001). *N. punctiforme* is amenable to genetic manipulations (Cohen et al. 1994), including targeted gene replacement (Campbell et al. 1998). The combination of genome sequence information, genetic tractability and expression of multiple phenotypic traits makes *N. punctiforme* ideal for detailed molecular characterization of a bacterium with a complex life cycle.

Vegetative cells of *N. punctiforme* can mature in four developmental directions (Figs. 1, 2). First, the cells can successively grow and divide to perpetuate the vegetative cell cycle when nutrients are unlimited (Figs. 1, 2A). Of the three vegetative cell developmental alternatives, the differentiation of $N_2$-fixing heterocysts in the absence of combined nitrogen is the most highly studied developmental event in cyanobacteria and is commonly recognized as a taxonomic trait (Fig. 2B). Nitrogenase, the enzyme catalyzing $N_2$ reduction to $NH_4^+$ is oxygen-sensitive (Wolk et al. 1994). Therefore, $N_2$ fixation appears incompatible with oxygenic photosynthesis, the unifying physiological characteristic of cyanobacteria. Highly specialized, terminally differentiated, microoxic heterocysts are the evolutionary solution to the incompatibility dilemma in all filamentous cyanobacteria in the orders Nostocales and Stigonemateles (Castenholz and Waterbury 1989). In the second developmental alternative, some or all vegetative cells of *N. punctiforme* can transiently differentiate into spore-like structures (Fig. 2C), called akinetes in cyanobacteria (Castenholz and Waterbury 1989). Akinetes are more resistant than vegetative cells to cold and desicca-

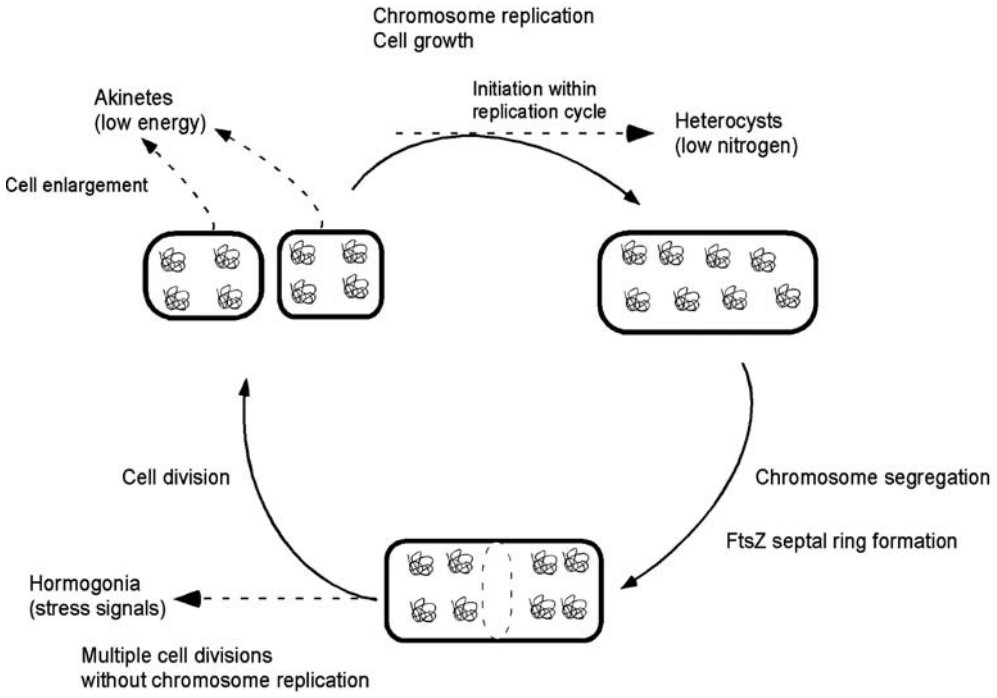


**Fig. 1** Schematic of the *Nostoc punctiforme* vegetative cell cycle and points where specific developmental events are hypothesized to depart from the cell cycle. *N. punctiforme* does not form branching filaments; thus, its life cycle of polar vegetative cell growth, DNA replication and chromosome segregation, followed by cell division in a transverse plane, is modeled as similar to unicellular bacteria. Physiological processes are denoted adjacent to the cell cycle or developmental event. In general, cyanobacteria have multiple copies of the chromosome per cell. Only four copies are depicted here, but *N. punctiforme* is likely to have more than four. The hypothesized developmental departure points leading to a developmental destination are represented by *dashed lines*; and *phrases in parentheses* represent the environmental signal initiating the developmental event. Heterocyst differentiation requires DNA replication after cell division, but akinetes appear to have the same DNA content as vegetative cells. Therefore, akinetes are depicted as departing from the cycle after cell division and prior to DNA replication, while heterocysts are depicted as departing at an unknown point during DNA replication (*the unrooted dashed line*). Hormogonium differentiation involves cell division uncoupled from DNA replication and, therefore, the departure of hormogonia coincides with septal ring formation and cell division

tion (Adams and Duggan 1999). Akinete differentiation is confined to the heterocyst-forming cyanobacteria. The third developmental alternative is the transient differentiation of vegetative filaments into motile filaments called hormogonia (Fig. 2D). Hormogonium filaments lack heterocysts, do not fix $N_2$ and function in short-distance dispersal (Tandeau de Marsac 1994). Some, but not all, heterocyst-forming and heterocyst-non-forming filamentous cyanobacteria can differentiate hormogonia (Castenholz and Waterbury 1989). Specifically, hormogonium differentiation has been used to distinguish the morphologically similar genera *Nostoc* (positive) and *Anabaena* (negative; Rippka et al. 1979). The fact that differentiation of motile hormogonia is often lost in culture minimizes its taxonomic application, except in feral samples or recent isolates. A schematic of the hypothetical *N. punctiforme* vegetative cell cycle and the postulated relationship of the three developmental alternatives to cell cycle events are depicted in Fig. 1. The schematic is based more on deductive reasoning than experimental fact and is presented as a working model.

The four developmental directions of a *N. punctiforme* vegetative cell are numerically unparalleled in the prokaryotic world. Other bacteria have at most two or three developmental choices, such as those that can sporulate and/or form motile swarmer or gliding cells, along with vegetatively competent prosthecate or undifferentiated cells, or subsurface and aerial mycelia and spores (Kaiser and Losick 1993; Roberts et al. 1996). Moreover, heterocyst, akinete and hormogonium differentiation occur only following signals of a specific environmental change, in contrast to the obligatory formation of swarmer cells by prosthecate bacteria (Roberts et al. 1996). Therefore, all three developmental alternatives can be eliminated by mutation and the *N. punctiforme* mutants maintained for analysis under the permissive growth conditions. How the environmental signals are perceived, integrated and elicit three distinct differentiation events is of fundamental biological interest.

An objective of this review is to briefly summarize knowledge of cellular differentiation resulting from analyses of *N. punctiforme* and related cyanobacteria, such as *Anabaena* sp. strain PCC 7120, with which genetic protocols for filamentous cyanobacteria were developed (Thiel 1994). *Anabaena* sp. strain PCC 7120 has received the most attention as an experimental system for analysis of heterocyst differentiation (Golden and Yoon 1998; Adams 2000; Wolk 2000). *Anabaena* sp. strain PCC 7120 is genotypically a *Nostoc* species (Rippka and Herdman 1992) that, presumably due to mutation during prolonged

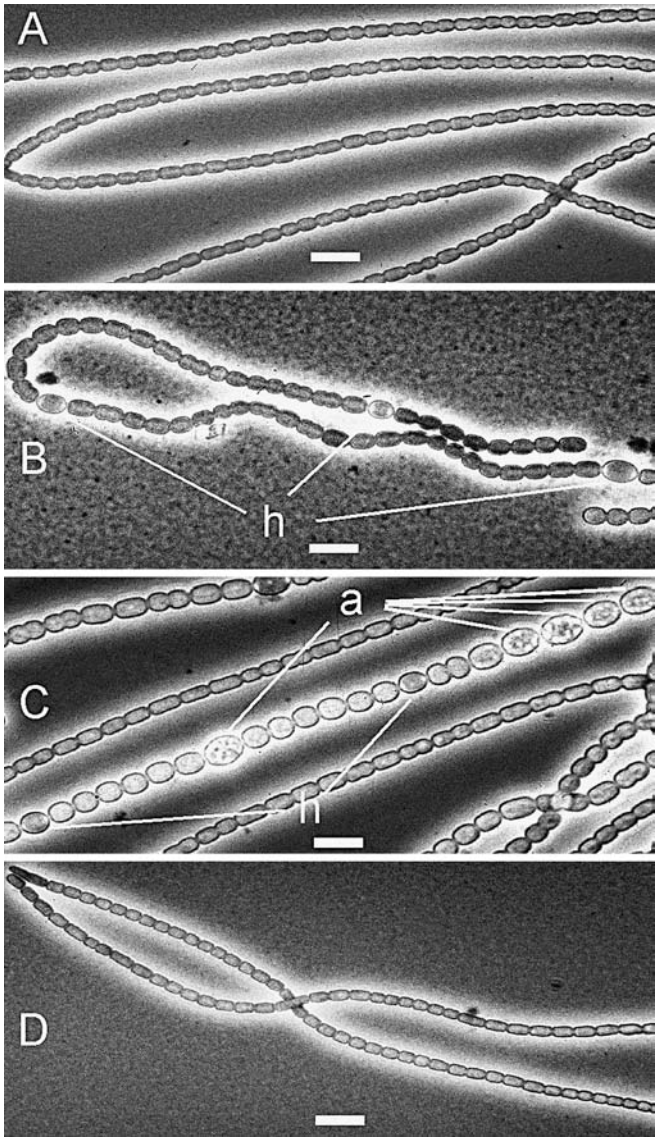


**Fig. 2A–D** Phase contrast photomicrographs of *N. punctiforme*, illustrating its vegetative cell developmental alternatives. **A** $NH_4^+$-Grown filaments consisting of entirely undifferentiated vegetative cells. **B** A $N_2$-grown filament from a mid- to late exponential phase culture showing three heterocysts (*h*) present in a nonrandom spacing pattern in the filaments. **C** An early stationary phase $N_2$-grown filament, showing akinete (*a*) differentiation starting near the midpoint of the interval between two heterocysts (*h*). The $N_2$ filament is flanked by two $NH_4^+$-grown filaments in this slide, made by immediately mixing differently grown cultures. **D** Hormogonium filaments, showing the smaller size and different shape of the cells, relative to vegetative filaments. *Bars* 10 μm

passage in culture, now has a much more limited phenotype than any taxonomically defined *Nostoc* species; it does not differentiate akinetes or hormogonia. The genome sequence of *Anabaena* sp. strain PCC 7120 is complete (Kaneko et al. 2001). All heterocyst-related genes currently identified in *N. punctiforme* are present in *Anabaena* sp. strain PCC 7120 and vice versa (Meeks et al. 2001).

In most studies of developmentally competent cyanobacteria, the experimental focus has been on responses of axenic cultures (Tandeau de Marsac 1994; Wolk et al. 1994; Adams and Duggan 1999). *N. punctiforme* forms symbiotic associations with specific representatives of the major phylogenetic groups of spore- and seed-producing plants, including bryophyte hornworts (Meeks 1998), gymnosperm cycads (source for isolation; Rippka et al. 1979) and the angiosperm *Gunnera* (Rai et al. 2000). Symbiotic associations, such as that with the hornwort *Anthoceros punctatus*, are of interest because the plant partner influences the differentiation of hormogonia (Campbell and Meeks 1989; Cohen and Meeks 1997) and heterocysts (Meeks and Elhai 2002); the symbiotic *Nostoc* shifts to a largely heterotrophic metabolic mode (Meeks 1998).

## Heterocyst differentiation

Physiologically, heterocysts may be considered as sinks of reduced carbon and sources of reduced nitrogen in the filaments. The processes giving rise to this physiological state involve differential gene expression as vegetative cells differentiate into heterocysts. Synthesis of certain enzyme systems in photosynthesis and $CO_2$ assimilation, such as phycobiliproteins, the photosystem II $O_2$-evolving complex and ribulose bisphosphate carboxylase, are repressed, while $N_2$ reduction (nitrogenase) and respiratory $O_2$ consumption are induced or enhanced. Coupled with the synthesis of a bilayered polysaccharide and glycolipid envelope that retards diffusion of gases and the translocation of ions and other hydrophilic solutes, some of these physiological changes result in the microoxic cytoplasm essential for nitrogenase expression and function (for details see Wolk et al. 1994).

It has been estimated that about 140 (Wolk 2000) to over 1,000 (Lynn et al. 1986) genes are involved in heterocyst differentiation and function. A number of regulatory and structural genes of heterocysts have been identified (summarized in Wolk 2000). However, the mechanisms of transcriptional regulation are not well defined in heterocyst-forming cyanobacteria (Curtis and Martin 1994) and the molecular mechanisms of induction of heterocyst differentiation are unknown. From sequencing projects and genetic analyses, it appears that elements of the *ntr* system, prevalent in nitrogen control in Proteobacteria (Merrick and Edwards 1995), and alternative sigma subunits are not obligatory elements in regulation of heterocyst differentiation (Meeks and Elhai 2002).

Heterocyst differentiation is a terminal event, but the physiological life-span of heterocysts has yet to be precisely defined and may vary with growth conditions. By definition, terminal heterocyst differentiation following cell division is a basic form of programmed cell death, or apoptosis. We do not suggest that the mechanism of apoptosis in cyanobacteria is the same as that of eukaryotic organisms, but the cellular consequences are identical.

The spaced pattern of heterocysts in the filament is perhaps the most intriguing aspect of their differentiation

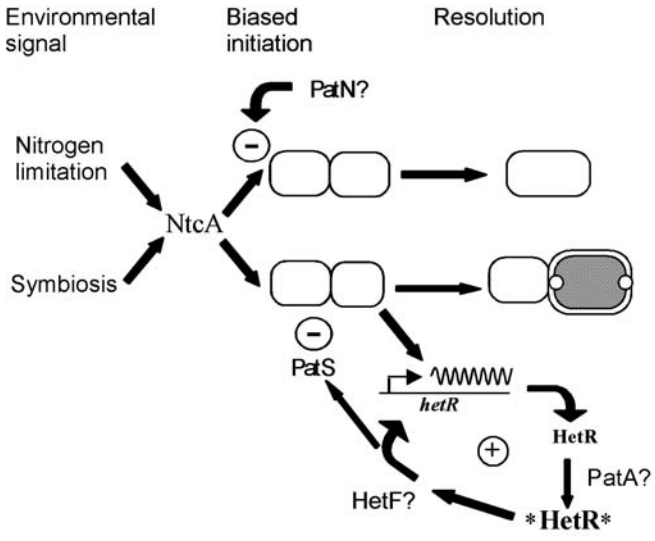


**Fig. 3** Schematic depiction of a hypothetical two-stage working model of the initiation of heterocyst differentiation and establishment of the spacing pattern. Upon nitrogen limitation or symbiotic interaction, NtcA is activated and sends a signal to all cells. Cells in an undefined, but appropriate stage of the cell cycle may be prevented from differentiating by PatN. Cells in the appropriate stage initiate development by activating the transcription of *hetR*. HetR is concurrently modified (**HetR**), perhaps mediated by PatA, which further activates the transcription and/or accumulation of HetR. HetF is required for the cell specific auto-stimulation of *hetR* transcription. Activated HetR also stimulates the synthesis of PatS and, through direct or indirect means, PatS inhibits neighboring cells from continuing their development and these cells then regress to the vegetative cell state. Such a competitive interaction resolves a differentiating cluster of cells into a single heterocyst at any developmental site

and three separate phenomena may be considered: (1) establishment, (2) maintenance and (3) disruption of pattern. A *N. punctiforme* filament cultured with combined nitrogen consists of a chain of vegetative cells (Fig. 2A). When the combined nitrogen source is exhausted, heterocysts appear singly in the filament, at a frequency of about 8% of the total cells, establishing a nonrandom spacing pattern (Fig. 2B). The spacing pattern is maintained, usually at a lower heterocyst frequency, as the vegetative cells in the interval between two heterocysts grow and divide, using fixed nitrogen produced by the heterocysts; a new heterocyst emerges at a point approximately midway in the expanding vegetative cell interval. The pattern can be naturally disrupted by symbiotic association with a photosynthetic eukaryotic partner (Meeks 1998; Rai et al. 2000), in which the heterocyst frequency is enhanced to range from 25% to more than 60% of the total cells and the vegetative cell interval between flanking heterocysts decreases (Meeks and Elhai 2002).

This section of the review focuses on the initiation of differentiation in the establishment of pattern and the symbiotic disruption of pattern, yielding enhanced heterocyst frequencies. Thorough discussions of known genes and processes involved in heterocyst maturation and function can be found in three references (Wolk et al. 1994; Adams 2000; Wolk 2000).

Models to explain the establishment of a nonrandom pattern of differentiated cells assume that either only certain cells are physiologically competent to initiate differentiation (Wilcox et al. 1973), or that any cell at random can initiate differentiation (Wolk and Quine 1975). In either case of initiation, the models hypothesize that the ultimate pattern is determined by the action of a diffusible inhibitor produced by differentiating heterocysts.

A two-stage initiation and resolution model was recently proposed in which all cells sense the signal to differentiate, but only some cells respond to the signal, resulting in a biased initiation of differentiation (Meeks and Elhai 2002; Fig. 3). Further, this biased initiation occurs in clusters of two or more contiguous cells spaced non-randomly in the filaments. The mechanism of biased initiation is not known, but it is assumed to be associated with the physiological state of the cells. Heterocyst differentiation is blocked by inhibitors of DNA replication (Adams and Duggan 1999); thus, bias could hypothetically be related to the position of the cells in the cell cycle when the signal is perceived (Meeks and Elhai 2002). This hypothesis is the basis for depicting heterocyst differentiation as emerging from the DNA replication portion of the cell cycle in Fig. 1. The earliest signal of nitrogen limitation is activation of the global nitrogen regulator, NtcA (Herrero et al. 2001). Activated NtcA then functions as a transcriptional activator of itself and of a variety of enzyme systems of nitrogen acquisition whose transcription is enhanced in the absence of ammonium, including catalytic and regulatory proteins of heterocyst differentiation (Herrero et al. 2001).

Biased initiation is followed by competitive resolution of the cluster of differentiating cells, such that only a single heterocyst is generally present at any site in the filament. This can be visualized as the result of a competitive interaction between one or more positive regulatory elements (HetR in Fig. 3) and one or more negative regulatory elements (PatS in Fig. 3). High HetR activity would tend to commit cells to differentiation, whereas high PatS would tend to decrease HetR and lead to regression. The developmental direction is also influenced by ancillary positive and negative regulatory elements, whose role is to enhance or inhibit the activity of the primary positive and negative regulatory elements. We suggest that eight of ten known mutants, described below, that block or alter the pattern of heterocyst spacing are likely involved in the resolution stage of pattern formation.

Four mutants have been isolated that fail to initiate heterocyst differentiation and a fifth mutant differentiates a low frequency of heterocysts. These phenotypes are characteristic of a defect in a positive activator. Two of the mutations are in genes, *ntcA* and *hanA*, that have global cellular roles, are not specific to heterocysts and are thought not to be involved in the resolution stage of pattern formation. NtcA was introduced above; it is in the Crp family of bacterial transcriptional activators and functions as a nitrogen-dependent regulator in cyanobacteria (Herrero et al. 2001). Recent evidence indicates that NtcA senses nitrogen status as levels of 2-oxoglutarate (Muro-

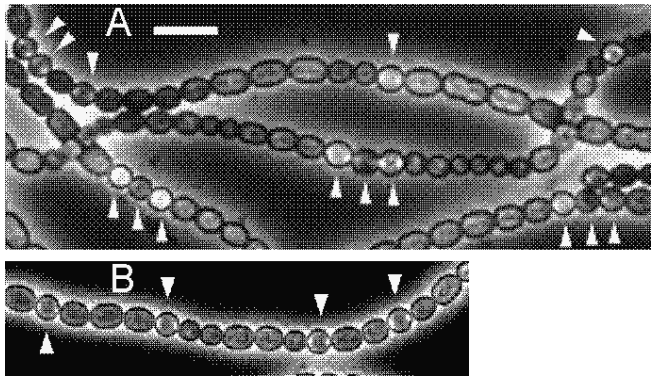


**Fig. 4** Phase contrast photomicrographs illustrating the pattern of (**A**) multiple contiguous heterocysts and (**B**) multiple singular heterocysts of *N. punctiforme* mutants. **A** The *patU* mutant; **B** the *patN* mutant. *Arrowheads* indicate heterocysts. *Bar* 10 μm

Pastor et al. 2001; Vazquez-Bermudez et al. 2002). While *hanA* mutants fail to initiate heterocyst differentiation, the mutant phenotype is highly pleiotrophic, indicating multiple cellular roles (Khudyakov and Wolk 1996). *hanA* encodes a DNA-binding protein analogous to HU. We model NtcA and HanA as instigating and/or relaying the initiation signal to elements of the developmental cascade. Two other mutations in this class are in genes, *hetR* and *hetF*, which when present *in trans* in a high copy can yield clusters of two, three or four multiple contiguous heterocysts (MCH; see Fig. 4A) in a nonrandom spacing pattern, a condition whereby HetR activity predominates over PatS activity in the model. HetR is a marvelously complex protein. Induced transcription of *hetR* is dependent on intact copies of *ntcA* (Herrero et al. 2001; Wong and Meeks 2001) and itself (Black et al. 1993). The protein may also be modified, has autoprotease activity (Zhou et al. 1998) and is localized to differentiating cells and mature heterocysts (Buikema and Haselkorn 2001; Wong and Meeks 2001). The mechanism of induction of the differentiation cascade by HetR is unknown. HetF is essential to the autoinduced transcription of *hetR* and the localization of HetR to differentiating cells (Wong and Meeks 2001). Other than a putative transmembrane domain, HetF has no obvious motifs or functional domains. Mutations in *patA* result in heterocyst differentiation only at the ends of filaments (Liang et al. 1992). When *hetR* is overexpressed in a *patA* mutant, heterocysts differentiate only at the ends of the filaments, indicating that PatA, a complex response regulator protein, likely operates in the same pathway as HetR, possibly in modification of HetR (Liang et al. 1992).

Five mutants have been isolated that result in the differentiation of multiple heterocysts in the absence of $NH_4^+$, suggesting a negative regulatory role for the respective gene products. Four of the mutations, *patS*, *hetN*, *patB* and *patU*, result in MCH, while mutations in *patN* uniquely yield a higher frequency of heterocysts (i.e. multiple) that are located singly in the filaments, with correspondingly shortened vegetative cell intervals (called multiple singular heterocysts or MSH, in contrast to MCH; Fig. 4B). *patS* encodes a 13- (Meeks et al. 2001) or 17-amino acid peptide (Yoon and Golden 1998). Both cellular overexpression in trans of *patS* and exogenous provision of a chemically synthesized C-terminal pentapeptide of PatS lead to repression of heterocyst differentiation in nitrogen-deprived cultures (Yoon and Golden 1998). PatS exemplifies the characteristics of the principle inhibitor involved in resolution. PatS mutants ultimately express a spacing pattern similar, but not identical, to the wild type (Yoon and Golden 2001), implying that a different mechanism controls maintenance of pattern. The original *hetN* mutants were highly pleiotrophic, with different mutations yielding different phenotypes (Black and Wolk 1994). Transcriptionally controlled overexpression of *hetN* leads to repression of heterocyst differentiation, but the controlled mutant MCH phenotype is apparent only in the second generation of heterocysts that differentiate following nitrogen starvation (Callahan and Buikema 2001). HetN, which has similarity to a β-ketoacyl synthase, may be more involved in maintenance of the pattern than is PatS. Upon nitrogen starvation, mutations in *patU* result in an initial cluster of four to eight cells differentiating through the proheterocyst stage at a single site that, ultimately and with delayed kinetics, resolves to a variety of singlet, doublet and triplet MCH (Wong and Meeks, unpublished data; Fig. 4A). PatU, like PatN (below), has no similarity to any sequences in the database other than *N. punctiforme* and *Anabaena* sp. strain PCC 7120. The *patB* mutant has a complex phenotype and was originally defined as a MSH pattern (Liang et al. 1993). However, recent observations indicate that a *patB* mutant has a MCH phenotype if the mutation is a deletion or frame shift (K. Jones, personal communication). PatB contains ferredoxin and DNA-binding motifs, but its role in heterocyst differentiation remains obscure. The MSH pattern has been reproducibly observed in the free-living state only in the *patN* mutant (Wong and Meeks, unpublished data; Fig. 4B). The *patN* phenotype can be repressed by the exogenous PatS pentapeptide and overexpression of *patN* does not lead to suppression of heterocyst differentiation (Wong and Meeks, unpublished data). Therefore, PatN is likely to function in a pathway parallel to PatS and conceivably could be involved in biased initiation (Fig. 3).

In symbiosis, *N. punctiforme* undergoes distinct physiological and morphological changes. Relative to the free-living state, the rates of growth, $CO_2$ fixation and $NH_4^+$ assimilation are repressed about 5-fold, while $N_2$-fixation is enhanced about 4-fold (Meeks 1998). The most dramatic morphological change is a 3- to 5-fold increase in heterocyst frequency. Two fundamental questions with mechanistic implications are whether the symbiotic heterocyst spacing pattern primarily resembles MCH or MSH and what is the environmental signaling cascade yielding enhanced heterocyst frequencies.

The heterocyst frequency of *Nostoc* in the *Anthoceros punctatus* association is approximately 25%, with an average vegetative cell interval of 3.2 cells (Wong, cited in Meeks and Elhai 2002). This is similar to the frequency

and MSH pattern of the *patN* mutant (Fig. 4B). However, in the cycad and *Gunnera* associations (Rai et al. 2000), a gradient of increasing heterocyst frequencies is observed from the tip of the root or stem toward the base, respectively. In the areas nearest to the stem or root tip, the heterocysts are present at single sites in the filaments at about 15% of the cells, but MCH and heterocyst frequencies approaching 60% of the cells are observed in the more distal portions. Increased rates of $N_2$ fixation parallel the increase in singly spaced heterocysts within the gradient, but the rate declines with the progressive increase in MCH and heterocyst frequency. These observations imply that the frequency of functional $N_2$-fixing heterocysts in symbiosis is 25–35% of the total cells in a MSH pattern. The MCH pattern is likely a consequence of later rounds of heterocyst differentiation by vegetative cells adjacent to a nonfunctional heterocyst (Meeks and Elhai 2002).

Since a high frequency of heterocysts in a MSH pattern in symbiosis only appears distal from the root or stem tip, where development is initiated in the cycad and *Gunnera* associations, this sequence of differentiation events is not the same as establishment of the pattern following combined nitrogen deprivation in free-living growth. Rather, high symbiotic heterocyst frequencies may result from an alteration in the mechanisms that maintain the free-living pattern.

Physiological evidence from studies of mutants of *N. punctiforme* implies that the signal initiating heterocyst differentiation in symbiosis with *A. punctatus* is not specifically nitrogen limitation. Rather, a plant signal may supercede the nitrogen signal (Campbell and Meeks 1992). Moreover, heterocyst differentiation occurs at a high frequency, while concurrently 80% of the $N_2$-derived $NH_4^+$ is translocated from heterocysts and excreted from vegetative cells of *Nostoc* in the *A. punctatus* association (Meeks 1998). Mutations in *hetR* and *hetF* block differentiation in both symbiotic and free-living growth states, indicating a common developmental pathway, at least downstream of HetR (Wong and Meeks 2002). Because NtcA is required for transcriptional regulation of genes expressed early and late during heterocyst development and function (Golden and Yoon 1998; Herrero et al. 2001), if the symbiotic signal enters the common pathway prior to HetR, it is most likely at or before activation of NtcA (Fig. 3).

## Akinete differentiation

Akinetes are generally recognized by their larger size, relative to vegetative cells, and conspicuous granulation (Fig. 2C) due to high concentrations of glycogen and cyanophycin, a nitrogen-storage polymer consisting of nearly equimolar amounts of arginine and aspartate (Wolk et al. 1994). However, size and granulation are not absolute characteristics (Adams and Duggan 1999); perhaps the most consistent property of akinetes is a greater resistance to cold than vegetative cells. Akinetes are neither as metabolically quiescent nor as resistant to various environmental extremes as are endospores of Gram-positive bacteria (Adams and Duggan 1999). They maintain significant metabolic activity (Thiel and Wolk 1983) and typically contain amounts of DNA, RNA and protein similar to vegetative cells (Sutherland et al. 1979). The similarity in DNA content is a reason akinete differentiation is depicted departing from the vegetative cell cycle after cell division, but prior to the chromosome-replication stage (Fig. 1).

Akinetes are most often seen in cultures in the stationary phase of growth, which could be a consequence of nutrient or light limitation. They can be induced within 2 weeks of culture by specific starvation for phosphate in *N. punctiforme* (Wong and Meeks 2002). A mutant strain of *N. punctiforme* lacking glucose-6-phosphate dehydrogenase activity differentiates akinetes within 4 days of dark incubation in the presence, but not in the absence of fructose (Summers et al. 1995). These collective observations are consistent with cellular energy limitation as a primary signal for akinete differentiation.

Light and nutrient conditions favorable for growth appear to be the major stimulus for akinete germination (Adams and Duggan 1999). These conditions are generally achieved by dilution into fresh medium. Germination is most often accompanied by localized lysis of the akinete wall and outgrowth of a short filament, with the remaining akinete wall present as a conspicuous cup-shaped structure. However some strains, including *N. punctiforme* (Meeks, unpublished data), appear to dissolve the entire akinete wall (Adams and Duggan 1999).

A number of physiological, structural and genetic properties contribute to the hypothesis that akinetes were evolutionary precursors to heterocysts (Wolk et al. 1994). Akinetes are only seen in heterocyst-forming cyanobacteria, although many heterocyst-forming cyanobacteria do not form akinetes (Castenholz and Waterbury 1989), especially those in long term culture. In a species-dependent manner, akinetes initiate differentiation either adjacent to existing heterocysts, as in *Anabaena cylindrica* (Adams and Duggan 1999), or at a point approximately equidistant in the vegetative cell interval between two heterocysts, as in *N. punctiforme* (Fig. 2C). It is noteworthy that a vegetative cell adjacent to a heterocyst has a low probability of differentiating into a heterocyst, while one midway in the interval between heterocysts has the highest probability. An inductive or repressive signal of akinete differentiation produced by heterocysts would seem to differ in the two cases and not be universal. Additional akinetes then differentiate in a successive pattern, starting with the vegetative cell(s) adjacent to the initial akinete.

Akinetes have a polysaccharide outer layer that is identical in composition to that of the heterocyst envelope and is not present in vegetative cells (Wolk et al. 1994). Akinetes are also reported to contain glycolipids characteristic of heterocysts (Soriente et al. 1993). The bilayered polysaccharide and glycolipid envelope of the heterocyst serves to dramatically retard diffusion of gases (Wolk et al. 1994). Because akinetes maintain a rate of oxygen consumption (Thiel and Wolk 1983) and lack the polar pore of the heterocyst envelope, the components must be organized differently in the two cell types.

Three heterocyst genes have been associated with akinetes. Consistent with a common polysaccharide layer, mutations in *hepA*, essential to its formation in heterocysts (Wolk et al. 1994), result in abnormal akinete envelopes of *A. variabilis* (Leganés 1994). Overexpression of *devR*, encoding a response regulatory protein that is likely involved in heterocyst polysaccharide synthesis, results in enhanced akinete differentiation in *N. punctiforme* (Campbell et al. 1996). This could be the consequence of cross-talk interference between phosphorelay signal transduction circuits of heterocyst and akinete differentiation. Mutations in *hetR* (Fig. 3) were reported to block both heterocyst and akinete differentiation in *N. ellipsosporum* (Leganés et al. 1994). However, a *hetR* mutant of *N. punctiforme* forms large akinete-like, chilling-resistant cells following phosphate starvation (Wong and Meeks 2002). The akinete-like cells of the *N. punctiforme hetR* mutant lack the granulation characteristic of wild-type akinetes. Thus, the genetic data are consistent with common envelope and perhaps cytoplasmic (cyanophycin and/or glycogen) storage properties of heterocysts and akinetes, but there is currently no unequivocal evidence for a common regulatory pathway of their differentiation.

A protein and its corresponding structural gene that is enriched in akinetes was recently identified in *A. variabilis* (Zhou and Wolk 2002). The protein (AvaK) has no obvious structural motifs, no sequence similarity to known genes, nor an assigned role in differentiation or akinete function. The *N. punctiforme* genome contains four open reading frames with sequence similarity to AvaK, with the closest match having 72% identity and 84% similarity. Transcription of the *avaK* homologue is induced in *N. punctiforme* coincidental with akinete differentiation. However, substantial expression is observed in cultures lacking akinetes grown in the presence of fructose and in light or dark (Argueta, Yuksek and Summers, unpublished data). This could imply that the protein functions in metabolic processes or storage components common to heterotrophic growth and akinete differentiation in *N. punctiforme*.

## Hormogonium differentiation

Hormogonia are identified as relatively short gliding filaments, lacking heterocysts, with cells that are smaller and generally of a different shape than cells in vegetative filaments (Fig. 2D). Hormogonia of some strains contain gas vesicles, but many lack such structures (Tandeau de Marsac 1994). Hormogonia are a transient, non-growth state. In addition to short-distance dispersal, they are the infective units of cyanobacterial symbiotic associations (Meeks 1998).

The smaller-sized cells of hormogonia result from cell divisions that are not accompanied by an increase in cell biomass; there is no significant net synthesis of DNA (Herdman and Rippka 1988), protein or chlorophyll (Campbell and Meeks 1989) during the differentiation and active gliding periods of the hormogonium cycle. This non-growth physiology is analogous to that of swarmer cells of *Caulobacter crescentus* (Roberts et al. 1996). However, extensive uncoupling of cell division from DNA replication is unique. Cyanobacteria, in general, contain multiple copies of the chromosome in each cell; consequently, even in the absence of DNA replication, hormogonium cells are likely to receive one or more copies of the chromosome (Tandeau de Marsac 1994). The cessation of net macromolecular synthesis, coupled with the synthesis of a few specific proteins during hormogonium formation (see below), is similar to the general stress response exhibited by many organisms, for example heat shock (Yura and Nakahigashi 1999). Based on these presumptive properties, hormogonium differentiation is depicted as a stress-induced reiteration of the septum formation/cell division stages in the vegetative cell cycle (Fig. 1).

The working model of the hormogonium cycle in heterocyst-forming strains is that differentiation is initiated by a variety of environmental changes that may be positive or negative for growth. The environmental changes are presumably detected by preexisting sensory proteins that signal a stress state to the cells (Meeks and Elhai 2002). The signals include nutrient and salt concentration (Herdman and Rippka 1988), light quality (Damerval et al. 1991) and symbiotic interactions (Campbell and Meeks 1989). The cells respond to the stress signals by repression of most macromolecular synthesis, initiation of multiple cell divisions, reorganization of the peptidoglycan-based cell wall, fragmentation of the parental filament at the junctions between heterocysts and vegetative cells and synthesis or activation of a motility system. Although initiation of differentiation is inhibited by chloramphenicol (Campbell and Meeks 1989), it is not clear how much differential protein synthesis occurs during the hormogonium cycle or whether the primary response is by activation/inactivation of existing proteins. Currently, only genes encoding a sigma subunit and a carboxyl terminal protease in *N. punctiforme* (Campbell et al. 1998), gas vesicle proteins (Damerval et al. 1991), cell division proteins and pili in *Calothrix* sp. strain PCC 7601 (Doherty and Adams 1999) are reported as transcriptionally upregulated during hormogonium differentiation. However, none of these is directly involved in initiation of differentiation.

Hormogonium filaments maintain gliding activity for 48–72 h, after which they differentiate heterocysts at the ends of filaments and resume net synthesis of macromolecular components as they enter the vegetative cell cycle (Campbell and Meeks 1989). Cultures of *N. punctiforme* which have differentiated hormogonia to an extensive degree require a vegetative growth period that includes several doublings before cultures are again able to differentiate a substantial number of hormogonia (Campbell and Meeks, unpublished data).

Symbiotic interactions positively and negatively influence hormogonium differentiation and also influence behavior. Symbiotic induction of hormogonium differentiation is in response to plant release of a hormogonium-inducing factor (HIF; Campbell and Meeks 1989), but the chemical identity of the factor is unknown. Plants produce

undefined chemotactic signals that attract the motile hormogonia (Knight and Adams 1996). Plants also produce factors that inhibit hormogonium differentiation (hormogonium repressing factors, HRF); the presence of HRF overrides HIF activity (Cohen and Meeks 1997).

Activity of a HRF was first implied by a mutant of *N. punctiforme* that continually produces hormogonia in the presence of *Anthoceros punctatus*. The mutant is normal when cultured apart from the plant partner (Cohen and Meeks 1997). Since hormogonia do not grow, continual differentiation is lethal by extinction if kept in the constant presence of the HIF signal. The target genes have organizational and sequence similarities to genes of hexuronic acid catabolism in heterotrophic bacteria (Campbell and Meeks, unpublished data). We hypothesize that the target genes are involved in HRF-induced synthesis of a metabolite inhibitor of hormogonium differentiation, rather than a carbon catabolic function. Hormogonium and heterocyst differentiation cannot occur simultaneously; therefore, the advantages to repression of hormogonium formation may be to shift the developmental direction away from hormogonia, allowing heterocyst formation once *N. punctiforme* has entered the symbiotic growth state. Nitrogen fixation, which requires heterocyst differentiation, is presumably the selective pressure in evolution of these symbiotic associations.

## Conclusions

The genes identified thus far as having a role in the induction of heterocyst differentiation, except for *ntcA*, are either unique to heterocyst-forming cyanobacteria, or contain motifs or domains that have no obvious relationship to a differentiation event, based on what is known in other bacteria. There appear to be no common models of bacterial developmental pathways to follow, as there are in many metabolic systems, e.g. organization and function of the nitrogenase gene cluster (Wolk et al. 1994). We hypothesize that heterocyst, akinete and hormogonium differentiation involve complex developmental regulatory cascades, as complex as *Bacillus* or *Myxococcus* sporulation, but that the regulatory elements and circuits have uniquely evolved in cyanobacteria. If so, the elements will not be readily identifiable through purely comparative bioinformatic analysis of the sequenced genomes of organisms, such as *N. punctiforme* and *Anabaena* sp. strain PCC 7120. The unique elements will require identification through functional analyses. *N. punctiforme* with its extensive phenotypic traits and growth condition-dependent multiple developmental alternatives is an exemplary candidate for global genome expression assays in such analyses.

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