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Heterocyst and akinete differentiation in cyanobacteria

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SUMMARY

Cyanobacteria are an ancient and morphologically diverse group of photosynthetic prokaryotes. They were the first organisms to evolve oxygenic photosynthesis, and so changed the Earth's atmosphere from anoxic to oxic. As a consequence, many nitrogen-fixing bacteria became confined to suitable anoxic environmental niches, because the enzyme nitrogenase is highly sensitive to oxygen. However, in the cyanobacteria a number of strategies evolved that protected nitrogenase from oxygen, including a temporal separation of oxygenic photosynthesis and nitrogen fixation and, in some filamentous strains, the differentiation of a specialized cell, the heterocyst, which provided a suitable microaerobic environment for the functioning of nitrogenase. The evolution of a spore-like cell, the

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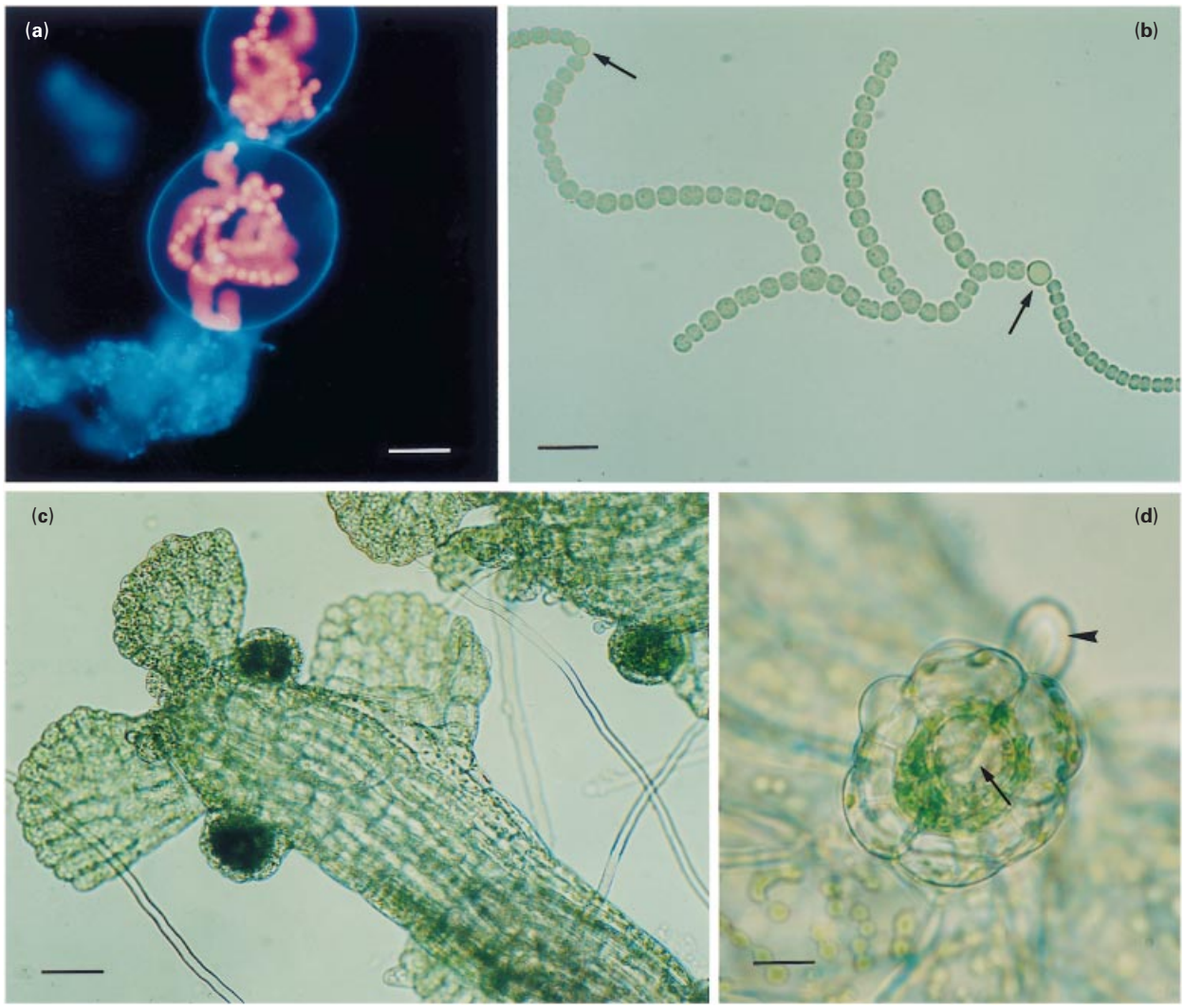

Fig. 1. (a) Fluorescence micrograph of a *Nostoc* sp. stained with calcofluor white. The filaments are contained within a discrete, spherical sheath of polysaccharide that shows blue fluorescence as a result of the calcofluor staining. Because the calcofluor has not penetrated this outer sheath, the filaments inside show only pink autofluorescence. However, towards the bottom left of the picture the outer sheath has ruptured and the cells have lysed allowing the calcofluor to stain polysaccharide associated with the cell debris. (b) A symbiotic cyanobacterium isolated from the liverwort *Blasia*, showing true branching of filments. Arrows indicate heterocysts. (c) *Blasia* thallus showing three symbiotic cyanobacterial colonies within structures known as auricles. The plant was infected in the laboratory with two *Nostoc* strains with different pigmentation, one brown and one blue-green. The former has colonized the two auricles to the left, the latter the single auricle to the right. (d) Symbiotic colony in *Blasia* auricle. The colony is at an early stage of development and is seen as the dark-green area between the inner slime papilla (arrow) and the outer wall of the auricle. An outer slime papilla can also be seen (arrowhead). Scale bars represent 25 μm (a), 20 μm (b), 90 μm (c) and 15 μm (d). *Photographs in* (*b*), (*c*) *and* (*d*) *by Slobodan Babic*.

akinete, almost certainly preceded that of the heterocyst and, indeed, the akinete may have been the ancestor of the heterocyst. Cyanobacteria have the capacity to differentiate several additional cell and filament types, but this review will concentrate on the heterocyst and the akinete, emphasizing the differentiation and spacing of these specialized cells.

Key words: cyanobacteria, heterocyst, akinete, nitrogen fixation, differentiation.

I. INTRODUCTION

Fossil evidence has revealed that both unicellular and filamentous cyanobacteria were in existence at least 3.5 billion yr ago (Schopf, 1994, 1996). Remarkably, these counterparts of today's oscillatoriacean and chroococcacean cyanobacteria have shown little if any morphological change in the intervening period. This immensely slow rate of evolution probably owes much to the wide ecological

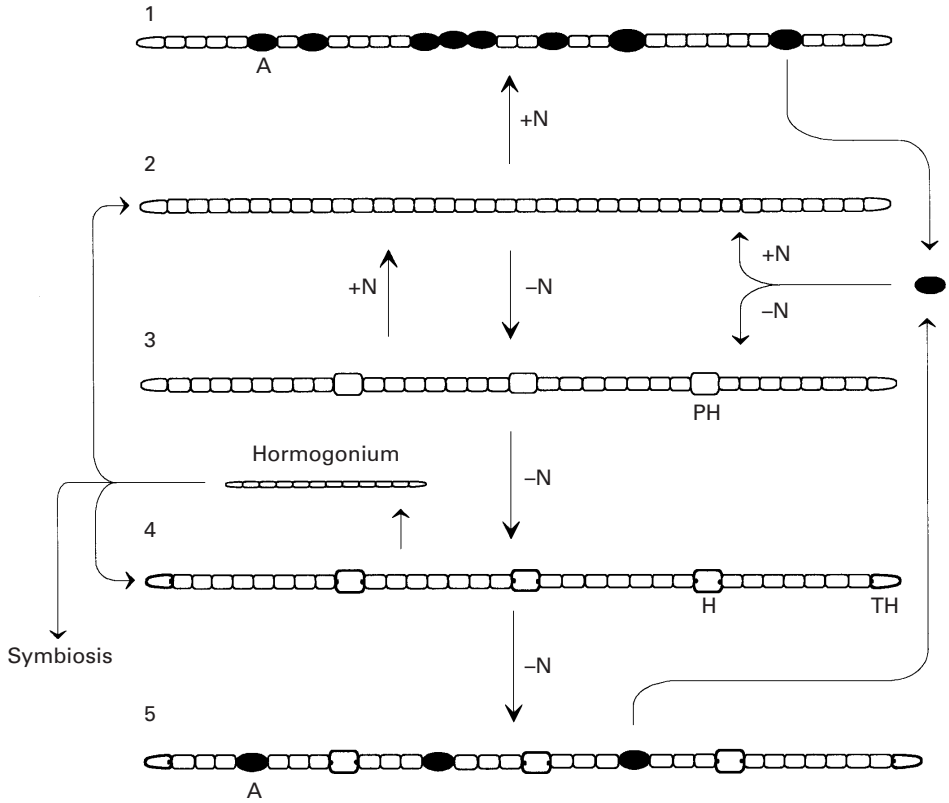

Fig. 2. Schematic diagram illustrating some of the possibilities for morphological development in filamentous, heterocystous cyanobacteria. When grown in the presence of a source of combined N the filament consists entirely of vegetative cells (2). At the end of the exponential growth phase when light becomes limiting, some vegetative cells may differentiate into the spore-like cells, akinetes (A) which, in the absence of heterocysts, are randomly placed within the filament (1). In the absence of combined N the vegetative filament differentiates the highly specialized N_2-fixing cells, heterocysts, at regular intervals within the filament (H) and in terminal positions (TH). Heterocysts are characterized by their thickened cell walls, relatively agranular cytoplasm, and polar bodies at the point of attachment to vegetative cells, there being two in heterocysts within the filament, but only one in terminal heterocysts (4). During their development heterocysts pass through an intermediate stage, the proheterocyst (PH) which unlike the mature cell, does not have the thickened cell wall and polar bodies, and is able to dedifferentiate in the presence of combined N (3). When akinetes develop in N_2-fixing cultures they do so at locations with a precise spatial relationship to the heterocysts, such as mid-way (5). Akinetes can germinate and give rise to filaments with or without heterocysts, depending on the availability of combined N. Hormogonia (HO) are short, motile, undifferentiated filaments that develop as a result of a variety of environmental stimuli, and provide a means of dispersal. Their formation usually involves the rapid division of vegetative cells without concomitant growth, followed by fragmentation of the filament to release heterocysts (which no longer function) and motile hormogonia. The latter can give rise to heterocystous or nonheterocystous filaments depending on the availability of combined N. Hormogonia can also serve as the infective agents in the establishment of symbiotic associations with a wide range of plants (e.g. Fig. 1c). *Adapted from Adams* (*1992a*).

tolerance of cyanobacteria, which is itself a result of their early evolution. Many of the phenotypic characteristics of existing cyanobacteria, such as synthesis of UV-absorbing pigments and extracellular polysaccharides, efficient DNA repair mechanisms, and phototactic motility, would have given them great advantages on the primitive earth when the UV flux was considerably higher than today (Schopf, 1994, 1996). Cyanobacteria were the first organisms to employ oxygenic photosynthesis, and their burgeoning growth during the Precambrian began to change the Earth's atmosphere from anoxic to oxic. This created problems for bacterial photosynthesis employing bacteriochlorophyll, the biosynthesis of which is inhibited by molecular oxygen, and would have resulted in the cyanobacteria supplanting anoxygenic photoautotrophs throughout much of the Earth's photic zone.

The increasing levels of oxygen in the atmosphere also created a problem for bacteria capable of nitrogen fixation, because the enzyme responsible, nitrogenase, is highly sensitive to oxygen (Gallon, 1992). For many N_2-fixing strains this resulted in their confinement to suitable anoxic environmental niches, whereas the cyanobacteria, generating oxygen intracellularly, had to evolve alternative strategies to protect nitrogenase from the toxic byproduct of their own metabolism. In some cases there evolved a temporal separation of oxygenic photosynthesis and N_2 fixation, with the former occurring during

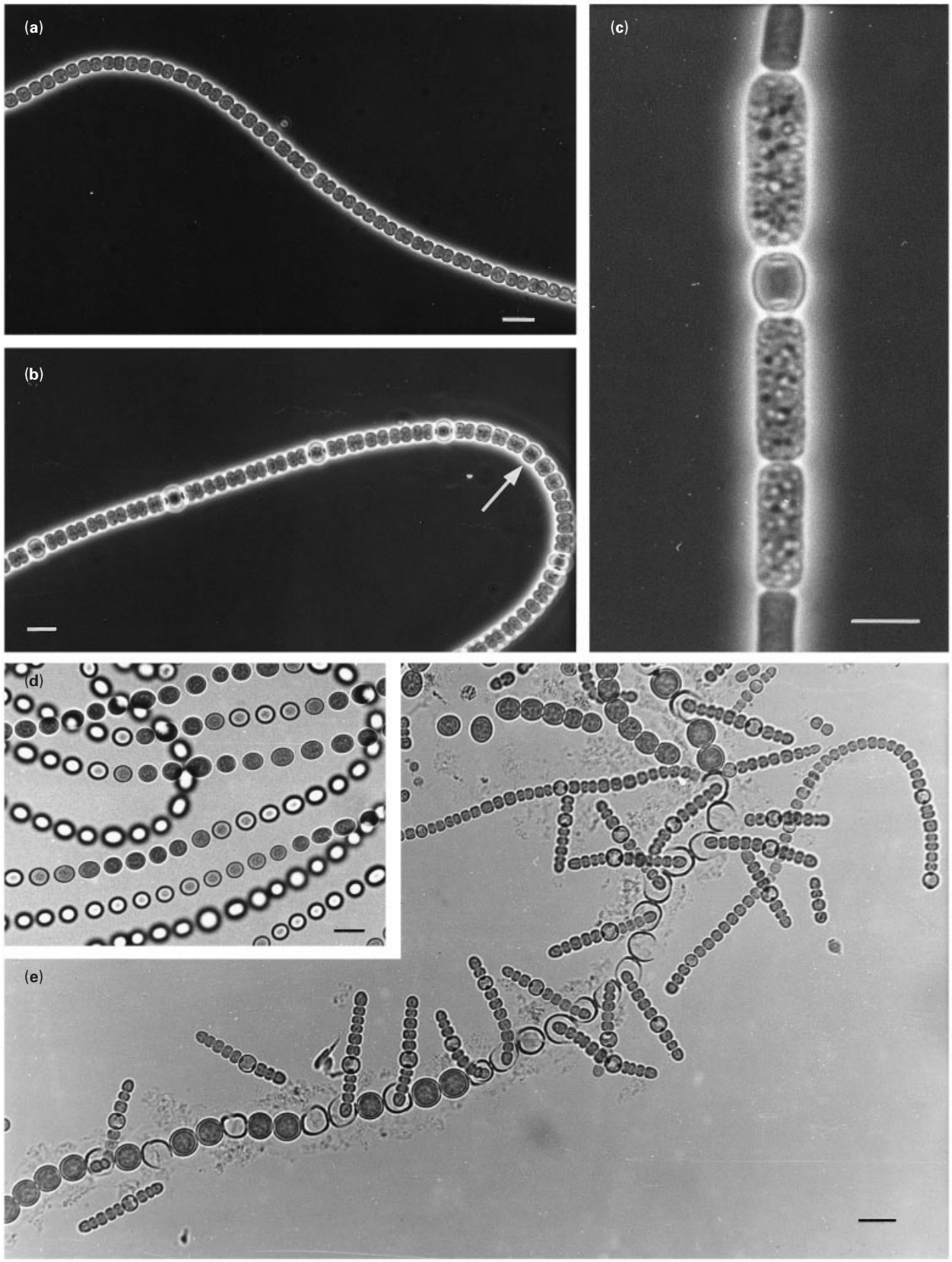

Fig. 3. Photomicrographs illustrating some of the differentiated cell types of cyanobacteria. (a) *Anabaena* sp. strain CA grown in the presence of nitrate, which completely suppresses heterocyst development. (b) *Anabaena* sp. strain CA grown in the absence of combined N, showing the regular spacing of heterocysts, which are the sites of N_2 fixation, and a developing proheterocyst (arrow). (c) *A. cylindrica* showing large, granular akinetes developing immediately adjacent to a heterocyst. The two akinetes below the heterocyst show a characteristic gradient of maturity with the largest and oldest being closer to the heterocyst. (d) An old culture of nitrate-

the day and the latter at night (Gallon, 1992; Fay, 1992; Bergman *et al.*, 1997). However, the filamentous nature of many of the early cyanobacteria permitted the evolution of an alternative strategy, the commitment of a small number of cells to the highly specialized task of providing a suitable environment for N_2 fixation. These cells are known as heterocysts, and are the subject of this review. The ability to form heterocysts probably evolved over 3 billion years ago, yet this was relatively late in the history of filamentous cyanobacteria (Giovannoni *et al.*, 1988). The akinete, a specialized spore-like cell, will also be considered here, because its evolution almost certainly preceded that of the heterocyst – indeed, the akinete may well have been the ancestor of the heterocyst (Wolk *et al.*, 1994; Adams, 1997).

The development and maintenance of heterocysts may involve 600–1000 genes, representing 15–25% of the genome (Lynn *et al.*, 1986). It is only in recent years that some of these heterocyst-specific genes have been isolated and characterized, thanks to advances in the genetic techniques available to analyse heterocyst-forming cyanobacteria. These techniques were developed primarily in *Anabaena* sp. strain PCC 7120, and have recently been adapted for the genetic analysis of symbiotically competent cyanobacteria (Cohen *et al.*, 1994). The genetic tools required for the molecular analysis of cyanobacteria have been reviewed recently (Haselkorn, 1991; Buikema & Haselkorn, 1993; Thiel, 1994; Wolk, 1996; Cohen *et al.*, 1998). More general information on cyanobacteria can be found in several books (Carr & Whitton, 1982; Fay & Van Baalen, 1987; Bryant, 1994; Whitton & Potts, 1999). This review will concentrate on the differentiation and spacing of heterocysts and akinetes, but the following articles contain information on some of the other differentiated cell and filament types of cyanobacteria, including baeocytes (Waterbury & Stanier, 1978; Herdman & Rippka, 1988; Waterbury, 1989), hairs (Whitton, 1987) and hormogonia (Herdman & Rippka, 1988; Tandeau de Marsac & Houmard, 1993; Tandeau de Marsac, 1994).

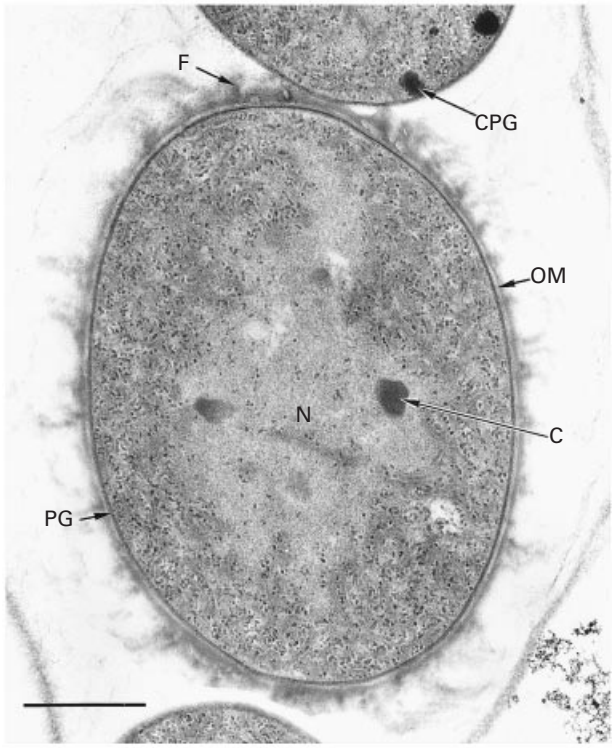

Fig. 4. Electron micrograph of a thin, longitudinal section of a filament of *Nostoc* sp. strain LBG1. The cell shown is a proheterocyst at a very early stage of development, showing little change from the neighbouring vegetative cells other than the deposition of loose, fibrous material (F) external to the normal cell envelope. Abbreviations: N, nucleoplasmic region; C, carboxysome; CPG, cyanophycin granule; PG, peptidoglycan layer; OM, outer membrane. Scale bar represents 1 μm. *Electron microscopy by D. Ashworth. Reproduced, with permission, from Adams (1992a).*

II. THE CYANOBACTERIA

Cyanobacteria are a diverse group of Gram-negative prokaryotes. They vary in diameter from <1 μm to >100 μm, and exhibit a range of morphological diversity rare among the prokaryotes (Fig. 1a,b; Figs 2–7). They form symbiotic associations with a wide range of eukaryotic partners, including plants such as bryophytes (Fig. 1c,d), cycads, the water-fern *Azolla*, and the angiosperm *Gunnera* (Bergman *et al.*, 1992; Bergman *et al.*, 1996; Meeks, 1998; Adams, 1999). Cyanobacteria are photoautotrophs that employ oxygenic photosynthesis of the type found in the chloroplasts of eukaryotic algae and higher plants (Castenholz & Waterbury, 1989; Tandeau de Marsac & Houmard, 1993). This form of photosynthesis, coupled with the organism's size and morphology, resulted in their classification as

grown *Anabaena* sp. strain CA in which all vegetative cells have transformed into spherical akinetes. Although the akinetes have become separated, as a result of pressure created by the coverslip being placed over the sample, the line of the original filaments can still be seen. Dilution of such a culture leads to germination of the akinetes. If the medium used for dilution of the akinetes does not contain a source of combined N, the short filaments that emerge from the akinete coats each contain a heterocyst (e). However, if the medium contains nitrate then the filaments remain undifferentiated. Scale bars represent 10 μm. Panels (a), (b) and (c) produced using phase contrast optics. *Panels* (*a*), (*b*) *and* (*d*) *reproduced, with permission, from Adams* & *Carr* (*1981b*); *panel* (*e*) *reproduced, with permission, from Adams* (*1992a*); *panel* (*c*) *reproduced, with permission, from Nichols* & *Adams* (*1982*).

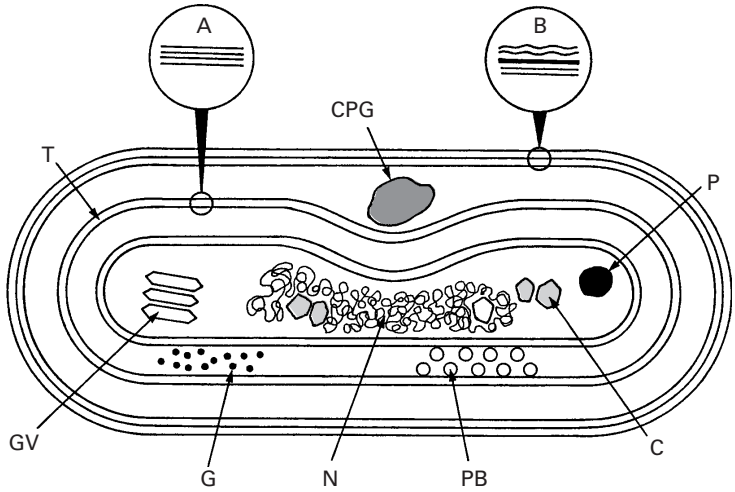

Fig. 5. Schematic diagram of a thin section of a cyanobacterial cell. Abbreviations: C, carboxysome; CPG, cyanophycin granule; T, thylakoid; P, polyphosphate granule; N, nucleoplasmic region; G, glycogen granules; PB, phycobilisome; GV, gas vesicle. Inset (A) is an enlarged view of a thylakoid, showing the paired unit membranes. Inset (B) is an enlarged view of the cell envelope, showing the outer membrane (top), the peptidoglycan layer and the cytoplasmic membrane. *Adapted from Stanier & Cohen-Bazire (1977).*

blue-green algae. However, after their prokaryotic nature was appreciated in the 1970s, the name cyanobacteria was increasingly accepted.

Approx. 50% of strains are facultative photoheterotrophs, capable of using light for energy and organic compounds as a carbon source. Perhaps 20% of these are also capable of chemoheterotrophic growth in the dark, when both energy and C are derived from organic compounds, although under these conditions growth is usually much slower than in the light (Castenholz & Waterbury, 1989; Tandeau de Marsac & Houmard, 1993). A small number of cyanobacteria are also capable of anoxygenic photosynthesis in which H_2S, rather than H_2O, supplies the reducing power (Padan & Cohen, 1982; Schmidt, 1988).

Although the cell wall of cyanobacteria is of the Gram-negative type, the thickness of the peptidoglycan layer, together with its degree of cross-linking and the presence of covalently linked polysaccharide, are more characteristic of Gram-positive cells (Weckesser & Jurgens, 1988). The cell wall consists of two unit membranes, the cytoplasmic membrane and the outer membrane, separated by the electron-opaque peptidoglycan layer, which is usually 1–10 nm thick (Stanier, 1988; Weckesser & Jurgens, 1988; Castenholz & Waterbury, 1989; Figs 5, 6). In cyanobacteria such as *Oscillatoria princeps*, which has cells 100 μm in diameter, the peptidoglycan layer can be 200 nm thick, and is usually traversed by large pores (approx. 70 nm in diameter) that bring the cytoplasmic membrane into contact with the outer membrane (Fig. 6). The walls of all cyanobacteria contain small diameter (5–13 nm) pores, the distribution of which varies greatly. In the longitudinal wall of filamentous strains the pores can be distributed over the whole surface, or in a ring around the cross walls, when they are referred to as junctional pores (Fig. 6). The cell septum may contain a single tiny central pore, or many pores (microplasmodesmata). Many cyanobacteria possess additional layers external to the outer membrane. The most apparent is a sheath, consisting mostly of polysaccharide, although greater than 20% by weight can be polypeptides (Castenholz & Waterbury, 1989). The consistency of this layer varies considerably, explaining its variety of names, such as glycocalyx, sheath, capsule, mucilage and slime. Many sheaths show a fibrillar structure and can be extremely tough, sometimes enclosing more than one cell or filament (Fig. 1a).

The photosynthetic machinery of cyanobacteria is contained on, and within, the thylakoid membranes (Stanier, 1988; Castenholz & Waterbury, 1989). These consist of two unit membranes separated by an electron-transparent space of approx. 3–5 nm, and are usually 60–70 nm from adjacent thylakoids (Fig. 5). In many cyanobacteria the thylakoids are arranged in rows of 3–6 that run parallel both to each other and to the cell wall. However, less-ordered arrangements also occur, and during heterocyst development the thylakoids become more convoluted (Fig. 7). The Chl*a*–protein complexes, the photosynthetic reaction centres, carotenoids and the electron transport system are all contained within the thylakoids, whereas the major light-harvesting pigments, the phycobiliproteins, are found within rows of hemidiscoidal structures, known as phycobilisomes, attached to the surface of the thylakoids (Fig. 5; Bryant, 1991). The phycobiliproteins are largely responsible for the characteristic blue-green, red and brown colours of cyanobacteria.

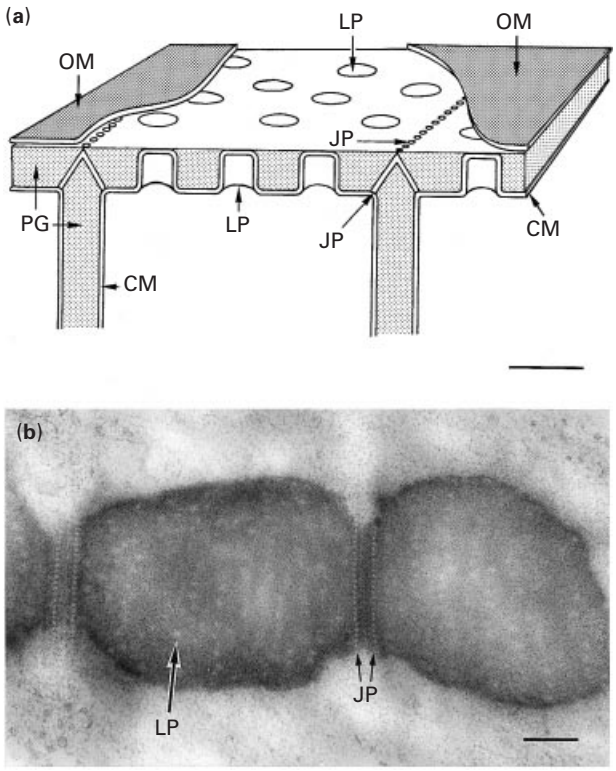

Fig. 6. (a) Diagram of the cell wall of *Oscillatoria* sp. The peptidoglycan layer (PG) is perforated by large pore pits (LP) up to 70 nm in diameter, which bring the cytoplasmic membrane (CM) into contact with the outer membrane (OM). The large pore pits are seen in cross-section in the lower half of the diagram, and appear as circles when viewed from above in the top half of the diagram. Junctional pores (JP) traverse the peptidoglycan between cells, and meet at the surface, appearing as a single row of small circles when viewed from above in the top half of the diagram. Scale bar represents 0.1 μm. *Adapted from Halfen* (*1979*). (b) Electron micrograph of a thin, longitudinal section of an unidentified member of the order Oscillatoriales. The large pore pits can be seen as bright circles scattered throughout the peptidoglycan. The plane of the section is within the peptidoglycan layer, which means that the junctional pores appear as parallel rows of small, closely positioned circles at the cell cross walls, rather than the single rows that would be seen at the surface of the peptidoglycan. Scale bar represents 0.2 μm. *Electron microscopy by Denise Ashworth*.

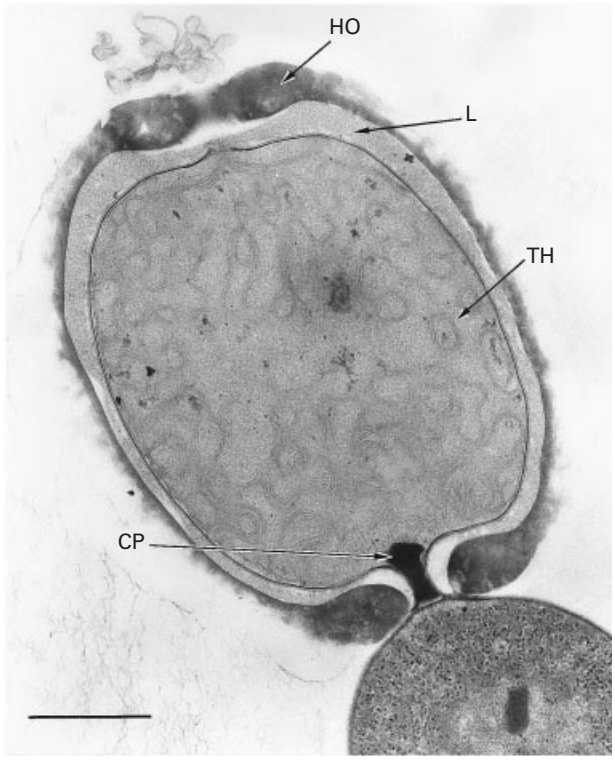

Fig. 7. Electron micrograph of a thin, longitudinal section of a filament of *Nostoc* sp. strain LBG1. The cell is a mature heterocyst showing the presence of the laminated (L) and homogeneous (HO) layers external to the normal cell wall. The junction between the heterocyst and the neighbouring vegetative cell is very narrow and this polar region shows the characteristic deposition of cyanophycin (CP). The relatively agranular nature of the cytoplasm of the heterocyst can be seen when compared with the vegetative cell at the bottom of the picture. Abbreviation: TH, thylakoids. Scale bar represents 1 μm. *Electron microscopy by D. Ashworth. Reproduced, with permission, from Adams* (*1992a*).

III. THE HETEROCYST

1. *Function and metabolism*

The function of the heterocyst is to provide a suitable microaerobic environment for nitrogenase, and to supply combined N to the aerobic vegetative cells in the rest of the filament (Adams & Carr, 1981a; Wolk, 1982; Adams, 1992a, 1997; Wolk *et al*., 1994; Golden & Yoon, 1998). Heterocysts are formed by cyanobacteria of subsections IV (order Nostocales) and V (order Stigonematales), which are all filamentous, although members of the genus *Chlorogloeopsis* (subsection V) can grow as unicells or aseriate clumps (Rippka *et al*., 1979, 1981; Castenholz, 1989a,b; Castenholz & Waterbury, 1989; Bergey's Manual, 1994). In many cyanobacteria, single heterocysts develop at intervals, within (and often at the ends of) the filament of vegetative cells, forming a one-dimensional pattern (Figs 2, 3). The highly differentiated nature of heterocysts makes them reliant on attached vegetative cells for metabolic support (Wolk *et al*., 1994), and this is presumably why no unicellular strains have the capacity for heterocyst development.

The creation of a microaerobic interior to the heterocyst requires a series of major modifications to the original vegetative cell (Wolk *et al*., 1994). It acquires additional cell wall layers (see section III.2) that decrease the diffusion of gases, including oxygen. The photosynthetic generation of oxygen is eliminated by the loss of photosystem II activity (although some of the components of PSII may still be present; Wolk *et al*., 1994). Any remaining traces of oxygen, and any that diffuses into the cell along with N_2, are removed by several mechanisms, the most important of which is probably respiration. Because they lack Rubisco, heterocysts do not fix

Table 1. *Summary of genes required for heterocyst development*

Gene	Homology (H) Possible role of gene product (R)	Induction time (h) following N-stepdown	Mutant phenotype	References
hetR	H: None R: Autoregulatory gene essential for heterocyst formation	2.0	Het$^-$; overexpression or supernumerary copies of *hetR* results in MCH	Buikema & Haselkorn, 1991a,b; Black *et al.*, 1993
hanA	H: *E. coli* DNA-binding protein HU R: Necessary for heterocyst differentiation	ND	Het$^-$, Fox$^-$, resistant to phage A-4(L)	Nagaraja & Haselkorn, 1994; Khudyakov & Wolk, 1996
patS	H: None R: Potentially encodes a 17-amino acid peptide. Diffusible inhibitor of heterocyst differentiation. Essential for the control of heterocyst development and pattern	Threefold increase in expression during 6 h period after N-stepdown	*patS* mutants display an increased frequency of heterocysts and abnormal pattern. Overexpression of *patS* suppresses heterocyst development	Golden & Yoon, 1998; Yoon & Golden, 1998
hetC	H: Bacterial ABC protein exporters	4–5	Het$^-$, although a spaced pattern observed by fluorescence microscopy, suggesting early stages of differentiation have occurred	Khudyakov & Wolk, 1997
hetP	H: None R: Required for normal heterocyst development	4–5	Het$^-$, although a spaced pattern observed by fluorescence microscopy. Extra copies of *hetP* causes MCP	Fernandez-Pinas *et al.*, 1994; Wolk, 1996
patA	H: Response regulator component of two component regulatory systems R: Required for differentiation of intercalary heterocysts. Proposed role in heterocyst pattern formation	3–6	Heterocysts form only at the ends of filaments	Liang *et al.*, 1992
devA	H: ATP-binding component of ABC transporter proteins R: In glycolipid export	6–14	Heterocyst maturation is blocked; mutant fails to synthesize normal envelope glycolipid layer	Maldener *et al.*, 1994
devB	H: Membrane fusion proteins of ABC protein transporters R: In glycolipid export	ND	As above	Fiedler *et al.*, 1998a,b
devC	H: Membrane-spanning proteins of ABC protein transporters R: In glycolipid export	ND	As above	Fiedler *et al.*, 1998a,b

devR	H: Receiver portion of two component regulatory systems R: Essential for normal heterocyst maturation	Constitutive expression with respect to culture N-status	Fox$^-$ mutants unable to complete normal heterocyst development (although heterocyst glycolipids and polysaccharides are synthesized)	Campbell *et al.*, 1996
hepK	H: Sensory protein-histidine kinases of two-component regulatory systems	ND	Induction of *hepA* and synthesis of the heterocyst envelope polysaccharide is blocked	Zhu *et al.*, 1998
hepC	H: None R: In envelope polysaccharide synthesis	ND	Mutation of *hepC* results in constitutive expression of *hepA* and blocks the synthesis of heterocyst envelope polysaccharide	Zhu *et al.*, 1998
hepA	H: ABC transporters R: Necessary for the normal deposition of the heterocyst envelope polysaccharide	4–7	Aberrant polysaccharide layer of the heterocyst envelope	Holland &Wolk, 1990; Wolk *et al.*, 1993
hepB	H: Glycosyl transferases R: Necessary for the normal deposition of the heterocyst envelope polysaccharide	ND	Envelope polysaccharide absent except for short spurs at heterocyst poles	Wolk *et al.*, 1988. (*hepB*, GenBank accession no. ASU68035; Kong & Wolk, unpublished).
patB	H: Transcriptional regulators, such as Fnr	12	Heterocysts develop slowly, but more numerous in older cultures compared to the wild type	Liang *et al.*, 1993
hetN	H: NAD(P)H-dependent oxidoreductases involved in fatty acid biosynthesis R: May be involved in glycolipid production	6–12	Insertions within *hetN* produce MCH phenotype, although these strains are unstable, giving rise to a Het$^-$ phenotype	Ernst *et al.*, 1992; Black & Wolk, 1994; Bauer *et al.*, 1997
hetM (also known as *hglB*)	H: NAD(P)H-dependent oxidoreductases that are involved in fatty acid biosynthesis R: Required for synthesis of heterocyst glycolipids	6–12	Fox$^-$ mutant deficient in heterocyst-specific glycolipids	Ernst *et al.*, 1992; Black & Wolk, 1994; Bauer *et al.*, 1997
hetI	H: NAD(P)H-dependent oxidoreductases involved in fatty acid biosynthesis	ND	Attempts at *hetI* inactivation unsuccessful, suggesting that it is essential for vegetative growth	Black & Wolk, 1994
hglE	H: Polyketide and fatty acid synthases R: Essential for formation of heterocysts	6–10	Fox$^-$ mutant deficient in heterocyst glycolipids	Campbell *et al.*, 1997
hglK	H: None R: Required for transport/assembly/deposition of heterocyst envelope glycolipids	Unclear as degraded mRNA observed	Fox$^-$; lacks normal glycolipid layer, although heterocyst-specific glycolipids are synthesized	Black *et al.*, 1995
ntcA (protein also known as BifA and VF1)	H: Crp family of regulatory proteins R: DNA-binding protein essential for heterocyst differentiation, N_2-fixation, nitrate assimilation. *ntcA* is autoregulatory and positively regulates the activity of N assimilatory genes	Constitutive expression, but peak observed 12 h following N-stepdown	Het$^-$, unable to grow on nitrate or N_2, but can grow on ammonium-containing media	Frias *et al.*, 1993, 1994; Wei *et al.*, 1993, 1994; Ramasubramanian *et al.*, 1994, 1996; Jiang *et al.*, 1997

In row H (homology), 'None' indicates that no homology has been found to any known proteins in the databases.
Abbreviations: MCH, multiple contiguous heterocysts; MCP, multiple contiguous heterocysts; ND, not determined.

CO_2, and must therefore rely on vegetative cells for a supply of fixed C to support N_2 fixation. N_2 is fixed to NH_3, which is assimilated by the glutamine synthetase–glutamate synthase (GS–GOGAT) pathway, to form glutamine and glutamate (Fay, 1992; Gallon, 1992; Wolk *et al.*, 1994), although it is still not clear which nitrogenous compound is exported to the vegetative cells (Wolk *et al.*, 1994).

2. *Heterocyst structure*

(a) *Overview*. Heterocysts have a number of characteristic features readily seen by light microscopy – they are usually larger than vegetative cells, with less granular cytoplasm, thickened cell walls, and refractile polar bodies at the point of contact with adjacent cells (Fig. 3). The cell wall becomes thickened by the deposition of three extra layers external to the normal cell envelope (Wolk *et al.*, 1994). The innermost is the laminated layer, consisting of glycolipid, the next is the homogeneous layer, consisting of polysaccharide, and the outermost is the fibrous layer, which is probably uncompacted strands of the same polysaccharide. Deposition of the fibrous layer is one of the earliest morphological changes visible in electron micrographs (Fig. 4), and is followed by deposition of the homogeneous layer and finally the laminated layer (Fig. 7). These additional layers, particularly the glycolipid, decrease gas diffusion into the heterocyst, and so help to maintain an microaerobic interior (Walsby, 1985; Murry & Wolk, 1989).

The importance of the heterocyst envelope can be seen in the many mutants of cyanobacteria that are unable to fix N_2 aerobically, but can do so anaerobically (Fox^- mutants; Ernst *et al.*, 1992). These mutants frequently have faults in the heterocyst envelope that render it ineffective as a oxygen barrier. The isolation of such Fox^- mutants has permitted the identification of a number of genes involved in the biosynthesis and organization of the heterocyst polysaccharide and glycolipid wall layers (Table 1).

(b) *The polysaccharide* (*homogeneous*) *layer*. Three genes, *hetA*, *hetB* (later renamed *hepA* and *hepB*, respectively; Ernst *et al.*, 1992) and *hepC*, are required for the synthesis or stabilization of the polysaccharide layer (Wolk *et al.*, 1988; Holland & Wolk, 1990; Zhu *et al.*, 1998). HepA resembles ATP-binding transport proteins (Khudyakov & Wolk, 1997). Its transcription is dependent on *hetR* (a gene essential for heterocyst development; see section III.5.(a); Black & Wolk, 1994), and begins at about 5–7 h following the removal of nitrate, several hours before morphological differentiation of heterocysts first becomes apparent by light microscopy (Holland & Wolk, 1990; Wolk *et al.*, 1993). Analysis of other mutants displaying defects in the heterocyst polysaccharide layer (Ernst *et al.*, 1992) has identified additional genes, such as *devT* (I. Maldener *et al.*, unpublished) and *hepK* (Zhu *et al.*, 1998). The *hepK* gene product shows particular similarity to the *E. coli* ArcB protein, which is involved in the repression of genes of aerobic metabolism under anaerobic conditions, and in the activation of specific genes under oxygen-limiting conditions. HepK may therefore play a role in sensing the decreasing oxygen potential within developing heterocysts as they mature (Zhu *et al.*, 1998).

(c) *The glycolipid* (*laminated*) *layer*. The *hglE* gene encodes a multidomain protein involved in the synthesis of heterocyst glycolipids (Campbell *et al.*, 1997). In *Anabaena* sp. strain PCC 7120 the gene *hglK* is thought to encode a protein needed for the transport and/or assembly of heterocyst glycolipids (Black *et al.*, 1995). A cluster of genes, *hglD*, *hglC* and *hglB* (*hglB* is also known as *hetM*; Black & Wolk, 1994), are also involved in the synthesis of heterocyst glycolipids (Bauer *et al.*, 1997). The products of *devA*, and two closely linked genes, *devB* and *devC* (Maldener *et al.*, 1994; Fiedler *et al.*, 1998a,b, 1999), are thought to function as an ABC-transporter involved in the export of heterocyst-specific glycolipids because, although the heterocyst envelopes of *devABC* mutants lack the laminated glycolipid layer, the cells do contain heterocyst-specific glycolipids (Fiedler *et al.*, 1998a,b, 1999).

(d) *The septum and microplasmodesmata*. As heterocyst development progresses, the septum separating it from the adjacent vegetative cell decreases considerably in diameter, until it becomes a narrow pore channel (Fig. 7). The number of plasma bridges (microplasmodesmata) traversing the septum also decreases three- to fivefold (Fay, 1992). These plasma bridges are about 8 nm in diameter and presumably represent the route for the intercellular transport of metabolites, although not for gas diffusion, because the permeability of the pore region to gases is considerably less than that of the heterocyst wall (Walsby, 1985). It is clear that intercellular communication has a critical role in the placing of heterocysts within each filament (see section III.10) and its importance has been demonstrated by introducing, into cells of *Anabaena* sp. strain PCC 7120, a replicating plasmid carrying the gene encoding the tobacco mosaic virus movement protein (Zahalak *et al.*, 1995). The movement protein, which can alter the structure and function of the microplasmodesmata in plants, prevents heterocyst differentiation.

3. *Nitrogen regulation and heterocyst development*

Heterocystous cyanobacteria express the genes for heterocyst development and N_2 fixation only when a suitable source of combined N becomes limited.

In the presence of ammonium they are unable to assimilate alternative sources of N, such as nitrate, N_2, or ammonium itself. This is due to the ammonium-induced repression of the systems for nitrate and ammonium transport and assimilation, N_2 fixation and heterocyst development (Flores & Herrero, 1994; Flores *et al*., 1999). Full expression of these systems requires the DNA-binding protein NtcA (Vega-Palas *et al*., 1990, 1992; Luque *et al*., 1994), encoded by the *ntcA* gene, which is widespread among cyanobacteria including heterocystous strains (Frías *et al*., 1993; Wei *et al*., 1993). NtcA belongs to a family of bacterial regulatory proteins typified by the cAMP receptor protein, Crp (Vega-Palas *et al*., 1992; Wei *et al*., 1993). An *ntcA* mutant of *Anabaena* sp. strain PCC 7120 requires ammonium for growth and is unable to induce *hetR*, develop heterocysts, or synthesize nitrogenase and enzymes of the nitrate reduction system (Frías *et al*., 1994; Wei *et al*., 1994).

NtcA from *Anabaena* sp. strain PCC 7120 interacts *in vitro* with promoter regions of *xisA* (which encodes an excisase; see section III.4.(e)), *glnA* (which encodes glutamine synthetase), *rbcLS* (which encodes Rubisco), *nirA* (which encodes nitrite reductase) and *nifH* (which encodes dinitrogenase reductase) (Frías *et al*., 1993; Ramasubramanian *et al*., 1994), and also binds upstream of the *ntcA* gene, implying autoregulation (Ramasubramanian *et al*., 1996). NtcA also binds *in vitro* to the promoter region of *gor*, a gene encoding glutathione reductase (Jiang *et al*., 1997). This interaction is regulated *in vitro* by a redox-dependent mechanism, implying that NtcA responds not only to the external N status but also to cellular redox changes, and this may be of particular importance during heterocyst differentiation as the heterocyst develops a microaerobic interior for the protection of nitrogenase (Jiang *et al*., 1997).

Ammonium assimilation in cyanobacteria occurs largely via the GS–GOGAT pathway (Flores & Herrero, 1994; Flores *et al*., 1999). In enteric bacteria, the synthesis and activity of GS is controlled by the two-component Ntr (nitrogen regulation) system in which the *glnB* gene product, the P_{II} protein, transmits the N status of the cell to the Ntr proteins (Merrick & Edwards, 1995). P_{II}-like proteins have been characterized from both unicellular and filamentous cyanobacteria (Tsinoremas *et al*., 1991; Liotenberg *et al*., 1996; García-Domínguez & Florencio, 1997; Hanson *et al*., 1998), and seem to be modified by phosphorylation rather than the uridylylation found in enteric bacteria (Forchhammer & Tandeau de Marsac, 1994, 1995; García-Domínguez & Florencio, 1997; Zhang & Libs, 1998). Moreover, the phosphorylation state of P_{II} appears to be governed by the N and C status of the cell, implying that P_{II} is involved in balancing C and N metabolism (Forchammer & Tandeau de Marsac, 1995; Liotenberg *et al*., 1996; Forchammer & Hedler, 1997). The *glnB* gene was recently cloned from the heterocystous cyanobacterium *Nostoc punctiforme* strain ATCC 29133, but could not be insertionally inactivated, implying that it is essential for growth (Hanson *et al*., 1998). The purified *N. punctiforme* P_{II} protein was phosphorylated *in vitro* by a *Synechococcus* PCC 7942 P_{II} kinase, although no modified forms of the P_{II} protein were detected *in vivo*.

Nitrate and nitrite assimilation in cyanobacteria involves active uptake, facilitated by a membrane-transporter complex encoded by the gene cluster *nrtABCD* (Cai & Wolk, 1997a,b; Frías *et al*., 1997). Intracellular nitrate is reduced to nitrite by nitrate reductase, and the resulting nitrite is reduced to ammonium by the action of nitrite reductase (Flores & Herrero, 1994; Flores *et al*., 1999). The genes encoding nitrite and nitrate reductases, *nirA* and *narB*, respectively, are transcriptionally activated within 30 min of the removal of ammonium from the growth medium (Cai & Wolk, 1997b). Mutants bearing a transposon within *nirA* and *nrtC*, and between *nrtD* and *narB*, retain the ability to differentiate heterocysts and fix N_2, indicating that they do not block the induction of *hetR*. Similarly, a *hetR* mutation does not prevent the induction of genes required for nitrite and nitrate assimilation (Cai & Wolk, 1997a).

Mutations affecting N uptake and metabolism result in heterocyst development on nitrate-containing media (Bagchi & Singh, 1984; Martin-Nieto *et al*., 1991). Similarly, inactivation of the *Anabaena* PCC 7120 *moeA* gene, which is required for the synthesis of the molybdenum-containing cofactor molybdopterin, results in a loss of nitrate reductase activity and a consequent production of heterocysts in the presence of nitrate (Ramaswamy *et al*., 1996).

4. *Heterocyst development*

(a) *The proheterocyst*. The development of a heterocyst represents a considerable metabolic expense and a loss of division capacity (heterocysts are unable to divide), making it essential that only the required number of vegetative cells are committed to differentiation. This requires flexibility in the developmental process, and this is provided by an intermediate stage, the proheterocyst, which can dedifferentiate under the appropriate conditions. Although clearly differentiated from the vegetative cell, the proheterocyst lacks the fully thickened walls and the polar bodies typical of the mature heterocyst (compare Figs 4 and 7). This flexibility in the differentiation process is also essential in forming the pattern of heterocysts seen in many cyanobacteria (Figs 2, 3 and sections III.9 and III.10).

Heterocyst development in cyanobacteria such as *Anabaena* and *Nostoc* is repressed in the presence of ammonium or nitrate (Figs 2, 3). Following the

transfer of filaments to medium free of combined N, the first visible sign of differentiation is the development of proheterocysts, beginning at approx. 6–8 h (in strains with doubling times of 15–20 h) and reaching a peak of 5–10% of total cells by 12–15 h. Proheterocysts then begin to mature and acquire the thickened walls and polar bodies of heterocysts, which consequently increase in frequency to a maximum at 25–30 h, while the proheterocyst frequency declines to a relatively constant 1–2%. The development of a mature heterocyst therefore takes approximately one cell cycle.

(b) *Proteolysis associated with heterocyst development*. Heterocyst differentiation involves the temporal synthesis and degradation of a large number of proteins (Fleming & Haselkorn, 1973, 1974). In cultures such as those transferred from medium containing combined N to medium lacking combined N, the stimulus to develop heterocysts is clearly N starvation. Considerable new protein synthesis is required for each heterocyst and the N for this must come from internal reserves. These reserves consist of the major light-harvesting pigments phycocyanin and phycoerythrin, constituting up to 50% of total cell protein (Glazer, 1987; Tandeau de Marsac & Houmard, 1993), and cyanophycin, a copolymer of arginine and aspartic acid that is unique to cyanobacteria (Simon, 1987). During periods of N_2 starvation in *Anabaena* spp., phycocyanin is degraded by a specific protease, phycocyaninase (Foulds & Carr, 1977; Wood & Haselkorn, 1980; Thiel, 1990), and phycocyanin gene expression is switched off, probably as a result of transcriptional regulation (Johnson *et al*., 1988; Wealand *et al*., 1989). The enzymes for cyanophycin synthesis and breakdown are more active in *Anabaena* heterocysts than in vegetative cells (Gupta & Carr, 1981), prompting the speculation that cyanophycin may serve as a dynamic reservoir of newly assimilated N that can be used for biosynthesis (Carr, 1988). In addition, a calcium-requiring serine protease, capable of degrading many vegetative cell proteins, has been reported in *Anabaena* spp. (Wood & Haselkorn, 1979; Lockau *et al*., 1988), although it is not essential for heterocyst development (Maldener *et al*., 1991; Tandeau de Marsac & Houmard, 1993).

The importance for heterocyst development of these different forms of proteolysis is unclear. It is possible that the non-specific Ca^{2+}-stimulated protease(s) induced during the early stages is involved more in the removal of vegetative proteins, rather than the provision of amino acids for *de novo* protein synthesis, because the products of this early proteolysis are mostly released from the cells (Thiel, 1990). The specific degradation of phycocyanin which occurs rather later during heterocyst development may be a more likely source of amino acids for new proteins. In actively growing, N_2-fixing cultures, new heterocysts develop midway between existing ones, so maintaining the pattern. In this situation, in which N starvation of the culture as a whole does not occur, it is not yet clear if localized depletion of intracellular combined N, or some other trigger, provides the stimulus for heterocyst development.

(c) *RNA polymerase sigma factors*. Cell differentiation in prokaryotes such as *Myxococcus xanthus* (Apelian & Inouye, 1990), *Bacillus subtilis* (Stragier & Losick, 1990) and *Streptomyces coelicolor* (Chater, 1989) involves the modification of RNA polymerase activity by alternative sigma factors. However, no developmentally specific sigma factors have been identified in cyanobacteria (Golden & Yoon, 1998). In *Anabaena* PCC 7120 the *sigA* gene encodes the principal vegetative cell sigma factor transcribed during growth under both N-replete and N_2-fixing conditions (Brahamsha & Haselkorn, 1991; Ramasubramanian *et al*., 1995). Two additional sigma factor genes, *sigB* and *sigC*, in *Anabaena* sp. strain PCC 7120 are not expressed under N-replete growth conditions, although both are expressed transiently following the removal of combined N from the growth medium (Brahamsha & Haselkorn, 1992). Neither gene alone is essential for heterocyst differentiation or N_2 fixation, and *sigB* and *sigC* double mutants are capable of heterocyst differentiation and growth on N_2. Three additional sigma factor genes, *sigD*, *sigE* and *sigF*, are also not essential for heterocyst development (Khudyakov & Golden, unpublished). The alternative sigma factor, SigH, is present in the symbiotically competent cyanobacterium *N. punctiforme*, but mutations in the *sigH* gene have no effect on heterocyst development, although they do increase the efficiency of infection of the host plant, the bryophyte *Anthoceros punctatus* (Campbell *et al*., 1998).

(d) *Developmental regulation of heterocyst cell wall and nitrogenase gene expression*. Formation of the thickened heterocyst cell wall can be inhibited by inducing heterocyst differentiation under anaerobic conditions, using an argon atmosphere and adding DCMU to inhibit the photosynthetic production of oxygen (Rippka & Stanier, 1978). Even under these conditions the Mo–Fe protein of nitrogenase is not found in vegetative cells, but is localized to the incompletely formed heterocysts (Murry *et al*., 1984), and the nitrogenase activity is highly sensitive to oxygen, because the heterocysts lack the thickened cell walls required to exclude the gas (Rippka & Stanier, 1978). N starvation and anaerobiosis, therefore, do not induce nitrogenase synthesis in vegetative cells, implying that the expression of *nif* genes in heterocystous cyanobacteria has both environmental and developmental components, re-

quiring N starvation, anaerobiosis and completion (or at least partial completion) of heterocyst development.

This developmental control of *nif* gene expression has been elegantly demonstrated by Elhai and Wolk (1990) using the *luxAB* genes, encoding bacterial luciferase, as transcriptional reporters. Bacterial luciferase catalyses the oxidation of an aldehyde (such as *n*-decanal) and $FMNH_2$, with the production of light. When promoterless *luxAB* genes are placed downstream from a strong cyanobacterial promoter, such as that for the Rubisco large and small subunits (P_{rbcLS}), their expression can be driven from the heterologous promoter (Elhai & Wolk, 1990). Expression of the cyanobacterial gene can therefore be followed by the emission of light, enabling the expression of specific genes in a single cell to be detected. In this way, Elhai and Wolk (1990) were able to provide visual confirmation of the existing biochemical data, showing that in *Anabaena* PCC 7120 *glnA* is expressed in both heterocysts and vegetative cells, rbc_{LS} in vegetative cells only, and the nitrogenase structural genes, *nifHDK*, in heterocysts only. In the mutant strain *Anabaena* PCC 7118, which fails to produce heterocysts but does fix N_2 under anaerobic conditions, expression of P_{nifHDK}*luxAB* is found only in morphologically distinct cells that occur in a spaced pattern and probably represent partially developed heterocysts (Elhai & Wolk, 1990). This confirms that expression of *nifHDK* is under developmental, and not purely environmental, control.

In *Anabaena variabilis* ATCC 29413 there are three clusters of genes that control N_2 fixation, *nif*1, *nif*2 and *vnf* (Schrautemeier *et al*., 1995; Thiel *et al*., 1999). The first of these is the homologue of the *nif* cluster in *Anabaena* PCC 7120, and is similarly interrupted by an 11 kb excision element, but lacks the 55 kb element (see section III.4.(e)). The *nif*1 cluster is only expressed in heterocysts, even under strictly anaerobic conditions (Thiel *et al*., 1995). However, the *nif*2 cluster, which lacks both the 11-kb and 55-kb elements, is expressed in vegetative cells, but only under anaerobic conditions, and in heterocysts (Thiel *et al*., 1995, 1997). The *vnf* cluster codes for an alternative nitrogenase system containing a vanadium cofactor, instead of the usual molybdenum cofactor, and is expressed in the absence of molybdenum, but only in heterocysts (Thiel, 1993, 1996).

(e) *Genome rearrangements associated with heterocyst development*. During the late stages of heterocyst differentiation in *Anabaena* PCC 7120 three developmentally regulated DNA rearrangements occur in the genes *nifD* (encoding the α-subunit of dinitrogenase), *fdxN* (encoding a bacterial-type ferredoxin) and *hupL* (encoding the large subunit of an uptake hydrogenase) (Haselkorn, 1992; Tandeau de Marsac & Houmard, 1993; Apte & Prabhavathi, 1994; Matveyev *et al*., 1994; Apte & Nareshkumar, 1996; Böhme, 1998; Golden & Yoon, 1998). These rearrangements involve the excision of an 11-kb element from *nifD* (Golden *et al*., 1988), a 55-kb element from *fdxN* (Golden *et al*., 1988), and a 10.5-kb element from *hupL* (Matveyev *et al*., 1994; Carrasco *et al*., 1995). These excisions are brought about by three excisases encoded by genes carried on the respective elements: *xisA* on the 11-kb element (Lammers *et al*., 1986); *xisF* on the 55-kb element (Carrasco *et al*., 1994); and *xisC* on the 10.5-kb element (Carrasco *et al*., 1995). They create the three functional operons *nifHDK*, *nifB-fdxN-nifS-nifU*, and *hupSL*, and leave the three elements as circular DNA molecules with no known function. The rearrangement of the *fdxN* element is now known also to require two overlapping genes, *xisH* and *xisI*, located downstream of *xisF* (Ramaswamy *et al*., 1997). These genes show no sequence similarity to any in the databases.

There is evidence that, although these excisions are required for the restoration of function to the respective operons, they have no role in heterocyst development or spacing. For example, insertional inactivation of *xisA*, or deletion of the 11-kb element, prevents *nifK* expression (from the *nifH* promoter), but has no effect on heterocyst development or spacing (Tandeau de Marsac & Houmard, 1993). Indeed, the *nifHDK* genes are contiguous in some heterocystous cyanobacteria, including some *Nostoc* strains (Meeks *et al*., 1994) and *Fischerella* ATCC 27929, as they are in all non-heterocystous strains (Tandeau de Marsac & Houmard, 1993). Inactivation of the *xisF* gene prevents N_2 fixation, but has no effect on heterocyst differentiation and pattern (Carrasco *et al*., 1994). Indeed, both the 11-kb (Oxelfelt *et al*., 1998; Böhme, 1998) and the 55-kb (Haselkorn, 1992; Thiel *et al*., 1999) elements are absent from the genomes of a number of *Anabaena* and *Nostoc* strains.

5. *Genes essential for heterocyst development*

(a) *hetR*. Of prime importance in the regulation of heterocyst development is the gene *hetR*, which was first identified by complementation of a heterocyst-deficient mutant of *Anabaena* PCC 7120 (Buikema & Haselkorn, 1991a,b; Wolk *et al*., 1994, 1999; Golden & Yoon, 1998). The presence of wild-type *hetR* in multiple copies stimulates the formation of heterocysts in the presence of nitrate, and the formation of clusters of two to five heterocysts in its absence (Buikema & Haselkorn, 1991b). In the presence of combined N, *hetR* is transcribed at a low level (Buikema & Haselkorn, 1991b; Black *et al*., 1993). Following removal of the combined N source, induction of *hetR* begins within 2 h, and by 3.5 h its transcription is strongly enhanced within spatially

separated cells, which are presumably the developing heterocysts (Black *et al*., 1993). This pattern of *hetR* induction has recently been confirmed at the protein level by immunoblotting (Zhou *et al*., 1998a). Expression of *hetR* requires a functional HetR protein, implying that the gene is positively autoregulatory (Black *et al*., 1993). HetR has no apparent DNA binding motifs and no similarities to any previously characterized protein have been found (Buikema & Haselkorn, 1991b). However, recent evidence implies that HetR acts as an unusual serine-type protease (possibly requiring Ca^{2+} *in vivo*), possibly degrading repressors of genes to be switched on and activators of genes to be switched off during heterocyst differentiation (Zhou *et al*., 1998b, 1999).

(b) *Protein phosphorylation and the regulation of* hetR *activity*. Increasing evidence implies that phosphorylation-based network systems are involved in the regulation of several aspects of cyanobacterial physiology and metabolism (Mann, 1994; McCartney *et al*., 1997), although it is not yet clear how important such systems are for heterocyst development. Certainly, some of the genes required for heterocyst development encode proteins that resemble members of bacterial two-component regulatory systems (Liang *et al*., 1992; Campbell *et al*., 1996; Zhu *et al*., 1998), and differences in the isoelectric point of native and recombinant HetR (expressed in *E. coli*) may imply that the protein is modified, possibly by phosphorylation (Zhou *et al*., 1998a). In recent years a family of genes encoding proteins similar to eukaryotic-type protein kinases have been cloned from *Anabaena* sp. strain PCC 7120 (Zhang, 1993, 1996; Zhang & Libs, 1998; Zhang *et al*., 1998). Deletion of one of these genes, *pknA*, leads to a pleiotropic phenotype that includes a decreased frequency of heterocysts under N_2-fixing conditions (Zhang, 1993). Another member of this multigene family, *pknD*, is required for growth under N_2-fixing conditions, but is not essential for heterocyst development (Zhang & Libs, 1998).

Phosphoprotein phosphatases, which catalyse protein dephosphorylation, have also been identified in cyanobacteria, including *Nostoc commune* UTEX584, which possesses a dual-specificity protein serine/threonine-tyrosine phosphatase (IphP; Potts *et al*., 1993). More recently, protein tyrosine phosphatase (and protein tyrosine kinase) activities have been identified in *Anabaena* sp. strain PCC 7120 (McCartney *et al*., 1997), and a gene, *prpA*, encoding a serine/threonine phosphatase, has been cloned from the same organism (Zhang *et al*., 1998). A eukaryotic-type protein kinase gene, *pknE*, is found downstream of *prpA*, and inactivation of these two genes results in mutants capable of normal growth on ammonium or nitrate, but with severely impaired growth under N_2-fixing conditions (Zhang *et al*., 1998). Both mutants display heterocysts with aberrant structures and a low level of nitrogenase activity, implying that the activity of both PrpA and PknE is necessary to maintain the correct level of protein phosphorylation in order to control processes such as heterocyst structural development and N_2 fixation (Zhang *et al*., 1998).

(c) *hetR in nonheterocystous cyanobacteria*. Regions of genomic DNA hybridizing to *hetR* have been found in nonheterocystous N_2-fixing cyanobacteria (Buikema & Haselkorn, 1991a; Janson *et al*., 1998). The *hetR* gene has been cloned and sequenced from several species of the filamentous cyanobacteria *Trichodesmium* and *Symploca* PCC 8002 (Janson *et al*., 1998), which has the remarkable ability to fix N_2 during daylight, concurrent with oxygenic photosynthesis (Bergman *et al*., 1997; Zehr *et al*., 1999). The protection conferred to nitrogenase within the apparently undifferentiated filaments has been given much attention (Fay, 1992; Gallon, 1992; Bergman *et al*., 1997; Capone *et al*., 1997; Zehr *et al*., 1999). Nitrogenase is restricted to a limited number of cells within colonies of *Trichodesmium* spp., implying a spatial separation between photosynthesis and N_2 fixation (Janson *et al*., 1994; Fredriksson & Bergman, 1995, 1997; Lin *et al*., 1998). Similar observations have recently been made with *Symploca* sp. strain PCC 8002, in which N_2 fixation occurs in the light period of a light–dark cycle, and nitrogenase protein is localized to 9% of cells, which, unlike *Trichodesmium*, contain Rubisco and presumably fix CO_2 (Fredriksson *et al*., 1998). Whether *hetR* has a role in the patterned distribution of the N_2-fixing cells of *Trichodesmium* or in *Symploca* remains to be established. However, this is clearly not the case in *Spirulina platensis*, a nonheterocystous filamentous cyanobacterium that does not fix N_2, but which was recently shown to contain a protein that cross-reacts with anti-HetR antibodies (Zhou *et al*., 1998a).

(d) *Other heterocyst-specific genes* (Table 1; Wolk *et al*., 1999). In addition to the interruption of the *hetR* gene, various other mutations lead to a Het^- phenotype. For example, the gene *hanA* is essential for the initiation of heterocyst differentiation (Khudyakov & Wolk, 1996). Interruption of the gene produces a Het^- mutant that is resistant to cyanophage A-4(L). The gene encodes a protein, related to the *E. coli* DNA-binding protein HU, which is found only in vegetative cells, and which is degraded during heterocyst differentiation to be replaced by a new DNA-binding protein of slightly greater molecular mass (Nagaraja & Haselkorn, 1994). HU protein may be involved in the transcriptional regulation of *hetR*, and the Het^- phenotype of *hanA* mutants probably results from impaired expression of the *hetR* gene (Khudyakov & Wolk, 1996).

Strain N10, a transposon-induced mutant of

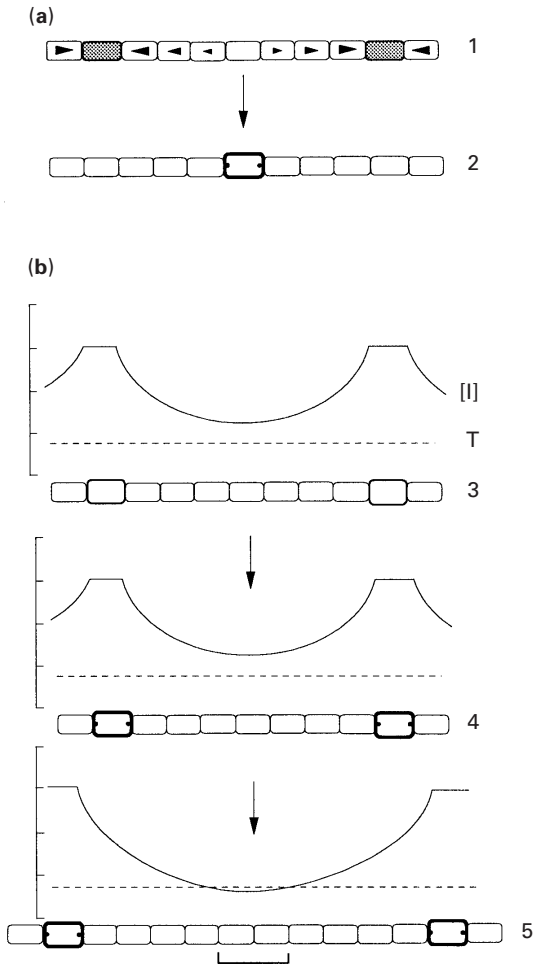

Fig. 8. Diagrammatic representation of two alternative models (Wolk, 1989) to explain the development of a spaced pattern of heterocysts in a filamentous cyanobacterium, following the removal of combined N from the medium. In the first model (a) the first cells to respond to N starvation (the two shaded cells in filament 1) activate amino acid pumps that draw the products of proteolysis (which is associated with heterocyst development) from adjacent cells, which in turn activate pumps and drain their neighbours of combined N. In this way pump activation is propagated along the filament, but the magnitude of the pumps decreases with distance from the initial cell. The arrowheads in each cell indicate the direction of flow of fixed N and their size indicates the magnitude of the pump drawing combined N from that cell. In this model the cells that will differentiate are not the ones that first respond to N deprivation (the shaded cells in filament 1), but are those that are being pumped from both directions (the middle cell of the interval in filament 1) and are therefore the most severely N starved. In the second model (b) the first cells to respond to N deprivation (the two slightly larger cells in filament 3) arise at semi-random locations (see Fig. 10) and produce an inhibitor of heterocyst development [I] that diffuses along the filament. The inhibitor is degraded, or lost, as it diffuses, establishing a decreasing concentration gradient, indicated by the solid line above the filament. The horizontal dashed line (T) represents a threshold intracellular concentration of inhibitor, below which a cell can differentiate and above which it cannot. The production of inhibitor prevents neighbouring cells from differentiating, and the first cells to respond to N starvation therefore develop into heterocysts (filament 4). Cell division will eventually widen the number of vegetative cells between the heterocysts and result in cells (in this case the two indicated by the square bracket) at the centre of the interval falling below threshold and becoming candidates for differentiation (filament 5). The scales to the left of each panel are for reference only. A candidate for the hypothetical inhibitor, I, has recently been identified as the peptide PatS (section III.8). *Adapted from Adams (1992a).*

Anabaena PCC 7120 defective in the *hetN* gene, has a Het$^-$ phenotype (Ernst *et al.*, 1992; Black & Wolk, 1994). Sequence analysis of DNA contiguous with *hetN* revealed two further genes, *hetI* and *hetM* (Black & Wolk, 1994). Most insertions within *hetN* lead to the production of multiple contiguous heterocysts, whereas multiple copies of the gene repress heterocyst formation (Black & Wolk, 1994; Bauer *et al.*, 1997), despite the fact that *hetN* is not transcribed until at least 6 h following the removal of combined N from the growth medium (Bauer *et al.*, 1997). The proteins encoded by *hetN*, *hetM* and *hetI* resemble NAD(P)H-dependent oxidoreductases involved in the biosynthesis of fatty acids, *nod* factor, poly-β-hydroxybutyrate and polyketides.

Two further genes, *hetP* (Ferández-Piñas *et al.*, 1994) and *hetC* (Khudyakov & Wolk, 1997), are important for heterocyst development. Mutants deficient in each of these genes fail to produce proheterocysts, although a pattern of spaced, weakly fluorescent cells is evident 2 d following the removal of combined N, implying that differentiation is arrested at a very early stage (Khudyakov & Wolk, 1997; Wolk, 1996). As with *hetR*, multiple copies of *hetP* result in the production of multiple heterocysts, although unlike *hetR*, the gene is not autoregulatory, and supernumerary copies of *hetP* do not induce heterocyst formation in the presence of combined N (Fernández-Piñas *et al.*, 1994).

The *hetP* gene has no known function and no similarities to existing proteins in the databases (Fernández-Piñas *et al.*, 1994), whereas the protein encoded by *hetC* has extensive similarity to members of a class of bacterial ATP-binding cassette (ABC) membrane transport proteins involved in the export of pore-forming toxic polypeptides (Khudyakov & Wolk, 1997). The gene encoding the ABC transporter is usually linked to the gene encoding the protein that is exported, prompting the suggestion that HetC may export HetP (Wolk, 1996). Transcription of *hetC* requires both HetR and a functional HetC protein, implying that *hetC*, like *hetR* (Black *et al.*, 1993), is autoregulatory (Khudyakov & Wolk, 1997). The role of *hetC* remains unclear, although it is essential at an early stage of differentiation, implying that it must regulate the expression of later-acting, heterocyst-specific genes. Localized transcription of *hetR* is visible by 3.5 h following N deprivation (Black *et al.*, 1993), whereas induction of *hetC* and *hetP* does not occur before 4 h, making it

unlikely that the products of *hetC* and *hetP* are involved in the development of a spaced pattern of heterocysts.

Mutation of the *devR* (developmental regulation) gene in the symbiotic cyanobacterium *Nostoc* sp. strain ATCC 29133, results in a Fox^- phenotype (Campbell *et al.*, 1996). The mutant differentiates heterocysts with a defective heterocyst envelope, despite being able to synthesize heterocyst glycolipids and polysaccharides. The sequence of DevR is most like that of the single-domain response regulators such as Spo0F (Grossman, 1991) and CheY (Parkinson, 1993), which lack a DNA binding domain and interact with another protein, rather than activate transcription (Parkinson & Kofoid, 1992). DevR may therefore interact with another protein, either as part of a phosphorelay system similar to that of Spo0F, or in the activation of an enzyme that is involved in the organization or assembly of the heterocyst outer envelope. It seems unlikely that DevR is involved in signalling the N status within cells, because *devR* mutants have a Het^+ Fix^+ phenotype repressed by combined N, similar to the wild-type strain (Campbell *et al.*, 1996).

6. *Heterocyst spacing*

(a) *Patterns of heterocyst differentiation.* The necessary interchange of nutrients between heterocysts and vegetative cells dictates that the former develop in positions that ensure the most efficient production and distribution of fixed N to the latter. This is reflected in the characteristic locations of heterocysts in different cyanobacteria (Rippka *et al.*, 1979, 1981; Castenholz & Waterbury, 1989). For example, in the genera *Nostoc* and *Anabaena*, heterocysts occur mostly at spaced locations within the filament (at intercalary positions; Figs 2, 3), whereas in *Cylindrospermum* they are found only terminally. As cell division increases the number of vegetative cells between heterocysts (the interheterocyst interval), the regular spacing and frequency is maintained by the differentiation of a new heterocyst at the centre of each interval once the number of vegetative cells in that interval has approximately doubled (Fig. 8). The existing pattern of heterocysts therefore regulates the position of newly developing cells. However, a culture grown in the presence of combined N, and therefore lacking any heterocysts, will develop a regular heterocyst pattern *de novo* following transfer to medium free of combined N (see sections III.9 and III.10).

(b) *Genes involved in heterocyst spacing.* Table 1 contains a summary of genes involved in heterocyst spacing. Although mutants displaying altered heterocyst patterns have been available for many years, they have so far contributed little to our understanding of the differentiation process (Wolk *et al.*, 1994). Only two genes, *patA* and *patB*, have been implicated in the regulation of pattern formation. Mutants in *patA* grow slowly in the absence of combined N, and develop only terminal heterocysts (Liang *et al.*, 1992). The *patA* mutation also suppresses the multiple heterocyst phenotype produced by extra copies of the wild-type *hetR* gene, suggesting that the PatA and HetR proteins are components of the same regulatory circuit. The C-terminal domain of the predicted PatA protein strongly resembles that of CheY and other response regulators in bacterial two-component signal-transduction systems (Liang *et al.*, 1992; Buikema & Haselkorn, 1993). PatA does not contain a known DNA-binding motif, and may therefore interact directly with another protein. Although the *patA* gene is not necessary for vegetative cell growth, it is transcribed at a low level under N-replete conditions, and this increases between 3 and 6 h following removal of combined N (Liang *et al.*, 1992). This pattern of expression is very similar to that of *hetR* (although the *patA* transcript is much less abundant than that of *hetR*), and supports the argument that PatA and HetR are components of an environment-sensing regulatory system (Liang *et al.*, 1992).

patB mutants grow rapidly in the presence of combined N, but slowly under N_2-fixing conditions, and heterocyst development is delayed, although in older cultures the heterocysts are spaced more closely than in the wild-type strain (Liang *et al.*, 1993). PatB resembles a group of transcription regulators that contain an iron-binding domain near their N-termini and a DNA-binding site near their C-termini. Members of this group include the Fnr protein of *E. coli* and FixK of *Bradyrhizobium japonicum* (Liang *et al.*, 1993). PatB may therefore act as a sensor of redox potential, or of the concentration of iron within the cell, and regulate the transcription of heterocyst-specific genes. There is no detectable expression of *patB* in cells grown in the presence of combined N, but transcription begins from 3–6 h following its removal.

(c) *Disruption of heterocyst pattern.* The double (adjacent) heterocysts produced in *Anabaena cylindrica* following exposure to high intensities of illumination (see section III.9.(a)) do not, strictly speaking, represent an alteration in heterocyst pattern, because the number of vegetative cells between heterocysts (considering each double heterocyst as a single unit) is little changed, implying that the inhibitory zones around heterocysts are not affected (Adams, 1992a). However, the frequency of heterocysts and the regularity of their spacing can be altered experimentally. In some mutants, highly irregular patterns of heterocysts can be seen, with as many as six to eight adjacent to each other (Wilcox *et al.*, 1975a; Adams, 1992a; Wolk *et al.*, 1994).

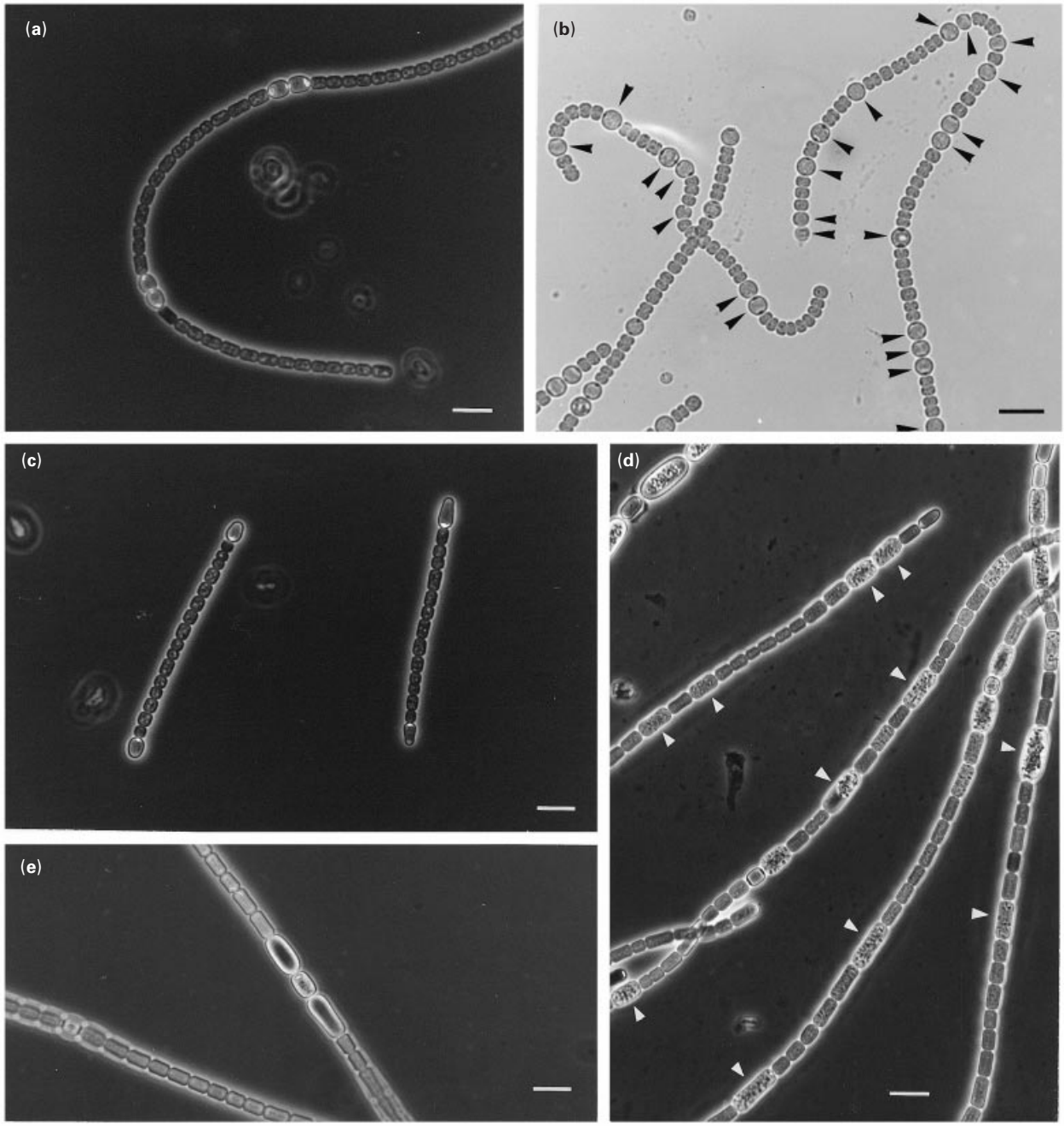

Fig. 9. Photomicrographs of *Anabaena* spp. showing alterations to the normal heterocyst pattern. (a) *A. cylindrica* showing double heterocysts induced by incubation at high light intensity for a short period following removal of combined N from the growth medium. (b) *Anabaena* sp. strain CA grown in the absence of nitrate but with 7-azatryptophan (1×10^{-5} M). The effect of the analogue on heterocyst pattern can be clearly seen when this figure is compared with Fig. 3b. Some of the heterocysts are marked by arrowheads, and many adjacent heterocysts can be seen. (c) *A. cylindrica* showing two short filaments with terminal heterocysts at both ends; these are not normally formed by this cyanobacterium, and were induced by fragmentation of filaments following transfer to medium lacking combined N. (d) and (e) N_2-fixing culture of *A. cylindrica* incubated with the arginine analogue canavanine (4×10^{-5} M). In (e) akinetes, devoid of their characteristic cyanophycin granules, can be seen in their usual location adjacent to a heterocyst. In (d) some akinetes are present adjacent to heterocysts, whereas large numbers (arrowheads) are in positions remote from heterocysts. All micrographs are phase contrast except (b) which is bright field. Bars represent 10 μm. Panels (a) and (c) reproduced, with permission, from Adams & Carr (1981b); Panel (e) reproduced, with permission, from Nichols *et al.* (1980).

A similar pattern alteration can be produced by incubation of cyanobacteria with the tryptophan analogue, 7-azatryptophan (Fig. 9b; Mitchison & Wilcox, 1973; Bothe & Eisbrenner, 1977; Adams, 1992a). When a plasmid carrying the *hetR* gene is transferred to wild-type *Anabaena* PCC 7120, a high frequency of heterocysts is produced, usually in pairs, but sometimes with up to five in a row (Buikema & Haselkorn, 1993). Finally, mutation of *patS* leads to an elevated heterocyst frequency and

the formation of multiple contiguous heterocysts (Yoon & Golden, 1998; see section III.8).

7. *Filament fragmentation and the regression of developing heterocysts*

The observation that fragmentation of *Anabaena* filaments into short chains of cells results in an overall increase in the number of heterocysts led Wolk (1967) to conclude that heterocysts (and proheterocysts) release a diffusible inhibitor of differentiation that prevents neighbouring cells from differentiating. Consequently, separation from the source of the inhibitor (by filament breakage) results in a higher proportion of vegetative cells developing into heterocysts. Later, Wilcox *et al.* (1973a,b) demonstrated that proheterocysts revert to the vegetative form when the filament is broken on one or both sides of the developing cell. They argued that a certain number of vegetative cells are necessary for the removal of the inhibitory substance, and that breakage of the filament prevents the proheterocyst from dispersing the inhibitor, thereby increasing the inhibitor concentration in the vicinity (Wilcox *et al.*, 1973b). This increase would presumably promote regression and a return to vegetative growth. The probability of a proheterocyst regressing is correlated with its stage of development and with the size of the filament fragment. Thus, the smaller the fragment the more advanced is the stage at which regression will occur (Wilcox *et al.*, 1973b).

As with the deliberate breakage of filaments, mutations that lead to extensive fragmentation also disrupt heterocyst formation (Buikema & Haselkorn, 1991a; Ernst *et al.*, 1992; Bauer *et al.*, 1995). For example, mutation of the gene *fraC* causes the production of short filaments, consisting of fewer than 15 cells in N-containing media, that form a small number of mature heterocysts and become almost unicellular when deprived of combined N (Bauer *et al.*, 1995). Most proheterocysts detach from the filaments before completing their development, and then revert to vegetative cells. *fraC* encodes a unique protein that is unlikely to be directly involved in heterocyst development or function (Bauer *et al.*, 1995). Inactivation of the gene *fraH*, which encodes a proline-rich protein with no significant sequence similarity to any other previously characterised protein, also results in a fragmentation phenotype following N starvation. Under these conditions, numerous detached mature heterocysts are observed in the culture medium and any remaining vegetative cells grow very slowly (Bauer *et al.*, 1995).

8. *The nature of the heterocyst inhibitor*

Most models that attempt to account for the patterned formation of heterocysts include the concept of a heterocyst inhibitor (Wolk, 1989; Adams, 1992a; Wolk *et al.*, 1994; see section III.10). Diffusion of the inhibitor away from the heterocyst and its loss from, or degradation within, vegetative cells would establish a decreasing gradient, and a vegetative cell could only differentiate if the concentration of inhibitor within it fell below a threshold value (Wolk, 1975, 1989, 1991; Fig. 8). Compounds once considered candidates for the role of heterocyst inhibitor, but since discounted, include ammonia, glutamine and glutamate (Adams 1992a; Wolk *et al.*, 1994). Interestingly, the tryptophan analogue DL-7-azatryptophan has been shown to induce heterocyst differentiation in undifferentiated cultures of *Anabaena* sp. strain CA (ATCC 33047) in the presence of nitrate (Stacey *et al.*, 1979; Bottomley *et al.*, 1980) and in other *Anabaena* spp. grown in the presence of ammonium chloride (Adams, 1992b). Moreover, addition of the amino acid tryptophan to N_2-fixing cultures of *A. cylindrica* inhibits heterocyst development (D. G. Adams & P. S. Duggan, unpublished), implying an involvement of tryptophan or its metabolism in the control of differentiation. A similar conclusion was reached by Adams (1978), Bottomley *et al.* (1980) and Chen *et al.* (1987).

The most encouraging evidence for the existence of an inhibitor of differentiation was obtained recently by Yoon and Golden (1998), who identified a gene, *patS*, that blocks heterocyst differentiation when present in multiple copies or when overexpressed. Interruption of the gene results in a mutant that forms heterocysts in the presence of combined N, and multiple contiguous heterocysts in its absence. *patS* encodes a peptide of just 17 amino acids, of which only the five C-terminal amino acids seem essential for heterocyst suppression (Yoon & Golden, 1998). Indeed, addition to the growth medium of a submicromolar concentration of a synthetic pentapeptide based on these final five amino acids completely inhibits heterocyst differentiation. PatS therefore has many of the features required for the proposed diffusible inhibitor that regulates heterocyst pattern.

9. *Cell selection during differentiation and pattern formation*

(a) *Cell division.* As far as is known, all vegetative cells have the genetic capacity to become heterocysts, and by light microscopy all cells in a filament grown in the presence of combined N appear remarkably similar. The *de novo* establishment of a regular pattern of heterocysts therefore requires an efficient system of cell selection based on rules for distinguishing between apparently identical cells. The mechanism for this is unknown, but there is evidence that cell division, the cell cycle, DNA replication and competition between developing cells are all important.

The subject of cell division symmetry in filamentous cyanobacteria has been controversial. In at least some *Anabaena* spp. division is asymmetrical and heterocysts develop from only the smaller daughter cell (Wilcox *et al*., 1975b; Adams, 1992a). Related observations have been made with *A. cylindrica*, in which incubation at a high light intensity for a short period following the induction of heterocyst development leads to a three- to fourfold increase in the frequency of symmetrical cell divisions, and results in the production of adjacent (double) heterocysts (Fig. 9a; Adams, 1992a). In *Chlorogloeopsis fritschii* the first detectable response to N starvation is an alteration in the pattern of cell division from mostly symmetrical in the presence of combined N to mostly asymmetrical during N starvation (Foulds & Carr, 1981). Although this change in cell division precedes heterocyst development, its significance for the process is unclear because *C. fritschii* often grows as clumps of cells with no readily discernible heterocyst pattern (Castenholz, 1989b).

Asymmetric cell division may therefore provide a means for cell selection during heterocyst development, and evidence for this is discussed in section III.10. It is not clear if this mechanism applies to all filamentous cyanobacteria that produce intercalary heterocysts, because there are reports that in some cases, such as *Anabaena* PCC 7120, cell division is symmetrical (Buikema & Haselkorn, 1993). However, in cultures of this cyanobacterium grown in the authors' laboratory the majority of cell divisions are asymmetrical.

(b) *DNA replication and the cell cycle*. The use of specific inhibitors has shown that DNA, RNA and protein synthesis are required for heterocyst development, and that a heterocyst fulfils the requirement for each of these at different stages of its formation (Adams, 1992a). The requirement for DNA synthesis has led to the suggestion that DNA replication serves as a timer that ties heterocyst differentiation to the cell cycle (Adams & Carr, 1989; Adams, 1992a). The need for a cell to be at a specific stage of its cycle before it can differentiate provides an explanation for the relatively long period taken (over half the generation time) to establish the full complement of 5–10% proheterocysts following the removal of combined N from the medium. This period would represent the time needed to allow all the cells required to form the initial pattern to reach the correct stage of their cycle and begin to differentiate.

(c) *Competition*. Initial selection of cells for differentiation may rely on asymmetrical cell division and position in the cell cycle, but this alone is unlikely to be sufficient to generate a regular pattern of heterocysts, because the first cells to initiate differentiation may sometimes be too close to other developing cells (see section III.10). There must therefore be a mechanism that allows competition between these cells so that some regress and return to vegetative growth, whereas others continue to differentiate. Evidence for such a competitive mechanism was obtained in the induced regression experiments of Wilcox *et al*. (1975b) (see also Adams & Carr (1981b) and section III.7). Competition is probably especially important between cell siblings because of their inherent similarities. Treatments that disrupt heterocyst spacing, such as incubation with 7-azatryptophan or at high light intensity, probably do so by interfering with competition. Selection of 'candidates' for differentiation, by cell cycle and cell division symmetry, should not be seen as separate processes from competition; they are aspects of the same process. For example, incubation at high light intensity increases the symmetry of cell divisions in *A. cylindrica* and so diminishes the ability of the resulting cell siblings to compete effectively with each other, with the result that both may differentiate.

10. *Models for heterocyst differentiation and pattern control*

Filaments of N_2-fixing cultures of *Anabaena* spp. or *Nostoc* spp. possess a regular spacing of single heterocysts and, as cell division increases the number of vegetative cells (the interval) between heterocysts, new heterocysts eventually develop at approximately the mid-point of each interval, so maintaining the pattern. However, cultures grown in the presence of combined N, and therefore consisting entirely of vegetative cells, must form (within one generation time) a pattern of single heterocysts when transferred to N-free media. Two models (often referred to as the 'altruistic' and the 'selfish' models) have been used to explain how this is achieved (Fig. 8; Wolk, 1989, 1991; Adams, 1992a; Wolk *et al*., 1994).

In the first (altruistic) model, following the removal of combined N, developing heterocysts establish zones of inhibition around themselves by producing a diffusible inhibitor of heterocyst development (Fig. 8b). A vegetative cell can only commence heterocyst differentiation if the concentration of inhibitor within it is below a threshold level. The second (selfish) model proposes that the first cells to respond to N deprivation establish high-efficiency uptake systems to scavenge combined N from their neighbours, which respond in like fashion. This model was first proposed by Haselkorn (1978), who suggested that the amino acids liberated by proteolysis might serve as diffusible inhibitors of differentiation and that, in response to N starvation, cells would establish amino acid pumps to scavenge fixed N from adjacent cells (Wolk, 1989; Fig. 8a). These cells would themselves respond by activating

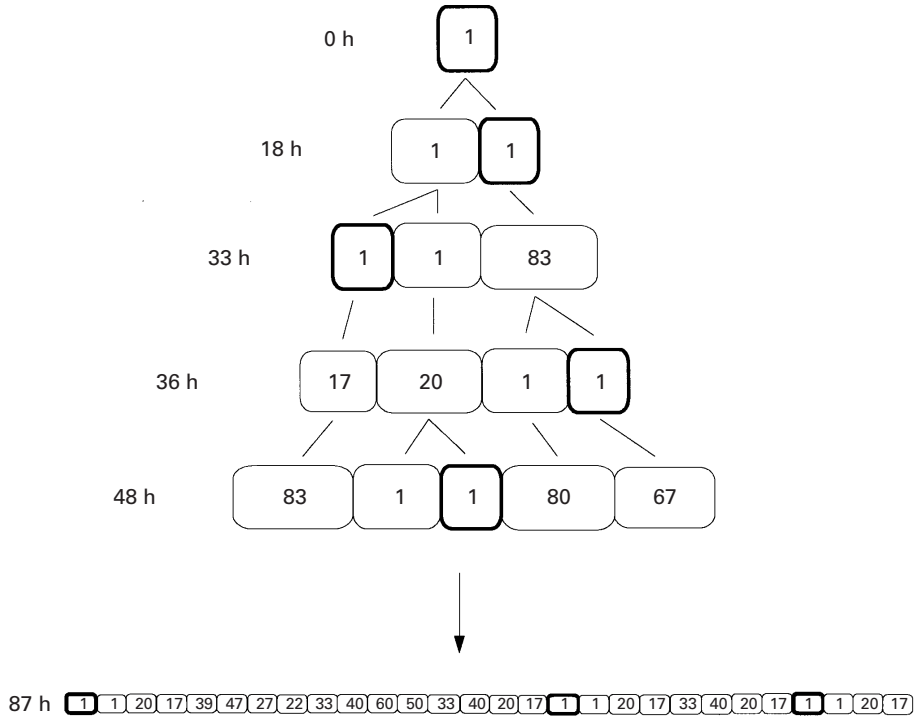

Fig. 10. Model to explain the way in which cell lineage can generate candidates for heterocyst development. The basis for this model is the observations made in *Anabaena* spp. by Mitchison & Wilcox (1972) and Wilcox *et al*. (1973a,b), who showed that: cell division is asymmetrical; the smaller daughter takes 20% longer to reach division than the larger daughter; if a cell arises as the left (or as the right) daughter, its small daughter will be the left (or the right) cell after division; and heterocysts only arise from small daughter cells. By applying these simple rules to a single cell, it is possible to generate an imaginary filament in which the lineage of each cell is known. The starting point (0 h) is the single cell at the top of the figure, which is a newly-formed small daughter cell. The figure inside each cell indicates that cell's position (as a percentage) in its cell cycle, starting at 1 immediately after division, and dividing once more when 100 is reached. The mean generation time has been taken as 16.5 h, which means that a large daughter cell divides every 15 h, and a small daughter every 18 h. The small daughters immediately after division (at 1% of their cell cycle) are indicated by a thicker line around the cell. After 18 h the small daughter divides to produce a small and a large daughter. After a further 15 h the larger of the cells divides to generate large and small progeny, and because it arose as the left cell of a division, its smaller daughter will be the left cell. At this point the original small daughter has reached 83 in its cell cycle; it divides at 36 h, and the resulting small daughter is the right of the two siblings. This process is repeated until by 87 h a filament of 28 cells is produced. If this filament is induced to differentiate, by the removal of combined N, the small daughter cells at the start of their cell cycles are potential candidates for differentiation. The positioning of these cells forms the basis of a spaced pattern that with fine tuning, could generate the final regular heterocyst pattern.

their own pumps from the next cell, and so on along the filament. These two models make different predictions of which cells will differentiate: in the first model it will be the first to respond to N starvation (Fig. 8b); whereas in the second, it will be those mid-way between, which are being drained of fixed N from both sides, and therefore have no alternative but to generate their own fixed N (Fig. 8a). At present there is no conclusive evidence to support either model, although there are reasons to believe that the first is the more likely (Adams, 1992a), particularly in the light of the recent discovery of the probable identity of the diffusible heterocyst inhibitor (PatS; see section III.8).

What these models do not address is the problem of why particular cells begin to differentiate in the first place. However, experimental evidence provided by Wilcox and co-workers provides the basis for a model to explain why only certain cells in any particular filament are capable of differentiating at a given time. The starting points for this model are the observations made in *Anabaena* spp. by Mitchison and Wilcox (1972) and Wilcox *et al*. (1973a,b) showing that: cell division is asymmetrical; the smaller daughter takes 20% longer to reach division than the larger daughter; if a cell arises as the left (or as the right) daughter, its small daughter will be the left (or the right) cell after division; and heterocysts arise only from small daughter cells. By applying these simple rules to a single cell, it is possible to generate an imaginary filament in which the lineage of each cell is known (Fig. 10). It is then possible to predict which cells will be capable of differentiation at any time. For example, after 87 h the original cell in Fig. 10 has generated a filament in which three cells are newly formed small daughters and would therefore be candidates for differentiation if combined N was removed at, or just prior to, this point.

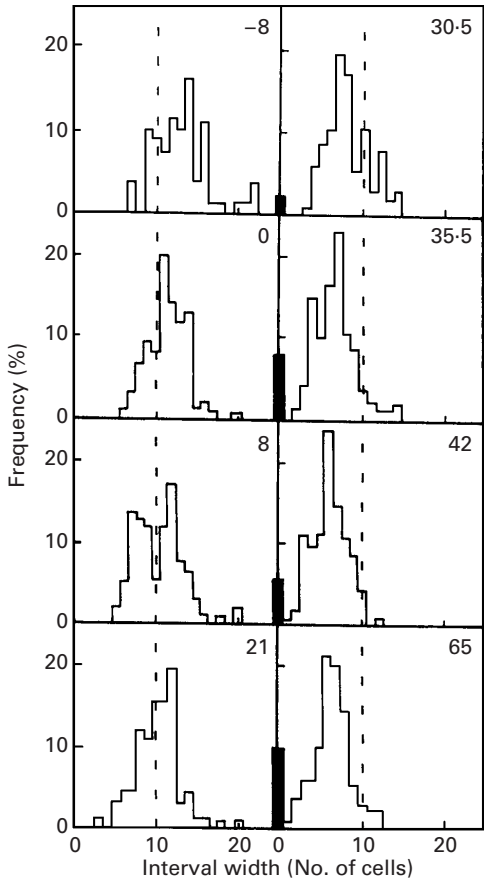

Fig. 11. Effect of 7-azatryptophan on interheterocyst intervals in an N_2-fixing culture of *Anabaena cylindrica*. The histograms illustrate the range and frequency of interheterocyst interval widths, estimated at the times (in hours) indicated by the figure to the top right of each panel, in a culture to which 7-azatryptophan (final concentration 5×10^{-5} M) was added at time zero. The filled blocks represent zero intervals (i.e. adjacent heterocysts). The vertical dashed line in each panel provides a reference point. Incubation with 7-azatryptophan causes new heterocysts to develop closer to existing heterocysts, so causing a shift in the distribution of intervals towards the lower end. Zero intervals, caused by the presence of adjacent heterocysts, are first seen at 30.5 h, although at this stage there are no 1- or 2-cell, and very few 3-cell intervals. This observation is difficult to reconcile with the simple inhibitor-diffusion model shown in Fig. 8b. *Reproduced, with permission, from Adams (1992b).*

These candidates provide the beginnings of a spaced pattern. The necessary 'fine tuning' of this preliminary pattern will begin as soon as these cells begin to differentiate, and it may be at this stage that additional factors such as DNA replication, competition and the inhibitor PatS come into play. Because of the inherent similarity of daughter cells, it may be that both begin to differentiate, resulting in increased *hetR* expression, but invariably the smaller daughter will be the one to continue differentiation, and will compete with any other small daughters nearby.

By artificially inducing regression of proheterocysts by filament fragmentation, Wilcox *et al.* (1973a,b) demonstrated that the early pattern of proheterocysts was fluid and could be altered by competition of cells that were too close to each other. This may occur by the diffusion of an inhibitory substance, as shown in Fig. 8b. However, this model fails to explain the influence of 7-azatryptophan on heterocyst pattern (Adams, 1992b; Fig. 11). Incubation of N_2-fixing cultures of *Anabaena* spp. with this amino acid analogue causes a gradual decline in the mean number of cells between heterocysts (Fig. 11), implying an equivalent decrease in the inhibitory zone around each heterocyst. The model in Fig. 8 would predict that intervals should continue to decrease until one-cell intervals are reached, after which zero intervals (i.e. adjacent heterocysts) would occur. However, adjacent heterocysts are produced long before any one-cell, or even two-cell intervals are found (Adams, 1992b; Fig. 11). A remarkably similar distribution of intervals is produced by a *patS* mutant (Yoon & Golden, 1998; see section III.8). This preponderance of double heterocysts can be explained if azatryptophan, or the loss of PatS, diminishes the ability of sibling cells to compete with each other, so that both continue to differentiate, rather than just the smaller cell.

IV. THE AKINETE

1. *Properties of akinetes*

Akinetes (from the Greek 'akinetos', meaning 'motionless') are thick-walled, resting cells produced by many strains of subsections IV (order Nostocales) and V (order Stigonematales), usually as cultures approach stationary phase. They were first described (Carter, 1856; de Bary, 1863; Rabenhorst, 1865) long before the discovery of the bacterial endospore (Cohn, 1877; Koch, 1877). They do not resemble the endospore structurally, and are not heat resistant, but they are resistant to cold and desiccation, and can germinate to produce new filaments under suitable conditions (Nichols & Adams, 1982; Herdman, 1987, 1988; Adams, 1992a). Akinetes therefore provide cyanobacteria with a means of overwintering and surviving dry periods, although there have been conflicting opinions about their contribution to the spring regrowth of cyanobacteria in natural bodies of water (Herdman, 1987). However, their ability to survive cold and desiccation is generally far greater than that of vegetative cells. For example, akinetes of *A. cylindrica* can survive for 5 yr in the dark in the dry state, whereas vegetative cells survive no longer than 2 wk under similar conditions (Yamamoto, 1975), and akinetes of *Nostoc* PCC 7524 survive in the dark at 4°C for 15 months, but vegetative cells lose viability within 7 d (Sutherland *et al.*, 1979). However, the akinetes of planktonic cyanobacteria, such as *Anabaena circinalis*, may be no more desiccation tolerant than the vegetative cells (Fay, 1988).

Akinetes of *Cyanospira* spp. can germinate with high efficiency after 7 yr in a desiccated state (Sili *et al*., 1994), and viable akinetes have been isolated from sediments as old as 64 yr (Livingstone & Jaworski, 1980).

2. *Structure, composition and metabolism*

Akinetes are generally larger (sometimes very much larger) than vegetative cells, with a thickened cell wall, and a multilayered extracellular envelope (Figs. 3c–e; Nichols & Adams, 1982; Herdman, 1987, 1988). The mean cellular content of the N reserve material, cyanophycin, in akinetes of *Nostoc* PCC 7524 is eightfold higher than that in vegetative cells (Sutherland *et al*., 1979). Indeed, the cytoplasm of differentiating akinetes accumulates both glycogen and granules of cyanophycin (Fig. 3c; Simon, 1987), although this process is not specific for akinete development, because vegetative cells also accumulate glycogen and cyanophycin after the end of exponential growth (Herdman, 1987). In addition, both incubation of *A. cylindrica* with the arginine analogue, canavanine (Nichols *et al*., 1980), and mutation of the arginine biosynthesis gene, *argL*, in *Nostoc ellipsosporum* (Leganés *et al*., 1998), can result in the production of akinetes lacking cyanophycin (Fig. 9e). Indeed, the akinetes of *Cyanospira* spp. naturally lack cyanophycin (Sili *et al*., 1994).

The mean cellular content of RNA, DNA and protein is similar in vegetative cells and akinetes of *Nostoc* PCC 7524 (Sutherland *et al*., 1979), whereas the akinetes of *A. cylindrica* contain the same amount of RNA, but more than twice as much DNA, and ten times as much protein as vegetative cells (Simon, 1977). These high values are probably a consequence of the increased size of the *A. cylindrica* akinetes (Fig. 3c), which are up to ten times the volume of the vegetative cells (Fay, 1969). Whereas many of the metabolic activities of akinetes, including CO_2 fixation and N_2 fixation, are very low, or undetectable (Rai *et al*., 1985; Rao *et al*., 1987; Sarma & Ghai, 1998), the rate of respiration is often elevated (Herdman, 1987), although this may depend on age, because respiratory capacity is lost in older akinetes (Chauvat *et al*., 1982). Isolated akinetes of *Nostoc spongiaeforme* retain some degree of metabolic activity, being capable of synthesizing protein and lipid, and of respiring in the dark and evolving oxygen in the light (Thiel & Wolk, 1983).

3. *Relationship to heterocysts*

Although produced only by heterocystous cyanobacteria, akinetes can develop in some strains when heterocyst development has been repressed by the presence of combined N. For example, in old cultures of *Anabaena* sp. strain CA, in which heterocyst development has been suppressed by the presence of sodium nitrate, every cell in a filament can transform into an akinete (Fig. 3d). Nevertheless, the presence of heterocysts exerts an influence on the positioning of akinetes, in a manner that is characteristic of the cyanobacterial strain. For example, in *Anabaena* sp. strain CA akinetes develop randomly in the absence of heterocysts, but midway between heterocysts when these are present. Akinetes develop immediately adjacent to heterocysts in *A. cylindrica* (Fig. 3c), but several cells away from heterocysts in *Anabaena circinalis* and some other planktonic species (Fay *et al*., 1984; Li *et al*., 1997). In most cases akinetes develop in strings, showing a gradient of decreasing maturity away from the first to develop (Fig. 3c). It is difficult to formulate a single mechanistic model to explain these extremes of akinete placement in relation to heterocysts. The need to accumulate large amounts of combined N in the form of cyanophycin might explain their occurrence next to heterocysts in *A. cylindrica*, but not their presence mid-way between heterocysts in *Anabaena* sp. strain CA and *Nostoc* PCC 7524.

The position of akinetes can be altered by the amino acid analogues 7-azatryptophan and canavanine. Incubation of *Nostoc* PCC 7524 with 7-azatryptophan causes both heterocysts and akinetes to develop further from the centre of the heterocyst interval than in control cultures (Sutherland *et al*., 1979). This led the authors to conclude that a common control mechanism existed for both heterocysts and akinetes in *Nostoc* PCC 7524. When N_2-fixing cultures of *A. cylindrica* are incubated with the arginine analogue canavanine, akinetes develop in random positions between heterocysts, in addition to their usual adjacent positions (Figs. 9d,e; Nichols *et al*., 1980; Adams, 1992a). The formation of akinetes at random positions suggests that canavanine is acting upon vegetative cells rather than heterocysts. The authors concluded that, during exponential growth, vegetative cells were inhibited from becoming akinetes by some aspect of their physiology, and that the function of the heterocyst was to remove or negate this inhibition in adjacent cells, without directly stimulating their development. A similar model had been proposed by Wolk (1965). While this model can help to explain the spatial relationship between heterocysts and akinetes in *A. cylindrica*, it is hard to see how it can do so in *Nostoc* PCC 7524 in which akinetes develop midway between heterocysts.

4. *Factors that influence akinete differentiation*

The major, although not the only, trigger for akinete development is light limitation. This is apparent from the observation that, in the presence of excess inorganic nutrients in the medium, akinetes differentiate at the end of the exponential growth phase, when increasing culture density results in self-

shading (Fay, 1969; Fay *et al.*, 1984; Wyman & Fay, 1986; Nichols & Adams, 1982; Herdman, 1987, 1988). Indeed, there is a direct correlation between the light intensity at which *A. cylindrica* is grown and the cell density at which akinete formation begins (Nichols *et al.*, 1980). The involvement of light energy limitation can be shown with a facultative photoheterotroph such as *Nostoc* PCC 7524 in which the exponential phase can be prolonged and akinete formation delayed by the addition of a usable C source such as sucrose (Sutherland *et al.*, 1979).

Limitation of a wide variety of nutrients, notably phosphate, has also been implicated as a trigger of akinete development (Nichols & Adams, 1982; Herdman, 1987, 1988). In *A. circinalis*, phosphate limitation appears to be the major trigger, whereas limitation for N, inorganic C, iron, trace elements or light have no effect (Vandok & Hart, 1996). In a range of planktonic *Anabaena* spp. temperature is important for triggering akinete differentiation (Li *et al.*, 1997), whereas in *Anabaena doliolum* (Rao *et al.*, 1987) and *Anabaena torulosa* (Sarma & Khattar, 1993), a critical C:N ratio appears to be important. It may be that these diverse stimuli induce a common physiological trigger – perhaps decreased cell division – which results in akinete development, but nothing is known of this at present.

5. *Extracellular signals*

An intriguing form of extracellular signalling has been reported for akinete-containing cultures of *Cylindrospermum licheniforme*, which excrete a compound (C_7H_5OSN) that stimulates akinete formation in young cultures of the same cyanobacterium (Fisher & Wolk, 1976; Hirosawa & Wolk, 1979a,b). It is not clear whether this compound functions as an inter- or intrafilamentous signal for akinete formation. There is the interesting possibility that it may become concentrated in a natural body of water during desiccation or stagnation, and trigger the development of akinetes to survive the impending drought (Fisher & Wolk, 1976). Unfortunately, limited attempts to detect similar activities in other cyanobacteria have been unsuccessful (Nichols & Adams, 1982).

6. *Akinete germination*

Increased light intensity seems to be the major stimulus for akinete germination, and this can be achieved experimentally by dilution of an akinete-containing culture with fresh or used medium (Herdman, 1987, 1988). Germination is generally most efficient when both photosystems I and II are active, and when the latter is inhibited, for example by the presence of DCMU, both cyclic photophosphorylation and respiration are required (Herdman 1987, 1988). *Nodularia spumigena* akinetes can germinate at relatively low light intensities (<9 μE m^{-2} s^{-1}), or in red light (Huber, 1985). Akinetes of *A. doliolum* lack photosynthetic pigments (Rai *et al.*, 1985) and consequently, the energy requirements for their germination are met, at least initially, by the aerobic oxidation of C reserves, until oxygenic photosyntheis commences 24 h after initiation of germination (Rai *et al.*, 1988). With occasional exceptions (Neely-Fischer *et al.*, 1989), germination does not occur in the dark, even when strains capable of chemoheterotrophic growth are supplied with the appropriate sugar (Chauvat *et al.*, 1982). Light and phosphate, but not N, are required for germination of akinetes of *A. circinalis* (Van Dok & Hart, 1997) and *N. spumigena* (Huber, 1985).

Release of the germinating akinete from the surrounding envelope generally occurs by one of three mechanisms (Nichols & Adams, 1982; Herdman, 1987). Most commonly the envelope remains intact, apart from a pore at one end, through which the germling emerges, having first undergone one or two cell divisions, or less commonly, prior to any cell division (Skill & Smith, 1987; Sili *et al.*, 1994; Fig. 3e). Empty akinete envelopes are frequently seen following germination (Fig. 3e). Occasionally the germling is released by dissolution of the entire akinete envelope. Akinetes germinating in the absence of combined N usually develop a single heterocyst at a time and at a position characteristic of the particular cyanobacterium. For example, in *Nostoc* PCC 7524 (Sutherland *et al.*, 1985b), *Anabaena* PCC 7937, *Nostoc* PCC 6720 (Skill & Smith, 1987), and *Cyanospira capsulata* (Sili *et al.*, 1994) the first heterocyst develops in a terminal position when the germling is three cells long. In other cyanobacteria a single heterocyst forms, at the 6–7 cell stage, near the centre of the germling in *Anabaena* CA (Nichols & Adams, 1982; see Fig. 3e), or in a terminal position in *Cyanospira rippkae* (Sili *et al.*, 1994).

Because of the difficulty of synchronizing germination in a population of akinetes, there have been relatively few biochemical studies of the process (Sutherland *et al.*, 1985a,b; Rai *et al.*, 1988; Sili *et al.*, 1994). One of the most comprehensive accounts of akinete germination was obtained for *Nostoc* PCC 7524 (Sutherland *et al.*, 1985a,b). Following initiation of germination, by dilution of the akinetes in fresh medium lacking combined N, protein synthesis begins immediately and continues for 11 h, RNA synthesis continues throughout germination, whereas DNA synthesis does not begin until 80 min after initiation, but is continuous thereafter (see Herdman, 1987). This DNA synthesis is not essential, because germination continues when DNA synthesis is inhibited by phenethyl alcohol. However, heterocyst development is inhibited by this compound, and nitrate must be present in the medium to supply the combined N normally

provided by heterocysts late in germination. Under these conditions short filaments of 10 vegetative cells are produced, presumably because the akinetes contain 10 genome equivalents of DNA (Sutherland *et al*., 1979), thus allowing cell division to continue in the absence of DNA replication, until each newly formed vegetative cell contains one genome copy.

The N for the protein synthesis that occurs during the early stages of akinete germination does not come from the two obvious N reserves, phycocyanin and cyanophycin, because the latter is not degraded until 6 h and this is associated with a period of phycocyanin synthesis. Nor does it come from N_2 fixation, which does not commence until the first heterocysts mature at 19 h. An alternative N source must therefore supply protein synthesis during the early stages of germination, and this may be the thick peptidoglycan layer which is degraded during germination to release a lipopolysaccharide-like laminated layer which accumulates inside the akinete envelope (Sutherland *et al*., 1985a,b). A similar conclusion was drawn by Sili *et al*. (1994) for germinating akinetes of *Cyanospira* spp. which do not contain cyanophycin.

Many of the enzymes of inorganic N metabolism have low or zero activity in the akinetes of *A. doliolum* (Rai *et al*., 1988). However, their activities increase rapidly during germination, in the order glutamate/pyruvate transaminase, aspartate dehydrogenase, glutamine synthetase and nitrate reductase. Glutamine synthetase activity increases to a value characteristic of vegetative filaments, whereas the other enzymes peak at a higher value and decline to that of vegetative filaments.

7. *Genes involved in akinete differentiation*

Most of our scant knowledge of the genes involved in akinete development derives from work on heterocyst formation. An intact *hetR* gene is required for akinete differentiation, and *hetR* is actively expressed in akinetes (Leganés *et al*., 1994). At least in *N. ellipsosporum*, therefore, HetR is required for both akinete and heterocyst differentiation, supporting the view that heterocysts may have evolved from akinetes, or that the two cell types may share a common ancestor (Wolk *et al*., 1994). Further evidence for this is found in *A. variabilis* ATCC 29413, in which *hepA* is required for the synthesis of both the akinete and the heterocyst envelope polysaccharide (Leganés, 1994). Introduction of a plasmid containing wild-type *devR* into cells of *Nostoc* sp. strain 29133 results in a stimulation of akinete formation in both ammonium-supplemented and N_2-fixing cultures, implying that akinete and heterocyst differentiation may be influenced by similar phosphorelay systems and that some cross-talk may occur between the two processes (Campbell *et al*., 1996).

V. CONCLUSION

Our understanding of heterocyst differentiation has advanced significantly in recent years, yet there are many unanswered questions. Of particular interest is the relationship between the cell cycle and heterocyst development. There is circumstantial evidence that vegetative cells can only differentiate at a particular stage of the cell cycle (see sections III.9, III.10), yet conclusive data are lacking. Elhai and co-workers are attempting to provide the necessary data by studying mutants of *Anabeana* PCC 7120 that fragment to short filaments upon deprivation of combined N (e.g. see section III.7). One such mutant, Ucl5, which grows as filaments of mostly two cells on nitrate-containing medium, fragments to single cells when nitrate is removed. The mutant is unable to form functional heterocysts, but does show normal expression of three markers: phycocyanin breakdown, polysaccharide deposition and expression of a *hetR*::*luxAB* fusion (J. Elhai, pers. comm.). The proportion of the single cells expressing these markers is the same as that in filaments of the wild type upon N stepdown, implying that only a subset of cells can initiate differentiation at any time. When Ucl5 was subjected to a period of darkness, to synchronize its DNA replication, the proportion of cells showing polysaccharide deposition after N stepdown rose and fell over the course of the cell cycle. Results such as these offer hope of clarifying the relationship between a cell's position in its cell cycle and its capacity to become a heterocyst.

Evidence for cell cycle involvement in heterocyst development has also been obtained from wild-type *A. cylindrica* in which the normal timing of proheterocyst and heterocyst development is disrupted when filaments are starved of combined N following the induction of synchronous DNA replication (D. G. Adams, unpublished). DNA replication itself has been suggested as a possible timer for heterocyst differentiation (Adams & Carr, 1989; see section III.9.(b)), and a link between replication and gene expression could be provided by the hemi-methylated state of newly replicated DNA. Ethionine, which prevents methylation of protein and of DNA (Alix, 1982), blocks both *hetR* induction and heterocyst development in *Anabaena* spp. (P. S. Duggan & D. G. Adams, unpublished). Further studies of the relationship between DNA replication, its methylation state, and heterocyst differentiation, may prove fruitful.

Perhaps the most exciting recent discovery concerning heterocyst development is that of *patS* (Haselkorn, 1998; Yoon & Golden, 1998; see section III.8), which opens the way for rapid advances in our understanding of heterocyst pattern control. In the light of such newly acquired molecular genetic data it is interesting to re-examine some of the older published data on heterocyst development and

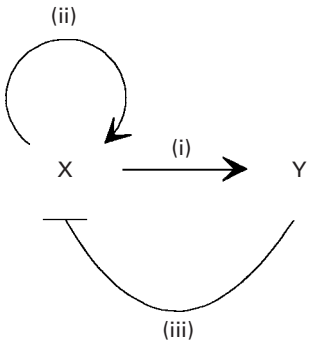

Fig. 12. Interactive model of heterocyst pattern control proposed by Wilcox *et al.* (1973b). X is a non-diffusible compound produced by developing heterocysts; its concentration expresses the state of differentiation of a cell, and it has an autocatalytic component (ii) that effectively drives development. Y is a diffusible inhibitor of differentiation whose rate of synthesis is dependent on the concentration of X (i), but which also inhibits the synthesis of X (iii). The fate of a cell, whether to continue differentiation or regress to vegetative growth, will depend on the relative values of [X] and [Y]. There is considerable similarity between the suggested characteristics of X and Y and those recently determined for HetR and PatS, respectively.

pattern. For example, in the interactive model for heterocyst development proposed by Wilcox (Wilcox *et al.*, 1973a; Fig. 12), the properties of the autocatalytic component X (which expresses a cell's degree of differentiation), and the diffusible inhibitor Y (the production of which requires X), bear striking similarity to those of HetR and PatS, repectively. HetR provides the forward impetus for heterocyst differentiation, whereas PatS suppresses the process. The PatS peptide is produced by proheterocysts at a very early stage of their development, and is probably processed, perhaps by the proteolytic activity of HetR (section III.5.(a)), to release the active COOH-terminal peptide, which diffuses via the filament's periplasmic space, creating the necessary gradient of inhibition around the developing cells (Yoon & Golden, 1998). The PatS-producing cells must be refractory to its influence, perhaps because the unprocessed peptide has no effect on *hetR* gene expression. It may be that a vegetative cell's developmental fate is decided by the ratio between active PatS and HetR.

Heterocysts and akinetes represent the most extreme forms of cellular differentiation in cyanobacteria, but in nonheterocystous cyanobacteria there is increasing evidence for more subtle types of cellular specialization, for N_2 fixation and for desiccation survival. For example, some intriguing questions are posed by the nonheterocystous, filamentous cyanobacteria *Trichodesmium* spp. and *Symploca* spp., in which N_2 fixation occurs during the light period of a light–dark cycle, and the nitrogenase protein is localized to a small proportion of the cells (Fredriksson *et al.*, 1998; Lin *et al.*, 1998; see section III.5.(c)). Strains of both of these genera possess homologues of *hetR*, which is essential for heterocyst development in other cyanobacteria. It remains to be seen how these cyanobacteria compartmentalize nitrogenase and protect it from oxygen, and whether *hetR* has a role in the patterned distribution of the N_2-fixing cells. However, this cannot be the case in *S. platensis*, which has neither the capacity to form heterocysts, nor to fix N_2, but which contains a protein that cross-reacts with anti-HetR antibodies (Zhou *et al.*, 1998a).

Great resistance to desiccation is often found in the unicellular cyanobacteria of the genus *Chroococcidiopsis*, which often occur as the sole photosynthetic organisms in extremely arid deserts (Friedmann & Ocampo-Friedmann, 1984, 1985). Spore-like cells, with thick, multilayered envelopes, have been reported in laboratory desiccated cultures (Grilli Caiola *et al.*, 1993), in desiccated samples on hot desert rocks (Grilli Caiola *et al.*, 1996), and in liquid cultures grown under N-limited or N-starved (but not N-replete) conditions (Billi & Grilli Caiola, 1996). It remains to be seen whether these cells truly represent a functional equivalent of the akinete, providing a dormant state for survival of prolonged N starvation, desiccation, or temperature extremes.

Many aspects of cellular differentiation in cyanobacteria require intercellular signalling or, indeed, interfilament signalling. The development of akinetes is responsive to extracellular cyanobacterial chemical signals (see section IV.5), and in heterocyst differentiation the first clear evidence for an intrafilamentous signal has been provided by the discovery of PatS (see section III.8). Another example is seen in the hormogonia produced by cyanobacteria of the orders *Nostocales* and *Stigonematales* (section III.1). Hormogonia are short, motile filaments, that provide a means of dispersal, and serve as the infective agents in the establishment of many cyanobacteria–plant symbioses (Bergman *et al.*, 1996; Adams, 1999). The differentiation and chemotaxis of these hormogonia is responsive to chemical signals from plants. Our understanding of these examples of signalling is rudimentary. There remain major questions concerning the nature of both the signals that pass within and between filamentous cyanobacteria, and the signal transduction mechanisms that presumably sense and respond to these signals. The molecular genetic techniques required to answer these questions are now available and an exciting period of discovery lies ahead.

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