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A nanopore array in the septal peptidoglycan hosts gated septal junctions for cell-cell communication in multicellular cyanobacteria

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ABSTRACT

Some filamentous cyanobacteria are phototrophic bacteria with a true multicellular life style. They show patterned cell differentiation with the distribution of metabolic tasks between different cell types. This life style requires a system of cell-cell communication and metabolite exchange along the filament. During our study of the cell wall of species *Nostoc punctiforme* and *Anabaena* sp. PCC 7120 we discovered regular perforations in the septum between neighboring cells, which we called nanopore array. AmiC-like amidases are drilling the nanopores with a diameter of 20 nm, and are essential for communication and cell differentiation. NlpD-like regulators of AmiC activity and septum localized proteins SepJ, FraC and FraD are also involved in correct nanopore formation. By focused ion beam (FIB) milling and electron cryotomography we could visualize the septal junctions, which connect adjacent cells and pass thru the nanopores. They consist of cytoplasmic caps, which are missing in the *fraD* mutant, a plug inside the cytoplasmic membrane and a tube like conduit. A destroyed membrane potential and other stress factors lead to a conformational change in the cap structure and loss of cell-cell communication. These gated septal junctions of cyanobacteria are ancient structures that represent an example of convergent evolution, predating metazoan gap junctions.

1. Introduction

Cell-to-cell adhesion and communication are the two principle prerequisites for the operation of a multicellular organism. Traditionally, multicellularity is considered as a typical property that evolved in Eukaryotes. However, it already evolved in the bacterial kingdom, where it emerged independently in various phyla. The most highly organized multicellular bacteria are found in the phylum of the cyanobacteria, where filaments may consist of hundreds of tightly interconnected cells. These cells communicate via a sophisticated cell-cell communication system, which is the focus of the present review. In filamentous cyanobacteria of the order Nostocales and Stigonematales, specialized cells may differentiate, which perform specific metabolic tasks that are interconnected in a homeostatic network of the entire multicellular filament (Mullineaux et al., 2008; Flores and Herrero, 2010, Maldener et al., 2014, Flores et al., 2016; Herrero et al., 2016). Under nitrogen-limiting conditions these cyanobacteria differentiate N_2-fixing heterocysts in a semi-regular pattern, which supply the vegetative cells along the filament with nitrogen-fixation products in form of glutamine and dipeptide β-aspartyl-arginine (Thomas et al., 1977; Martin-Figueroa et al., 2000). Vegetative cells, in turn, fix CO_2 via oxygenic photosynthesis and provide heterocysts with sucrose as a carbon source (Jüttner, 1983; Cumino et al., 2007). In addition to metabolites, signal molecules need to be exchanged to guaranty the correct pattern of differentiated cells along the filament (Yoon and Golden, 1998). In order to get from one cell to the adjacent one, molecules have to cross the septal barrier, which consists of two cytoplasmic membranes, the septal peptidoglycan (PG) and the periplasmic space (Fig. 1A, B). The outer membrane of the gram-negative cell wall does not enter the septa and envelopes the entire filament continuously. Therefore, it was suggested that the flow of metabolites along the filaments may involve the continuous periplasmic space that lies between the outer and the cytoplasmic membrane (Mariscal et al., 2007; Wilk et al., 2011). However, by using fluorescent dyes, Mullineaux and coworkers revealed that small molecules could directly diffuse through the septal barrier by a so-far unknown mechanism and structures (Mullineaux et al., 2008). Several septum-localized proteins, that were suggested to play a role in connecting the neighboring cells, are necessary for intercellular communication, since mutants deficient in the respective genes, termed *sepJ* (also known as *fraG*), *fraC* and *fraD*, are strongly affected in rate of molecular exchange (Mullineaux et al., 2008; Merino-Puerto et al., 2011; Flores et al., 2016).

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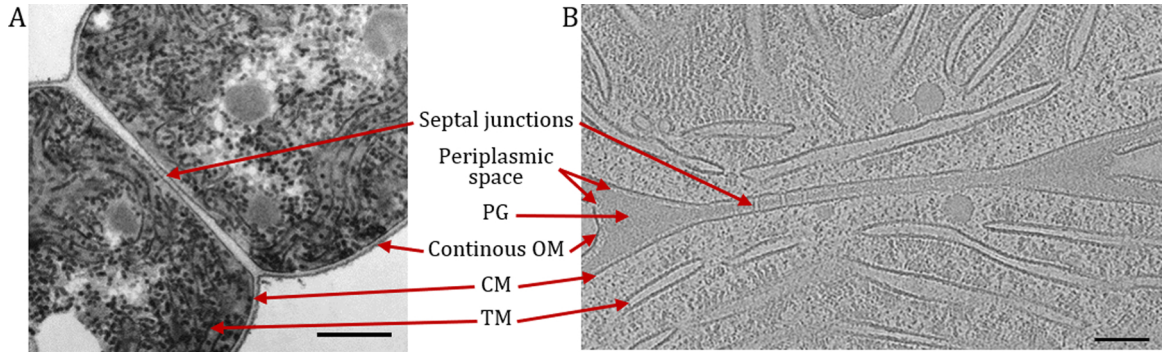

Fig. 1. Overview of the septal region of *Anabaena* wild-type cells. (A) Electron micrograph of a ultrathin section of chemically fixed cells and (B) electron cryotomogram of two adjacent cells in a wild-type filament. (A) Scale bar 500 nm. (B) The filaments were milled with a focused ion beam (FIB) prior to imaging to yield a 200 nm thin lamella and a projection of a 13.5 nm thick slice is shown. Scale bar 100 nm. Modified from (Weiss et al., 2018). OM, outer membrane; PG, peptidoglycan; CM, cytoplasmic membrane; TM, thylakoid membrane.

2. The septal cell wall of

It has been recognized since years that cyanobacteria possess a PG combining features of gram-positive and gram-negative bacteria, even though they exhibit an outer membrane. The PG layer is considerably thicker than in *Escherichia coli* (2–6 nm), reaching a thickness from 10 nm to 50 nm in filamentous species (Hoiczyk and Baumeister, 1995; Palinska and Krumbein, 2000). Perforations in the PG of gliding species like *Oscillatoria princeps* have been recognized at the boundary of the septal cross walls and termed junctional pores. They are potentially sites for slime extrusion involved in gliding motility.

The architecture of the peptidoglycan cell wall inside the septum was unclear until recently. Previous chemical and microscopical studies indicated that cell-connecting structures exist in the septum. After harsh chemical treatment, pores in the cross walls were visible (Metzner, 1955), and by freeze-fracture electron microscopy structures termed 'microplasmodesmata' were initially described (Giddings and Staehelin, 1981). These were considered to be homologue to plant plasmodesmata, connecting the cytoplasm of all cells along the filament. Furthermore, the precise arrangement of septal PG layers was controversially interpreted for long. It has been suggested that each cell is surrounded by an own layer of peptidoglycan and that in the septal region the two layers of the neighboring cells are connected by proteins such as the SepJ or FraC/FraD proteins (Herrero et al., 2016; Wilk et al., 2011). This assumption seemed reasonable, since mutation of SepJ or Fra proteins resulted in a fragmentation of the filaments (Nayar et al., 2007; Flores et al., 2007; Merino-Puerto et al., 2011). If this assumption was true, the newly formed septal peptidoglycan should be split by cell-wall amidases, in a similar way as cell wall amidases in unicellular bacteria (see below) cleave the septal cell wall during cell division to enable daughter cell separation. This model imposes the question, how in filamentous cyanobacteria daughter cell separation is prevented to form a multicellular filament. To answer this question, the function of amidases in filamentous cyanobacteria was studied.

N-acetylmuramyl-L-alanine amidases are cell wall lytic enzymes, which cleave the amide bond between the N-terminal L-Ala residue of the peptide and Mur*N*Ac (Höltje, 1995). The *E. coli* amidases have a broad functional redundancy and can be replaced by orthologues. Only when at least two of the three orthologues *amiA*, *amiB* and *amiC*, are disrupted, the resulting *E. coli* mutant does not divide correctly and forms filaments through impaired daughter cell-separation (Heidrich et al., 2002; Priyadarshini et al., 2007). AmiA and AmiC seem to be especially important for septum cleavage in unicellular gram-negative bacteria. In the genomes of filamentous cyanobacteria (which do not separate their daughter cells), genes for AmiC type amidases are found, whose function was unknown until then. Indeed, all strains of the order Nostocales harbor in their genome a tandem arrangement of two *amiC* homologues, *amiC1* and *amiC2*. We first created mutants in the *amiC* genes of *Nostoc punctiforme* (*N. punctiforme* hereafter) and investigated their phenotype. Only mutation of *amiC2* resulted in viable progeny whereas *amiC1* appeared to be essential (Lehner et al., 2011). Although not essential, the mutant in *amiC2* displayed a remarkable phenotype as it showed an aberrant filament morphology and lost the ability to differentiate any specialized cell types(Fig. 2A), (Lehner et al., 2011).

To gain a more detailed insight into the cell wall structure of the *amiC2* mutant in comparison to the wild type, PG sacculi were purified and studied by transmission electron microscopy (TEM). In the purification protocol of sacculi, all biomolecules are solubilized except the peptidoglycan mesh, which retained the cell shape as an empty case. Strikingly, in all preparations, the PG sacculi of filaments appeared as a continuous structure except for occasional raptures due to mechanical forces (Fig. 2C). This indicates that the septal PG is not split in two halves but it appears as a continuous disc-shaped layer. Recent high-resolution cryotomograms of the septal region confirm this conclusion (Fig. 1B). The septal PG is thickened at the outer circumference and is more rigid than the lateral cell wall as visualized by sacculi preparations (Fig. 2C). Frequently, the junction between the lateral wall and the septal PG discs was disrupted so that isolated septal cell wall discs could be obtained (Fig. 2B). In such septal discs, isolated from the wild type, we discovered an array of semi-regular arranged pores, which we termed 'nanopore array'. In different preparations, we found between 80 to 150 nanopores, each 20 nm in diameter. Strikingly, the nanopore array was completely absent in the *amiC2* mutant septa (Fig. 2B), (Lehner et al., 2013). Formation of pores into PG was a so far unknown property of AmiC homologs. Remarkably, the *amiC2* mutant did not show any intercellular molecular exchange as shown by FRAP (fluorescence recovery after photobleaching) studies (Fig. 2D), (Lehner et al., 2011). Since the mutant was not able to transfer molecules from cell to cell and did not differentiate different cell forms, we concluded that filamentous cyanobacteria communicate along the filament via cell-cell junctions, which pass thru the nanopores (Lehner et al., 2013; Maldener and Forchhammer, 2015). These septal junctions might correspond to electron-dense structures that traversed the entire periplasmic space and PG connecting the adjacent cells as visualized by ultra thin sections of chemically fixed cells in electron micrographs of septal regions (Fig. 1A)

2.1. The cell wall amidase AmiC2 from N. punctiforme

Immunofluorescence showed that, like in *E. coli*, native AmiC2 localizes to the maturing septum in *N. punctiforme* (Büttner et al., 2016). Furthermore, GFP-fusions to AmiC1 and AmiC2 were constructed and expressed under their native promoter to visualize the dynamics of their localization. In agreement with the previous finding, GFP-tagged

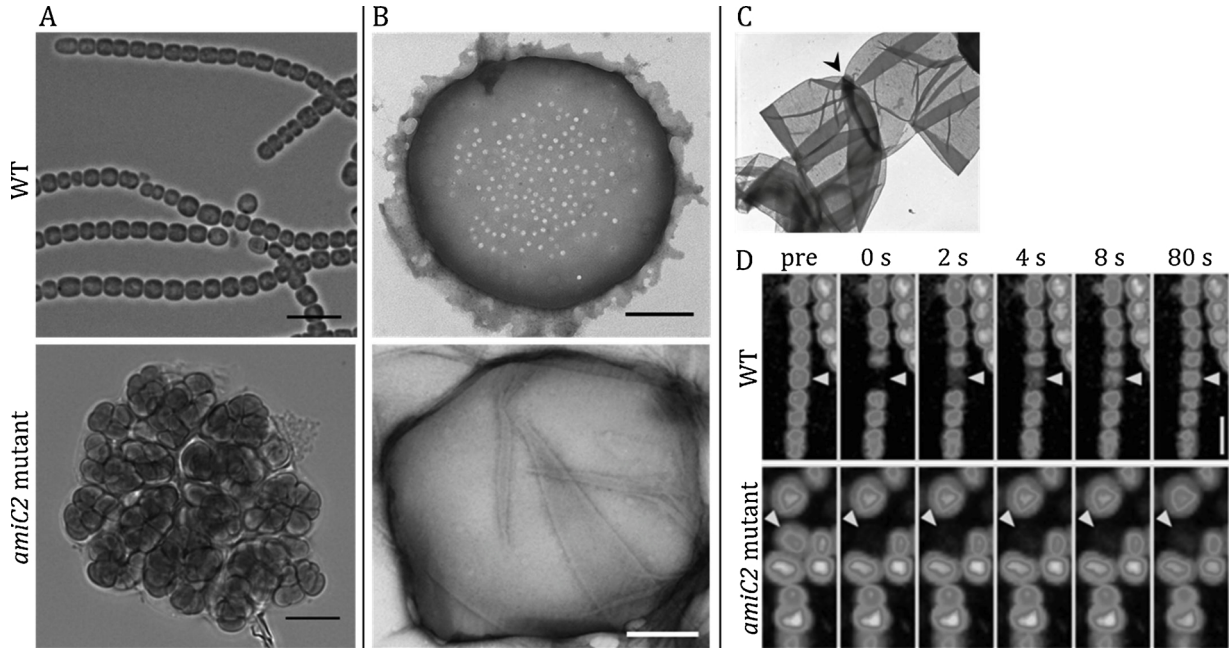

Fig. 2. Phenotype of the *amiC2* mutant of *Nostoc punctiforme*. (A) The *amiC2* mutant shows aberrant filaments. Scale bar 10 µm. (B) Purified septal PG revealed a missing nanopore array in the mutant. Scale bar 300 nm. (C): Purified continous PG sacculi of a *N. punctiforme* filament. The arrowhead points to septal PG. (D) Time series of a FRAP study is shown. The arrow points to the bleached cell. The *amiC2* mutant shows no recovery of calcein fluorescence. Scale bar 5 µm. Figure modified from (Lehner et al., 2011, 2013).

AmiC1 and AmiC2 localized to the newly formed septa, and disappeared in the older septum of *N. punctiforme* (Lehner et al., 2011; Büttner et al., 2016). This indicated that the amidases are required during septum maturation for the purpose of nanopore formation.

To investigate these new properties on the enzymatic level, the catalytic domain of AmiC2 (AmiC2-cat) was purified and characterized in more detail (Büttner et al., 2016). The high-resolution crystal structure (1.12 Å) of AmiC2-cat differed significantly from known structures of cell splitting and PG recycling amidases (Fig. 3). A wide and shallow binding cavity allows easy access of the substrate to the active site, which harbors an essential zinc ion and is homologous to AmiC$^{E.coli}$. However, an inhibitory α-helix, which shields the catalytic site as found in the *E. coli* AmiC structure (Yang et al., 2012), is absent.

In line with the structural data, the AmiC2-cat protein exhibited strong cell wall hydrolytic activity in *in vitro* dye release assays, without the need to add activator proteins, as in the case of *E. coli* AmiC (Büttner et al., 2016). There, the amidase is only active when incubated with the LytM factor NlpD, which leads to a conformational change of the α-helix to expose the catalytic site of the amidase (Uehara et al., 2010). In addition to the non-canonical catalytic site, AmiC2 from *N. punctiforme* has two N-terminal AMIN domains. AMIN domains are involved in septal targeting and PG binding in *E. coli* (Rocaboy et al., 2013). Mutants with truncated versions of AmiC2, lacking both or only one of the AMIN domains, confirmed that these domains are responsible for localization to the septal cell wall in *N. punctiforme* (Faulhaber, 2017). A flexible proline-rich region between the catalytic and the AMIN domains modulates the amidase activity, as shown by *in vitro* studies of truncated versions. The presence of this proline-rich modulator domain diminished the AmiC-Cat activity by more than 50% and could represent a regulatory site, corresponding to the inhibitory α-helix of

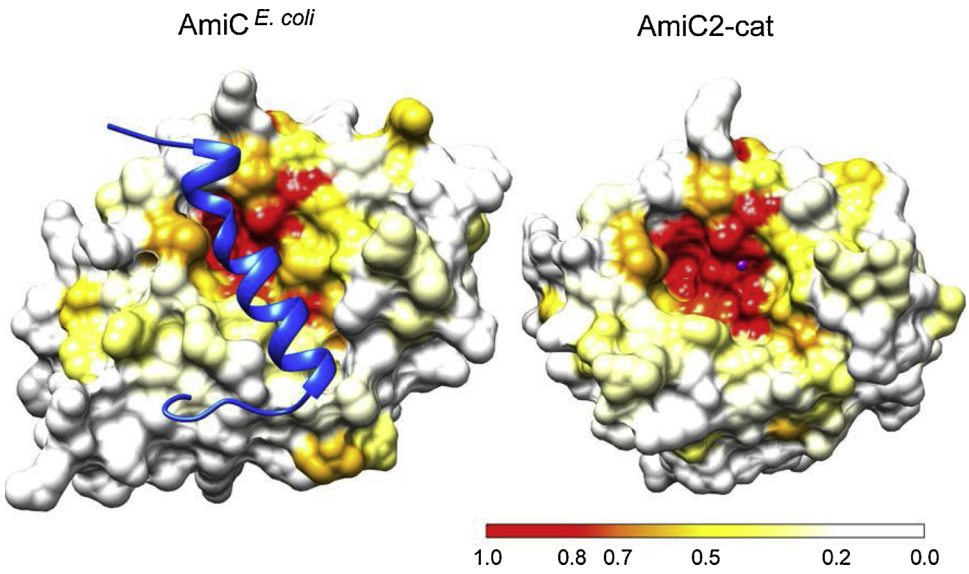

Fig. 3. Surface conservation of AmiC2-cat (right), and AmiC *E. coli* (left). AmiC2-cat, a member of the Amidase_3 family, displays conservation in the zinc-binding region. However, the self-inhibitory α-helix covering the active site in AmiC$^{E. coli}$ is missing. The color bar depicts the level of conservation. Adapted from (Büttner et al., 2016).

AmiC from *E. coli* (Faulhaber, 2017). Together, the unique biological function of AmiC2 to drill precise pores into the septal PG points towards a highly controlled enzyme activity, distinct from other amidases (Büttner et al., 2016).

2.2. The AmiC-like amidases in Anabaena sp

After the in-depth study of AmiC2 in *N. punctiforme,* we investigated the function of homologous genes in the species *Anabaena* sp. PCC 7120 (*Anabaena* sp. hereafter), which is a model organisms for cell-cell communication and cell differentiation and which provides easier handling and molecular techniques. *Anabaena* sp. mutants in the genes *amiC1* (*alr0092*) and *amiC2* (*alr0093*), which are clustered on the genome as in *N. punctiforme* (see above), showed different phenotypes. The impossibility to create a fully segregated double mutant showed that they are functional redundant (Berendt et al., 2012; Bornikoel et al., 2017). In contrast to *N. punctiforme*, the filaments of the *amiC1* mutant in *Anabaena* sp. looked normal. However, this mutant could not grow on N_2, even though it formed heterocysts. Compared to the wild type, the mutant heterocysts showed a decreased nitrogen fixation activity measured by the acetylene reduction assay, suggesting that the heterocysts were at least partially functional and producing micro-oxic conditions to allow nitrogenase activity. Taken together this implies, that the products of nitrogen fixation can probably not be delivered to the vegetative cells within the filament due to impaired cell-cell connections (Bornikoel et al., 2017). Indeed, the *amiC1* mutant in *Anabaena* sp. showed a reduced number of nanopores and a strongly reduced fluorescent tracer exchange during FRAP measurements, confirming the importance for cell-cell communication during diazotrophic growth. In contrast, the *Anabaena* sp. *amiC2* mutant showed a wild-type phenotype and could communicate normally

In a fully segregated *amiC1* mutant, *amiC2* could not be completely knocked out, and *vice versa*, suggesting that at least one of the two enzymes is required for normal growth in *Anabaena* sp. (Bornikoel et al., 2017). In contrast to the single mutants, cell and filament morphology was affected in both types of double mutants, suggesting that AmiC-type amidases are involved in cell division and in correct placement of the septal plane in *Anabaena* sp. as well (Bornikoel et al., 2017). In the fully segregated *amiC1* mutant with most genome copies bearing a disruption of *amiC2*, heterocyst differentiation was completely inhibited. Furthermore, aberrantly shaped cells of this *amiC1-amiC2* double mutant could not exchange the fluorescent tracer calcein (Bornikoel et al., 2017).

The reduced (in normal shaped filaments) and abolished (in aberrantly shaped filaments) cell-cell communication, as measured by FRAP, correlated well with irregular shaped and sized nanopores in the mutant (Bornikoel et al., 2017). In a fully segregated *amiC2* mutant with partially knocked out *amiC1*, also aberrant filaments showed cell-cell communication and heterocysts were differentiated. However, molecular tracer exchange was reduced, which could be correlated with a reduced number of nanopores (Bornikoel et al., 2017).

3. A LytM like regulator of amidase activity

In *E. coli,* the LytM domain proteins EnvC and NlpD are known to positively regulate the activity of amidases (Uehara et al., 2009, 2010, Yang et al., 2012). A homologue of NlpD could be identified in the genome of *Anabaena* sp. as the gene product Alr3353, which represents a candidate interaction partner of AmiC1. Inactivation of the *alr3353* gene resulted in a mutant that displayed significantly fewer nanopores and, as a consequence, a lower rate of fluorescent tracer exchange between cells. The mutant was not able to grow with N_2 as sole nitrogen source, although heterocysts were formed. A GFP-fused version of Alr3353 was introduced to *Anabaena* sp., where it localized mainly to fully developed intercellular septa of vegetative cells. The purified protein bound to PG and enhanced the hydrolytic activity of AmiC1 *in vitro*. Further biochemical studies showed that Alr3353 directly interacts with AmiC1 from *Anabaena* sp. as well as to AmiC2 from *N. punctiforme in vitro* (Bornikoel et al., 2018). These data indicate that the LytM factor Alr3353 is involved in the regulation of nanopore formation by enhancing the activity of AmiC1/AmiC2.

4. Septal proteins and septal junctions

As described above, we suggested that the nanopores are traversed by cell-cell connections to mediate communication and metabolite exchange along the filaments(Fig. 5). These structures were known as microplasmodesmata, septosomes and channels but finally, several groups including ours, agreed on the term ‘septal junctions’ (Mariscal, 2014; Flores et al., 2016). Temperature dependence of the intercellular exchange rates measured in FRAP experiments using various fluorescent tracers indicated that the communication process takes place by simple diffusion, without active transport involved (Nieves-Morión et al., 2017). The septal junctions thus have the properties of channels, which resemble cell-cell connections in eukaryotes. In metazoan, gap junctions connect the cells in the multicellular tissues. These are proteinaceous structures that span the gap between the cytoplasmic membranes of two neighboring cells and have cap structures at the outward openings to gate the channels. By contrast, plants connect their cells by plasmodesmata allowing molecule exchange between all living cells (Nicolas et al., 2017). The plasmodesmata are direct connections where the cytoplasmic membranes of neighboring cells are fused resulting in a functional symplast in plants.

Up to now, the structure and protein composition of the septal junctions was unknown. However, the septal proteins FraC, FraD, and SepJ (also known as FraG) were likely candidates for components of septal junctions since they are important for filament integrity especially in combined nitrogen-depleted medium (Bauer et al., 1995; Flores et al., 2007; Nayar et al., 2007; Merino-Puerto et al., 2010; Mariscal et al., 2011; Flores et al., 2016). Beside the fragmenting phenotype, mutants in the *fra* genes were not able to grow under nitrogen-fixing conditions, although heterocysts were formed. A functional link between these septum proteins and AmiC1 was demonstrated, since mutants showed a highly reduced number of nanopores, and AmiC1 was necessary for correct focused localization of SepJ in the center of the septum (Nürnberg et al., 2015). Intriguingly, mutation of *amiC* in a *fra*-mutant background phenotypically complemented filament fragmentation. This suggested that the fragmentation phenotype may not result from a direct involvement of these proteins in tethering the neighboring cells, but rather from an uncontrolled AmiC activity that lysed the septal cell wall and thereby leads to filament fragmentation. Furthermore, all mutants in Fra proteins and AmiC1 showed a diminished intercellular exchange of fluorescent probes corresponding to their diminished number of nanopores (Nürnberg et al., 2015; Mullineaux et al., 2008; Mariscal et al., 2011; Merino-Puerto et al., 2011).

Recently electron cryotomography (ECT) imaging of cryo-focused ion beam-milled (FIB) *Anabaena* sp. cells was used to get deeper insights in the *in situ* structure of septal junctions (Weiss et al., 2018). Indeed, images of channel-like structures, spanning the septal space were obtained (Fig. 1B). By subtomogram averaging, the *in situ* architecture of these septal junctions could finally be resolved (Fig. 4). Unexpectedly, the septal junctions have cap and plug structures on both ends that superficially resemble the cap structures of gap junctions. However, in contrast to gap junctions, the septal caps display a five-fold symmetry. Another difference between the cyanobacterial septal junction and gap junctions concerns the channel itself: in gap junctions, the channel is composed of two half-channels with a fixed length. By contrast, the length of the channel is variable in septal junctions and it precisely matches the thickness of the septal PG. This implies a flexible organization of the septal junction channel, which could be built of assembled smaller subunits like in pili (Weiss et al., 2018).

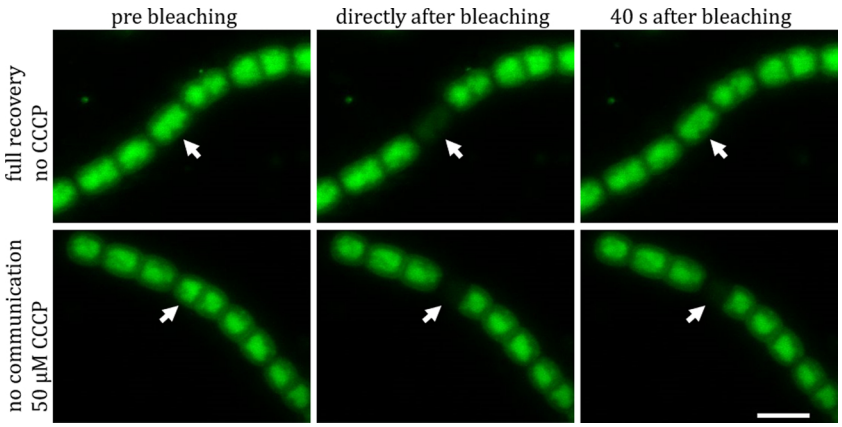

Fig. 4. The switch between closed and open septal junction occurs via a structural rearrangement of the cap. Septal junctions switch from open (A–C) to closed state (D–F) upon disruption of the membrane potential by treatment of the cells with 50 µM CCCP. Subtomogram averages of FIB-milled cryotomograms (A, D) are displayed as surface representations viewed from the side (B, C) or from the top (C, F). Scale bars 10 nm. Figure modified from (Weiss et al., 2018).

The presence of cap and plug structures at both sides of the septal junction channel facing into the cytoplasm of the connected cells suggested that the septal junctions may be gated, as it is the case in gap junctions. Indeed, by treating the filaments with compounds that disrupt the membrane potential, cell-cell communication could be abolished. After recovering the cells in fresh medium, the molecular diffusion was restored, even if recovery was performed in the presence of a protein synthesis inhibitor. Septal junctions imaged from cells treated with CCCP displayed a remarkable conformational change. The slit-like openings in the cap structure were no more visible in this conformation. This suggests that this observed conformational change is responsible for the gating of the septal junctions.

The gating properties were also assayed with mutants in the septal proteins FraD, FraC, and AmiC1. Gating was lost in the *fraCfraD* double mutant and the *fraD* single mutant. Imaging the septal junctions of these mutants showed that the cap and plug parts were missing (Weiss et al., 2018). Therefore, the integral membrane proteins FraD and/or FraC could themselves represent proteins of the septal junction cap and plug structure or are essential in positioning of the cytoplasmic domains. The few septal junctions that could be imaged in the *amiC1* mutant of *Anabaena* sp. showed the wild- type structure (Weiss et al., 2018). This confirms that the AmiC proteins are in fact not components of the septal junctions but are rather involved in the processing of the septal PG. The residual nanopores in the *amiC1* mutant are likely formed by redundant amidases like AmiC2 (Bornikoel et al., 2017) and AmiC3 (Zheng and O'Shea, 2017) and finally filled by septal junctions. However, up to now the process of septal junction formation is not known.

When cells within a cyanobacterial filament deteriorate due to predatory attack, physical stress, or revised senescence, such as the terminally differentiated heterocysts, they have to avoid leakage of the cytoplasmic symplast. Therefore, a closing mechanism of the cell-cell joining septal junctions appears necessary to avoid uncontrolled leakage of the filament. Nürnberg showed, that fluorescent tracer-loaded wild-type filaments stay fluorescent after fragmentation by sonication, meaning the filaments do not bleed out and the septal junctions in the new terminal cell are closed (Nürnberg, 2015). Heterocysts, who have a limited lifetime, loose cell communication during early stages of senescence, before they detach from the filament (Nürnberg et al., 2015). The gating of the septal junctions provides a straightforward explanation for these features. Beyond its role in protecting the integrity of the cells in the filaments upon injury, the gating of the septal junctions could have physiological significance for growth under nitrogen-fixing conditions. The inability of *fraCfraD* mutants to grow diazotrophically could be due to the lack of septal junction gating. The cap and plug structure might be involved in controlling the metabolites that are allowed to diffuse through the channels. Loss of such control might impede the sophisticated exchange of metabolites, which is necessary between vegetative cells and heterocysts.

Nanopore array and septal junctions together represent an ancient

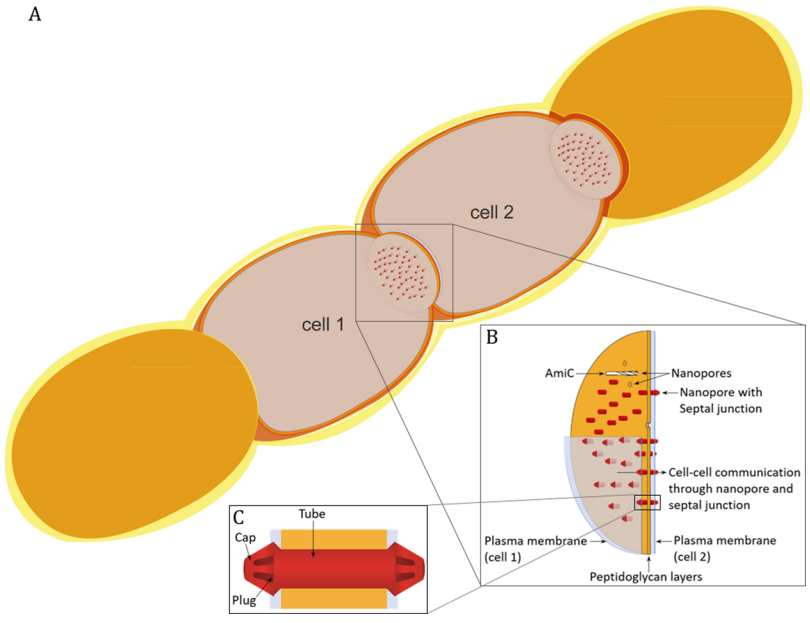

Fig. 5. Model of the cell-cell communication structure in filamentous cyanobacteria. (A) Schematic depiction of the septal PG disks in two sectioned cells of a filament. (B) Detailed view of the septal PG between vegetative cells, showing the nanopore array drilled by AmiC. Nanopores containing septal junction complexes allow the exchange of molecules through the septal peptidoglycan. The Fra proteins may be part of the cap/plug module of the septal junctions. Figure adapted and modified from (Bornikoel et al., 2017).

structure evolved in filamentous cyanobacteria, to enable a lifestyle based on multicellularity and represent an example of convergent evolution of a gated cell-cell connection, which predates the evolution of metazoan gap junctions by more than a billion years.

Competing interest statement

The authors claim no conflict of interest.

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