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Review Article

REVIEW ARTICLE

The multicellular nature of filamentous heterocyst-forming cyanobacteria

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One sentence summary: The organismic unit in heterocyst-forming cyanobacteria is a filament of interconnected cells; to gain an understanding of multicellularity in these organisms, the authors analyze and discuss their filament structure, theoretical models of heterocyst pattern formation, and their distinct cell division characteristics.
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ABSTRACT

Cyanobacteria carry out oxygenic photosynthesis, play a key role in the cycling of carbon and nitrogen in the biosphere, and have had a large impact on the evolution of life and the Earth itself. Many cyanobacterial strains exhibit a multicellular lifestyle, growing as filaments that can be hundreds of cells long and endowed with intercellular communication. Furthermore, under depletion of combined nitrogen, filament growth requires the activity of two interdependent cell types: vegetative cells that fix CO_2 and heterocysts that fix N_2. Intercellular molecular transfer is essential for signaling involved in the regulation of heterocyst differentiation and for reciprocal nutrition of heterocysts and vegetative cells. Here we review various aspects of multicellularity in cyanobacterial filaments and their differentiation, including filament architecture with emphasis on the structures used for intercellular communication; we survey theoretical models that have been put forward to understand heterocyst patterning and discuss the factors that need to be considered for these models to reflect the biological entity; and finally, since cell division in filamentous cyanobacteria has the peculiarity of producing linked instead of independent cells, we review distinct aspects of cell division in these organisms.

Keywords: *Anabaena*; cell differentiation; cyanobacteria; heterocysts; intercellular communication; multicellularity

INTRODUCTION

The emergence of oxygenic photosynthesis in cyanobacterial ancestors at about 3 Gyr ago (Gya) was one of the major revolutions in the history of life on Earth. Cyanobacterial activity was responsible for the accumulation of oxygen in the atmosphere and surface waters that led to the Great Oxygenation Event (GEO) 2.4–2.3 Gya, which started to change the Earth's environment and challenged the existent forms of life to cope with, or in most cases to take advantage of, the available oxygen (see Lyons, Reinhard and Planavsky 2014). This trend would eventually lead to the development of oxygenic respiration and animals with a high respiratory rate. Moreover, ancestral cyanobacteria gave rise by symbiosis to the plastids of all extant algae and plants. Thus, the evolution of eukaryotic organisms relied to a great degree on ancient cyanobacteria (see Ku *et al*. 2015). Currently, plastids, which perform cyanobacterial-like photosynthesis, and extant cyanobacteria, which inhabit almost all natural habitats, contributes most of the primary production that is at the basis of the trophic chains in the biosphere. In addition, cyanobacteria are dominant N_2 fixers in the oceans, introducing assimilable nitrogen into the biosphere. In view of their remarkable role, the title 'The cyanobacteria, life's microbial heroes' given by

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A. H. Knoll to a chapter of his book on the history of life on Earth appears to be most justified (Knoll 2004; see also Knoll 2008).

Multicellularity can be defined as a form of biological organization in which a permanent cell aggregate exhibits an activity more complex than that of the individual cells. Multicellularity is a major evolutionary innovation that marks a new level of organization, together with a shift of the unit of selection from single cells to an organism (Szathmary and Smith 1995), and is a requisite for the development of adaptive complexity. Possible scenarios for the emergence of multicellular life forms include cell clustering and cooperative behavior of unicellular organisms, as well as the appearance of physical factors or mutations that may have prevented the separation of dividing cells (Bonner 1998). To survive, the selective advantages of an aggregated life form must exceed likely shortcomings such as increased competition for local resources, reduced mobility, and the presence of non-cooperator or cheater cells that reap the benefits of cooperation but do not bear its costs, leading cooperators to extinction and thus to the celebrated 'tragedy of the commons' (Hardin 1968; West *et al*. 2006). Although other aggregative forms are found (e.g. in magnetotactic bacteria, see Abreu *et al*. 2013), a simple form of multicellular organization is found when an organism grows as chains of cells. Bacteria that grow taking the form of filaments can be found in different phylogenetic groups. To give just a few examples, other than the cyanobacteria, the Actinobacteria (Flärdh and Buttner 2009), *Desulfobulbaceae* (Pfeffer *et al*. 2012) and *Lachnospiraceae* (Thompson *et al*. 2012) contain filamentous forms, and bacteria of the genera *Chloroflexus* (Pierson and Castenholz 1974), *Beggiatoa* (Strohl and Larkin 1978) and *Entotheonella* (Wilson *et al*. 2014) are filamentous. The structure of such filaments can, however, be very different. Thus, whereas *Streptomyces* forms hyphae, the filamentous cyanobacteria form chains of cells.

Extant cyanobacteria present a large morphological diversity comprising unicellular forms and filaments with different degrees of complexity, including uniseriate and multiseriate organizations and three recognized developmental alternatives. Cell differentiation in filamentous cyanobacteria includes that of micro-oxic cells, called heterocysts, specialized in the fixation of atmospheric nitrogen (N_2), which takes place under conditions of nitrogen deprivation and allows performance of the extremely O_2-sensitive N_2-fixation machinery in oxic environments; of resting cells called akinetes; and of short reproductive filaments made of small cells called hormogonia (Flores and Herrero 2014). Additionally, necridia resulting from programmed cell death have a role in the process of hormogonia release from mature filaments (Claessen *et al*. 2014; Nürnberg *et al*. 2014). Besides that, cells specialized in calcium accumulation, called calcicytes, have been described to occur in the filamentous cyanobacterium *Mastigocoleus testarum* that excavates and grows into calcium carbonates (Guida and García-Pichel 2016). Fossil akinetes have been reported in *ca*. 2 Gyr-old rocks, indicating that morphological diversity of cyanobacteria was already present at that time, several hundred million years before the emergence of eukaryotes (see Knoll 2008).

On morphological grounds cyanobacteria are classified into five sections or taxonomic orders (Rippka *et al*. 1979; Castenholz 2001). Section I (order Chroococcales) includes unicellular cyanobacteria that reproduce by binary fission or by budding, and Section II (Pleurocapsales) includes cyanobacteria that reproduce by multiple fission (through the formation of small reproductive cells called baeocytes) or by multiple and binary fission. Sections III (Oscillatoriales), IV (Nostocales) and V (Stigonematales) include filamentous cyanobacteria in which growth of the trichome occurs mainly by intercalary cell division and that reproduce by random trichome breakage. In cyanobacteria of Section III, the filaments are made of a single cell type and cell division takes place in a plane perpendicular to the trichome's long axis producing uniseriate filaments and hormogonia in some strains. Cyanobacteria of Sections IV and V have the capacity for cell differentiation into heterocysts and, in many but not all strains, into akinetes and/or hormogonia. Members of Sections IV and V are distinguished by cell division. Whereas in Section IV cyanobacteria cell division takes place perpendicular to the long axis of the filament, in Section V cyanobacteria some divisions take place at a different angle, giving rise to filaments with true branches. Phylogenetic analysis based on 16S rRNA sequences and concatenated protein sequences has led to the construction of evolutionary trees with extant species organized in a few clades, which have been compared to Sections I–V based on morphology (Shih *et al*. 2013; Sánchez-Baracaldo, Ridgwell and Raven 2014; Schirrmeister, Gugger and Donoghue 2015). Among these five taxonomic units, only Sections IV and V jointly are monophyletic (Giovannoni *et al*. 1988; Shih *et al*. 2013; Schirrmeister, Gugger and Donoghue 2015). Multicellular cyanobacteria are identified with filamentous strains, especially those capable of cell differentiation, which constitute the main subject of this review. However, although traditionally considered unicellular, some representatives of Section I form characteristic cell aggregates enclosed in laminar sheaths, and some representatives of Section II form aggregates of firmly adhered vegetative cells enclosed by an additional fibrous layer (Rippka *et al*. 1979). It would be worth investigating whether these cell aggregates exhibit some social characteristics of multicellularity.

FILAMENTOUS CYANOBACTERIA

Filamentous cyanobacteria are among the oldest multicellular organisms on our planet. The cyanobacteria constitute a monophyletic group and, although the last universal ancestor of the cyanobacterial phylum was most likely unicellular, both 16S rRNA and genome-scale phylogenetic analyses support an ancient occurrence of multicellular cyanobacteria, already present by the end of the Archean eon before the GOE (Schirrmeister, Antonelli and Bagheri 2011; Schirrmeister *et al*. 2013; Schirrmeister, Gugger and Donoghue 2015; Shih *et al*. 2013). Indeed, multicellularity could have led to increased metabolic performance favoring diversification and extension of cyanobacteria in Nature, thereby increasing oxygen production and leading to the GOE. In contrast to unicellular organisms that may form multicellular aggregates in response to an environmental cue or stress, filamentous cyanobacteria display undifferentiated multicellular lifestyles despite their costs and in the absence of stress, supporting the notion that differentiation is not a driving force for the evolution of multicellularity nor its obligatory consequence. The possible advantages that multicellularity confers to filamentous cyanobacteria are manifold: defense against predation by protists (Matz and Kjelleberg 2005), more efficient acquisition of resources and nutrient utilization (Claessen *et al*. 2014), sharing of information among individuals (Lachmann, Sella and Jablonka 2000), reduced interactions with non-cooperative competitors (Pfeiffer and Bonhoeffer 2003), and lastly, the benefits of division of labor as a result of the evolution of differentiation in a clonal population (Hamilton 1964a,b).

Filamentous cyanobacteria inhabit almost every niche in our planet. In the oceans, the availability of fixed nitrogen is recognized as one main factor limiting productivity, and

biological N_2 fixation is an important source of nitrogen in oceans. Without dismissing the substantial contribution made by culturable and unculturable unicellular cyanobacteria, the Section III representative *Trichodesmium* is probably the most abundant N_2 fixer in the open oceans (Zehr 2011). *Trichodesmium* fixes N_2 under oxic conditions during the day. Although it does not form heterocysts, the possibility that N_2 fixation takes place, perhaps transiently, in specialized cells of the filament—thus invoking cell-to-cell communication—has been debated (see Finzi-Hart *et al*. 2009). Interestingly, *Trichodesmium* requires large quantities of iron for N_2 fixation, and its organization in colonies of bundled filaments appears to be important for trapping and dissolving particulate sources of iron, thus increasing cellular iron uptake (Rubin, Berman-Frank and Shaked 2011). On the other hand, heterocyst-forming cyanobacteria are common in estuarine blooms, but occur mainly in symbiosis with unicellular algae, including diatoms, in open oceans (Zehr 2011). In terrestrial habitats, filamentous heterocyst-forming cyanobacteria can be found in extreme environments such as geothermal springs, hot deserts and Polar regions, including the dry deserts of Antarctica, perhaps the most hostile places for life on Earth. They also inhabit coastal tidal sand flats and hypersaline lagoons, forming part of microbial mats, and are constituents of the phytoplankton of rivers, lakes and reservoirs, sometimes forming blooms. In soils, filamentous cyanobacteria are found both on and below the surface, even playing the role of early colonizers in degraded soils, forming part of surface crusts of semideserts, and contributing to the soil fertility in semi-arid regions and wetland rice fields throughout the world (see Whitton and Potts 2000). In addition, filamentous cyanobacteria, frequently heterocyst formers, establish symbiotic associations with representatives of many phyla of plants and animals both on land and in aquatic environments (Adams 2000).

Due to continued research efforts, considerable insight has been gained into the dissection of cyanobacterial cell differentiation, particularly heterocyst differentiation, at a molecular level. The focus has been placed in recent years on intercellular communication and the concept of multicellularity in filamentous cyanobacteria, especially in the model strain *Anabaena* sp. PCC 7120 (here referred to as *Anabaena*). In the present review we survey the features and mechanisms that have allowed multicellularity in cyanobacteria to emerge and be maintained, including structural features that allow communication between cells in the filament, and cell division resulting in linked rather than independent cells. We further discuss the roles these structural features play under conditions of stress that lead to differentiation and division of labor, and to cell–cell coupling of gene expression along a multicellular filament. We also survey theoretical approaches that describe developmental pattern formation in these organisms.

STRUCTURE OF THE CYANOBACTERIAL FILAMENT

Cyanobacteria bear a Gram-negative-type cell envelope, which is characterized by the presence of an outer membrane outside of the peptidoglycan mesh (Hoiczyk and Hansel 2000; Hahn and Schleiff 2014). Heterocyst-forming cyanobacteria are the filamentous cyanobacteria for which the most structural information is available, although some Section III representatives have also been investigated in detail, for instance *Plectonema boryanum* (Smoker *et al*. 1989) or *Trichodesmium erythraeum* (see, e.g. van Baalen and Brown 1969).

Structure of the filament of vegetative cells

In filamentous cyanobacteria, the outer membrane does not enter into the septum between consecutive vegetative cells, and is therefore continuous along the filament. Although thoroughly recognized only recently (Flores *et al*. 2006), the presence of a continuous outer membrane was evident in the early electron microscopy literature (Ris and Singh 1961). Evidence is now available for Section III (see, e.g. Hoiczyk and Baumeister 1995), Section IV (see, e.g. Giddings and Staehelin 1978; Plominsky *et al*. 2015), and Section V (Nierzwicki-Bauer, Balkwill and Stevens 1984) representatives. The presence of a continuous outer membrane determines the existence of a structurally continuous periplasm, which is the space that lies between the cytoplasmic and outer membranes (Flores *et al*. 2006; Wilk *et al*. 2011). The periplasm contains the peptidoglycan mesh, which in cyanobacteria appears to be thicker than in better investigated Gram-negative bacteria such as *Escherichia coli* (Hoiczyk and Hansel 2000). In *Anabaena* the peptidoglycan mesh may consist of two interlinked peptidoglycan layers that surround each cell in the filament (Hahn and Schleiff 2014). The peptidoglycan mesh from adjacent cells appears to be chemically fused in many of the septa in a filament, allowing the isolation of murein sacculi that are several cell units long (Dunn and Wolk 1970; Lehner *et al*. 2011; Mariscal *et al*. 2016). In isolated murein sacculi, the septal peptidoglycan can be seen as a disk of higher density than the rest of the sacculus (Lehner *et al*. 2013).

Cyanobacteria are generally characterized by containing intracellular membranes, the thylakoids, which harbor the photosynthetic apparatus (Liberton and Pakrasi 2008). Only the unicellular *Gloeobacter violaceous*, which diverged very early from other extant cyanobacteria (Schirrmeister, Antonelli and Bagheri 2011; Shih *et al*. 2013), lacks intracellular membranes and carries out the photosynthetic reactions in specialized patches of the cytoplasmic membrane (Rexroth *et al*. 2011). Three different arrangements have been noted for the thylakoids in different cyanobacterial groups: thylakoids parallel to the cytoplasmic membrane (found in some cyanobacteria of Section III and in others such as the unicellular *Synechococcus* spp.), radial thylakoids (found e.g. in the unicellular *Synechocystis* spp., as well as in some cyanobacteria of Section III), and somewhat irregularly distributed thylakoids (found in the heterocyst formers). These arrangements have been used for taxonomic purposes (see Büdel and Kauff 2012; Flores and Herrero 2014).

The filaments of many strains of heterocyst-forming cyanobacteria can be hundreds of cells long. It has recently been shown that the components of the cell envelope (the peptidoglycan mesh and the outer membrane) are important in *Anabaena* for producing long filaments. Mutants of genes encoding proteins involved in peptidoglycan biosynthesis or remodeling, in lipopolysaccharide biosynthesis or in outer membrane protein assembly (Omp85-type proteins) generally produce filaments shorter than those produced by the wild type (Burnat, Schleiff and Flores 2014b; Videau *et al*. 2015b). These results provide evidence for the importance of the cell envelope in filamentation.

Structure of the heterocyst

When grown without a source of combined nitrogen, the filaments of heterocyst-forming cyanobacteria contain, in addition to the vegetative cells that perform oxygenic photosynthesis, cells specialized for the fixation of N_2, the heterocysts (Fay 1992; Wolk 1996). Heterocysts show a distinct pigmentation and

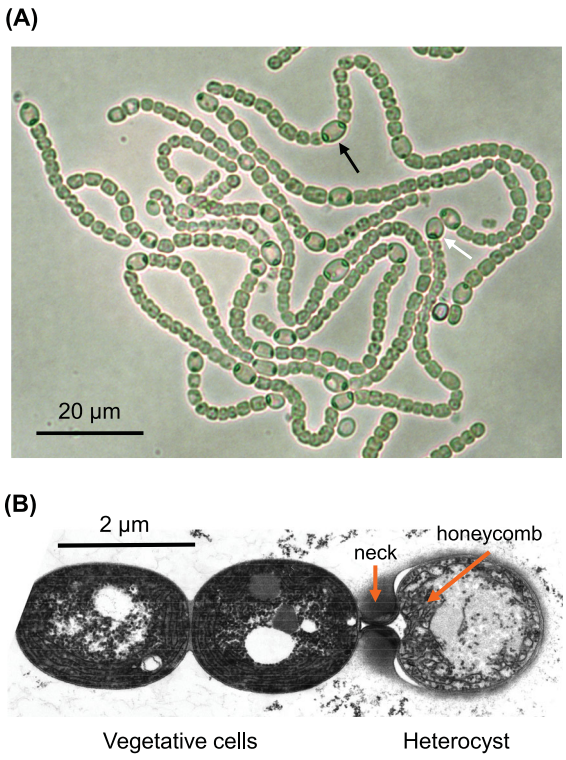

Figure 1. Filaments of cells in the heterocyst-forming cyanobacterium *Anabaena* sp. strain PCC 7120. (**A**) Light micrograph of diazotrophically grown filaments showing vegetative cells and heterocysts. One intercalary (black arrow) and one terminal (white arrow) heterocyst are indicated. Micrograph by Mireia Burnat (CSIC, Sevilla, Spain). (**B**) Transmission electron micrograph of a fragment of a filament showing two vegetative cells and one terminal heterocyst. The arrows point to the heterocyst 'neck' and 'honeycomb' structures. (Sample prepared and transmission electron microscopy performed as described in Merino-Puerto *et al.* (2011a); micrograph by V. Merino-Puerto, CSIC, Sevilla, Spain, and I. Maldener, Universität Tübingen, Germany.) Note the cyanophycin plugs at the heterocyst poles in (A). The place that the cyanophycyn plug occupies in the heterocyst neck is seen as an empty space in (B).

contain an extra cell envelope (Fig. 1). The heterocyst-specific cell envelope is composed of two chemically different layers deposited outside of the outer membrane, one made of glycolipids (heterocyst glycolipid; Hgl) and the other made of polysaccharide (heterocyst envelope polysaccharide; Hep) (see Herrero, Picossi and Flores 2013, and references therein). Whereas the Hgl layer appears to have a reduced permeability to gases, the Hep layer has been suggested to have a protective role for the Hgl layer (Xu, Elhai and Wolk 2008). Consistently, during heterocyst differentiation, Hep is deposited in the cell envelope prior to Hgl (reviewed in Nicolaisen, Hahn and Schleiff 2009a).

A distinct feature of heterocysts is the presence of structures known as 'necks' at their poles (Fig. 1B). At the heterocyst poles, the cytoplasm is narrowed and the Hgl and Hep layers are thicker than in the rest of the heterocyst (Lang and Fay 1971). Notably, a neck is only formed in a heterocyst pole next to an adjacent vegetative cell, so that terminal heterocysts have only one neck (Fig. 1B). The neck's cytoplasm is filled with cyanophycin in a structure known as the 'cyanophycin plug' ('polar plug' or 'polar nodule'; Sherman, Tucker and Sherman 2000). The presence of the neck makes the heterocyst–vegetative cell septum narrower than a typical septum between vegetative cells. It has been suggested that these are the sites of gas exchange between vegetative cells and heterocysts, their narrowness limiting gas entry into the heterocyst (Walsby 2007). A gene, *conR*, whose inactivation notably widens the heterocyst–vegetative cell septa, has been described (Fan *et al.* 2006; Mella-Herrera, Neunuebel and Golden 2011). ConR belongs to the LytR-CpsA-Psr (LCP) family of proteins (Hübscher *et al.* 2008), which in Gram-positive bacteria have roles in attaching teichoic acids and polysaccharides to peptidoglycan (Kawai *et al.* 2011) and in glycosylation of a cell wall protein (Wu *et al.* 2014). It is possible that ConR is involved in maturation of the cell envelope at the polar regions of the heterocysts, and that this process is required to make a narrow heterocyst neck.

During heterocyst differentiation, granular inclusions, including carboxysomes and glycogen granules, are lost from the cytoplasm (Lang and Fay 1971; Valladares *et al.* 2007), thylakoids are rearranged, and a special system of highly contorted intracellular membranes, known as the 'honeycomb', is built close to the heterocyst poles (Lang and Fay 1971) (Fig. 1B). The 'honeycomb' membranes appear to have a role in respiration (Valladares *et al.* 2007) and photosynthesis (Kumazaki, Akari and Hasegawa 2013).

Cell–cell joining structures

In early transmission electron microscopy (TEM) studies of heterocyst-forming cyanobacteria, the presence of some structures connecting adjacent cells in the filament was noticed (Wildon and Mercer 1963a,b). They were seen as thin structures perpendicular to the cytoplasmic membranes present in the intercellular septa (see Fig. 2A), and were afterwards termed 'microplasmodesmata' (Lang and Fay 1971). These structures were also observed in freeze-fracture electron microscopy studies as protrusions and pits on the protoplasmic and exoplasmic fracture faces, respectively, of the membranes at the intercellular septa (Giddings and Staehelin, 1978, 1981). These authors reported that microplasmodesmata have an outside diameter of less than 20 nm and that they occur in a circular area at the center of the septum. About 100–250 microplasmodesmata were present in septa between vegetative cells (Giddings and Staehelin 1981), and about 50 in septa between vegetative cells and heterocysts (Giddings and Staehelin 1978). More recently, structures described to be proteinaceous and termed 'septosomes' have been observed by electron tomography to traverse the septal peptidoglycan (Wilk *et al.* 2011). Depending on the method of staining used, septosomes can be positively stained (5.5 nm in diameter, 18 nm in length) or negatively stained (14 nm in diameter, 26 nm in length). Finally, structures traversing the septal peptidoglycan have been observed by cryo-electron tomography of whole filaments of *Anabaena* and termed 'channels' (Omairi-Nasser, Haselkorn and Austin 2014). The channels are reported to be 12 nm long and 12 nm in diameter in the septa between vegetative cells, and 21 nm long and 14 nm in diameter in the septa between vegetative cells and heterocysts. As mentioned above, septal peptidoglycan disks can be observed in isolated murein sacculi from filaments of heterocyst-forming cyanobacteria. These disks show perforations (see Fig. 2B) that have been termed 'nanopores' (Lehner *et al.* 2013) and that likely are the same structures described as peptidoglycan 'pores' in the early electron microscopy literature (Metzner 1955). In the central part of the septal peptidoglycan disk between vegetative cells, an array of about 155 nanopores (each about 20 nm in diameter) has been described in *Nostoc punctiforme* (Lehner *et al.* 2013) and an array of about 75 nanopores (each about 15 nm in diameter) in *Anabaena* (Nürnberg *et al.* 2015).

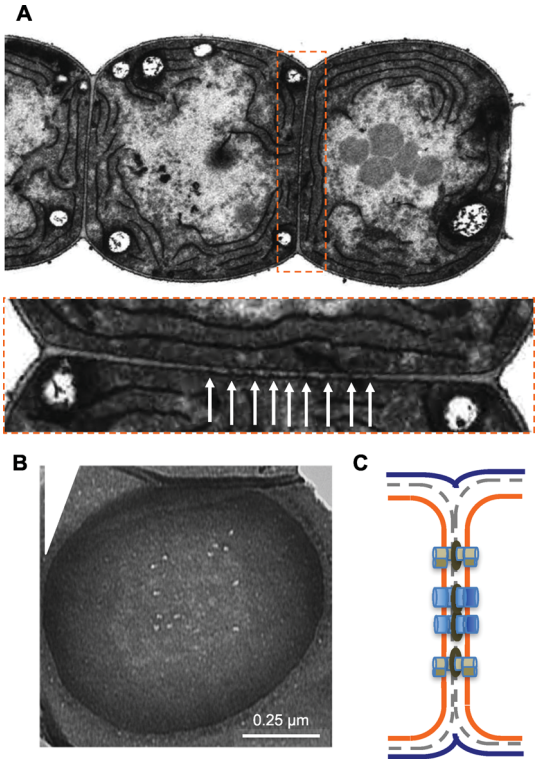

Figure 2. Septal structures in *Anabaena* sp. strain PCC 7120. (**A**) Part of a filament showing vegetative cells and highlighting the septum between two adjacent cells. Arrows point to septal junctions. Top micrograph, magnification 8000×. (TEM performed by Iris Maldener, Universität Tübingen, Germany, as described; Flores *et al.* 2007.) (**B**) Transmission electron micrograph of a peptidoglycan septal disk, where nanopores (perforations in the septal peptidoglycan) are visible. (Murein sacculi isolated and TEM performed by Mercedes Nieves-Morión and Vicente Mariscal, CSIC, Sevilla, Spain, as described; Mariscal *et al.* 2016.) (**C**) Schematic diagram of a septum between vegetative cells reflecting the current hypothesis on the composition of septal junctions. Blue structures, multidomain SepJ protein complexes; pistachio structures, complexes containing multidomain FraD and cytoplasmic-membrane FraC proteins. Dark blue line, outer membrane; discontinuous grey line, peptidoglycan; orange line, cytoplasmic membrane. Nanopores are represented as brownish-green ovals. Note that the outer membrane does not enter into the septum.

It is hypothesized that in heterocyst-forming cyanobacteria, proteinaceous septal structures, now termed 'septal junctions' (Mariscal 2014; Mullineaux and Nürnberg 2014; Flores *et al.* 2016), traverse the septal peptidoglycan connecting adjacent cells (Fig. 2C). The septal junctions would correspond to the microplasmodesmata observed by TEM and freeze-fracture electron microscopy, and to the positively stained septosomes. The perforations in the septal peptidoglycan, which could harbor septal junctions, would correspond to the negatively stained septosomes, to the channels observed by cryo-electron tomography and to the nanopores of the septal peptidoglycan disks observed in isolated murein sacculi (Fig. 2B). Summarizing available data, septal junctions may have a size of 12–26 nm in length and 5.5–14 nm in diameter, and the peptidoglycan nanopores that accommodate them may have a diameter up to about 20 nm.

SEPTAL PROTEINS

Many heterocyst-forming cyanobacteria grow as chains of cells that can be hundreds of cells long, but mutant strains that grow making short filaments, or which make filaments that fragment when transferred from medium containing combined nitrogen to medium lacking any source of combined nitrogen, have been isolated from *Anabaena* (Buikema and Haselkorn 1991; Ernst *et al.* 1992). Genes mutated in some of these strains have been identified and named *fraC*, *fraH* (Bauer *et al.* 1995) and *sepJ* (Flores *et al.* 2007) (also known as *fraG*; Nayar *et al.* 2007).

SepJ

Mutants bearing an inactivated *sepJ* gene make short filaments (mean size, about seven cells per filament) when growing in medium containing combined nitrogen, and they fragment to produce filaments with a mean of about two cells per filament when transferred to medium lacking any source of combined nitrogen (Flores *et al.* 2007; Nayar *et al.* 2007; Mariscal *et al.* 2011). Heterocyst differentiation is arrested at an early stage in these mutants. A SepJ–green fluorescent protein (GFP) fusion protein carrying the GFP fused close to the C-terminus of SepJ localizes to the cell poles in a focused region in the center of the septa between vegetative cells (Fig. 3) (Flores *et al.* 2007; Mariscal *et al.* 2011). In the septa between vegetative cells and heterocysts, two GFP fluorescence foci can be seen, one closest to the vegetative cell and the other closest to the heterocyst (Flores *et al.* 2007; Flores and Herrero 2010), and in some micrographs the latter can be seen extending towards the heterocyst neck (Omairi-Nasser *et al.* 2015; Flores *et al.* 2016). In summary, SepJ is a septal protein that is needed to keep cells bound to each other, making long filaments both in the presence and, mainly, in the absence of combined nitrogen. Consistently, expression of the *sepJ* gene can be detected in filaments grown with combined nitrogen, although it increases moderately upon N step-down and is higher in diazotrophically grown filaments than in the presence of combined nitrogen (Flores *et al.* 2007). This increased expression appears to concentrate in heterocysts (Nayar *et al.* 2007).

In *Anabaena*, SepJ is a 751-amino-acid protein that is predicted to have three well-differentiated domains: a coiled-coil domain in the N-terminal region (residues 28–207); a central linker domain (residues 208–411) rich in Pro, Ser and Thr residues; and an integral membrane domain in the C-terminal region (residues 412–751) (Fig. 4A). This general structure is strongly conserved in SepJ from heterocyst-forming cyanobacteria, although the linker domain is very variable in length and amino acid sequence. Inspection of SepJ from 20 different heterocyst formers shows linker domains of 67 to 317 amino acids (mean, 205 amino acids). However, the linker is conserved in amino acid composition, generally containing high percentages of Pro (frequently 15 to 20%) and Ser (10 to 20%), in addition to Thr (6 to 15%) and, in some cases, Glu (around 10%). Inspection of SepJ from different heterocyst formers also reveals strong conservation in the 26 N-terminal amino acids and a high conservation in the coiled-coil domain, which consists of two strongly predicted coiled-coil motifs (Fig. 4B; CC1 and CC2 in Fig. 4A). Indeed, antibodies raised against the coiled-coil domain of *Anabaena* label the intercellular septa in *Mastigocladus laminosus* (Nürnberg *et al.* 2014), which is a true-branching (Section V) heterocyst-forming cyanobacterium phylogenetically distant from *Anabaena* (Shih *et al.* 2013). Regarding the integral membrane domain, two subdomains can be distinguished (IM1 and IM2 in Fig. 4A). The last eight transmembrane segments (TMSs) constitute IM2 and are strongly conserved in all available SepJ sequences except that of *Cylindrospermopsis raciborskii* (Plominsky *et al.* 2015). (In this cyanobacterium, the *sepJ* gene appears to be split into two genes, *Crc_03186* encoding a protein

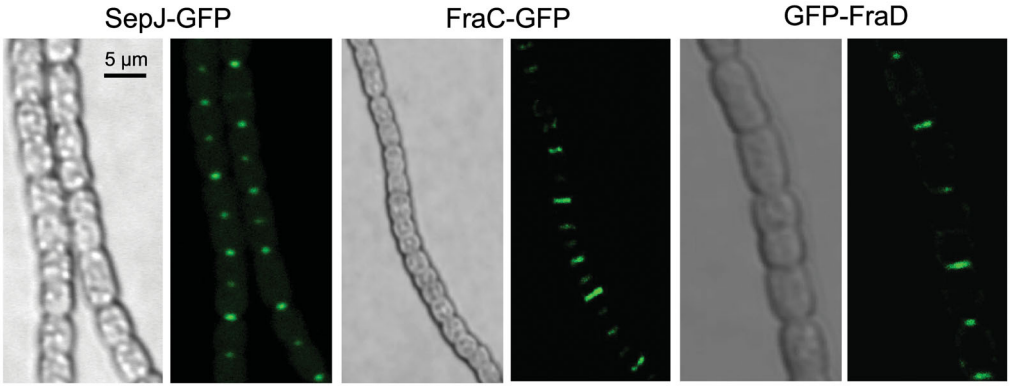

Figure 3. Septal localization of SepJ, FraC and FraD in *Anabaena*. Bright field (left) and GFP fluorescence (right) micrographs of filaments of *Anabaena* sp. strain CSAM137 (*sepJ-gfp*), CSVT13 (*fraC-gfp*) and CSVT21 (*gfp-fraD*) grown in nitrate-containing medium. (Micrographs by Mercedes Nieves-Morión and Victoria Merino-Puerto, CSIC, Seville, Spain.)

that contains the coiled-coil and linker domains and an integral membrane domain made of six TMSs, and *Crc_03185* encoding a protein that consists of two TMSs that correspond to the last two TMSs of *Anabaena* SepJ.) The IM2 subdomain contains the sequences responsible for similarity of SepJ to proteins in the drug/metabolite exporter (DME) family (Transporter Classification Database (TCDB) number 2.A.7.3; http://tcdb.org/). Subdomain IM1 is predicted to contain one, two or three TMSs in SepJ from different cyanobacteria. Because there is evidence that the C-terminus of SepJ is cytoplasmic (Flores *et al*. 2007; Ramos-León *et al*. 2015), an odd number of TMSs would position the N-terminal, extra-membrane part of SepJ (including the coiled-coil and linker domains) in the periplasm, whereas an even number of TMSs would leave it in the cytoplasm. This is an important issue for understanding SepJ function (Flores *et al*. 2016).

Although in the absence of structural information bioinformatics analysis has not solved the topology of SepJ, available experimental evidence suggests a periplasmic location of the extra-membrane part of SepJ. First, SepJ has been shown to interact through its linker domain with a periplasmic domain of FtsQ, a protein that establishes protein–protein interactions in the divisome (Ramos-León *et al*. 2015; see below). Second, SepJ has been shown to interact with SjcF1, a peptidoglycan-binding protein, in an interaction that appears to be mediated by a periplasmic SH3 domain of SjcF1 (Rudolf *et al*. 2015; see below). Finally, immunogold localization of the SepJ coiled-coil domain in an *Anabaena* strain overproducing SepJ clearly localizes that domain in the septa between vegetative cells (see Omairi-Nasser *et al*. 2015). Thus, SepJ is an integral membrane protein that is located at the cell poles and likely has a large periplasmic section. Because the coiled-coil domains are generally involved in protein–protein interactions, the coiled-coil domains of SepJ proteins from adjacent cells have been hypothesized to interact (Flores *et al*. 2007). Additionally, the high abundance of Pro and Ser/Thr residues in the linker domain, which resembles the amino acid composition of cell wall-interacting proteins in plants, is suggestive of possible interactions with the peptidoglycan mesh. In any case, as shown with the bacterial two-hybrid system (Ramos-León *et al*. 2015), the SepJ protein is strongly involved in self-interactions likely forming multimers.

The 'microplasmodesmata' observed by freeze-fracture electron microcopy were also described for filamentous non-heterocyst-forming cyanobacteria (Giddings and Staehelin 1981). It is therefore of interest that BLAST searches indicate that SepJ is also present in this type of cyanobacteria (Nürnberg *et al*. 2014). Whereas we previously deduced that the filamentous non-heterocyst-forming cyanobacteria contained a special type of SepJ protein lacking a linker domain (Mariscal *et al*. 2011; Nürnberg *et al*. 2014), it is now clear that a linker domain is always present in SepJ, although sometimes it is very short. For instance, in the case of SepJ from *Arthrospira platensis* NIES-39, it is only about 40 amino acids in length. Nonetheless, these short linker domains maintain the characteristic abundance in Pro and Ser residues (22 and 15%, respectively, in the case of SepJ from *A. platensis*). Finally, BLAST searches also identify a homologue of SepJ in many unicellular cyanobacteria (Nürnberg *et al*. 2014), but rather than SepJ, these are DME permeases of these organisms (Escudero, Mariscal and Flores 2015).

The Fra proteins

The *fraC* gene is part of a gene cluster that contains an operon of genes, *fraC–fraD–fraE*, that are required to make long filaments under nitrogen deprivation (Merino-Puerto *et al*. 2010) (Fig. 5A). The cluster also contains, located downstream from the operon in the opposite orientation, *fraF*, whose inactivation results in increased frequencies of long filaments, implying that FraF is needed to restrict filament length (Merino-Puerto, Herrero and Flores 2013). The *fraC* operon is highly conserved in filamentous cyanobacteria, except for the Pseudanabaenas that represent a phylogenetically distinct group of Section III cyanobacteria (Shih *et al*. 2013), and the *fraC–fraD–fraE←fraF* cluster (where the arrow indicates a change in orientation) is frequently conserved in heterocyst-forming cyanobacteria. Whereas the *fraC* operon is expressed at a low and constitutive level, *fraF* is induced by nitrogen deprivation producing mRNAs that overlap as antisense RNAs *fraE* and, partly, *fraD* (Fig. 5A; Merino-Puerto, Herrero and Flores 2013; Ehira and Ohmori 2014). The role of the increased expression of *fraF* under nitrogen deprivation has not been discerned yet, since the effect of inactivation of *fraF* increasing filament length is observed in the presence as well as in the absence of combined nitrogen (Merino-Puerto, Herrero and Flores 2013). In contrast, the increase in filament length that results from eliminating the antisense RNA is observed only in the absence of combined nitrogen (Merino-Puerto, Herrero and Flores 2013). A *fraF* inactivation mutant is specifically impaired in diazotrophic growth on solid medium.

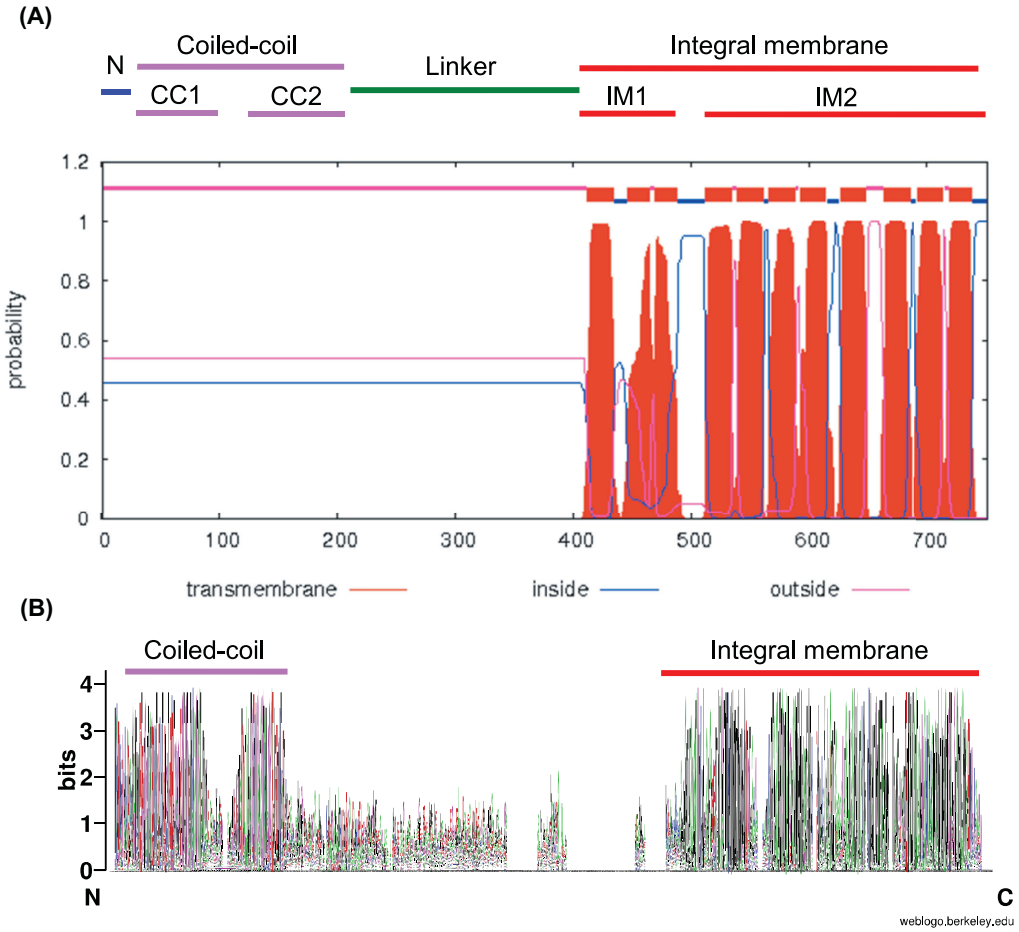

Figure 4. Primary structure of the SepJ protein. (**A**) Predicted topology of SepJ from *Anabaena* sp. strain PCC 7120. The general structure of the protein, containing coiled-coil, linker and integral membrane domains, is shown. Subdomains CC1 (residues 28–95), CC2 (residues 127–207), IM1 (residues 412–488) and IM2 (residues 512–751), as well as the presence of a strongly conserved N-terminal sequence (N), are noted. Prediction of transmembrane segments was performed by the TMHMM program at Expasy (http://www.expasy.org/tools/). (**B**) Weblogo of amino acid sequence conservation in SepJ from 37 heterocyst-forming cyanobacteria (done with default parameters at http://weblogo.berkeley.edu/). Note the strong conservation of the N-terminal, coiled-coil and integral membrane domains. Protein length does not correspond to the *Anabaena* protein shown in (A) because of the variable length of the linker domain in different cyanobacteria.

FraC, FraD and FraE are predicted integral membrane proteins (Fig. 5B), and FraC–GFP and GFP–FraD fusions (the order in protein names indicates N to C position in the polypeptide) have shown localization in the cell poles at the intercellular septa, although localization is less focused than that of SepJ–GFP (Merino-Puerto *et al.* 2010) (Fig. 3). FraD has a long extramembrane section with a coiled-coil domain (Fig. 5B), and immunogold labeling has shown this section to be located between the cytoplasmic membranes of the two adjacent cells in the septum (Merino-Puerto *et al.* 2011a). Whereas neither FraC nor FraD shows homology to proteins in the databases outside of the cyanobacteria, FraE is homologous to the permease components of some ABC transporters. In particular, it is most similar to the type IV pilus biogenesis protein PilI from *Myxococcus xanthus* (TCDB number 3.A.1.144.5; http://tcdb.org/), which is a component of an ABC exporter necessary for pilus assembly and pilus subunit (PilA) export (Wu *et al.* 1998). The *fraE* mutant has a distinct phenotype showing negligible nitrogenase activity, whereas the *fraC* and *fraD* mutants show nitrogenase activity lower than the wild type but detectable (Merino-Puerto *et al.* 2010). Structurally, whereas the *fraE* mutant makes heterocysts that lack a well-formed heterocyst neck, the *fraC* and *fraD* mutants make heterocysts showing heterocyst necks in which the septal region of the heterocyst cytoplasmic membrane is withdrawn towards the heterocyst interior (Merino-Puerto *et al.* 2011a) (Fig. 5D). The phenotypic similarity of *fraC*, *fraD* and double *fraC fraD* mutants, and the coincident location of FraC–GFP and GFP–FraD at the intercellular septa suggest that FraC and FraD could work together in filamentation (Merino-Puerto *et al.* 2011a).

FraF is a pentapeptide-repeat protein with a structure, as predicted by Phyre2 (www.sbg.bio.ic.ac.uk/phyre2/), very similar to that of HetL (Ni *et al.* 2009) (Fig. 5C). Proteins of this type adopt a highly regular four-sided, right-handed β helical structure. A FraF–GFP fusion shows a cytoplasmic location and higher level in heterocysts than in vegetative cells (Merino-Puerto, Herrero and Flores 2013). Whereas the mechanism through which FraF restricts filament length is unknown, the presence of *fraF* in a filamentation gene cluster may indicate a role in counteracting the effect of positive filamentation proteins such as FraC, FraD and FraE. Of note, in a heterocyst-forming cyanobacterium in which the *fraC* operon is apparently not conserved, *Fischerella muscicola* strain PCC 7414, a *fraE*←*fraF* gene pair is found (https://img.jgi.doe.gov/cgi-bin/m/main.cgi). A number of

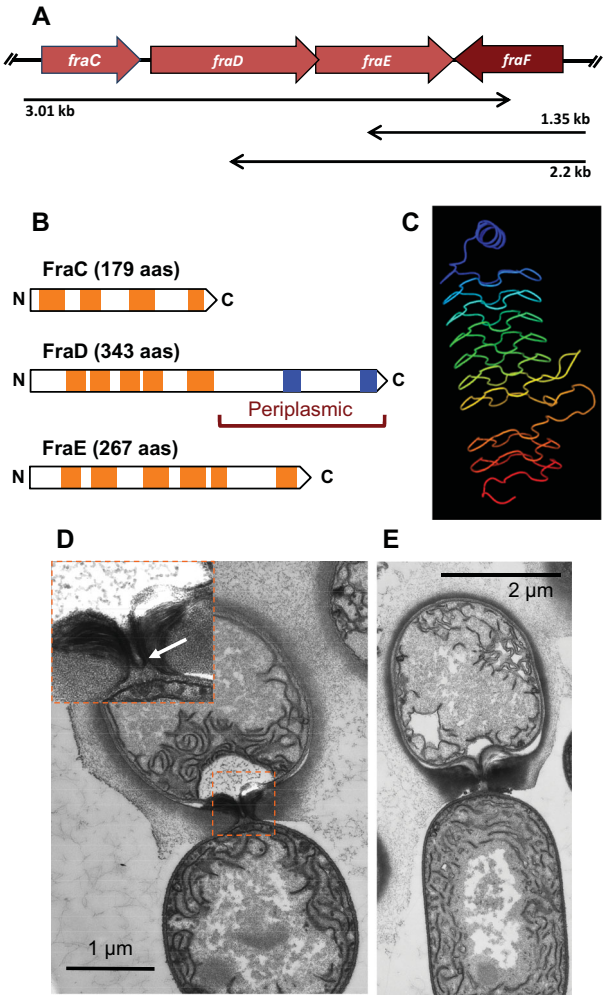

Figure 5. The *fra* gene cluster and encoded Fra proteins. (**A**) Scheme of the *fra* genomic region in *Anabaena* sp. strain PCC 7120. The transcripts that have been identified in this region (Merino-Puerto, Herrero and Flores 2013) are indicated. (**B**) Schemes of the FraC, FraD and FraE proteins. Orange boxes denote transmembrane segments (approximate location as deduced by MEMSAT-SVM [http://bioinf.cs.ucl.ac.uk/psipred/]). Blue boxes denote coiled-coil motifs. (**C**) 3D model of the FraF protein predicted by Phyre2. Color code: blue to red, N-terminal to C-terminal sequences. (**D**) Transmission electron micrograph of a fragment of a filament of *Anabaena* sp. strain CSVT2 (Δ*fraD*) showing one vegetative cell and one terminal heterocyst. The arrow points to the retracted cytoplasmic membrane at the heterocyst 'neck'. (**E**) Transmission electron micrograph of a fragment of a filament of *Anabaena* sp. strain CSVT4 (Δ*fraH*) showing one vegetative cell and one terminal heterocyst. (Samples prepared and transmission electron microscopy performed as described in Merino-Puerto *et al.* (2011a,b). Micrographs by V. Merino-Puerto, CSIC, Sevilla, Spain, and I. Maldener, Universität Tübingen, Germany.)

heterocyst differentiation proteins such as HglK (Black, Buikema and Haselkorn 1995), HetL (Liu and Golden 2002) and PatL (Liu and Wolk 2011) are also pentapeptide repeat proteins. It has been conjectured that since the pentapeptide repeat domain has the structure of a rectangular parallelepiped, pentapeptide repeat proteins may interact by stacking (Liu and Wolk 2011). Possible interactions between these pentapeptide repeat proteins or between them and filamentation proteins are, however, yet to be investigated.

The *fraH* gene is in a chromosomal location distant from the other *fra* genes (Kaneko *et al.* 2001). *fraH* is induced under nitrogen deprivation (Merino-Puerto *et al.* 2010), resulting in levels of the FraH protein that are higher in diazotrophically grown than in nitrate-grown filaments (Stenjö *et al.* 2007). The increased expression of *fraH* under nitrogen deprivation may be related to a specific role of FraH in heterocyst differentiation (Merino-Puerto *et al.* 2011b). FraH is a 289-amino-acid protein composed of three domains: an N-terminal region with a putative double zinc ribbon domain (residues 4–48), a central domain (residues 58–215) rich in Pro (27.8%), and a C-terminal putative forkhead-associated (FHA) domain (residues 204–260). The zinc ribbon and FHA domains are known molecular interaction domains (the latter frequently involving phosphoproteins), and the Pro-rich domain is reminiscent of plant cell wall extensins. FraH may have one TMS, and a FraH–GFP fusion localizes as spots in the periphery of vegetative cells and, conspicuously, at the heterocyst poles in a location similar to that of the 'honeycomb' membranes (Merino-Puerto *et al.* 2011b). Notably, a *fraH* inactivation mutant lacks the heterocyst 'honeycomb' membrane system (Fig. 5E) (Merino-Puerto *et al.* 2011b). Finally, *fraH* is generally found in the genomes of heterocyst-forming cyanobacteria (and frequently in other cyanobacteria) downstream of the gene encoding 6-phosphogluconolactonase of the pentose phosphate pathway, *alr1602* in *Anabaena*. Downstream of *fraH*, genes encoding a protease and a peptidoglycan hydrolase, both involved in cell wall remodeling, are frequently found (https://img.jgi.doe.gov/cgi-bin/m/main.cgi). In summary, FraH is a protein likely involved in protein–protein interactions that may have a role in the differentiation of the heterocyst polar regions. This may be related to the observation that the filament fragmentation phenotype of *fraH* mutants involves the frequent release of heterocysts from the filaments (Bauer *et al.* 1995).

HETEROCYST DIFFERENTIATION AND PATTERNING

Heterocyst differentiation takes place mainly in response to the external cue of combined nitrogen deprivation. During differentiation, the structural changes described earlier are accompanied by extensive modifications of cellular metabolism. Thus, the phototropic metabolism of the mother vegetative cell is transformed to a photoheterotrophic metabolism that facilitates the function of the nitrogenase complex, which is expressed in the differentiated heterocyst. All these structural and metabolic changes result from the establishment of a pattern of gene expression that conspicuously differs from that taking place in the vegetative cells (for more extensive reviews on this topic, see Xu, Elhai and Wolk 2008; Kumar, Mella-Herrera and Golden 2010; Herrero, Picossi and Flores 2013). Gene expression during heterocyst differentiation is orchestrated by two main transcription factors, NtcA and HetR. Whereas HetR is specifically required for cellular differentiation (Flaherty, Johnson and Golden 2014), NtcA is a global regulator of universal distribution in cyanobacteria that belongs to the CRP family of transcription factors and responds primarily to the cue of nitrogen deficiency (Picossi, Flores and Herrero 2014). Thus, NtcA is activated by binding of 2-oxoglutarate (Valladares, Flores and Herrero 2008; Zhao *et al.* 2010), which accumulates in cyanobacteria under nitrogen deprivation (Muro-Pastor, Reyes and Florencio 2001). At advanced stages of differentiation transcription activation by NtcA is aided by the small protein PipX (Valladares *et al.* 2011; Camargo *et al.* 2014). The *ntcA* and *hetR* genes are subject to positive mutual regulation and to auto-regulation, and

together they activate or repress many heterocyst differentiation genes in a hierarchical manner (see Herrero, Picossi and Flores 2013 for a detailed description). Among the activated genes, *patS* and *hetN* have been identified to play a role in the establishment of the pattern of heterocyst distribution along the filament, which in *Anabaena* consists of linear arrays of heterocysts separated by about 10–15 vegetative cells. The heterocysts are terminal cells that do not divide. Notably, under steady-state diazotrophic growth, the heterocyst pattern is maintained by differentiation of new heterocysts at about the center of the vegetative cell intervals, which increase through vegetative cell division.

In *Anabaena* the *patS* gene is transcribed at low levels during growth in the presence of ammonium, and its expression is activated early during differentiation in small cell clusters. Inactivation of *patS* increases the total number of heterocysts and leads to the multiple contiguous heterocysts (Mch) phenotype, whereas its over-expression in vegetative cells abolishes differentiation (Yoon and Golden 1998, 2001; Corrales-Guerrero, Flores and Herrero 2013; Corrales-Guerrero *et al.* 2015). The primary *patS* gene product is a peptide of 17 amino acids that is processed in the producing cells to render a morphogen made of a C-terminal peptide—which could consist of five, six or eight amino acids—that is transferred to neighboring cells inhibiting their differentiation (Yoon and Golden 1998, 2001; Corrales-Guerrero, Flores and Herrero 2013; Hu *et al.* 2015). Indeed, a gradient of PatS has been detected in cells that flank producing cells, with the amount of the morphogen decreasing with distance (Corrales-Guerrero, Flores and Herrero 2013). In *Anabaena* inactivation of the *hetN* gene, which is expressed in the heterocysts, also leads to a Mch phenotype, although this phenotype is delayed with regard to that produced by *patS* inactivation (Black and Wolk 1994; Higa *et al.* 2012; Corrales-Guerrero *et al.* 2014b). The *hetN* gene encodes a protein that includes an internal ERGSGR peptide that is identical to the C-terminal hexapeptide of PatS, and could also generate an intercellular signal that would affect heterocyst differentiation negatively, involving the residues in common with PatS (Higa *et al.* 2012; Corrales-Guerrero *et al.* 2014b). However, the whole HetN protein appears to have an enzymatic function that could be related to photosynthesis/respiration in the heterocysts, consistent with its location to thylakoid membranes and its similarity to fatty acid oxidoreductases (Black and Wolk 1994; Corrales-Guerrero *et al.* 2014b). In addition, in *Anabaena*, a peptide of 84 amino acids, PatC, has been reported to act in long-range maintenance of the heterocyst pattern (Corrales-Guerrero, Flores and Herrero 2014a). However, the possible intercellular movement of this peptide has not been studied yet.

Besides regulation at the level of gene expression, the HetR transcription factor is subject to post-transcriptional regulation. HetR can be phosphorylated with a negative impact of phosphorylation on the accumulation of HetR tetramers, which could represent the transcriptionally active form of HetR (Valladares, Flores and Herrero 2016). On the other hand, regulation of the turnover of HetR has been reported, with several factors including HetF, a predicted protease, and PatA, with similarity to the CheY response regulator (Risser and Callahan 2008), as well as PatS and HetN, participating in this regulation. Indeed, negative regulation of the HetR protein levels by PatS and HetN has been suggested to determine gradients of HetR along the filament with concentrations increasing away from heterocysts (Risser and Callahan 2009).

PATHWAYS FOR INTERCELLULAR COMMUNICATION

In both developing and fully differentiated filaments of heterocyst-forming cyanobacteria, an exchange of substances, including nutrients and regulators, takes place, as will be detailed below. These exchanges are crucial for differentiation and nutrition in the diazotrophic filament. However, intercellular communication likely occurs also in the undifferentiated filament of heterocyst formers as evidenced by the correlation of gene expression fluctuations between nearby cells (Corrales-Guerrero *et al.* 2015), in non-heterocyst formers, and along hormogonia. As an example, motility of filamentous cyanobacteria on surfaces, which is important to find favorable light and nutritional conditions, and motility of hormogonia, which is needed to establish symbiotic associations with plants, have been considered to involve the coordinated activity of molecular motors in the different cells of a filament, likely requiring cell-to-cell communication (Khayatan, Meeks and Risser 2015; Wilde and Mullineaux 2015). The structural information discussed in previous sections suggests two possible pathways for intercellular molecular exchange in the cyanobacterial filaments: the continuous periplasm and direct cell–cell joining structures.

A functionally continuous periplasm

The question of whether a structurally continuous periplasm is also functionally continuous was addressed in *Anabaena* by expressing a periplasmic GFP in developing heterocysts. The GFP (molecular mass, 27 kDa) could be observed in the periphery of cells away from the producing cell but, importantly, a GFP engineered to be anchored to the cytoplasmic membrane remained exclusively in the producing cell (Mariscal, Herrero and Flores 2007; Flores and Herrero 2010) (Fig. 6). These results imply that the GFP diffuses along the filament's periplasm, corroborating that the periplasm can be functionally continuous. However, the GFP is not evenly distributed along the filament suggesting that barriers, likely corresponding to the peptidoglycan mesh, exist (Mariscal, Herrero and Flores 2007). The possible presence of barriers has been emphasized by Zhang *et al.*, who described lack of diffusion of GFP and of the smaller fluorescent protein iLOV (13 kDa) in the periplasm (Zhang *et al.* 2008, 2013). In the case of GFP, the use of a heterologous targeting signal by Zhang *et al.* could have represented a problem for localization in the periplasm as a free protein, whereas in the case of iLOV, which uses a flavin cofactor, the cofactor could have not been retained in the periplasm. In any case, contrasting results from different laboratories could arise from different growth conditions, which may result in peptidoglycan with a more relaxed or stretched structure (Vollmer and Seligman 2010). However, there is agreement that the peptidoglycan mesh is unlikely to be an effective barrier for small molecules including metabolites (Mariscal, Herrero and Flores 2007; Zhang *et al.* 2008).

For communication through the periplasm, specific cytoplasmic membrane transporters (exporters and importers) for substances exchanged intercellularly should be present in vegetative cells and heterocysts. Many transporters are present in the cytoplasmic membrane of a cyanobacterium such as *Anabaena* (Hahn and Schleiff 2014), including transporters for amino acids, oxoacids and sucrose (Nicolaisen *et al.* 2009b; Pernil, Herrero and Flores 2010b; Pernil *et al.* 2015), and some amino acid transporters are needed for optimal diazotrophic growth (Picossi *et al.*

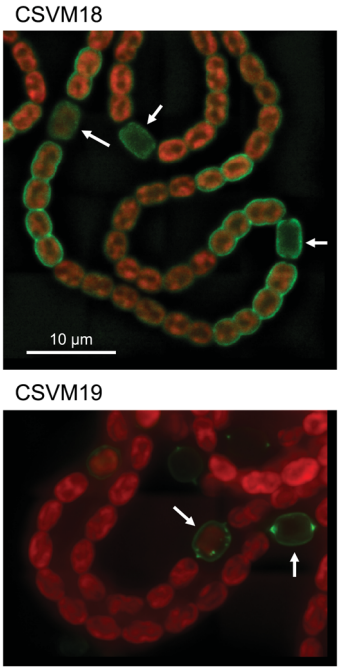

Figure 6. Functionally continuous periplasm in *Anabaena*. The *gfp–mut2* gene was expressed from the *patS* promoter in developing heterocysts producing a GFP protein that is released into the periplasm (strain CSVM18, upper) or a GFP protein that is anchored to the membrane (strain CSVM19, lower) (for details, see Mariscal, Herrero and Flores 2007). Overlay of cyanobacterial autofluorescence (red) and GFP fluorescence (green) is shown. Cells producing the GFP are indicated by arrows. Note that in strain CSVM18 the GFP fluorescence is observed not only in cells expressing the GFP but also in adjacent cells. (Micrograph by Vicente Mariscal, CSIC, Sevilla, Spain.)

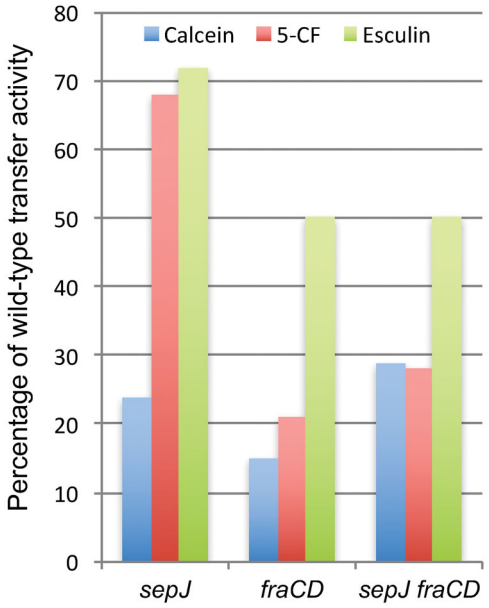

Figure 7. Intercellular transfer of fluorescent tracers in *Anabaena sepJ* and *fraC fraD* mutants. The recovery rate constant (*R*) for the transfer of the indicated tracers between vegetative cells of nitrate-grown filaments is represented as a percentage of the wild-type values for *sepJ*, *fraC fraD* and *sepJ fraC fraD* mutants. Data redrawn from Nürnberg *et al.* (2015).

2005; Pernil *et al.* 2008; see below). If the periplasm were a significant communication conduit between cells, the outer membrane of heterocyst-forming cyanobacteria would be expected to be an effective barrier for intercellularly exchanged substances. Indeed, mutants of some outer membrane components of *Anabaena* exhibit increased permeability for sucrose and glutamate, implying that the outer membrane represents a permeability barrier for these metabolites (Nicolaisen *et al.* 2009b). This feature could contribute to retention in the periplasm of the transferred metabolites, hampering their leakage to the external medium. Although the outer membrane proteome of *Anabaena* includes some porins, these proteins are now known to have size- and charge-based selectivity, and porins through which intercellularly exchanged metabolites could be lost from the filament may be absent (Hahn and Schleiff 2014). These reflections are consistent with the idea that the periplasm could be a communication conduit in the filament (Flores *et al.* 2006), though no direct evidence for such a role is available.

Septal junctions and intercellular communication

Here we describe recent advances in the understanding of direct cell-to-cell communication in the filaments. A major contribution to this topic was the introduction of fluorescent probes to test intercellular molecular exchange (Mullineaux *et al.* 2008). The fluorescein derivatives calcein and 5-carboxyfluorescein (5-CF) are provided to the filaments as acetoxymethyl ester derivatives that are hydrophobic and readily permeate into the cells, where they are hydrolyzed by cytoplasmic esterases producing fluorescent, hydrophilic substances. Because calcein and 5-CF are retained within the cells, fluorescence recovery after photobleaching (FRAP) experiments can then be performed (Mullineaux *et al.* 2008; Mariscal *et al.* 2011). In these experiments, the fluorescence of a given cell is bleached and its recovery is quantified over time, normally for less than 1 min. Importantly, the fluorescence recovered in the bleached cell corresponds to fluorescence lost from neighboring cells, indicating that recovery results from intercellular movement of the probe (Mullineaux *et al.* 2008). Additionally, movement always takes place down the concentration gradient of the fluorescent probe, which is indicative of diffusion. This in turn suggests the presence of communication conduits between the cells in the filament.

Calcein (623 Da) and 5-CF (376 Da) are negatively charged molecules that represent artificial probes. A fluorescent probe, esculin (a coumarin β-glucoside, 340 Da), that is an analog of sucrose, an important vehicle of reduced carbon in the filament (see below), has now been introduced (Nürnberg *et al.* 2015). In contrast to calcein or 5-CF, esculin is incorporated into cells by sugar transporter(s), but esculin-loaded filaments can also be subjected to FRAP analysis showing that esculin can move from cell to cell in a similar way as the fluorescein probes (Nürnberg *et al.* 2015).

Mutants lacking SepJ, FraC and/or FraD have been used in FRAP analysis with calcein, 5-CF and esculin, and all of them are impaired in intercellular exchange of the probes (Mullineaux *et al.* 2008; Mariscal *et al.* 2011; Merino-Puerto *et al.* 2010, 2011a; Nürnberg *et al.* 2015). These results indicate that SepJ, FraC and FraD are needed to build intercellular molecular transfer structures. However, whereas mutants lacking FraC and/or FraD are most strongly impaired in the transfer of calcein and 5-CF, mutants lacking SepJ are more affected in the transfer of calcein than of 5-CF or esculin (Fig. 7), which led to the suggestion that at least two types of conduits may be present between cells (Merino-Puerto *et al.* 2011a; Nürnberg *et al.* 2015).

Mutants lacking SepJ and/or FraC and FraD also show a decreased number of nanopores in the septal peptidoglycan disks, about 10% of the number in wild-type *Anabaena* (Nürnberg *et al.* 2015). This observation suggests that SepJ and FraC/FraD are together needed to make a normal number of nanopores. An appealing hypothesis is that SepJ and FraC/FraD contribute to the formation of 'septal junctions', which would traverse the septal peptidoglycan mesh through the nanopores (Fig. 2C). Different types of 'septal junctions' may exist with regard to protein composition. In this scenario, SepJ-related 'septal junctions' would be the main conduits responsible for the intercellular exchange of calcein, with FraC and FraD contributing to the proper localization of SepJ (Merino-Puerto *et al.* 2010). On the other hand, FraC/FraD-related junctions would also mediate intercellular molecular exchange, since *fraC fraD* mutants are more affected in the intercellular transfer of fluorescent probes than *sepJ* mutants (Fig. 7). Finally, a *sepJ fraC fraD* mutant still shows substantial activity of intercellular transfer of probes (Fig. 7) and about 7% the number of nanopores observed in the wild type (Nürnberg *et al.* 2015). Hence, a pathway or mechanism of intercellular molecular exchange independent of SepJ and the Fra proteins may additionally exist. Although direct communication through septal junctions appears to be well sustained, currently available evidence is based on the use of fluorescent probes (calcein, 5-CF and esculin), of which only one (esculin) is an analog of a physiologically relevant substrate, sucrose (Nürnberg *et al.* 2015). Thus, the pathway(s) through which physiological substrates, including nutrients and regulators, are exchanged between cells in the filament await identification. Nonetheless, significant information regarding regulators is being gained (see below).

In this section, we have covered information indicating that both the continuous periplasm and cell–cell joining complexes could be involved in intercellular molecular exchange in the filaments of heterocyst-forming cyanobacteria. A unifying hypothesis is that septal junctions are main mediators of cell–cell communication, and that material (including metabolites and regulators) leaked from the septal junctions is retained in the periplasm, from where it can then be recovered by the cells using cytoplasmic membrane transporters.

INTERCELLULAR MOLECULAR TRANSFER AND SIGNALING

In the filament of heterocyst-forming cyanobacteria, intercellular molecular exchange and communication is of particular importance during diazotrophic growth, when different physiological and metabolic processes are compartmentalized in different cell types. This compartmentalization imposes a requirement for the intercellular exchange of metabolic substances and the transfer of regulatory factors.

Transfer of metabolites

In addition to the intercellular exchange of fluorescent probes, solid support for the intercellular exchange of metabolites is available. Support came from the cell-specific localization of different enzymes of anabolic or catabolic pathways related to carbon and nitrogen utilization in either vegetative cells or heterocysts. First, the system for N_2 fixation operating in oxic environments, including the Mo–nitrogenase complex (Peterson and Wolk 1978; Bergman, Lindblad and Rai 1986; Elhai and Wolk 1990) and auxiliary proteins such as those of pathways for the provision of reductant and protection of nitrogenase from oxygen (Masepohl *et al.* 1997; Happe, Schütz and Böhme 2000; Valladares *et al.* 2003; Zhao, Ye and Zhao 2007; Ermakova *et al.* 2013, 2014), is confined to the heterocysts. This implies that products of N_2 fixation generated in heterocysts are transferred to vegetative cells (Wolk *et al.* 1974). The ammonium resulting from N_2 reduction by nitrogenase is incorporated into carbon skeletons, mainly through the glutamine synthetase (GS)–glutamate synthase (GOGAT) pathway (Wolk *et al.* 1976). Heterocysts contain high amounts of GS, but lack glutamate synthase (Thomas *et al.* 1977; Martín-Figueroa, Navarro and Florencio 2000). Thus glutamate, the substrate for ammonium incorporation by GS, should be provided to heterocysts by vegetative cells. The glutamine produced by GS in heterocysts is to some extent conveyed to the vegetative cells, where it is converted to glutamate, from which nitrogen is distributed to most nitrogen-containing cellular compounds. Indeed, isolated heterocysts have been shown to produce glutamine when supplemented with appropriate substrates (Thomas *et al.* 1977). In addition, a part of the organic nitrogen produced in heterocysts is transiently incorporated into the reservoir polymer cyanophycin, which, as described earlier, is conspicuously accumulated at the heterocyst polar regions (Fig. 1). Cyanophycin is catabolized in two steps: first cyanophycinase yields β-aspartyl-arginine, which is thereafter hydrolyzed to aspartate and arginine by isoaspartyl dipeptidase. Whereas cyanophycinase is much more abundant in heterocysts than in vegetative cells (Gupta and Carr 1981; Picossi *et al.* 2004), isoaspartyl dipeptidase is considerably more abundant in the latter (Burnat, Herrero and Flores 2014a). Indeed, isolated heterocysts release substantial amounts of β-aspartyl-arginine (Burnat, Herrero and Flores 2014a). The implication is that β-aspartyl-arginine produced from cyanophycin in heterocysts is transferred to vegetative cells. Compartmentalization extends to arginine catabolism proteins, since agmatinase, and hence the arginine decarboxylase pathway, has been shown to be present at higher levels in vegetative cells than in heterocysts (Burnat and Flores 2014).

In the heterocyst, the available assimilatory power (reductant and ATP) is mainly directed to nitrogenase for N_2 fixation, whereas CO_2 fixation is drastically decreased by lack of ribulose bisphosphate carboxylase/oxygenase (RubisCo). Hence, heterocysts acquire reduced carbon compounds from vegetative cells to serve as a source of reductant and energy. Alanine dehydrogenase is mainly expressed in heterocysts (Pernil, Herrero and Flores 2010a), supporting the idea of alanine transfer from vegetative cells to heterocysts (Jüttner 1983). Also, the confinement of the invertase InvB to heterocysts highlights the crucial role of catabolism of sucrose transferred from vegetative cells (López-Igual, Flores and Herrero 2010; Vargas *et al.* 2011). In summary, during diazotrophic growth, at least glutamine and β-aspartyl-arginine dipeptide appear to be transferred as nitrogen vehicles from heterocysts to vegetative cells, whereas glutamate, alanine and sucrose appear to represent reduced carbon vehicles donated from vegetative cells to heterocysts.

Although no specific pathway for intercellular movement of metabolites has yet been identified, intercellular exchange of the sucrose analog esculin dependent on putative septal junction proteins (especially FraC/FraD) has been observed, suggesting that sucrose moves through these conduits (Nürnberg *et al.* 2015; see Fig. 7). However, as mentioned above, putative inner membrane import system(s) for sucrose, which can be probed with esculin (Nürnberg *et al.* 2015), occur in *Anabaena* (Nicolaisen *et al.* 2009b; López-Igual *et al.* 2012). An ATP-binding domain (MalK) and a permease domain (GlsP) of ABC transporters and an MFS

transporter (HepP) that mediate esculin uptake have been identified (M. Nieves-Morión, S. Lechno-Yossef, C.P. Wolk and E. Flores, unpublished). Mutants of these transporters are impaired in diazotrophic growth, raising the possibility of a role in the intercellular transfer of sucrose. Furthermore, studies on the substrate, cell specificity and mutant phenotypes of amino acid transporters located in the cytoplasmic membrane have suggested the possible participation of some of these transporters in the diazotrophic growth of *Anabaena*. The ABC transporter N-I for hydrophobic amino acids and Gln is required for optimal diazotrophic growth and is specifically expressed in vegetative cells (Picossi *et al*. 2005). The ABC transporter N-II for acidic and neutral polar amino acids, including Gln, is expressed in both vegetative cells and heterocysts and is also required for optimal diazotrophic growth (Pernil *et al*. 2008). N-I and N-II could participate in the uptake by vegetative cells of Gln produced by heterocysts. On the other hand, because N-II is responsible for Glu uptake by isolated heterocysts (Pernil *et al*. 2008), it could also be involved in the uptake by heterocysts of Glu released from vegetative cells. A possible role of sucrose and amino acid transporters in diazotrophic growth would imply a step of substrate localization in the periplasm during intercellular transfer. Whether this is an obligatory step in the transfer or, as considered above, a result of metabolite leakage from septal junctions is unknown. Clearly, more studies are necessary to elucidate the pathways for intercellular exchange of carbon and nitrogen in the diazotrophic filament, as well as the role that membrane transporters may play in those pathways.

Transfer of regulators

In addition to the intercellular transfer of metabolites, the development of the diazotrophic filament of heterocyst-forming cyanobacteria involves movement of signaling molecules for the establishment and maintenance of a spatial pattern of heterocysts. Several recent reports have dealt with the molecular actors that could participate in the intercellular movement of compounds that regulate heterocyst distribution along a filament. The HetC protein, which is homologous to ABC exporters, is required for heterocyst differentiation and is localized to membranes in the heterocyst poles (Khudyakov and Wolk 1997; Corrales-Guerrero, Flores and Herrero 2014a). Besides the transport domain, HetC includes a putative peptidase domain whose deletion severely impairs differentiation (Corrales-Guerrero, Flores and Herrero 2014a). Epistasis analysis of the effects of inactivation or deletion of *hetC* and the genes *patS* and *hetN* encoding signaling molecules is consistent with a role of HetC in the processing and export of the PatS morphogen from heterocysts (Corrales-Guerrero, Flores and Herrero 2014a). However, this may not be the only function of HetC. It is noteworthy that the requirement for HetC can be fully compensated by moderate over-expression of HetP. HetP, which is encoded downstream of *hetC*, is also required for differentiation and is localized to the polar regions of heterocysts (Fernández-Piñas, Leganés and Wolk 1994; Corrales-Guerrero, Flores and Herrero 2014a). Whether HetP function involves PatS (and HetN) and is related to intercellular transfer is not known at present.

The involvement of HetC in the transfer of PatS and HetN has also been studied through their effects on degradation of the HetR transcription factor along a filament of cells (Videau *et al*. 2015a; see the section 'Heterocyst differentiation and patterning' above). The reporter used in this study was a form of HetR with reduced activity fused to cyan fluorescent protein (CFP), introduced in all the cells of the filament in a plasmid that also expressed yellow fluorescent protein (YFP) from the *patS* gene promoter (used to detect prospective heterocysts that would express *patS* and *hetN* from their native loci). It was observed that the spatial range of HetR–CFP fluorescence loss was reduced to zero in *hetC*, *hetC patS*, and *hetC hetN* genetic backgrounds at times when morphological signals of differentiation were observed for each strain. An involvement of HetC in the transfer of PatS and HetN from heterocysts to the neighboring vegetative cells was deduced.

Evidence for intercellular communication along filaments has recently been obtained using a novel quantitative approach based on the statistical analysis of fluctuations in gene expression in individual cells and the correlation of these fluctuations along a filament, as detected with a translational *hetR–gfp* fusion (a construct in which *gfp* is fused to the 5′ region of *hetR*; Corrales-Guerrero *et al*. 2015). It was found that correlations in expression extend to about two to three cells under nitrogen-replete conditions and that this length scale does not appreciably vary at early times after nitrogen deprivation. Furthermore, evidence was provided supporting the notion that these correlations are due primarily to cell–cell communication and not only to cell division effects. In addition to the wild type, fluctuations in the expression of *hetR–gfp* were studied in mutant backgrounds, and focus was placed on the involvement of putative septal junction proteins in the transfer of PatS and HetN. As mentioned above, these regulators seem to act on HetR, which additionally is autoregulated. Thus, the fact that the coupling of correlations of the expression of *hetR–gfp* between cells along the filament, both in the presence of ammonium and at early times upon N step-down, was increased with regard to the wild type by deletion of *patS*, but decreased by deletion of *sepJ*, suggests a functional relationship between their products, PatS and SepJ, and could be explained by PatS being transferred through SepJ-dependent conduits (Corrales-Guerrero *et al*. 2015). At early times after nitrogen deprivation, the behavior of a *hetN* mutant or of a *fraC fraD* mutant was similar to that of the wild type. In contrast, the behavior of *sepJ* and *fraC fraD* mutants became comparable at later times, suggesting that FraC/FraD could also participate in the movement of regulatory signals (e.g. a HetN signal) along the filament (Corrales-Guerrero *et al*. 2015).

The movement of PatS and HetN-derived inhibitors has also been studied in genetic mosaics built in *Anabaena* filaments (Rivers, Videau and Callahan 2014). The constructs were generated by randomly introducing plasmids encoding the genes *yfp* and *hetN* or *patS* expressed from heterologous inducible promoters in some cells (source cells) of a parental strain with deleted *patA* and *hetF* genes, in which the HetR turnover is decreased. In this strain, HetR fused to CFP (reporter) is also expressed from a heterologous inducible promoter. Also in this case, the loss of fluorescence from CFP in cells away from source cells was used to estimate the range of the inhibitory signal. The signal range of HetN, but not of PatS, was found to be shortened by inactivation of *sepJ*, which was taken to suggest an involvement of SepJ in the intercellular transfer of a HetN-derived signal (Rivers, Videau and Callahan 2014).

Because *sepJ* deletion mutants conspicuously fragment under nitrogen deprivation and arrest heterocyst differentiation at an early stage, it has not been possible to study export from heterocysts in those mutants. However, the pattern of heterocyst differentiation was studied in strains overexpressing *sepJ* in combination to *patS* or *hetN* deletions (Mariscal *et al*. 2016). In the wild-type background, over-expression of *sepJ* decreased the number of heterocysts and increased the size of vegetative-cell intervals between heterocysts, whereas the *patS* deletion was

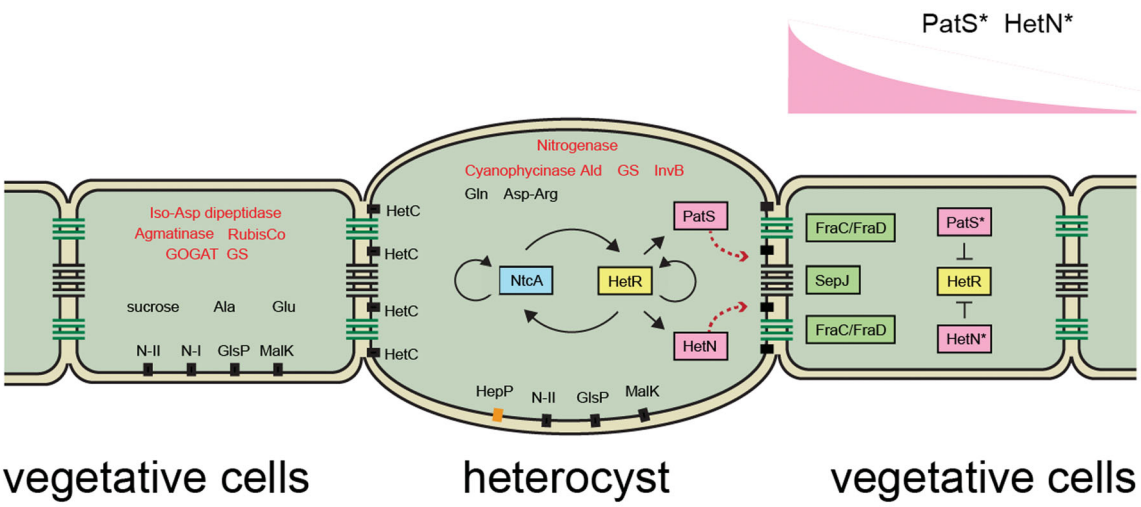

Figure 8. Scheme of part of a filament of *Anabaena* showing one heterocyst and adjacent vegetative cells and highlighting enzymes, transporters, metabolites and regulators thought to be involved in the multicellular physiology of this cyanobacterium. Two types of septal junction complexes are distinguished, those related to SepJ and those related to FraC/FraD. Black squares indicate ABC transporters, designated by their names in the literature (N-I, N-II, HetC) or by the name of their only known components (GlsP, MalK). Orange square, MFS transporter HepP. The regulatory circuit involving transcription factors NtcA and HetR and regulators PatS and HetN is depicted; PatS* and HetN* denote PatS- and HetN-processed regulators, and their concentration gradient in the filament is schematically represented (upper right). Enzymes (in red): Ald, alanine dehydrogenase; GOGAT, glutamine-oxoglutarate amidotransferase; GS, glutamine synthetase; InvB, invertase; Iso-Asp dipeptidse, isoaspartyl dipeptidase; RubisCo, ribulose 1,5-bisphosphate carboxylase/oxygenase. For simplicity, the constriction of the heterocyst cytoplasm at the heterocyst–vegetative cell junction (the heterocyst 'neck') is not shown.

epistatic over *sepJ* overexpression. Notably, *sepJ* overexpression increased the frequency of contiguous heterocysts in the wild-type or Δ*patS* backgrounds, which could indicate a lower accumulation of the inhibitors in the cells adjacent to the producing cells. These results are consistent with an increased transfer of PatS (and of a HetN-derived signal) between vegetative cells involving SepJ. In summary, indications have been obtained for the involvement of the ABC exporter HetC on the transfer of the PatS morphogen and of a HetN-derived signal from the heterocysts to the vegetative cells, and of SepJ in the transfer of those regulators, at least between vegetative cells. However, direct characterization of the transport processes is a task for future research. A scheme of the compartmentalization of enzymes, transporters, metabolites and regulators relevant for multicellularity in *Anabaena*, as discussed above, is presented in Fig. 8.

THEORETICAL DESCRIPTIONS OF DEVELOPMENTAL PATTERN FORMATION

Various analytical, numerical and computational approaches have been used to model developmental pattern formation in filamentous cyanobacteria. The necessity for models to capture essential features and mechanisms, and yet to remain tractable, forces the choice of as few as possible dynamical variables and unknown parameters (Economou and Green 2014; Kirk, Babtie and Stumpf 2015).

Early models: a diffusible inhibitor

An early scheme aiming at describing the pattern of near regular intervals of vegetative cells separating consecutive heterocysts was formulated before any identification of specific genes in *Anabaena* was made (Wilcox, Mitchison and Smith 1973). This scheme was based on the original insight of Fogg, who proposed that the diffusion of an inhibitory substance produced by heterocysts is responsible for this regularity (Fogg 1949). According to this scheme, a substance X whose concentration specifies the state of development of a cell towards a heterocyst state promotes its own synthesis and that of its inhibitor Y. In contrast to the diffusing inhibitor Y, X does not diffuse. It was further assumed that the inhibitor produced in heterocysts was destroyed in vegetative cells, setting up a gradient around heterocysts, and that when Y drops below a threshold level, development in a group of vegetative cells begins. Asymmetrical cell division and competitive interactions in this group prevent the formation of more than one heterocyst. The scheme was proposed for its plausibility but not framed mathematically, so no quantitative comparisons with experimental data were made, in spite of its intuitive appeal. Evidence that heterocysts inhibit the differentiation of vegetative cells in their vicinity was provided by Wolk (1967), who made a statistical study of the formation of new heterocysts in fragmented filaments. Further support for the notion that heterocysts act as sources of a diffusible inhibitory substance rather than as sinks was provided by Wolk and Quine (1975), who constructed one-dimensional tessellations commonly used to study crystal growth processes to model the distribution of interval lengths between heterocysts, both analytically and by computer simulations. These tessellations consisted of spatial domains that grew out of nuclei generated stochastically both in space and time, until they touched. The lengths between interval centers played the role of inter-heterocyst intervals, and different domain growth mechanisms corresponded to different mechanisms for the propagation of the inhibitor along a filament. They found that model distributions approximated experimentally measured ones when the inhibitor moves along a filament by diffusion.

Models based on reaction–diffusion schemes

The evidence for activator and diffusing inhibitor factors, diffusive transport, the lack of a characteristic organismal size—filament length can vary from a small number to hundreds of cells—and the fact that patterns in filamentous cyanobacteria

arise from a homogeneous state suggested early on an analogy with the mechanism of pattern formation originally envisioned by Turing in his study of morphogenesis (Turing 1952). In the Turing model, the stability of a homogeneous state of two reacting chemical species u and v, both of which can diffuse is examined. The system is governed by a pair of reaction–diffusion equations:

$$\frac{\partial u}{\partial t} = f(u, v) + D_u \nabla^2 u$$

$$\frac{\partial v}{\partial t} = g(u, v) + D_v \nabla^2 v$$

Here f and g, the reaction terms, are non-linear functions of u and v, and D_u and D_v the respective diffusion coefficients. A linear stability analysis shows that the homogeneous state may become unstable against the formation of stationary spatial patterns, when one of the species acts as an activator and the other as an inhibitor, and when the diffusion constant of the inhibitory chemical largely exceeds that of the activator (Turing 1952; Segel and Jackson 1972) (the precise condition is cast in terms of the diffusion lengths of both chemicals). Many patterns can arise for the same value of model parameters and the pattern that actually appears is sensitively dependent on initial conditions and fine tuning of parameters (Levin and Segel 1976; Mimura and Murray 1978; Castets *et al.* 1990; Koch and Meinhardt 1994; Baurmann, Gross and Feudel 2007; Butler and Goldenfeld 2009). Importantly, the characteristic length scale of the spatial patterns is intrinsic and independent of the size of the domain over which the equations are defined, like developmental patterns in filamentous cyanobacteria.

In spite of various commonalities, there are substantial differences between Turing's instability and the formation of developmental patterns in cyanobacteria, which we now discuss. The Turing instability requires diffusion of *both* the activator and the inhibitor species, with large differences in the two diffusivities. In fact, it can be shown mathematically that the instability cannot appear in any two-component reaction–diffusion model in which only one of the two species diffuses (Ermentrout and Lewis 1997; Cantini *et al.* 2014). Of note, no evidence for diffusion of the activator HetR between cells has been reported. It has been shown in recent years that stochastic Turing patterns may arise when only one species undergoes diffusion, provided that the discreteness of molecular species, dubbed intrinsic or demographic noise, is significant (Butler and Goldenfeld 2009, 2011; Biancalani, Fanelli and Di Patti 2010; Woolley *et al.* 2011; Fanelli, Cianci and Di Patti 2013). However, these patterns are transient and fluctuating in time, whereas patterns in cyanobacteria are not: once the commitment point to differentiation has been crossed, at *circa* 8 h after nitrogen deprivation under laboratory conditions (see, e.g. Yoon and Golden 2001), heterocysts are terminally formed. The equations for the Turing model are defined on a continuum spatial support of fixed size, whereas cyanobacterial developmental patterns are intrinsically discrete and filaments continually grow by cell growth and division. The typical length scale of developmental patterns in cyanobacteria is only of the order of 10 cells (Haselkorn 1998), far from any continuum approximation. Moreover, the typical time scale for differentiation of a heterocyst is comparable to that of cell division of vegetative cells in growing filaments, under naturalistic conditions. Filament growth through cell division may lead to non-trivial effects beyond dilution, such as an increase in intrinsic noise due to unequal binomial partition of molecular species that are present in small numbers upon cell division (Swain, Elowitz and Siggia 2002). Lastly, patterns in cyanobacteria are quite robust, in contrast to the fine tuning of parameters required to observe a Turing instability. Given these differences, claims as to the adequacy of the two-component Turing model to describe development in filamentous cyanobacteria (see, e.g. Hu *et al.* 2015) must be re-examined, unless three or more molecular species are considered (see, e.g. Hata, Nakao and Mikhailov 2014; Anma, Sakamoto and Yoneda 2012).

Following Turing's original use of reaction–diffusion systems, numerous studies based on the same formalism have been proposed, which we now survey. Meinhardt and Gierer demonstrated pattern formation for specific reaction–diffusion equations involving diffusion of both activator and inhibitor substances, and whose reaction terms included autocatalysis of the activator (Gierer and Meinhardt 1972; Meinhardt 2008). No identification of activator or inhibitor substances with specific genes or metabolites in cyanobacteria was made, and only a qualitative comparison between the patterns obtained from the equations and those observed in experiments was carried out. Extension of Meinhardt and Gierer's model to a growing domain, in order to account for filament growth through cell division and the discrete nature of cyanobacterial filaments, was carried out using Lindenmayer or L-systems (Hammel and Prusinkewicz 1996; Coen *et al.* 2004). Originally designed to simulate plant and fractal growth (Lindenmayer 1968), L-systems comprise recursive algorithms in which the state of a system is replaced in parallel by the next state using a set of predefined rules, as opposed to a sequential process. Pattern formation similar to that observed in experiments was generated by this scheme, though no quantitative comparison with experiment was attempted.

In contrast to *de novo* developmental patterns that necessarily arise from fluctuations in gene expression between cells in undifferentiated filaments, the problem of pattern maintenance is different. New heterocysts must form against the backdrop of morphogen gradients, and positional information as envisioned by Wolpert must be interpreted (Wolpert 1969). A model of pattern maintenance has been proposed by Zhu, Callahan and Allen (2010), who incorporated the effects of the HetR inhibitor HetN, in addition to the effects of PatS. In their model, the concentration of HetN is high at heterocysts, and it diffuses to neighboring cells, forming a bowl-shaped profile between neighbor heterocysts. When HetN levels in the middle region decrease sufficiently through vegetative cell division, HetR accumulation is promoted, triggering in turn local PatS production and inhibiting the increase of HetR levels in nearby cells. To reproduce pattern maintenance as observed in experiments, the model requires that diffusion of either HetN or a HetN-derived signal is small enough relative to filament growth that levels near the middle of vegetative-cell intervals can drop to allow for HetR levels to grow. Numerical solution of the model was carried out with a diffusion constant of HetN ~10 times smaller than that of PatS (Zhu, Callahan and Allen 2010).

Two reaction–diffusion models aiming to describe both *de novo* formation and maintenance of patterns have recently been proposed (Torres-Sánchez, Gómez-Gardeñes and Falo 2015; Muñoz-García and Ares 2016). The model by Torres-Sánchez, Gómez-Gardeñes and Falo (2015) involves differential equations for four dynamical variables: non-diffusive HetR and NtcA, as well as PatS and combined nitrogen, the latter two diffusing from cell to cell. In addition, the model included external noise of the same amplitude in all the dynamical equations. However, neither the late-stage HetN inhibitor or a product thereof, which has been reported to be involved in pattern maintenance

(Callahan and Buikema 2001; Li, Huang and Zhao 2002; Higa *et al.* 2012; Corrales-Guerrero *et al.* 2014b), nor filament growth through cell division of vegetative cells was included. The model reproduced some dynamical features of NtcA and HetR observed in experiments during development from a homogeneous state. Agreement between experimental heterocyst spacing distributions and calculated ones was obtained by tuning the amplitude of the external noise. The model of Muñoz-García and Ares (2016) includes HetR, PatS, HetN and fixed nitrogen as dynamical variables. Notably, this model includes stochasticity in both gene expression and cell division through the use of coupled Langevin equations. The authors reported agreement with heterocyst spacing distributions both for the wild type and for mutants in which PatS and HetN function were inactivated, and that both HetN and fixed nitrogen products produced at heterocysts are necessary to maintain developed patterns. However, a role for PatS in pattern maintenance was not considered. The numerical solutions also reproduce a preponderance of even-numbered vegetative cell intervals between heterocysts, as observed in experiments.

Other theoretical approaches

Not all models of developmental pattern formation in filamentous cyanobacteria have been based on reaction–diffusion schemes (Hammel and Prusinkewicz 1996; Coen *et al.* 2004; Allard, Hill and Rutenberg 2007; Gerdtzen *et al.* 2009; Brown and Rutenberg 2012a,b, 2014; Ishihara *et al.* 2015). In a series of works, Rutenberg and coworkers have emphasized fixed nitrogen as a dynamical variable and its transport along filaments, but neglected proteins or peptides in the developmental network such as HetR, PatS and HetN, to model fixed nitrogen and heterocyst spacing distributions (Allard, Hill and Rutenberg 2007; Brown and Rutenberg 2012b). They found qualitative agreement with measured distributions of fixed nitrogen near heterocysts (Wolk *et al.* 1974; Popa *et al.* 2007). The possible effects of periplasmic transport of fixed nitrogen in addition to cytoplasmic transport through septa between cells were studied. Even though involvement of the periplasm in fixed nitrogen transport could not be ruled out, the results were consistent with a small effect. The assumption of a gradient in cell growth away from heterocysts, explicitly built into the equations, has not been tested in experiments yet. More recently, the same authors incorporated fixed nitrogen storage as an important variable in the formation and maintenance of developmental patters, and included effects of lateral inhibition that model PatS and HetN action (Brown and Rutenberg 2014). As in their previous works, stochastic cell–cell differences in initial cell lengths and cellular growth rates were included, but not other sources of stochasticity. According to the model, a smaller cell size leads to faster depletion of fixed nitrogen through storage exhaustion, increasing the likelihood that small cells will differentiate into heterocysts. This leads to the prediction that faster growth will lead to earlier commitment through local nitrogen depletion. Furthermore, the inclusion of PatS and HetN effects, albeit modeled in a non-graded, Boolean fashion, led to the prediction that multiple-contiguous heterocyst clusters in a Δ*hetN* mutant are built around older heterocysts, a prediction that remains to be tested. The model captured heterocyst spacing and commitment time distributions.

Gerdtzen *et al.* (2009) implemented a scheme in which filaments are modeled as a finite circular array of discrete cell reactors, and in which temporal evolution is modeled using the iterative application of a perceptron-type rule: an algorithm based on functions that can determine whether an input represented by a vector of numbers belongs to one class or another. The perceptron-type rule maps the concentrations of NtcA, HetR and PatS between consecutive discrete times, in a threshold-dependent way. The threshold defines the limit of activation of each of the three genes in the simplified network. While the model exhibited robustness to perturbations in interaction parameters between pairs of the three proteins, it displayed high sensitivity to the choice of threshold, and it required fine-tuning of parameters (e.g. their transport factor) to reproduce the observed mean spacing between adjacent heterocysts. It captured qualitatively observed effects of deleting or over-expressing either PatS or HetR. No cell growth or cell–cell variability was considered, except for randomness in the initial values of the concentrations of the three proteins.

Ishihara *et al.* (2015) implemented a cellular automata scheme involving stochastic cell division, differentiation and increase of cell age in vegetative cells, as well as lateral inhibition from heterocysts flanking a given segment of vegetative cells. This scheme was developed in order to describe two stages in heterocyst pattern formation that they distinguished in their experimental cell lineage data. In their model, a vegetative cell is more likely to either divide or differentiate as the cell ages after a cell division. Using parameters derived from their experimental data, they determined first the steady state age distribution of vegetative cells and second the distribution of vegetative segment lengths, and concluded that while differentiation is initially independent of cell age, it becomes dependent on cell age at later stages.

The picture that emerges from this survey is that there is still no clear consensus as to what ingredients and mechanisms are essential in formulating a minimal and yet realistic model of development in filamentous cyanobacteria. Spatial patterns that bear similarities with those observed experimentally can be generated with ease, independently of the theoretical approach used. However considerable degeneracy remains in that seemingly realistic pattern formation can be obtained with such a different choice of basic variables and mechanisms. One unavoidable conclusion is that comparison with heterocyst spacing distributions is not sufficient to rule out given mechanisms or variables over others. The paucity of experimental data other than heterocyst spacing distributions has certainly impeded more quantitative comparisons between theory and experiment. The advent of quantitative gene expression data at the level of individual cells along filaments, mapping of spatial profiles under various experimental conditions and mutant backgrounds as well as spatial correlations should lead to more stringent comparisons between models and experimental data, and should help constrain and refine extant models. Future models should also take into account other factors in addition to those already considered, such as newly identified genes whose function and spatiotemporal activation are being elucidated, as well as fluxes of fixed carbon and other metabolites. The fact that cellular growth and division take place on time scales similar to differentiation may hint at more subtle roles for growth and cell division, besides simple dilution of molecular species.

Lastly, while some studies have explored the consequences of stochasticity in cellular growth, differentiation (Brown and Rutenberg 2014; Ishihara *et al.* 2015) and environmental noise (Torres-Sánchez, Gómez-Gardeñes and Falo 2015), no models have incorporated cell-to-cell stochastic variations in gene expression or noise explicitly, except for a recent reaction–diffusion model (Muñoz-García and Ares 2016). Noise can be considerable: for example, the typical cell-to-cell variation in the concentrations of the vast majority of proteins in isogenic

populations of *E. coli* is ~30% (Taniguchi *et al.* 2010). The important role that noise plays in the dynamics of differentiation in the unicellular organism *Bacillus subtilis* has been amply demonstrated (Suel *et al.* 2007). As discussed earlier, a first experimental exploration of noise in *Anabaena* has measured the transcriptional noise from a chromosomal translational fusion *hetR–gfp* in backgrounds in which genes encoding HetR inhibitors or septal proteins and HetR positive autoregulation were deleted, but only under nitrogen-replete conditions and at early times after nitrogen step-down (Corrales-Guerrero *et al.* 2015). A full study of how cellular variations in expression change during the developmental process remains to be done. Most likely, variations such as these can be important determinants in the cellular decision of whether to differentiate or not, in addition to other factors such as biased inheritance of PatN, whose presence has been hypothesized to modulate the competency of a vegetative cell to initiate heterocyst differentiation (Risser, Wong and Meeks 2012), the interpretation of a morphogen gradient such as a HetR-inhibiting peptide or fixed nitrogen (Wolpert 1969), or variations in cellular growth (Brown and Rutenberg 2014).

CELL DIVISION TO PRODUCE A FILAMENT OF COMMUNICATING CELLS

The distinct features of the cyanobacterial filament, which consists of strings of cells with individual inner membranes and cytoplasms, but with a continuous outer membrane and a periplasm shared by all the cells of the filament, call for distinct mechanisms of cell division, different from those of common unicellular bacteria. Originally, mutation in some cell division elements of ancestral unicellular cyanobacteria could have led to a primordial filament that would have represented a substrate for evolution. Indeed, the idea that transitions between unicellular and multicellular forms have repeatedly occurred during cyanobacterial evolution (Schirrmeister, Antonelli and Bagheri 2011) invokes relatively simple changes in cytokinesis. Septal junction structures mediating direct communication of the cytoplasms of adjacent cells in a filament could be among the features that a primordial cyanobacterial filament later acquired during evolution.

Cell division proteins in unicellular bacteria and cyanobacteria

Cell division in cyanobacteria has mainly been studied in two unicellular model strains, the rod-shaped *Synechococcus* sp. PCC 7942 (*S. elongatus*) and the spherical *Synechocystis* sp. PCC 6803 (hereafter *Synechocystis*). However, since most of the extant unicellular and filamentous cyanobacteria, including *S. elongatus* and *Synechocystis* sp. PCC 6803, have filamentous progenitors (Schirrmeister, Antonelli and Bagheri 2011; Schirrmeister, Gugger and Donoghue 2015; Shih *et al.* 2013), the specific features of cell division in these two organisms could not represent those of the ancestral unicellular cyanobacterium that developed into primordial filaments.

In the great majority of bacteria studied so far, cell division is initiated by the polymerization of the tubulin homolog FtsZ underneath the cytoplasmic membrane, at the future site of division (Kirkpatrick and Viollier 2011). FtsZ, which has no membrane-interacting domain, is directed to the membrane by a variety of tethers (Adams and Errington 2009; Du, Park and Lutkenhaus 2015; Rowlett and Margolin 2014). FtsZ polymerization modulated by its GTPase activity, tether polymerization, and filament interactions and sliding lead to the establishment of an encircling Z-ring that drives membrane bending and constriction (Erickson, Anderson and Osawa 2010; Szwedziak *et al.* 2014). The Z-ring also represents a scaffold for the recruitment of further division components that extend to the periplasm, leading to the formation of the divisome. This later promotes peptidoglycan remodeling for the synthesis of the new polar caps of the daughter cells (Typas *et al.* 2012; Natale, Pazos and Vicente 2013; Egan and Vollmer 2015) and may also coordinate chromosome segregation (Reyes-Lamothe, Nicolas and Sherratt 2012; Männik and Bailey 2015). Lastly, the divisome recruits proteins that contribute to outer membrane constriction and cell separation (Gerding *et al.* 2007).

Amino acid sequence comparisons have indicated that cyanobacteria bear cell division proteins some of which are homologous to those of well-studied unicellular Gram-negative bacteria and others to those of Gram-positive bacteria; additionally, cyanobacteria have some cell division proteins that are only found in cyanobacteria and in some chloroplasts, which as mentioned above are of cyanobacterial origin (Miyagishima, Wolk and Osteryoung 2005; Chauvat and Cassier-Chauvat 2014). FtsZ is present in both unicellular and filamentous cyanobacteria. Regarding FtsZ anchoring to the inner membrane, no homolog of the common FtsA tether, or of ZipA, which is present in gammaproteobacteria, can be recognized in cyanobacterial genomes. In *Synechocystis* a homolog of SepF, which stimulates bundling of FtsZ polymers and has recently been shown to function as an FtsZ tether in the Gram-positive bacterium *Bacillus subtilis* (Duman *et al.* 2013), has been shown to interact with FtsZ and increase the stability of FtsZ polymers (Marbouty *et al.* 2009a). The cyanobacterial-specific ZipN (also known as Ftn2) protein exhibits a central role in the recruitment of downstream cytokinetic proteins, reminiscent of FtsA (Marbouty *et al.* 2009b). Divisome proteins found to interact with ZipN in *Synechocystis* include homologs of the essential *E. coli* FtsI protein, a monofunctional murein transpeptidase involved in septal peptidoglycan synthesis (Typas *et al.* 2012), and of the central interacting components FtsQ of *E. coli* (DivIB of *Bacillus*) (van den Ent *et al.* 2008) and DivIVA of Gram-positive bacteria (Oliva *et al.* 2010), as well as the cyanobacterial-specific factor ZipS (also known as Ftn6), which is of unknown function.

Cell division proteins in heterocyst-forming cyanobacteria

Besides FtsZ, Ftn2, Ftn6, SepF, FtsQ and FtsI, the genome of *Anabaena* encodes homologs of the essential divisome components FtsK, FtsE and FtsW (Koksharova and Wolk 2002; Miyagishima, Wolk and Osteryoung 2005). In *E. coli*, FtsK is required for the recruitment of subsequent divisome components and has a role in proper chromosome resolution and segregation (Männik and Bailey 2015); FtsE is an ATP-binding subunit of an ABC transporter-like complex (Yang *et al.* 2011); and FtsW has been described to be a flippase for the lipid II murein precursor, although this is controversial (see Sham *et al.* 2014). More recently, FtsW has been proposed to be a divisome-linked peptidoglycan glycosyltransferase (Meeske *et al.* 2016). Importantly, however, *Anabaena* appears to lack FtsB, FtsL and FtsN (although recently a protein structurally similar to FtsB has been named CyDiv and claimed to represent an FtsB analog; Mandakovic *et al.* 2016). FtsQ, FtsB and FtsL are conserved together in many bacteria with diverse shapes and cell-division modes (González *et al.* 2010). In *E. coli*, FtsQLB form a subcomplex that directly recruits FtsW and FtsI and, through FtsN and likely FtsA (also absent in

Anabaena), coordinates the initiation of septal peptidoglycan remodeling and cell constriction (Busiek and Margolin 2014; Liu *et al.* 2015; Tsang and Bernhardt 2015).

Although not annotated in all the cyanobacterial genomes present in Cyanobase (http://genome.microbedb.jp/cyanobase/), which encompasses 32 unicellular and five filamentous (two Section III and three Section IV) strains, *ftsQ* can be detected in all of them (see Chauvat and Cassier-Chauvat 2014; González *et al.* 2010), whereas *ftsB*, *ftsL* or *ftsN* homologs have been detected in none. Homologs of *ftsQ*, but not of *ftsB*, *ftsL* and *ftsN*, have been detected in six Section V strains and the Section IV *Scytonema hofmanni* (Dagan *et al.* 2013). No *ftsB*, *ftsL* or *ftsN* is detected, either, in the cyanobacterial genomes (>100 available) at the Integrated Microbial Genomes resource (JGI), including the unicellular organism *Gloeobacter violaceus*, which represents the first diverging lineage of the cyanobacterial phylum. In cyanobacteria FtsQ could, directly or indirectly, recruit FtsW and FtsI, and trigger septal peptidoglycan synthesis without the participation of FtsA, FtsB, FtsL or FtsN.

Distinct septal peptidoglycan processing

Late events during cell division in *Anabaena* include septal peptidoglycan synthesis and degradation, as supported by the essential roles of *ftsI* (Burnat, Schleiff and Flores 2014b) and *ftsW* (our unpublished observations) and the participation of murein amidases of the AmiC type (Bernhardt and de Boer 2003; Berendt *et al.* 2012). Heterocyst-forming cyanobacteria bear a gene cluster encoding two AmiC amidases, AmiC1 and AmiC2, which are very similar to each other (Zhu *et al.* 2001; Lehner *et al.* 2011; Berendt *et al.* 2012). One of these amidases is necessary for heterocyst differentiation, but the picture is different for *Nostoc punctiforme* and *Anabaena*. In *N. punctiforme*, whereas *amiC1* is an essential gene, inactivation of *amiC2* produces viable cells that cannot enter into differentiation processes (Lehner *et al.* 2011). In *Anabaena*, *amiC2* (also named *hcwA* for heterocyst cell wall related) was described as necessary for proper heterocyst differentiation (Zhu *et al.* 2001). However, it has been described more recently that *Anabaena amiC2* (*hcwA*) is dispensable, whereas *amiC1* is also dispensable for vegetative growth but needed for heterocyst differentiation (Berendt *et al.* 2012). AmiC1 would thus play a role in *Anabaena* similar to that of AmiC2 in *N. punctiforme* (Berendt *et al.* 2012). The AmiC proteins preferentially localize to the intercellular septa of the filaments, principally in septa in the process of formation (Lehner *et al.* 2011; Berendt *et al.* 2012; Büttner *et al.* 2016). In spite of the requirement of AmiC amidase activity in heterocyst-forming cyanobacteria, as mentioned above septal peptidoglycan of the daughter cells appears to remain fused in the mature septa (Lehner *et al.* 2011; Mariscal *et al.* 2016). To a certain extent, the structure of the *Anabaena* filament resembles that of *E. coli* mutants lacking the three Ami amidases (AmiA, AmiB and AmiC). These mutants form long chains of cells with compartmentalized cytoplasms and thickened rings of incompletely split peptidoglycan at the septa, which result from impaired septal peptidoglycan hydrolytic processing in the presence of septal peptidoglycan synthesis, and a shared outer membrane (Heidrich *et al.* 2001; Priyadarshini, de Pedro and Young 2007).

Nostoc punctiforme amiC2 and *Anabaena amiC1* mutants are strongly hindered in calcein transfer (Lehner *et al.* 2011; Berendt *et al.* 2012) and lack observable nanopores (Lehner *et al.* 2013; Maldener, Summers and Sukenik 2014). An appealing hypothesis is that the AmiC proteins have the distinct activity of drilling nanopores in the septal peptidoglycan around the septal junction protein complexes that connect adjacent cells in the filament (see above). Remarkably the SepJ protein, a likely component of septal junctions, is recruited to the divisome during cell division by direct interaction with FtsQ (Ramos-León *et al.* 2015). Once recruited, a linkage of the septal junction proteins to the septal peptidoglycan would contribute to their maintenance at the septum after the divisome is disassembled. Thus, in *Anabaena* septal peptidoglycan remodeling could produce a septal peptidoglycan disk thicker than the lateral peptidoglycan, and traversed by perforations likely harboring septal junction complexes, a unique array in bacteria (Lehner *et al.* 2013; Nürnberg *et al.* 2015). It would be interesting to identify which other components participate in this step of cell division, and how they (and peptidoglycan synthases and hydrolases involved) are recruited to the divisome.

One factor affecting the architecture of the intercellular septa in *Anabaena* is the recently described SjcF1 protein (Rudolf *et al.* 2015). SjcF1 is a novel type of peptidoglycan-binding protein identified in *Anabaena* that contains two peptidoglycan-binding domains and an SH3 domain for protein–protein interactions (Rudolf *et al.* 2015). An SjcF1-like protein is generally present in filamentous cyanobacteria, and in *Anabaena* is located in the periphery of the cells with increased levels at the intercellular septa. An *Anabaena sjcF1* mutant is impaired in the intercellular transfer of calcein and, notably, shows an altered morphology of the septal peptidoglycan nanopores, which are wider and of a more variable diameter than in the wild type (Rudolf *et al.* 2015). Thus, whereas an AmiC protein is needed to form the nanopores, SjcF1 appears to be needed to restrict their size to about 20 nm in diameter. Interestingly, SjcF1 appears to interact through its SH3 domain with FraC and SepJ, corroborating a relationship between these proteins and the formation of nanopores.

It will be of interest also to discern whether the distinct step of septal peptidoglycan construction in heterocyst-forming cyanobacteria already leads to termination of cell division before outer membrane constriction, or whether further steps take place. In any case, at one step intercellular septal junction proteins could be placed in the intercellular septa, leaving the periplasm between daughter cells unsealed, which through successive cell division events would lead to a periplasm shared by all the cells of the filament.

OUTLOOK

Filamentous, heterocyst-forming cyanobacteria are emerging as a paradigm of multicellular bacteria, not only because they are stably constituted by cell aggregates, but also because the cells in these aggregates communicate, exchanging nutrients and regulators. These properties transfer the organismic unit from the individual cell to the filament of cells. Although intercellular exchanges have been classically considered in the context of heterocyst differentiation and diazotrophy, it is likely that novel differentiation processes and instances of intercellular communication will be uncovered as new cyanobacterial representatives are studied. The cells in the filament are enclosed in a continuous outer membrane that defines the existence of a continuous periplasm, which is therefore a compartment shared between cells. Proteinaceous septal junctions traverse the septal peptidoglycan and join adjacent cells, allowing communication between their cytoplasms. Proteins SepJ and FraC/FraD appear to be involved in the formation of two different types of septal junction complexes, but the full conformation of these

complexes is unknown. Moreover, the specific roles of other proteins that influence filament length, such as FraE and FraF, are yet to be determined. As a result of intercellular exchange of regulators and a complex program of gene expression (not covered in detail in this review), some vegetative cells in the filament differentiate into heterocysts. The identification of the precise pathways for movement of regulators (such as those related to PatS and HetN) and nutrients (including sucrose, amino acids and the dipeptide β-aspartyl arginine) between the cells of the filament, inclusive of vegetative cells and heterocysts, requires further research. Nonetheless, recent evidence suggests a role of SepJ in the intercellular transfer of the regulators, and of septal junctions in general in tranfer of sucrose. In differentiating heterocysts, the putative processing of PatS and export of the PatS morphogen by the peptidase/ABC transporter protein HetC are of high interest but need further insight. Theoretical descriptions that adequately capture the essential features of developmental patterns in multicellular cyanobacteria, which should stem from a much more intimate contact between theory and experiment, are still to be developed. Future models should include elements that have been largely neglected in the past, such as cell growth and division, noise in gene expression, detailed comparisons with data obtained from mutant strains and, lastly, an analysis of the robustness of theoretical predictions to uncertainties in model parameters. Particularly valuable for the development of theoretical models would be experiments using systems biology methodologies in which filaments are followed in real time as they develop, and gene expression is monitored at the level of individual cells. Finally, distinct processes in the last steps of cell division must ensure the correct localization of septal junction complexes and divisome disassembly without periplasm sealing between adjacent cells. Such processes, which are needed to produce a filament of cells capable of communication, are still to be fully resolved.

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