An amidase is required for proper intercellular communication in the filamentous cyanobacterium *Anabaena* sp. PCC 7120

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Channels that cross cell walls and connect the cytoplasm of neighboring cells in multicellular cyanobacteria are pivotal for intercellular communication. We find that the product of the gene *all1140* of the filamentous cyanobacterium *Anabaena* sp. PCC 7120 is required for proper channel formation. *All1140* encodes an amidase that hydrolyses purified peptidoglycans. An *All1140-GFP* fusion protein is located at the Z-ring in the periplasmic space during most of the cell cycle. An *all1140-null mutant* (M40) was unable to grow diazotrophically, and no mature heterocysts were observed in the absence of combined nitrogen. Expression of two key genes, *hetR* and *patS*, was studied in M40 using GFP as a reporter. Upon nitrogen step-down, the patterned distribution of green fluorescent cent cells in filaments seen in the wild type were not observed in mutant M40. Intercellular communication in M40 was studied by measuring fluorescence recovery after photobleaching (FRAP). Movement of calcein (622 Da) was aborted in M40, suggesting that the channels connecting the cytoplasm of neighboring cells are impaired in the mutant. The channels were examined with electron tomography; their diameters were nearly identical, 12.7 nm for the wild type and 12.4 nm for M40, suggesting that *AmiC3* is not required for channel formation. However, when the cell wall sacculi isolated by boiling were examined by EM, the average sizes of the channels of the wild type and M40 were 20 nm and 12 nm, respectively, suggesting that the channel walls of the wild type are expandable and that this expandability requires *AmiC3*.

T
he occurrence of multicellular organisms is one of the most significant steps in evolution (1, 2). One of the advantages of multicellularity is that an organism can differentiate specialized cells for different functions (3, 4). In prokaryotes, there are several independently evolved groups of multicellular organisms, including Actinobacteria, Myxobacteria, and the cyanobacteria (5). The cyanobacteria are a group of eubacteria that carry out oxygenic photosynthesis. They have the most diversified morphology among prokaryotes, ranging from unicellular cells to multicellular filaments with true branches (6). *Anabaena* sp. PCC 7120 (*Anabaena* 7120) is a filamentous cyanobacterium that can form heterocysts, providing an excellent model for studying cell differentiation and pattern formation (7–9). The importance of intercellular material exchange is evident in that heterocysts provide a micro-oxic environment for nitrogen fixation and supply nitrogenous compounds to the vegetative cells, whereas the vegetative cells perform oxygenic photosynthesis and supply sugars as energy and carbon skeleton to the heterocysts (7).

The heterocyst pattern is dependent upon intercellular communication according to Turing’s activator–inhibitor model, which requires the inhibitor to be diffusible (10, 11). Although many genes are involved in the regulation of heterocyst formation (7–9, 12, 13), *hetR* and *patS* are most important in heterocyst pattern formation. HetR is a transcription factor that controls the expression of other genes involved in heterocyst differentiation (14–18). The *patS* gene encodes a 17-aa peptide whose C-terminal pentapeptide is the inhibitor (19). The C-terminal peptide (RGSGR) prevents HetR from binding to DNA targets (16), leading to the suppression of heterocyst differentiation. In the case of heterocyst pattern formation, current evidence supports the view that the short peptide (E)RGSGR moves from heterocysts and proheterocysts to neighboring cells (19–23).

The detailed route of the PatS peptide movement between the cells has not been determined. Although it could move through the periplasmic space that is continuous and shared by all cells along the filaments of *Anabaena* 7120 (21, 24–27), we think that the PatS peptide and other metabolites move along the filaments through intercellular channels (26, 25–30). A recent electron tomography (ET) study has clearly established that channels penetrate the rigid peptidoglycan (PG) layer that separates cells in the filaments (30). The presence of nanopore pits on the PG septa between two cells (29) also strongly implies that there are cytoplasmic connections between two neighboring cells. The nanopores are located in the central areas of the septa. Formation of the nanopores on the septa between the cells requires amidases in both *Anabaena* 7120 and *Nostoc punctiforme* (29, 31, 32).

*N*-Acetymuramyl-l-alanine amidases (Ami) cleave an amide bond between the *N*-acetyluramic acid backbone (MurNAc) and *l*-alanine of the peptidoglycan (33). There are five subgroups

Significance

The filamentous cyanobacterium *Anabaena* has become a widely studied model to determine the molecular mechanisms involved in establishing and maintaining the pattern of heterocyst differentiation in response to the removal of fixed nitrogen from the environment. Heterocysts develop from vegetative cells, usually spaced about 10 cells apart, converting an oxic cell capable of division into an anoxic factory for nitrogen fixation that does not divide. Genetic analysis to elucidate the mechanisms of intercellular material exchange between heterocysts and vegetative cells is in an early phase.

Here we show that an amidase is involved in the function of channels that penetrate the rigid peptidoglycan walls that separate cells in the filaments.
of these enzymes in *Escherichia coli*: periplasmic AmiA, AmiB, AmiC, AmiD, and cytoplasmic AmpD (34–37). In *Anabaena* 7120, a mutant of amiC1 (alr0092) is unable to form heterocysts and loses intercellular communication. Two studies of the adjacent homologous gene amiC2 (alr0093) reported different results. Zhu et al. (38) showed that a mutant lacking alr0093 could not form mature heterocysts, whereas Berendt et al. (32) reported that a mutant lacking alr0093 showed no observable phenotype. An amiC2 mutant of *N. punctiforme* ATCC 29133 showed irregular cell-division planes and lacked both cell differentiation and intercellular communication through the cytoplasm (31). Recently, the 3D structure of AmiC2 from *N. punctiforme* was determined, and some structural features of the enzyme suggest that it has unique roles in cell-wall remodeling (39). Here, we show that *all1140*, encoding a different amidase-C, is required for intercellular material exchange in *Anabaena* 7120 and the differentiation of heterocysts.

### Results

**Inactivation of *all1140* (AmiC3) of *Anabaena* sp. PCC7120.** In a screen of a mutant library of *Anabaena* 7120 for genes that are involved in heterocyst formation, we found that an insertion mutant of *all1140* was incapable of diazotrophic growth. The gene *all1140* and five other genes in *Anabaena* 7120 (alr0092, alr0093, all2494, all4998, and all4999) encode proteins that belong to the N-acetylmuramoyl-l-alanine amidases based on a cyanobacterial genome database search (cyanoBase; genome.microbedb.jp/cyanoBase). All the amidases except All4294 have an AmiC domain. The genes alr0092 and alr0093 encode AmiC1 and AmiC2, respectively; their catalytic AmiC domain is located at the C terminus (32). The protein encoded by *all1140* has an AmiC catalytic domain located at the N terminus along with two adjacent PG-binding domains at the C terminus (Fig. 1A), and the domain arrangement in All1140 is conserved in heterocystous cyanobacteria (Fig. S1). The AmiC domain of All1140 is 36.1% and 33.7% identical to that of AmiC1 and AmiC2, respectively, and is 32.3% identical to AmiC in *E. coli*.

*All1140* protein hydrolyzes PG. Recombinant All1140 was produced in *E. coli* and purified (Fig. S2A). The recombinant protein was incubated with PG isolated from *Staphylococcus*, *E. coli*, and *Anabaena* 7120, and the hydrolyzed products were analyzed by liquid chromatography. The results (Fig. 1B–D) show that the recombinant All1140 has PG hydrolysis activity; we named this protein “AmiC3.” A mutant of *Anabaena* 7120 was constructed in which the *all1140* gene was replaced by the streptomyacin-resistance (*Sm*<sup>4</sup>) cartridge (Fig. S2C), confirmed by Southern hybridization (Fig. S2B). The mutant strain is named “M40.”

Growth of the wild-type and M40 strains in BG11 (with nitrate) was measured (Fig. S3A). M40 had a longer lag time and a somewhat slower growth rate than the wild-type strain. Under these experimental conditions, the doubling time of M40 was 31.9 ± 1.3 h, whereas the doubling time of the wild-type strain was 27.1 ± 0.7 h. M40 was not able to grow when N<sub>2</sub> was the sole nitrogen source, and no heterocysts could be detected after a nitrogen step-down (Fig. 2A). The growth phenotype of the M40 mutant was largely restored to that of the wild-type strain when M40 was complemented with a wild-type *all1140* gene (C40). C40 had a doubling time of 28.8 ± 1.7 h, and it restored the ability to develop heterocysts in a semiregular distribution along
Subcellular Localization of AmiC3-GFP. Although there is no signal peptide or other domain that might target AmiC3 to periplasmic space, proteomic analysis showed that AmiC3 (All1140) existed in the outer membrane fraction of Anabaena 7120 (40). In E. coli, AmiC is located in the periplasm, and it is recruited to the Z-ring during cell division (41). To study the subcellular location of AmiC3, we constructed an all1140-gfp (AmiC3-GFP) fusion and transformed it into the wild-type and M40 strains. We first isolated different cell fractions and used immunoblotting to determine which fractions contained the fusion protein. In strain C40G, obtained by complementing M40 with the all1140-gfp fusion, the majority of AmiC3-GFP was found in outer membrane/Pg fractions; a minor portion of AmiC3-GFP could be detected in the cytoplasmic fraction (Fig. 3A and Fig. S4). In one control, a strain that expressed the gfp gene from the rbcL (ribulose-1,5-bisphosphate carboxylase/oxygenase; Rubisco) promoter, GFP was detected only in the cytoplasmic fraction, demonstrating that various membranes were not contaminated with GFP. Fig. 3B shows the subcellular location of AmiC3-GFP in the filaments of these strains grown in BG11 medium (with nitrate). The GFP fluorescence images in strain C40G (Fig. 3B) show that AmiC3-GFP forms a ring structure at midcell positions in growing cells, possibly a result of association with the Z-rings in cell division (42–45). The AmiC3-GFP rings at the septa between two separated or nearly separated daughter cells were double-layered, suggesting that the AmiC3-GFP rings were split by the formation of septa. The diameter of the AmiC3-GFP rings became smaller as two daughter cells separated, and the ring eventually disappeared from the septal regions. In the absence of combined nitrogen, C40G formed heterocysts (Fig. 3C) and grew diazotrophically. In nitrogen-deprived cultures of C40G, the AmiC3-GFP fluorescence rings were observed on both sides of a proheterocyst, whereas no AmiC3-GFP ring was seen on either side of mature heterocysts (Fig. 3C). Because the septal ring is dependent upon FtsZ. We compared the AmiC3-GFP ring with the FtsZ-GFP ring. Although both rings are located in the septal regions (Fig. 3D), the AmiC3 ring persisted longer in cell cycles. Another difference was observed in the stationary phase: FtsZ-GFP was located mostly in the cytoplasm, whereas AmiC3-GFP was located mostly in the periplasmic space (Fig. 3E).

Intercellular Molecule Movement and Septal Nanopores (Channels). To determine whether AmiC3 is required for intercellular communication, we investigated whether small molecules are able to move between the cells in Anabaena 7120. A nonfluorescent acetoxymethylester (AM) derivative of calcein was loaded into the cytoplasm of Anabaena 7120 (28) where it was converted into hydrophilic green-fluorescent calcein (622.5 Da) (46). The fluorescence recovery after photobleaching (FRAP) method was used to determine the capacity of the wild-type and M40 strains to support calcein movement (Fig. 4). In the calcein-loaded filaments of the wild-type strain, fluorescence of a bleached cell did not recover during the entire observation period of 80 s. We also investigated fluorescence recovery when the cells were loaded with fluorescent esculin, which has a molecular mass of 340.3 Da, smaller than calcein (Fig. 4B). Fast recovery of esculin fluorescence with a t1/2 of 2 s was observed in the wild-type strain. The degree of the recovery was ∼50%. In M40, a small (18%) recovery of esculin fluorescence was observed with a recovery t1/2 of 9 s (Table S2).

The lack of intercellular movement of calcein in M40 was investigated further using EM. In transmission EM (TEM), images of thin sections after chemical fixation show that the channels that physically connect two vegetative cells are present in both the wild-type and M40 strains (Fig. 5A and B). Connections between a vegetative cell and heterocyst were observed also (Fig. S5). Next, high-pressure freezing combined with ET was used to investigate the channels of the wild-type and M40 strains; the results are shown in Fig. 5 C and D. The septal...
nanopores that are required for the formation of channels between cells are present as opaque areas of PG wall in perpendicular sections of the septa. Our measurements show an average diameter of 12.7 nm and 12.4 nm for the wild-type and M40 strains, respectively. The channels could be observed best when the images are rotated 90° (Fig. 5 C and D, 2). The septal nanopores were examined further with isolated cell-wall sacculi from Anabaena 7120 strains according to Lehner et al. (29); results are shown in Fig. 5 C and D. Although arrays of nanopores are observed in the septa of both the wild-type and M40 strains (Fig. 5 E, 2 and F, 2), the sizes of nanopores in the isolated sacculi of these two strains are quite different: The average diameter of the nanopores in the M40 strain is 11.7 nm, similar to that obtained by ET, whereas the average diameter of the nanopores of the wild-type strain is 20.1 nm, significantly larger than that obtained by ET.

FRAP in Nonheterocystous Strains of Cyanobacteria. In a survey of the ability to recover fluorescence after photobleaching in filamentous cyanobacteria, we found two nonheterocystous strains that showed no calcein fluorescence recovery when a cell was bleached: Phormidium sp. and Oscillatoria sp. (Table 1 and Fig. S6). Septal nanopores were examined with the sacculi isolated from these two strains and a Microcoleus sp. strain, which showed fast fluorescence recovery (Fig. S6). The average diameters of the septal nanopores of Phormidium sp. and Microcoleus sp. were 12.5 nm (Fig. 6A) and 20.0 nm (Fig. 6B), respectively (Table 1). The nanopore in Oscillatoria sp. was unique in that it had only one central nanopore on a septum, and the diameter of the nanopore was 23.3 nm (Fig. 6C).

Discussion

Like AmiC1 and AmiC2, AmiC3 of Anabaena 7120 had an AmiC domain, and its amidase activity was demonstrated by its ability to hydrolyze PG isolated from E. coli, Staphylococcus aureus, and Anabaena 7120 (Fig. 1). Computer modeling (Fig. S7) predicts that the catalytic domain of AmiC3 is very similar to that of the N. punctiforme AmiC2 (39). Fractionation of cellular proteins revealed that the AmiC-GFP fusion protein is located in the periplasmic space (Fig. 3A), even though there is no signal peptide in the primary sequence of AmiC3. Confocal microscopy demonstrated that AmiC3-GFP is associated with the septal rings (Fig. 3B). A similar situation was found for the AmiC protein of E. coli, which has no signal peptide but is located in the periplasmic space (41). In Anabaena 7120, AmiC3 is a persistent component of the septal ring: The association of AmiC3-GFP with the septal rings can be observed for nearly the entire period of septum formation. The behavior of the AmiC3-GFP ring during the cell cycle (Fig. 3B) indicates that AmiC3 is tightly associated with inward-growing cell septa even after the disassembly of the FtsZ-ring (Fig. 3D). It also shows that AmiC3 in Anabaena 7120 performs its function in a restricted area: the newly formed PG layer of the cell septa. The M40 mutant lacking the all1140 gene encoding Ami3 showed no fluorescence recovery after a cell loaded with calcein was photobleached (Fig. 4), indicating that cellular communication is impaired in the mutant.

Because the cyanobacterial cells have a rigid PG layer in cell walls, the formation of the channels for material exchange between cells would require the formation of nanopores in septa, and therefore it is not surprising that amidases are involved in these processes. An early study with freeze-fracture EM showed that some filamentous cyanobacteria had channels between the cells (47). These channels were called “microplasmodesmata,” but their relationship to plant connections was not established in terms of detailed structure or function. Several recent studies demonstrated that nanopores or channels are indeed present in the septa of heterocystous cyanobacteria (30). These studies agreed that there is an array of nanopores in the septum, but the reported size of the nanopore differed in these studies. A diameter of 12 nm was found when ET was used to study the channels crossing the septum in Anabaena 7120 (30). When sacculi of

Fig. 4. FRAP of Anabaena 7120 strains. The wild-type and mutant M40 strains were loaded with calcein (A, 1) or esculin (B, 1) before photobleaching. As indicated by arrowheads, a cell was photobleached, and its fluorescence was monitored continuously. Images before photobleaching (Pre) and at 0, 4, 20, and 80 s after photobleaching are shown. A, 2 and B, 2 show the kinetics of fluorescence intensities as a function of the recovery time of filaments loaded with calcein and esculin, respectively. (Scale bars: 5 μm.)
N. punctiforme were isolated by boiling and were examined by EM, the observed diameter of septal nanopores was 20 nm (29). Because the size of nanopores is important for their function, we compared the two methods. We found that the different nanopore sizes reported by various laboratories do exist in the wild-type Anabaena 7120 when the two different methods mentioned above were used to observe the nanopores. However, when M40 lacking AmiC3 was studied, very similar sizes of nanopores were observed with the same two methods (Fig. 5). These results indicate that the function of AmiC3 is not directly involved in pore drilling as is AmiC2 of N. punctiforme. More importantly, we found that the septal walls of the wild-type and M40 strains respond differently to the treatment during saccule isolation. The septal wall of the wild-type strain was expandable, and the expandability was dependent upon AmiC3. Apparently, the ability to expand is important for proper channel formation to allow molecules with certain mass to move between the cells. That some fluorescence recovery was observed when the cells of the M40 strain were loaded with esculin (Fig. 4) supports this suggestion. It is also interesting that Phormidium sp. (Fig. 6) and

Table 1. Measurement of diameters of septal nanopores and fluorescence recovery times of filamentous cyanobacteria

<table>
<thead>
<tr>
<th>Species</th>
<th>Diameter of nanopores, nm</th>
<th>Recovery time $t_{1/2}$, s</th>
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<tbody>
<tr>
<td>Anabaena sp.7120 WT</td>
<td>$12.7 \pm 1.3$ (ET)*</td>
<td>-</td>
</tr>
<tr>
<td>Anabaena sp.7120 M40</td>
<td>$12.4 \pm 0.7$ (ET)*</td>
<td>-</td>
</tr>
<tr>
<td>Anabaena sp.7120 WT</td>
<td>$20.07 \pm 0.42$ (sacculi)$^1$</td>
<td>$2.17 \pm 1.14$</td>
</tr>
<tr>
<td>Anabaena sp.7120 M40</td>
<td>$11.66 \pm 0.53$ (sacculi)$^1$</td>
<td>$&gt;80$</td>
</tr>
<tr>
<td>Anabaena cylindrica</td>
<td>$25.55 \pm 0.46$</td>
<td>$2.06 \pm 0.93$</td>
</tr>
<tr>
<td>Microcoleus vaginatus</td>
<td>$19.95 \pm 0.81$</td>
<td>$3.13 \pm 2.67$</td>
</tr>
<tr>
<td>Oscillatoria lutea</td>
<td>$23.32 \pm 0.52$</td>
<td>$&gt;80$</td>
</tr>
<tr>
<td>Phormidium foveolarum</td>
<td>$12.49 \pm 0.45$</td>
<td>$&gt;80$</td>
</tr>
</tbody>
</table>

Two methods were used in measurement of the nanopore diameters: observation of isolated sacculi with TEM and ET of cryopreserved filaments. For determination of fluorescence recovery time, filaments were loaded with calcein, and fluorescence recovery was measured after a cell was photobleached. The strains used were obtained from the Institute of Hydrobiology (FACHB), Chinese Academy of Sciences, Wuhan, China.

*Data from ET images of samples frozen under high pressure.
$^1$Data from TEM images of purified PG sacculi.
expression of other downstream genes for heterocyst formation. However, even though hetR was up-regulated in the absence of combined nitrogen in the M40 strain (Fig. 2), the patS gene, which is a downstream gene regulated by hetR, was not up-regulated, and no morphological differentiation was observed (Fig. 2). Overexpression of hetR, which usually induces multiple contiguous heterocyst formation (15), did not induce the formation of hetR. Because (Fig. S3) in the M40 strain. Because the molecular mass of the short C-terminal peptide of PatS ([E]RGSGR) is similar to that of calcein, its movement from cell to cell could be impeded in the M40 strain.

How do we explain the inability of the mutant strain M40, which fails to make the enzyme AmiC3, to differentiate heterocysts? Differentiation requires the activity of HetR (14). The hetR gene is transcribed in response to a regulatory cascade that begins with deprivation of fixed nitrogen, in particular a reduction in the ratio of glutamine to 2-oxoglutarate. The latter activates NtcA, which activates transcription of nrrA (49), which in turn activates transcription of hetR. Thus, HetR is produced in all the cells of the filament of Anabaena 7120. Therefore, one would expect that all cells should differentiate. However, one gene that is immediately activated by HetR is patS; its product, the protein PatS, whose C-terminal hexapeptide, ERGSGR, is released by proteolysis, binds to HetR, and causes it to dissociate from target DNA (8, 16, 19). HetR freed from DNA is destroyed rapidly by protease. Therefore, the critical gradient that determines the pattern of differentiated cells should be a gradient of PatS peptide whose concentration is highest next to a heterocyst and lowest halfway between two heterocysts (23, 28). The full PatS protein must be made in the heterocyst, where there is abundant active HetR. Logic suggests that the protease that generates the inhibitory peptide from PatS is located in or near the channels that connect heterocysts to vegetative cells. Our FRAP experiments indicate that the channels through the peptidoglycan layer are a correct size to transport the PatS peptide. To complete this model, we must conclude that the channels made in the mutant M40 strain are defective in PatS processing and transport, so inhibitory PatS levels remain in all cells. Without export of PatS there can be no functional HetR and no heterocysts.

Experimental Procedures

Gene Inactivation and Complementation. All enzymes were purchased from Promega and used according to instructions. To construct amiC3 (all1140) mutants, a DNA fragment containing amiC3 was amplified by the PCR with primers P3 and P4, using total genomic DNA as template. All primers used in PCR are listed in Table S3; all PCR products were confirmed by sequencing. The PCR-generated fragment was cloned in pGEM-T vector (Promega) to generate plasmid pTv-amiC3. The plasmid was inversely amplified by PCR with primers P5 and P6. The generated fragment was ligated with a blunt-ended Sm′-cartridge encoding resistance to streptomycin. The resulting plasmid pAmiC3-Sm was digested with BglII and PstI, and the recovered fragment then was cloned into pRL277 (50) for transformation of Anabaena 7120 by conjugation. Segregation of the mutant amiC3 was confirmed by Southern hybridization as described by Huang et al. (16). Total genomic DNA of Anabaena 7120 was isolated with the E.Z.N.A. Plant DNA Miniprep Kit (Omega Biotek) and then was digested with Clal and EcoRI. The digested fragments were separated on a 1.0% agarose gel before transfer onto nitrocellulose paper for hybridization. The DNA probe was synthesized by random primer extension using the template amplified with primers P7 and P8. The mutant with all1140 mutation was named M40. For complementation of M40, the amiC3 gene plus a 600-bp upstream section were amplified by PCR using primers P9 and P10 followed by insertion into a chromosome.

Fig. 6. Analyses of septal nanopores from filamentous nonheterocystous cyanobacteria by EFM. (A–C) Images from Phormidium foveolatum (A), Microcoleus vaginatus (B), and Oscillatoria lutea var. contorta (C), respectively. (A, 1, B, 1, and C, 1) Isolated cell wall sacculi. (A, 2, B, 2, and C, 2) EM images of septa with nanopores. (A, 3, B, 3, and C, 3) Enlarged images of a nanopore in A, 2, B, 2, and C, 2, respectively. (Scale bars: 500 nm in A and B; 20 nm in C)

Microcoleus sp. have 12-nm and 20-nm nanopores on septa of isolated sacculi, respectively. The former displays no calcein fluorescence recovery, but the latter does. In addition to nanopore size, the number of nanopores on septa is also important. In Oscillatoria sp., which has just one nanopore at the center of the septum, no calcein fluorescence recovery was detected even though the nanopore has a diameter of 23 nm (Fig. 6).

The ability of a vegetative cell to differentiate and form heterocysts for nitrogen fixation represents an advanced feature of multicellularity in prokaryotes. Among many genes that are involved in heterocyst formation, hetR together with patS plays a central role in the process. The initiation of heterocyst differentiation requires up-regulation of hetR (48), which in turn regulates the
To express the hetR gene from the petE promoter, the petE promoter (15) amplified with P19 and P20 was cloned into pAM505, generating plasmid pAM505-Pets. The hetR gene amplified with P21 and P22 was cloned into the SacI and EcoRI sites of pAM505-Pets, and the resultant plasmid pAM505-Pets-hetR was transformed into the wild-type and M40 strains separately. Confocal images of Anabaena 7120 were obtained with a Zeiss LSM 710 NLO DuoScan System confocal microscope using a Plan-Apochromat 63×/1.40 iil differential interference contrast (DIC) M27 objective. Photosynthetic pigment fluorescence images (excitation: 561 nm; emission detection: 600-650 nm) and GFP fluorescence images (excitation: 488 nm; emission detection: 495-540 nm) were recorded.

Localization of AmiC3 and FtsZ. To localize AmiC3, a gfp gene was fused to the C-terminal part of amiC3 as follows. A gfp gene was amplified with PCR by primers P11 and P12 using the plasmid pAM1951 (19) as template before it was ligated to pAM505 to generate plasmid pAM505-gfp. The PamiC3-amiC3 was amplified using primers P9 and P13 and was cloned into the BamHI and SalI sites of pAM505-gfp-2. The resultant plasmid, pAM505-PamiC3-amiC3-gfp, was transformed into the M40 strain by conjugation to generate the Ca040 str. FtsZ was localized according to Sakr et al. (43). First, the ftsZ gene was amplified using primers P23 and P24 by PCR with pJana 7120 C120 genomic DNA as a template and was cloned into the SacI and Sall sites of pAM505-Pets. Second, the ftsZ gene amplified with PCR by primers P11 and P12 was digested with SalI and EcoRI and then ligated to pAM505-Pets-ftsZ; the resultant plasmid pAM505-Pets-ftsZ gfp was transformed into wild-type Anabaena PCC 7120.

The cell suspensions were stained on agar-coated glass slides (1.5% [wt/vol] Bacto-Agar in growth medium). All measurements were carried out at room temperature (∼25 °C). 3D-SIM images were obtained on an N-SIM imaging system (Nikon) equipped with a 100×/1.49 NA oil-immersion objective (Nikon) and four laser beams (488 and 561 nm). Image stacks with an interval of 0.120,240.48 μm were acquired and computationally reconstructed to generate superresolution optical serial sections with twofold extended resolution in both x,y and z directions. The reconstructed images were processed further for maximum-intensity projections and 3D rendering with NIS-Elements AR 4.2.0.0 (Nikon).

To determine which cellular compartment contains AmiC3, cell fractionation was performed with the strain Ca040 and a strain expressing gfp according to Böltger et al. (52) and Moslavac et al. (40). Proteins in different fractions were analyzed by immunoblotting using antibodies against GFP.

Calcine Labeling and FRAP. Calcine labeling was done according to Mullineaux et al. (28) and Lehner et al. (31). For calcine labeling, cultures were grown until the OD750 was 0.6. Then 0.5 mL of culture was harvested by gentle centrifugation and was washed three times with fresh BG11 medium (6). The pellet was resuspended in 0.5 mL fresh BG11 medium and was mixed with 20 μL of calcine-AM (1 mg/mL in dimethylsulfoxide; Invitrogen Molecular Probes). The suspension was incubated at 30 °C in the dark for 90 min, and cells were washed three times with fresh BG11 medium. The suspension was incubated for another 90 min in the dark before imaging. For FRAP, the suspensions were spotted onto agar-coated glass slides (1.5% [wt/vol] Bacto-Agar with growth medium). All measurements were carried out at room temperature (∼25 °C). FRAP experiments were performed on an UltraView VoX spinning disk confocal microscope (PerkinElmer) with a 100×/1.4 NA oil-immersion objective and a solid state 50-mW 488-nm laser line. Calcine fluorescence was imaged with 488-nm excitation and 525/50-nm emission filters. After three prebleach images were recorded using 10% laser power, a region of interest was bleached using a single iteration with the 488-nm line operating at 100% laser power, which lasted 50–80 ms depending on the bleach region size. Fluorescence recovery was monitored at low laser intensity (10% of a 50-nW laser) over 80 s at 0.5-s intervals. FRAP data were analyzed using Velocity software.

Esculin Labeling and FRAP. For esculin labeling (53), cell cultures were grown on BG11 medium until the OD750 was 0.6. Then 0.5 mL of culture was harvested by gentle centrifugation and was washed three times with fresh BG11 medium. The cell pellet was resuspended in 0.5 mL fresh BG11 and mixed with 15 μL of saturated (5 mM) aqueous esculin hydrate solution (Sigma-Aldrich). The suspension was incubated at 30 °C in the dark for 30 min, and cells were washed three times with fresh BG11 medium. The suspension was incubated for another 15 min in the dark before imaging. For FRAP, the suspensions were spotted onto agar-coated glass slides (1.5% [wt/vol] Bacto-Agar with growth medium). FRAP experiments were performed as described above for calcine, except that the bleach used a 430-nm line and emission was recorded at 474/501 nm.

EM and Cryo-EM. Peptidoglycan sacculi were prepared by the method of de Pedro et al. (54) and Lehner et al. (29). EM of purified PG sacculi was done according to Priyadarshini et al. (59) and Lehner et al. (31). The samples of ultrathin sections were handled according to Findlater et al. (56) and were observed with a JEM-1010 electron microscope (JEOL). Cryopreservation of Anabaena 7120 strains and EM tomography were performed as described by Omairi-Nasser et al. (30).

The experimental procedures of strains and culture conditions, protein purification, and enzyme assays are provided in SI Experimental Procedures.

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