Cyanobacterial Heterocysts

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Advanced article

Article Contents

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Cyanobacteria are phototrophic bacteria carrying out oxygen-producing photosynthesis. Indeed, cyanobacteria were the inventors of oxygenic photosynthesis carried out by eukaryotic algae and plants. Besides showing the capability of building their cellular carbon from carbon dioxide, available in the atmosphere, several strains of cyanobacteria have also acquired the ability to fix molecular dinitrogen (N_2). As the enzyme responsible for nitrogen fixation (nitrogenase) is highly sensitive towards oxygen, nitrogen fixation and oxygenic photosynthesis cannot take place simultaneously in cyanobacterial cells. To solve this problem, some filamentous strains are able to restrict N_2 fixation to a special cell type, the heterocyst. Heterocysts are specialised, morphologically distinct, terminally differentiated cells that develop, in the absence of alternative sources of combined nitrogen, mostly in a semiregular pattern along the filament. Thus, a filament containing heterocysts provides division of labour between photosynthetic carbon dioxide fixation (in vegetative cells) and anaerobic N_2 fixation (in heterocysts). These cyanobacteria represent true multicellular organisms with profound morphological cell differentiation and sophisticated intercellular communication systems.

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Introduction

Diazotrophic cyanobacteria, which fix molecular nitrogen, are photosynthetic microorganisms that gain their energy from sunlight and their carbon and nitrogen from air and water. Being able to utilise carbon dioxide, nitrogen and water as macronutrients, these organisms can occupy niches not accessible to other microorganisms that require reduced carbon compounds and chemically less inert, bound, nitrogen species for growth. Fixation of nitrogen is a highly energy-demanding process catalysed by an extremely oxygen-labile enzyme, nitrogenase. Organisms that fix nitrogen have evolved a variety of strategies to maintain an active nitrogenase in the presence of oxygen, and cyanobacteria are even able to reconcile photosynthetic oxygen production with nitrogen fixation (Fay, 1992; Gallon, 1992). Some cyanobacteria bypass the oxygen problem by making their nitrogenases only in the dark when photosynthesis is inactive and the intracellular partial pressure of oxygen (Po_2) is lowered by respiration. In this case the two processes are temporally separated. Several strains of multicellular cyanobacteria can modify a small percentage of their cells for the task of nitrogen fixation, thereby generating a spatial separation of the processes. In some genera the functional specialisation of nitrogen-fixing cells is accompanied by a morphological differentiation of about every tenth cell of a filament into heterocysts, producing a semiregular pattern of morphologically and metabolically different cell types. Prospective heterocysts form a special envelope that limits the entrance of oxygen. Additionally they enhance their respiratory activity and switch-off the oxygen-releasing activity of photosystem II. This allows the mature heterocyst to generate a microoxic environment suitable for the functioning of nitrogenase. Because developing heterocysts lose the ability to fix carbon dioxide, adjacent vegetative cells have to provide reduced compounds for the generation of reductants for respiration and nitrogen fixation. In turn, heterocysts supply vegetative cells with the needed fixed

nitrogen, probably in the form of amino acids. Having two different cell types (photosynthetic vegetative cells and specialised nitrogen-fixing heterocysts) these cyanobacteria can be considered as true multicellular organisms. Both cell types of the trichome depend on each other and must communicate with respect to exchange of metabolites as well as signalling molecules. This communication could operate via the continuous periplasm or via cell-to-cell connections.

Heterocysts undergo a terminal differentiation and become unable to reproduce by cell division. In diazotrophically growing filaments the semiregular pattern of heterocysts is maintained by differentiation of new heterocysts at approximately equal distances between two preexisting ones. The possibility to grow filaments without heterocysts by supplying them with combined nitrogen has facilitated the isolation and characterisation of mutants specifically affected in heterocyst development and function. The phenotype of mutants range from absence of heterocysts to the presence of aberrant or supernumerary heterocysts (MCH). Through the use of mutants, such complex processes as sensing and responding to environmental signals, control of differentiation, intercellular communication and biological pattern formation became amenable to analysis. **See also**: Cyanobacteria; Nitrogen Fixation; Nitrogenase Complex; Photosynthesis; Photosynthesis and Respiration in Cyanobacteria; Photosynthesis: Light Reactions; Photosynthesis: The Calvin Cycle

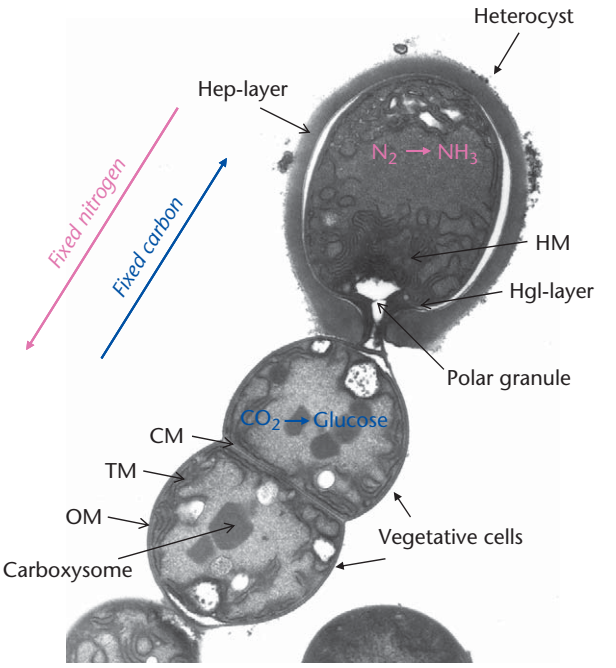

Figure 1 Ultrastructure of *Anabaena* strain PCC 7120. The structures of a terminal heterocyst and two vegetative cells visualised by transmission electron microscopy.

Structure of Mature Heterocysts

The differences in ultrastructure of vegetative cells and heterocysts (**Figure 1**) reflect the strategy of heterocyst-forming cyanobacteria to reconcile two incompatible processes: oxygenic photosynthesis and oxygen-sensitive nitrogen fixation.

Heterocyst envelope

To limit the entrance of oxygen, mature heterocysts have a special envelope that consists of a laminated layer containing *h*eterocyst *gl*ycolipids (Hgl) and a protective homogeneous layer of *h*eterocyst *e*nvelope *p*olysaccharides (Hep) (**Figure 1**). Hgl is composed of a hydrophilic sugar moiety (glucose, galactose and mannose) linked via a glycosidic bond to a C_{26} or C_{28} polyhydroxy or ketohydroxy hydrocarbon chain; polyhydroxy and ketohydroxy Hgl are products of distinct biosynthetic pathways. Interactions of the hydrophobic chains result in the formation of lipid monolayers of 4 nm width with low permeability for gases and solutes. Heterocyst envelope polysaccharides consist of concatamers of an oligosaccharide that has a tetrasaccharide backbone (25% mannose, 75% glucose) and side-chains comprising strain-specific combinations of mannose, glucose, galactose, xylose and arabinose as terminal residues. Mutants defective in the synthesis or extracellular deposition of Hep and Hgl exhibit a highly oxygen-sensitive nitrogenase that is synthesised only under microaerobic growth conditions (reviewed in Awai *et al*., 2009; Nicolaisen *et al*., 2009). **See also**: Polysaccharides

Membranes

In heterocysts, four types of lipid bilayers are observed that differ in composition and function.

i. Intracellular thylakoids contain photosynthetic pigments and components of the photosynthetic and respiratory electron transport chain. However, they differ structurally and functionally from those in vegetative cells (**Figure 1**). They lack photosystem II activity and the amount of phycobiliproteins, the major light harvesting pigments of photosystem II, is reduced. The remaining pigments serve as antennae of photosystem I. The primary function of heterocyst thylakoids appears to be the provision of extra ATP (adenosine triphosphate) for nitrogenase by means of cyclic photophosphorylation. **See also**: Photosystem I; Photosystem II

ii. Photomicrographs show a paucity of absorption of chlorophyll towards the poles of heterocysts, in the region of the 'honeycomb' membrane, named after its distinct regular structure. In the dark, the product of oxidation of diaminobenzidine, which reacts with haemoproteins, is accumulated preferentially in this region. This was taken as evidence for enhanced respiratory activity in this membrane region.

iii. The cytosol is surrounded by the cytoplasmic membrane. This membrane lacks photosynthetic pigments. Membrane energisation is provided by hydrogen ion-translocating respiratory electron transport chains and, in some strains, by hydrogen ion-translocating ATP hydrolases. The cytoplasmic membrane contains numerous proteins, permeases, that function in solute transport. In heterocysts, the presence of specific carriers facilitating diffusion of newly fixed nitrogen from heterocysts to the periplasmic space has been shown.

iv. The outer membrane is composed of *li*po*po*ly*s*accharides (LPS), phospholipids and proteins. In filamentous cyanobacteria this membrane surrounds not individual cells but the entire filament. During cell division the outer membrane does not enter the septum formed between the new daughter cells. Thereby, a continuous compartment, the periplasmic space, is generated in which solutes are able to diffuse along the filament without passing through cells. In many cyanobacteria the outer membrane is decorated with amorphous extracelluar polysaccharides, mucilaginous sheaths or proteinaceaous S-layers. Around heterocysts, the outer membrane serves as basis of the heterocyst envelope and thus contains specific proteins involved in the build-up of the heterocyst envelope layers. **See also**: Lipopolysaccharides

Communication between heterocysts and vegetative cells

To allow fluxes of nutrients and gases, the heterocyst envelope remains open at the junctions with vegetative cells but the connection is reduced to a narrow septum in contrast to the broad septa between two adjacent vegetative cells (**Figure 1**). At their poles heterocysts accumulate large amounts of refractive, electron-dense material, the polar granules (**Figure 1**). They contain cyanophycin, a high-molecular weight copolymer of arginine and aspartic acid that serves as a nitrogen reserve in cyanobacteria. In heterocysts the polar granule may have a specific function in sequestering newly fixed nitrogen that otherwise might lead to a negative feedback regulation of nitrogenase. In addition, it could serve as reserve pool for fixed nitrogen to be transferred to the vegetative cells.

Regarding heterocystous cyanobacteria as true multicellular organisms, one substantial question is how cell-to-cell communication and coordination inside the filament takes place. Communication is critical for two different aspects. One is the exchange of metabolites, necessary to meet the needs of the two cell types. The other is the diffusion of regulatory molecules that ensures the correct pattern of heterocyst and avoids formation of multiple adjacent heterocysts in a filament.

Basically two different mechanisms are considered and experimental evidence for each of them has been obtained. One mechanism is based on the presence of extracellular conduits and the other one is based on the existence of proteinaceous cell-to-cell bridges. Most likely both mechanisms are involved in intracellular exchange. A continuous periplasmic space represents a good possible extracellular candidate route for substances that have to move along the filament from one cell to another. Since the outer membrane is continuous along the filament, the periplasmic space should also be continuous, thus surrounding all cells of the trichome (Flores *et al*., 2006). Green fluorescence protein (GFP, 26 kDa) can diffuse rapidly along the filament, only after having been translocated over the cell membrane into the periplasmic space, an observation that supports the operation of a 'periplasmic route' (Mariscal *et al*., 2007). Instead smaller markers, as the small fluorophore calcein (623 Da), show rapid flow also from cytoplasm to cytoplasm of all cells of the filament via unspecified intercellular connections traversing the septum, supporting the idea of the presence of cell-to-cell conduits (Mullineaux *et al*., 2008; **Figure 2**).

Potential intracellular channels, known in the literature as microplasmodesmata, can be seen in **Figure 2a**. They could be formed by septum-localised proteins as SepJ, also known as FraG, and other proteins. Membrane-bound SepJ has a large extracellular domain, which could traverse the septum to connect with an SepJ complex (or another protein) at the adjacent cell. Localisation of GFP translationally fused with the cytoplasmic domain of SepJ as one central fluorescence dot in the septa is in line with this hypothesis (**Figure 2b**). The small fluorophore calcein cannot move between cells in an SepJ-mutant. The fragmenting phenotype of fra-mutants could be due to the absence of connections in the septum that, in the wild-type strain, stabilise the filament and enable the transport of small solutes (Bauer *et al*., 1995; Nayar *et al*., 2007; Merino-Puerto *et al*., 2010; Flores *et al*., 2007; Mullineaux *et al*., 2008).

Thus, the emerging picture might well include a combination of two routes, i.e. a periplasmic extracellular route and connections between two adjacent cells. A model is depicted in **Figure 2c**. **See also**: Cell Junctions; Fluorescence Microscopy; Genetic Engineering: Reporter Genes; Green Fluorescent Protein (GFP)

Heterocyst Function

In an aerobic environment, heterocysts provide a microoxic compartment suitable for the functioning of the highly oxygen-labile nitrogenase. The ammonium produced is immediately incorporated into amino acids by the reaction of glutamine synthetase/glutamine-oxoglutarate-aminotransferase (GS/GOGAT pathway). Interestingly, the ammonium acceptor glutamate is provided by vegetative cells.

Nitrogenase and *nif* genes

Nitrogen fixation is catalysed by dinitrogenase, a heterotetramer of two polypeptides encoded by the genes *nifD* and *nifK*. Dinitrogenase is supplied with electrons by dinitrogenase reductase, a dimer of a polypeptide encoded

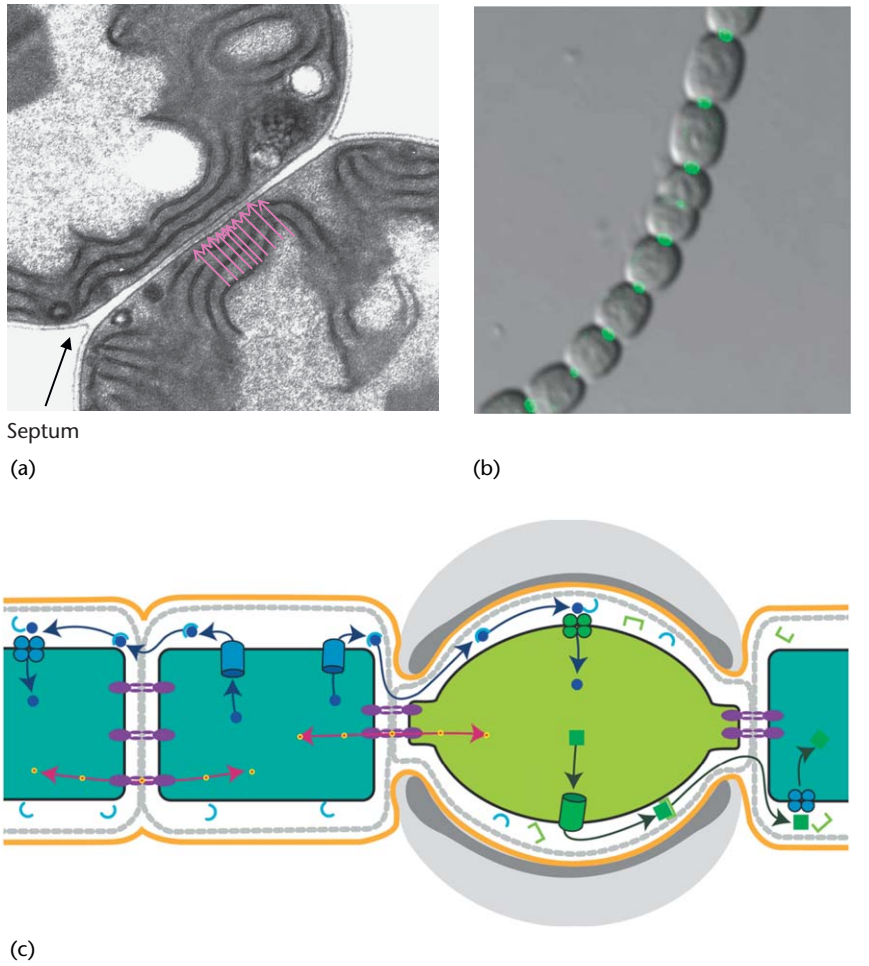

Figure 2 Intracellular solute exchange could occur via a periplasmic route or with the aid of cell-to-cell connections in a filament of *Anabaena*. (a) Electron micrograph of the septum between two vegetative cells of an *Anabaena* filament. Purple arrows point to electron dense material traversing the septum through the periplasmic space that could present putative cell-to-cell bridges. (b) Filament of *Anabaena* sp. PCC 7120 (strain CSAM137; Flores *et al.*, 2007) expressing an SepJ-GFP fusion protein. GFP is found in the middle of the septum between two cells. Courtesy of Vicente Mariscal, CSIC and Universidad de Sevilla, Spain. (c) Scheme of intracellular and periplasmatic routes of solutes between the cells of a heterocystous filament: Barrels represent exporter, as putative sugar transporters of vegetative cells, tetrads represent importers of solutes, as amino acids imported by heterocysts. Protein complexes are localised in the septum, putatively composed of SepJ and Fra proteins that allow the transport of small solutes (small yellow dots) between adjacent cells of the filaments. Half circles and half squares represent binding proteins and dots represent solutes that diffuse through the periplasmic space. Artwork of (c) by Ingeborg Schleip is gratefully acknowledged.

by *nifH*. Dinitrogenase and dinitrogenase reductase together comprise the oxygen-labile nitrogenase. For synthesis of apoproteins and cofactors, and for the assembly of both, a number of additional enzymes encoded by other *nif* genes are required. In mature heterocysts these genes are organised in multicistronic operons located in a large, contiguous *nif* gene cluster and at least one distant operon (Wolk *et al.*, 1994). **See also**: Nitrogen Fixation; Nitrogenase Complex

For a still unknown reason, the *nif* gene cluster of vegetative cells of many strains of *Anabaena* and *Nostoc* spp. is interrupted by an insertion of deoxyribonucleic acid (DNA) elements that must be precisely excised. In the case of *Anabaena* sp. PCC 7120, such element consists of 11 kb of DNA interrupting the *nifD* open reading frame. Some strains have an additional 55 kb excision element located in *fdxN*. Both elements are excised during heterocyst differentiation but remain as circular DNA in the mature heterocyst.

In contrast to the usual molybdenum-containing nitrogenase, some heterocyst-forming cyanobacteria can synthesise alternative nitrogenases that are expressed under specific environmental conditions. *Anabaena variabilis* strain ATCC 29413 can form a vanadium-nitrogenase in which molybdenum is replaced by vanadium. The vanadium-nitrogenase and enzymes required for its synthesis are encoded in the *vnf* gene cluster. They are expressed in heterocysts in the absence of molybdenum only. This strain also harbours genes for a second molybdenum-nitrogenase, organised in the *nif-II* gene cluster, which can be expressed in all cells of the filament but under strictly anaerobic conditions only (Thiel *et al.*, 1995).

Regeneration of reductants and ATP in heterocysts

Because heterocysts cannot fix carbon dioxide, vegetative cells have to supply reduced carbon compounds for heterocyst functioning. The imported solutes, presumably sucrose, are a source of electrons for nitrogen fixation and respiratory activity, and of building blocks for envelope materials.

In heterocysts, NAD(P)H is regenerated by oxidation of hexose phosphates via the oxidative pentose phosphate cycle (**Figure 3**). The cycle operates in the oxidative mode both in dark and in the light. The key enzyme of this cycle, glucose-6-phosphate dehydrogenase, was shown to be essential for diazotrophic growth and is highly active in heterocysts. Triose phosphates, if not directly imported from vegetative cells, can be removed from the oxidative pentose phosphate cycle and converted to pyruvate via the Entner–Doudoroff pathway. Pyruvate is required for generation of reductant for nitrogenase under iron-limiting growth conditions, and to generate acetyl-CoA for synthesis of fatty acids and Hgl (Böhme, 1998).

A *f*erredoxin *N*ADPH oxido*r*eductase (FNR) transfers electrons from NADPH to a special ferredoxin, which then delivers electrons directly to dinitrogenase reductase. Light is not needed for this reaction. In addition, pyridine nucleotides are oxidised by a type-I or mitochondrial-type *N*AD(P)H *deh*ydrogenase (NDH). From there, electrons pass several components of an electron transport chain and build up a pH gradient (ΔpH) required for ATP synthesis. The chain ends at either of two terminal electron acceptors, a cyt aa_3-type oxidase that transfers electrons to oxygen, or the oxidising site of photosystem I. Light is required to transfer electrons from there to the reducing site of photosystem I and, via ferredoxin, to nitrogenase and thence nitrogen. To increase the ΔpH and, hence, the ATP/e^- ratio, electrons can be redirected from the reducing site of photosystem I to NDH in a process called cyclic photophosphorylation (**Figure 3**).

ATP is regenerated from ADP (adenosine diphosphate) by a vectorial ATP synthase (reversible F_0F_1-ATPase). This enzyme utilises the energy stored in the ΔpH for generation of the energy-rich phosphate bond. In heterocyst extracts, substrate-level phosphorylation by a glycolytic pathway was not observed.

Oxygen protection of nitrogenase

Heterocysts provide a microoxic environment required for nitrogenase function by repressing oxygen production

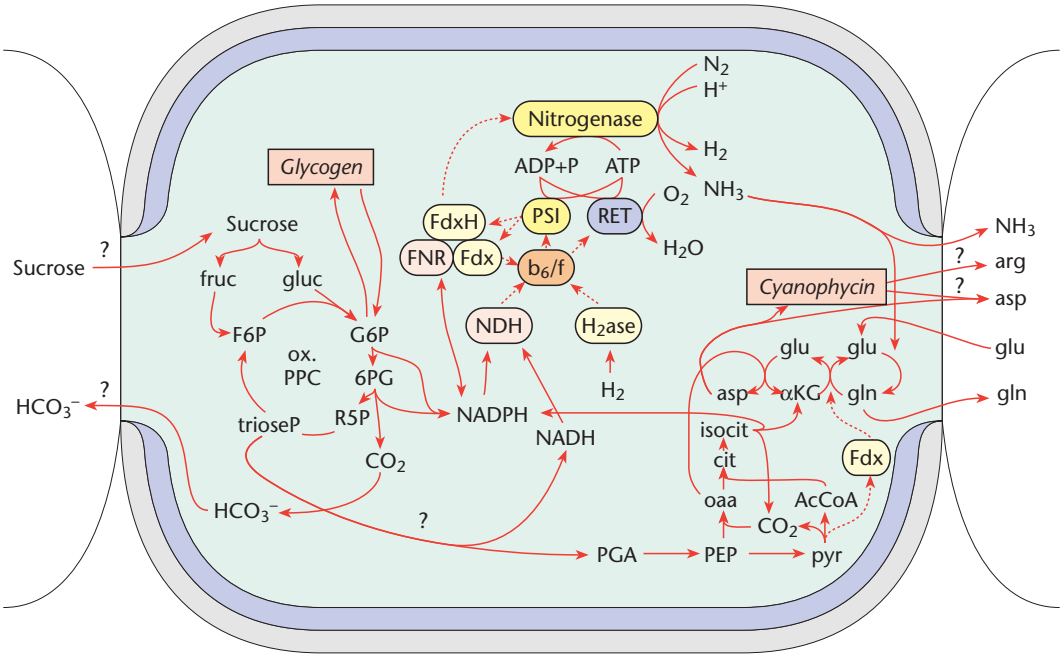

Figure 3 Fluxes of carbon, nitrogen and reductant in heterocysts. Heterocysts act as a sink for carbohydrates (sucrose?) from vegetative cells and as a source of fixed nitrogen (glutamine, NH_4^+, aspartate?) to vegetative cells. Solid lines represent fluxes of carbon and nitrogen; dashed lines refer to fluxes of reducing equivalents; question marks indicate uncertainties. Enzymes, enzyme complexes and components of the electron transport chains are circled; storage compounds are in italic; metabolites are depicted in regular letters. Abbreviations: AcCoA, acetyl-coenzyme A; arg, arginine; asp, aspartate; b_6/f, cytochrome b_6/*f* complex; cit, citrate; fruc, fructose; F6P, fructose 6-phosphate; Fdx, vegetative cell-type ferredoxin; FdxH, heterocyst-specific ferredoxin; FNR, ferredoxin: $NADP^+$ oxidoreductase; G6P, glucose 6-phosphate; 6PG, 6-phosphogluconate; gln, glutamine; glu, glutamate; gluc, glucose; H_2ase, uptake hydrogenase; isocit, isocitrate; αKG, α-ketoglutarate; NDH, NAD(P)H dehydrogenase; oaa, oxaloacetate; ox. PPC, oxidative pentose phosphate cycle; P, inorganic phosphate; PEP, phosphoenolpyruvate; PGA, 3-phosphoglycerate; PSI, photosystem I; pyr, pyruvate; R5P, ribulose 5-phosphate; RET, respiratory electron transport; trioseP, triose phosphate. Not all intermediates are depicted. Modified from Wolk *et al.* (1994, Figure 4). Copyright © Kluwer Academic Publishers 1994, with kind permission.

during photosynthesis, by forming a barrier to diffusion of gases, and by enhanced respiratory activity. Auxiliary means to minimise the costs of nitrogen fixation are operating a switch-off mechanism for nitrogenase that ensures prevalence of oxygen-removing processes over nitrogenase activity, and providing a specific pathway for degradation of oxygen-damaged nitrogenase proteins.

It is important to realise that, in an aerobic environment, the interior of heterocysts is not completely free of oxygen. Nitrogen and oxygen are physically so similar that there is no obvious way to allow the former to enter a cell while the latter is wholly excluded. An approximately 10-fold lower diffusion of gases into heterocysts than into vegetative cells was measured (Walsby, 1985). Any biochemical mechanism for removing (reacting with) the oxygen that enters the cell has a nonzero k_m (oxygen), and so, in the presence of a constant influx of oxygen, cannot render the interior of the heterocyst anaerobic.

To remove permeant oxygen, heterocysts enhance respiration even in the light. This is accomplished in *Anabaena* sp. PCC 7120 by two alternative cytochrome *c* oxidases, encoded by the *coxII* and *coxIII* gene, respectively. Both genes are upregulated in heterocysts and are not expressed in vegetative cells where a different cytochrome *c* oxidase functions in oxygen reduction in the dark (Valladares *et al*., 2003; Jones and Haselkorn, 2002).

The oxidase competes with nitrogenase for reductants derived from photosynthates. If the need for reductants of both enzymes exceeds the supply, then the internal P_{O_2} will rise. Putatively, this results in a signal to switch-off nitrogenase activity. The oxygen-induced, reversible inactivation of nitrogenase is accompanied by a modification of the dinitrogenase reductase. If the internal P_{O_2} increases to a concentration that damages nitrogenase irreversibly, subunits become modified by ubiquitin and are rapidly degraded in a high-molecular weight protein complex (Durner and Böger, 1995).

Heterocyst Development

If no source of combined nitrogen is available, heterocysts develop in semiregular intervals along a filament of vegetative cells. The frequency of heterocysts is related to the proliferation of the entire filament. In a growing filament, the pattern is maintained by differentiation of new heterocysts at approximately equal distances between two previously existing ones. To study the formation and maintenance of this pattern and of heterocyst differentiation, mutants have been isolated that grow well in the presence of combined nitrogen but are deficient in their ability to *f*ix nitrogen in presence of *ox*ygen (Fox^- phenotype). Fox mutants show diverse morphologies that provide further hints about the processes affected by the mutation (Wolk *et al*., 1994). For details about genetic techniques see Cohen *et al*. (1998).

Heterocysts, a model for biological pattern formation

Heterocysts appear nonrandomly along a nitrogen-starved filament. The resulting pattern of spaced heterocysts is a simple example of a multicellular pattern. One of the most striking features of this process is the ability of developing heterocysts to suppress differentiation in adjacent cells long before they can supply them with fixed nitrogen.

Several models have been proposed to explain this feature (Wolk *et al*., 1994). Here we concentrate on a model that describes the stabilisation of gradients of chemicals promoting morphogenesis (Meinhardt, 1994). The model is based on solutions of two differential equations describing the turnover and diffusion of two substances, the morphogens. One functions as a slowly diffusing short-range activator, the other as rapidly diffusing long-range inhibitor of morphogenesis. The activator promotes its own synthesis (local self-enhancement, autocatalysis) but also the production of the inhibitor. The inhibitor suppresses the activator. If the inhibitor/activator ratio drops below a threshold value, the activator can escape from this control and start to catalyse its own synthesis and that of inhibitor. If the newly formed inhibitor remains at the place of production, it will regain control over the activator. However, if rapid diffusion drains the inhibitor from the place of production, the activator synthesis is stabilised. In neighbouring cells, the entry of inhibitor will prevent formation of activator. In more distant areas, diffusion of the inhibitor may not be sufficient to compensate for losses of inhibitor by turnover, and so new centres of activation can form. If two existing centres move apart by elongation or division of intervening cells, the new centre forms at approximately equal distance between two previously existing ones.

If the model is applied to heterocyst formation, we expect that the activator is a regulatory protein and the inhibitor is a small diffusible molecule. Both should be produced in a prospective heterocyst. The inhibitor should be exported to the periplasmic space, in which it can diffuse along the filament to neighbouring cells. Continuous localised production and export of the inhibitor will prevent formation of new heterocysts in the vicinity of an existing one.

Early events and pattern formation

Early events in heterocyst development comprise sensing of nitrogen deprivation in the filament, localisation of prospective heterocysts, the activation of primary heterocyst-specific genes and the silencing of genes exclusively expressed in vegetative cells. Mutants defective in the former two processes are suppressed in *het*erocyst formation, the Het^- phenotype, or exhibit supernumerary (MCH) or irregularly spaced heterocysts, the Pat phenotype (referring to the heterocyst *pat*tern).

The *ntcA* gene was found essential for heterocyst formation and nitrogen fixation. Upon mutation of *ntcA* an

Het$^-$ phenotype is produced. The gene encodes NtcA, a protein that belongs to the cyclic AMP receptor protein (CRP) family of prokaryotic regulatory proteins. In cyanobacteria, NtcA functions in global nitrogen control by ammonia (Luque *et al*., 1994). During nitrogen deprivation, binding of NtcA to its own promoter leads to an enhanced transcription of *ntcA*. In addition to being autoregulated at the level of gene expression, transcriptional regulation exerted by NtcA is modulated in response to the C-to-N balance of the cells and 2-oxoglutarate has been shown to exert a positive effect on NtcA function both *in vivo* and *in vitro* (Tanigawa *et al*., 2002; Valladares *et al*., 2008; Vázquez-Bermúdez *et al*., 2003). 2-Oxoglutarate is the substrate of GOGAT and thus the acceptor molecule for fixed nitrogen. Low nitrogen results in high level of 2-oxoglutarate and vice versa, and the concentration of this substance is an indication of the C/N balance in cyanobacteria.

By binding to promoters of several other genes, NtcA then stimulates, in all cells of the filament, the synthesis of diverse proteins involved in acquisition of different nitrogen-containing compounds, but also activates localised expression of *hetR*, *hetC*, *devBCA* and *nifHDK* in prospective or mature heterocysts. Thus, NtcA acts as a molecular switch that enables nitrogen-starved filaments to explore a number of alternative nitrogen sources including molecular nitrogen.

NtcA is essential but not sufficient for heterocyst development. Two genes, *hetR* and *patS* have attracted considerable attention because the mutant phenotypes and properties of the gene products indicate that they are involved in the formation of the heterocyst pattern. The gene *hetR* was identified in a mutant unable to form heterocysts (Buikema and Haselkorn, 1991; Black *et al*., 1993). When expressed from additional copies in the wild type, it caused heterocyst formation in the presence of bound nitrogen and multiple heterocysts under nitrogen-fixing conditions (MCH-phenotype). The gene *patS* of a wild-type strain was identified because extra copies cloned in a replicating plasmid suppressed heterocyst formation, whereas an inactivation of the genes caused the MCH-phenotype (Yoon and Golden, 1998). This suggested that the products of *hetR* and *patS* represent counteractive morphogenes – a heterocyst activator and inhibitor, respectively. The putative heterocyst activator gene, *hetR*, is the earliest gene known to be upregulated in spatially separated cells after nitrogen withdrawal. For this localised expression, a functional copy of the gene is required, indicating that HetR is involved in an autocatalytic process (Black *et al*., 1993). Unlike NtcA, HetR seems to be specifically involved in cell differentiation, and is not required for growth in the presence of combined nitrogen. *hetR* encodes a unique protein with no known homologues. Protease (Shi *et al*., 2006; Zhou *et al*., 1998) and DNA-binding (Huang *et al*., 2004) activities have been described for HetR but the relationship, if any, between those activities and the regulatory effects exerted by HetR remains to be demonstrated. There seems to be a correlation between HetR protein turnover and heterocyst differentiation (Risser and Callahan, 2008; Zhou *et al*., 1998).

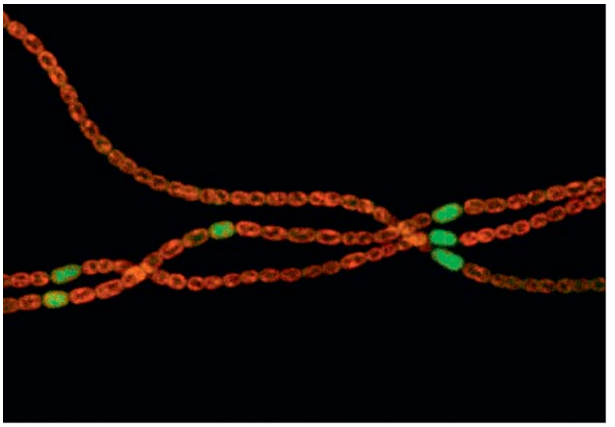

Figure 4 Increased expression of GFP from the *ntcA* promoter in (pro)heterocysts 8 h after nitrogen step-down. The micrograph is an overlay of red (autofluorescence) and green (GFP fluorescence) channels (see also Olmedo-Verd *et al*., 2006).

Expression of both *ntcA* and *hetR* is induced in those cells that are differentiating (**Figure 4**) and depends on each other (Muro-Pastor *et al*., 2002; Olmedo-Verd *et al*., 2006). The observation that the two products involved in the early regulatory loop, NtcA and HetR, are autoregulatory is relevant in the context of initiation of development because, as previously discussed, this kind of behaviour stabilises developmental decisions (Wolk *et al*., 1994). Transcription of many other genes whose products are involved in different stages of differentiation is altered in *ntcA* or *hetR* mutants but because these two gene products are at the very top of the sequence that leads to the differentiation of functional heterocysts, it is difficult to determine whether they exert a direct or indirect effect at the transcriptional level. Some promoters that are expressed in mature heterocysts, such as the P_1 promoter of *glnA* (encoding glutamine synthetase) or the P_1 promoter of the *ntcA* gene, show a direct dependence on NtcA (Olmedo-Verd *et al*., 2008; Valladares *et al*., 2004), indicating that NtcA is in fact directly involved not only in the developmental decision (whether or not to differentiate) but also in the transcriptional activity taking place in mature heterocysts.

The gene of the putative heterocyst inhibitor, *patS*, encodes a small peptide, PatS. In mutants forming supernumerary heterocysts, a pentapeptide, Arg-Gly Ser-Gly Arg, corresponding to the last five amino acids of PatS was sufficient to reduce the number of heterocysts *in vivo* and to inhibit DNA-binding activity of HetR *in vitro*. The expression of PatS as well as of a second inhibitor, HetN, also containing the pentapeptide, is HetR dependent. Both inhibitors of heterocyst formation are thought to diffuse to the neighbouring vegetative cells, establishing a concentration gradient. Genetic and cytological evidence has been obtained that this gradient promotes the decay of the activator HetR (Risser and Callahan, 2009).

Heterocyst differentiation

Nitrate and ammonia suppress the initiation of heterocyst formation. Differentiation is initiated once a pattern of prospective heterocysts is established. The selected cell, a proheterocyst, undergoes successive morphological changes until, in the mature heterocyst, nitrogenase is synthesised. At some point during differentiation the process becomes irreversible. The cell loses the ability to divide and enters terminal differentiation.

Nitrogen starvation induces the proteolytic degradation of the reserve polymer cyanophycin and also of the phycobiliproteins, in all cells of the filament. Heterocyst-specific enzymes involved in this response have not been identified so far. The first visible (in light and electron micrographs) event in heterocyst development is the deposition of polysaccharides on top of the pre-existing cell wall. At the same time the constriction of the broad septum between the proheterocyst and the adjacent cell is observed. The carboxysomes (paracrystalline aggregates of ribulose-bisphosphate carboxylase) disappear and the laminated layer of glycolipids gets formed between the outer homogenous layer and the outer membrane of the cell wall. Finally the reorganisation of intracellular membranes occurs (**Figure 1**).

Numerous Fox$^-$ mutants were isolated that were unable to *dev*elop mature-looking heterocysts (Dev$^-$ phenotype) or that synthesised aberrant forms of the *h*eterocyst *en*velope (Hen$^-$ phenotype) (Fan *et al*., 2005). Dev$^-$ and Hen$^-$ mutants are able to synthesise nitrogenase under anaerobic assay conditions (Fix$^+$ phenotype).

Mutational inactivation of three clustered genes *devA*, *devB* and *devC*, resulted in arrested heterocyst differentiation. Transmission electron microscopy revealed that in these mutants the laminated layer, formed by Hgl, is missing and that intracytoplasmic membranes do not reorganise. However, Hgl synthesised after nitrogen withdrawal was identified in lipid extracts. The *devBCA* cluster encodes an exporter (ABC-transporter) possibly functioning in the export of glycolipids or of factors/proteins required for assembly of the laminated layer (Fiedler *et al*., 1998). Heterocysts seem to express several of such specific transport systems (Black *et al*., 1995; Fan *et al*., 2005). Outer membrane protein TolC of *Anabaena*, coded by *hgdD*, is possibly involved in the modifications of the heterocyst envelope, since mutation of that gene shows the same phenotype as mutants of the DevBCA transporter (Maldener *et al*., 2003; Moslavac *et al*., 2007).

Other mutants can seemingly form mature heterocysts but are unable to express nitrogenase under any experimental condition. They exhibit a Fix$^-$ phenotype. Often mutations are found in *nif* genes but some mutants inherit intact *nif* genes. The latter were found to be unable to rearrange the major *nif* gene cluster of vegetative cells. The removal of the excision elements, catalysed by excisases, is a prerequisite to the formation of intact open reading frames of two *nif* operons. Hence, mutations in *xis* genes lead to Fix$^-$ phenotypes. However, mutant strains cured of the elements are seemingly unaffected in diazotrophic growth. The evolutionary origins of these elements and the specific functions of these gene rearrangements are still unknown.

Environmental and developmental signalling

Multiple external and internal signals are involved in heterocyst development by affecting gene activation temporally and spatially. During heterocyst differentiation, single genes or group of genes are transcriptionally activated at different times after nitrogen step-down (Cai and Wolk, 1997; Ehira *et al*., 2003; Campbell *et al*., 2007). The timing is due to sensing of and response to intracellular changes caused by the differentiation process itself. Signals could involve calcium ions that influence HetR activity or redox control of regulator's activities, like protein phosphorylation. Several protein kinases and phosphatases have been identified that play a role in early and later steps of heterocyst differentiation (Zhang *et al*., 1998; Wang *et al*., 2002). Recently, two protein phosphatases have been identified that are required for the mutual regulation of *ntcA* and *hetR* in *Anabaena* sp. (Jang *et al*., 2009). However, in no case has the complete molecular basis of cyanobacterial signal transduction been worked out.

Protein phosphorylation as part of two-component systems or eukaryotic-like phosphorylation cascades is involved in signalling during heterocyst formation. This has been shown for the activation of HepA by the DevR/HepK two-component system (Zhou and Wolk, 2003). PatA, involved in activation of HetR is a response regulator related to patterning of heterocysts (Liang *et al*., 1992).

The PII N-signal protein has been well characterised in unicellular cyanobacteria (Forchhammer, 2004). A mutant of *glnB*, encoding PII protein, was difficult to obtain in heterocystous cyanobacteria. Although in *Nostoc punctiforme* PII seems to be essential (Hanson *et al*., 1998), in *Anabaena* sp. PCC 7120 *glnB* mutants could be obtained by different approaches (Zhang *et al*., 2007; Paz-Yepes *et al*., 2009). The mutants were able to form heterocysts, however were impaired in diazotrophic growth. The role of PII in diazotrophic growth is not clear yet.

The dissection of heterocyst development with molecular tools holds the promise that heterocysts will become a prime bacterial model for pattern formation, cell-to-cell communication and for terminal differentiation.

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