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Is the periplasm continuous in filamentous multicellular cyanobacteria?

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Filamentous, heterocyst-forming cyanobacteria are multicellular organisms in which individual cells exchange nutrients and, presumably, regulatory molecules. Unknown mechanisms underlie this exchange. Classical electron microscopy shows that filamentous cyanobacteria bear a Gram-negative cell wall comprising a peptidoglycan layer and an outer membrane that are external to the cytoplasmic membrane, and that the outer membrane appears to be continuous along the filament of cells. This implies that the periplasmic space between the cytoplasmic and outer membranes might also be continuous. We propose that a continuous periplasm could constitute a communication conduit for the transfer of compounds, which is essential for the performance of these bacteria as multicellular organisms.

Metabolic intercellular traffic in multicellular bacteria

The characteristic of being multicellular seems to have arisen repeatedly in the evolutionary history of life. An obvious characteristic of many eukaryotes, multicellularity is also found in some bacteria [1]. For instance, there are bacterial species in which cells are organized in trichomes or strings of cells that constitute their units of growth. Such organisms have been grouped into diverse phylogenetic lines including *Chloroflexus*, actinomycetes and cyanobacteria [2]. A central question in multicellularity is whether the different cells that comprise an organism communicate with each other and, if so, how this communication takes place. In the case of filamentous, heterocyst-forming [nitrogen (N_2)-fixing] cyanobacteria, individual cells in the filament exchange compounds that include metabolites (which can be used as nutrients) and, probably, regulatory molecules as well. The route for exchange is unknown but two basic possibilities can be formulated: (i) a continuous cytoplasm between cells enables the transfer of material by diffusion, as has been widely assumed; or (ii) the different cells exchange metabolites to and from an extracytoplasmic route, with cytoplasmic membrane permeases mediating export and import. This is an important topic that has scarcely been investigated and is largely ignored in the literature. Here, we propose that the filamentous, N_2-fixing cyanobacteria have a continuous periplasm through which different compounds can be exchanged between cells.

Morphological diversity of cyanobacteria

A characteristic of the prokaryotes known as cyanobacteria is that they perform oxygenic photosynthesis. A widely used taxonomy for cyanobacteria proposes the division of these organisms into five taxonomic sections [3]: section I, unicellular cyanobacteria that divide by binary fission or budding; section II, unicellular cyanobacteria that divide by multiple fission or by both multiple and binary fission; and section III, cyanobacteria that form filaments that grow by intercalary cell division and filament breakage and do not show differentiation of specialized cell types. Sections IV and V comprise filamentous cyanobacteria that develop heterocysts (see later); section IV cyanobacteria show division in one plane but section V cyanobacteria show division in more than one plane, giving rise to truly branched filaments. Whereas section III includes cyanobacteria that are not monophyletic, sections IV and V together constitute a monophyletic group [4] in which cellular differentiation has evolved.

When deprived of fixed nitrogen, some cells of the filament differentiate into heterocysts: N_2-fixing cells in which the thick envelope, heightened respiration and cessation of photosynthetic production of oxygen (O_2) provide a microoxic environment for the synthesis and function of the oxygen-sensitive enzyme nitrogenase [5]. In some species, other environmental conditions induce the development of akinetes (spores) and/or hormogonia [6]. These are short, motile filaments of cells of reduced size that have a role in dispersal or, in some species, in the establishment of symbioses with plants [7]. Figure 1 shows the different cell types in two species of filamentous cyanobacteria.

Cyanobacterial cell walls

Once thought to be algae because of their chlorophyll-*a*-dependent photosynthesis, electron microscopy studies showed that cyanobacteria are prokaryotes with walls that bear a close structural resemblance to the walls of Gram-negative bacteria [8,9]. Outside of the cytoplasmic membrane, the cell wall comprises a layer of peptidoglycan (murein), which can vary extensively in thickness among

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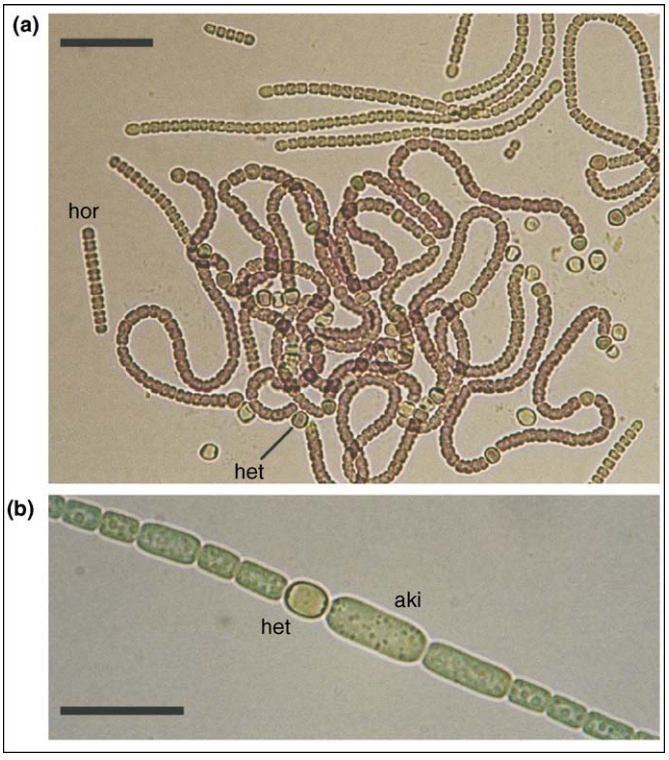

Figure 1. Different cell types in filamentous cyanobacteria. **(a)** Light micrograph of a sample from a culture of the filamentous cyanobacterium *Nostoc* sp. strain PCC 9203 grown without combined nitrogen. In addition to vegetative cells, heterocysts (het) and hormogonia (hor) are seen. Scale bar = 30 μm. **(b)** Light micrograph of a filament of *Anabaena cylindrica* grown without combined nitrogen. The filament contains vegetative cells, a heterocyst and an akinete (aki). Scale bar = 20 μm. Micrographs courtesy of José E. Frías (Servicio de Cultivos Biológicos, Centro de Investigaciones Científicas Isla de la Cartuja, C.S.I.C.–Universidad de Sevilla, Seville, Spain).

taxa, and an outer membrane. Some cyanobacterial strains also bear an S-layer (a surface layer attached to the outermost portion of the cell wall), similar to many other bacteria [10].

The cyanobacterial outer membrane has not been intensively studied but it is known to exhibit special features such as the presence of carotenoids and some proteins that are distinct from those of well-characterized Gram-negative species such as enterobacteria or *Pseudomonas* spp. [10]. Although several iron-uptake-related proteins commonly found in bacterial outer membranes are also present in cyanobacteria, typical bacterial porins similar to OmpC, OmpD, OmpF or PhoE are not found in these organisms (http://www.kazusa.or.jp/cyanobase/). Instead, an outer membrane protein called SomA has been characterized in two unicellular cyanobacteria, *Synechococcus* sp. strains PCC 6301 and PCC 7942 [11,12]. SomA has some porin-like properties and bears an N-terminal domain, similar to that of S-layer proteins, which might anchor it to peptidoglycan. Genomic sequences predict that each of the unicellular cyanobacteria *Synechocystis* sp. strain PCC 6803, *Thermosynechococcus* sp. strain BP-1 and *Gloeobacter violaceus* strain PCC 7421, and the heterocyst-forming *Anabaena* sp. strain PCC 7120 has four to six SomA homologues. Cyanobacteria also have one or more homologues of the outer-membrane protein Omp85 but these are more similar to the chloroplast outer-membrane protein Toc75 than to any other bacterial protein [13]. An important advance in the knowledge of cyanobacterial outer membranes is expected from the analysis of proteins that have recently been identified in the proteome of the outer membranes of *Synechocystis* sp. strain PCC 6803 [14] and *Anabaena* sp. strain PCC 7120 [15].

A continuous outer membrane

How does the outer membrane relate topologically to the cells in a cyanobacterial filament? Electron micrographs from an early paper in 1961, which described the structure of filamentous cyanobacteria, suggest that a structural element corresponding to the outer membrane extends into the periphery of the filament without entering the septa between consecutive cells [16]. Although not much attention has been paid to this aspect, recent examples have confirmed that the outer membrane does not enter the septum between two consecutive cells in the filaments of, for example, the section III cyanobacterium *Phormidium uncinatum* [17] or the heterocyst-forming *Anabaena* sp. strain PCC 7120 [18]. Figure 2 shows the septum between two consecutive cells in a filament of *Anabaena* sp. strain PCC 7120. Consistent with the concept that the outer membrane is continuous along the filament, Figure 2a–c clearly shows that the outer membrane does not enter the septum.

Are there plasmodesmata-like structures in filamentous cyanobacteria?

Nothing similar to plant plasmodesmata (cytoplasmic bridges delimited by cytoplasmic membranes) has ever been shown in cyanobacteria to the best of our knowledge. Pore structures have been observed in the peptidoglycan sacculus of some cyanobacteria [19] but these seem to be part of the junctional pore complex organelle (which spans the cell wall and is involved in slime secretion for gliding motility [20]) and, as such, they should not be construed as plasmodesmata. However, the term 'microplasmodesmata' has been used in the cyanobacterial literature to denote some material observed in the septum in the form of thin strands that are perpendicular to the cytoplasmic membranes [21] (Figure 2). These strand-like structures were identified as the pits and protrusions seen in electron micrographs of freeze-fracture preparations of cells of *Anabaena cylindrica* [22], and could correspond to integral membrane-protein complexes that are also found in unicellular bacteria [23].

Whether the so-called microplasmodesmata represent proteinaceous structures (like gap junctions) that provide tiny pores for the passage of small molecules from cell to cell is unknown. (Note that open reading frames that encode proteins homologous to gap junction proteins are not found in the sequenced cyanobacterial genomes.) Alternatively, this material could correspond to cell-to-cell anchoring structures. Fragmentation mutants of *Anabaena* sp. strain PCC 7120 that form short filaments and that might lack proteins essential for the integrity of cell junctions have been described [18]. In summary, available data do not clarify whether any structures traverse the septa between cells and permit intercellular communication, but do show that the outer membrane is continuous along the filament.

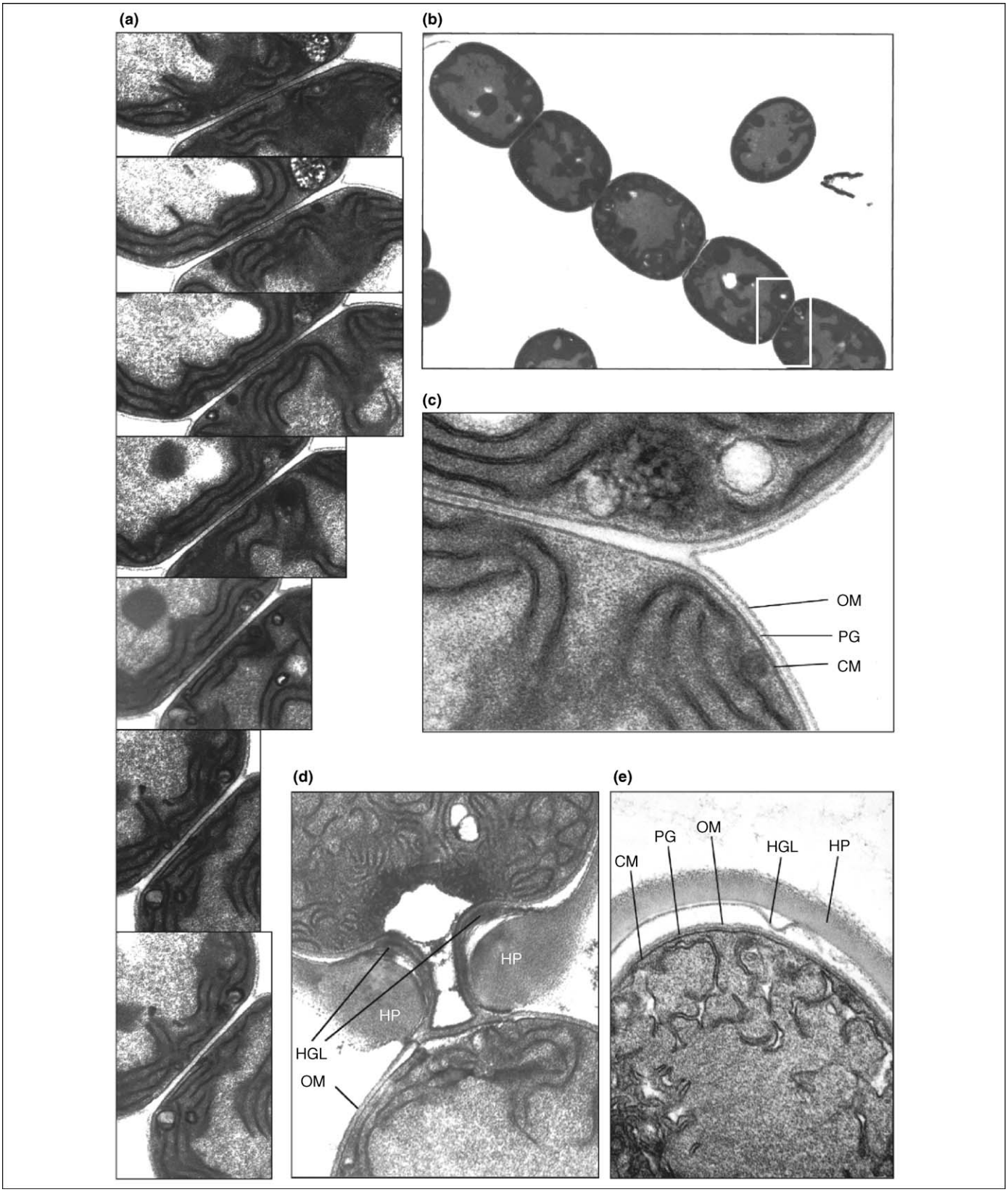

Figure 2. The cell wall structure of *Anabaena*. **(a)** Electron micrographs of serial sections (60–90 nm apart) of a filament of *Anabaena* sp. strain PCC 7120 (original magnification × 20 000). **(b)** Lower magnification (× 3150) electron micrograph of the same *Anabaena* filament [the white frame denotes the area magnified in part (a)]. **(c)** Further magnification (× 50 000) of the septum shows the cytoplasmic membrane (CM), peptidoglycan layer (PG) and outer membrane (OM). In the septum, electron-dense material is observed that runs parallel to the cytoplasmic membranes, which might represent the peptidoglycan layer(s) of the two adjacent cells. **(d)** A partial vegetative cell (bottom) and partial heterocyst (top) show continuity of the outer membrane and the presence of heterocyst glycolipid (HGL) and heterocyst polysaccharide (HP) in the envelope at the heterocyst neck. The white area in the neck corresponds to the location of the cyanophycin granule (a polymer of aspartate and arginine), which was lost during sample preparation. **(e)** Micrograph of a heterocyst section shows HGL and HP layers outside the CM, PG and OM layers. The protoplast has contracted during sample preparation to leave an empty space. Samples were prepared as described in Ref. [34] (for parts a–d) and Ref. [36] (for part e), and examined with a Zeiss EM10C microscope at 80 kV (parts a–d) and a JEOL 100CX microscope at 100 kV (part e).

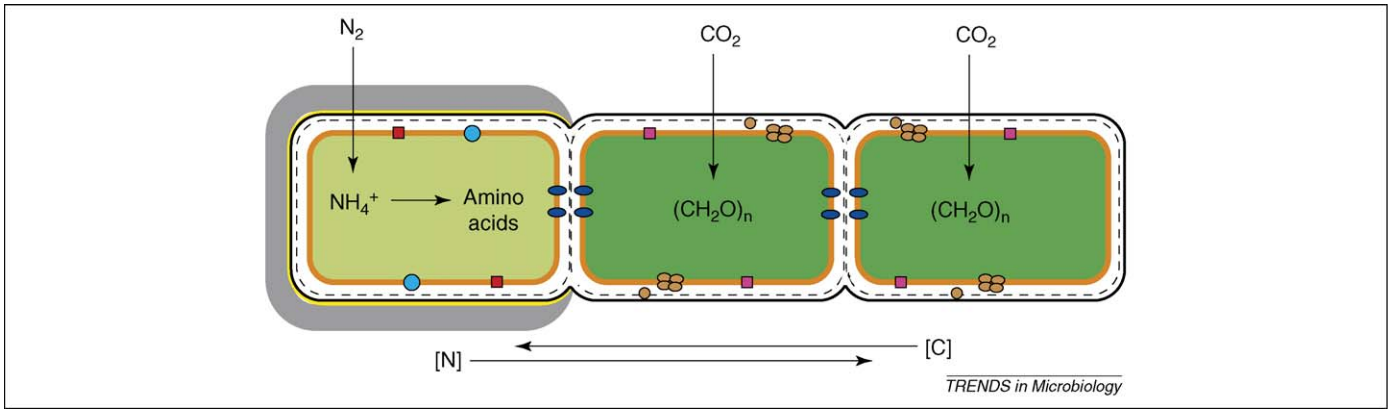

Figure 3. Scheme of a cyanobacterial N_2- and CO_2-fixing filament with a heterocyst (far left) and vegetative cells (not to scale). The lighter green heterocyst cytoplasm indicates the different pigment content of the two cell types. The heterocyst fixes N_2 to produce ammonium and amino acids, the major form in which fixed nitrogen moves to vegetative cells. Vegetative cells fix CO_2 to produce sugars, which are probably involved in transfer of reduced carbon to heterocysts (for a review, see Ref. [37]). The outer membrane (black line) is continuous along the filament and the peptidoglycan layer (discontinuous line) is porous. In the heterocyst, a laminated glycolipid layer (yellow line) and an amorphous polysaccharide layer (grey) are deposited outside the outer membrane. The cytoplasmic membrane (orange line) contains permeases and transport complexes but the distribution of these between vegetative cells and heterocysts is largely undetermined. Key to putative membrane transporters: red squares, heterocyst cytoplasmic membrane exporters; light-blue circles, heterocyst importers; pink squares, vegetative cell cytoplasmic membrane exporters; brown complexes, ABC-type uptake transporters; single brown circles, uptake system binding proteins. Protein structures (blue ovals) could exist in the septa between cells and might also have a transport function. For simplicity, the constriction of the heterocyst cytoplasm at the heterocyst–vegetative cell junction is not shown.

The case for a functional continuous periplasm along the cyanobacterial filament

The continuity of the outer membrane has an interesting corollary: the periplasmic space (the space that is delimited by the cytoplasmic and outer membranes) might, from a structural point of view, be continuous along the filament. If this compartment is continuous, it could provide a function based on structural continuity. The periplasm (the substance that fills the periplasmic space [23]) comprises a large collection of macromolecules including proteins that are elements of transport systems for uptake (e.g. the substrate-binding proteins of ABC-type transporters) or export. It has been suggested that the periplasm is so crowded with proteins that it forms a gel [24]. Nonetheless, a recent elegant determination of protein diffusion in *Escherichia coli* (using the green fluorescent protein, which was engineered to be exported as a free protein in the periplasm) provided evidence that the periplasm is a relatively fluid environment [25]. It remains to be determined whether this is also the case in cyanobacteria.

When fixing N_2, the heterocyst-forming cyanobacteria rely on intercellular exchange of metabolites for growth. Reduced carbon moves from vegetative cells to heterocysts [26] and N_2-derived fixed nitrogen moves from heterocysts to vegetative cells [27]. In a cyanobacterium such as *Anabaena* sp. strain PCC 7120, the heterocysts represent approximately one in every 10–20 cells, appearing in a semi-regular pattern. Therefore, fixed nitrogen must move from the heterocyst over a distance of five to ten cells. The mechanism of movement is unknown but we have suggested that transfer of amino acids released from the heterocysts could take place through the periplasmic space [28,29]. Substrate-binding proteins of ABC-type transporters might have an important role in retaining the mobile amino acids in the periplasm and donating them to the membrane complexes of these transporters for uptake by vegetative cells. In addition, there are developmental regulatory interactions that seem to require communication between cells [30,31] and these could also involve transfer of regulatory molecules through the periplasmic space. In particular, PatS-5, the C-terminal pentapeptide (Arg-Gly-Ser-Gly-Arg) of PatS, could move from developing heterocysts and inhibit differentiation of nearby cells [32]. Interestingly, exogenously added PatS-5 inhibits differentiation, thus demonstrating that the cells can take up PatS-5, which suggests that it does not simply diffuse through cytoplasmic connections.

To provide a conduit for the movement of molecules between different kinds of cells in the N_2-fixing filament, the periplasm should be functionally continuous, not only between vegetative cells but also between vegetative cells and heterocysts. The outer membrane also seems to be continuous between heterocysts and vegetative cells ([21,33]; Figure 2d). This continuity is possible because the heterocyst envelope, composed of a glycolipid layer and a polysaccharide layer, is deposited outside of the outer membrane ([21,33–35]; Figure 2e).

Figure 3 shows a scheme of a filamentous heterocyst-forming cyanobacterium, which highlights the cell-wall

Box 1. Outstanding questions

- The outer membrane is continuous along the cyanobacterial filament but the peptidoglycan layer surrounds each cell in the filament. How is the growth of the different cell-wall components regulated in filamentous cyanobacteria?
- Are there septa-specific protein complexes that help to keep cells together to form a filament? If such complexes exist, could they permit the transfer of small ions and molecules between cells?
- The periplasm seems to be continuous along the cyanobacterial filament. Is it a major metabolic conduit between heterocysts and vegetative cells?
- If intercellular transfer of metabolites involves an extracytoplasmic step, what is the complement of cytoplasmic membrane exporters and uptake systems in each cell type (i.e. vegetative cells and heterocysts) of an N_2-fixing filament?
- If the periplasm is an extracytoplasmic metabolic conduit along the filament, must the outer membrane porins be more restrictive in heterocyst-forming cyanobacteria than in some other bacteria to prevent the loss of important metabolites from the filament?
- If intercellular movement of compounds takes place through the periplasm, does it occur just by diffusion or is it facilitated by some unknown mechanism?

structural features discussed here and indicates the possible presence of different types of cytoplasmic membrane transporters in heterocysts and vegetative cells. If intercellular transfer of metabolites involves an extracytoplasmic step, both exporters and uptake systems should exist, and they should be different in vegetative cells and heterocysts to mediate a directional movement of different metabolites resulting in net fluxes of carbon and nitrogen. In vegetative cells, an ABC-type uptake system for neutral amino acids (called Nat) is the only transporter that has so far been implicated in the physiology of intercellular transport of nitrogen in the diazotrophic filament [29].

Concluding remarks and future perspectives

Heterocyst-forming cyanobacteria represent true multicellular organisms in which interdependent cells with specialized functions exchange metabolites and regulatory molecules to achieve the overall performance of the whole. However, the mechanism for this exchange is unknown (Box 1). Although little work has been done in this area recently, current knowledge of the structure of the cyanobacterial cell wall establishes the continuity of the outer membrane along the filament, implying the existence of a periplasm that is common to all cells in the filament. We suggest that a continuous periplasm is essential for the multicellular character of these organisms. Whether it indeed represents a communication conduit between spatially separated cells remains to be experimentally tested. We believe that this is an important issue to be addressed in future research.

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