A network of filament-forming proteins maintains multicellular shape in the cyanobacterium *Anabaena* sp. PCC 7120

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Abstract

The determinants of bacterial cell shape are extensively studied in unicellular forms. Nonetheless, the mechanisms that shape bacterial multicellular forms remain understudied. Here we study coiled-coil rich proteins (CCRPs) in the multicellular cyanobacterium *Anabaena* sp. PCC 7120 (hereafter *Anabaena*). Our results reveal two CCPRs, Alr4504 and Alr4505 (termed LfiA and LfiB for linear filament), which assemble into a heteropolymer *in vivo* and *in vitro*. Two additional CCRPs, Alr0931 (termed CypS for cyanobacterial polar scaffold) and All2460 (termed CeaR for cyanobacterial elongasome associated regulator), form a polar proteinaceous scaffold and are associated with MreB activity, respectively. Deletion mutants of these CCRPs are characterized by impaired trichome (i.e. cyanobacterial filament) and cell shape and decreased viability. All four CCRPs interacted with each other, with the septal junction protein SepJ and all but CypS interacted with MreB. Our results indicate that filament-forming CCRPs are present in cyanobacteria and that they, likely in cooperation with SepJ and MreB, could form a proteinaceous network that stabilizes the *Anabaena* trichome. We propose that this network is essential for the manifestation of the linear trichome phenotype in *Anabaena*.

Importance

The phylum Cyanobacteria is characterized by a large morphological diversity, ranging from coccoid or rod-shaped unicellular species to species forming multicellular morphology, which comprise several cells connected into a linear form. Despite this diversity, very few molecular mechanisms underlying the cyanobacterial morphological diversity are known. Among these, the cytoskeletal proteins FtsZ and MreB are important regulators of cyanobacterial cell shape and viability. The multicellular phenotype of cyanobacteria has been linked also to the septal junctions, which comprise a pretentious complex dividing between neighboring cells in the linear form. In our research we identified and characterized four proteins that are involved in cell and trichome shape regulation in multicellular cyanobacteria. We show that two of those proteins are interdependent for polymerization, revealing a novel feature for prokaryotic

filament-forming proteins. Our study leads to a broader understanding of the underlying principles of cyanobacterial morphological diversity.

Introduction

Bacterial multicellularity ranges from transient associations, such as colonies and biofilms to permanent multicellular forms[1]. The basic characteristics of prokaryotic organisms that are considered as multicellular are mechanisms of cell-cell adhesion and intercellular communication[2]. Biofilms are considered as transient forms of prokaryotic multicellularity as they lack a reproducible multicellular[3]. The key hallmarks of permanent bacterial multicellularity are morphological differentiation and a well-defined and reproducible shape, termed patterned multicellularity[3]. Unlike biofilms, patterned multicellular structures are the result of either coordinated swarming or developmental aggregation behavior as in myxobacteria[4]. Additional factors include cell division, proliferation and cell differentiation as in sporulating actinomycetes[5] and cyanobacterial trichomes[6]. In myxobacteria as well as in actinomycetes, it has been shown that patterned multicellular traits are dependent on the coordinated function of different coiled-coil-rich proteins (CCRPs). Similar to eukaryotic intermediate filaments (IFs)[7], many bacterial CCRPs were shown to perform cytoskeletal functions through their ability to self-assemble into filaments *in vitro* and *in vivo*[8–11]. However, unlike FtsZ or MreB[12,13], the prokaryotic tubulin and actin homologs[14], bacterial filament-forming CCRPs do not require additional co-factors for polymerization *in vitro*[15]. For example, in *Myxococcus xanthus*, the coordinated swarming and aggregation into fruiting bodies is mediated by its gliding motility which strictly depends on the filament-forming CCRP AglZ[16]. AglZ is organized in a large multi-protein complex that governs gliding motility in synergy with MreB, which still retained its peptidoglycan (PG) synthesis function but was co-opted for gliding motility in *M. xanthus*[17]. Actinobacteria, such as *Streptomyces* species, grow by building new cell wall (i.e. PG) only at the cell poles, independent of MreB[18], which is strikingly different from how most other bacteria grow[19]. This characteristic polar growth mode is organized by a cytoskeletal network of at least three CCRPs - DivIVA, Scy and FilP – that form the polarisome[9]. Two of those, FilP and Scy,

self-assemble into filaments *in vitro*[9,11,20] thereby fulfilling a major criterion of IF-like cytoskeletal proteins[14]. *In vivo*, however, Scy does not form filaments and instead acuumulates as foci at future branching points[9], while FilP localizes as gradient-like caudates at the hyphael tips[21] instead of forming distinct filaments as observed for the CCRP crescentin from *Caulobacter crescentus*[8]. Although of essential importance for growth and cell shape, the polarisome is not directly involved in the hallmark patterned multicellular trait of Actinobacteria. In contrast, patterned multicellularity in Actinobacteria is governed by the highly reproducible and coordinated formation of Z-ring ladders during sporulation[3,22].

Cyanobacteria are characterized by a large phenotypic diversity, ranging from unicellular species to complex multicellular cyanobacteria of which some can undergo irreversible cell differentiation[23]. Multicellular cyanobacteria that differentiate multiple cell types and form trichomes are considered the peak of prokaryotic complexity and their biology has been studied in the context of cytoplasmic continuity, intercellular communication, and cell differentiation[24]. Species of the Nostocaceae are characterized by the formation of linear trichomes, where equally interspaced heterocysts (specialized cells for nitrogen fixation) are differentiated upon nitrogen starvation in a highly reproducible pattern[24]. Studies of multicellular growth in the model multicellular cyanobacterium *Anabaena* showed that FtsZ is an essential protein that localizes to future septum sites in a typical Z-ring structure, while MreB determines the cell shape of single cells within an *Anabaena* trichome but is dispensable for trichome viability[25,26]. Deletion of MreB[26] or of a class B penicillin-binding-protein (PBP)[27] resulted in swollen and rounded cell morphotypes, a phenotype commonly associated with defects in PG biogenesis[28]. As a true-multicellular organism, *Anabaena* contains functional analogs to the eukaryotic gap-junctions, termed septal junctions, which facilitate intercellular communication[29] by direct cell connections and likely function by gating[30,31]. These structures involve the septum localized proteins SepJ, FraC and FraD[31–33] and a nanopore array in the septal PG[34]. The importance of SepJ, FraC and FraD for multicellularity in *Anabaena* is highlighted by a defect in trichome integrity and a resulting loss of multicellularity under diazotrophic growth conditions in strains lacking any of the three genes[32,33]. Besides the

canonical cytoskeletal proteins FtsZ and MreB, no other cytoskeletal proteins have been described in cyanobacteria that could explain the diverse morphotypes. Thus, here we study the contribution of CCRPs to the *Anabaena* phenotype. For this purpose, we predicted *Anabaena* CCRPs with presumed IF-like functions and evaluated their structural properties and cellular localization using *in vivo* and *in vitro* approaches.

Results

Prediction of CCRP candidates in *Anabaena*

To predict potential filament-forming proteins, we performed a computational survey of the *Anabaena* genome for CCRPs putatively having IF-like function[8,10,11,16]. *Anabaena* CCRPs were filtered according to the presence of a central rod-domain, which is characteristic to eukaryotic IF and prokaryotic IF-like proteins[35]. Similar to Bagchi *et al*. (2008), who identified the filament-forming CCRP FilP in *Streptomyces coelicolor*, we defined the presence of a rod-domain as 80 amino acids in coiled-coil conformation. This analysis resulted in the identification of 186 rod domain-containing CCRPs (for a full list of predicted CCRPs see Supplementary File 1). The predicted 186 CCRPs were further filtered to include only hypothetical proteins of unknown function, yielding a set of 13 candidates for further analysis (Supplementary Fig. 1 and Supplementary Table 1). The distribution of homologs to these 13 candidates in cyanobacteria showed that eight *Anabaena* CCRPs have homologs in multicellular cyanobacteria as well as in unicellular cyanobacteria while five have homologs present only in multicellular cyanobacteria (Fig. 1a; Supplementary Table 1).

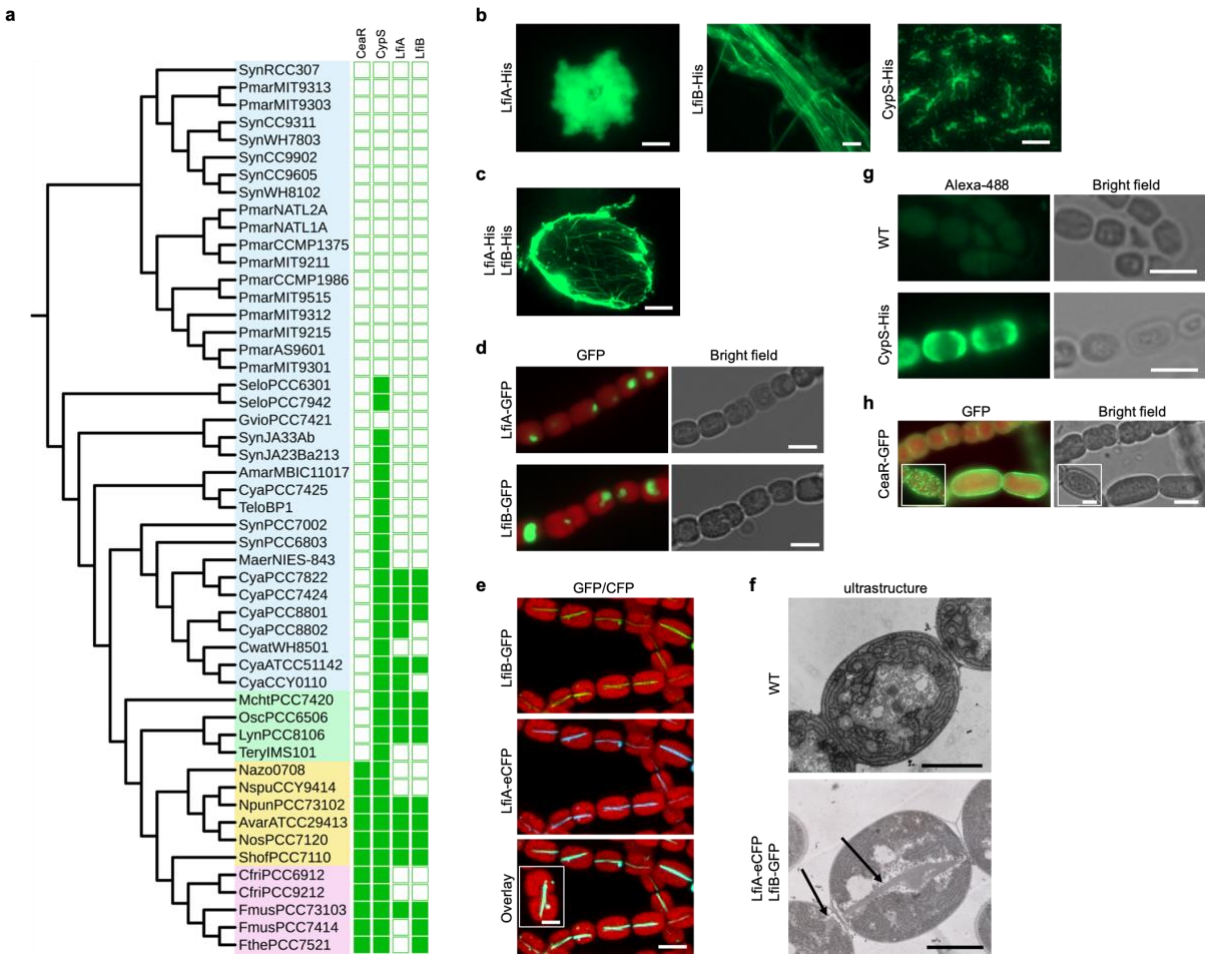

Fig. 1: Cyanobacterial CCRPs polymerize *in vitro* and *in vivo*

(**a**) Distribution of protein candidate homologs in cyanobacteria. Organism names include the genus first letter and species first three letters. The presence of homologous genes is marked by a green rectangle. Organism names are shaded according to cell or colony morphology; blue: unicellular, green: filamentous, yellow: filamentous and heterocyst forming, pink: heterocyst forming and true branching or multiseriate filaments. Homologs accession numbers are supplied in Supplementary File 2. (**b**-**c**) Epifluorescence micrographs of NHS-Fluorescein-stained *in vitro* structures formed by purified and renatured (**b**) LfiA-His (1 mg ml_{-1}), LfiB-His (0.5 mg ml_{-1}) and CypS-His (0.5 mg ml_{-1}) or (**c**) co-renatured LfiA-His and LfiB-His (0.25 mg ml_{-1} each) in 25 mM HEPES, pH 7.4 (LfiB), HLB (LfiA and co-renatured LfiA/B) or PLB (CypS) renaturation buffer. Note: although LfiB formed somewhat filamentous structures *in vitro*, the vast majority of LfiB clumped into aggregates, reminiscent of GroEL1.2 (Supplementary Fig 2). (**d**) Merged GFP-fluorescence and chlorophyll autofluorescence (red) and bright field micrographs of *Anabaena* WT cells expressing LfiA-GFP or LfiB-GFP from P_{petE}. (**e**) Merged GFP and/or eCFP fluorescence micrographs of *Anabaena* cells co-expressing LfiA-eCFP and LfiB-GFP from P_{petE} and grown in $BG11_0$. Localization of LfiA/B grown in BG11 is depicted in Supplementary Fig. 7. Inlay shows that LfiA/B filaments only cross not yet fully divided cells. (**f**) Electron micrographs of ultrathin sections of *Anabaena* WT and *Anabaena* cells co-expressing LfiA-eCFP and LfiB-GFP. Black arrows indicate electron-dense structures coinciding with the LfiA/B heteropolymer observed in Fig. 1e. (**g**) Alexa Fluor 488 and bright field micrographs of anti-His immunofluorescence staining of *Anabaena* WT and *Anabaena* cells expressing CypS-His from P_{petE}. Polar sites loaded with CypS-His plugs coincide with sites of retracted chlorophyll autofluorescence observed prior to immunofluorescence (Supplementary Fig. 6b). (**h**) Merged GFP fluorescence and chlorophyll autofluorescence and bright field micrographs of *Anabaena* WT cells expressing CeaR-GFP from P_{petE}. Notably, no enlarged cells were identified in the Δ*ceaR* mutant strain expressing CeaR-GFP from P_{ceaR} (Supplementary Fig. 8a), indicating that CeaR levels in WT cells are tightly regulated. Inlay shows patchy and cell periphery-localized CeaR-GFP. N-terminal YFP translational fusion of CCRPs did not reveal coherent structures, suggesting that the N-terminus is essential for protein localization. Scale bars: 5 µm, (e) 1.25 µm, (f) 1.6 µm or (h inlay) 2.5 µm.

In vitro and in vivo filamentation of CCRP candidates

Out of the 13 candidates, four CCRPs showed self-association and protein filamentation properties, including LfiA together with LfiB, CypS and All4981 (that will be investigated in a separate report). The remaining nine candidates failed to form filamentous structures *in vivo* and *in vitro* and were excluded from further analysis. An exception is CeaR whose *in silico* prediction suggested similarities to the well-characterized prokaryotic IF-like protein crescentin[8] (Supplementary Table 1). Notably, none of those four proteins has homologs in the SynProCya clade of unicellular cyanobacteria (Fig. 1a), hinting for a function in more complex cyanobacterial morphotypes. To evaluate the ability of the four candidates to self-associate, we ectopically expressed C and N-terminally tagged (His_6, YFP, eCFP or GFP) recombinant proteins and investigated *in vitro* polymerization properties and *in vivo* localization pattern. The assembly of CCRPs into filaments *in vitro* was tested by fluorescence microscopy using the NHS-Fluorescein dye, which was previously successfully used to visualize *in vitro* FtsZ protein filaments[36]. Similar to previous investigations of filament-froming CCRPs[8,9,20], we purified His_6-tagged CCRPs by Ni-NTA affinity chromatography under denaturing conditions and renatured them by dialysis followed by NHS-fluorescein staining. We note that the His_6-tag has previously been shown to have no impact on the *in vitro* polymerization properties of the CCRP FilP[20]. As a positive control for our approach we used crescentin. The NHS-fluorescein staining of crescentin revealed an extensive filamentous network in our *in vitro* assay (Supplementary Fig. 2), showing that our approach is applicable for filament-forming CCRPs. As negative controls we included empty vector-carrying BL21 (DE3) cells, GroEL1.2 from *Chlorogloeopsis fritschii* PCC 6912 (known to self-interact[37]) and the maltose binding protein (MBP), all of which were tested negatively for filament formation *in vitro* using our approach. While neither, the cell-free extract nor the highly soluble MBP protein formed any discernible structures *in vitro*, GroEL1.2 aggregates could be indicative for an uncontrolled oligomerization (Supplementary Fig. 2). Additionally, we observed similar clumps of protein aggregates from other negatively tested *Anabaena* CCRPs, suggesting that this is a common observation for putative oligomerizing proteins.

LfiA and LfiB are interdependent for polymerization *in vitro* and *in vivo*

Since the candidate proteins were annotated as hypothetical proteins, we initially investigated and confirmed the transcription of all four genes under standard (BG11) and diazotrophic ($BG11_0$) growth conditions (Supplementary Fig. 3a,b). An additional inspection of the genomic loci suggested that *lfiA* and *lfiB* are encoded in an operon structure, however, RT-PCR data indicated that they are not co-transcribed (Supplementary Fig. 3a,c). Applying our *in vitro* polymerization assay to renatured and purified LfiA revealed amorphous non-filamentous protein aggregates while LfiB assembled into aggregated and sheet-like structures (Fig. 1b). We note, however, that the vast majority of LfiB protein precipitated into clumps of aggregates upon renaturation, suggesting that LfiB has only a partial capacity to form filaments or is unstable *in vitro*. Inspired by the close genomic localization of *lfiA* and *lfiB*, we next tested for co-polymerization of both proteins. Upon co-renaturation, LfiA and LfiB co-assembled into a meshwork of protein heteropolymers (Fig. 1c). While both, LfiA and LfiB renatured alone formed aggregates in the dialysis tubes that were detectable with the naked eye (similar to GroEL1.2), the co-renatured LfiA/B sample remained in solution, a known property of eukaryotic IFs[38]. We also observed that this co-filamentation is dosage-dependent as only equal amounts of LfiA and LfiB resulted in distinct protein filaments *in vitro* (Supplementary Fig. 4). These data indicate that LfiA and LfiB are interdependent for filamentous assembly *in vitro*.

To examine the *in vivo* localization pattern of LfiA and LfiB, we initially expressed translational GFP fusions of both proteins from the replicative pRL25C plasmid, which is commonly used in experimental work in *Anabaena*[26,39,40]. The expression of LfiA-GFP and LfiB-GFP from their respective native promoters (as predicted using BPROM[41]) revealed no discernible expression of LfiB-GFP (Supplementary Fig. 5a). Consequently, we investigated the *in vivo* localization of both proteins from the copper-regulated *petE* promoter (P_{petE}), which has previously been used to study the localization of FtsZ and MreB in *Anabaena*[26,39,40]. We generally observed that the P_{petE}-driven gene expression does not always lead to expression

of the fusion protein in every cell under standard growth conditions. Notably, this was not observed under diazotrophic growth conditions ($BG11_0$) or upon supplementation with additional $CuSO_4$. The expression of LfiA-GFP and LfiB-GFP from P_{petE} in *Anabaena* independently did not reveal filamentous structures (Fig. 1d). However, upon co-expression of LfiA-eCFP and LfiB-GFP from P_{petE}, a distinct filamentous structure along the longitudinal cell axis could be observed (Fig. 1e). To confirm that the localization of LfiA-GFP and LfiB-GFP is not affected by the wildtype (WT) *lfiA* or *lfiB* alleles, we localized both proteins individually in a Δ*lfiA*Δ*lfiB* double mutant; this revealed the same localization pattern as in the WT (Supplementary Fig. 5b), suggesting that co-polymerization is a dosage-dependent process. . We further validated the *in vivo* co-polymerization of LfiA and LfiB by heterologous expression in *E. coli*, which also revealed an interdependent polymerization pattern (Supplementary Fig. 5c 7). The intracellular localization of the LfiA/B heteropolymer in *Anabaena* suggests that the polymer is either anchored at the cell poles or specifically broken up during cell division, as LfiA/B filaments were never observed to cross cell-cell borders and only traversed through not yet fully divided cells (Figs. 1e inlay and 1f).

CypS localizes to the cell poles in *Anabaena*

Applying our *in vitro* polymerization assay to CypS revealed that CypS assembled into star-like filamentous strings (Fig. 1b). The expression of CypS-GFP in *Anabaena* WT cells from the predicted native promoter (P_{cypS}; using BPROM) did not reveal coherent fluorescence signals (Supplementary Fig. 6a). When expressed from P_{petE}, CypS-GFP was localized to the cytosol and the cell envelope (Supplementary Fig. 6a). The same localization to the cell envelope and the cytoplasm was also observed upon expression of CypS-GFP from P_{cypS} in a *ΔcypS* mutant background (Supplementary Fig. 6a), suggesting that CypS concentration is strictly controlled in the cells. Notably, CypS-GFP only partially complemented the Δ*cypS* mutant swollen cell phenotype (Supplementary Fig. 6a). Consequently, we examined whether the addition of a C-terminal His_6-tag can reconstitute the WT phenotype and found that CypS-His_6 expressed from P_{petE} can complement the Δ*cypS* mutant (Supplementary Fig 9a,c). To localize CypS-His_6 in

the cell, we performed anti-His immunofluorescence staining and found that CypS-His_6 forms plugs at the cell poles (Fig. 1g) that appeared to displace the thylakoid membranes (Supplementary Fig. 6b). Notably, similar polar plugs could also be observed for CypS-GFP but only after additional induction of protein expression (Supplementary Fig. 6a), demonstrating that the GFP-tag only partially interferes with CypS localization. Further induction of CypS-His_6 expression led to the formation of swollen cells (Supplementary Fig. 6b), indicating that CypS has morphogenic properties. Since we found that CypS forms polar plugs and that the LfiA/B filament generally ended at the cell poles, we tested for a possible interplay between LfiA/LfiB and CypS and localized LfiA/LfiB in the Δ*cypS* mutant. This revealed that in the absence of CypS,LfiA/B could no longer form linear filaments (Supplementary Fig. 7), indicating that CypS might aide in the anchorage of LfiA/B to the poles.

CeaR is morphogenic and is localized to the cell envelope and the Z-ring

Attempts to overexpress CeaR-His_6 in *E. coli* for the *in vitro* polymerization assay were not successful, possibly due to the N-terminal transmembrane domains (TMDs; Supplementary Fig. 1). Removal of the CeaR N-terminal TMDs enabled overexpression in *E. coli,* but nonetheless no filamentous CeaR *in vitro* structures were observed. We note that genomes of unicellular cyanobacteria do not have a homologous gene to *ceaR* (Fig. 1a), and furthermore, unlike CypS, LfiA and LfiB, recombinant expression of CeaR-GFP in *Synechocystis* was unsuccessful. This indicates that CeaR function is specific to the multicellular cyanobacterial phenotype. Expression of a functional CeaR-GFP fusion protein (functionality of the fusion protein is shown in Supplementary Fig. 11b,d,e) from P_{ceaR} and from P_{petE} in *Anabaena* WT showed that the protein localized to the cell periphery in a patchy pattern (Fig. 1h, Supplementary Fig. 8a), yet it also accumulated at the septa or at the Z-ring. Z-ring or septal localization was found in 25% of cells (589 out of 2301 counted cells) carrying P_{petE}::*ceaR-gfp* and in 17% of cells (206 out of 1237 counted cells) carrying P_{ceaR}::*ceaR-gfp*. In addition, we observed that the expression of CeaR-GFP from P_{petE} led to a swollen cell phenotype in a large proportion of cells (1754 (76%) of 2301 counted cells) and a similar proportion of swollen cells

when CeaR-GFP was expressed from P_{ceaR} (789 (64%) of 1237 counted cells; Fig. 1h, Supplementary Fig. 8a). A similar swelling of cells was also identified in *Anabaena* WT cells expressing untagged CeaR from P_{ceaR} (435 (32%) of 1346 counted cells; Supplementary Fig. 8a). Expression of both, CeaR-GFP or untagged CeaR in the Δ*ceaR* mutant strain did not induce cell swelling (Supplementary Figs. 8a and 9a). The localization pattern of CeaR indicates that CeaR is associated with the FtsZ-driven divisome. In agreement with this, CeaR-GFP localization to the Z-ring was lost upon deletion of the N-terminal TMDs from CeaR (Supplementary Fig. 8a). This indicates that membrane anchorage is key for proper CeaR function and localization.

Anabaena **CCRP deletion strains show defects in trichome shape and viability**

To further study the function of the four CCRPs, we generated Δ*cypS,* Δ*ceaR* and a double Δ*lfiA*Δ*lfiB* mutant strain and examined their phenotype. Notably, single Δ*lfiA* or Δ*lfiB* mutant strains could not be generated, suggesting that the presence of only one of those proteins is lethal for *Anabaena*. Our results show that the Δ*cypS* and Δ*lfiA*Δ*lfiB* mutants were characterized by altered trichome and cell shape phenotypes and reduced trichome viability (Fig 2a, Supplementary Fig. 10a,b). Unlike the linear trichome growth pattern of the *Anabaena* WT, both Δ*cypS* and Δ*lfiA*Δ*lfiB* mutant strains showed zigzagged trichomes (Fig. 2a, Supplementary Fig. 9d). Additionally, Δ*cypS* and Δ*lfiA*Δ*lfiB* cells were significantly larger and significantly more round in comparison to the WT (Fig. 2c), reminiscent of a Δ*mreB* mutant[26]. The defect phenotype of the Δ*cypS* and Δ*lfiA*Δ*lfiB* mutant strains could be complemented with pRL25C carrying P_{cypS}::*cypS* and P_{petE}::*cypS-his*$_6$ or $P_{lfiA/lfiB}$::*lfiA-lfiB*, respectively (Supplementary Fig. 9a-c). We also observed a slight decrease in cell volume in older Δ*ceaR* mutant cultures (>3 weeks) that exhibited shortened trichomes (Fig. 2c, Supplementary Figs. 9d and 11c). This is in accordance with our observation of cell volume increase upon CeaR-GFP overexpression in *Anabaena* WT (Figs. 1h, Supplementary Fig. 8a).

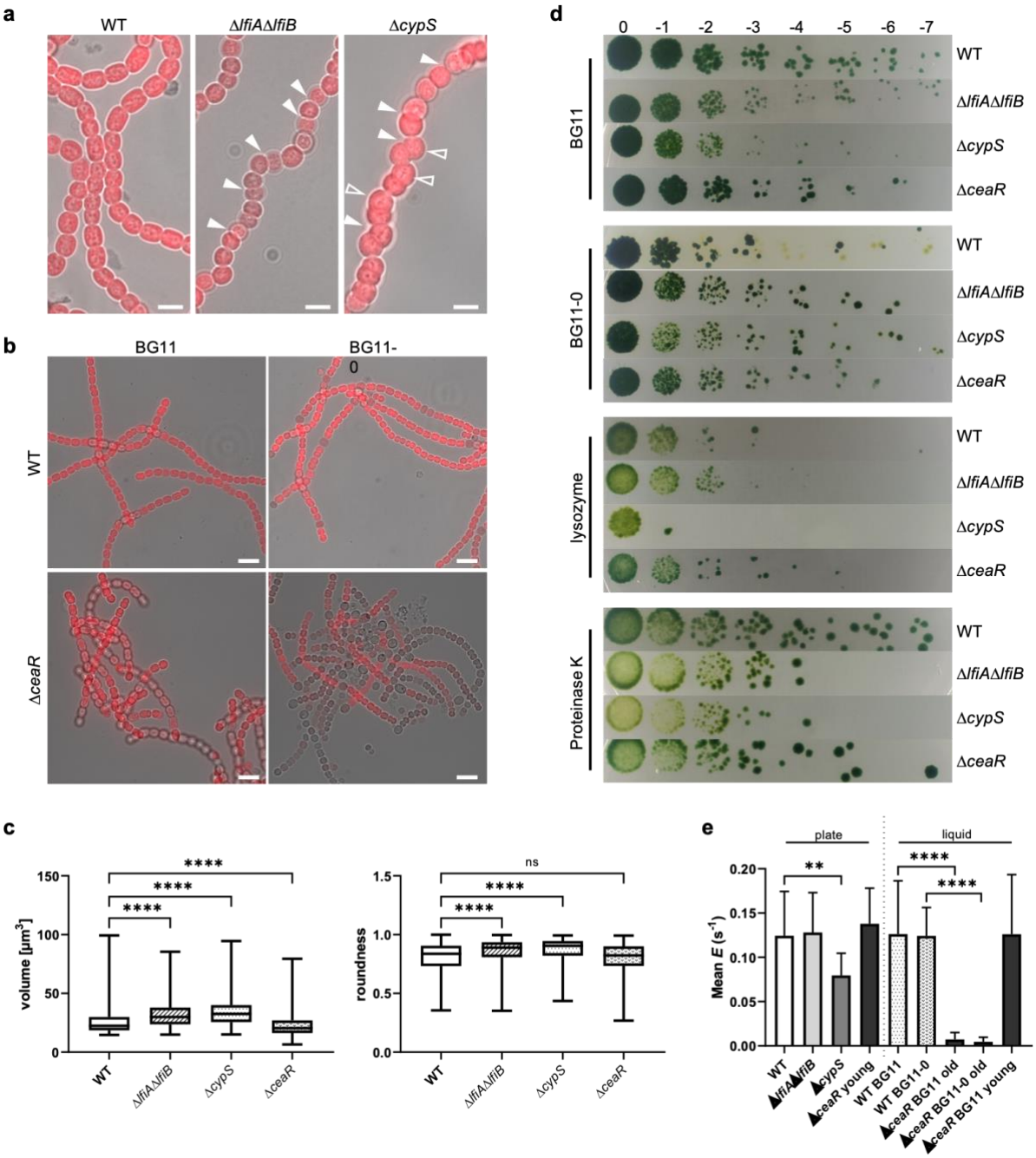

Fig. 2: *Anabaena* CCRP mutants reveal altered trichome and cell shape phenotypes

(**a**-**b**) Merged chlorophyll autofluorescence and bright field micrographs of (**a**) *Anabaena* WT, Δ*lfiA*Δ*lfiB* and Δ*cypS* mutants grown on BG11 plates and (**b**) *Anabaena* WT and Δ*ceaR* mutant grown in BG11 and 5 d after transfer into $BG11_0$. Similar to what was observed upon transfer into $BG11_0$, reduced Δ*ceaR* filament length was also observed during prolonged cultivation on BG11 plates (Supplementary Figs. 9d and 11c).. White triangles indicate zigzagged growth and translucent triangles show swollen cells. Scale bars: (a) 5 µm and (b) 10 µm. (**c**) Cell roundness and volume of *Anabaena* WT, Δ*lfiA*Δ*lfiB*, Δ*cypS* and Δ*ceaR* mutants measured with Fiji imaging software A perfect circle is defined as roundness of 1. *Anabaena* WT: n=537; Δ*ceaR*: n=796; Δ*lfiA*Δ*lfiB*: n=404; Δ*ceaR*: n=369. (**d**) *Anabaena* WT, Δ*lfiA*Δ*lfiB*, Δ*cypS* and Δ*ceaR* mutants were spotted onto BG11, $BG11_0$ or BG11 plates supplemented with lysozyme or Proteinase K in triplicates of serial dilutions of factor 10 and grown until no further colonies arose in the highest dilution (n=2). (**e**) Mean exchange coefficients (*E*) of fluorescence recovery after photobleaching (FRAP) experiments from calcein-labelled *Anabaena* WT and CCRP mutants. Liquid *Anabaena* WT and Δ*ceaR* cultures were grown in BG11 and partially transferred to $BG11_0$ 1 d prior labelling. Plate grown *Anabaena* WT, Δ*lfiA*Δ*lfiB* and Δ*cypS* mutant strains were grown on BG11 plates. Data present the number of recordings of bleached cells (n). *Anabaena* WT BG11 plate: n=21; *Anabaena* WT liquid BG11: n=10; *Anabaena* WT liquid $BG11_0$: n=11;

Δ*cypS*: n=23; Δ*lfiA*Δ*lfiB*: n=17; Δ*ceaR* liquid BG11: n=16; Δ*ceaR* liquid $BG11_0$: n=6. Representative FRAP micrographs are shown in Supplementary Fig. 12. Representative fluorescence recovery curves are shown in Supplementary Fig. 13. Values indicated with * are significantly different from the WT. *: P<0.05, **: P<0.01, ***: P<0.001, ****: P<0.0001. ns indicates no significant difference (using one-way ANOVA with Dunnett's multiple comparison test).

The round and swollen cell phenotypes of the Δ*cypS* and Δ*lfiA*Δ*lfiB* mutant strains are indicative of an impairment in cell wall integrity and/or defects in PG biogenesis as well as an elevated sensitivity to turgor pressure[28,42]. Consequently, we tested the sensitivity of the CCRP mutants to cell wall degrading enzymes and osmotic stressors. This showed that the Δ*cypS* mutant had an elevated sensitivity to lysozyme, suggesting a defect in cell wall integrity in both mutants. No increased sensitivity to Proteinase K was identified in any of the mutants (Fig 2d). . An increased sensitivity to lysozyme has previously been associated with a defect in elongasome function[26], suggesting that CypS could be associated with the *Anabaena* elongasome. Furthermore, Δ*cypS* and Δ*lfiA*Δ*lfiB* mutants were unable to grow in liquid culture (Supplementary Fig. 10a), with Δ*cypS* mutant cells readily bursting upon transfer to liquid culture (Supplementary Fig. 10b), hinting for an elevated sensitivity to fluid shear stress or turgor pressure. In contrast, the Δ*ceaR* mutant was unaffected by the presence of cell wall stressors (Fig. 2d) and grew well in BG11 growth medium (Fig. 2b). However, upon nitrogen stepdown (i.e., transfer into $BG11_0$), the Δ*ceaR* mutant readily fragmented into shorter trichomes that aggregated into large-scale cell clumps (Fig. 2b, Supplementary Figs. 10a and 11d,e). Cells in those clumps also gradually lost their chlorophyll auto-fluorescence signal (an indicator for cell viability) and ultimately died within a few days (Fig 2b, Supplementary Fig. 11a,b,d), revealing a viability defect of the Δ*ceaR* mutant under diazotrophic conditions. The defect in trichome viability could be complemented with pRL25C carrying P_{ceaR}::*ceaR* or P_{ceaR}::*ceaR-gfp* (Supplementary Fig. 11b,d,e). Notably, however, heterocyst-development was not impaired in the Δ*ceaR* mutant nor in the Δ*cypS* and Δ*lfiA*Δ*lfiB* mutants.

Previous studies showed that mutants of genes involved in intercellular communication in *Anabaena* show a similar, albeit more pronounced fragmentation phenotype (producing unicellular forms) when transferred from BG11 to $BG11_0$ due to the loss of diazotrophy[32,33]. Proper nutrient exchange through septal junctions is essential for *Anabaena* viability especially

under diazotrophic growth[24]. Consequently, we investigated the level of intercellular communication using FRAP experiments of calcein stained CCRP mutants. Our results show that the Δ*lfiA*Δ*lfiB* mutant is not impaired in intercellular solute diffusion while diffusion is reduced in the Δ*cypS* mutant and virtually absent in the Δ*ceaR* mutant grown in liquid medium (Fig. 2e, Supplementary Figs. 12 and 13). Notably, solute diffusion was not decreased in young Δ*ceaR* mutant cells (i.e. cultures up to 10 days; Fig. 2e), indicating that the effect of *ceaR* knockout on cell-cell communication is culture age dependent. This age-dependent effect on cell-cell communication is also reflected by the different Δ*ceaR* mutant trichome lengths in young (10 days) and older (> 3 weeks old) cultures. While young Δ*ceaR* mutant trichomes showed normal trichome lengths, trichomes from older Δ*ceaR* mutant cultures were shortened (Supplementary Figs. 9d,e and 11c). To further investigate the underlying cause for the impaired cell-cell communication, we isolated sacculi and observed that the Δ*ceaR* and the Δ*cypS* mutant partially contained significantly larger septal disks with decreased nanopore counts compared to the WT (Supplementary Fig. 14a-c). We suggest that the decreased nanopores per septal disk may be responsible for the decrease in solute diffusion in both mutants. In addition, we observed that some nanopores in the Δ*ceaR* mutant strain were large and irregular (Supplementary Fig. 14B), which as well could contribute to the altered efficiency in solute diffusion.

Anabaena **CCRPs affect MreB localization**

The swollen cell phenotype of an *Anabaena* Δ*mreB* mutant has been previously reported to have no effect on intracellular structures[26]. To assess whether the altered cell and trichome shape of *Anabaena* CCRP mutant strains had any effect on intracellular arrangements, we compared ultrathin sections of *Anabaena* WT and CCRP mutants. Except for Δ*ceaR* mutant cells that contained linear seemingly void structures, which did not represent thylakoid membranes, intercellular ultrastructures of the CCRP mutants were unaffected regardless of their impact on trichome viability and shape (Supplementary Fig. 15). Using epifluorescence microscopy, the void linear structures in the Δ*ceaR* mutant were observed as prominent red

autofluorescence signals (Fig. 3d, Supplementary Fig. 11e and 9e), as such, we named them red fluorescent filaments, whose nature is yet to be elucidated. Nonetheless, the observed cell wall and cell and trichome defects indicated that CypS, LfiA/LfiB and CeaR function is related to PG biogenesis, possibly through association with FtsZ or MreB. To test for a link with the FtsZ-driven divisome, we visualized Z-ring placement in the WT and the mutants by immunofluorescence. However, no alterations in Z-ring placement were observed, indicating that Z-ring formation is unaffected in the mutants (Supplementary Fig. 16). To test for an association with the elongasome, we localized a functional P_{petE}::*gfp*-mreB fusion[26] in *Anabaena* WT and the CCRP mutants. Unlike in the previously reported P_{petE}::*gfp-mreBCD* overexpression strain[26], we never saw polar aggregates in our GFP-MreB-expressing strain (Fig. 3a). This suggests that the previously observed aggregates in the GFP-MreBCD strain[26] are specific to the *mre* operon overexpression rather than the overexpression of *mreB* only. In contrast, we observed GFP-MreB filaments throughout the cells without any directional preference (Fig. 3a). Expression of GFP-MreB in the CCRP mutants revealed considerable alterations of GFP-MreB localization. Even though GFP-MreB filaments were present in the Δ*lfiA*Δ*lfiB* mutant strain, we only detected those in non-rounded cells that seemingly had a WT-like phenotype (Fig 3b inlay; 245 (24%) of 1040 cells counted), whereas in rounded/swollen cells of zigzagged trichomes, the GFP-MreB signals were restricted to the cell poles (Fig. 3b; 795 (76%) of 1040 counted cells). This observation suggests that LfiA/B are involved in proper localization of MreB. Similar to the deletion of *lfiA/B*, deletion of *cypS* seemed to recruit MreB-GFP to the poles but unlike in the Δ*lfiA*Δ*lfiB* mutant, GFP-MreB filaments were still detectable in cells of the Δ*cypS* mutant with a polar GFP-MreB signal (Fig. 3c). In contrast, in the Δ*ceaR* mutant, GFP-MreB only localized as aggregate-like patches and never formed filamentous strings as seen in the WT (Fig. 3d). This suggests that CeaR is important for proper MreB filament-formation. The negative effect of the absence of CeaR on GFP-MreB was even more evident during growth in liquid culture. There, ectopic expression of GFP-MreB led to a prominent swelling of Δ*ceaR* mutant cells and a zigzagged trichome shape (Fig. 3d). Despite being expressed from the P_{petE}, GFP-MreB signal intensity was strongly elevated in these cells

(cells in liquid from Fig. 3d were excited with 1/5 of the excitation time used for the other strains), which suggests a role of CeaR in MreB turnover regulation. An association of CeaR with MreB is further reinforced by a banded and helical-like assembly of CeaR-GFP in *E. coli* (Supplementary Fig. 8b), resembling the localization of MreB in *E. coli*[43].

PG biogenesis is altered in *Anabaena* CCRP mutants

To further assess the function of *Anabaena* CCRPs in PG biogenesis, we stained active sites of PG synthesis by fluorescently labeled vancomycin (Van-FL). While Δ*lfiA*Δ*lfiB* and Δ*cypS* mutants showed a similar Van-FL staining pattern compared to the WT (Fig. 3e), both mutants had a significantly reduced staining intensity (Fig 3f). Hence, it is likely that CypS and LfiA/LfiB are linked to PG biogenesis. In contrast, the Δ*ceaR* mutant is characterized by an increased mean intensity of Van-FL staining (Fig. 3f) and staining is often observed not only in the septal wall, as in the WT, but also in the lateral cell wall (Fig. 3e).

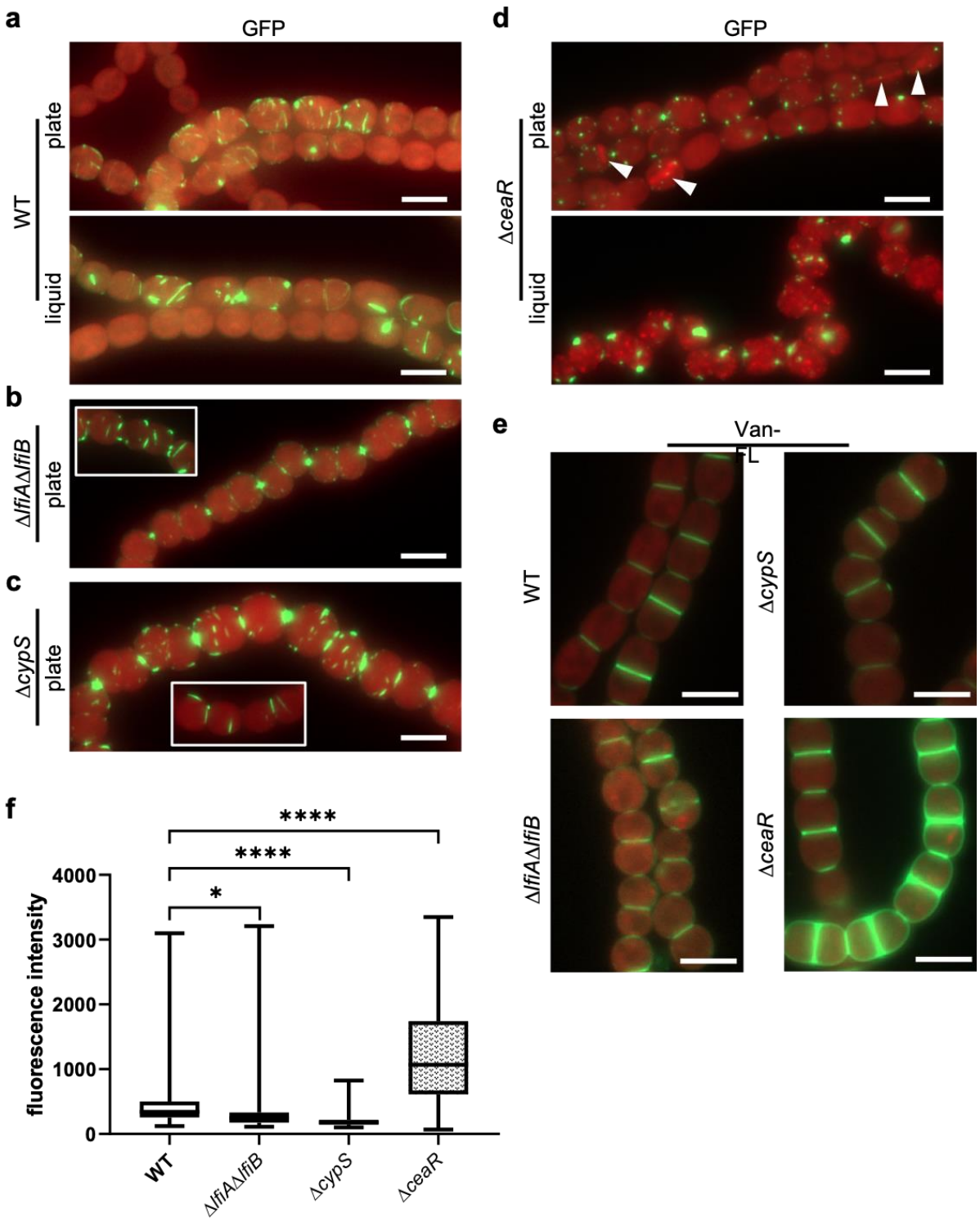

Fig. 3: CCRPs affect MreB localization and PG biogenesis

(**a**-**d**) Merged GFP fluorescence and chlorophyll autofluorescence micrographs of *Anabaena* WT, Δ*lfiA*Δ*lfiB*, Δ*cypS* and Δ*ceaR* mutants expressing GFP-MreB from P_{petE}. Cells were either grown on BG11 plates or in BG11 liquid medium. White triangles indicate red fluorescent filaments within Δ*ceaR* mutant cells. Exposure time for GFP fluorescence excitation was 70 ms except for 14 ms in the Δ*ceaR* mutant grown in liquid BG11. As indicated by the abnormal trichome morphology that resembled the Δ*lfiA*Δ*lfiB*, Δ*cypS* mutant trichome phenotypes (which were unable to grow in liquid culture), GFP-MreB-expressing Δ*ceaR* mutant only survived for a few days in liquid culture. Also: expression of GFP-MreB from its native promoter never resulted in any successfully transformed *Anabaena* CCRP mutant clones while it could be transformed into *Anabaena* WT. (**e**) Merged BODIPY™ FL Vancomycin (Van-FL) fluorescence and chlorophyll autofluorescence micrographs of *Anabaena* WT, Δ*lfiA*Δ*lfiB*, Δ*cypS* and

Δ*ceaR* mutants stained with 5 µg ml^{-1} Van-FL. As a result of the low Van-FL staining and for better visibility, Van-FL fluorescence intensity in Δ*lfiA*Δ*lfiB* and Δ*cypS* mutants was artificially increased about twofold after image acquisition (note: this increase was not used for the fluorescence intensity measurement in (**f**)). (**f**) Analysis of the arithmetic mean fluorescence intensities of cells from Fig. 3e. For all measurements, Van-FL fluorescence intensity from cell septa was recorded using 130 ms exposure time from an area of 3.52 μm^2. Sample size (n) was 200 stainings for each strain. Values indicated with * are significantly different from the WT. *: P<0.05, **: P<0.01, ***: P<0.001, ****: P<0.0001. ns indicates no significant different to the WT (using one-way ANOVA with Dunnett's multiple comparison test). Scale bars: 5 µm.

Anabaena **CCRPs have the capacity to form an interconnected network and interact with SepJ and MreB**

As our *Anabaena* CCRP mutant strains displayed similar phenotypes and the four *Anabaena* CCRPs were all seemingly linked to MreB localization or PG biogenesis, we next investigated whether the four proteins interact with each other and with other known morphological determinants in *Anabaena*. Using bacterial two hybrid assays (BACTH), we found that all our four CCRPs were able to self-interact (Fig 4a). Additionally, all four CCRPs could cross-interact with each other and we found that LfiA, LfiB and CeaR but not CypS, interacted with MreB. Furthermore, CeaR weakly interacted with FtsZ (Fig. 4a, Supplementary Fig. 17), which agrees with the Z-ring-like localization of CeaR-GFP in *Anabaena* (Fig. 1h, Supplementary Fig. 8a). All four CCRPs were identified as interaction partners of the septal junction protein SepJ, but not with FraC and FraD (Fig. 4a, Supplementary Fig. 17), two other septal junction proteins[44]. Since coiled-coil motifs are well-known protein-protein interaction domains[45,46], they are putatively prone for false-positive results in the interaction assays. However, interactions of coiled-coil containing proteins are usually considered to be specific[45,47–49]; nonetheless, we further tested the interaction specificity of our four CCRPs – Cyps, LfiA, LfiB and CeaR – in the bacterial two-hybrid assay by including Alr3364, another *Anabaena* CCRP (Supplementary Fig. 1), as a negative control in our screening. Our results show that Alr3364 only weakly interacted with LfiA and LfiB and failed to interact with CypS and CeaR (Supplementary Fig. 18). This confirms that the comparably strong interactions observed between CypS, LfiA, LfiB and CeaR are indeed specific interactions. We attempted to further confirm our interaction results with affinity co-elution experiments. However, we found that Ni-NTA-bound *Anabaena* CCRPs readily precipitated upon transfer from denaturing to native buffer conditions,

precluding further co-elution studies. Additionally, we observed that non-denaturing conditions failed to purify overexpressed CCRPs from *E. coli*, confirming their inherent insoluble nature, a property known to eukaryotic IFs[35]. Instead, we surveyed for further interaction partners by anti-GFP co-immunoprecipitation experiments of *Anabaena* cells expressing CeaR-GFP or LfiA-GFP and analyzed co-precipitated proteins by LC-MS/MS analytics (full list of possible interactiors in Supplementary File 3). This analysis confirmed that LfiA and LfiB interact with each other *in vivo* and validated the association of CeaR and LfiA with MreB as well as the interaction of CeaR with SepJ in *Anabaena* (Fig. 4b). Corroborating a role of CeaR in PG biogenesis and MreB function, CeaR was also found to be associated with three penicillin binding proteins (Fig. 4b), which are known regulators of PG synthesis and are part of the elongasome[50]. Furthermore, both, CeaR and LfiA, co-precipitated ParA, and CeaR was additionally found to be associated with MinD (Fig. 4b). Both ParA and MinD belong to a protein family of Walker-A-type ATPases and mediate plasmid and chromosome segregation[51]. To test for a similar function in our CCRPs, we compared the DNA distribution among the CCRP mutant cells as measured by distribution of 4′,6-Diamidin-2-phenylindol (DAPI) staining intensity (Supplementary Fig. 19). For that, we calculated the width of the DAPI focal area as the range of DAPI staining around the maximum intensity focus (±10 grey intensity in arbitrary units). This revealed that the staining focal area size was significantly different among the four tested strains ($P=3.14x10^{-41}$, using Kruskal-wallis). Post-hoc comparison showed that the focal area size in the *ΔceaR* mutant was larger than the others, and the area size in *Anabaena* WT was not significantly different than Δ*cypS*. The DAPI signal observed in the Δ*lfiA*Δ*lfiB* mutant appears as the most condensed, and indeed, the Δ*lfiA*Δ*lfiB* mutant focal DAPI area was smallest in comparison to the other strains (alpha=0.05, using Tukey test; Supplementary Fig. 19a,b). Unlike the *ΔceaR* mutant and the WT, DAPI signals in the Δ*lfiA*Δ*lfiB* and Δ*cypS* mutant strains were also observed between two neighboring cells (Supplementary Fig 19a), indicating that DNA distribution is not properly executed in those strains.

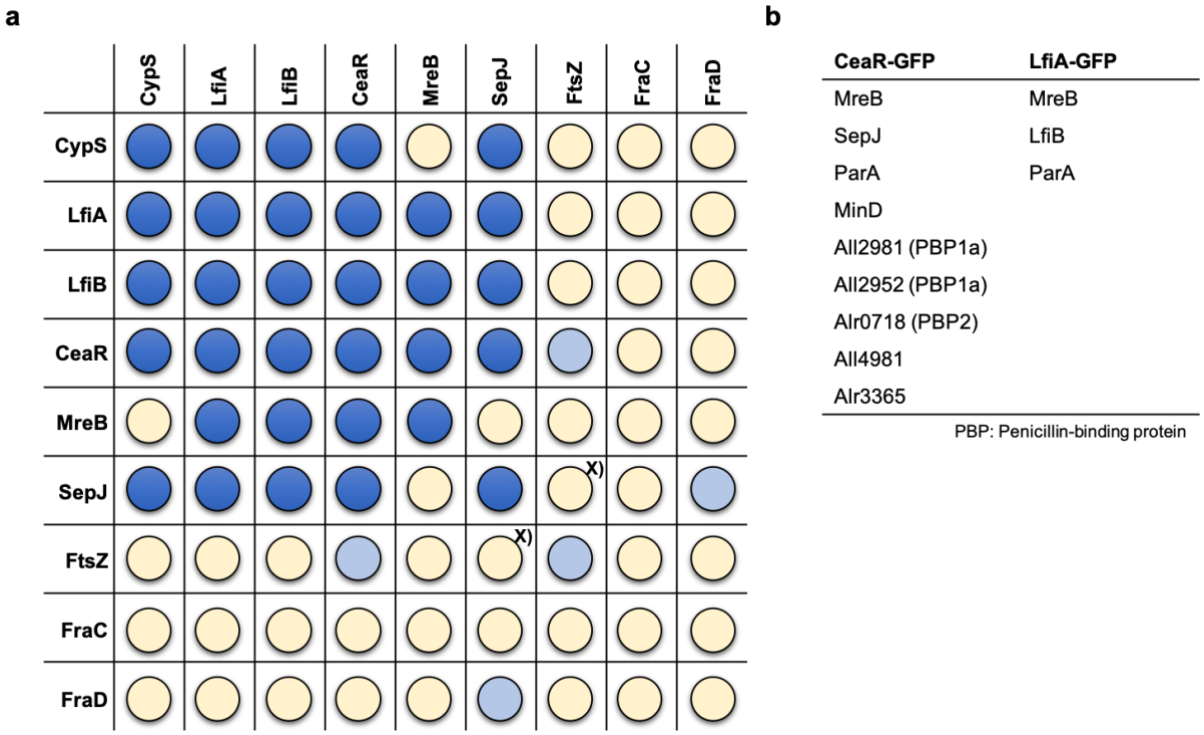

Fig. 4: *Anabaena* filament-forming CCRPs form a putative proteinaceous network that links the septal junction protein SepJ and MreB

(**a**) Graphical visualization of beta-galactosidase assay results of *E. coli* BTH101 cells co-expressing indicated translational fusion constructs of *Anabaena* proteins with the T18 and T25 subunit, investigating all possible pair-wise combinations. Corresponding data are shown in Supplementary Fig. 19. Blue spots indicate comparably strong verified interactions (arbitrary chosen as >500 Miller units mg^{-1}) while light blue dots mark comparably moderate interactions (arbitrary chosen as <500 Miller units mg^{-1}) and no interactions are depicted with yellow-colored dots. The here shown readout of interaction strengths is selected based on the highest identified beta-galactosidase activity from T25 and T18 translational fusions of all possible pair-wise combinations (see Supplementary Fig. 17 for precise data of all the possible combinations). Dots marked with "X" were previously reported by Ramos-León *et al.* (2015)[52]. (**b**) Excerpt of the identified specific interactors of CeaR-GFP and LfiA-GFP. The full list is listed in Supplementary File 3. Notably, CeaR interacts with All4981, another filament forming protein in *Anabaena* (covered in a separate report) as well as Alr3365 that lies directly downstream of *alr3364* in the *Anabaena* genome, which we also identified in our screening for CCRPs in *Anabaena* (Supplementary Fig. 1).

Discussion

Here we provide evidence for the capacity of three *Anabaena* CCRPs - CypS, LfiA together with LfiB - to form polymers *in vitro* and *in vivo*. The characterization of multiple CCRPs in our study was possible thanks to the easy to use and comparably high-throughput approach for the screening of novel filament-forming CCRPs using the NHS-Fluorescein dye. Our approach supplies an alternative for the examination of protein-filament formation by electron microscopy; instead it allows for a simplified protocol for the detection of protein filaments using fluorescence microscopy. While the previously described prokaryotic filament-forming CCRPs formed homopolymers[8,10,11,16], LfiA and LfiB assemble into a heteropolymer. Furthermore, LfiA

and LfiB have the capacity to co-polymerize in a heterologous *E. coli* system, similar to other known CCRPs such as Scc from *Leptospira biflexa*[53] or crescentin[54]. We note, however, that the results from our *in vivo* experiments of LfiA/LfiB co-polymerization are based on artificial expression of the two CCRPs. We hypothesize that the absence of a LfiA/LfiB heteropolymer in strains expressing LfiA-GFP or LfiB-GFP alone (with the WT alleles still present) may be due to a dosage-dependent effect, where the presence of unequal concentration of LfiA and LfiB in the cell leads to protein aggregates. Our observation of LfiA-GFP or LfiB-GFP aggregates when they were expressed alone in the Δ*lfiA*Δ*lfiB* mutant strain supports the dosage effect hypothesis. Also, in our *in vitro* polymerization assay, LfiA and LfiB only formed clear filamentous structures when both proteins are present in equal concentrations. Furthermore, the genomic neighbourhood of LfiA and LfiB suggests that the LfiA/B heteropolymer formation is relying on co-translational assembly (e.g., as observed for LuxA/LuxB[55]). Co-translational assembly of the WT LfiA/B would lead to an efficient binding of the two subunits such that the expression of one unit only in excess (i.e., LfiA-GFP or LfiB-GFP alone) would lead to the formation of aggregates.

Our results indicate that the four CCRPs described here could form an interconnected network in *Anabaena*. The network might be anchored to the cell poles through the interaction with the septal junction protein SepJ. Together with the cell shape-determining protein MreB, *Anabaena* CCRPs could possibly contribute to the cell shape and relay trichome shape-stabilizing properties to neighboring cells in the trichome, thereby maintaining the linear *Anabaena* phenotype. The interaction of *Anabaena* CCRPs with SepJ and the septal localization of CeaR-GFP and CypS-His$_6$ as well as the pole-to-pole LfiA-eCFP/LfiB-GFP filament hint for an involvement of Anabaena CCRPs in septal junction function. As such, proper septal junction functionality would not only be important for trichome integrity and divisome function[32,52,56], but also for trichome shape in *Anabaena*. Hence the four CCRPs are likely involved in trichome integrity, similarly to the integral membrane proteins SepJ and FraC/FraD. The *Anabaena* CCRPs might constitute stabilizing platforms or scaffolds for other proteinaceous structures, similarly to the stabilizing function of the eukaryotic cytoskeleton for

cell-cell contacts (i.e. desmosomes)[57]. Specifically, the polar localization of CypS and the loss of LfiA-eCFP/LfiB-GFP filament localization in the Δ*cypS* mutant suggest a polar scaffolding or anchorage function of CypS. A similar localization pattern was previously described for the CCRPs Scy from *S. coelicolor* and the bactofilins BacA and BacB from *C. crescentus* that showed non-filamentous polar or patchy localization and still assembled into filaments *in vitro*[15]. Both, Scy and BacA/B act as polar assembly platforms or scaffolds that mediate the localization of FilP and DivIVA in the case of Scy[9] and Pbpc in the case of BacA/B[58]. Furthermore, LfiA shares *in silico* predicted structural similarities with the spectrin repeats of plectin (Supplementary Table 1), a well-described eukaryotic cytolinker protein. Plectins link the three eukaryotic cytoskeletal systems (actin filaments, microtubules and IFs), thereby contributing to the resistance to deformation of vertebrate cells[57]. They furthermore stabilize desmosomes and are hence directly involved in cell-cell contact integrity[59]. An analogous cytolinker function of LfiA could explain why LfiB alone did not form protein filaments and suggests that LfiB requires LfiA as the linking protein for polymerization. Based on the structural similarity to spectrin, it is also conceivable that LfiA and LfiB possess similar functions as α and β-spectrin. Together, spectrin α/β-heteropolymers produce a cell shape-maintaining interconnected cytoskeletal network (the so called spectre) below the plasma membrane of erythrocytes[60]. Furthermore, similar to LfiA/B, spectrins are directly linked to the actin cytoskeleton[57]. This link of LfiA/B to the actin-like MreB cytoskeleton is also indicated by the altered localization of GFP-MreB in the Δ*lfiA*Δ*lfiB* mutant strain. The observed PG staining pattern where PG staining was strongly elevated in the Δ*ceaR* mutant strain also hints for a function of CeaR to (down)regulate MreB or elongasome function (hence its name: cyanobacterial elongasome associated regulator). In the *ΔcypS* and Δ*lfiA*Δ*lfiB* mutants, PG staining was decreased, and as such, CypS and LfiA/B might indirectly act to positively regulate PG biogenesis. These observations would further suggest an association of CypS with the elongasome, despite the failure of CypS to directly interact with MreB (Fig. 4a). MreB and the elongasome are the main determinants of the PG exoskeleton, which provides the cell with structural integrity and resistance to turgor pressure[61]. Notably, both Δ*cypS* and Δ*lfiA*Δ*lfiB*

mutant strains showed a swollen cell phenotype and were unable to grow in liquid culture, hinting for a defect in MreB or elongasome function as a similar swollen cell phenotype has previously described for an *Anabaena* Δ*mreB* mutant[26]. The association of *Anabaena* CCRPs with proper elongasome function is further supported by the elevated sensitivity of the Δ*cypS* mutant strain to lysozyme, similar to the Δ*mreB* mutant strain[26]. However, lysozyme sensitivity is only indicative of cell wall defects and outer membrane defects or other factors might influence this sensitivity. An interaction of prokaryotic filament-forming CCRPs with MreB and PG synthesis has been previously described in other bacteria. Examples are the gliding motility in *M. xanthus,* where a multi-protein complex, including the filament-forming CCRP AglZ and MreB were found to coordinate type A-motility[17]. Similarly, the curved morphotype of *C. crescentus* is induced by crescentin, which modulates PG biogenesis by exuding local mechanical forces to the cell membrane[15,62]. Although CeaR-GFP co-localized with the Z-ring *in vivo* and interacted with FtsZ and the divisome protein SepJ, whose subcellular localization is dependent on FtsZ[52], no alteration of Z-ring placement was detected in the Δ*ceaR* mutant, as well as in the Δ*cypS* and Δ*lfiA*Δ*lfiB* mutant strains. This suggests an independence of the four *Anabaena* CCRPs from FtsZ. However, both, the Δ*cypS* and the Δ*ceaR* mutant revealed decreased cell viability and growth rates, respectively, and both mutants possessed septal disks that were significantly larger than the septal disks in the WT. This could suggest that both, CypS and CeaR might be involved in divisome function downstream of Z-ring formation, either through affecting FtsZ turnover or through an altered PG synthesis, which is supported by the altered PG biogenesis identified on both mutant strains and the interaction of CeaR with several PBPs. Future studies will thus try to unravel whether CypS and CeaR might be associated with other components of the divisome besides FtsZ.

The conserved combination of all four CCRPs in heterocystous cyanobacteria that form linear trichomes (or false branching; Fig. 1a) highlights that the linear trichome formation has a selective advantage. Both Δ*cypS* and Δ*lfiA*Δ*lfiB* mutants had a zigzagged phenotype and were unable to grow in liquid culture. The zigzagged mutants provide more accessible surface for the acting mechanical forces in liquid[63], including fluid shear stress[64], ultimately resulting in

forces that cannot be endured by the abnormal mutant trichomes. Notably, while the selective advantage of cell shape is considered to be mostly a manifestation of biotic and abiotic selective factors in the cell environment[65], the selective advantage of multicellular shapes is likely related to the efficiency of intercellular communication and transport[63]. Indeed, the results of our FRAP experiments show that the efficiency of diffusion in the zigzagged Δ*cypS* mutant trichome is reduced. Furthermore, the Δ*ceaR* mutant failed to grow in diazotrophic conditions where transport of metabolites in the filament is considered essential for *Anabaena* viability. A similar observation has been made for knockout mutant strains of SepJ, FraC and FraD that are essential for *Anabaena* multicellularity[32,33]. Additionally, the decrease in solute diffusion in the Δ*ceaR* and Δ*cypS* mutants suggests that CeaR and CypS could be involved in the buildup or stabilization of the septal junctions. This notion is further supported by the abnormal nanopore formation observed in some Δ*ceaR* mutant septa and the decreased nanopore abundance in the larger septal disks of the Δ*cypS* and the Δ*ceaR* mutants. Furthermore, septal localization of CypS-His_6 and CeaR-GFP as well as the direct interaction of both proteins with SepJ in the BACTH assay and the interaction of CeaR-GFP with SepJ in Anabaena WT provides further support for an involvement in septal junction functionality. Our results thus indicate that CeaR is important for *Anabaena* multicellularity while CypS, LfiA and LfiB serve as regulators of *Anabaena* patterned multicellularity. The evolution of patterned multicellularity is considered an important step towards a sustainable division of labor and the development of cell differentiation[3]. Our study provides evidecne for a role of filament-forming CCRPs in the evolution and maintenance of cyanobacterial multicellular forms.

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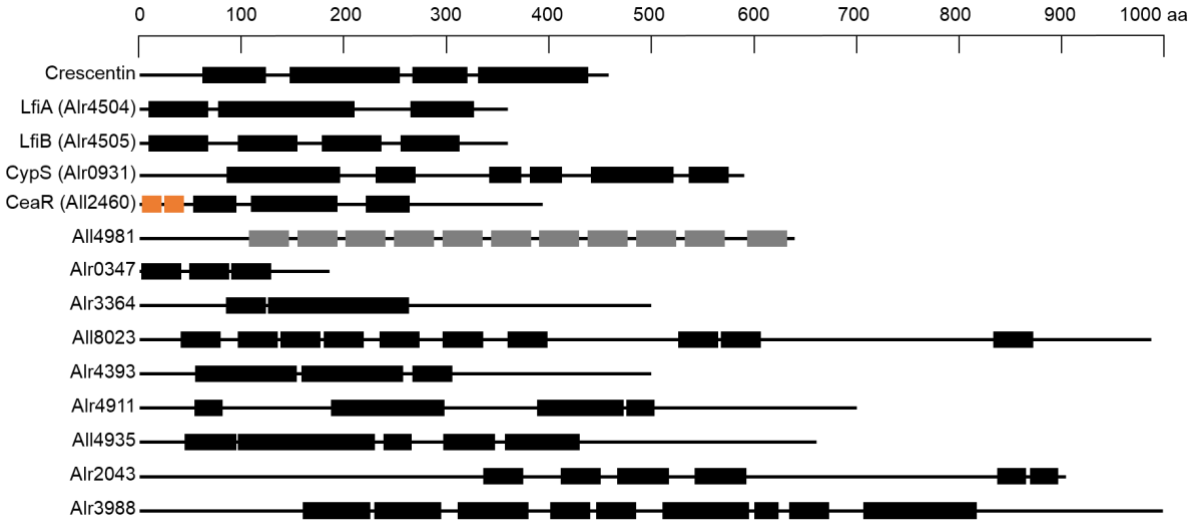
Author contribution

BLS and KS designed the study. BLS established and performed the experimental work with contributions from MT and JW. CW and TD performed comparative genomics analysis. DJN and AKK performed FRAP assays and AKK and IM carried out ultrathin structures and nanopore analyses. AOH and AT analyzed protein samples by mass spectrometry. BLS, TD and KS drafted the manuscript with contributions from all coauthors.

Competing interests

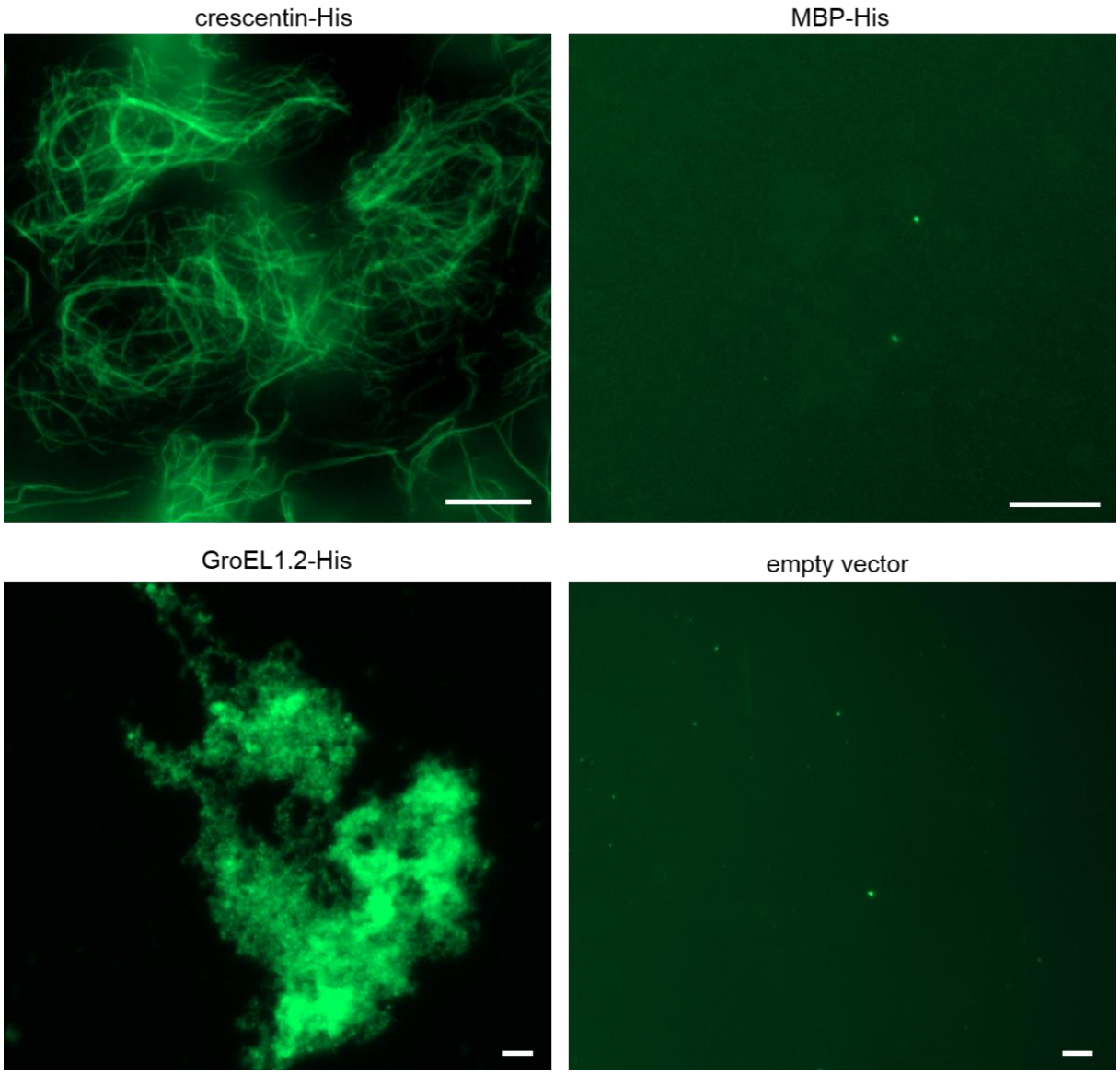
The authors declare no competing interests.

Supplementary information



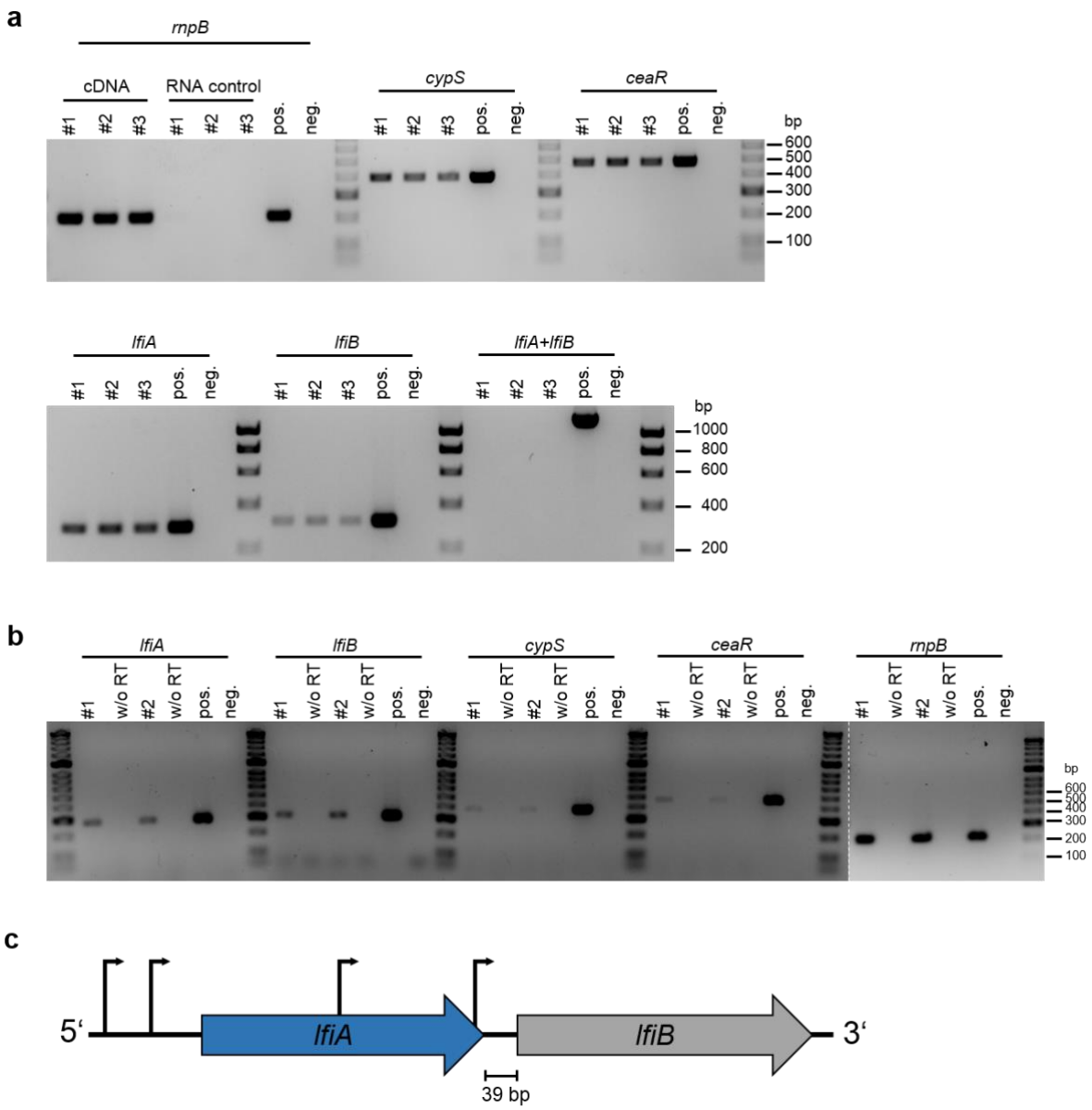
Supplementary Fig. 1:Domain architecture of IF-like protein candidates

Depiction of coiled-coil domains of cyanobacterial CCRP candidates and Crescentin from *Caulobacter crescentus* as identified by the COILS algorithm[66] with a window width of 21. The scale on top is given in amino acid residues (aa) and amino acid sequences in coiled coil conformation are depicted by black bars, transmembrane domains are shown in orange bars, while non-coiled-coil sequences are represented by black lines. Tetratricopeptide repeats (TRPs) are shown as grey bars. Cyanobacterial proteins are given as cyanobase locus tags.



Supplementary Fig. 2: *In vitro* polymerization assay controls

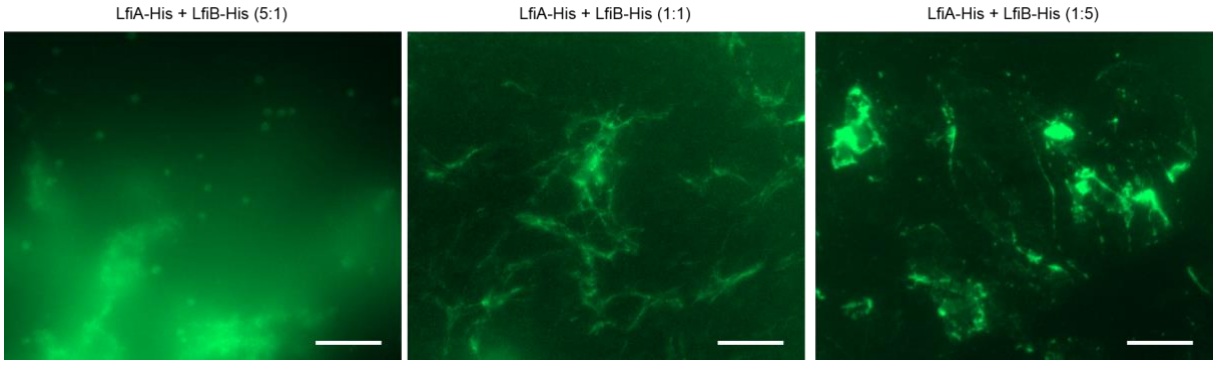
NHS-fluorescein fluorescence micrographs of purified and renatured Crescentin-His, MBP-His and GroEL1.2 from *Chlorogloeopsis fritschii* PCC 6912 (0.5 mg ml^{-1} each) as well as purified cell-free extracts of *E. coli* BL21 (DE3) carrying empty vector (pET21a(+)) in HLB. Notably, GroEL1.2, able to self-interact[37], collapses into indistinct aggregates, showing that oligomerizing proteins do not form filaments in our assay. Proteins and cell-free extracts (empty vector) were dialyzed in a step-wise urea-decreasing manner and stained with an excess of NHS-Fluorescein. Scale bars: 10 µm.



Supplementary Fig. 3: *Anabaena* CCRPs are expressed at standard growth conditions

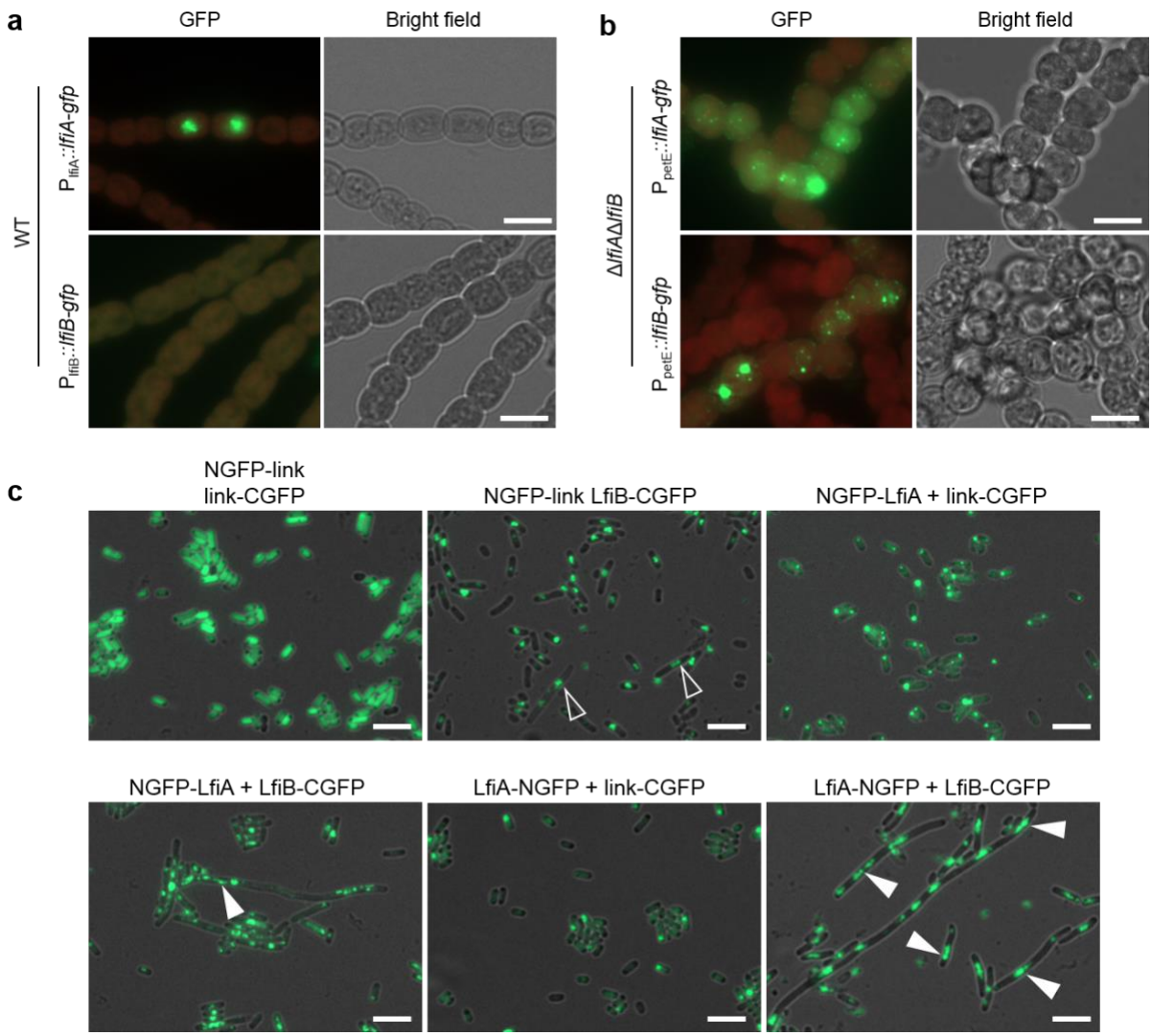
(a,b) RT-PCR of whole RNA from *Anabaena* WT cultures grown in (a) BG11 or (b) $BG11_0$ liquid medium from (a) three or (b) two independent biological replicates. Gene transcripts were verified using internal gene primers (*rnpB*: #1/#2; *cypS*: #3/#4; *ceaR*: #5/#6; *lfiA*: #7/#8; *lfiB*: #9/#10; *lfiA* and *lfiB*: #7/#10). As negative control (neg), PCR reactions were performed with water instead of cDNA or RNA and as a positive control (pos) *Anabaena* gDNA was included. PCR fragments were resolved on a 2% agarose gel in TAE buffer. For each RT-PCR reaction, 100 ng cDNA was used. Absence of residual genomic DNA in DNase I-treated samples was verified with (a) 100 ng DNase I-treated RNA (RNA control) or (b) 100 ng DNase I-treated RNA that was subjected to cDNA synthesis reaction lacking reverse transcriptase (w/o RT). No common transcript for *lfiA* with *lfiB* was detected, suggesting that both proteins are not encoded in an operon.

(c) Depiction of the genomic environment of *lfiA* (blue) and *lfiB* (grey) within the *Anabaena* genome and their respective *in silico* predicted promoters depicted by black arrows (as predicted by BPROM[41]). Promoters of *lfiA* are predicted to reside 204 bp and 543 bp upstream of the open reading frame (ORF) and promoters of *lfiB* are located 22 bp and 450 bp upstream of the ORF, thereby residing within the *lfiA* ORF.



Supplementary Fig. 4: Co-polymerization of LfiA and LfiB is dosage-dependent

NHS-fluorescein micrographs of purified and co-renatured LfiA-His and LfiB-His in HLB. LfiA-His and LfiB-His were combined in different ratios, either with a fivefold excess of LfiA-His (left image; corresponding to 0.25 mg ml^{-1} LfiA-His and 0.05 mg ml^{-1} LfiB-His), a fivefold excess of LfiB-His (right image; corresponding to 0.25 mg ml^{-1} LfiB-His and 0.05 mg ml^{-1} LfiA-His) or an equal concentration of LfiA-His and LfiB-His (central image; 0.25 mg ml^{-1} each). Proteins were dialyzed in a stepwise urea-decreasing manner and stained with an excess of NHS-Fluorescein. Fine heteropolymers only form when equal concentrations of LfiA-His and LfiB-His are present. In concert with the partial self-polymerization capacity of LfiB-His (Fig. 1b), certain filamentous structures are also detected in the LfiB-His excess samples. However, most protein still precipitated under those conditions. Scale bars: 10 µm.

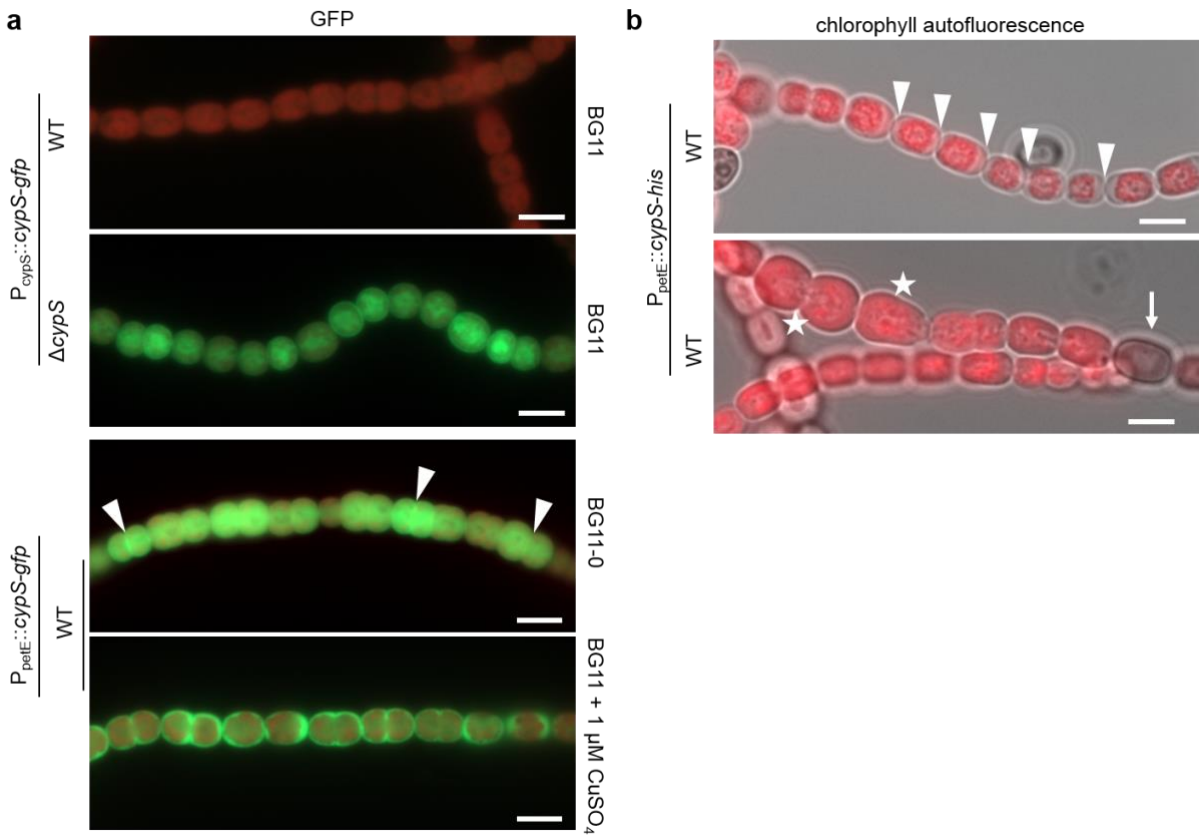


Supplementary Fig. 5: Heterologous expression of LfiA and LfiB

(a) Merged GFP fluorescence and chlorophyll autofluorescence (red) and bright field micrographs of *Anabaena* WT cells expressing LfiA-GFP or LfiB-GFP from P_{lfiA} and P_{lfiB}. No expression of LfiB-GFP is detectable from P_{lfiB} while expression of LfiA-GFP from P_{lfiA} leads to similar patchy clumps within the cells as observed from P_{petE} in Fig. 1d. Scale bars: 5 µm.

(b) Merged GFP fluorescence and chlorophyll autofluorescence and bright field micrographs of Δ*lfiA*Δ*lfiB* mutant strain expressing LfiA-GFP or LfiB-GFP from P_{petE}. Cells were grown on BG11 growth plates. For expression of LfiA-GFP, BG11 plates were supplemented with 1 µM $CuSO_4$. This experiment shows that LfiA-GFP and LfiB-GFP from P_{petE} (Fig. 1d) expression and localization in *Anabaena* WT is not affected by native LfiA or LfiB present in the WT background. Scale bars: 5 µm.

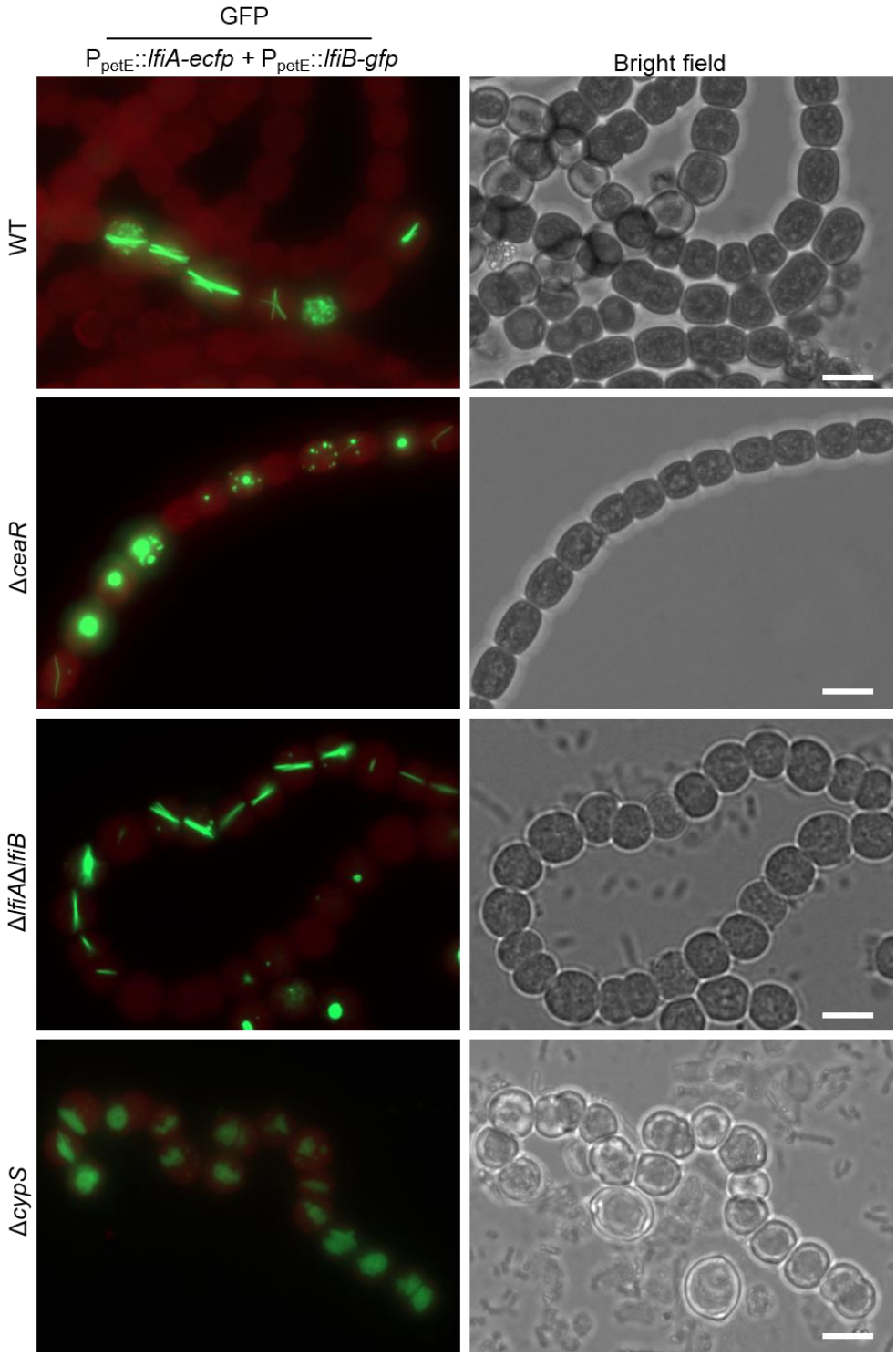
(c) Detection of protein-protein interactions with the GFP-fragment reassembly assay[67]. Merged GFP fluorescence and bright field micrographs of *E. coli* BL21(DE3) cells co-expressing NGFP-link (empty pET11a-link-NGFP) and -link-CGFP (empty pMRBAD-link-CGFP), NGFP-link and LfiB-CGFP, NGFP-LfiA and link-CGFP, NGFP-LfiA and LfiB-CGFP, LfiA-NGFP and link-CGFP or LfiA-NGFP and LfiB-CGFP. Cells were grown to an OD_{600} of 0.5, induced with 0.2% L-arabinose and 0.05 mM IPTG and incubated for 48 h at 20 °C. Transparent triangles point to structures resembling LfiB-His *in vitro* polymers. White triangles indicate FilP-GFP-like[11] filamentous structures that resemble structures indicated with translucent triangles but span longer distances. Co-expression of both, LfiA and LfiB leads to an elongated cell phenotype. FilP-like structures and elongated cells can already be seen upon co-expression of NGFP-LfiA with LfiB-CGFP but only upon co-expression of LfiA and LfiB with C-terminal GFP-fragments leads to a clear filamentous cell phenotype and abundant intracellular filamentous structures. This suggests that the N-terminus is important for heteropolymerization. Scale bars: 5 µm.



Supplementary Fig. 6: CypS *in vivo* localization is tag orientation-dependent

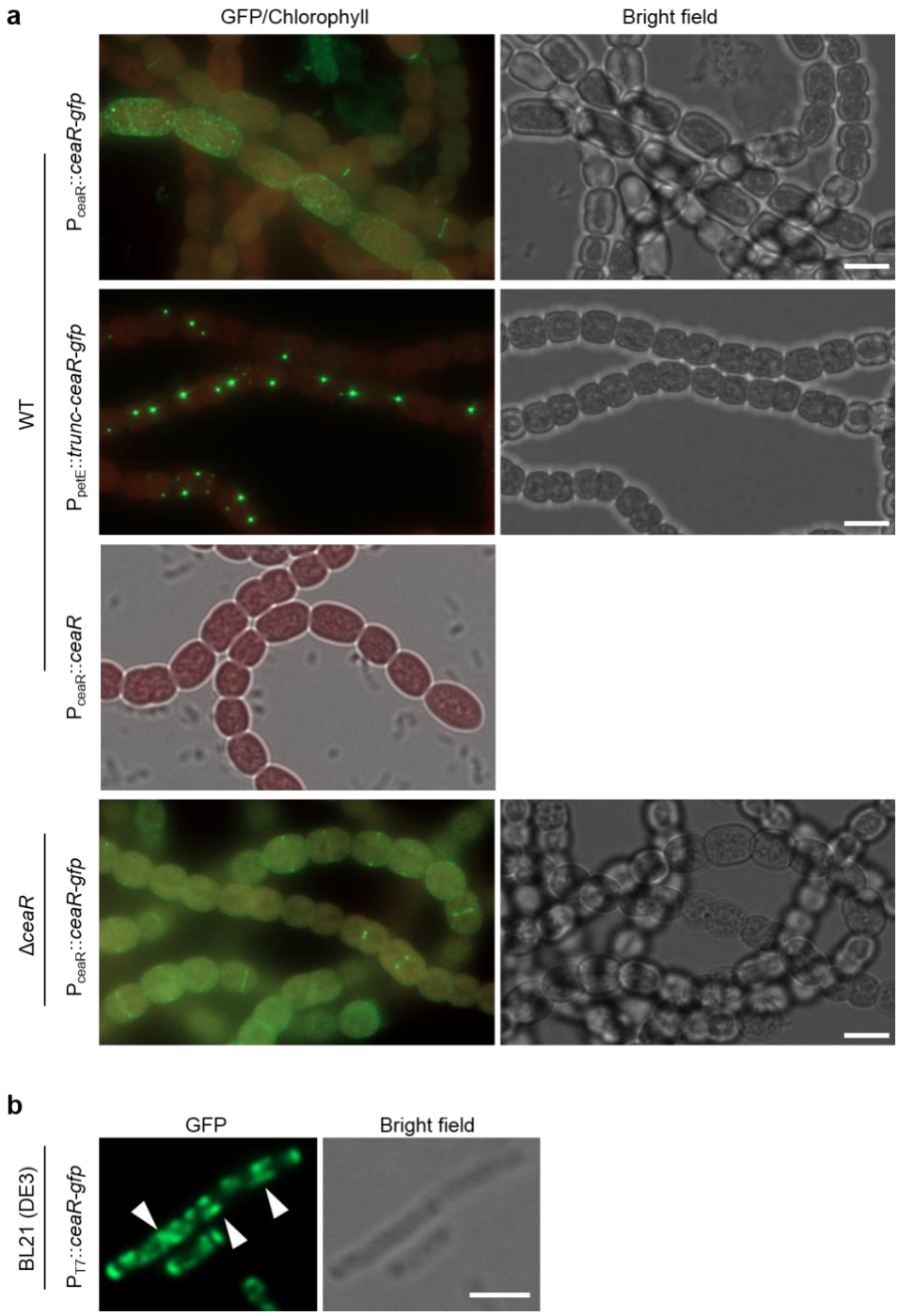
(a) Merged GFP fluorescence and chlorophyll autofluorescence micrographs of *Anabaena* WT and Δ*cypS* mutant strain expressing CypS-GFP from P_{petE} or P_{cypS}. Strains carrying P_{cypS}::*cypS-gfp* were grown in BG11, while the strains carrying P_{petE}::*cyps-gfp* are grown in indicated media with or without $CuSO_4$ supplementation. White triangles indicate membrane localization, which is most pronounced in dividing cells. Further induction of protein expression shows polar localization of CypS-GFP, similar to CypS-His. In the presence of native *cypS* (i.e. *Anabaena* WT), no CypS-GFP expression was detected from P_{cyps}, indicating that CypS dosage is tightly controlled in the WT. Based on the comparably strong autofluorescence of cyanobacteria, this could explain why the presumed few CypS-GFP molecules could not be detected when expressed from P_{cypS}. Control is likely exerted at the transcriptional level as overexpression from P_{petE} still produces detectable protein in the WT background. Scale bars: 5 µm.

(b) Merged bright field and chlorophyll autofluorescence micrographs of *Anabaena* WT cells expressing CypS-His from P_{petE} grown in $BG11_0$ supplemented with 0.25 µM $CuSO_4$ (upper image) or with 2.5 µM $CuSO_4$ for 2 d (lower image). White triangles mark cell septa with the most prominent retraction of chlorophyll signal away from the poles. White stars indicate swollen cells. White arrow points to a heterocyst. Note that the areas devoid of chlorophyll signal are occupied by CypS-His plugs (Fig. 1g), suggesting that CypS forms a dense proteinaceous meshwork at the cell poles. Scale bars: 5 µm.



Supplementary Fig. 7: *Anabaena* CCRPs affect LfiA/B *in vivo* localization

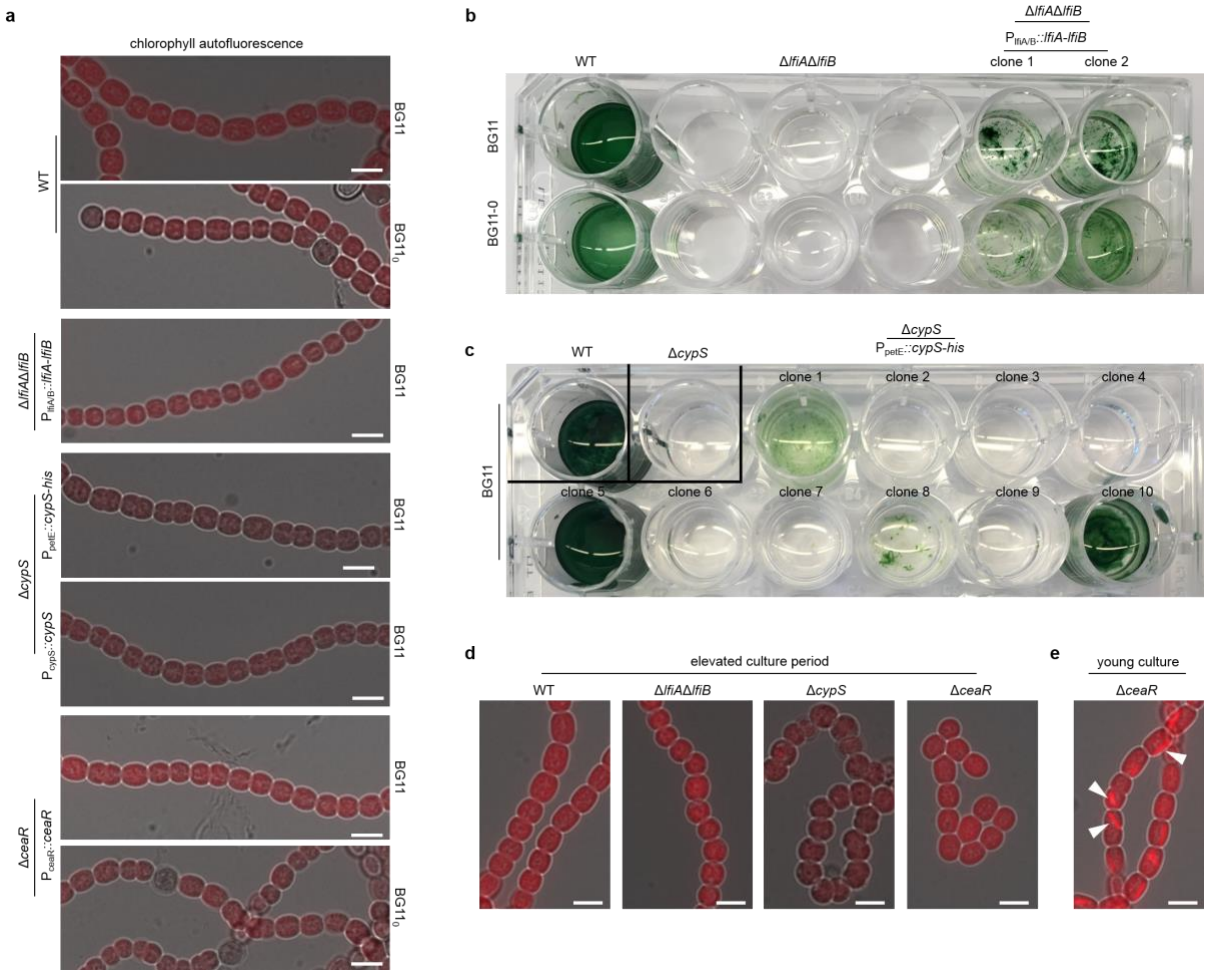
Mergend GFP-fluorescence and chlorophyll autofluorescence and bright field micrographs of *Anabaena* WT and *Anabaena* mutant strains co-expressing LfiA-eCFP and LfiB-GFP from P_{petE}. First, second and fourth images are maximum intensity projections of a Z-stack. Localization of the LfiA/B filament is slightly altered in the Δ*ceaR* mutant strain and fully deranged in the Δ*cypS* mutant strain. This suggests that CypS is involved in LfiA/B polar attachment, possibly by providing a proteinaceous scaffold for LfiA/B anchorage. Also, Δ*cypS* mutant strain expressing LfiA/B showed a decrease in filament viability. Colonies arose upon transformation with the LfiA/B-expressing construct but did not grow upon re-streaking on fresh plates. Lack of fluorescence signal in some of the depicted cells is likely due to the phenotypic variation of copy numbers of the pRL25C plasmid in different cells within an *Anabaena* filament[68]. Scale bars: 5 µm.



Supplementary Fig. 8: *In vivo* localization of CeaR-GFP in *Anabaena* and *E. coli*

(a) Merged GFP-fluorescence and chlorophyll autofluorescence and bright field micrographs of *Anabaena* WT or Δ*ceaR* mutant strain expressing CeaR-GFP, CeaR-GFP without the N-terminal transmembrane domain (first 52 aa removed; *trunc-ceaR*) or CeaR form P_{petE} or P_{ceaR}. Additional expression of CeaR-GFP from P_{ceaR} or P_{petE} and CeaR from P_{ceaR} induces a swollen cell phenotype. This phenomenon is not present upon expression of CeaR-GFP or CeaR (Supplementary Fig. 9a) from P_{ceaR} in the Δ*ceaR* mutant strain, indicating that *ceaR* expression or protein level is tightly regulated in *Anabaena* WT. Expression of truncated CeaR-GFP in *Anabaena* WT cells was induced for 1 d with 0.2 µM $CuSO_4$. Scale bars: 5 µm.

(b) GFP-fluorescence and bright field micrographs of *E. coli* BL21 (DE3) cells expressing CeaR-GFP. Cells were grown till an OD_{600} of 0.5 and induced for 48 h at 20 °C with 0.05 mM IPTG. White triangles indicate banded and helical localization of CeaR-GFP. Scale bar: 5 µm.

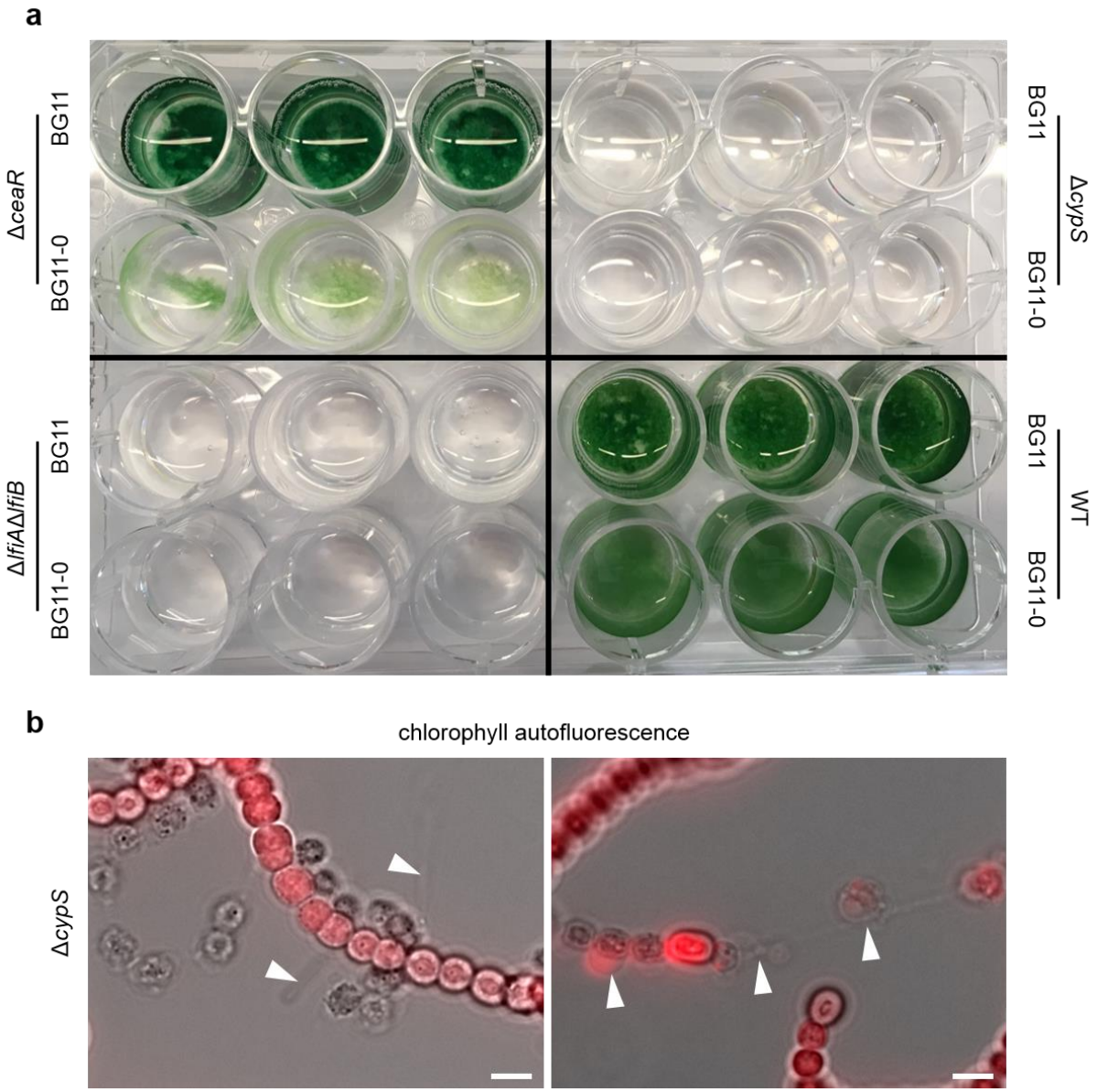


Supplementary Fig. 9: Mutant phenotype complementation and culture age-dependency of *Anabaena* mutant phenotypes

(a) Morphological complementation of *Anabaena* CCRP mutant strains as a result of native expression of *lfiA-lfiB*, *cypS* and *ceaR* from pRL25C. Notably, CypS-His expressed from P_{petE} also complemented the morphological defect of the Δ*cypS* mutant strain and rescues the linear *Anabaena* filament shape. The ability to complement the mutant phenotypes using the pRL25C plasmid shows that pDU1-based plasmids can be successfully employed to rescue WT phenotypes despite their variation in the relative copy number[68]. Scale bars: 5 µm.

(b,c) Complementation of (b) Δ*lfiA*Δ*lfiB* and (c) Δ*cypS* mutant strains by expressing *lfiA-lfiB* from $P_{lfiA/B}$ or *cypS-his* from P_{petE} from the replicative pRL25C plasmid. Note, not all tested clones successfully complemented the mutant growth defects in liquid culture, likely due to the phenotypic variation caused by the copy number variation of pRL25C[68,69].

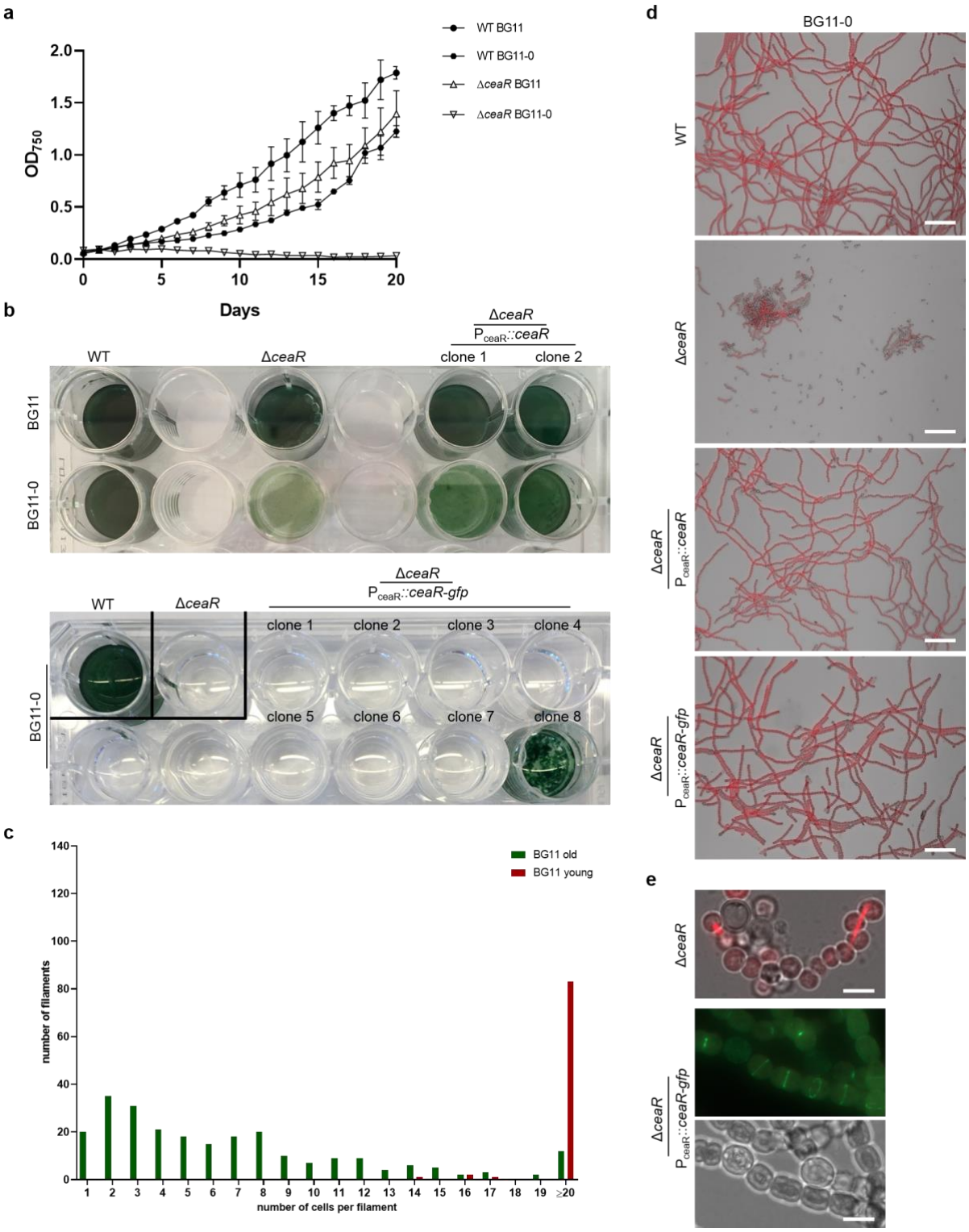
(d) Merged bright field and chlorophyll autofluorescence micrographs of (a) *Anabaena* WT and Δ*lfiA*Δ*lfiB*, Δ*cypS* and Δ*ceaR* mutant strains grown on BG11 plates for an elevated time period (about 3 weeks) or (d) Δ*ceaR* mutant strain grown on BG11 plates for about one week. White triangles indicate red fluorescent filaments. Note: a decreased viability of the Δ*cypS* mutant strain is evident by a decreased chlorophyll autofluorescence signal. Scale bars: 5 µm.



Supplementary Fig. 10: *Anabaena* CCRP mutant strains show growth defects in liquid culture

(a) *Anabaena* WT, Δ*cypS,* Δ*lfiA*Δ*lfiB* and Δ*ceaR* mutant strains were grown on BG11 plates, transferred to liquid BG11 and $BG11_0$ medium and incubated for 12 d at standard growth conditions without shaking. The Δ*ceaR* mutant strain can somewhat manage to survive in $BG11_0$ liquid medium without agitation. Nevertheless, prolonged incubation usually led to cell death. On the contrary, Δ*ceaR* mutant cells are not viable when grown in liquid media with agitation.

(b) Merged bright field and chlorophyll autofluorescence micrographs of Δ*cypS* mutant strain resuspended in BG11 liquid medium from BG11 plates. Cells were visualized immediately after transfer. White triangles indicate material released from cells upon cell rupture. Scale bars: 5 µm.



Supplementary Fig. 11: Fragmentation and decreased viability of the *ΔceaR* mutant strain

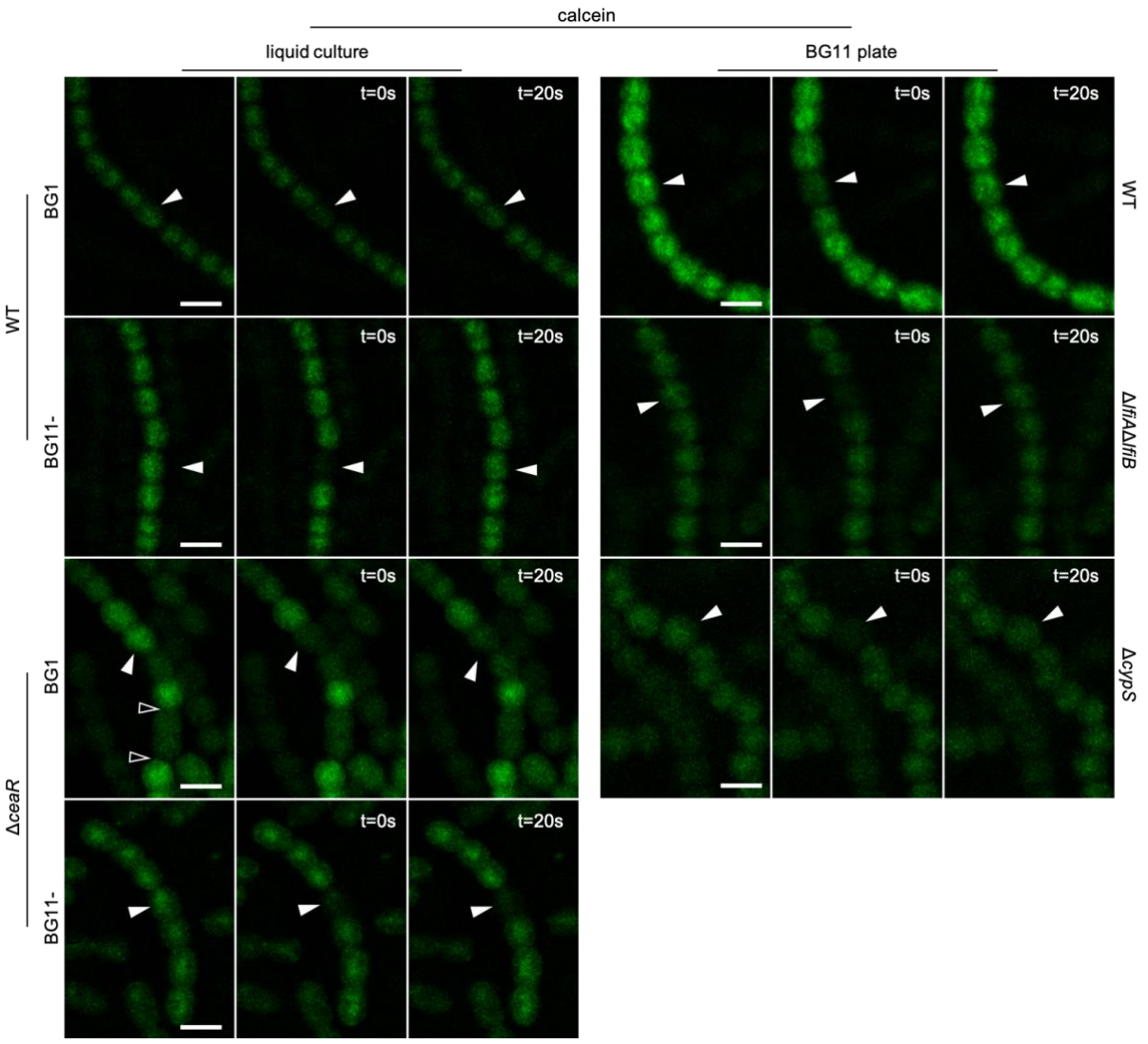
(a) *Anabaena* WT and Δ*ceaR* mutant strain were grown in BG11, washed three times in BG11 or $BG11_0$, adjusted to an OD_{750} of 0.1 and then grown in triplicates at standard growth conditions. OD_{750} values were recorded once a day for 20 d. Error bars show the standard deviation (n=3).

(b) *Anabaena* WT grows in BG11 and $BG11_0$ while the Δ*ceaR* mutant strain only grows in BG11. Growth in $BG11_0$ can, however, be rescued using the pRL25C plasmid bearing P_{ceaR}::*ceaR* or P_{ceaR}::*ceaR-gfp*, showing that the CeaR-GFP fusion protein is active.

(c) Filament length (number of cells per filament) of Δ*ceaR* mutant strain from young and older cultures grown in BG11 liquid medium. Filament length of filaments with up to 19 cells were individually counted while filaments with with more than 20 cells are listed with ≥20.

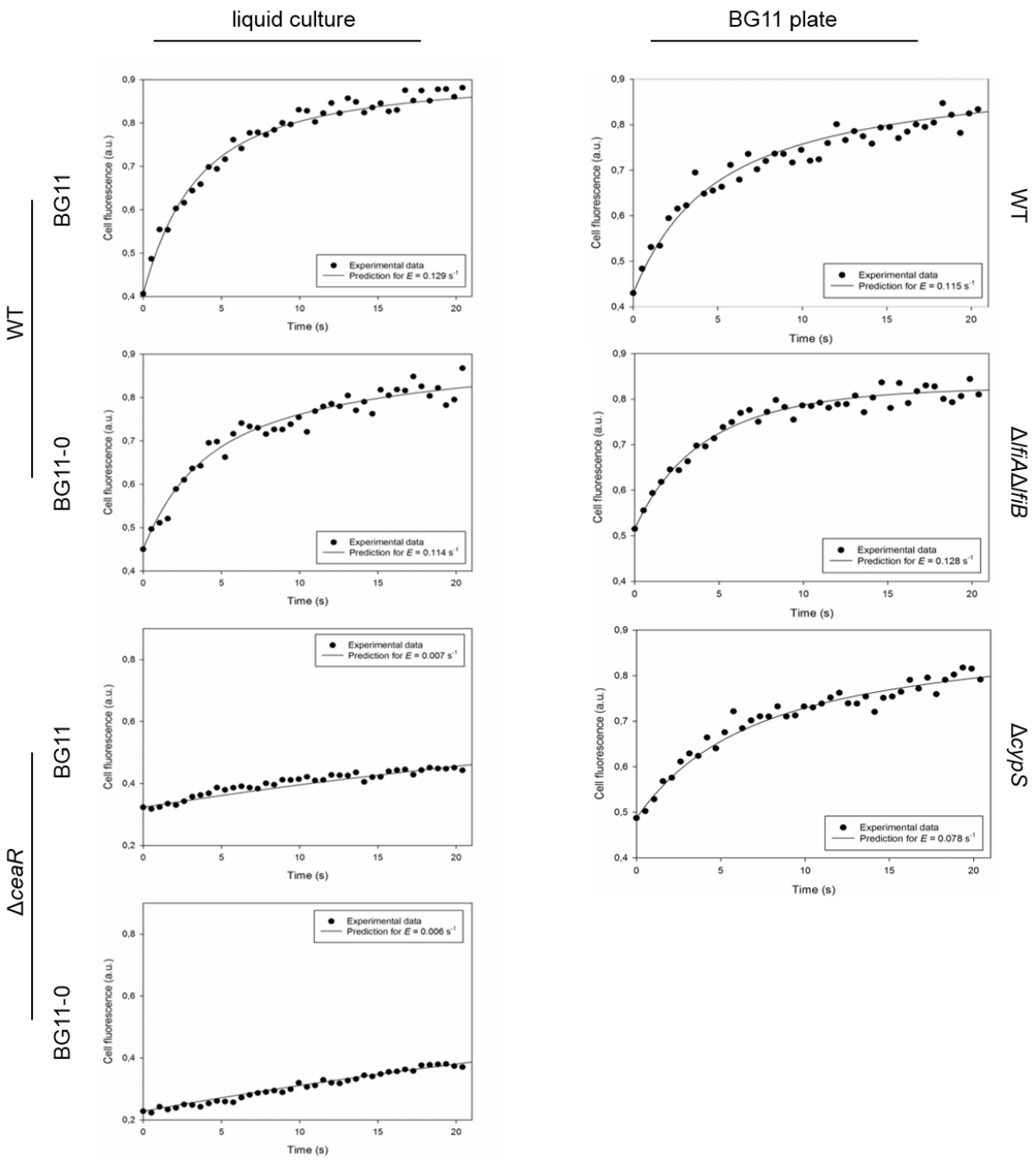
(d) Merged bright field and chlorophyll autofluorescence micrographs of *Anabaena* WT, Δ*ceaR* mutant and the Δ*ceaR* mutant carrying a pRL25C plasmid bearing P_{ceaR}::*ceaR* or P_{ceaR}::*ceaR-gfp*. Micrographs show cells from Supplementary Fig. 11b 48 h after transfer to $BG11_0$. The Δ*ceaR* mutant fragments into short filaments that clump together with cells losing their chlorophyll auto-fluorescence signal. However, the Δ*ceaR* mutant fragmentation/aggregation can be fully complemented with a pRL25C plasmid bearing P_{ceaR}::*ceaR* or P_{ceaR}::*ceaR-gfp*.

(e) Micrographs of Δ*ceaR* mutant and Δ*ceaR* mutant expressing CeaR-GFP from P_{ceaR} on the pRL25C plasmid 48 h after transfer to $BG11_0$.



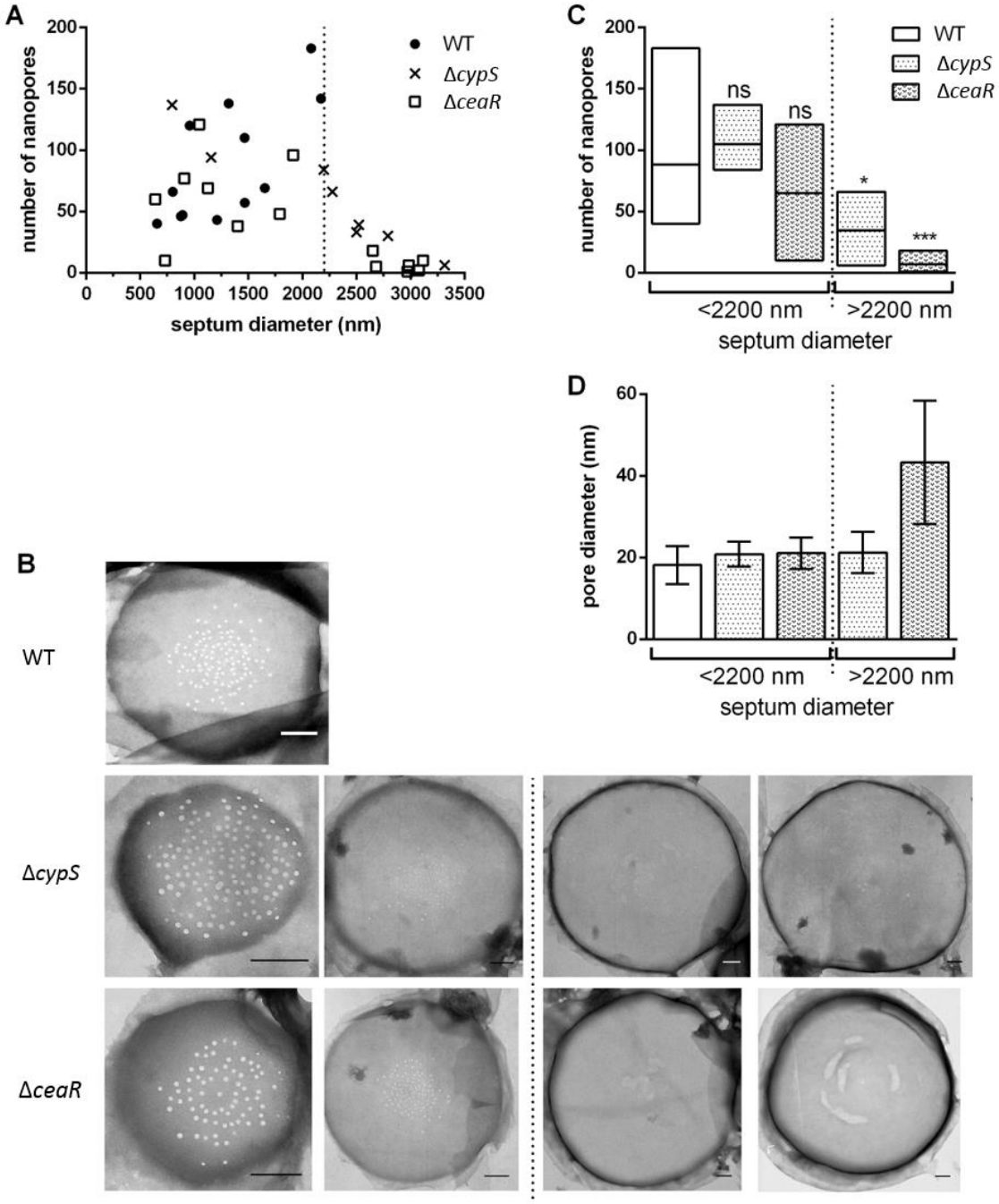
Supplementary Fig. 12: *Anabaena* CCRP mutant strains display defects in cell-cell solute diffusion

Representative calcein fluorescence micrographs depicting intercellular molecular exchange following laser-based bleaching of calcein fluorescence in *Anabaena* WT or Δ*ceaR* mutant strain grown in liquid BG11 and liquid $BG11_0$ as well as in *Anabaena* WT and Δ*lfiA*Δ*lfiB* and Δ*cypS* mutant strains grown on BG11 plates. White triangles indicate bleached cells. Translucent triangles show diffusion barriers present in the Δ*ceaR* mutant strain. Fluorescence images show respective cells prior bleaching, immediately after bleaching (t=0) and after 20 seconds after bleaching (t=20s). Images show representative examples. Scale bars: 5 µm.



Supplementary Fig. 13: Exchange coefficients of FRAP assays

Fluorescence recovery curves with their predicted exchange coefficient values (*E*)[29] for selected bleached cells of *Anabaena* WT or Δ*ceaR* mutant strain grown in liquid BG11 and liquid BG11$_0$ as well as in *Anabaena* WT and Δ*lfiA*Δ*lfiB* and Δ*cypS* mutant strains grown on BG11 plates. Fluorescence values are given in arbitrary units (a.u.)[29] over a time course of 20 s post bleaching.



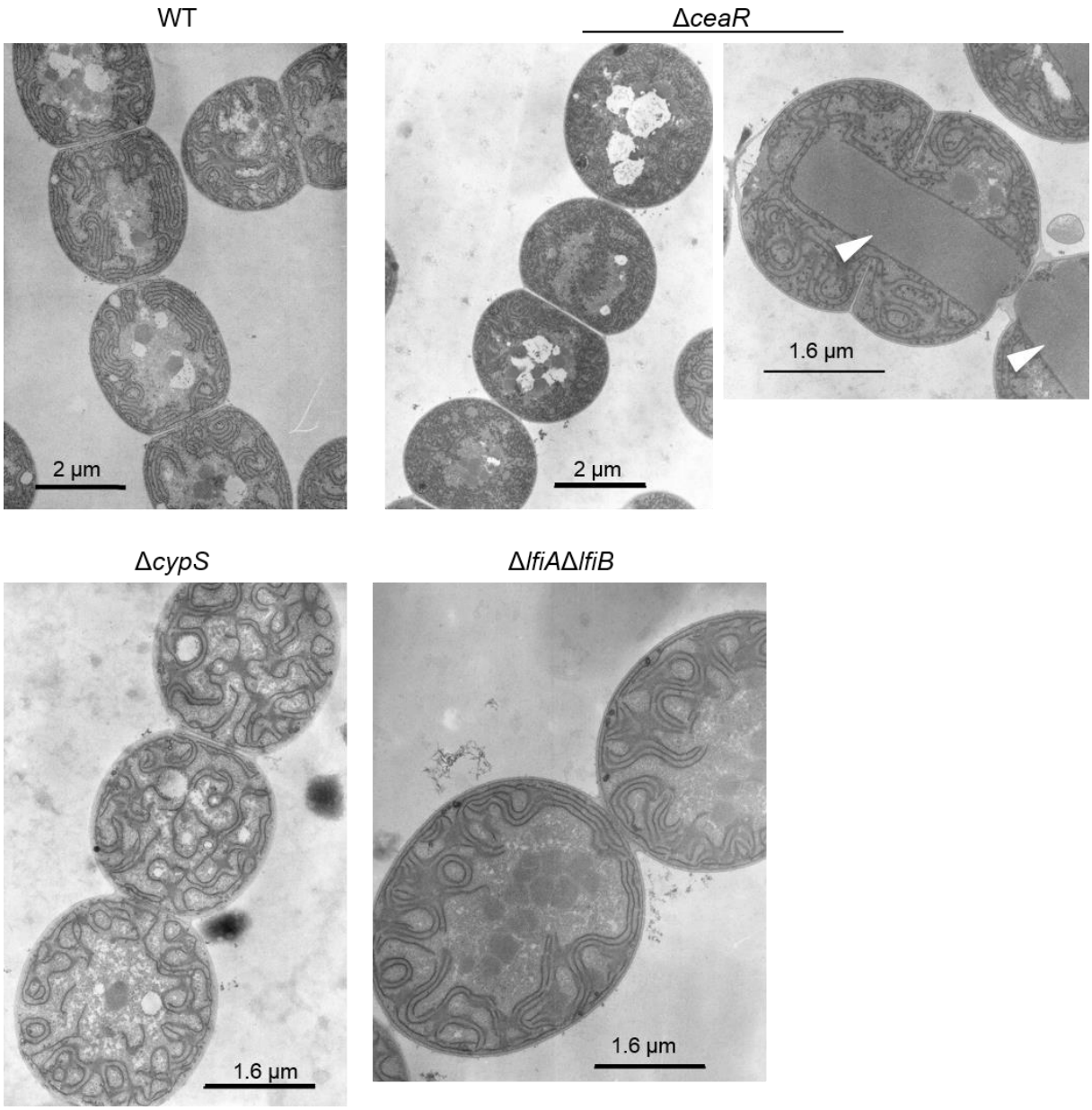
Supplementary Fig. 14: Septal nanopore array

(a) The number of nanopores is shown correlated to the septum size. In contrast to the WT, *Anabaena* CCRP mutants show a subset of large septa (>2200 nm) with few nanopores.

(b) Representative transmission electron microscopy images of indicated strains are shown. The dotted line divides the septa into a diameter of <2200 nm (left) and >2200 nm (right) derived from (a). Scale bars: 250 nm.

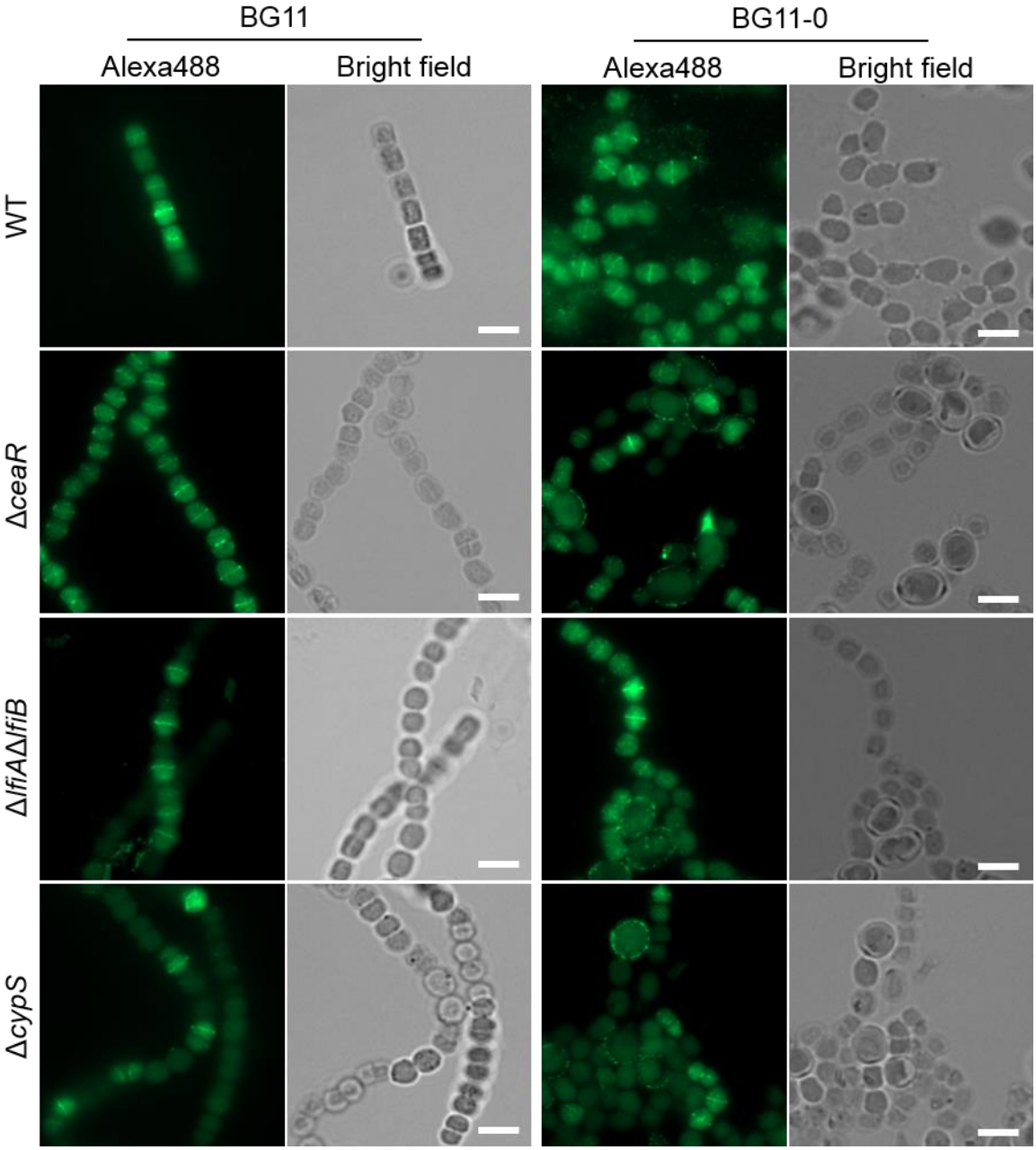
(c) Number of pores per septum (</> 2200 nm). Student's t-test (mutants vs. WT). P-values are indicated (ns: not significant, *P<0.05, ***P<0.001). P-values were calculated from the following number of septa: n (WT) = 12; n (Δ*cypS* <2200 nm) = 3; n (Δ*ceaR* <2200 nm) = 8; n (Δ*cypS* >2200 nm) = 5; n (Δ*ceaR* >2200 nm) = 6. WT data were derived from Bornikoel, *et al*[70].

(d) Nanopore diameter. Floating bars show the mean value from the number of analyzed nanopores. Sample size was n (WT) = 1061; n (Δ*cypS* <2200 nm) = 315; n (Δ*ceaR* <2200 nm) = 371; n (Δ*cypS* >2200 nm) = 174; n (Δ*ceaR* >2200 nm) = 42.



Supplementary Fig. 15: Ultrastructure of *Anabaena* WT and CCRP mutant strains

Ultrathin sections of *Anabaena* WT and *Anabaena* CCRP mutant strains grown on BG11 plates. White triangles indicate unusual structures in the Δ*ceaR* mutant that coincide with the observed red fluorescent filaments in the Δ*ceaR* mutant visualized by live cell fluorescence microscopy (Fig. 3d, Supplementary Fig. 9e).



Supplementary Fig. 16: FtsZ localization is unaffected in *Anabaena* CCRP mutant strains

Alexa Flour-488 fluorescence and bright field micrographs of *Anabaena* WT and Δ*ceaR*, Δ*lfiA*Δ*lfiB* and Δ*cypS* mutant strains grown on BG11 or $BG11_0$ growth plates. Cells were subjected to immunofluorescence staining using anti-FtsZ primary antibody and Alexa Fluor-488 coated secondary antibody essentially as described by Ramos-León *et al.*[52]. Note that the decreased abundance of Z-rings in the Δ*cypS* mutant could be attributed to the decreased viability of the Δ*cypS* mutant (Fig. 2d) or could be an artifact due to unequal permeabilization of the cells during the experimental procedure. In fact, we noticed that permeabilization using the method from Ramos-León *et al* (2015) is not always complete. Scale bars: 5 µm.

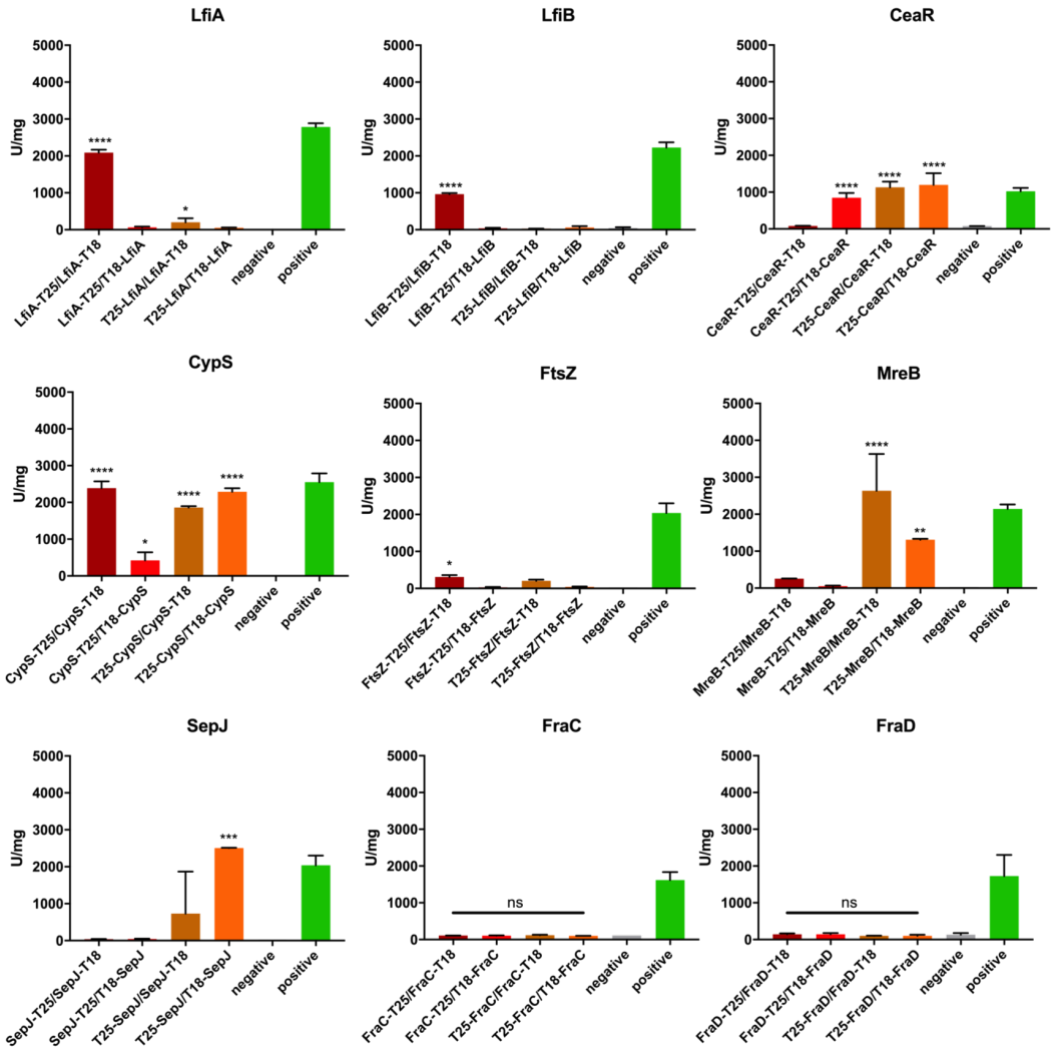
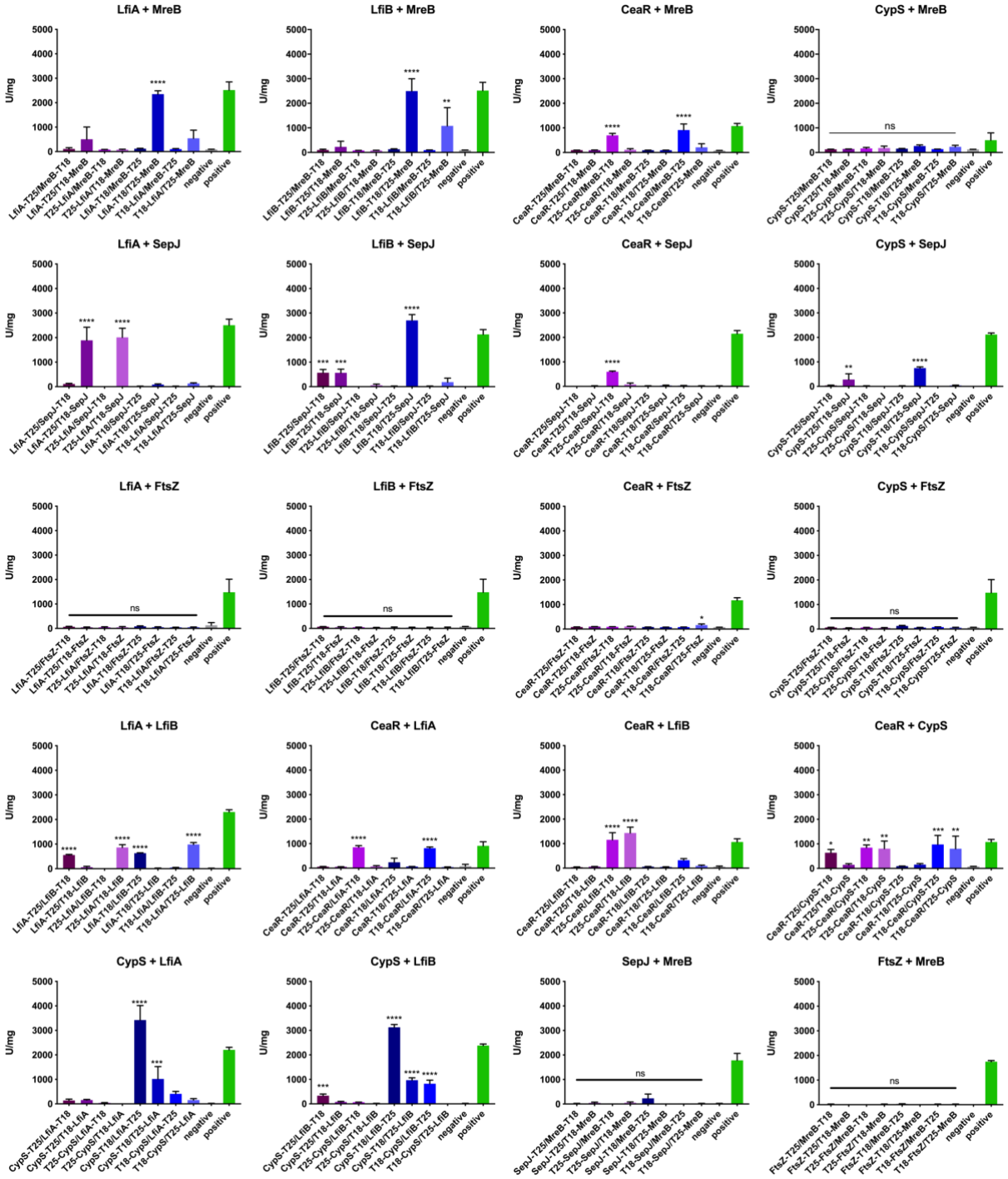


Figure description follows on page 45.



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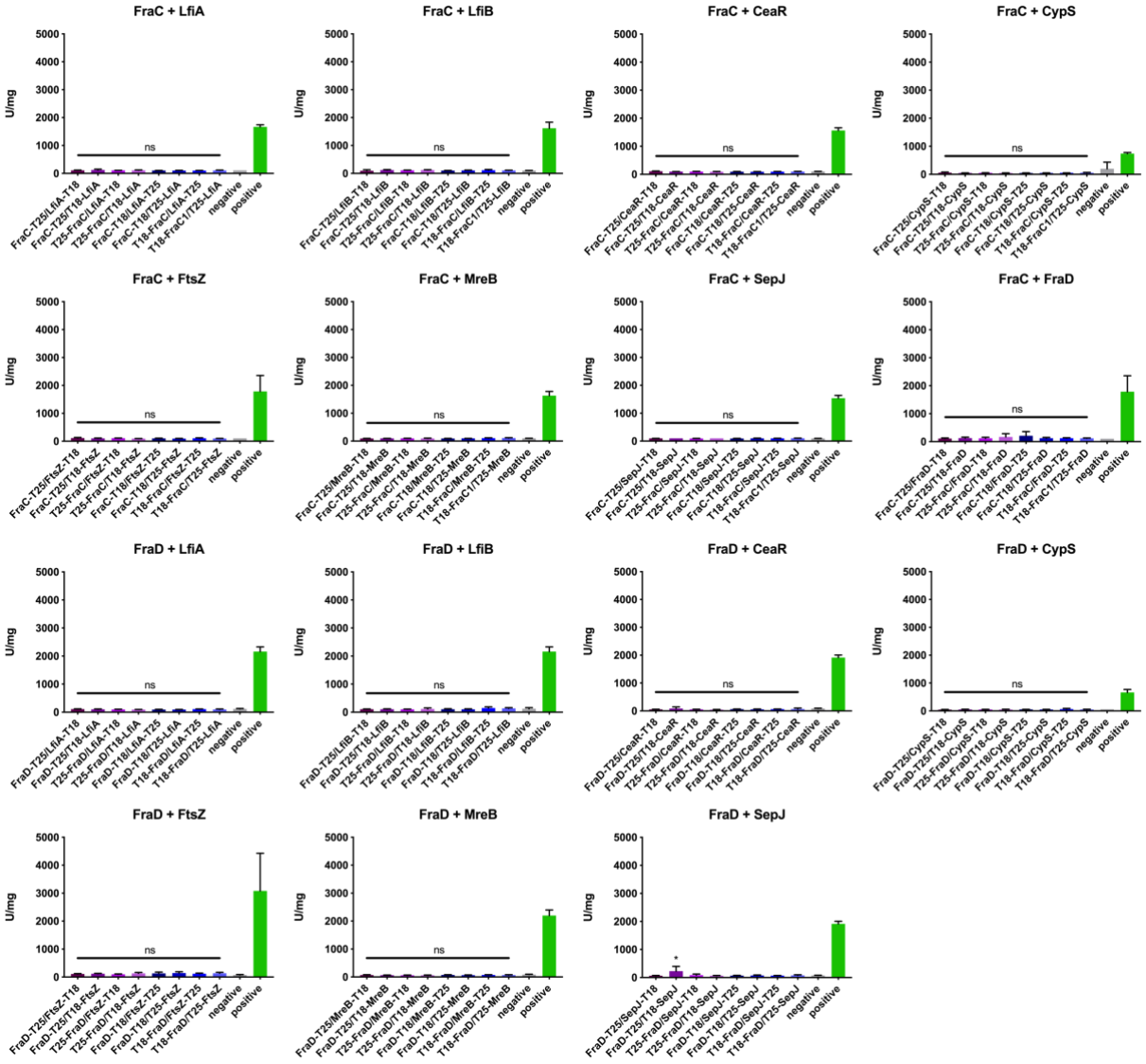
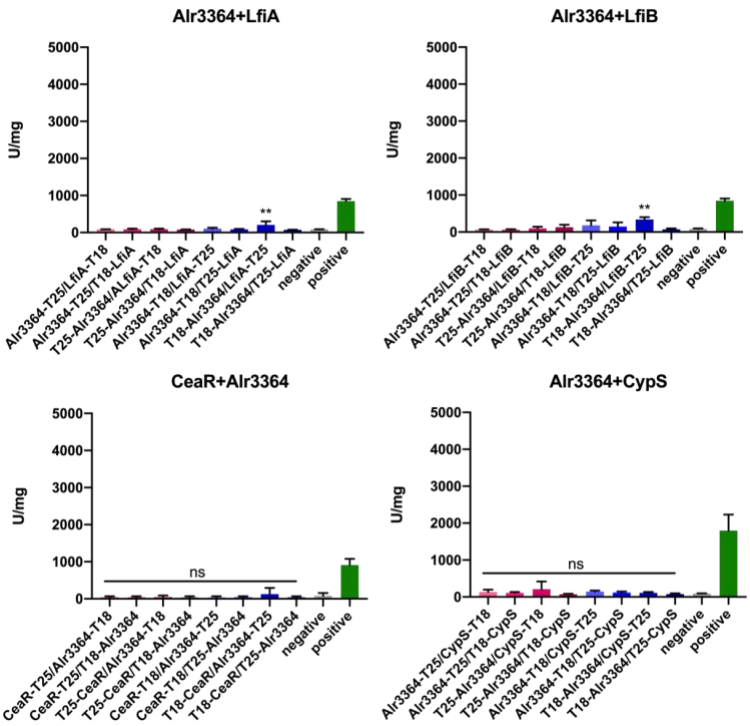


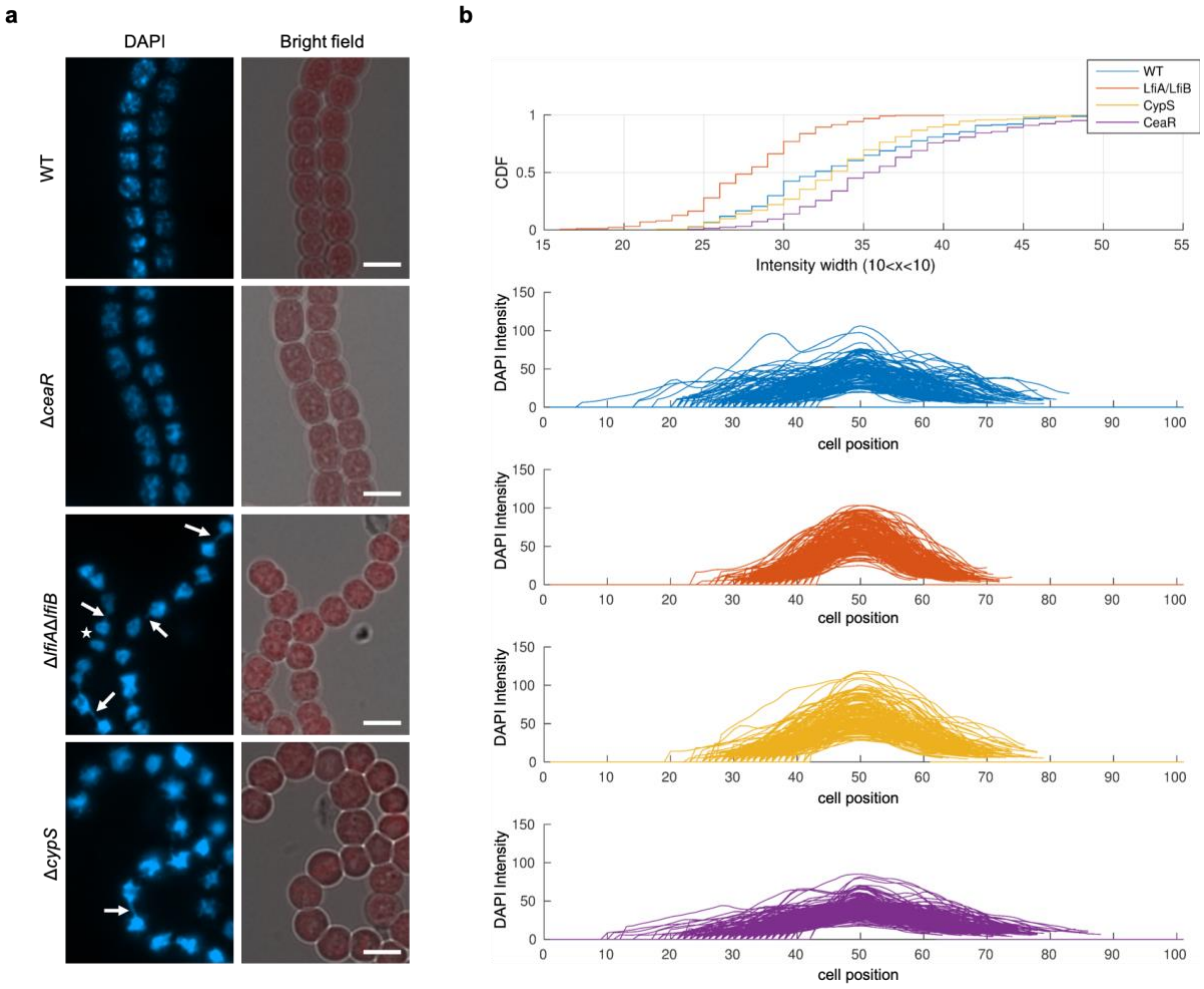
Supplementary Fig. 17: *Anabaena* possess a complex filament-forming CCRP network that is linked to a septal junction protein

Beta-galactosidase assays of *E. coli* BTH101 cells co-expressing indicated T25 and T18 translational fusions of all possible pair-wise combinations. *E. coli* cells carrying the respective plasmids were subjected to beta-galactosidase assay as described by Karimova *et al.*[71] in triplicates from three independent colonies grown for 2 d at 20 °C. Quantity values are given in Miller Units per milligram LacZ of the mean results from three independent colonies. Negative: N-terminal T25 fusion construct of the respective protein co-transformed with empty pUT18C. Positive: Zip/Zip control. Error bars indicate standard deviations (n=3). *: P<0.05, **: P<0.01, ***: P<0.001, ****: P<0.0001 (Dunnett's multiple comparison test and one-way ANOVA).



Supplementary Fig. 18: Interaction of *Anabaena* IF-like CCRPs is specific in a bacterial two hybrid system

Beta-galactosidase assays of *E. coli* BTH101 cells co-expressing indicated T25 and T18 translational fusions of all possible pair-wise combinations. *E. coli* cells carrying the respective plasmids were subjected to beta-galactosidase assay as described by Karimova *et al.*[71] in triplicates from three independent colonies grown for 2 d at 20 °C. Quantity values are given in Miller Units per milligram LacZ of the mean results from three independent colonies. Negative: N-terminal T25 fusion construct of the respective protein co-transformed with empty pUT18C. Positive: Zip/Zip control. Error bars indicate standard deviations (n=3). Values indicated with * are significantly different from the WT. **: P<0.01 (Dunnett's multiple comparison test and one-way ANOVA).



Supplementary Fig. 19: Condensation of intracellular DNA in Anabaena CCRPs

(a) DAPI fluorescence and merged bright field and chlorophyll autofluorescence micrographs of *Anabaena* WT and Δ*ceaR*, Δ*lfiA*Δ*lfiB* and Δ*cypS* mutant strains grown on BG11 growth plates. Cells were resuspended in BG11 and incubated with 10 µg ml_{-1} DAPI (final concentration). White arrows indicate strings of DNA that traverse from one cell to the other. Notably, no such strings are observed in dividing cells (white star), suggesting that it is an effect that occurs after complete cell division. Scale bars: 5 µm.

(b) Plot profile showing a cumulative distribution function (CDF) of the DAPI signal intensities of pixels (grey value) along *Anabaena* WT and *Anabaena* mutant cells (n=151 for each strain) in arbitrary units (a.u.) and arranged to the respective peak maxima. The focal area size in the Δ*lfiA*Δ*lfiB* mutant was smallest in comparison to the other strains, Δ*ceaR* was larger than the others, and the area size in WT was not significantly different than Δ*cypS* (alpha=0.05, using Tukey test). Notably, the comparison of cell size among the strains reveals a similar result: the Δ*lfiA*Δ*lfiB* mutant cell size was smallest in comparison to the other strains, Δ*ceaR* was larger than the others, and the area size in WT was not significantly different than the Δ*cypS* mutant ($P=1.75x10_{-54}$, using Kruskal-wallis; alpha=0.05, using Tukey test). Consequently, we compared the area of the focal DAPI staining decided by the cell size among the strains. This reveals that this ratio is smallest in Δ*ceaR*, largest in Δ*cypS* and not significantly different between Δ*lfiA*Δ*lfiB* and the WT.

847 **Supplementary Table 1: Characteristics of protein candidates**

Locus tag	Genus	Subsection	Homolog distribution	*In silico* structural simillarity prediction (I-TASSER)	Conserved domains	Others 848
crescentin	*C. crescentus*	n/a	n/a	Cytoplasmic domain of bacterial cell division protein EzrA	SMC_N, CCDC158	Validated IF-like protein[54,75–77]
cypS (*alr0931*)	*Anabaena*	IV	I, II, III, IV, V	Cytoplasmic domain of bacterial cell division protein EzrA	SMC_N, CCDC158 DUF3084, Neuromodulin_N	
all4981	*Anabaena*	IV	III, IV, V	TTC7B/Hyccin Complex or Clathrin coat	TPR	4 bp overlap to *all4982*
lfiA (*alr4504*)	*Anabaena*	IV	I, II, III, IV, V	Spectrin repeats 7, 8, and 9 of the plakin domain of plectin	SMC_N	*lfiB* localized downstream of *lfiA*
lfiB (*alr4505*)	*Anabaena*	IV	I, II, III, IV, V	Cytoplasmic domain of bacterial cell division protein EzrA	SMC_N, DUF3552	
ceaR (*all2460*)	*Anabaena*	IV	III, IV, V	Cytoplasmic domain of bacterial cell division protein EzrA	SMC_N, TerB_C, CALCOCO1, Spc7	Two N-terminal TMHs
alr0347	*Anabaena*	IV	I, II, III, IV, V	*Bacillus subtilis* Smc coiled-coil middle fragment	Filament superfamily	
alr3364	*Anabaena*	IV	II, III, IV, V	Cytoplasmic domain of bacterial cell division protein EzrA	SMC_N, FtsK, DUF4696	
all8023	*Anabaena*	IV	IV	Human ATR-ATRIP complex	SMC_N, Pentapeptide, Yjbl	
alr4393	*Anabaena*	IV	I, II, III, IV, V	Cytoplasmic domain of bacterial cell division protein EzrA	SMC_N, DUF3084	
alr4911	*Anabaena*	IV	I, II, III, IV, V	Structure of the Smc head domain with a coiled-coil and joint derived from *Pyrococcus yayanosii*	SMC_N, P-loop_NTPase DNA_S_dndD, Spc7, SbcC	
all4935	*Anabaena*	IV	IV	Cytoplasmic domain of bacterial cell division protein EzrA	DUF4114, DUF3084	
alr2043	*Anabaena*	IV	IV, V	Human ATR-ATRIP complex (replication stress response)	SMC_N, Tubulin_2	
alr3988	*Anabaena*	IV	I, II, III, IV, V	Two dynein tail domains bound to dynactin and BICDR1	SMC_N, SbcC	

Note: Structural similarities and conserved domains are inferred from *in silico*-based prediction tools and are not based on actual experimentally identified protein structures but simply serve as a mean to identify similarities between known filament-forming proteins and candidate CCRPs. The identified similarities might be subject to prediction bias due to the repeated nature of coiled-coil motifs. The first column indicates the respective locus tags of protein candidates and Crescentin. The second and third column indicate the respective subsection of the corresponding genus according to Rippka *et al.* (1979)[23]. Column four lists the subsections that contain homologous proteins to the respective candidate. Column five contains proteins predicted to be structurally similar to the protein candidates in the PDB (Protein Data Bank) based on I-TASSER[72–74]. The sixth column indicates predicted sub-domains of protein candidates identified by BLAST CDS. Column seven states other features of interest. Abbreviations: (TMH) Transmembrane helix; (DUF) Domain of unknown function; (CCDC158) Coiled-coil domain-containing protein 158; (SMC) Structural maintenance of chromosomes; (SbcC) DNA repair exonuclease SbcCD ATPase; (CALCOCO1) Calcium binding and coiled-coil domain; (TRP): Tetratricopeptide repeat; (Spc7) Spc7 kinetochore protein; (TerB_C) TerB-C occurs C-terminal of TerB in TerB-N containing proteins, putative metal chelating; (Filament superfamily) Intermediate filament protein; (FtsK) DNA segregation ATPase FtsK; (Pentapeptide) Pentapeptide repeats often found in many cyanobacterial proteins with unknown function (predicted to be a β-helix); (Yjbl) Uncharacterized protein containing pentapeptide repeats; (DNA_S_dndD) DNA sulfur modification protein DndD; (Tubulin_2 superfamily) Tubulin like; Many of the residues conserved in Tubulin (pfam00091) are also conserved in this family; (P-loop_NTPase) P-loop containing Nucleoside Triphosphate Hydrolases superfamily. n/a: not applicable.

Material and methods

Bacterial strains and growth conditions

Anabaena WT was obtained from the Pasteur Culture Collection (PCC) of cyanobacteria (France). Cells were grown photoautotropically in BG11 or without combined nitrogen ($BG11_0$) at constant light with a light intensity of 30 µmol m^{-2} s^{-1}. When appropriate, 5 µg ml^{-1} spectinomycin (Sp), 5 µg ml^{-1} streptomycin (Sm) or 30 µg ml^{-1} neomycin (Nm) was added to strains carrying respective plasmids or chromosomal insertions. In some cases, basal copper-regulated *petE*-driven expression of gene candidates in *Anabaena* cells was lethal or growth inhibiting, therefore these strains were grown in BG11 without copper and protein expression was later induced by the addition of $CuSO_4$ at indicated concentrations to the culture. *E. coli* strains DH5α, DH5αMCR, XL1-blue and HB101 were used for cloning and conjugation by triparental mating. BTH101 was used for BACTH system and BL21 (DE3) was used for expression of His_6- and GFP-tagged proteins in *E. coli*. All strains were grown in LB medium containing the appropriate antibiotics at standard concentrations. Supplementary Tables 2-5 list all used bacterial strains, plasmids and oligonucleotides.

Prediction of coiled-coil rich proteins

Genome sequence of *Anabaena* (GCA_000009705.1) was analyzed by the COILS algorithm[66] as described by Bagchi *et al.*[11]. The algorithm was run with a window width of 21 and the cut-off for amino acids in coiled-coil conformation was set to ≥ 80 amino acid residues. The resulting set of protein candidates was further manually examined with online available bioinformatic tools (NCBI Conserved Domain Search, NCBI BLAST, TMHMM Server, PSORTb, I-TASSER). Protein candidates exhibiting BLAST hits involved in cytoskeletal processes or similar domain architectures as known IF proteins like CreS, FilP, vimentin, desmin or keratin were selected, and enzymatic proteins as well as proteins predicted to be involved in other cellular processes were excluded.

Distribution of homologs in cyanobacteria

Cyanobacteria species tree is according to Dagan *et al.*[78] with the root of the tree as recently inferred by Tria, Landan and Dagan[79]. Homologs to the *Anabaena* proteins were detected by amino acid sequence similarity using stand-alone BLAST[80] ver. 2.2.26. Protein sequences that were found as BLAST hits with a threshold of E-value ≤ $1x10^{-5}$ were further compared to the *Anabaena* protein by global alignment using needle[81]. Hits having ≥30% identical amino acids in the global alignment were considered as homologs. The phylogenetic tree was visualized with iTOL[82].

Genomic DNA and RNA isolation and cDNA synthesis

Genomic DNA (gDNA) from *Anabaena* was isolated using the GeneJET Plant Genomic DNA Purification Kit (Thermo Fischer Scientific) and the DNeasy Plant Mini Kit (QIAGEN) according to the manufacturer's instructions from 10 ml cyanobacterial cultures.

RNA from *Anabaena* WT was isolated using the Direct-zol™ RNA MiniPrep Kit (Zymo Research) according to the manufacturer's instructions. RNA was isolated in technical triplicates from 10 ml cultures. Isolated RNA was treated with DNA-free™ Kit (2 units rDNAs/reaction; Thermo Fischer Scientific) and 200 ng RNA was reverse transcribed using the qScript™ cDNA Synthesis Kit (Quanta Biosciences). RT-PCR of cDNA samples for *rnpB*, *cypS*, *ceaR*, *lfiA*, *lfiB* and *lfiA*+*lfiB* was done using primer pairs #1/#2, #3/#4, #5/#6, #7/#8, #9/#10, #7/#10, respectively.

Transformation

Transformation of chemically competent *E. coli* was performed by the standard heat shock procedure[83]. *Anabaena* was transformed by triparental mating according to Ungerer and Pakrasi[84]. Briefly, 100 µl of overnight cultures of DH5α carrying the conjugal plasmid pRL443 and DH5αMCR carrying the cargo plasmid and the helper plasmid pRL623, encoding for three methylases, were mixed with 200 µl *Anabaena* culture (for transformation into *Anabaena* mutant strains, cells were scraped from the plate and resuspended in 200 µl BG11). This

mixture was directly applied onto sterilized nitrocellulose membranes placed on top of BG11 plates supplemented with 5% LB medium. Cells were incubated in the dark at 30 °C for 6-8 h with subsequent transfer of the membranes to BG11 plates. After another 24 h, membranes were transferred to BG11 plates supplemented with appropriate antibiotics.

Plasmid construction

Ectopic expression of *Anabaena* protein candidates was achieved from a self-replicating plasmid (pRL25C[85]) under the control of the copper-inducible *petE* promoter (P_{petE}) or the native promoter (predicted by BPROM[41]) of the respective gene. All constructs were verified by Sanger sequencing (Eurofins Genomics).

Initially, we generated pTHS1 (pRL25C, P_{petE}::*lfiA-gfp)*, which served as template for many other pRL25C-based plasmids employed in this study. For this, P_{petE} and *lfiA* were amplified from *Anabaena* gDNA using primers #11/#12 and #13/#14, respectively. *gfpmut3.1* was amplified from pJET1.2 containing P_{petE}-*gfp*[86] using primers #15/#16. This *gfpmut3.1* (hereafter *gfp*) is deprived of its internal NdeI site and contains a 5' linker sequence of 12 alternating codons encoding for alanine and serine. The PCR fragments were next ligated into PCR-amplified pRL25C (using primers #17/#18) by Gibson assembly.

For pTHS2 (pRL25C, P_{petE}::*lfiB-gfp*) and pTHS3 (pRL25C, P_{petE}::*ceaR-gfp*)*, lfiB* and *ceaR* were amplified from *Anabaena* gDNA using primers #19/#20 or #21/#22, respectively and ligated into PCR-linearized pTHS1 (using primers #23/#24; thereby removing only *lfiA* and leaving P_{petE} and *gfp* in the vector) by Gibson assembly.

For pTHS4 (pRL25C, P_{petE}::*cypS-gfp*), *cypS* was amplified by PCR from *Anabaena* gDNA using primers #36/#38, introducing NdeI and SacI sites, and then ligated into pJET1.2-P_{petE}::*gfp*, thereby generating P_{petE}::*cypS-gfp* which is flanked by a 5' BamHI site and a 3' EcoRI site. The P_{petE}::*cypS-gfp* fragment was excised by BamHI and EcoRI and ligated into BamHI and EcoRI-digested pRL25C.

For pTSH5 (pRL25C, P_{petE}::*trunc-ceaR-gfp*), pTHS3 was amplified using primers #148/#148 and re-ligated using Quick Ligase (NEB). Thereby, the first 153 bp from *ceaR* were removed

For pTHS6 (P_{petE}::*cypS-his*), *cypS-his* was amplified from pTHS8 using primers #25/#26 and ligated into PCR-linearized pRL25C (using primers #24/#27) by Gibson assembly.

For pTHS7 (pRL25C, P_{petE}-*lfiA*-ec*fp,* P_{petE}-*lfiB-gfp*), P_{petE}-*lfiA* was amplified from pTHS1 using primers #28/#29 and ligated into ClaI-digested pRL25C by Gibson assembly together with *myc-link-ecfp* (initially amplified with primers #30/#31, purified and then again amplified with primers #32/#33). This construct was digested by BamHI and ligated by Gibson assembly with PCR-amplified P_{petE}::*lfiB-gfp* from pTHS2 (using primers #34/#35).

pET21a(+) plasmids bearing C-terminal His-tag translational fusions of CCRPs were generated by restriction-based cloning techniques. For this, *cypS*, *lfiA*, *lfiB* or *ceaR* were amplified by PCR from *Anabaena* gDNA using primers #36/#37, #39/#40, #41/#42 or #43/#44, respectively, introducing NdeI and XhoI sites. NdeI and XhoI-digested fragments were then ligated into pET21a(+) using Quick Ligase (NEB). This procedure yielded plasmids pTHS8, pTHS9, pTHS10 and pTSH11, respectively.

pET21a(+) bearing C-terminal *gfp* translational fusions were generated based on pTHS12 (pET21a(+), P_{T7}::*cypS-gfp*). For this, *cypS* was amplified by PCR from *Anabaena* gDNA with primers 36#/#38, introducing NdeI and SacI sites, and ligated into NdeI and SacI-digested pJET1.2 bearing P_{petE}-*gfp*. *cypS-gfp* was excised by NdeI and EcoRI and ligated into NdeI and EcoRI-digested pET21a(+), generating pTHS12. For pTHS13 (pET21a(+), P_{T7}::*ceaR-gfp*), *ceaR-gfp* was amplified by PCR from pTHS3 using primers #47/#48 and ligated into PCR-linearized pTHS12 (primers #49/#50) by Gibson assembly.

GFP-fragment reassembly plasmids were generated by Gibson assembly. For this aim, *lfiA* was amplified by PCR from *Anabaena* gDNA with primer 51/52 or 53/54 and ligated into XhoI and BamHI or NheI digested pET11a-link-NGFP, generating pTHS15 or pTHS16. *lfiB* was

amplified by PCR from *Anabaena* gDNA with primers 55/56 and ligated into NcoI and AatII digested pMRBAD-link-CGFP, generating pTHS17.

Clonings for bacterial two-hybrid plasmids were done using Gibson assembly of PCR-linearized pKNT25, pKT25, pUT18 or pUT18C plasmids, using primers #57/#58 for pKNT25 and pUT18, primers #59/#60 for pKT25 and primers 61/62 for pUT18C. For each gene, three primer combinations were used for amplification from *Anabaena* gDNA. The first primer pair was always used for cloning of the respective gene into pKNT25 and pUT18 while the second and third primer pairs were used for cloning into pKT25 or pUT18C, respectively: *cypS* (primers #63/#64, #65/#66 or #67/#68), *lfiA* (primers #69/#70, #71/#72 or #73/#74), *lfiB* (primers #75/#76, #77/#78 or #79/#80), *ceaR* (primers #81/#82, #83/#84 or #85/#86), *sepJ* (primers #87/#88, #89/#90 or #91/#92), *ftsZ* (primers #93/#94, #95/#96 or #97/#98), *mreB* (primers #99/#100, #101/#102 or #103/#104), *fraC* (primers #105/#106, #107/#108 or #109/#110) and *fraD* (primers #111/#112, #113/#114 or #115/#116). This yielded plasmids pTHS17-pTHS52, respectively.

Like for P_{petE}-driven expression, native expression of *Anabaena* CCRPs was mediated from the pRL25C plasmid. For pTHS56 (pRL25C, P_{cypS}::*cypS-gfp*), P_{cypS} was amplified from *Anabaena* gDNA using primers #150/#151 and ligated into BamHI and EcoRI-digested pRL25C by Gibson assembly together with *cypS-gfp*, which was amplified from pTHS4 using primers #152/#153.

For pTHS57 (pRL25C, P_{ceaR}::*ceaR-gfp*), P_{ceaR} was amplified from *Anabaena* gDNA using primers #154/#155 and ligated into BamHI and EcoRI-digested pRL25C by Gibson assembly together with *ceaR-gfp*, which was amplified from pTHS3 using primers #156/#153.

For pTHS58 (pRL25C, P_{lfiA}::*lfiA-gfp*), P_{lfiA} was amplified from *Anabaena* gDNA using primers #157/#158 and ligated into BamHI and EcoRI-digested pRL25C by Gibson assembly together with *lfiA-gfp*, which was amplified from pTHS1 using primers #159/#153.

For pTHS59 (pRL25C, P_{lfiB}::*lfiB-gfp*), P_{lfiB} was amplified from *Anabaena* gDNA using primers #160/#161 and ligated into BamHI and EcoRI-digested pRL25C by Gibson assembly together with *lfiB-gfp*, which was amplified from pTHS2 using primers #162/#153.

For pTHS60 (pRL25C, P_{cypS}::*cypS-his*), P_{cypS} was amplified from *Anabaena* gDNA using primers #150/#151 and ligated into BamHI and EcoRI-digested pRL25C by Gibson assembly together with *cypS-his*, which was amplified from pTHS8 using primers #152/#163.

For pTHS61 (pRL25C, P_{ceaR}::*ceaR*), P_{ceaR}::*ceaR* was amplified from *Anabaena* gDNA using primers #154/#164 and ligated into BamHI and EcoRI-digested pRL25C by Gibson assembly.

For pTHS58 (pRL25C, $P_{lfiA/B}$::*lfiA-lfiB*), $P_{lfiA/B}$::*lfiA-lfiB* was amplified from *Anabaena* gDNA using primers #157/#165 and ligated into BamHI and EcoRI-digested pRL25C by Gibson assembly.

Anabaena mutant strain construction

All *Anabaena* mutant strains were generated using the pRL278-based double homologous recombination system employing the conditionally lethal *sacB* gene[87]. For this, 1500 bp upstream and downstream of the gene to be replaced were generated by PCR from *Anabaena* gDNA. Upstream and downstream regions of *cypS* and *ceaR*, were amplified using primers #121/#122 and #123/#124 or #125/#126 and #127/#128, respectively. Upstream region of *lfiA* was amplified using primers #129/#130 and downstream region of *lfiB* was amplified using primers #131/#132. The respective upstream and downstream homology regions flanking the CS.3 cassette (amplified with primer #119/#120 from pCSEL24) were then inserted into PCR-amplified pRL278 (using primer #117/#118) by Gibson assembly, yielding pTHS55, pTHS56 and pTHS57, respectively. *Anabaena* transformed with those plasmids was subjected to several rounds of re-streaking on new plates (about 5-8 rounds for each strain). To test for fully segregated clones, colony PCRs were performed. For this, *Anabaena* cells were resuspended in 10 µl sterile H_2O of which 1 µl was used for standard PCR with internal gene primers #3/#4, #5/#6 and #7/#10 for Δ*cypS*, Δ*ceaR* and Δ*lfiA*Δ*lfiB,* respectively. Correct placement of the CS.3 cassette was then further confirmed using primers CS.3 cassette primers with primers

binding outside of the 5' and 3' flanks used for homologous recombination (#137/#118 and #117/#138 for Δ*cypS*, #135/#118 and #117/#136 for Δ*ceaR* and #133/#118 and #117/#134 for Δ*lfiA*Δ*lfiB*).

Growth curve analysis

For analysis of mutant viability, growth curves of *Anabaena* WT and Δ*ceaR* mutant strain were performed. For this, cells were grown in BG11 liquid medium, washed three times by centrifugation (6500 x *g*, RT, 3 min) in BG11 or $BG11_0$, adjusted to an OD_{750} of 0.1 and then grown in triplicates at standard growth conditions in 15 ml culture volumes. OD_{750} values were recorded once a day for 24 d.

Fluorescence microscopy

Bacterial strains grown in liquid culture were either directly applied to a microscope slide or previously immobilized on a 2% low-melting agarose in PBS (10 mM Na_2HPO_4, 140 mM NaCl, 2.7 mM KCl, 1.8 mM KH_2PO_4, pH 7.4) agarose pad and air dried before microscopic analysis. Epifluorescence was done using an Axio Imager.M2 light microscope (Carl Zeiss) equipped with Plan-Apochromat 63x/1.40 Oil M27 objective and the AxioCam MR R3 imaging device (Carl Zeiss). GFP, Alexa Fluor 488 and BODIPY™ FL Vancomycin (Van-FL) fluorescence was visualized using filter set 38 (Carl Zeiss; excitation: 470/40 nm band pass (BP) filter; emission: 525/50 nm BP). Chlorophyll auto-fluorescence was recorded using filter set 15 (Carl Zeiss; excitation: 546/12 nm BP; emission: 590 nm long pass). When applicable, cells were previously incubated in the dark at RT for about 5 min with 10 µg ml^{-1} DAPI (final concentration) to stain intracellular DNA. For visualization of DAPI fluorescence filter set 49 (Carl Zeiss; excitation: G 365 nm; emission: 455/50 nm) was employed. For confocal laser scanning microscopy, the LSM 880 Axio Imager 2 equipped with a C-Apochromat 63x/1.2 W Korr M27 objective and an Airyscan detector (Carl Zeiss) was used and visualization of GFP, eCFP and chlorophyll auto-fluorescence was done using Zen black smart setup settings. *E. coli* BL21 (DE3) cells expressing C-terminally GFP-tagged protein candidates were were grown over night in LB and then diluted 1:40 in the same medium the following day. Cells were grown for 2 h at 37 °C,

1028 briefly acclimated to 20 °C for 10 min and induced with 0.05 mM IPTG at 20 °C. Protein
1029 localization of GFP-tagged proteins was then observed after indicated time points of cells
1030 immobilized on an agarose pad.

1031 Transmission electron microscopy and sacculi preparation

1032 For ultra-structure analysis, *Anabaena* filaments were fixed with 2.5% glutaraldehyde,
1033 immobilized in 2% agaorse, treated with 2% potassium permanganate and dehydrated through
1034 a graded ethanol series. The fixed cells were infiltrated by ethanol:EPON (2:1 to 1:2 ratio) and
1035 embedded in pure EPON. Ultrathin sections were prepared with a Leica UC6iUltramicrotome,
1036 transferred to formvar coated copper grids and post-stained with uranyl acetate and lead
1037 citrate[88]. Micrographs were recorded at a Philips Tecnai10 electron microscope at 80 kV.

1038 Peptidoglycan sacculi were isolated from filaments grown on BG11 agar plates by the method
1039 of Kühner *et al.*[89] with the following modifications: Cells were sonicated (Branson Sonifier 250;
1040 duty cycle 50%, output control 1, 2 min) prior to boiling in 0.1 M Tris-HCl pH 6.8 with 3% SDS.
1041 After incubation in a sonifier waterbath, the samples were incubated with α-Chymotrypsin (600
1042 µg ml^{-1}) at 37 °C over night in 50 mM Na_3PO_4 buffer pH 6.8. After inactivation of the enzyme,
1043 the sample was sonified again and loaded on a formvar/carbon film coated copper grid
1044 (Science Services GmbH, Munich) and stained with 1 % (w/v) uranyl acetate as described
1045 previously[90]. Images were taken with a Philips Tecnai10 electron microscope at 80 kV.

1046 Calcein labelling and fluorescence recovery after photobleaching (FRAP) experiments

1047 *Anabaena* WT and mutant strains were either grown on BG11 plates and resuspended in BG11
1048 or directly taken from liquid cultures, washed several times in BG11, resuspended in 0.5 ml
1049 BG11 and incubated with 10 µl calcein-AM (1 mg ml^{-1} in DMSO). The cells were incubated in
1050 the dark at 30 °C for 1 hour and then subjected to four washing steps with 1 ml BG11.
1051 Subsequently, cells were resuspended in a small volume of BG11, spotted on BG11 agar
1052 plates (1 % w/v) and air dried. Samples were visualized by using an inverted confocal laser
1053 scanning microscope (Leica TCS SP5) with a x63 oil immersion objective (HCX PL APO 63x

1.40-0.60 OIL CS). Fluorescence was excited at 488 nm and emission monitored by collecting across a window of 500 to 530 nm with a maximally opened pinhole (600 µm). FRAP experiments were carried out by an automated routine as previously described (Mullineaux *et al.* EMBO). After recording an initial image, selected cells were bleached by increasing the laser intensity by a factor of 5 for two subsequent scans and the fluorescence recovery followed in 0.5 s intervals for 30 s was recorded using the Leica LAS X software. Exchange coefficients (*E*) were then calculated according to Mullineaux *et al*. and Nieves-Morión *et al.*[29,69].

BODIPY™ FL Vancomycin (Van-FL) staining

Van-FL staining of BG11-grown filaments of the *Anabaena* WT and mutant strains was essentially performed as previously described by Lehner *et al*.[34] and Rudolf *et al*.[91]. Briefly, cells were resuspended in BG11 medium, washed once in BG11 by centrifugation (6500 x *g*, 4 min, RT) and incubated with 5 µg ml^{-1} Van-FL (dissolved in methanol). Cells were incubated in the dark for 1 hour at 30 °C, washed three times with BG11 and immobilized on an agarose pad. Van-FL fluorescence signals were then visualized using epifluorescence microscopy with an excitation time of 130 ms. Arithmetic mean fluorescence intensities were then recorded from the septa between two cells with a measured area of 3.52 μm^2 using the histogram option of the Zen blue 2.3 software (Carl Zeiss).

Data analysis

Cell length, volume and roundness were determined using the imaging software ImageJ[92], a perfect circle is defined to have a roundness of 1. Cell volume was calculated based on the assumption of an elliptic cell shape of *Anabaena* cells using the Major Axis and Minor Axis values given by ImageJ and the formula for the volume of an ellipse ($V = \frac{4}{3}\pi abc$):

- $V = \frac{4}{3}\pi\left(\left(\frac{Major\ Axis}{2}\right)^2 \frac{Minor\ Axis}{2}\right)$

Distribution of DAPI fluorescence signals was done in ImageJ with the Plot Profile option along 151 single cells with rectangle tool. The resulting grey values were arranged according to the

maximum intensity focus and the width of the DAPI focal area was calculated as the range of DAPI staining around the maximum (±10 grey value in arbitrary units).

Bacterial two-hybrid and beta galactosidase assays

Chemically competent *E. coli* BTH101 cells were co-transformed with 5 ng of plasmids carrying the respective T18 and T25 translational fusion constructs, plated onto LB plates supplemented with 200 µg ml^{-1} X-gal, 0.5 mM IPTG, Amp, Km and grown at 30°C for 24-36 h. Interactions were quantified by beta-galactosidase assays from three independent colonies. For this aim, cultures were either grown over night at 30 °C or for two days at 20 °C in LB Amp, Km, 0.5 mM IPTG and beta-galactosidase activity was recorded as described in the manufacturer's instructions (Euromedex; BACTH System Kit Bacterial Adenylate Cyclase Two-Hybrid System Kit) in a 96 well plate according to Karimova, Davi and Ladant[71].

GFP-fragment reassembly assay

Chemically competent *E. coli* BL21 (DE3) were co-transformed with indicated plasmid combinations, plated on LB Amp, Km and grown over night at 37 °C. Liquid overnight cultures of single colonies of the respective plasmid-bearing *E. coli* strains were then diluted 1:40 in the same medium the following day. Cells were grown for 2 h at 37 °C, briefly acclimated to 20 °C for 10 min and protein expression was induced with 0.05 mM IPTG and 0.2% L-arabinose. Pictures of induced cultures grown at 20 °C were taken after 48 h of cells immobilized on an agarose pad.

Co-immunoprecipitation

About 20-30 ml of the respective *Anabaena* culture was pelleted by centrifugation (4800 x *g*, 10 min, RT), cells were washed twice by centrifugation (4800 x *g*, 10 min, RT) with 40 ml PBS and then resuspended in 1 ml lysis buffer (PBS-N: PBS supplemented with 1% NP-40) supplemented with protease inhibitor cocktail (PIC; cOmplete™, EDTA-free Protease Inhibitor Cocktail, Sigma-Aldrich). Cells were lysed using the VK05 lysis kit (Bertin) in a Precellys® 24 homogenizer (3 strokes for 30 seconds at 6500 rpm) and cell debris was pelleted by

centrifugation (30 min, 21,100 x *g*, 4 °C). 50 µl µMACS anti-GFP MicroBeads (Miltenyi Biotec) was added to the resulting cell-free supernatant and incubated for 1 h at 4 °C with mild rotation. Afterwards, the sample was loaded onto µColumns (Miltenyl Biotec), washed two times with 1 ml lysis buffer and eluted in 50 µl elution Buffer (50 mM Tris HCl pH 6.8, 50 mM DTT, 1% SDS, 1 mM EDTA, 0.005% bromphenol blue, 10% glycerol; Miltenyl Biotec). Until further use, samples were stored at -80 °C.

Mass spectrometry analysis

Coomassie stained gel bands were excised and protein disulfide bonds were reduced with 10 mM dithiotreitol at 56 °C for 45 min and alkylated with 55 mM iodoacetamide at RT for 30 min in the dark. The gel bands were washed 50 mM ammonium bicarbonate and subsequently dehydrated with acetonitrile. 10 µl trypsin (5 ng μl^{-1} in 25 mM ammonium bicarbonate) were added and the gel bands were rehydrated for 5 min at 37 °C. Samples were digested over night at 37 °C. Prior to peptide extraction samples were acidified with 10% formic acid. After transferring the supernatant into a new Eppendorf tube, 5% formic acid was added to the gel bands and incubated for 10 min. Subsequently the samples were sonicated for 1 min in ice-cooled water and the supernatant was combined with the one from the step before. Two additional extraction steps with 60% acetonitrile/1% formic acid and 100% acetonitrile were performed in the same manner. The combined supernatants were dried in the SpeedVac and the samples were reconstituted in 30 µL 3% acetonitrile/0.1% trifluoroacetic acid. LC-MS/MS analysis was performed using a Dionex U3000 nanoUHPLC coupled to a Q Exactive Plus mass spectrometer (both from Thermo Scientific). The LC-MS/MS parameters were as follows: Six microliter were injected and loaded on a trap column (Acclaim Pepmap 100 C18, 10 mm × 300 µm, 3 µm, 100 Å, Dionex) and washed for 3 min with 2% ACN/0.05% TFA at a flow-rate of 30 µL min^{-1}. separation was performed using an Acclaim PepMap 100 C18 analytical column (50 cm × 75 µm, 2 µm, 100 Å, Dionex) with a flow-rate of 300 nL/min and following eluents: A (0.05% FA) and B (80% ACN/0.04% FA); linear gradient 5-40% B in 60 min, 50-90% B in 5 min, 90% B for 10 min, 90-5% B in 1 min and equilibrating at 5% B for 11 min. Ionization was

performed with 1.5 kV spray voltage applied on a non-coated PicoTip emitter (10 µm tip size, New Objective, Woburn, MA) with the source temperature set to 250°C. MS data were acquired from 5 to 85 min with MS full scans between 300 and 1,800 m/z at a resolution of 70,000 at m/z 200. The 10 most intense precursors with charge states ≥2+ were subjected to fragmentation with HCD with NCE of 27%; isolation width of 3 m/z; resolution, 17,500 at m/z 200. Dynamic exclusion for 30 s was applied with a precursor mass tolerance of 10 ppm. Lock mass correction was performed based on the polysiloxane contaminant signal of 445.120025 m/z. Additional wash runs were performed between samples from gel bands to reduce carry over while cytochrome C was used to monitor mass accuracy and LC quality control. The acquired MS/MS data were searched with the SequestHT algorithm against the entire reviewed Uniprot protein database of *Nostoc* sp. PCC 7120 including plasmids (6922 sequences in total). Static modifications applied were carbamidomethylation on cysteine residues, while oxidation on methionine residues was set as dynamic modification. Spectra were searched with full enzyme specificity. A MS mass tolerance of 10 ppm and a MS/MS tolerance of 0.02 Da was used. Proteins were identified with at least three unique peptides with a FDR confidence ≤ 0.01 (high).

Immunofluorescence

Immunolocalization of CypS-His and FtsZ in *Anabaena* was essentially performed as described by Ramos-León *et al.*[52]. For visualization of FtsZ, *Anabaena* WT and mutant strains were streaked from growth plates (BG11 and $BG11_0$ plates), resuspended in a small volume of distilled water and air-dried on Polysine® adhesion slides (Menzel) at RT followed by fixation and permeabilization with 70% ethanol for 30 min at -20 °C. Cells were allowed to air dry for 30 min at RT and then washed two times with PBST (PBS supplemented with 0.1% (v/v) Tween-20) for 2 min. Unspecific binding sites were blocked for 30 min at RT with blocking buffer (1x Roti®-ImmunoBlock in PBST; Carl Roth) and afterwards rabbit anti-FtsZ (Agrisera; 1:150 diluted) antibody in blocking buffer was added to the cells and incubated for 1.5 h at RT in a self-made humidity chamber followed by five washing steps with PBST. 7.5 µg ml^{-1} Alexa Fluor 488-conjugated goat anti-rabbit IgG (H+L) secondary antibody (Thermo Fischer

Scientific) in blocking buffer was added to the cells and incubated for 1 h at RT in the dark in a self-made humidity chamber. Subsequently, cells were washed five times with PBST, air dried and mounted with ProLong™ Diamond Antifade Mountant (Thermo Fischer Scientific) overnight at 4 °C. Immunolocalization of FtsZ was then analyzed by epifluorescence microscopy. Similarly, *in vivo* localization of CypS-His expressed in *Anabaena* was evaluated by immunolocalization of $BG11_0$ grown liquid cultures, induced with 0.25 µM $CuSO_4$ for two days, and compared to *Anabaena* WT cells using mouse anti-His primary antibody (1:500 diluted; Thermo Fischer Scientific).

Spot assays

Spot assays were essentially performed as described by Dörrich *et al.*[93]. *Anabaena* WT and mutant strains were grown on BG11 growth plates, resuspended in BG11 liquid medium and adjusted to an OD_{750} of 0.4. Cells were then spotted in triplicates of 5 µl onto the respective growth plates containing either no additives (BG11 or $BG11_0$), 50 µg ml^{-1} Proteinase K or 100 µg ml^{-1} lysozyme in serial 1/10 dilutions and incubated under standard growth conditions until no further colonies arose in the highest dilution.

Protein purification and *in vitro* filamentation assays

For protein purification, *E. coli* BL21 (DE3) cells carrying His-tagged protein candidates were grown in overnight cultures at 37 °C and 250 rpm. The next day, overnight cultures were diluted 1:40 in the same medium and grown at 37 °C until they reached an OD_{600} of 0.5-0.6. Protein expression was induced with 0.5 mM IPTG for 3-4 h at 37 °C and 250 rpm. Afterwards, cell suspensions of 50 ml aliquots were harvested by centrifugation, washed once in PBS and stored at -80 °C until further use. For *in vitro* filamentation assays, cell pellets were resuspended in urea lysis buffer (ULB: 50 mM NaH_2PO_4, 300 mM NaCl, 25 mM imidazole, 6 M urea; pH 8.0) and lysed in a Precellys® 24 homogenizer (3x 6500 rpm for 30 s) using the 2 ml microorganism lysis kit (VK01; Bertin) or self-packed Precellys tubes with 0.1 mm glass beads. The resulting cell debris was pelleted by centrifugation at 21,000 x *g* (30 min, 4 °C) and the supernatant was incubated with 1 ml HisPur™ Ni-NTA resin (Thermo Fischer Scientific)

for 1 h at 4°C in an overhead rotator. The resin was washed 5 times with 4x resin-bed volumes ULB and eluted in urea elution buffer (UEB: ULB supplemented with 225 mM imidazole). Total protein concentration was measured using the Qubit® 3.0 Fluorometer (Thermo Fischer Scientific) and generally adjusted to 0.5-1.0 mg ml^{-1} before dialysis. Filament formation of purified proteins was induced by overnight dialysis against polymerization buffer (PLB: 50 mM PIPES, 100 mM KCl, pH 7.0; HLB: 25 mM HEPES, 150 mM NaCl, pH 7.4; or 25 mM HEPES pH 7.5) at 20 °C and 180 rpm with three bath changes using a Slide-A-Lyzer™ MINI Dialysis Device (10K MWCO, 0.5 ml or 2 ml; Thermo Fischer Scientific). Purified proteins were stained with an excess of NHS-Fluorescein (dissolved in DMSO; Thermo Fischer Scientific) and *in vitro* filamentation was analyzed by epifluorescence microscopy.

Data availability

All data generated or analyzed during this study are included in this published article (and its supplementary information files). The datasets generated during and/or analyzed during the current study are available from the corresponding authors on reasonable request.

Supplementary Table 2: Cyanobacterial strains used in this study

Strain	Genotype	Resistance Marker	Source
Anabaena sp. PCC 7120	WT		Pasteur culture collection of Cyanobacteria (PCC)
BLS1	*Anabaena* Δ*cypS*::CS.3	Sp, Sm	This study
BLS2	*Anabaena* (Δ*lfiA*Δ*lfiB*)::CS.3	Sp, Sm	This study
BLS3	*Anabaena* Δ*ceaR*::CS.3	Sp, Sm	This study

Sp = spectinomycin, Sm = streptomycin

Supplementary Table 3: *E. coli* strains used in this study

Strain	Genotype	Resistance	Source
DH5αMCR	F- *endA1 supE44 thi-1* λ_- *recA1 gyrA96 relA1 deoR* Δ(*lacZYA-argF*)U169 Φ80d*lac*ZΔM15 *mcrA* Δ(*mrr* hsdRMS *mcrBC*)		Grant *et al.*,1990 (94)
BL21 (DE3)	F_- *ompT gal dcm lon hsdS*$_B$(r_B–m_B–) λ(DE3 [*lacI lacUV5-T7p07 ind1 sam7 nin5*]) [*malB*+]$_{K-12}$(λ_S)		Studier *et al.*, 1986 (95)
BTH101	F-, *cya-99*, *araD139*, *galE15*, *galK16*, *rpsL1* (*Str*$_r$), *hsdR2, mcrA1*,*mcrB1*	Sm	Euromedex
XL1-blue	endA1 gyrA96(nal$_R$) thi-1 recA1 relA1 lac glnV44 F'[::Tn10 proAB+ lacI$_q$ Δ(*lacZ*)M15] *hsdR17*(r_{K-} m_{K+})	Tet	Stratagene
HB101	F- *mcrB mrr hsd*S20(r_{B-} m_{B-}) *rec*A13 *leuB*6 *ara*-14 *pro*A2 *lac*Y1 *gal*K2 *xyl*-5 mtl-1 rpsL20(Sm$_R$) glnV44 λ_-	Sm	Boyer & Roulland-Dessoix, 1969 (96)

Tet = tetracycline

Supplementary Table 4: Plasmids used in this study

Name	Description	Resistance	Source
pET21a(+)	Bacterial vector for expressing N- terminal T7 and/or C-terminal His6-tagged proteins in *E. coli*	Amp	Novagen
pRL25C	Shuttle cosmid vector for cyanobacteria and *E. coli*	Km, Nm	Wolk *et al.*, 1988 (85)
pRL623	Methylation plasmid	Cm	Wolk *et al.*, 1988 (85)
pRL443	Conjugation plasmid	Amp	Wolk *et al.*, 1988 (85)
pRL278	Suicide vector used for homologous recombination in cyanobacteria; contains *sacB* for positive selection of double recombination events	Km, Nm	Wolk *et al.*, 1988 (85)
pKNT25	P_{lac}::-*T25*	Km	Euromedex
pKT25	P_{lac}::*T25*-	Km	Euromedex
pUT18	P_{lac}::-*T18*	Amp	Euromedex
pUT18C	P_{lac}::*T18*-	Amp	Euromedex
pKT25-zip	pKT25; P_{lac}::*T25-zip*	Km, Nm	Euromedex
pUT18C-zip	pUT18C, P_{lac}::*T18-zip*	Amp	Euromedex

pET11a-link-NGFP	IPTG-inducible expression vector for translational fusion of target gene with a N-terminal *gfp* fragment in *E. coli*	Amp	Wilson *et al.,* 2004 (67)
pMRBAD-link-CGFP	L-arabinose-inducible expression vector for translational fusion of target gene with a C-terminal *gfp* fragment in *E. coli*	Km	Wilson *et al.,* 2004 (67)
pAM5084	P_{trc}::*ecfp-kaiC*	Amp	Cohen *et al.,* 2014 (97)
pCSEL24	Integrates into the *nucA-nuiA* region of *Anabaena*	Amp, Sm, Sp	Olmedo-Verd *et al.,* 2006 (98)
pJET1.2-P*petE-gfp*	pJET1.2 vector containing P_{petE}::*gfp*a)	Amp	Stucken *et al.,* 2012 (86)
pTHS1	pRL25C, P_{petE}::*lfiA-gfp*	Km, Nm	This study
pTHS2	pRL25C, P_{petE}::*lfiB-gfp*	Km, Nm	This study
pTHS3	pRL25C, P_{petE}::*ceaR-gfp*	Km, Nm	This study
pTHS4	pRL25C, P_{petE}::*cypS-gfp*	Km, Nm	This study
pTHS5	pRL25C, P_{petE}::*trunc-ceaR-gfp* (truncated *ceaR* without the N-terminal transmembrane domain; first 156 base pairs removed)	Km, Nm	This study
pTHS6	pRL25C, P_{petE}::*cypS-his*	Km, Nm	This study
pTHS7	pRL25C, P_{petE}::*lfiA*-ec*fp*b), P_{petE}::*lfiB-gfp*	Km, Nm	This study
pTHS8	pET21a(+),P_{T7}::*cypS-his*	Amp	This study
pTHS9	pET21a(+), P_{T7}::*lfiA-his*	Amp	This study
pTHS10	pET21a(+), P_{T7}::*lfiB-his*	Amp	This study
pTHS11	pET21a(+), P_{T7}::*ceaR-his*	Amp	This study
pTHS12	pET21a(+); P_{T7}::*cypS-gfp*	Amp	This study
pTHS13	pET21a(+), P_{T7}::*ceaR-gfp*	Amp	This study
pTHS14	pET11a-link-NGFP, P_{T7}::*ngfp-lfiA*	Amp	This study
pTHS15	pET11a-link-NGFP, P_{T7}::*lfiA-ngfp*	Amp	This study
pTHS16	pMRBAD-link-CGFP, P_{ara}::*lfiB-cgfp*	Km	This study
pTHS17	pKNT25, P_{lac}::*cypS-T25*	Km, Nm	This study
pTHS18	pKT25, P_{lac}::*T25-cypS*	Km, Nm	This study
pTHS19	pUT18, P_{lac}::*cypS-T18*	Amp	This study
pTHS20	pUT18C, P_{lac}::*T18-cypS*	Amp	This study
pTHS21	pKNT25, P_{lac}::*lfiA-T25*	Km, Nm	This study
pTHS22	pKT25, P_{lac}::*T25-lfiA*	Km, Nm	This study
pTHS23	pUT18, P_{lac}::*lfiA-T18*	Amp	This study
pTHS24	pUT18C, P_{lac}::*T18-lfiA*	Amp	This study
pTHS25	pKNT25, P_{lac}::*lfiB-T25*	Km, Nm	This study
pTHS26	pKT25, P_{lac}::*T25-lfiB*	Km, Nm	This study
pTHS27	pUT18, P_{lac}::*lfiB-T18*	Amp	This study
pTHS28	pUT18C, P_{lac}::*T18-lfiB*	Amp	This study
pTHS29	pKNT25, P_{lac}::*ceaR-T25*	Km, Nm	This study
pTHS30	pKT25, P_{lac}::*T25-ceaR*	Km, Nm	This study
pTHS31	pUT18, P_{lac}::*ceaR-T18*	Amp	This study
pTHS32	pUT18C, P_{lac}::*T18-ceaR*	Amp	This study

pTHS33	pKNT25, P_{lac}::*sepJ-T25*	Km, Nm	This study
pTHS34	pKT25, P_{lac}::*T25-sepJ*	Km, Nm	This study
pTHS35	pUT18, P_{lac}::*sepJ-T18*	Amp	This study
pTHS36	pUT18C, P_{lac}::*T18-sepJ*	Amp	This study
pTHS37	pKNT25, P_{lac}::*ftsZ-T25*	Km, Nm	This study
pTHS38	pKT25, P_{lac}::*T25-ftsZ*	Km, Nm	This study
pTHS39	pUT18, P_{lac}::*ftsZ-T18*	Amp	This study
pTHS40	pUT18C, P_{lac}::*T18-ftsZ*	Amp	This study
pTHS41	pKNT25, P_{lac}::*mreB-T25*	Km, Nm	This study
pTHS42	pKT25, P_{lac}::*T25-mreB*	Km, Nm	This study
pTHS43	pUT18, P_{lac}::*mreB-T18*	Amp	This study
pTHS44	pUT18C, P_{lac}::*T18-mreB*	Amp	This study
pTHS45	pKNT25, P_{lac}::*fraC-T25*	Km, Nm	This study
pTHS46	pKT25, P_{lac}::*T25-fraC*	Km, Nm	This study
pTHS47	pUT18, P_{lac}::*fraC-T18*	Amp	This study
pTHS48	pUT18C, P_{lac}::*T18-fraC*	Amp	This study
pTHS49	pKNT25, P_{lac}::*fraD-T25*	Km, Nm	This study
pTHS50	pKT25, P_{lac}::*T25-fraD*	Km, Nm	This study
pTHS51	pUT18, P_{lac}::*fraD-T18*	Amp	This study
pTHS52	pUT18C, P_{lac}::*T18-fraD*	Amp	This study
pTHS53	pRL278 containing 1500 bp upstream and downstream of *cypS* flanking the CS.3 cassette	Nm, Km, Sm, Sp	This study
pTHS54	pRL278 containing 1500 bp upstream and downstream of *ceaR* flanking the CS.3 cassette	Nm, Km, Sm, Sp	This study
pTHS55	pRL278, containing 1500 bp upstream of *lfiA* and 1500 bp downstream of *lfiB* flanking the CS.3 cassette	Nm, Km, Sm, Sp	This study
pTHS56	pRL25C, P_{cypS}::*cypS-gfp*	Nm, Km,	This study
pTHS57	pRL25C, P_{ceaR}::*ceaR-gfp*	Nm, Km,	This study
pTHS58	pRL25C, P_{lfiA}::*lfiA-gfp*	Nm, Km,	This study
pTHS59	pRL25C, P_{lfiB}::*lfiB-gfp*	Nm, Km,	This study
pTHS60	pRL25C, P_{cypS}::*cypS-his*	Nm, Km,	This study
pTHS61	pRL25C, P_{ceaR}::*ceaR*	Nm, Km,	This study
pTHS62	pRL25C, $P_{lfiA/B}$::*lfiA-lfiB*	Nm, Km,	This study

Km = kanamycin, Nm = neomycin, Amp = ampicillin; Cm = chloramphenicol

- a) Modified gfpmut3.1[86] in which the internal NdeI site was removed by replacing CAT by the synonymous CAC codon. The GFP is N-terminally preceded by 12 alanine and serine residues. Abbreviated: *gfp*.
- b) eCFP from Cohen *et al.* (2014)[97] was adjusted for C-terminal translational fusion instead of N-terminal fusion. For this, a N-terminal Myc sequences followed by a seven amino acid linker (GSGSGSG) and an additional stop codon at the C-terminus were added.

Supplementary Table 5: Oligonucleotides used in this study

#	Given name	Sequence (5' - > 3')
1	rnpB_intern_A	TGCTGGATAACGTCCAGTGC
2	rnpB_intern_B	GGTTTACCGAGCCAGTACCTC
3	Nos295_intern_A	CAAAGTCAGGCGATGAGTGA
4	Nos295_intern_B	GGAACCGCATTACCAGAAGT
5	Nos842_intern_A	TCGGGCAGAAATTACCCAGT
6	Nos842_intern_B	TGCCATTCTTCAGGCAAAGC
7	Nos903_intern_A	TCAGCTAGACGTAAAGAGTGGC
8	Nos903_intern_B	TAATTCTGCTGGGAATGCAGC
9	Nos904_intern_A	TGGAATTAGCGAAGGGGTGG
10	Nos904_intern_B	TGTTCATAGCCATCTGTTGCCA
11	petE_903_Fwd	GAGATTATCAAAAAGGATCCCAGTACTCAGAATTTTTTGCTGAGGTACT
12	petE_903_Rev	TTGAGTGCAACTGTCGTCATGGCGTTCTCCTAACCTGTAGTTTTATTTTT
13	pRL25-Nos903_Fwd	CTACAGGTTAGGAGAACGCCATGACGACAGTTGCACTCAAAGATAG
14	pRL25-Nos903_Rev	GCACTAGCAGATGCACTAGCTTTAGCCGTAGAACTATCAAAAGCTCTCATTGC
15	GFP_903_Fwd	TTGATAGTTCTACGGCTAAAGCTAGTGCATCTGCTAGTGCTAGT
16	GFP_903_Rev	CTTTCGTCTTCAAGAATTCTTTATTTGTATAGTTCATCCATGCCATGTGTAATCC
17	pRL25c-903_V_F	TGGATGAACTATACAAATAAAGAATTCTTGAAGACGAAAGGGCC
18	pRL25c-903_V_R	GCAAAAAATTCTGAGTACTGGGATCCTTTTTGATAATCTCATGACCAAAATCC
19	Nos904-2A	CTACAGGTTAGGAGAACGCCATGGCAGTCAAAAAGTTAACAGACAAAAAC
20	Nos904_2B	GCACTAGCAGATGCACTAGCTTTATTTTTCACTTGACTTTTTGCCTGTTCTAAAGC
21	Nos842_2A	TACAGGTTAGGAGAACGCCATGCAACAAGTCATAGTAAGTAATCGATT
22	Nos842_2B	CACTAGCAGATGCACTAGCGGATGCGTATCTAGCTATTAGATGTTC
23	pRL25c_NEB_Fwd	GCTAGTGCATCTGCTAGTGCTAGTG
24	pRL25c_NEB_Rev	GGCGTTCTCCTAACCTGTAGTTTTATTTTTCT
25	Nos295His_2A	TACAGGTTAGGAGAACGCCATGCTGTATTTAGCAGAAGTACAAAAG
26	Nos295His_2B	CCTTTCGTCTTCAAGAATTCTTCAGTGGTGGTGGTGGTG
27	MBP7_1A	AGAATTCTTGAAGACGAAAGGGCC
28	petE_2A	ACTACCGCATTAAAGCTTATCAGTACTCAGAATTTTTTGCTGAGGTAC
29	Nos903_2B	TTCGCTGATAAGCTTCTGTTCTTTAGCCGTAGAACTATCAAAAGCTCTC
30	Linker_eCFP_3A	GGCTCTGGATCGGGTTCAGGAATGGTGAGCAAGGGCGAG
31	eCFP_3B	CTGCTGCTTACTTGTACAGCTCGTCCATGCC

32	MYC_Linker_3A2	GAACAGAAGCTTATCAGCGAAGAAGATCTGGGCTCTGGATCGGGTTCAG
33	eCFP_3B2	TCATGTTTGACAGCTTATCATTTACTTGTACAGCTCGTCCATGCC
34	petE_BamHI_2A	TTGGTCATGAGATTATCAAAAAGCAGTACTCAGAATTTTTTGCTGAGG
35	GFP_BamHI_2B	ATTGATTTAAAACTTCATTTTTAATTTAAAAGTTATTTGTATAGTTCATCCATGCCATGTG
36	Nos295_NdeI_Fwd	GCTACATATGCTGTATTTAGCAGAAGTACAAA
37	Nos295_XhoI_wo_R	GCTACTCGAGAGATGCCAACAACTCAGG
38	Nos295_SacI_wo_R	GCTAGAGCTCAGATGCCAACAACTCAGG
39	Nos903_NdeI_F	GCTACATATGACGACAGTTGCACTCA
40	Nos903_XhoI_R_w/o	GCTACTCGAGTTTAGCCGTAGAACTATCAAAAGC
41	Nos904_NdeI_F	GCTACATATGGCAGTCAAAAAGTTAACAGAC
42	Nos904_XhoI_wo_R	GCTACTCGAGTTTATTTTTCACTTGACTTTTTGCCT
43	Nos842_NdeI_F	GCTACATATGCAACAAGTCATAGTAAGTAATCG
44	Nos842_XhoI_wo_R	GCTACTCGAGGGATGCGTATCTAGCTATTAGATG
45	Nos903_pET_2A	GTTTAACTTTAAGAAGGAGATATACATATGACGACAGTTGCACTCAAAG
46	Nos904_pET_2A	GTTTAACTTTAAGAAGGAGATATACATATGGCAGTCAAAAAGTTAACAGAC
47	Nos842_pET_2A	GTTTAACTTTAAGAAGGAGATATACATATGCAACAAGTCATAGTAAGTAATCG
48	GFP_pET21a_2B	AGTGGTGGTGGTGGTGGTGTTTGTATAGTTCATCCATGCCATGTGTAATC
49	pET21a_1A	CACCACCACCACCACCAC
50	pET21a_1B	ATGTATATCTCCTTCTTAAAGTTAAACAAAATTATTTCTAGAGG
51	903_split_A	AAGGTGGCTCTGGCTCTGGCTCGAGCATGACGACAGTTGCACTCAAAG
52	903_split_B	CGGGCTTTGTTAGCAGCCGTTATTTAGCCGTAGAACTATCAAAAGCTCTC
53	903_split_A2	TTAACTTTAAGAAGGAGATATACATATGACGACAGTTGCACTCAAAG
54	903_split_B2	CCATGGTGATGGTGGTGATGAGATGCACTAGCTTTAGCCGTAGAACTATCAAAAGCTCT
55	904_split_A	TTTAACTTTAAGAAGGAGATATACCATGGCAGTCAAAAAGTTAACAGACA
56	904_split_B	TTACCGCTTCCACCCGACGTTTTATTTTTCACTTGACTTTTTGCCTGTTC
57	N-term_1A	GAGGATCCCCGGGTACC
58	N-term_1B	TAGAGTCGACCTGCAGGCA
59	pKT25_1A	CCCCGGGTACCTAAGTAAGTAAG
60	pKT25_1B	ATCCTCTAGAGTCGACCCTGC
61	pUT18C_1A	CCGAGCTCGAATTCATCGAT
62	pUT18C_1B	TACCCGGGGATCCTCTAGAGT
63	MB_5A	TGCCTGCAGGTCGACTCTAATGCTGTATTTAGCAGAAGTACAAAAG
64	MB_5B	TCGGTACCCGGGGATCCTCAGATGCCAACAACTCAGGC
65	MB_6A	AGGGTCGACTCTAGAGGATATGCTGTATTTAGCAGAAGTACAAAAGC
66	MB_6B	CTTACTTAGGTACCCGGGGAGATGCCAACAACTCAGGC
67	MB_8A	TCTAGAGGATCCCCGGGTAATGCTGTATTTAGCAGAAGTACAAAAG

68	MB_8B	TCGATGAATTCGAGCTCGGAGATGCCAACAACTCAGGC
69	MB_17A	TGCCTGCAGGTCGACTCTAATGACGACAGTTGCACTCAAAG
70	MB_17B	TCGGTACCCGGGGATCCTCTTTAGCCGTAGAACTATCAAAAGCTCTC
71	MB_18A	AGGGTCGACTCTAGAGGATATGACGACAGTTGCACTCAAAG
72	MB_18B	CTTACTTAGGTACCCGGGGTTTAGCCGTAGAACTATCAAAAGCTCTC
73	MB_20A	TCTAGAGGATCCCCGGGTAATGACGACAGTTGCACTCAAAG
74	MB_20B	TCGATGAATTCGAGCTCGGTTTAGCCGTAGAACTATCAAAAGCTCTC
75	MB_21A	TGCCTGCAGGTCGACTCTAATGGCAGTCAAAAAGTTAACAGACAA
76	MB_21B	TCGGTACCCGGGGATCCTCTTTATTTTTCACTTGACTTTTTGCCTGTTC
77	MB_22A	AGGGTCGACTCTAGAGGATATGGCAGTCAAAAAGTTAACAGACAA
78	MB_22B	CTTACTTAGGTACCCGGGGTTTATTTTTCACTTGACTTTTTGCCTGTTC
79	MB_24A	TCTAGAGGATCCCCGGGTAATGGCAGTCAAAAAGTTAACAGACAA
80	MB_24B	TCGATGAATTCGAGCTCGGTTTATTTTTCACTTGACTTTTTGCCTGTTC
81	MB_25A	TGCCTGCAGGTCGACTCTAATGCAACAAGTCATAGTAAGTAATCGAT
82	MB_25B	TCGGTACCCGGGGATCCTCGGATGCGTATCTAGCTATTAGATGTTC
83	MB_26A	AGGGTCGACTCTAGAGGATATGCAACAAGTCATAGTAAGTAATCGAT
84	MB_26B	CTTACTTAGGTACCCGGGGGGATGCGTATCTAGCTATTAGATGTTC
85	MB_28A	TCTAGAGGATCCCCGGGTAATGCAACAAGTCATAGTAAGTAATCGAT
86	MB_28B	TCGATGAATTCGAGCTCGGGGATGCGTATCTAGCTATTAGATGTTC
87	MB_41A	TGCCTGCAGGTCGACTCTAATGGGGCGATTTGAGAAGC
88	MB_41B	TCGGTACCCGGGGATCCTCACCTTCTGCATTGGCAGG
89	MB_42A	AGGGTCGACTCTAGAGGATATGGGGCGATTTGAGAAGC
90	MB_42B	CTTACTTAGGTACCCGGGGACCTTCTGCATTGGCAGG
91	MB_44A	TCTAGAGGATCCCCGGGTAATGGGGCGATTTGAGAAGC
92	MB_44B	TCGATGAATTCGAGCTCGGACCTTCTGCATTGGCAGG
93	MB_49A	ATGCCTGCAGGTCGACTCTAATGACACTTGATAATAACCAAGAGCTTACC
94	MB_49B	CTCGGTACCCGGGGATCCTCATTTTTGGGTGGTCGCCGTC
95	MB_50A	CAGGGTCGACTCTAGAGGATATGACACTTGATAATAACCAAGAGCTTACC
96	MB_50B	TACTTACTTAGGTACCCGGGGATTTTTGGGTGGTCGCCGTC
97	MB_52A	CTCTAGAGGATCCCCGGGTAATGACACTTGATAATAACCAAGAGCTTACC
98	MB_52B	TATATCGATGAATTCGAGCTCGGATTTTTGGGTGGTCGCCGTC
99	MB_53A	ATGCCTGCAGGTCGACTCTAATGGGGCTTTTTAGGAACTTTCG
100	MB_53B	CTCGGTACCCGGGGATCCTCCATATTTCGAGATCGTCCGCTAAAAAC
101	MB_54A	CAGGGTCGACTCTAGAGGATATGGGGCTTTTTAGGAACTTTCG
102	MB_54B	TACTTACTTAGGTACCCGGGGCATATTTCGAGATCGTCCGCTAAAAAC
103	MB_56A	CTCTAGAGGATCCCCGGGTAATGGGGCTTTTTAGGAACTTTCG
104	MB_56B	TATATCGATGAATTCGAGCTCGGCATATTTCGAGATCGTCCGCTAAAAAC
105	MB_69A	ATGCCTGCAGGTCGACTCTAATGTTTGAAGATTTGACTATACCCAGG
106	MB_69B	CTCGGTACCCGGGGATCCTCCCTATTACGTATCAATAAAATAATAGTTATAGCGGTG
107	MB_70A	CAGGGTCGACTCTAGAGGATATGTTTGAAGATTTGACTATACCCAGG

108	MB_70B	TACTTACTTAGGTACCCGGGGCCTATTACGTATCAATAAAATAATAGTTATAGCGGTG
109	MB_72A	CTCTAGAGGATCCCCGGGTAATGTTTGAAGATTTGACTATACCCAGG
110	MB_72B	ATATCGATGAATTCGAGCTCGGCCTATTACGTATCAATAAAATAATAGTTATAGCGGTG
111	MB_73A	ATGCCTGCAGGTCGACTCTAGTGAATTTATTATTTAAAGACCTTTTCGGAATATT
112	MB_73B	CTCGGTACCCGGGGATCCTCCTGCTGCGGTGGCGCTG
113	MB_74A	GGGTCGACTCTAGAGGATGTGAATTTATTATTTAAAGACCTTTTCGGAAT
114	MB_74B	TACTTACTTAGGTACCCGGGGCTGCTGCGGTGGCGCTG
115	MB_76A	CTAGAGGATCCCCGGGTAGTGAATTTATTATTTAAAGACCTTTTCGGAAT
116	MB_76B	TATATCGATGAATTCGAGCTCGGCTGCTGCGGTGGCGCTG
117	pRL271_Fwd	GAGCTCGCGAAAGCTTGCATG
118	pRL271_Rev	CTCGAGATCTAGATATCGAATTTCTGCCAT
119	CS.3_Fwd	GATCCGTGCACAGCACCTTG
120	CS.3_Rev	TTATTTGCCGACTACCTTGGTGATCT
121	295KO_2A	ATTCGATATCTAGATCTCGAGACTCAACATAATCATCGGTATATACCGAAAT
122	295KO_2B	CAAGGTGCTGTGCACGGATCACCGTTCTTCCTCTTGTGTACTTGA
123	295KO_4A	CCAAGGTAGTCGGCAAATAACAATTCAAAATTCAAAATTCAAAATATTTAGGACTTACG
124	295KO_4B	ATGCAAGCTTTCGCGAGCTCTGTAAATTTCTCACTAAGTGATGGATCAACACT
125	842KO_2A	ATTCGATATCTAGATCTCGAGATGGATAATCCAGCAATGTCGGC
126	842KO_2B	AAGGTGCTGTGCACGGATCATTGCTGATTTTTAGCGTAGTTAAGCTTT
127	842KO_4A	CAAGGTAGTCGGCAAATAAAATTTAATATCCCTAGCTCATCGTAAAATTTTTATAAAAATATG
128	842KO_4B	ATGCAAGCTTTCGCGAGCTCTTTAAAACTAGAACTATGAACTAGCTCGCTAAAC
129	903KO_2A	ATTCGATATCTAGATCTCGAGAAGCAACGGCAACGCC
130	903KO_2B	AAGGTGCTGTGCACGGATCATTTCAACTCCCTTGATTAGATAATGATTAATCGAG
131	904KO_4A	CAAGGTAGTCGGCAAATAAAATACAAATAATAAAAATAAATAAAAAGACGTAACGAAAATTACG
132	904KO_4B	TGCAAGCTTTCGCGAGCTCGTAGTGGGTTTCGCACAAGCTATC
133	903KO_Seq_A	TGCGAATTCCAGTAGGTCTTGGTAA
134	904KO_Seq_B	GGTGGCGCAGAAGTATTTTTG
135	842KO_Seq_A	TCAACAGTCAACAGTCAATAGTGAAGG
136	842KO_Seq_B	TTCATCTACACCGATATCTTGACCC
137	295KO_Seq_A	GCCATCCTAGCTCTGATTTGATC
138	295KO_Seq_B	CAGGGTTATCGGTAAGGAATCG
139	Fragment1.FOR	GATTTCGAACCCGGGGTACCACCTGTAGAGAAGAGTCCCTGAATATCAA
140	Fragment1.REV	TTTTTCGTATTTTCCCTCATTGAATTAATCTCCTACTTGACTTTATGAGTTGGGA
141	Fragment4.FOR	TGGATGAACTATACAAATAAACCGGTGTTTGGATTGTCGG
142	Fragment4.REV	CCCTGCAGGTCGAGGAATTCGCTGTCGAAGTTGAACATCAGTAAGC
143	Nos903_pIGA_2A	TAAAGTCAAGTAGGAGATTAATTCAATGACGACAGTTGCACTCAAAG
144	Fragment3.FOR	ATTTAATGACTGCCTTAGTCGCTAGTGCATCTGCTAGTGCTAGT

145	Fragment3.REV	CCGACAATCCAAACACCGGTTTATTTGTATAGTTCATCCATGCCATGTGTAATCC
146	Vector.FOR	TGATGTTCAACTTCGACAGCGAATTCCTCGACCTGCAGGG
147	Vector.REV	AGGGACTCTTCTCTACAGGTGGTACCCCGGGTTCGAAATCG
148	842_petE_F	CTACAGGTTAGGAGAACGCCATGGATAAGCGACGGAGGAAT
149	petE_842_R	CTCCGTCGCTTATCCATGGCGTTCTCCTAACCTGTAGTTTTATTTTTCT
150	p295_25C_long_A	TTTTGGTCATGAGATTATCAAAAAGATTGACGCAGCATGGC
151	pNos295_Rev	ACCGTTCTTCCTCTTGTGTACT
152	Nos295_pNos295_A	CACAAGAGGAAGAACGGTGTGCTGTATTTAGCAGAAGTACAAAAG
153	GFP_25C_R	AGGCCCTTTCGTCTTCAAGTTATTTGTATAGTTCATCCATGCCATGTGT
154	p842_25C_long_A	TTTTGGTCATGAGATTATCAAAAAGTCTCTCTATCCCCAAGTACAATTTCTCC
155	pNos842_2B	CTTGTTGCATATTGCTGATTTTTAGCG
156	Nos842_pNos842_3A	AATCAGCAATATGCAACAAGTCATAGTAAGTAATCGATTTATTTTAG
157	p903_25C_long_A	TTTTGGTCATGAGATTATCAAAAAGACCCGACACTCTTGAGG
158	pNos903_2B	ACTGTCGTCATATTTCAACTCCCTTG
159	Nos903_pNos903_3A	GGAGTTGAAATATGACGACAGTTGCACTCAAAG
160	pNos904_25C_F	ATTTTGGTCATGAGATTATCAAAAAGAGAAATATCAGCTAGACGTAAAGAGTGG
161	pNos904_2B	TGACTGCCATAAAAACCTCTATTTATTGC
162	Nos904_pNos904_3A	AGAGGTTTTTATGGCAGTCAAAAAGTTAACAGACAAAAAC
163	295_His_25C_R	GGCCCTTTCGTCTTCAAGTTAGTGGTGATGGTGATGATGAGATGC
164	Nos842_25C_B	GAGGCCCTTTCGTCTTCAAGTCAGGATGCGTATCTAGCTATTAGATG
165	Nos904_25C_Ba	GGCCCTTTCGTCTTCAAGTTATTTATTTTTCACTTGACTTTTTGCCTGT

Employed enzymatic cut sites are underlined.

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