Transcriptional regulation of development in heterocyst-forming cyanobacteria

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ABSTRACT

Filamentous, heterocyst-forming cyanobacteria are among the simplest multicellular systems in Nature. In the absence of combined nitrogen, the filaments consist of vegetative cells that fix CO₂ through oxygenic photosynthesis and micro-oxic heterocysts specialized for the fixation of N₂ in a proportion of about 10 to 1. The development of a heterocyst-containing filament involves differentiation of vegetative cells into heterocysts in a process that requires a distinct gene expression program. Two transcription factors are strictly required, the CRP-family NtcA and HetR. NtcA directly activates the expression of multiple genes during heterocyst differentiation –in some cases assisted by coactivators including HetR– and in mature heterocysts, whereas HetR is needed to build high NtcA levels in differentiating heterocysts and directly activates some particular genes. A few other regulators of gene expression participate at specific differentiation steps, and a specific transcription factor, CnfR, activates *nif* gene expression under the micro-oxic conditions of the heterocyst.

1. Introduction

Multicellularity is a form of organization widely found in living organisms including evidently eukaryotes but also prokaryotes. Two properties characterize multicellularity: cell-cell binding and cell-cell communication. Additionally, organisms take advantage of multicellularity to specialize cells in different functions, so that cellular differentiation is another hallmark of multicellularity. Proterozoic microfossils frequently show a multicellular form, and filamentous cyanobacteria showing cellular differentiation have been traced back to about 2.45 – 2.1 billion years ago [1]. Cyanobacteria are a phylogenetically coherent group of organisms characterized by performing oxygenic photosynthesis, which allows a mainly photoautotrophic mode of growth [2]. Additionally, many cyanobacteria can fix atmospheric N_2 , making these organisms capable to grow with very simple nutritional sources –air and water with a few mineral salts. However, because the N_2 -fixation machinery is very sensitive to oxygen [3], cyanobacteria need to separate N_2 fixation from oxygenic photosynthesis. Whereas some unicellular and filamentous cyanobacteria separate these two incompatible processes temporally, restricting N_2 fixation to the dark period of diel cycles [3], some filamentous cyanobacteria form cells specialized for N_2 fixation. Thus, in filaments of cells that fix CO_2 through oxygenic photosynthesis, some of these cells (usually less than 10 %) differentiate into N_2 -fixing heterocysts when no combined nitrogen is available [4]. Other developmental options found in some heterocyst-forming cyanobacteria include the differentiation of akinetes, a type of spores that resist drought and cold, and the production of hormogonia, short motile filaments frequently made of small-sized cells that serve a dispersal function [5, 6]. Knowledge of the transcriptional regulation for the differentiation of akinetes and hormogonia is however scarce [6]. This review will focus on the transcriptional regulatory mechanisms that operate in heterocyst differentiation. We shall first briefly introduce the structure of the cyanobacterial filament and the morphology and biochemistry of the heterocyst, then describe the mechanisms of regulation of gene expression that operate during the process of differentiation and in the mature N_2 -fixing heterocyst, and finally discuss the early regulation that leads to a defined pattern of heterocyst distribution in the filament.

2. Cyanobacterial filaments and heterocysts

Cyanobacteria of the order Nostocales –taxonomic section IV in the classification of Rippka *et al.* [2]– are characterized by their ability to produce heterocysts when they lack a source of combined nitrogen [2]. These cyanobacteria grow as filaments that can be hundreds of cells

long, and heterocysts can be found intercalary or at the end in the filaments, although some strains make only terminal heterocysts [2]. Heterocysts are terminally differentiated cells, and commitment to complete differentiation is established after the spatial distribution pattern of heterocysts has already been settled [7, 8].

2.1. Structure of the cyanobacterial filament

The cyanobacteria are diderm bacteria, i.e., they bear a Gram-negative type of cell envelope. The cells in a cyanobacterial filament are surrounded by their cytoplasmic membrane and peptidoglycan layer(s), but the outer membrane does not enter into the septa between consecutive cells and, therefore, is continuous along the filament [9]. This implies that the periplasm –the space that lies between the cytoplasmic and outer membranes and contains the peptidoglycan– is also continuous and may be a communication conduit between the cells in the filament [10]. Consecutive cells in the filament are also joined by proteinaceous structures known as septal junctions [9, 11], which appear to traverse the septal peptidoglycan through perforations known as nanopores [12, 13]. These perforations are drilled in the peptidoglycan by AmiC-type amidases [12, 14]. In the model heterocyst-forming strain *Anabaena* sp. PCC 7120 (hereafter *Anabaena*), the SepJ (a.k.a. FraG), FraC and FraD proteins are localized at the cell poles in the intercellular septa of the filament [15, 16]. They are membrane proteins that, in the case of SepJ and FraD, have long extra-membrane sections that appear to be located outside of the cells in the intercellular septa, and at least SepJ is known to form multimers [17, 18]. Hence, they are candidate components of the septal junctions (see below).

2.2. Heterocyst morphology

Under the light microscope, the heterocysts are seen larger than the vegetative cells and show two refractile bodies at their poles (just one, proximal to the vegetative cells, in the case of terminal heterocysts) (Fig. 1A). Under the electron microscope, differences become evident in the global form of the heterocyst, in its envelope, and in its cytoplasm (Fig. 1B). The heterocyst poles adjacent to vegetative cells take a narrow form known as “neck”, which makes the area of contact between heterocysts and vegetative cells smaller than that between vegetative cells. The cell envelope contains extra material that is deposited outside the outer membrane and consists of an inner glycolipid (heterocyst-specific glycolipid, Hgl) layer and an outer polysaccharide (heterocyst envelope polysaccharide, Hep) layer. Three characteristics distinguish the heterocyst cytoplasm from the vegetative cell cytoplasm [6]: (i) intracellular membranes are reorganized forming a structure known as “honeycomb” around the heterocyst poles; (ii) glycogen granules and carboxysomes (the intracellular micro-compartments that

contain ribulose biphosphate carboxylase/oxygenase [RuBisCO, the CO₂-fixing enzyme]) disappear; and (iii) a large granule made of cyanophycin (multi-L-arginyl-poly [L-aspartic acid]) is deposited at the heterocyst poles adjacent to vegetative cells –these granules correspond to the refractile bodies observed by light microscopy.

2.3. Heterocyst biochemistry

The heterocyst has evolved to become a N₂-fixing factory. Hence, an essential goal of its biochemistry is to keep a micro-oxic environment appropriate for the expression and function of the N₂-fixing enzyme, nitrogenase, and accessory proteins. First, importantly, the oxygen-releasing photosystem II (PSII) is non-functional in the heterocyst (reviewed in [19]). Second, the extra envelope described above restricts gas diffusion contributing to reduce the amount of oxygen that enters in the heterocyst as air (78 % N₂, 21 % O₂) [20]; indeed, O₂ has been suggested to enter the heterocyst mainly through the vegetative cell-heterocyst connections [20]. Third, dedicated terminal respiratory oxidases located in the honeycomb membranes and cytoplasmic flavodiiron proteins consume O₂ contributing to create a micro-oxic environment [21, 22]. The nitrogenase reaction consumes high amounts of ATP and reductant, and the heterocyst keeps an electron transport chain and PSI activity that support photophosphorylation and the reduction of ferredoxin that can provide nitrogenase with electrons [19].

In the heterocyst, dinitrogen is reduced by the nitrogenase complex producing ammonium (and hydrogen as a byproduct). The ammonium resulting from the N₂-fixing reaction is incorporated by glutamine synthetase into glutamate producing glutamine [23]. Glutamine is, at least in part, transferred to vegetative cells in exchange for glutamate, which is not synthesized at high levels in the heterocyst [24, 25]. Further anabolic nitrogen metabolism in the heterocyst includes the biosynthesis of aspartate and arginine, which are incorporated by cyanophycin synthetase into cyanophycin that serves as a dynamic nitrogen reservoir [26, 27]. In addition to glutamine, β-aspartyl arginine, the product of cyanophycin degradation by cyanophycinase, is transferred to vegetative cells, in which β-aspartyl arginine is hydrolyzed by isoaspartyl dipeptidase releasing aspartate and arginine [28]. Hence, both glutamine and β-aspartyl arginine serve as nitrogenous nutrients for the vegetative cells. In turn, because the heterocyst does not accomplish the photosynthetic fixation of CO₂, the vegetative cells provide the heterocysts with reduced carbon in the form of a sugar, sucrose, that in *Anabaena* is split by an invertase –InvB– producing glucose and fructose, which support heterocyst metabolism [29, 30]. This exchange of reduced carbon by fixed nitrogen is at the basis of the multicellular behavior of heterocyst-forming cyanobacteria.

2.4. Intercellular molecular exchange

During heterocyst differentiation and in the diazotrophic filament, regulators and metabolites are transferred between cells. Intercellular molecular exchange in filamentous cyanobacteria has been probed with fluorescent markers including calcein, 5-carboxyfluorescein and the sucrose analog esculin [13, 31, 32]. The transfer has properties of simple diffusion, indicating the existence of direct connections between adjacent cells [31, 32]. Mutants of *Anabaena* lacking the septal proteins SepJ or FraC and FraD are impaired in the intercellular transfer of those fluorescent markers and make less nanopores than the wild type, suggesting that these proteins contribute to the formation of the septal junctions [13, 31]. Differences in the impairment of transfer of the various fluorescent markers in different mutants have suggested the existence of functionally-discernible SepJ- and FraCD-related septal junctions [13, 17]. Important for our discussion here is that septal junction complexes in the vegetative cell/heterocyst septa may have specific characteristics as compared to those in vegetative cell/vegetative cell septa. Thus, these structures appear to be longer in the former than in the latter [33], and the SepJ protein visualized as a SepJ-GFP fusion is seen as a single fluorescent spot between vegetative cells and as a double spot in the vegetative cell/heterocyst septa, implying a more spread localization in the latter than in the former [11].

3. Key transcription factors and transcriptional regulation mechanisms

Two genes whose inactivation completely abolish heterocyst differentiation are *ntcA* [34, 35] and *hetR* [36]. Whereas NtcA is a global regulatory transcription factor of cyanobacteria mediating responses to variations in the C-to-N balance of the cells, HetR is a transcription factor specifically found in filamentous cyanobacteria whose best-known role is in cell differentiation.

3.1. NtcA

NtcA is a cyanobacterial transcription factor that orchestrates the genetic responses to the C-to-N balance by directly regulating the expression of multiple genes involved in pathways for nitrogen assimilation, but also of a number of other metabolic pathways [37, 38]. Indeed, by means of chromatin-immunoprecipitation assays performed in *Anabaena*, an extraordinarily large number of NtcA-binding sites on DNA were identified that could be ascribed to more than 2,000 genes [38]. Besides genes involved in nitrogen scavenging and N₂ fixation, the NtcA targets included genes involved in DNA metabolism, transcription and translation, central metabolism

and, remarkably, many predicted regulatory genes, which should extend the range of NtcA-influenced cellular processes. NtcA belongs to the CRP (cAMP receptor protein) family of transcriptional regulators, presenting a domain architecture similar to that of the well-characterized *E. coli* CRP protein, including an N-terminal β -roll fold, which in CRP accommodates the effector cAMP, a central long dimerization helix and a C-terminal helix-turn-helix (HTH) motif for interaction with DNA [39] (Fig. 2A). These regulators are dimeric proteins, so that each subunit interacts with half of a palindromic recognition sequence in DNA.

NtcA can act as a transcriptional activator or repressor. For both activities, the NtcA dimer binds to palindromic DNA sites including the consensus sequence GTAN₈TAC [37, 38]. In many NtcA-activated promoters an NtcA-binding site is found separated by ca. 22 bp from a -10 promoter determinant of consensus sequence TAN₃T, thus conforming to the so-called Class II bacterial activated promoters, in which the regulator binds in place of a missing or degenerated -35 determinant. This promoter structure is frequently found in genes of unicellular cyanobacteria and, in heterocyst-forming strains, in vegetative cell-expressed genes as well as in genes expressed in mature heterocysts [40]. Besides this, NtcA can activate gene expression from DNA sites centered further upstream the -35 position, conforming to Class I activated promoters, or from promoters with very degenerated NtcA-binding sites, which are generally activated during heterocyst differentiation [41]. Finally, repression by NtcA has been shown involving binding sites downstream from promoter determinants, overlapping the -10 box or even within the regulated ORF [41].

Regarding metabolic signals, NtcA activity is influenced by 2-oxoglutarate (2-OG) [42]. Because cyanobacteria lack the enzyme 2-oxoglutarate dehydrogenase, the 2-OG cellular levels depend on its synthesis during CO₂ fixation and its consumption mainly to form glutamate by the glutamine synthetase/glutamate synthase pathway, thus representing an indicator of the cellular C-to-N balance [40]. Indeed, 2-OG has been shown to increase the binding of NtcA to DNA in many regulated promoters *in vitro*, although in contrast to CRP, NtcA can interact with DNA in the absence of the effector (see e.g. [37, 42, 43]). Structurally, the positive effect of 2-OG in binding is explained by its effect on dimer stabilization by decreasing the distance between the dimerization helices of each subunit, which in turn repositions the two second helices in the HTH DNA-binding domains, making the global conformation more suitable for interaction with DNA [39]. In the absence of the effector, the NtcA apo-protein already presents in its DNA-binding domain some exposed positively-charged residues that represent a DNA-binding patch. In addition, the conformation of the last helices in this domain allows some interaction with DNA, whereas these helices are buried in the apo-CRP [39].

Whereas a positive effect of 2-OG on NtcA binding to DNA *in vitro* has been found in all types of NtcA-activated promoters, although at variable extents, an additional and stringent requirement for 2-OG has been found for the step of promoter melting to form the tripartite NtcA-RNA polymerase-DNA open promoter complex, and thus for transcript production, at Class II NtcA-activated promoters [43]. However, no specific structural information is available for this 2-OG effect, although it is reminiscent of that of cAMP during Class II activation by CRP. Finally, the intrinsic capacity of NtcA to interact with DNA may be important for its action as a repressor under physiological conditions that determine low 2-OG levels. This appears to occur in the *cmpR* gene (encoding a LysR-type regulator of a bicarbonate transporter operon), which under conditions that determine low 2-OG levels is expressed at higher levels in an *ntcA* mutant than in the wild type [44].

3.2. HetR

For many years considered as a heterocyst-specific regulator, evidence is recently accumulating in support of HetR activity also in undifferentiated cells, consistent with the presence of HetR in non-heterocystous cyanobacteria as well as in heterocyst formers [45]. HetR is required for the expression of multiple genes involved in heterocyst differentiation, and binding of HetR to DNA from the promoter region of a few of them has been detected *in vitro*, including sequences from the promoters of *hetR*, *patS* and *hepA* ([46], see below) as well as of *hetP*, which has been implicated in the commitment to irreversible differentiation [47, 48]. A global study of HetR targets by chromatin immunoprecipitation identified 26 direct targets, of which only 10 % are related to known heterocyst-differentiation genes [49]. Finally, a role of HetR in repression of several gene promoters in vegetative cells has been reported [29, 50, 51].

Structurally, HetR represents a rather distinct type of transcription factor. The crystal structure of a dimer of HetR from the heterocyst-forming cyanobacterium *Fischerella* sp. shows a central DNA-binding region constituted by the N-terminal HTH motifs of both subunits, and new folds conforming two globular "flaps" and a "hood" over the central core, the latter formed by the two C-terminal parts [52] (Fig. 2B). However, HetR tetramers have been detected *in vivo*, at higher levels during heterocyst differentiation, leading to the proposal that the tetramer represents an active form of HetR [53]. This is consistent with (i) the fact that in crystals assembled by HetR in complex with long synthetic DNA targets, HetR tetramers formed by interaction of the flap domains were the predominant form [54], and (ii) the occurrence of tandem repeats of the HetR DNA-binding consensus sequence in several of the identified HetR targets [49].

3.3. Mechanisms of transcriptional activation by NtcA and HetR

The expression of both *ntcA* and *hetR* increase early during heterocyst differentiation, upon combined N step-down, in a mutually-dependent manner enhanced by positive autoregulation, with *hetR* induction preceding that of *ntcA* [55]. Remarkably, induction takes place in specific cells or cell clusters along the filament, marking the places of vegetative cell differentiation into heterocysts ([56-58], see below). The response regulator-like factor NrrA, which is directly induced by NtcA early upon N step-down from a canonical Class II promoter [59], has been reported to bind the *hetR* promoter region *in vitro* and, hence, to mediate the induction of *hetR* by NtcA [60].

Multiple genes whose products effect the morphological and biochemical differentiation of heterocysts (see below), including secondary regulators, are also activated upon combined N step-down at specific steps during differentiation [41, 61], and for some of them induction could be ascribed to specific cells along the filament (see e.g. [29, 62]). In contrast to *nrrA*, which is independent of HetR, *in vivo* activation of many other heterocyst-differentiation genes depends on both NtcA and HetR, as they are not induced in strains bearing inactivated *ntcA* or *hetR* genes. Mechanistically, a remarkable feature of heterocyst-differentiation genes is that they present complex promoter sequences including several successive promoters, which may include σ^{70} consensus-type promoters, likely directing a basal level of gene expression in all the cells of the filament before N step-down, Class II and Class I NtcA-activated promoters, and HetR-dependent promoters (see e.g. [41, 50, 63]). The binding affinity of NtcA for the different NtcA-activated promoters and the response to HetR are important in determining the temporal sequence of transcription activation during the differentiation process that, as a result of the cell-confined increase of *hetR* and *ntcA* expression, takes place in specific cells along the filament.

HetR appears to exert a positive effect on transcription activation by several mechanisms. As mentioned above, direct binding of HetR *in vitro* has been reported for only a few sequences upstream of heterocyst-differentiation genes [49], suggesting that the *in vivo* requirement could be indirect in many promoters, e.g., by the requirement of high levels of NtcA, attained by HetR-dependent activation of the *ntcA* gene, or of later secondary regulators. In the case of the proximal promoter of the *devBCA* operon involved in deposition of the Hgl layer (see below), HetR could not bind DNA by itself but was found to increase the binding of NtcA at degenerated NtcA-binding sequences, thus behaving as an NtcA-coactivator [50] (Fig. 2C). On the other hand, the *pipX* gene, which is activated directly by NtcA at late stages of heterocyst differentiation, encodes a small protein required for full diazotrophic growth and full

activation of further late genes [62]. The structure of a complex of the NtcA and PipX proteins from the unicellular cyanobacterium *Synechococcus elongatus* has been determined, leading to the suggestion that binding of PipX stabilizes the active conformation of NtcA [64]. With the *Anabaena* proteins, PipX has been shown to increase NtcA binding to DNA and transcription from NtcA-dependent promoters *in vitro*, thus representing a late NtcA-coactivator [65].

Regarding RNA polymerase sigma factors, NtcA-dependent transcription has been achieved *in vitro* with the *Anabaena* RNA polymerase holoenzyme including the principal group 1 σ^{70} -family factor SigA [43]. In *Anabaena*, the *sigA* gene is transcribed from one major, proximal promoter that was first described to display constant activity [66] and later to be repressed by NtcA after N step-down [67]. Additionally, several promoters induced upon removal of combined nitrogen contribute to expression of *sigA*. This array of promoters produces increased transcription of *sigA* in the differentiating cells, which is consistent with a bulk of NtcA-dependent transcription during differentiation. Further, *Anabaena* has multiple σ^{70} -family factors of groups 2 and 3 [68]. Although inactivation of none of them abolishes heterocyst differentiation or diazotrophic growth, some of the single and double mutants that have been isolated are affected in these processes [69]. Also, the *sigC* (group 2), *sigE* (group 2) and *sigG* (group 3) genes are responsive to N step-down, with successive increases of expression in the cells that are differentiating into heterocysts [70]. SigE is upregulated late during differentiation, and both NrrA [71] and NtcA [72] directly bind to sequences upstream of *sigE*. SigE is required for normal cellular differentiation and for expression of several heterocyst-specific genes including *nifH* (encoding a nitrogenase subunit), *fdxH* (encoding a putative electron donor to nitrogenase [73]), *hglE₂* (encoding a heterocyst-specific glycolipid biosynthesis protein [72]), and the *xfp* operon involved in glycolysis [71]. Thus, in spite of considerable redundancy, a succession of specific σ^{70} -family factors appears to cooperate with SigA during heterocyst differentiation.

Finally, HetR is a target of phosphorylation *in vitro* and phosphorylation interferes with the formation of HetR tetramers and HetR binding to DNA [53]. Consistently, overexpression in *Anabaena* of a heterocyst-specific kinase, PknE, blocks heterocyst differentiation in a process that appears to involve inactivation of HetR [74]. The *pknE* gene is induced late during differentiation [61] with a direct participation of HetR [74] and NtcA [38], the latter using a putative Class II promoter. These independent, *in vitro* and *in vivo* observations strongly suggest that phosphorylation of HetR is a physiological process, leading to the proposal that phosphorylation represents a mechanism of HetR inactivation at late stages of heterocyst differentiation, when a direct action of HetR would be no longer required [53, 74].

4. Regulation of gene expression in the course of heterocyst differentiation

The expression of genes involved in heterocyst differentiation and function is generally dependent on NtcA and HetR. Although regulation by these transcription factors may in some cases be indirect, evidence for direct regulation, especially by NtcA, is also available for many genes. Additionally, a number of other regulators, some of them transcription factors, have been shown to be involved in the expression of genes at specific stages of heterocyst differentiation.

4.1. Biosynthesis and deposition of the heterocyst envelope

Key steps in the morphological and metabolic differentiation of heterocysts are the deposition of the Hep and Hgl layers. During differentiation, the Hep layer is deposited before the Hgl layer, allowing the latter to lie beneath the former.

4.1.1. Hep layer

In the chromosome of *Anabaena*, numerous genes involved in Hep layer formation are clustered in a “gene expression island”, which covers ORFs *alr2825* to *alr2841* [75], and are induced under nitrogen deprivation [76]. Because they are tightly packed together, the genes *alr2825* to *alr2831* might constitute an operon, as might be the case for the genes *alr2835* (*hepA*) to *alr2841* as well [61]. In spite of the presence of numerous NtcA binding sites in this region [38], direct NtcA-activated expression has not been reported. On the other hand, induction in response to nitrogen deprivation of the *hepA* gene within this cluster requires HetR [56], which binds to a DNA fragment from the *hepA* promoter [46]. Direct regulation of *hepA* by HetR establishes a link between formation of the Hep layer and the developmental cascade of gene regulation that operates heterocyst differentiation (Fig. 3).

Expression of *hepA* is additionally dependent on HepK, a protein-histidine kinase of a two-component regulatory system that shows homology to bacterial proteins involved in acclimation to changing O₂ conditions [77]. Interestingly, a normal oxic atmosphere is required for differentiation of mature heterocysts [78], but it remains to be determined whether HepK actually mediates a response to O₂. A cognate response regulator of HepK is DevR [79], which can be phosphorylated but lacks a C-terminal effector domain (RR* in Fig. 3). This suggests that HepK/DevR participate in a phosphorelay transduction pathway that includes further protein components, in particular a possible DNA binding protein [80].

Three additional putative regulatory genes that are required for production of the Hep layer are *hepN* (*alr0117*) that encodes a histidine kinase that lacks a sensor domain (HK* in Fig. 3) [81, 82], *hepS* (*all2760*) that encodes a putatively membrane-anchored Ser-Thr kinase [82], and *henR* (*alr1086*) that encodes a response regulator containing a CheY-like receiver domain and a PPM-type (magnesium or manganese-dependent protein phosphatase that dephosphorylates Ser and Thr residues) phosphatase domain [82]. The effect of inactivation of some of these genes on global gene expression in *Anabaena* –analyzed with oligonucleotide microarrays– has suggested that HepS and HenR belong to the same regulatory system, and that HepN is part of the HepK/DevR regulatory pathway as well as of another undetermined pathway [83]. To which environmental cues the HepS kinase and the HenR phosphatase respond is unknown, although expression of *hepS* is activated under nitrogen deprivation [61].

In summary, genes involved in formation of the Hep layer are subjected to regulation within the cascade of gene expression that operates during heterocyst differentiation promoted at least by HetR, but also to pathway-specific regulation involving two-component regulatory system(s) and protein kinases/phosphatases. A main goal of future research should be to find out whether any of these pathway-specific regulators responds to oxygen, and a point of general interest is whether any of them are subjected to direct NtcA/HetR-dependent regulation.

4.1.2. Hgl layer

For a detailed discussion of the heterocyst envelope Hgl layer, the excellent review of Awai et al [84] is recommended. As is the case for the Hep layer, numerous genes involved in the formation of the Hgl layer are clustered in the chromosome of *Anabaena* (ORFs *all5341* to *all5359*), including genes involved in the biosynthesis of the glycolipids and genes involved in their deposition outside of the outer membrane [85, 86]. Further genes necessary for the formation of the Hgl layer are located outside this cluster, and these include the *devBCA* operon encoding and ABC exporter that, together with a TolC-like protein (HgdD), constitutes a Type I secretion system involved in the export of glycolipids outside of the outer membrane in the differentiating heterocyst [87]. Along the Hgl cluster, NtcA binds to several sites, including sites upstream of genes *all5341* (*hglT*) and *asr5350* as well as in the region between *all5347* and *all5348*, which could constitute activator sites [38]. On the other hand, as described above, the promoter of the *devBCA* operon is a well-characterized, complex NtcA-dependent promoter [50, 88, 89]. It contains one canonical Class II NtcA-activated promoter and one non-canonical promoter that appears to be activated by NtcA assisted by HetR [50].

Inactivation of the *hepS* and *henR* genes involved in formation of the Hep layer also down-regulates expression of genes involved in the formation of the Hgl layer such as *hglE_A*

(*alr5351*) [83]. On the other hand, DevH is a CRP-family transcriptional regulator whose expression is increased under nitrogen deprivation in an NtcA- and HetR-dependent mode [90]. Inactivation of *devH* affects specifically the expression of Hgl-biosynthesis genes such as *hglE_A* and the production of Hgl [91]. Whether HepS/HenR and DevH act in concert in the regulation of expression of *hgl* genes is unknown (discussed in [83]). *Anabaena* has two heterocyst-specific glycolipids. Production of the minor one appears to be dependent also on two Ser/Thr kinases, Pkn44 (Al1625) and Pkn30 (Al13691) [92], and production of the major one appears to be dependent on a specific protein phosphatase, PrpJ1 (Al1731), that also affects expression of *hglE_A* [93]. The possibility that Pkn44/Pkn30 and PrpJ1 belong to two different regulatory branches for production of the minor and major Hgls, respectively, acting downstream of DevH has been discussed [92].

In summary, expression of genes for the formation of the Hgl layer involves transcription factors including the well-known NtcA and HetR proteins discussed above and DevH. In the case of DevH, the mechanism of dependence on NtcA and HetR and the direct DNA targets remain to be elucidated. Additionally, protein kinases and phosphatases affect the formation of the Hgl layer as a whole and specifically the major and minor glycolipids of that layer. The environmental cues to which such kinases and phosphatases respond and how they affect glycolipid production remain to be determined (e.g., they could affect transcription factors or directly the activity of biosynthetic enzymes).

4.2. Oxygen-consuming enzymes and protection against ROS

Oxygen that enters the heterocyst is reduced by at least two types of enzymatic processes: respiration [4] and reduction by flavodiiron proteins [94]. Additionally, a ruberythrin, RbrA, protects against oxidative stress in the heterocyst [95]. In *Anabaena*, two terminal respiratory oxidases, Cox2 and Cox3, substitute in the heterocyst for the terminal respiratory oxidase, Cox1, expressed in vegetative cells [21]. Cox2 is an *aa₃*-type cytochrome *c* oxidase encoded by the *coxB2-coxA2-coxC2* operon and Cox3 appears to be an heme-copper quinol oxidase that is encoded by the *alr2729-alr2730-coxB3-coxA3-coxC3* operon. The contribution of Cox3 to protection of nitrogenase appears to be more important than that of Cox2 [21, 96]. The expression of both operons is strictly dependent on NtcA and HetR. The *cox3* promoter region has been characterized in detail and found to contain both Class I and Class II NtcA-dependent promoters in which NtcA appears to work assisted by PipX [65].

Flavodiiron proteins directly reduce O₂ to water without the production of deleterious reactive oxygen species (ROS) [97]. In *Anabaena*, two flavodiiron proteins, Flv1B and Flv3B, are

specifically expressed in the heterocysts [94], being Flv3B especially relevant for diazotrophic performance [22]. These flavodiiron proteins are the products of two contiguous genes, *all0178* (Flv3B) and *all0177* (Flv1B), which may constitute an operon expressed from an NtcA-dependent promoter [38].

Interestingly, scavenging of O₂ by terminal respiratory oxidases and flavodiiron proteins appears not to be redundant [38]. Whereas the terminal respiratory oxidases likely remove O₂ in the heterocyst poles, since respiratory activity is located at the honeycomb membrabes [98], the heterocyst flavodiiron proteins are cytoplasmic [94]. Photosystem I-generated reductants can also reduce O₂, but in this case producing ROS. In *Anabaena*, the rubrerythrin RbrA is a heterocyst-specific peroxidase that transfers electrons from NADPH to H₂O₂ with the participation of ferredoxin-NADP⁺ oxidoreductase [95]. The *rbrA* gene is expressed from two promoters one of which directs expression in the heterocysts, is dependent on HetR and binds HetR *in vitro* [95].

4.3. Particular heterocyst metabolism genes

The metabolism of the heterocyst is focused on the fixation of N₂ and the assimilation of the N fixed in that reaction. We shall address now the transcriptional regulation of *nif* genes and of nitrogen assimilation genes such as those encoding glutamine synthetase and cyanophycin metabolism enzymes, as well as other relevant genes.

4.3.1. *nif* gene expression

In diazotrophic bacteria, the nitrogenase structural genes, *nifH-nifD-nifK*, are generally clustered together with other nitrogen fixation genes –including, e.g., genes encoding nitrogenase electron donors such as specific ferredoxins– and nitrogenase maturation genes –such as FeMo-cofactor biosynthesis genes (99). The *Anabaena* genome bears a large *nif* gene cluster including *nifB-fdxN-nifS-nifU-nifH-nifD-nifK-nifE-nifN-nifX* and a few other genes downstream. Notably, *fdxN* and *nifD* are interrupted by long DNA elements that are excised during heterocyst differentiation [100]. After excision, the *nifHDK* operon is mainly expressed from a promoter located upstream from *nifB* [101], although the large mRNA produced is subjected to processing so that *nifHDK*, *nifHD* and *nifH* transcripts are produced (see e.g. [96]). In *Anabaena* and other diazotrophic cyanobacteria, including non-heterocystous strains, the *nifB* promoter is activated by CnfR [102], previously known as PatB [103]. The CnfR protein has N-terminal sequences for binding two 4Fe-4S clusters, resembling bacterial-type ferredoxins, and a C-terminal HTH motif for binding to DNA [103, 104]. It is thought that CnfR can activate *nif* gene expression in response

to a low-oxygen tension [104, 105], and putative CnfR-binding sites have been characterized in the promoter region of *nifB* [105, 106].

In *Anabaena*, expression of *cnfR* (*patB*) is induced in response to nitrogen deprivation [61, 103], exclusively in heterocysts [107]. Based on the presence of consensus NtcA-binding sites upstream of *cnfR*, it can be suggested that this gene is subjected to NtcA-dependent expression in both heterocyst-forming and N₂-fixing, non-heterocystous cyanobacteria [38, 105]. *Anabaena variabilis* harbors two *nif* gene clusters, *nif1* that is expressed in the heterocysts and *nif2* that is expressed in the vegetative cells under micro-oxic conditions (see e.g. [106]), and expression of the *nif2* cluster regulatory gene *cnfR2* has been shown to require NtcA [104]. Therefore, expression of *cnfR2* is activated when combined nitrogen is scarce, but the Fe-S clusters may be formed only when the oxygen tension is low resulting in a protein that can promote expression of the *nifB* operon only under micro-oxic conditions [104, 105].

4.3.2. Expression of *petH*

The *petH* gene encodes ferredoxin-NADP⁺ oxidoreductase that is expressed at much higher levels in heterocysts than in vegetative cells [108], consistent with a very relevant role of this enzyme in the distribution of electrons in the heterocyst [19]. In heterocysts, *petH* is expressed from an NtcA-dependent promoter to which NtcA directly binds [109]. No good NtcA-binding site is however found in this promoter, suggesting the involvement of an NtcA coactivator such as PipX.

4.3.3. Expression of *glnA*

Glutamine synthetase, which incorporates into glutamate the ammonium produced in the N₂-fixation reaction to synthesize glutamine, is present at higher levels in the heterocysts than in vegetative cells [110]. The *glnA* gene encoding glutamine synthetase is expressed at higher levels in the absence than in the presence of combined nitrogen, being transcribed from a complex promoter region [111]. The promoter most proximal to the gene is a Class II NtcA-dependent promoter that is used during growth with nitrate and under deprivation of combined N [34, 35]. This is the *glnA* promoter that is used in the heterocysts, and its dependence on NtcA has been corroborated by mutation of its NtcA-binding site [112].

4.3.4. Cyanophycin metabolism genes

As mentioned earlier, the dynamic N reservoir cyanophycin is accumulated conspicuously at the heterocyst poles. In *Anabaena*, the genes encoding the main cyanophycin synthetase (*all3879*,

cphA1) and cyanophycinase (*all3880*, *cphB1*) are clustered together. These genes are expressed as an operon (*cphB1-cphA1*) from a complex promoter region localized upstream of *cphB1*, and *cphA1* is also transcribed monocistronically from a complex promoter present in the intergenic region. In these regions, NtcA-dependent promoters comprising high- and low-affinity NtcA-binding sites are found which are active in heterocysts [113]. The gene *all3922*, encoding isoaspartyl dipeptidase that hydrolyzes the dipeptide β -aspartyl arginine, is induced under nitrogen deprivation [61] and is expressed preferentially in vegetative cells [28], consistent with the transfer of the dipeptide from the heterocysts to the vegetative cells.

4.3.5. Expression of RuBisCO

As mentioned earlier, the RuBisCO enzyme is not active in the heterocysts thus avoiding the investment of energy and reductant in the fixation of CO₂, a process confined to the vegetative cells. Lack of RuBisCO in the heterocysts results from repression of gene expression [114], and binding of NtcA to a DNA site overlapping the translation start of the *rbcLXS* operon encoding RuBisCO appears to represent a mechanism of repression [38, 115]. This mechanism of cell-specific repression of RuBisCO stresses the relevant role of NtcA in regulation of gene expression, not only by induction but also by repression, in the mature heterocyst.

5. Heterocyst patterning

Heterocyst distribution is not random in the cyanobacterial filaments, but instead it follows a relatively fixed pattern. In most strains of the order Nostocales, heterocysts are separated by ca. 10 to 15 vegetative cells along the filament. Importantly, this pattern is established early upon N step-down, implying that products of N₂ fixation are not required for generation of at least the initial pattern.

5.1. Coupled fluctuations of HetR expression

Several theoretical models have been put forward to try a mathematical description of the generation of the heterocyst distribution pattern, and most of those models are based on the operation of an activator and an inhibitor of differentiation with different intercellular diffusivities (revised in [116]). The HetR transcription factor has been considered as the activator in these models. As mentioned above, basal expression of *hetR* takes place in the filament even in the presence of ammonium, and expression fluctuations are coupled in 2-4 nearby cells influenced by positive autoregulation ([117]; J. Stavans, personal communication). Moreover,

this pattern persists during the initial stages of development upon N step-down [117], which could explain the classical observation that differentiation is first observed in small clusters of cells that are later resolved to single heterocysts [8]. Thus, coupled fluctuations of *hetR* expression after N step-down would build-up upon the basic pattern already latent under N-replete conditions. At intermediate stages of differentiation, an increase in autocorrelation functions of *hetR* expression is observed [117], which would result from increased *hetR* autoregulation in response to *ntcA* (and *nrrA*) induction.

5.2. The PatS morphogen and the ABC exporter HetC

The *patS* gene is induced early in specific cells, depending on HetR [46], and its overexpression abolishes heterocyst differentiation, whereas its inactivation leads to the formation of heterocysts in groups (the so called MCH, *multiple contiguous heterocysts* phenotype). Hence, it was suggested that a *patS* gene product effects a lateral inhibition of differentiation [118]. The primary product of the *patS* gene is a 17-amino acid peptide that is processed to render a smaller active peptide including the C-terminal part of the primary gene product [118, 119], likely consisting of the C-terminal seven residues [119, 120]. Nonetheless, a peptide consisting of only the five C-terminal amino acids of PatS can also suppress heterocyst differentiation when added to *Anabaena* cultures [118]. The active PatS peptide (which we will denote hereafter PatS*) is transferred to the neighboring cells to establish a concentration gradient with highest levels in the cells adjacent to the producing cell, in which the concentration remains negligible, and decreasing thereon, thus behaving as a morphogen [119]. The N-terminal half of the *patS* primary product is important for processing of the peptide and for conferring immunity against the inhibitory activity in the producing cell, which would involve peptide excretion [119].

The *hetC* gene encodes an ABC-type exporter that includes a putative peptidase domain required for heterocyst differentiation [121, 122]. *hetC* is expressed from a complex promoter region that includes a canonical NtcA-dependent promoter and a HetR- and NtcA-dependent promoter, similar to the promoter region of the *devBAC* operon [43, 89]. HetC localizes mostly to the heterocyst poles [122], and the possibility of its involvement in export of PatS* from the differentiating heterocysts has been considered [122, 123], as has also been considered the participation of the septal junction protein SepJ in the intercellular transfer of PatS* between vegetative cells [124]. Regarding the inhibitory activity of the morphogen, PatS* has been shown to interact with HetR [125, 126], to influence the turnover of HetR [127], and to inhibit HetR tetramer accumulation [53] and HetR binding to DNA [46]. All these observations are fully consistent with the reported isolation of a suppressor mutant of *patS* overexpression, which bore a mutation in the *hetR* gene [128]. Thus, at least one component of the inhibition of

heterocyst differentiation by PatS* is the inhibition of HetR activity. Figure 4 presents a hypothetical model in which (i) HetC participates in processing of PatS and transfer of PatS* from the (pro)heterocyst to its neighboring vegetative cell, and (ii) SepJ participates in spreading of PatS* between vegetative cells along the filament. How PatS* is prevented from moving back to the (pro)heterocyst through SepJ-related conduits is unknown, but may be related to the distinct architecture of the septal junctions in vegetative cell-heterocyst septa described above.

Besides being dependent on *hetR* auto-regulation, the spatial correlation of *hetR* expression fluctuations along the filament, both in the presence of ammonium and early after N step-down, is influenced by expression of *patS* and intercellular communication dependent on SepJ [117]. This implies the existence of a basal expression of *patS* in the presence of ammonium, consistent with the reported detection of *patS* transcription in the presence of combined nitrogen [8]. Thus, fluctuations in the expression of *hetR* encoding a positive regulator of differentiation, HetR positive autoregulation, lateral inhibition by PatS (whose synthesis depends on HetR), and cell-to-cell communication are fundamental components of the developmental process.

5.3. Other proteins influencing pattern formation

Finally, other protein factors –including HetL, HetN, PatA, PatL and PatN– have been described to influence the spatial pattern of heterocyst distribution. The *hetN* gene influences the pattern of heterocysts especially at advanced stages of differentiation [129, 130]. This effect likely involves an internal hexapeptide sequence identical to the C-terminal sequence of PatS [130, 131]. Indeed, at late, but not at early times after N step-down, expression of *hetN* has been shown to influence the coupling of *hetR* expression fluctuations through the filament [117]. At times when heterocyst differentiation is already completed, the FraC/FraD proteins involved in intercellular molecular exchange impact *hetR* expression correlations in neighboring cells in a manner similar to SepJ [117]. This effect of FraC/FraD could involve transfer of a HetN-derived product or other regulators or N₂ fixation-derived metabolites.

Whereas inactivation of *patN* results in an increased heterocyst frequency [132, 133], inactivation of *patA* [134] or *patL* [135] results in the preferential production of terminal heterocysts in the filaments (implying a decreased heterocyst frequency). On the other hand, overexpression of *hetL* increases heterocyst frequency [136]. PatL and HetL are pentapeptide repeat proteins of unknown function, PatA and PatL have been shown to interact with each other [135], and PatN has been shown to influence the expression of *patA* [132]. Significantly, the *patA* gene is transcribed in differentiating heterocysts from two NtcA-activated promoters

[137]. PatA is a response regulator that lacks a DNA binding domain and counteracts PatS and HetN inhibition of heterocyst differentiation [138].

6. Concluding remarks

Heterocyst differentiation from a vegetative cell involves changes in expression of several hundred genes [139], including repression and induction (thoroughly reviewed by Xu *et al.* [140]). Several transcription factors effecting this regulation have been identified mainly in the model organism *Anabaena* (Table 1). NtcA and HetR are absolutely needed for heterocyst differentiation, whereas other transcription factors participate in specific steps of the differentiation process. NtcA and HetR are however not equivalent. Whereas NtcA is a global transcription factor that activates numerous gene promoters at different steps of differentiation, and represses some promoters as well, HetR has been shown to bind to few DNA sequences possibly activating transcription. Additionally, the strict requirement for HetR may rely on (i) its effect on localized induction of *ntcA* in the developing heterocyst and (ii) a role as a coactivator of NtcA at specific promoters. An additional coactivator of NtcA is PipX, which being expressed late in heterocyst differentiation supports NtcA-dependent activation of late genes, including some that are expressed in mature heterocysts. In the mature heterocyst, the *rbcl* operon promoter is directly repressed by NtcA, and a highly-expressed gene such as *glnA* is transcribed from a canonical Class II NtcA-dependent promoter, raising the possibility that expression in the mature heterocyst no longer requires differentiation-specific transcription factors. This is consistent with an inactivation of HetR by phosphorylation as discussed above. Finally, in the mature heterocyst, expression of the *nif* genes requires a specific transcription factor (CnfR) that may link expression to the micro-oxic conditions prevailing in the differentiated cell.

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References

- [1] Tomitani A, Knoll AH, Cavanaugh CM, Ohno T, The evolutionary diversification of cyanobacteria: molecular-phylogenetic and paleontological perspectives., *Proc Natl Acad Sci USA* 103 (2006) 5442-7.
- [2] Rippka R, Deruelles J, Waterbury JB, Herdman M, Stanier RY, Generic assignments, strain histories and properties of pure cultures of cyanobacteria, *J Gen Microbiol* 111 (1979) 1-61
- [3] Gallon JR, The oxygen sensitivity of nitrogenase: a problem for biochemists and micro-organisms, *TIBS* 6 (1981) 19-23
- [4] Fay P, Oxygen relations of nitrogen fixation in cyanobacteria, *Microbiol Rev* 56 (1992) 340-73.
- [5] Flores E, Herrero A, Compartmentalized function through cell differentiation in filamentous cyanobacteria, *Nat Rev Microbiol* 8 (2010) 39-50.
- [6] Maldener I, Summers ML, Sukenik A, Cellular differentiation in filamentous cyanobacteria, in: Flores E, Herrero A (Eds.) *The Cell Biology of Cyanobacteria*, Caister Academic Press, Norfolk, UK (2014) pp. 263-291.
- [7] Bradley S, Carr NG, Heterocyst and nitrogenase development in *Anabaena cylindrica*, *J Gen Microbiol* 96 (1976) 175-84.
- [8] Yoon HS, Golden JW, PatS and products of nitrogen fixation control heterocyst pattern, *J Bacteriol* 183 (2001) 2605-13.
- [9] Wilk L, Strauss M, Rudolf M, Nicolaisen K, Flores E, Kühlbrandt W, Schleiff E, Outer membrane continuity and septosome formation between vegetative cells in the filaments of *Anabaena* sp. PCC 7120, *Cell Microbiol* 13 (2011) 1744-54.
- [10] Mariscal V, Herrero A, Flores E, Continuous periplasm in a filamentous, heterocyst-forming cyanobacterium, *Mol Microbiol* 65 (2007) 1139-45.
- [11] Flores E, Herrero A, Forchhammer K, Maldener I, Septal junctions in filamentous heterocyst-forming cyanobacteria, *Trends Microbiol* 24 (2016) 79-82.
- [12] Lehner J, Berendt S, Dörsam B, Pérez R, Forchhammer K, Maldener I, Prokaryotic multicellularity: a nanopore array for bacterial cell communication, *FASEB J* 27 (2013) 2293-300.
- [13] Nürnberg DJ, Mariscal V, Bornikoel J, Nieves-Mori6n M, Krauß N, Herrero A, Maldener I, Flores E, Mullineaux CW, Intercellular diffusion of a fluorescent sucrose analog via the septal junctions in a filamentous cyanobacterium, *mBio* 6 (2015) e02109.
- [14] Bornikoel J, Carri6n A, Fan Q, Flores E, Forchhammer K, Mariscal V, Mullineaux CW, Perez R, Silber N, Wolk CP, Maldener I, Role of two cell wall amidases in septal junction and nanopore formation in the multicellular cyanobacterium *Anabaena* sp. PCC 7120, *Front Cell Infect Microbiol* 7 (2017) 386.
- [15] Flores E, Pernil R, Muro-Pastor AM, Mariscal V, Maldener I, Lechno-Yossef S, Fan Q, Wolk CP, Herrero A, Septum-localized protein required for filament integrity and diazotrophy in the heterocyst-forming cyanobacterium *Anabaena* sp. strain PCC 7120, *J Bacteriol* 189 (2007) 3884-90.
- [16] Merino-Puerto V, Mariscal V, Mullineaux CW, Herrero A, Flores E, Fra proteins influencing filament integrity, diazotrophy and localization of septal protein SepJ in the heterocyst-forming cyanobacterium *Anabaena* sp., *Mol Microbiol* 75 (2010) 1159-70.
- [17] Merino-Puerto V, Schwarz H, Maldener I, Mariscal V, Mullineaux CW, Herrero A, Flores E, FraC/FraD-dependent intercellular molecular exchange in the filaments of a heterocyst-forming cyanobacterium, *Anabaena* sp., *Mol Microbiol* 82 (2011) 87-98.
- [18] Ramos-Le6n F, Mariscal V, Battchikova N, Aro EM, Flores E, Septal protein SepJ from the heterocyst-forming cyanobacterium *Anabaena* forms multimers and interacts with peptidoglycan, *FEBS Open Bio* 7 (2017) 1515-1526.
- [19] Magnuson A, Cardona T, Thylakoid membrane function in heterocysts. *Biochim Biophys Acta* 1857 (2016) 309-19.

- 664 [20] Walsby AE, Cyanobacterial heterocysts: terminal pores proposed as sites of gas exchange, Trends
665 Microbiol 15 (2007) 340-9.
- 666 [21] Valladares A, Herrero A, Pils D, Schmetterer G, Flores E, Cytochrome *c* oxidase genes required for
667 nitrogenase activity and diazotrophic growth in *Anabaena* sp. PCC 7120, Mol Microbiol 47 (2003)
668 1239-49.
- 669 [22] Ermakova M, Battchikova N, Richaud P, Leino H, Kosourov S, Isojärvi J, Peltier G, Flores E, Cournac L,
670 Allahverdiyeva Y, Aro EM, Heterocyst-specific flavodiiron protein Flv3B enables oxic diazotrophic
671 growth of the filamentous cyanobacterium *Anabaena* sp. PCC 7120, Proc Natl Acad Sci USA 111
672 (2014) 11205-10.
- 673 [23] Wolk CP, Thomas J, Shaffer PW, Austin SM, Galonsky A, Pathway of nitrogen metabolism after fixation
674 of ¹³N-labeled nitrogen gas by the cyanobacterium, *Anabaena cylindrica*, J Biol Chem 251 (1976)
675 5027-34.
- 676 [24] Thomas J, Meeks JC, Wolk CP, Shaffer PW, Austin SM, Formation of glutamine from [¹³N]ammonia,
677 [¹³N]dinitrogen, and [¹⁴C]glutamate by heterocysts isolated from *Anabaena cylindrica*, J Bacteriol 129
678 (1977) 1545-55.
- 679 [25] Martín-Figueroa E, Navarro F, Florencio FJ, The GS-GOGAT pathway is not operative in the
680 heterocysts. Cloning and expression of *glsF* gene from the cyanobacterium *Anabaena* sp. PCC 7120,
681 FEBS Lett 476 (2000) 282-6.
- 682 [26] Sherman DM, Tucker D, Sherman LA, Heterocyst development and localization of cyanophycin in N₂-
683 fixing cultures of *Anabaena* sp. PCC 7120 (Cyanobacteria), J Phycol 36 (2000) 932-941.
- 684 [27] Herrero A, Burnat M, Cyanophycin, a cellular nitrogen reserve material, in: Flores E, Herrero A (Eds.)
685 The Cell Biology of Cyanobacteria, Caister Academic Press, Norfolk, UK (2014) pp. 211-219.
- 686 [28] Burnat M, Herrero A, Flores E, Compartmentalized cyanophycin metabolism in the diazotrophic
687 filaments of a heterocyst-forming cyanobacterium, Proc Natl Acad Sci USA 111 (2014) 3823-8.
- 688 [29] López-Igual R, Flores E, Herrero A, Inactivation of a heterocyst-specific invertase indicates a principal
689 role of sucrose catabolism in heterocysts of *Anabaena* sp., J Bacteriol 192 (2010) 5526-33.
- 690 [30] Vargas WA, Nishi CN, Giarrocco LE, Salerno GL, Differential roles of alkaline/neutral invertases in
691 *Nostoc* sp. PCC 7120: Inv-B isoform is essential for diazotrophic growth, Planta 233 (2011) 153-62.
- 692 [31] Mullineaux CW, Mariscal V, Nenninger A, Khanum H, Herrero A, Flores E, Adams DG, Mechanism of
693 intercellular molecular exchange in heterocyst-forming cyanobacteria, EMBO J 27 (2008) 1299-308.
- 694 [32] Nieves-Mori6n M, Mullineaux CW, Flores E, Molecular diffusion through cyanobacterial septal
695 junctions, mBio 8 (2017) e01756-16.
- 696 [33] Omairi-Nasser A, Mariscal V, Austin JR 2nd, Haselkorn R, Requirement of Fra proteins for
697 communication channels between cells in the filamentous nitrogen-fixing cyanobacterium
698 *Anabaena* sp. PCC 7120, Proc Natl Acad Sci USA 112 (2015) E4458-64.
- 699 [34] Frías JE, Flores E, Herrero A, Requirement of the regulatory protein NtcA for the expression of
700 nitrogen assimilation and heterocyst development genes in the cyanobacterium *Anabaena* sp. PCC
701 7120, Mol Microbiol 14 (1994) 823-32.
- 702 [35] Wei TF, Ramasubramanian TS, Golden JW, *Anabaena* sp. strain PCC 7120 *ntcA* gene required for
703 growth on nitrate and heterocyst development, J Bacteriol 176 (1994) 4473-82.
- 704 [36] Buikema WJ, Haselkorn R, Characterization of a gene controlling heterocyst differentiation in the
705 cyanobacterium *Anabaena* 7120, Genes Dev 5 (1991) 321-30.
- 706 [37] Luque I, Flores E, Herrero A, Molecular mechanism for the operation of nitrogen control in
707 cyanobacteria, EMBO J 13 (1994) 2862-9.
- 708 [38] Picossi S, Flores E, Herrero A, ChIP analysis unravels an exceptionally wide distribution of DNA binding
709 sites for the NtcA transcription factor in a heterocyst-forming cyanobacterium, BMC Genomics 15
710 (2014) 22.

711 [39] Zhao MX, Jiang YL, He YX, Chen YF, Teng YB, Chen Y, Zhang CC, Zhou CZ, Structural basis for the
712 allosteric control of the global transcription factor NtcA by the nitrogen starvation signal 2-
713 oxoglutarate, *Proc Natl Acad Sci USA* 107 (2010) 12487-92.

714 [40] Luque I, Forchhammer K, Nitrogen assimilation and C/N balance sensing, in: Herrero A, Flores E (Eds.)
715 The Cyanobacteria: Molecular Biology, Genomics and Evolution, Caister Academic Press, Norfolk, UK
716 (2008) pp. 335-382.

717 [41] Herrero A, Picossi S, Flores E, Gene expression during heterocyst differentiation, *Adv Bot Res* 65
718 (2013) 281-329.

719 [42] Vázquez-Bermúdez MF, Herrero A, Flores E, 2-Oxoglutarate increases the binding affinity of the NtcA
720 (nitrogen control) transcription factor for the *Synechococcus glnA* promoter, *FEBS Lett* 512 (2002)
721 71-4.

722 [43] Valladares A, Flores E, Herrero A, Transcription activation by NtcA and 2-oxoglutarate of three genes
723 involved in heterocyst differentiation in the cyanobacterium *Anabaena* sp. strain PCC 7120, *J*
724 *Bacteriol* 190 (2008) 6126-33.

725 [44] López-Igual R, Picossi S, López-Garrido J, Flores E, Herrero A, N and C control of ABC-type bicarbonate
726 transporter Cmp and its LysR-type transcriptional regulator CmpR in a heterocyst-forming
727 cyanobacterium, *Anabaena* sp., *Environ Microbiol* 14 (2012) 1035-48.

728 [45] Zhang JY, Chen WL, Zhang CC, *hetR* and *patS*, two genes necessary for heterocyst pattern formation,
729 are widespread in filamentous nonheterocyst-forming cyanobacteria, *Microbiology* 155 (2009)
730 1418-26.

731 [46] Huang X, Dong Y, Zhao J, HetR homodimer is a DNA-binding protein required for heterocyst
732 differentiation, and the DNA-binding activity is inhibited by PatS, *Proc Natl Acad Sci USA* 101 (2004)
733 4848-53.

734 [47] Higa KC, Callahan SM, Ectopic expression of *hetP* can partially bypass the need for *hetR* in heterocyst
735 differentiation by *Anabaena* sp. strain PCC 7120, *Mol Microbiol* 77 (2010) 562-74.

736 [48] Videau P, Rivers OS, Hurd K, Ushijima B, Oshiro RT, Ende RJ, O'Hanlon SM, Cozy LM, The heterocyst
737 regulatory protein HetP and its homologs modulate heterocyst commitment in *Anabaena* sp. strain
738 PCC 7120, *Proc Natl Acad Sci USA* (2016) pii: 201610533.

739 [49] Flaherty BL, Johnson DB, Golden JW, Deep sequencing of HetR-bound DNA reveals novel HetR targets
740 in *Anabaena* sp. strain PCC7120, *BMC Microbiol* 14 (2014) 255.

741 [50] Camargo S, Valladares A, Flores E, Herrero A, Transcription activation by NtcA in the absence of
742 consensus NtcA-binding sites in an *Anabaena* heterocyst differentiation gene promoter, *J Bacteriol*
743 194 (2012) 2939-48.

744 [51] Videau P, Ni S, Rivers OS, Ushijima B, Feldmann EA, Cozy LM, Kennedy MA, Callahan SM, Expanding
745 the direct HetR regulon in *Anabaena* sp. strain PCC 7120, *J Bacteriol* 196 (2014) 1113-21.

746 [52] Kim Y, Joachimiak G, Ye Z, Binkowski TA, Zhang R, Gornicki P, Callahan SM, Hess WR, Haselkorn R,
747 Joachimiak A, Structure of transcription factor HetR required for heterocyst differentiation in
748 cyanobacteria, *Proc Natl Acad Sci USA* 108 (2011) 10109-14.

749 [53] Valladares A, Flores E, Herrero A, The heterocyst differentiation transcriptional regulator HetR of the
750 filamentous cyanobacterium *Anabaena* forms tetramers and can be regulated by phosphorylation,
751 *Mol Microbiol* 99 (2016) 808-19.

752 [54] Kim Y, Ye Z, Joachimiak G, Videau P, Young J, Hurd K, Callahan SM, Gornicki P, Zhao J, Haselkorn R,
753 Joachimiak A, Structures of complexes comprised of *Fischerella* transcription factor HetR with
754 *Anabaena* DNA targets, *Proc Natl Acad Sci USA* 110 (2013) E1716-23.

755 [55] Muro-Pastor AM, Valladares A, Flores E, Herrero A, Mutual dependence of the expression of the cell
756 differentiation regulatory protein HetR and the global nitrogen regulator NtcA during heterocyst
757 development. *Mol Microbiol* 44 (2002) 1377-85.

758 [56] Black TA, Cai Y, Wolk CP, Spatial expression and autoregulation of *hetR*, a gene involved in the control
759 of heterocyst development in *Anabaena*, *Mol Microbiol* 9 (1993) 77-84. Erratum in: *Mol Microbiol*
760 10 (1993) 1153.

761 [57] Olmedo-Verd E, Muro-Pastor AM, Flores E, Herrero A, Localized induction of the *ntcA* regulatory gene
762 in developing heterocysts of *Anabaena* sp. strain PCC 7120, J Bacteriol 188 (2006) 6694-9.

763 [58] Rajagopalan R, Callahan SM, Temporal and spatial regulation of the four transcription start sites of
764 *hetR* from *Anabaena* sp. strain PCC 7120, J Bacteriol 192 (2010) 1088-96.

765 [59] Muro-Pastor AM, Olmedo-Verd E, Flores E, All4312, an NtcA-regulated two-component response
766 regulator in *Anabaena* sp. strain PCC 7120, FEMS Microbiol Lett 256 (2006) 171-7.

767 [60] Ehira S, Ohmori M, NrrA directly regulates expression of *hetR* during heterocyst differentiation in the
768 cyanobacterium *Anabaena* sp. strain PCC 7120, J Bacteriol 188 (2006) 8520-5.

769 [61] Flaherty BL, Van Nieuwerburgh F, Head SR, Golden JW, Directional RNA deep sequencing sheds new
770 light on the transcriptional response of *Anabaena* sp. strain PCC 7120 to combined-nitrogen
771 deprivation, BMC Genomics 12 (2011) 332.

772 [62] Valladares A, Rodríguez V, Camargo S, Martínez-Noël GM, Herrero A, Luque I, Specific role of the
773 cyanobacterial PipX factor in the heterocysts of *Anabaena* sp. strain PCC 7120, J Bacteriol 193 (2011)
774 1172-82.

775 [63] Mitschke J, Vioque A, Haas F, Hess WR, Muro-Pastor AM, Dynamics of transcriptional start site
776 selection during nitrogen stress-induced cell differentiation in *Anabaena* sp. PCC7120, Proc Natl
777 Acad Sci USA 108 (2011) 20130-5.

778 [64] Llácer JL, Espinosa J, Castells MA, Contreras A, Forchhammer K, Rubio V, Structural basis for the
779 regulation of NtcA-dependent transcription by proteins PipX and PII, Proc Natl Acad Sci USA 107
780 (2010) 15397-402.

781 [65] Camargo S, Valladares A, Forchhammer K, Herrero A, Effects of PipX on NtcA-dependent promoters
782 and characterization of the *cox3* promoter region in the heterocyst-forming cyanobacterium
783 *Anabaena* sp. PCC 7120, FEBS Lett 588 (2014) 1787-94.

784 [66] Brahamsha B, Haselkorn R, Isolation and characterization of the gene encoding the principal sigma
785 factor of the vegetative cell RNA polymerase from the cyanobacterium *Anabaena* sp. strain PCC
786 7120, J Bacteriol 173 (1991) 2442-50.

787 [67] Muro-Pastor AM, Brenes-Álvarez M, Vioque A, A combinatorial strategy of alternative promoter use
788 during differentiation of a heterocystous cyanobacterium, Environ Microbiol Rep 9 (2017) 449-458.

789 [68] Imamura S, Asayama M, Sigma factors for cyanobacterial transcription, Gene Regul Syst Bio 3 (2009)
790 65-87.

791 [69] Khudyakov IY, Golden JW, Identification and inactivation of three group 2 sigma factor genes in
792 *Anabaena* sp. strain PCC 7120, J Bacteriol 183 (2001) 6667-75.

793 [70] Aldea MR, Mella-Herrera RA, Golden JW, Sigma factor genes *sigC*, *sigE*, and *sigG* are upregulated in
794 heterocysts of the cyanobacterium *Anabaena* sp. strain PCC 7120, J Bacteriol 189 (2007) 8392-6.

795 [71] Ehira S, Ohmori M, NrrA, a nitrogen-regulated response regulator protein, controls glycogen
796 catabolism in the nitrogen-fixing cyanobacterium *Anabaena* sp. strain PCC 7120, J Biol Chem 286
797 (2011) 38109-14.

798 [72] Mella-Herrera RA, Neunuebel MR, Kumar K, Saha SK, Golden JW, The *sigE* gene is required for normal
799 expression of heterocyst-specific genes in *Anabaena* sp. strain PCC 7120, J Bacteriol 193 (2011) 1823-
800 32.

801 [73] Schrautemeier B, Neveling U, Schmitz S, Distinct and differently regulated Mo-dependent nitrogen-
802 fixing systems evolved for heterocysts and vegetative cells of *Anabaena variabilis* ATCC 29413:
803 characterization of the *fdxH1/2* gene regions as part of the *nif1/2* gene clusters, Mol Microbiol 18
804 (1995) 357-69.

805 [74] Saha SK, Golden JW, Overexpression of *pknE* blocks heterocyst development in *Anabaena* sp. strain
806 PCC 7120, J Bacteriol 193 (2011) 2619-29.

807 [75] Huang G, Fan Q, Lechno-Yossef S, Wojciuch E, Wolk CP, Kaneko T, Tabata S, Clustered genes required
808 for the synthesis of heterocyst envelope polysaccharide in *Anabaena* sp. strain PCC 7120, J Bacteriol
809 187 (2005) 1114-23.

810 [76] Ehira S, Ohmori M, Sato N, Genome-wide expression analysis of the responses to nitrogen
811 deprivation in the heterocyst-forming cyanobacterium *Anabaena* sp. strain PCC 7120, DNA Res 10
812 (2003) 97-113.

813 [77] Zhu J, Kong R, Wolk CP, Regulation of hepA of *Anabaena* sp. strain PCC 7120 by elements 5' from the
814 gene and by *hepK*, J Bacteriol 180 (1998) 4233-42.

815 [78] Rippka R, Stanier R, The effect of anaerobiosis on nitrogenase synthesis and heterocyst development
816 by Nostocacean cyanobacteria, J Gen Microbiol 105 (1978) 83-94.

817 [79] Zhou R, Wolk CP, A two-component system mediates developmental regulation of biosynthesis of a
818 heterocyst polysaccharide, J Biol Chem 278 (2003) 19939-46.

819 [80] Hagen KD, Meeks JC, Biochemical and genetic evidence for participation of DevR in a phosphorelay
820 signal transduction pathway essential for heterocyst maturation in *Nostoc punctiforme* ATCC 29133,
821 J Bacteriol 181 (1999) 4430-4.

822 [81] Ning D, Xu X, *alr0117*, a two-component histidine kinase gene, is involved in heterocyst development
823 in *Anabaena* sp. PCC 7120, Microbiology 150 (2004) 447-53.

824 [82] Fan Q, Lechno-Yossef S, Ehira S, Kaneko T, Ohmori M, Sato N, Tabata S, Wolk CP, Signal transduction
825 genes required for heterocyst maturation in *Anabaena* sp. strain PCC 7120, J Bacteriol 188 (2006)
826 6688-93.

827 [83] Lechno-Yossef S, Fan Q, Ehira S, Sato N, Wolk CP, Mutations in four regulatory genes have interrelated
828 effects on heterocyst maturation in *Anabaena* sp. strain PCC 7120, J Bacteriol 188 (2006) 7387-95.

829 [84] Awai K, Lechno-Yossef S, Wolk CP, Heterocyst envelope glycolipids, in: Wada H, Murata N (Eds.) Lipids
830 in Photosynthesis: Essential and Regulatory Functions, Springer Science + Business Media B.V. (2009)
831 pp.179–202.

832 [85] Fan Q, Huang G, Lechno-Yossef S, Wolk CP, Kaneko T, Tabata S, Clustered genes required for synthesis
833 and deposition of envelope glycolipids in *Anabaena* sp. strain PCC 7120, Mol Microbiol 58 (2005)
834 227-43.

835 [86] Awai K, Wolk CP, Identification of the glycosyl transferase required for synthesis of the principal
836 glycolipid characteristic of heterocysts of *Anabaena* sp. strain PCC 7120, FEMS Microbiol Lett 266
837 (2007) 98-102.

838 [87] Staron P, Forchhammer K, Maldener I, Novel ATP-driven pathway of glycolipid export involving TolC
839 protein, J Biol Chem 286 (2011) 38202-10.

840 [88] Fiedler G, Muro-Pastor AM, Flores E, Maldener I, NtcA-dependent expression of the *devBCA* operon,
841 encoding a heterocyst-specific ATP-binding cassette transporter in *Anabaena* spp., J Bacteriol (2001)
842 3795-9.

843 [89] Muro-Pastor AM, Flores E, Herrero A, NtcA-regulated heterocyst differentiation genes *hetC* and *devB*
844 from *Anabaena* sp. strain PCC 7120 exhibit a similar tandem promoter arrangement, J Bacteriol
845 (2009) 5765-74.

846 [90] Hebbar PB, Curtis SE, Characterization of *devH*, a gene encoding a putative DNA binding protein
847 required for heterocyst function in *Anabaena* sp. strain PCC 7120, J Bacteriol 182 (2000) 3572-81.

848 [91] Ramírez ME, Hebbar PB, Zhou R, Wolk CP, Curtis SE, *Anabaena* sp. strain PCC 7120 gene *devH* is
849 required for synthesis of the heterocyst glycolipid layer, J Bacteriol 187 (2005) 2326-31.

850 [92] Shi L, Li JH, Cheng Y, Wang L, Chen WL, Zhang CC, Two genes encoding protein kinases of the HstK
851 family are involved in synthesis of the minor heterocyst-specific glycolipid in the
852 cyanobacterium *Anabaena* sp. strain PCC 7120, J Bacteriol 189 (2007) 5075-81.

853 [93] Jang J, Wang L, Jeanjean R, Zhang CC, PrpJ, a PP2C-type protein phosphatase located on the plasma
854 membrane, is involved in heterocyst maturation in the cyanobacterium *Anabaena* sp. PCC 7120, Mol
855 Microbiol 64 (2007) 347-58.

856 [94] Ermakova M, Battchikova N, Allahverdiyeva Y, Aro EM, Novel heterocyst-specific flavodiiron proteins
857 in *Anabaena* sp. PCC 7120, FEBS Lett 587 (2013) 82-7.

- [95] Zhao W, Ye Z, Zhao J, RbrA, a cyanobacterial rubrerythrin, functions as a FNR-dependent peroxidase in heterocysts in protection of nitrogenase from damage by hydrogen peroxide in *Anabaena* sp. PCC 7120, *Mol Microbiol* 66 (2007) 1219-30.
- [96] Valladares A, Maldener I, Muro-Pastor AM, Flores E, Herrero A, Heterocyst development and diazotrophic metabolism in terminal respiratory oxidase mutants of the cyanobacterium *Anabaena* sp. strain PCC 7120, *J Bacteriol* 189 (2007) 4425-30.
- [97] Allahverdiyeva Y, Isojärvi J, Zhang P, Aro EM, Cyanobacterial oxygenic photosynthesis is protected by flavodiiron Proteins, *Life (Basel)* 5 (2015) 716-43.
- [98] Murry MA, Olafsen AG, Benemann JR, Oxidation of diaminobenzidine in the heterocysts of *Anabaena cylindrica*, *Curr Microbiol* 6 (1981) 201-206.
- [99] Rubio LM, Ludden PW, Biosynthesis of the iron-molybdenum cofactor of nitrogenase, *Annu Rev Microbiol* 62 (2008) 93-111.
- [100] Haselkorn R, Organization of the genes for nitrogen fixation in photosynthetic bacteria and cyanobacteria, *Annu Rev Microbiol* 40 (1986) 525-47.
- [101] Ungerer JL, Pratte BS, Thiel T, RNA processing of nitrogenase transcripts in the cyanobacterium *Anabaena variabilis*, *J Bacteriol* 192 (2010) 3311-20.
- [102] Tsujimoto R, Kamiya N, Fujita Y, Transcriptional regulators ChlR and CnfR are essential for diazotrophic growth in nonheterocystous cyanobacteria, *Proc Natl Acad Sci USA* 111 (2014) 6762-7.
- [103] Liang J, Scappino L, Haselkorn R, The *patB* gene product, required for growth of the cyanobacterium *Anabaena* sp. strain PCC 7120 under nitrogen-limiting conditions, contains ferredoxin and helix-turn-helix domains. *J Bacteriol* 175 (1993) 1697-704.
- [104] Pratte BS, Thiel T, Homologous regulators, CnfR1 and CnfR2, activate expression of two distinct nitrogenase gene clusters in the filamentous cyanobacterium *Anabaena variabilis* ATCC 29413, *Mol Microbiol* 100 (2016) 1096-109.
- [105] Tsujimoto R, Kamiya N, Fujita Y, Identification of a cis-acting element in nitrogen fixation genes recognized by CnfR in the nonheterocystous nitrogen-fixing cyanobacterium *Leptolyngbya boryana*, *Mol Microbiol* 101 (2016) 411-24.
- [106] Vernon SA, Pratte BS, Thiel T, Role of the *nifB1* and *nifB2* promoters in cell-type-specific expression of two Mo nitrogenases in the cyanobacterium *Anabaena variabilis* ATCC 29413, *J Bacteriol* 199 (2017) e00674-16.
- [107] Jones KM, Buikema WJ, Haselkorn R, Heterocyst-specific expression of *patB*, a gene required for nitrogen fixation in *Anabaena* sp. strain PCC 7120, *J Bacteriol* 185 (2003) 2306-14.
- [108] Razquin P, Fillat MF, Schmitz S, Stricker O, Böhme H, Gómez-Moreno C, Peleato ML, Expression of ferredoxin-NADP⁺ reductase in heterocysts from *Anabaena* sp., *Biochem J* 316 (1996) 157-60.
- [109] Valladares A, Muro-Pastor AM, Fillat MF, Herrero A, Flores E, Constitutive and nitrogen-regulated promoters of the *petH* gene encoding ferredoxin:NADP⁺ reductase in the heterocyst-forming cyanobacterium *Anabaena* sp., *FEBS Lett* 449 (1999) 159-64.
- [110] Bergman B, Lindblad P, Pettersson A, Renström E, Tiberg E, Immuno-gold localization of glutamine synthetase in a nitrogen-fixing cyanobacterium (*Anabaena cylindrica*), *Planta* 166 (1985) 329-34.
- [111] Tumer NE, Robinson SJ, Haselkorn R, Different promoters for the *Anabaena* glutamine synthetase gene during growth using molecular or fixed nitrogen, *Nature* 306 (1983) 337-342.
- [112] Valladares A, Muro-Pastor AM, Herrero A, Flores E, The NtcA-dependent P1 promoter is utilized for *glnA* expression in N₂-fixing heterocysts of *Anabaena* sp. strain PCC 7120, *J Bacteriol* 186 (2004) 7337-43.
- [113] Picossi S, Valladares A, Flores E, Herrero A, Nitrogen-regulated genes for the metabolism of cyanophycin, a bacterial nitrogen reserve polymer: expression and mutational analysis of two cyanophycin synthetase and cyanophycinase gene clusters in heterocyst-forming cyanobacterium *Anabaena* sp. PCC 7120, *J Biol Chem* 279 (2004) 11582-92.

907 [114] Elhai J, Wolk CP, Developmental regulation and spatial pattern of expression of the structural genes
908 for nitrogenase in the cyanobacterium *Anabaena*, EMBO J 9 (1990) 3379-88.

909 [115] Picossi S, Flores E, Herrero A, The LysR-type transcription factor PacR is a global regulator of
910 photosynthetic carbon assimilation in *Anabaena*, Environ Microbiol 17 (2015) 3341-51.

911 [116] Herrero A, Stavans J, Flores E, The multicellular nature of filamentous heterocyst-forming
912 cyanobacteria, FEMS Microbiol Rev 40 (2016) 831-854.

913 [117] Corrales-Guerrero L, Tal A, Arbel-Goren R, Mariscal V, Flores E, Herrero A, Stavans J, Spatial
914 fluctuations in expression of the heterocyst differentiation regulatory gene *hetR* in *Anabaena*
915 filaments, PLoS Genet 11 (2015) e1005031.

916 [118] Yoon HS, Golden JW, Heterocyst pattern formation controlled by a diffusible peptide, Science 282
917 (1998) 935-8.

918 [119] Corrales-Guerrero L, Mariscal V, Flores E, Herrero A, Functional dissection and evidence for
919 intercellular transfer of the heterocyst-differentiation PatS morphogen, Mol Microbiol 88 (2013)
920 1093-105.

921 [120] Zhang L, Zhou F, Wang S, Xu X, Processing of PatS, a morphogen precursor, in cell extracts
922 of *Anabaena* sp. PCC 7120, FEBS Lett 591 (2017) 751-759.

923 [121] Khudyakov I, Wolk CP, *hetC*, a gene coding for a protein similar to bacterial ABC protein exporters,
924 is involved in early regulation of heterocyst differentiation in *Anabaena* sp. strain PCC 7120, J
925 Bacteriol 179 (1997) 6971-8.

926 [122] Corrales-Guerrero L, Flores E, Herrero A, Relationships between the ABC-exporter HetC and
927 peptides that regulate the spatiotemporal pattern of heterocyst distribution in *Anabaena*, PLoS One
928 9 (2014) e104571.

929 [123] Videau P, Rivers OS, Higa KC, Callahan SM, ABC transporter required for intercellular transfer of
930 developmental signals in a heterocystous cyanobacterium, J Bacteriol 197 (2015) 2685-93.

931 [124] Mariscal V, Nürnberg DJ, Herrero A, Mullineaux CW, Flores E, Overexpression of SepJ alters septal
932 morphology and heterocyst pattern regulated by diffusible signals in *Anabaena*, Mol Microbiol 101
933 (2016) 968-81.

934 [125] Feldmann EA, Ni S, Sahu ID, Mishler CH, Risser DD, Murakami JL, Tom SK, McCarrick RM,
935 Lorigan GA, Tolbert BS, Callahan SM, Kennedy MA, Evidence for direct binding between
936 HetR from *Anabaena* sp. PCC 7120 and PatS-5, Biochemistry 50 (2011) 9212-24.

937 [126] Hu HX, Jiang YL, Zhao MX, Cai K, Liu S, Wen B, Lv P, Zhang Y, Peng J, Zhong H, Yu HM, Ren YM, Zhang
938 Z, Tian C, Wu Q, Oliveberg M, Zhang CC, Chen Y, Zhou CZ, Structural insights into HetR-PatS
939 interaction involved in cyanobacterial pattern formation, Sci Rep 5 (2015) 16470.

940 [127] Risser DD, Callahan SM, Genetic and cytological evidence that heterocyst patterning is regulated by
941 inhibitor gradients that promote activator decay, Proc Natl Acad Sci USA 106 (2009) 19884-8.

942 [128] Khudyakov IY, Golden JW, Different functions of HetR, a master regulator of heterocyst
943 differentiation in *Anabaena* sp. PCC 7120, can be separated by mutation, Proc Natl Acad Sci USA 101
944 (2004) 16040-5.

945 [129] Callahan SM, Buikema WJ, The role of HetN in maintenance of the heterocyst pattern in *Anabaena*
946 sp. PCC 7120, Mol Microbiol 40 (2001) 941-50.

947 [130] Corrales-Guerrero L, Mariscal V, Nürnberg DJ, Elhai J, Mullineaux CW, Flores E, Herrero A, Subcellular
948 localization and clues for the function of the HetN factor influencing heterocyst distribution in
949 *Anabaena* sp. strain PCC 7120, J Bacteriol 196 (2014) 3452-60.

950 [131] Higa KC, Rajagopalan R, Risser DD, Rivers OS, Tom SK, Videau P, Callahan SM, The RGSGR amino acid
951 motif of the intercellular signalling protein, HetN, is required for patterning of heterocysts in
952 *Anabaena* sp. strain PCC 7120, Mol Microbiol 83 (2012) 682-93.

953 [132] Risser DD, Wong FC, Meeks JC, Biased inheritance of the protein PatN frees vegetative cells to
954 initiate patterned heterocyst differentiation, Proc Natl Acad Sci USA 109 (2012) 15342-7.

955 [133] Masukawa H, Sakurai H, Hausinger RP, Inoue K, Increased heterocyst frequency by *patN* disruption
956 in *Anabaena* leads to enhanced photobiological hydrogen production at high light intensity and high
957 cell density, *Appl Microbiol Biotechnol* 101 (2017) 2177-2188.

958 [134] Liang J, Scappino L, Haselkorn R, The *patA* gene product, which contains a region similar to CheY of
959 *Escherichia coli*, controls heterocyst pattern formation in the cyanobacterium *Anabaena* 7120, *Proc*
960 *Natl Acad Sci USA* 89 (1992) 5655-9.

961 [135] Liu J, Wolk CP, Mutations in genes *patA* and *patL* of *Anabaena* sp. strain PCC 7120 result in similar
962 phenotypes, and the proteins encoded by those genes may interact, *J Bacteriol* 193 (2011) 6070-4.

963 [136] Liu D, Golden JW, *hetL* overexpression stimulates heterocyst formation in *Anabaena* sp. strain PCC
964 7120, *J Bacteriol* 184 (2002) 6873-81.

965 [137] Bastet L, Boileau C, Bédu S, Janicki A, Latifi A, Zhang CC, NtcA regulates *patA* expression
966 in *Anabaena* sp. strain PCC 7120, *J Bacteriol* 192 (2010): 5257-9.

967 [138] Orozco CC, Risser DD, Callahan SM, Epistasis analysis of four genes from *Anabaena* sp. strain PCC
968 7120 suggests a connection between PatA and PatS in heterocyst pattern formation, *J Bacteriol* 188
969 (2006) 1808-16.

970 [139] Lynn ME, Bantle JA, Ownby JD, Estimation of gene expression in heterocysts of *Anabaena variabilis*
971 by using DNA-RNA hybridization, *J Bacteriol* 167 (1986) 940-6.

972 [140] Xu X, Elhai J, Wolk CP, Transcriptional and developmental responses by *Anabaena* to deprivation of
973 fixed nitrogen, in: Herrero A, Flores E (Eds.) *The Cyanobacteria: Molecular Biology, Genomics and*
974 *Evolution*, Caister Academic Press, Norfolk, UK (2008) pp. 383-422.

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976 Table 1. DNA-binding transcription factors in heterocyst differentiation and function.
977

Transcription factor	<i>Anabaena</i> ORF	Characteristics
NtcA	<i>alr4392</i>	CRP-family transcriptional regulator. Global transcription factor that mediates the cellular responses to changes in the C-to-N balance. Binds 2-oxoglutarate.
HetR	<i>alr2339</i>	Novel type of transcription factor absolutely required for heterocyst differentiation. Inhibited by the PatS morphogen.
NrrA	<i>all4312</i>	OmpR-family two-component response regulator. Induced by NtcA, it seems to mediate some NtcA responses.
DevH	<i>alr3952</i>	CRP-family transcriptional regulator. Needed for transcription of some genes encoding heterocyst glycolipid-biosynthesis proteins.
CnfR (a.k.a. PatB)	<i>all2512</i>	2 x [4Fe-4S]-binding protein and Xre family transcriptional regulator. Needed for <i>nif</i> gene expression.

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Figure legends

Figure 1. Filaments and heterocysts of *Anabaena* sp. strain PCC 7120. (A) Filaments consisting of vegetative cells and heterocysts. Black arrows, intercalary heterocysts; white arrow, terminal heterocyst. Micrograph by J. E. Frías (CSIC, Seville). (B) Transmission electron micrograph of a terminal heterocyst and adjacent vegetative cells. The honeycomb membranes, the heterocyst polysaccharide (Hep) layer and, just beneath the Hep layer, the heterocyst glycolipid (Hgl) layer are indicated. CG indicates the place of localization of the cyanophycin granule (at the heterocyst pole, next to the heterocyst “neck”), which is frequently lost during sample preparation. Carboxysomes are readily observed in vegetative cells. Micrograph courtesy of I. Maldener (Universität Tübingen).

Figure 2. Crystal structures of NtcA from *Anabaena* (A) and of HetR from *Fischerella* (B), and example of a heterocyst differentiation gene promoter region, that of the *devBCA* promoter from *Anabaena* (C). The NtcA dimer contains one molecule of 2-oxoglutarate bound (black arrow), and the HetR dimer is bound to a 24mer DNA target (bottom) and has also bound one Mg^{2+} ion (black arrow). Structures from Protein Data Bank in Europe (PDBe), number 3la7 (NtcA [39]) and number 4j00 (HetR [54]). (C) The *devBCA* operon is transcribed during heterocyst differentiation from two promoters, a distal Class II NtcA-dependent promoter that is independent of HetR, and a proximal NtcA- and HetR-dependent promoter that bears an imperfect NtcA-binding site but is activated by NtcA with a positive effect of HetR [50]. Orange boxes, -10 promoter hexamers; solid green box, consensus NtcA-binding site; hatched green box, degenerated NtcA-binding site.

Figure 3. General scheme of transcriptional activation during heterocyst differentiation including a selected set of target genes as examples. The process takes about 18 to 24 h to complete after N step-down, depending on lab conditions. Although no precise times are intended in the scheme, the genes presented left to right correspond to genes expressed early to late in the process. The left part of the scheme shows the NtcA-NrrA-HetR interactions. Grey central boxes include groups of genes involved in the indicated activities (*hetC* is included in pattern formation because of its proposed role in PatS transfer). The upper part captures the extensive direct regulation exerted by NtcA. Three examples of direct regulation by HetR are indicated in the lower-left part. Below the central boxes, other regulators that influence transcription, including regulators that may respond to O_2 , are shown. Block arrows represent

the indicated genes. Other shapes (ovals, squares, triangles, etc.) represent proteins. Abbreviations: HK, histidine kinase; RR, response regulator; STK, serine/threonine kinase; HTH, helix-turn-helix motif; -P, demonstrated or possible phosphorylation. Solid arrows represent direct regulation (i.e., demonstrated binding of the regulator to a promoter), dotted arrows represent possible or hypothetical direct regulation, and dashed arrows indicate indirect regulation or an unknown mechanism.

Figure 4. Hypothetical model of PatS production and intercellular transfer in *Anabaena*. The *patS* gene is induced at the beginning of heterocyst differentiation dependent on NtcA and HetR, producing a polypeptide (PatS) that is processed to render the active morphogen (PatS*). The peptidase/ABC exporter HetC appears necessary for PatS function; because PatS* does not accumulate in (pro)heterocysts, it can be suggested that it is actively exported from them. On the other hand, SepJ appears to be involved in the transfer of PatS* between vegetative cells suggesting transfer through the septal junctions. The activity of PatS* as a negative regulator of HetR is represented.

-N (high C/N)