

Compartmentalized function through cell differentiation in filamentous cyanobacteria

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Abstract | Within the wide biodiversity that is found in the bacterial world, Cyanobacteria represents a unique phylogenetic group that is responsible for a key metabolic process in the biosphere — oxygenic photosynthesis — and that includes representatives exhibiting complex morphologies. Many cyanobacteria are multicellular, growing as filaments of cells in which some cells can differentiate to carry out specialized functions. These differentiated cells include resistance and dispersal forms as well as a metabolically specialized form that is devoted to N₂ fixation, known as the heterocyst. In this Review we address cyanobacterial intercellular communication, the supracellular structure of the cyanobacterial filament and the basic principles that govern the process of heterocyst differentiation.

Oxygenic photosynthesis

A biological process that converts light energy into chemical energy and splits water to release oxygen.

Hypha

A branching cellular filament that forms the mycelium of actinobacteria and fungi.

Fruiting body

A cell aggregate where spores are formed in some myxobacteria and fungi.

Primary productivity

The production of organic compounds from CO₂.

Heterocyst

A terminally differentiated N₂-fixing cell found in some filamentous cyanobacteria.

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The bacterial world contains much metabolic biodiversity. If life was present on Earth at least 3.5 billion years ago, bacteria had notably diversified by around 2.3 billion years ago, by which time an astonishing range of metabolic capabilities, including oxygenic photosynthesis, had developed¹. Since then, bacteria have kept a genetic reservoir of the key redox-chemistry metabolic capabilities of the biosphere². Although commonly viewed as single-celled organisms, many bacteria are multicellular. Morphologically complex bacteria were probably present on Earth in the Archean eon (that is, before 2.5 billion years ago)^{1,3}. Multicellular forms are found in several different bacterial phyla, such as Actinobacteria, Cyanobacteria and Myxococcales. Actinobacteria develop hyphae, myxobacteria can construct a fruiting body and both produce spores. Cyanobacteria, however, exhibit a wider range of developmental forms.

As the first organisms to use oxygenic photosynthesis, cyanobacteria were key players in the early evolution of life on Earth. Oxygenic photosynthesis requires the coordinated action of two photosystems (PSI and PSII) that, working in series, generate the electrochemical potential needed to extract electrons from water, releasing O₂. The oxygenic photosynthetic apparatus probably evolved from a functional pairing of the two different types of photosystem that already existed at the time, in either the same or different organisms, along with the addition of the Mn₄Ca oxygen-evolving complex⁴. The production of oxygen by cyanobacteria was responsible for the

oxidation of Earth's atmosphere around 2.4 to 2.2 billion years ago; cyanobacteria also contributed substantially to primary productivity in the Proterozoic eon and are now important players in the carbon and nitrogen cycles of the biosphere⁵. Some cyanobacteria can grow heterotrophically, but most are characterized by their reliance on photosynthesis, which produces the 'assimilatory power' (ATP and reducing equivalents in the form of NADPH or reduced ferredoxin) that is used in the fixation of CO₂ (a process termed photoautotrophic metabolism) and the assimilation of other nutrients including nitrogen.

The cyanobacteria are a phylogenetically coherent group of organisms that also include chloroplasts^{6,7}. However, cyanobacteria have diversified through evolution, producing both unicellular and multicellular forms. The multicellular forms consist of trichomes (FIG. 1) that, in some strains, can contain hundreds of cells⁸. Most cyanobacteria divide by binary fission, but some exhibit alternatives to the standard mode of bacterial division, reproducing by budding or by multiple fission of enlarged cells^{8,9}. In some filamentous cyanobacteria, vegetative cells can differentiate into heterocysts (FIG. 1a), which are specialized for the fixation of N₂ gas¹⁰; a subset of these cyanobacteria can also form spores, which are known as akinetes⁸ (FIG. 1c). In the filaments of most heterocyst-forming cyanobacteria, such as *Anabaena* spp. and *Nostoc* spp., the cells divide in only one plane, but in the filaments of some other species, such as *Fischerella* spp., the cells can divide in more than one plane, producing branched filaments⁸ (FIG. 1b). Some filamentous

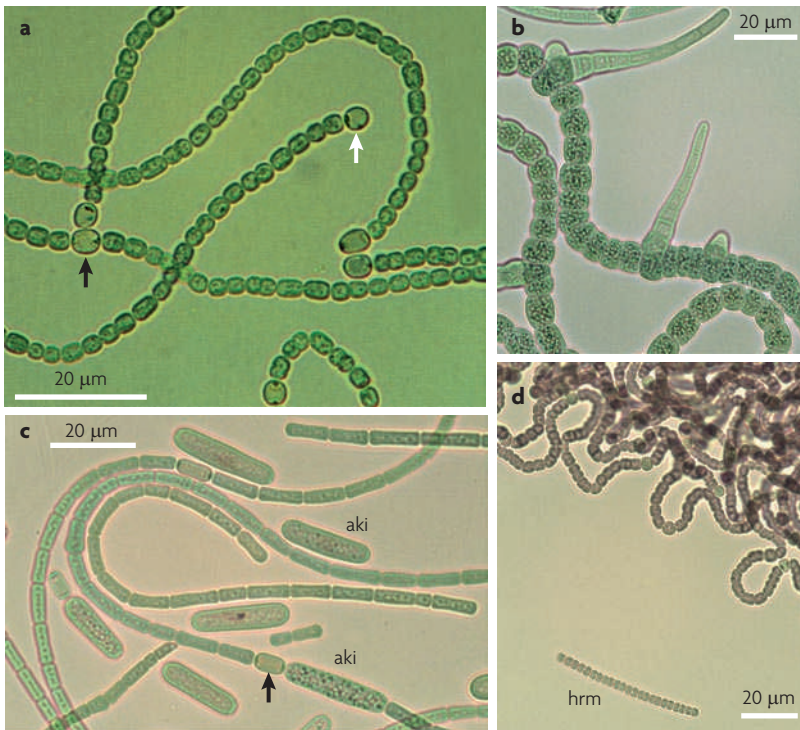


Figure 1 | Cell types in heterocyst-forming cyanobacteria. **a** | N_2 -grown filaments of *Anabaena* sp. PCC 7120 (also known as *Nostoc* sp. PCC 7120) showing terminal heterocysts (white arrow; note the presence of only one polar granule) and intercalary heterocysts (black arrow; note the presence of two polar granules) in addition to vegetative cells. **b** | Filaments of *Fischerella muscicola* showing true filament branches that result from cell division in more than one plane. **c** | N_2 -grown filaments of *Anabaena cylindrica*, with heterocysts (black arrow) and akinetes (aki) in addition to vegetative cells; akinetes adjacent to heterocysts in the filament and isolated akinetes (already released from the filament) can be observed. **d** | N_2 -grown *Nostoc* sp. PCC 9203 showing mature filaments of vegetative cells and heterocysts and one hormogonium (hrm; note the small size of its cells) that has moved away from the colony. Images courtesy of José E. Frías, CSIC and Universidad de Sevilla, Spain.

Akinete

A cyanobacterial spore that tolerates cold and desiccation but not heat.

Hormogonium

A short filament made of small cells that serve a dispersal function in some filamentous cyanobacteria.

Murein sacculus

Also known as peptidoglycan. A large polymer surrounding the bacterial cell as a mesh and consisting of amino sugars and amino acids.

Lipopolysaccharide

A major component of the outer membrane of Gram-negative bacteria, consisting of a lipid covalently bound to a long polysaccharide that faces the extracellular medium.

cyanobacteria also produce hormogonia, which are small motile filaments that can serve a dispersal function in some benthic species⁸ (FIG. 1d). Therefore, four different cell types can be found in some cyanobacteria: the vegetative cells of the mature filaments and the differentiated akinetes, heterocysts and hormogonial cells (FIG. 2).

Multicellularity involves at least three well-defined processes: cell–cell adhesion, intercellular communication and cell differentiation. In this Review, we reflect on some of the features of the filamentous, heterocyst-forming cyanobacteria that are relevant for multicellularity. These features include a special supracellular structure and the developmental process that produces a filament with two metabolically interdependent cell types, the photosynthetic vegetative cells and the N_2 -fixing heterocysts. *Anabaena* sp. PCC 7120 (also known as *Nostoc* sp. PCC 7120) and *Nostoc punctiforme* PCC 73102 are becoming model organisms in this field (BOX 1). Whereas *Anabaena* sp. PCC 7120 is an obligate autotroph in which some cells can differentiate into heterocysts, *N. punctiforme* PCC 73102 is a facultative heterotroph with a complex life cycle and exhibits all of the developmental possibilities described above.

Filament structure

Cyanobacteria bear a Gram-negative type of cell envelope containing an outer membrane that is external to the cytoplasmic membrane and to the murein sacculus, which is thicker than in most other well-characterized Gram-negative bacteria^{11,12}. Although evident in the early electron microscopy-based cytological literature^{13,14}, it has been realized only recently that the outer membrane of heterocyst-forming cyanobacteria does not enter the septum between adjacent cells in the filament, even though these cells are surrounded by their cytoplasmic membrane and peptidoglycan layers^{13–16} (FIG. 3C). Staining the outer membrane and cytoplasmic membrane of *Fischerella* spp. with a fluorescent dye also reveals that the outer membrane is continuous along the entire filament¹⁷, which might help to keep cells together in the filament.

The bacterial outer membrane is asymmetrical, bearing an inner leaflet that is rich in phospholipids and an outer leaflet that is rich in lipopolysaccharide (LPS)¹⁸. It also contains proteins including porins, which usually take the form of β -barrels with a central pore that allows the passage of small ions and molecules, making the outer membrane permeable to small molecules¹⁹. Porins have been noted in the outer-membrane proteome of *Anabaena* sp. PCC 7120 (REF. 20), but inspection of the genomic sequence of *Anabaena* sp. PCC 7120 fails to identify orthologues of the best characterized proteobacterial porins^{15,21}. In addition, in some cyanobacteria, including an *Anabaena* species, LPS lacks the typical components of the enterobacterial LPS, such as phosphate, 3-deoxy-D-manno-octulosonic acid and heptose^{12,22}. An *Anabaena* sp. PCC 7120 mutant that apparently does not produce LPS has been isolated²³, but the effect of the mutation on filament structure has not been reported. A better characterization of the cyanobacterial outer membrane would contribute to our understanding of its role in filament structure and physiology.

A continuous periplasm. The presence of a continuous outer membrane suggests that the periplasmic space, which lies between the cytoplasmic membrane and outer membrane, is also continuous¹⁵. Importantly, this seems to be the case not only along the string of vegetative cells but also between vegetative cells and heterocysts²⁴. The periplasm might therefore allow movement of molecules along the filament. Accordingly, a soluble, periplasmic GFP that is produced in differentiating *Anabaena* sp. PCC 7120 heterocysts can be found at a distance from the producing cells, suggesting that the GFP moves through the periplasm; by contrast, if the GFP is produced as a cytoplasmic membrane-anchored protein, it is found only in the producing cells²⁵ (FIG. 3a). However, barriers to diffusion of the 27 kDa GFP do seem to exist^{25,26}. The nature of these barriers is currently unknown, but they could correspond to the peptidoglycan entering the intercellular septa, which might have a size exclusion limit of 25 kDa to 50 kDa²⁵. Whether such barriers might also affect the diffusion of smaller molecules is unknown.

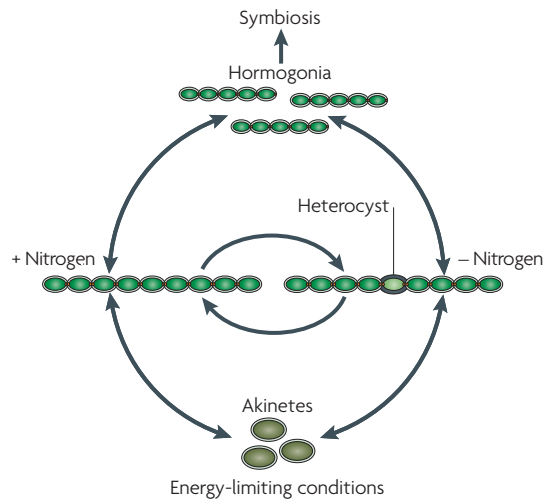


Figure 2 | The complex life cycles of some heterocyst-forming cyanobacteria. Some filamentous cyanobacteria, such as *Anabaena* sp. PCC 7120 (also known as *Nostoc* sp. PCC 7120), form heterocysts in response to deprivation of combined nitrogen; when filaments are re-supplied with combined nitrogen, heterocysts become spaced apart as intervening vegetative cells grow and divide. In other heterocyst-forming strains, some vegetative cells differentiate into akinetes in response to energy-limiting conditions; akinetes germinate under favourable conditions, producing growing filaments with or without heterocysts, depending on the availability of combined nitrogen. Some filamentous cyanobacteria, such as *Nostoc punctiforme* PCC 73102, also produce hormogonia, which are dispersal forms that can also function as infection units for the establishment of symbiotic associations with plants. The differentiation of hormogonia can be stimulated by some environmental conditions, such as nitrogen deprivation, and by a plant hormogonium-inducing factor⁶⁰; when hormogonia resume growth, they produce filaments with or without heterocysts, depending on the availability of combined nitrogen.

Cell-to-cell joining structures. *Anabaena* sp. PCC 7120 mutants showing filament fragmentation have been isolated, suggesting the existence of genes encoding proteins that are specifically involved in cell–cell adhesion^{27–29}. Two of these genes, *sepJ* (also known as *alr2338*) and the filament integrity protein gene (*fraC*), are predicted to encode integral cytoplasmic-membrane proteins^{29–31}. *SepJ* is also predicted to carry a long extracytoplasmic segment that bears coiled-coil domains³¹, which are known to participate in protein–protein interactions. A *SepJ*–GFP fusion protein localizes to the cell poles in the intercellular septa of the *Anabaena* sp. PCC 7120 filaments³¹ (FIG. 3b). The GFP part of this fusion protein is expected to reside on the cytoplasmic face of the cytoplasmic membrane; therefore, the fact that a single fluorescent spot is seen between adjacent vegetative cells suggests that the *SepJ* molecules from two adjacent cells are closely clustered together. However, the *SepJ*–GFP units seem to be more separated in the septa between vegetative cells and heterocysts, where separate fluorescent spots are observed³¹ (FIG. 3b). *SepJ* could be a component of

cell-to-cell joining structures in the heterocyst-forming cyanobacteria. Whether other proteins, such as *FraC*, contribute to such structures is unknown.

The *SepJ*-containing cell-to-cell joining structures could correspond to the thin structures that are perpendicular to the cytoplasmic membrane in the intercellular septa of the filament, as observed by electron microscopy (FIG. 3c) (see REF. 14 for an example in the early literature), which have been termed ‘microplasmodesmata’ (REF. 32). These structures, seen by freeze-fracture electron microscopy as pits and protrusions in the different leaflets of the cytoplasmic membranes of adjacent cells, are around five times as abundant in septa between vegetative cells as they are in septa between vegetative cells and heterocysts³³. Although this is a controversial issue, it should be noted that, because of their small size (less than 20 nm in outside diameter), these structures are unlikely to correspond to true plasmodesmata, which are cytoplasmic bridges delimited by cytoplasmic membrane³³. In addition, the lack of transfer of the cytoplasmic membrane-anchored GFP from heterocysts to adjacent vegetative cells (FIG. 3a) supports the idea that there is no cytoplasmic-membrane continuity between the two types of cells²⁵.

Differentiated cells

Heterocysts. N_2 fixation is carried out exclusively by prokaryotes (Bacteria or Archaea) in either an anoxic environment or under intracellular micro-oxic conditions that are appropriate for the expression and function of the N_2 -fixing enzyme, nitrogenase. This enzyme is encoded by the *nifHDK* operon and is extremely sensitive to oxygen. In a photosynthetic, oxygen-evolving cyanobacterial filament, the heterocyst provides a micro-oxic site for N_2 fixation. Therefore, in these organisms, compartmentalization of oxygenic photosynthesis and N_2 fixation in different cell types is the solution to the problem of incompatibility between the two processes.

The heterocyst (FIG. 4) carries a distinctive envelope consisting of a glycolipid laminated layer that is a permeability barrier for gases and, external to it, a thicker homogeneous layer of polysaccharide that apparently protects the glycolipid layer from physical damage³⁴. Consistent with the recent identification of a TolC-like protein, HgdD, needed for deposition of the glycolipid layer³⁵, these layers are found outside the outer membrane¹⁵. TolC family proteins are trimeric, pore-forming, outer-membrane β -barrel proteins that work in concert with cytoplasmic-membrane transporters to export materials out of Gram-negative bacteria. Cells with mutations in the *devBCA* genes³⁶, which encode a heterocyst-specific ABC transporter, or in *hgdD*³⁵ have similar phenotypes, suggesting that *DevBCA* could be the companion to HgdD. Many of the genes encoding proteins that may be involved in the biosynthesis of the heterocyst-specific glycolipid and polysaccharide layers are grouped in gene islands in the genome of *Anabaena* sp. PCC 7120 (REFS 37,38).

The organization of the intracytoplasmic membranes (thylakoids) is different in the heterocysts and in the vegetative cells, probably reflecting the particular bioenergetics

β -Barrel

A protein domain made of β -strands that forms a closed structure with an internal opening.

Anoxic

Containing no oxygen.

Micro-oxic

Containing very low amounts of oxygen.

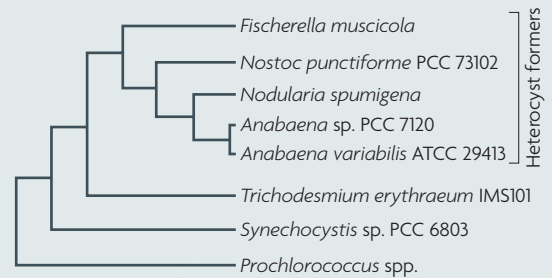
Box 1 | Genomes and genetics of heterocyst-forming cyanobacteria

The availability of an experimental system for DNA transfer that permits genetic analysis of heterocyst-forming cyanobacteria has been essential for the current understanding of these organisms, along with substantial genomic information, most of which can be found on [CyanoBase](#) and the [US Department of Energy Joint Genome Institute](#) website. The complete genome sequence is currently available for around 50 cyanobacterial strains, including 3 heterocyst formers: *Anabaena* sp. PCC 7120 (also known as *Nostoc* sp. PCC 7120) (which has a genome of 7.21 Mb and 6,223

predicted genes), *Anabaena variabilis* ATCC 29413 (which has a genome of 7.07 Mb and 5,724 predicted genes), and *Nostoc punctiforme* PCC 73102 (which has a genome of 9.06 Mb and 6,794 predicted genes). *Anabaena* sp. PCC 7120 and *Anabaena variabilis* ATCC 29413 are very closely related, but *N. punctiforme* PCC 73102 is more distant (see the figure; phylogenetic distances between strains are not depicted). In addition, the genome sequences of heterocyst-forming *Nodularia* and *Calothrix* strains are being completed by the [Gordon and Betty Moore Foundation Microbial Genome Sequencing Project](#). The genome sizes of these organisms are substantially larger than those of most sequenced unicellular cyanobacteria, which are in the range of about 1.7 Mb for the marine *Prochlorococcus* spp. strains to 3.97 Mb for the facultative photoheterotroph *Synechocystis* sp. PCC 6803. However, the sequenced cyanobacterial strain that is most closely related to the heterocyst formers, the marine filamentous *Trichodesmium erythraeum* IMS101, has a genome size of 7.75 Mb. A trend of increasing genome size is consistent with the notion that larger genomes are needed to support a multicellular organism than are needed to support a related single-celled organism¹⁰⁴.

Wolk and colleagues developed an experimental system to transfer DNA into heterocyst-forming cyanobacteria that was optimized for *Anabaena* sp. PCC 7120 (REF. 105). It is based on the transfer of plasmids from *Escherichia coli* to the cyanobacterium by means of conjugation. To increase efficiency, protection of the transferred DNA against resident restriction endonucleases is achieved by pre-methylation of the plasmid to be transferred, carried out in *E. coli*¹⁰⁶. The transferred plasmid can replicate in the cyanobacterium if it carries an appropriate origin of replication, but it can also integrate into the recipient's genome through homologous recombination if it carries homologous DNA. The different possible fates of the transferred DNA give multiple possibilities of genetic manipulation, from complementation of mutants to targeted mutagenesis and construction of strains carrying reporter gene fusions¹⁰⁷. This genetic system is also functional with *N. punctiforme* PCC 73102 and *A. variabilis* ATCC 29413¹⁰⁷.

Data for the figure is from published molecular sequence phylogenetic analyses^{5-7,63}.



of the heterocysts. The water-splitting activity of PSII is lost during heterocyst differentiation, favouring the establishment of an intracellular micro-oxic environment³⁹, but some oxygen will still enter the heterocyst (along with N₂), probably through its poles⁴⁰. The 'neck'-like structures at the heterocyst poles might diminish the surface of contact with the adjacent vegetative cells to decrease the amount of gas entering the heterocyst⁴⁰. Respiration consumes the oxygen that enters the heterocysts³⁹, and two heterocyst-specific terminal respiratory oxidases have been identified in *Anabaena* sp. PCC 7120 (REF. 41). Additionally, PSI-dependent photophosphorylation is important in heterocyst bioenergetics³⁹.

The heterocyst lacks ribulose 1,5-bisphosphate carboxylase-oxygenase and does not photosynthetically fix CO₂, relying instead on fixed carbon that is received from the neighbouring vegetative cells⁴². Reduced compounds are transferred to heterocysts, probably in the form of a sugar such as sucrose^{39,43}, and the oxidative pentose-phosphate pathway is important for sugar catabolism in the heterocyst⁴⁴. Ammonium is produced by the fixation reaction that is carried out by nitrogenase, but in N₂-fixing filaments it is immediately incorporated into glutamine and other amino acids⁴⁵. Heterocysts contain high levels of glutamine synthetase (which catalyses the ATP-dependent amidation of glutamate, producing glutamine) and lack glutamine-2-oxoglutarate amidotransferase (GOGAT) (which catalyses the reductive

transamidation of 2-oxoglutarate (2-OG), producing two glutamate molecules)^{39,46}. Isolated heterocysts that can fix N₂ therefore produce glutamine when incubated in the presence of glutamate⁴⁷. These observations suggest that there is a glutamate-glutamine exchange between vegetative cells and heterocysts that results in a net export of nitrogen from the heterocysts^{45,47}. In the heterocyst poles, at the cytoplasmic side of the heterocyst 'necks', granules of cyanophycin (multi-L-argininyl-poly-(L-aspartic acid)) accumulate⁴⁸. Interestingly, in terminal heterocysts the 'neck' and cyanophycin granule are formed only at the heterocyst pole close to the adjacent vegetative cell (FIGS 1, 4), another morphogenetic effect. The heterocyst's cyanophycin has been suggested to serve as a nitrogen buffer, transiently storing fixed nitrogen⁴⁹, although the accumulation of cyanophycin is not required for diazotrophic growth^{50,51}.

Akinetes. Akinetes are spore-like cells that are frequently larger than vegetative cells (FIG. 1) and that differentiate from these cells in response to environmental conditions such as light limitation or phosphate deprivation⁵², which probably result in a shortage of cellular energy. Consistently, in a glucose-6-phosphate 1-dehydrogenase (*zwf*) mutant of *N. punctiforme* PCC 73102, which is impaired in heterotrophy, akinetes differentiate when the filaments are placed in the dark in the presence of fructose⁵³. In N₂-fixing filaments, the position of

akinetes in relation to heterocysts is strain-specific⁵². For instance, in *Anabaena cylindrica* akinetes differentiate adjacent to heterocysts (FIG. 1). Akinetes show a reduced but existent metabolic activity⁵², accumulate carbon (glycogen) and nitrogen (cyanophycin) reserves⁵², and bear a thickened cell wall that shares some components with the heterocyst envelope^{54–56}. Genes that are induced during akinete differentiation have now been identified^{53,57}, and a global gene expression analysis carried out with *N. punctiforme* PCC 73102 has identified 497 genes (around 7% of the genome) that change their expression levels in differentiating akinetes⁵⁸. Many of the upregulated genes encode unassigned proteins, suggesting that they have novel functions, and many downregulated genes encode central metabolism proteins, which is consistent with the akinetes being in a non-growth state⁵⁸. Akinete germination takes place under conditions that are permissive for growth and frequently involves the release of a short-filament germling through a pore in the akinete envelope⁵².

Hormogonia. Hormogonia are short filaments that are made of small cells and show gliding motility (involving the action of pili or the production of slime) or gas vacuole-mediated buoyancy^{59,60}. They can be seen both at the edge of and at a distance from *Nostoc* spp. colonies grown on solid growth media (FIG. 1). No unique nutritional or physical factor is known to trigger hormogonium differentiation, although it can be stimulated by changes in multiple factors^{59,60}. The differentiation of hormogonia involves cell division without an increase in biomass, resulting in their characteristic small cells, and requires a distinct transcriptional programme^{58,59}. In differentiating *N. punctiforme* PCC 73102 hormogonia, the expression levels of 1,827 genes (around 25% of the genome) have been found to differ from the expression levels in vegetative cells⁵⁸. Half of the downregulated genes are predicted to encode central metabolism proteins, around half of the upregulated genes encode unassigned proteins and 22% of the upregulated genes encode proteins that are involved in adaptation functions, including signal transduction, taxis and motility⁵⁸. These data illustrate the role of hormogonia as dispersal units that, once formed, are released by fragmentation of the producing vegetative filament. After a few days hormogonia become sessile and start to grow, producing a new vegetative filament that may or may not bear heterocysts, depending on the availability of fixed nitrogen.

Hormogonia are the infection units in the establishment of symbiosis between heterocyst-forming cyanobacteria and plants^{59,60}, and some plant factors can stimulate hormogonia differentiation⁶⁰. The transcriptional programme of differentiation that is triggered by a bryophyte-derived hormogonium-inducing factor and differentiation that is induced by nitrogen deprivation only partially overlap in *N. punctiforme* PCC 73102 (REF. 61). In the hormogonia of this cyanobacterium, pili are important for plant infection, as shown by inactivation of *pil*-like genes⁶².

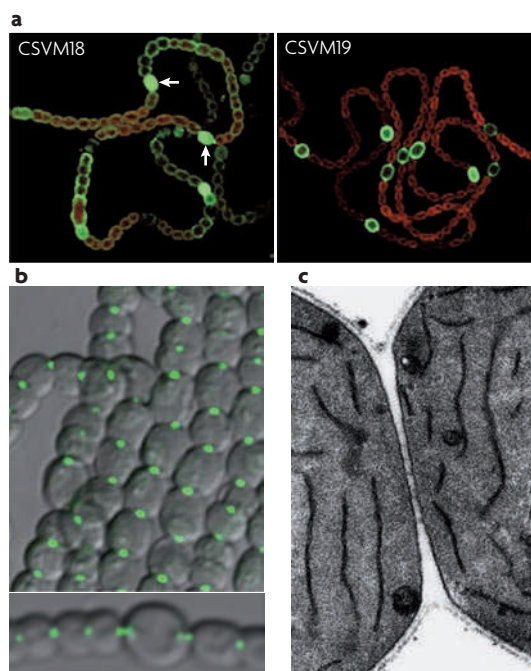


Figure 3 | Putative structures for intercellular communication. **a** | Periplasmic continuity between the heterocyst and the adjacent vegetative cells. The left micrograph shows *Anabaena* sp. strain CSVM18, the differentiating heterocysts (green cells indicated by white arrows) of which express GFP that is soluble in the periplasm (see REF. 25 for details). The right micrograph shows *Anabaena* sp. strain CSVM19, which expresses a periplasmic GFP that is anchored to the cytoplasmic membrane of the producing cells (green cells). Whereas the cytoplasmic membrane-anchored GFP remains in the producing cell, the soluble GFP is seen not only in producing cells but also in the periphery of non-producing cells, suggesting that the GFP moves through the periplasm. The micrographs are overlays of the cyanobacterial red autofluorescence and the GFP green fluorescence, captured by confocal microscopy, of filaments from colonies grown on solid medium without a source of combined nitrogen. **b** | Cell-to-cell joining structures. The top panel shows nitrate-grown filaments of *Anabaena* sp. strain CSAM137 expressing a protein consisting of SepJ (also known as Alr2338) fused to GFP; this fusion protein localizes to the cell poles in the intercellular septa³¹. The bottom panel shows a portion of a N_2 -grown filament of the same strain with a heterocyst, revealing double fluorescence spots at the heterocyst-vegetative cell junctions. The figures are overlays of bright field microscopy and GFP green fluorescence, captured by confocal microscopy. **c** | A transmission electron micrograph showing an intercellular septum in a filament of wild-type *Anabaena* sp. PCC 7120 (also known as *Nostoc* sp. PCC 7120), in which thin structures perpendicular to the cytoplasmic membranes of the two adjacent cells can be observed. Parts **a** and **b** courtesy of Vicente Mariscal, CSIC and Universidad de Sevilla, Spain. Part **c** courtesy of Iris Maldener, Universität Tübingen, Germany.

Evolutionary relationships. Heterocyst-forming cyanobacteria are monophyletic, suggesting that the heterocyst evolved only once. Akinetes have been preserved in old sedimentary rocks, providing evidence for the presence

Monophyletic
Concerning a group of organisms that have evolved from a common ancestor.

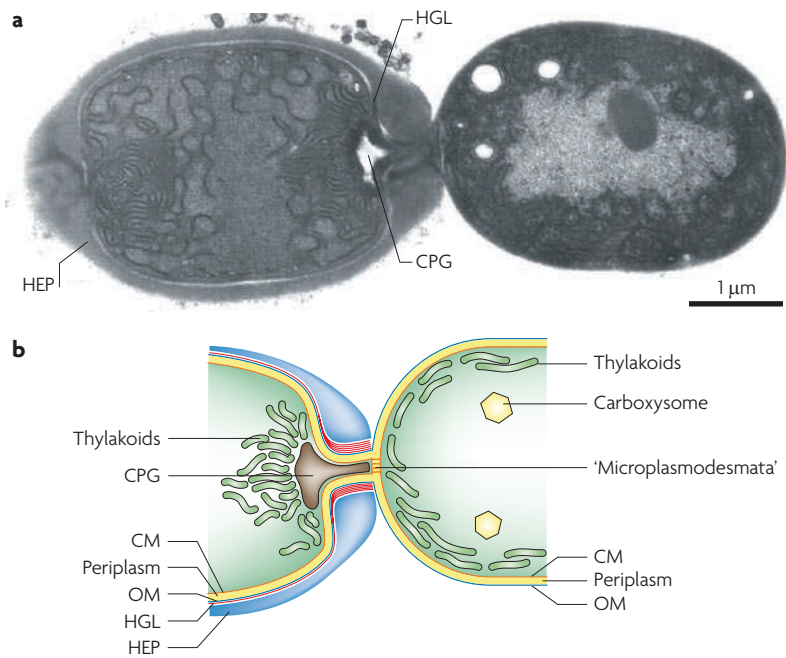


Figure 4 | The heterocyst and the heterocyst-vegetative cell septum. a | A transmission electron micrograph showing a terminal heterocyst (left) and its adjacent vegetative cell (right) from a filament of *Anabaena* sp. PCC 7120 (also known as *Nostoc* sp. PCC 7120). In the preparation of samples for electron microscopy, the cyanophycin granule (CPG) is frequently lost, leaving a white, empty space in the micrographs. **b** | Schematic of portions of a heterocyst and an adjacent vegetative cell. A continuous periplasm that is delimited by the cytoplasmic membrane (CM) and outer membrane (OM) and contains the peptidoglycan runs along the filament. In the septum between the two cells, as well as in the septa between vegetative cells along the filament, thin structures that have been called ‘microplasmodesmata’ are present. In the heterocyst, external to the outer membrane, specific glycolipid and polysaccharide layers are present. The different distributions of thylakoids in the two types of cells (peripheral in vegetative cells and polar in the heterocyst) and the presence of carboxysomes in vegetative cells but not in heterocysts are indicated. HEP, heterocyst polysaccharide layer; HGL, heterocyst glycolipid layer. Part **a** courtesy of Iris Maldener, Universität Tübingen, Germany.

of cyanobacteria with complex developmental processes by around 2.1 billion years ago⁶³. Whereas the akinetes are resistance cell forms, the function of the heterocyst is nutritional, and N₂ fixation in an oxic environment could have set the selection pressure for the evolution of heterocysts shortly after the oxidation of Earth’s atmosphere⁶³. The two differentiated cell types share some components of their special cell envelopes, suggesting that they are evolutionarily related³⁹. Whereas akinetes and hormogonia are transient cell forms that will produce growing filaments, heterocysts are terminally differentiated, non-growing cells that serve an altruistic function. Finally, comparison of the genomes of some algae and plants with those of cyanobacteria suggests that the endosymbiotic ancestor of the chloroplast was a heterocyst-forming cyanobacterium⁶⁴. As infection units, hormogonia could have had a key role in the original endosymbiosis, which took place around 1.5 billion years ago⁶⁵.

Heterocyst differentiation

Developmental processes in bacteria rely on two types of circuits for information sensing and processing: regulation of gene expression and generation of positional

information. Regulation of gene expression takes place in response to cell cycle progression or, in the case of adaptive cell differentiation processes, to external cues or metabolic conditions, and it frequently follows a temporal sequence. The generation of positional information is often linked to cell cycle progression and is responsible for the establishment of the spatial pattern characteristic of the developmental process. Moreover, these two circuits are usually interconnected at specific points.

Activation of gene expression. Heterocyst differentiation can be considered to be one extreme of the range of the organism’s responses to nitrogen stress, which are orchestrated by *NtcA*, a global transcriptional regulator found in all cyanobacteria^{66,67}. *NtcA* belongs to the cyclic AMP receptor protein family of regulators and binds to specific DNA sites that bear the signature sequence GTAN₈TAC, which are found in the promoter regions of multiple genes involved in nitrogen assimilation and related functions⁶⁸. The standard *NtcA*-dependent promoters bear an *NtcA*-binding site centred at around -41.5 nucleotides from the transcription start site and a -10 sequence in the form TAN₃T (REF. 66). *NtcA* perceives nitrogen stress by responding to 2-OG, which has been shown to increase *NtcA* binding to its targets in several regulated promoters (for examples, see REFS 69–71) and to be required for open-complex formation, and hence transcription, at the promoters of several *Anabaena* sp. PCC 7120 genes that are involved in heterocyst differentiation⁷².

Microarray analysis of transcript abundance at 3 hours, 8 hours and 24 hours after combined-nitrogen deprivation of nitrate-grown cells has shown significant changes for 495 upregulated and 196 downregulated genes in *Anabaena* sp. PCC 7120 (REFS 34, 73). The earliest induced genes increase the scavenging of traces of ammonium, nitrate, nitrite or urea, which are sources of combined nitrogen^{66,74}. If nitrogen stress persists, the cyanobacterium will enter the heterocyst differentiation process (FIG. 5). The differentiation of a single cell into a heterocyst is governed by a sequence of gene activation events triggered by the concerted action of two regulators: *NtcA*^{75,76} and heterocyst differentiation control protein (*HetR*)⁷⁷. At initiation, the two regulatory genes are induced in a mutually dependent manner, with induction of *hetR* preceding that of *ntcA*⁷⁸. Moreover, positive autoregulation seems to be crucial for increasing the expression of *hetR* and *ntcA* up to the levels that are required for the establishment of heterocyst differentiation^{71,79}. Increased expression of both *ntcA*⁸⁰ and *hetR*⁷⁹ is mainly localized to certain spatially distributed cells that eventually become heterocysts.

When heterocyst differentiation has been completed, the expression of many of the genes that have increased expression on combined-nitrogen deprivation return to initial levels^{34,73}. Around 30 of the transiently induced genes have putative or demonstrated regulatory functions affecting the expression of other genes (TABLE 1). However, inactivation of these genes has a far smaller effect on heterocyst differentiation than inactivation of *ntcA* or *hetR*, which prevents differentiation^{75–77}.

Many of the genes that act at different stages of the differentiation process or in the mature heterocyst, including *ntcA* and *hetR*, bear complex promoter regions that allow the start of transcription at several sites, each directed by a different promoter (FIG. 6). These include constitutive promoters with recognizable -10 sequence and -35 sequence hexamers (ensuring a low level of gene expression that is independent of the nitrogen regime), promoters that are activated by direct binding of NtcA, either to sites located at the standard -41.5 nucleotide position or further upstream, and promoters that are dependent on NtcA or on NtcA and HetR but that have no apparent NtcA-binding site or determinant other than a -10 sequence⁶⁷. Some of the promoters that are directly activated by NtcA can function in the absence of the accompanying promoters, as has been shown *in vivo* for the *ntcA* and glutamine synthetase (*glnA*) genes^{80,81}. Indeed, standard NtcA-dependent promoters such as those of the heterocyst differentiation gene *hetC* (TABLE 1), *devBCA* (discussed above) and *nrrA* (discussed below) can be activated *in vitro* in the presence of only RNA polymerase, NtcA and 2-OG⁷². We speculate that this kind of promoter could be activated on combined-nitrogen deprivation in all cells of the filament, although the increased levels of NtcA in the differentiating cells, which result from the localized increase of expression of *ntcA* at a certain time during differentiation⁸⁰, would potentiate direct activation by NtcA in those particular cells⁶⁷.

The requirement of NtcA for activation of the promoters that do not bear canonical NtcA-binding sites could be an indirect effect, which would call for the participation of additional regulators. NrrA, a nitrogen- and NtcA-regulated response regulator⁸², has been proposed to serve such a role in a *hetR* promoter^{73,83}. However, the fact that NrrA-deficient mutant strains still undergo heterocyst differentiation and the fact that induction of *nrrA* on combined-nitrogen deprivation occurs in all cells of the filament⁸², independent of HetR, suggest that the role of NrrA is not specifically related to heterocyst differentiation.

Finally, for the HetR-dependent promoters that do not show a recognizable promoter structure, the mechanism for activation of cell-specific transcription is unclear. HetR has been reported to bind DNA upstream of *hetR* and two other genes that are also activated during heterocyst differentiation, although the protein has no recognizable DNA-binding domain^{84,85}. However, conflicting results have been reported concerning the necessity of the protein dimerization and autoproteolytic activity of HetR for DNA binding and heterocyst differentiation^{85,86}. Indeed, unravelling the mechanism of action of HetR will be essential for understanding heterocyst differentiation at the molecular level.

Establishment of the spatial pattern of heterocysts. In the cyanobacterial filaments, heterocysts occupy strain-characteristic positions, a trait that arguably represents the simplest example of patterning in multicellular organisms. In diazotrophically grown *Anabaena* sp. PCC 7120, heterocysts are found at semiregular intervals along the filament, with 10–15 vegetative cells

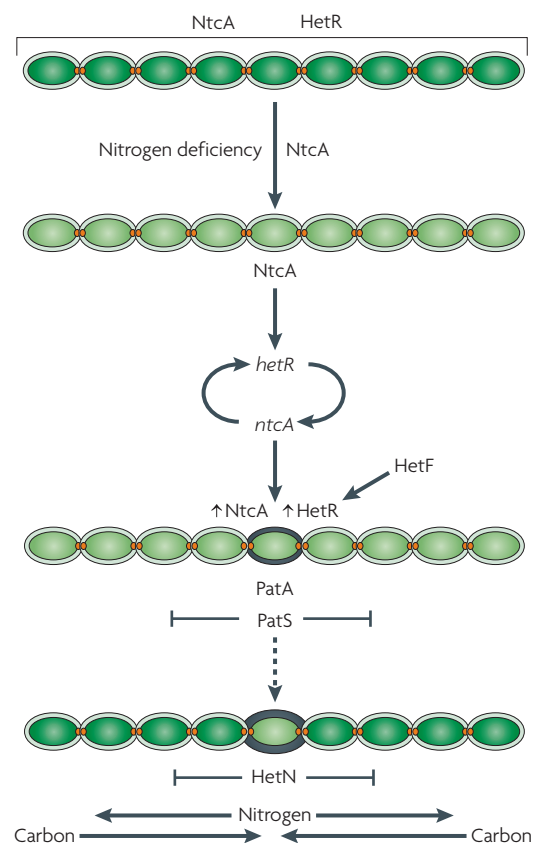


Figure 5 | Progress of heterocyst differentiation. The scheme represents a filament of a heterocyst-forming cyanobacterium grown with a source of fixed nitrogen, in which NtcA and heterocyst differentiation control protein (HetR) are present. In response to nitrogen deficiency, NtcA is activated, probably by interaction with its effector, 2-oxoglutarate. In some cells or cell clusters (as discussed in the main text), NtcA activates expression of *hetR*, and the NtcA–HetR amplification loop, which includes autoregulation of *ntcA* and *hetR*, results in increased levels of the two proteins. NtcA stays active possibly because of high levels of 2-oxoglutarate present in the heterocysts (including differentiating heterocysts), and HetR activity is positively influenced by heterocyst differentiation protein F (HetF). The differentiating heterocyst produces heterocyst inhibition-signalling peptide (PatS), which inhibits neighbouring cells from differentiating, and PatA, which counteracts the action of differentiation inhibitors in the differentiating cell. Finally, by 18 to 24 hours after the onset of nitrogen deprivation, a thick-walled, mature heterocyst has been formed, which fixes N_2 and produces other inhibitors such as ketoacyl reductase HetN-dependent inhibitor. In the N_2 -fixing filament, fixed carbon moves from vegetative cells to heterocysts, and fixed nitrogen moves from heterocysts to vegetative cells. Inter-cellular molecular exchange of metabolites and regulatory molecules could take place through the continuous periplasm or cell-to-cell connecting structures. Note the changing pigmentation of the vegetative cells (during the differentiation process, these cells lose phycobiliproteins, which are regained when nitrogen compounds produced by the heterocysts reach the vegetative cells) and the different pigmentation of the heterocyst (the phycobiliprotein content of which is lower than that of vegetative cells).

Table 1 | Further genes regulating heterocyst differentiation

Gene*	Protein function or motif	Role in heterocyst differentiation	References
Heterocyst differentiation and patterning			
<i>hetC</i>	ABC-type exporter	Commitment to differentiation	108–110
<i>ccbP</i>	Ca ²⁺ -binding protein	Ca ²⁺ balance	111
<i>asr1734</i>	Unknown	Response to nutrient stress	112
<i>hetZ</i>	Helix–turn–helix motif	Coordinating differentiation and pattern formation	113
Heterocyst maturation			
<i>devR</i>	Response regulator	Polysaccharide layer synthesis	114
<i>hepK</i>	DevR histidine kinase	Polysaccharide layer synthesis	115
<i>hepN</i>	Histidine kinase	Polysaccharide layer synthesis	116
<i>hepS</i>	Serine/threonine kinase	Polysaccharide layer synthesis	117
<i>devH</i>	CRP-type transcriptional regulator	Glycolipid synthesis	118
<i>prpJ</i>	Protein phosphatase	Glycolipid synthesis	119
<i>pkn44</i>	HstK family protein kinase	Glycolipid synthesis	120
<i>pkn30</i>	HstK family protein kinase	Glycolipid synthesis	120
<i>xisA</i>	Site-specific recombinase	<i>nifHDK</i> operon recreation	121
<i>patB</i>	Bacterial ferredoxin and helix–turn–helix motifs	Unknown	122,123
Transcription			
<i>sigC</i>	Group 2 σ -factor	Induced in differentiating heterocysts	124
<i>sigE</i>	Group 2 σ -factor	Induced in differentiating heterocysts	124
<i>sigG</i>	Group 2 σ -factor	Induced in differentiating heterocysts	124

*Many genes have been identified in *Anabaena* sp. PCC 7120 (also known as *Nostoc* sp. PCC 7120) and *Nostoc punctiforme* PCC 73102 mutants as being responsible for the inability of these mutants to fix N₂ under oxic conditions. Some genes were cloned by genetic complementation of mutants, and others were cloned by transposon mutagenesis that marked the inactivated gene. CRP, cAMP receptor protein; *hetC*, heterocyst differentiation protein C gene; *xisA*, excisase A.

separating 2 heterocysts. However, when filaments of vegetatively growing cells are subjected to combined-nitrogen deprivation, differentiation often begins in clusters of cells and proceeds up to a certain point, when only one of the cells in the cluster is committed to continue differentiation⁸⁷. Several genes have been identified that alter the pattern of heterocyst distribution in *Anabaena* sp. PCC 7120 filaments when they are mutated. Inactivation of *patS* (the heterocyst inhibition-signalling peptide gene), which is induced in the cells undergoing differentiation, leads to the formation of multiple contiguous heterocysts (the Mch phenotype) and short vegetative-cell intervals, whereas its overexpression suppresses differentiation⁸⁸. *patS* encodes a 17- or 13-amino-acid product, the carboxy-terminal pentapeptide (RGSGR; also known as PatS-5) of which suppresses differentiation when added to the external medium⁸⁸. It has been proposed that *PatS* could be exported from the producing cell to inhibit differentiation in its neighbours, leading to resolution of the initial clusters of differentiating cells⁸⁸. This model requires that the inhibitory activity is different in the producing and receptor cells. In contrast to the full *PatS*, *PatS-5* expression in differentiating heterocysts failed to restore a wild-type heterocyst pattern in a *PatS*-deficient mutant⁸⁹, which is consistent with *PatS* being processed during intercellular transfer.

In cells lacking a functional *patS* gene, the Mch phenotype is evident after 24 hours of combined-nitrogen deprivation, when the first round of differentiation is complete, but after 48 hours the heterocyst pattern in the mutants resembles that of the wild type. Consistently, expression of *patS* returns to basal levels following 24 hours of combined-nitrogen deprivation. By contrast, inactivation of the gene encoding ketoacyl reductase *HetN* produces no discernible effect during the first round of heterocyst differentiation but results in a Mch phenotype after 48 hours of combined-nitrogen deprivation^{90,91}. Expression of *hetN* is activated later than that of *patS* during the differentiation process and, as is the case for *patS*, overexpression of *hetN* suppresses heterocyst formation. Therefore, whereas *PatS* is involved in the *de novo* pattern formation, *HetN* participates in the maintenance of the pattern of heterocyst distribution once it has been established. It has been suggested that both *PatS* and *HetN* influence the activity of *HetR*⁹². Whether this influence is restricted to an effect on the reported DNA-binding activity of *HetR*, which is inhibited by *PatS-5* (REFS 84,85), remains to be discerned. Interestingly, *HetN* also carries an RGSGR pentapeptide in its sequence.

The *patA* gene encodes a protein with a chemotaxis protein Y-like phospho-acceptor domain and a PATAN (*PatA* N-terminus) domain that is possibly

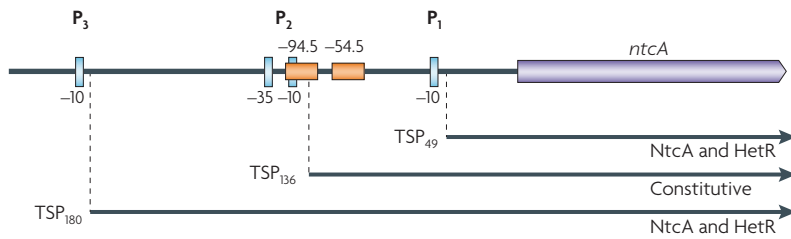


Figure 6 | A complex gene promoter region. Some *Anabaena* sp. PCC 7120 (also known as *Nostoc* sp. PCC 7120) genes that are expressed during heterocyst differentiation and in mature heterocysts bear complex promoter regions containing several promoters that generate different transcription start points (TSPs). The promoter of the regulatory *ntcA* gene is depicted as an example (the gene and promoter elements are not presented to scale). This gene carries three promoters that generate TSPs at 49, 136 and 180 nucleotides upstream from the gene translation start. P_1 is used at a low level in filaments grown in the presence of combined nitrogen and is transiently induced, depending on NtcA and heterocyst differentiation control protein (HetR), during heterocyst differentiation; it has a recognizable -10 promoter sequence and two NtcA-binding sites (orange boxes) that are centred at nucleotides -54.5 and -94.5 with regard to the TSP⁷¹. P_2 is constitutively active and has recognizable -10 and -35 promoter sequences. P_3 is transiently induced, depending on NtcA and HetR, during heterocyst differentiation but has no recognizable promoter elements other than a putative -10 promoter sequence.

involved in protein–protein interactions⁹³, and its inactivation leads to heterocyst differentiation mostly at the ends of the filament⁹⁴. Epistasis analysis with mutants that are altered in *patA*, *patS* and *hetN* shows that PatA is required only in the presence of wild-type *patS* or *hetN* alleles, suggesting a role for PatA in attenuation of the negative effects of PatS and, later, of HetN⁹⁵. The heterocyst differentiation gene *hetF* encodes a putative cysteine protease that is required for differentiation⁹⁶ and for activation of several HetR-dependent promoters⁹⁷. Although no direct action has been demonstrated yet, both PatA and HetF could influence the levels of the HetR protein⁹⁷.

Therefore, the correct spatial pattern of heterocyst distribution along the filament, so that mature heterocysts do not occupy adjacent positions, seems to rely on interference with the stream of gene transcription activation that sustains the differentiation of a given cell. However, molecular details of the equilibrium between positive and negative factors that determine which cell in a neighbourhood will differentiate are still largely unresolved. Two other important aspects of the pattern that are yet to be resolved at the molecular level are why, on perception of nitrogen stress, differentiation begins in clustered cells⁸⁷, and how the sites of the differentiation of new heterocysts are selected during sustained diazotrophic growth⁹⁸. Perhaps initiation by grouped cells responds to a requirement for a given stage in the cell cycle, which could be shared by neighbouring cells. The reported requirement of DNA replication⁶⁰ and cell division⁹⁹ for heterocyst differentiation supports this notion. In addition, differentiation of new heterocysts approximately midway between pre-existing heterocysts might involve regulation by nitrogenous compounds that are exported by the pre-existing, N_2 -fixing heterocysts.

Epistasis analysis

Investigation of the interaction between genes, such that a gene with a mutant phenotype that prevails is said to be epistatic over another related gene.

Diazotrophic physiology

The physiology related to the growth of an organism that uses N_2 as a source of nitrogen.

Molecular exchange in the filament

As mentioned above, during both heterocyst differentiation and diazotrophic growth, exchange of metabolites and regulatory molecules takes place between the cells of the filament. A technique for studying cell-to-cell communication in filamentous cyanobacteria has been recently developed and consists of loading a fluorescent tracer (calcein) into the cytoplasm of the cells, where it is stably retained¹⁰⁰. Fluorescence recovery after photobleaching (FRAP) experiments show rapid transfer of the calcein between the cytoplasm of the cells in filaments of *Anabaena* spp. As no specific permeases are expected to be present for an artificial substrate such as calcein, these results suggest the presence of direct cell-to-cell communication conduits. Calcein transfer is impaired in a SepJ-deficient mutant, suggesting that the proteinaceous cell-to-cell joining structures discussed above might be involved in molecular transfer directly between the cytoplasm of two adjacent cells¹⁰⁰. Whereas calcein (which is 623 Da in size) can be transferred, there is no evidence for intercytoplasmic exchange of GFP^{25,88}. Exchange of calcein is faster between vegetative cells than between vegetative cells and heterocysts, suggesting a different structure to the communication conduits between the different cell types¹⁰⁰. This is consistent with the different fluorescence patterns of SepJ–GFP at the junctions between heterocysts and vegetative cells (which have a double fluorescence spot) and between vegetative cells (which have a single spot), as discussed above. Unlike simple cytoplasmic bridges, proteinaceous structures could be regulated and therefore behave as gates rather than simple pores. Additionally, the possible processing of PatS to direct its action to neighbours of the producing cell, as discussed above, would not be compatible with cytoplasmic bridges that were freely open to diffusion.

Alternatively, intercellular molecular exchange could take place through the periplasm, with cytoplasmic membrane permeases mediating the export and import of different molecules in the different cell types. For example, if the periplasm were important for exchange of nutrients, sugar exporters would be expected to be present in the vegetative cells and sugar importers would be expected to be present in the heterocysts; similarly, amino acid exporters would be expected to be present in the heterocysts and amino acid importers would be expected to be present in the vegetative cells¹⁵. In *Anabaena* sp. PCC 7120, inactivation of two ABC-type transporters that mediate amino acid uptake, one of which is expressed only in vegetative cells, specifically impairs N_2 -dependent growth, indicating a role for these transporters in diazotrophic physiology^{101,102}. A role for the periplasm as a metabolic communication conduit would require the outer membrane to have a low permeability for the exchanged metabolites, which has been recently shown for sucrose and glutamate¹⁰³.

We suggest that a specific use of each of the two possible pathways for moving different types of compounds between the two cell types might be a key feature in cyanobacterial multicellularity. An important property of the paths for intercellular molecular exchange in the filament is that they should permit the formation of

gradients of nitrogenous compounds or regulatory molecules. Consumption by the vegetative cells could contribute to the establishment of gradients of molecules (such as nutrients) that originate in the heterocysts, but regulated gates could also influence the formation of such gradients.

Conclusions

Consistent with the description that reproduction in heterocyst-forming cyanobacteria takes place 'by random trichome breakage' (REF. 8), the filament (or trichome), which is now viewed as a string of cells encapsulated by the outer membrane, can be considered as an organismic unit in these bacteria. Heterocyst-forming cyanobacteria are unique in that they can form two different types of metabolically active cells, photosynthetic vegetative cells and N₂-fixing heterocysts, the activities of which are necessary and complementary for the growth of the organism. Specific mechanisms seem to

keep cells together in the cyanobacterial filament and to allow the intercellular exchange of nutrients that is necessary for growth. Of particular importance will be an increased understanding of the role of a shared periplasm and of SepJ-containing cell-to-cell connecting structures. Heterocyst differentiation from a vegetative cell also depends on intercellular communication, with exchange of regulatory molecules, such as a PatS-related compound, that influence the execution of a genetic differentiation programme. This programme is started as a nitrogen deprivation response that is mediated by NtcA, the global nitrogen-control transcription factor of cyanobacteria, and requires specific regulators, of which HetR is essential to differentiation. Knowledge of the mechanism of functional interactions between NtcA and HetR and of how other regulators inhibit differentiation of too many cells into heterocysts will be key to gaining a final understanding of this process that leads to a truly multicellular bacterium.

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Competing interests statement

The authors declare no competing financial interests.

DATABASES

Entrez Gene: <http://www.ncbi.nlm.nih.gov/gene>

[fraC](#) | [glnA](#) | [hetC](#) | [hetF](#) | [nrrA](#) | [patA](#) | [sepJ](#)

Entrez Genome Project: <http://www.ncbi.nlm.nih.gov/genomeprj>

[Anabaena](#) sp. PCC 7120 | [Anabaena variabilis](#) ATCC 29413 |

[Escherichia coli](#) | [Nostoc punctiforme](#) PCC 73102 |

[Synechocystis](#) sp. PCC 6803 | [Trichodesmium erythraeum](#)

[IMS101](#)

UniProtKB: <http://www.uniprot.org>

[HetN](#) | [HetR](#) | [NtcA](#) | [PatS](#)

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Enrique Flores and Antonia Herrero's homepage:

http://www.ibvf.cartuja.csic.es/Cianobac_Diferenciacion/Cyanobac_Diferenciacion.htm

CyanoBase: <http://genome.kazusa.or.jp/cyanobase>

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