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**TITLE**

# Intercellular transfer along the trichomes of the invasive terminal heterocyst forming cyanobacterium *Cylindrospermopsis raciborskii* CS-505.

**RUNNING TITLE**

Intercellular transfer along *C. raciborskii* CS-505

**CONTENTS CATEGORY**

Cell and Molecular Biology of Microbes

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## Abstract

*Cylindrospermopsis raciborskii* CS-505 is an invasive freshwater filamentous cyanobacterium that when grown diazotrophically may develop trichomes of up to 100 vegetative cells while differentiating only two end heterocysts, the sole sites for their $N_2$ fixation process. We examined the diazotrophic growth and intercellular transfer mechanisms in *C. raciborskii* CS-505. Subjecting cultures to a combined-nitrogen-free medium to elicit $N_2$-fixation, the trichome length remained unaffected while growth rates decreased. The structures and proteins for intercellular communication showed that while a continuous periplasmic space was apparent along the trichomes, the putative septal junction *sepJ* gene is divided into two open reading frames and lacks several transmembrane domains unlike the situation in *Anabaena*, differentiating a five-fold higher frequency of heterocysts. FRAP analyses also showed that the dyes calcein and 5-CFDA were taken up by heterocysts and vegetative cells, and that the transfer from heterocysts and 'terminal' vegetative cells showed considerably higher transfer rates than that from vegetative cells located in the middle of the trichomes. The data suggest that *C. raciborskii* CS-505 compensates its low-frequency heterocyst phenotype by a highly efficient transfer of the fixed nitrogen towards cells in distal parts of the trichomes (growing rapidly) while cells in central parts suffers (slow growth).

## Introduction

Cyanobacteria are organisms characterized by their oxygenic photosynthesis. Additionally, some representatives are also able to fix $N_2$ (diazotrophy) in the absence of combined nitrogen sources (Bergman *et al*., 1997; Flores & Herrero, 2010). Since the $N_2$-fixing enzyme nitrogenase is irreversibly inactivated by atmospheric concentrations of oxygen (Smith & Evans, 1971), this process is incompatible with oxygenic photosynthesis, and requires intracellular micro-oxic conditions. To perform these two physiological processes within the same organism, some multicellular cyanobacteria differentiate specialized cells along their trichomes termed heterocysts. Heterocysts develop highly specific metabolic and structural features in order to create micro-oxic conditions for the optimal operation of nitrogenase. These include a lack of photosystem II and carbon fixation activities, and deposition of extra polysaccharide and glycolipid wall layers (Flores & Herrero, 2010). Heterocysts in turn rely on carbon supplied from their neighboring vegetative cells to function (Wolk, 1968). Most cyanobacteria of the Nostocales clade (Sub-section IV) differentiate heterocysts in a regular pattern among the dominating vegetative cells (intercalary heterocysts) (Flores & Herrero, 2010). Differentiation of new heterocysts occurs in positions where combined nitrogen levels become limited, *i.e*. in vegetative cells at the midpoint between two heterocysts (Popa *et al*., 2007). This pattern forming mechanism includes, among other elements, the release of negative regulators from vegetative cells such as the heterocyst-differentiation inhibitor PatS (Yoon & Golden, 1998; Risser & Callahan, 2009; Higa *et al*., 2012; Corrales-Guerrero *et al*., 2013).

Intercellular transport along the trichome has primarily been examined in *Anabaena* sp. strain PCC 7120 (hereafter *Anabaena* PCC7120), in which three different ways of transfer have been described. Two involve direct cell-to-cell transport facilitated via septal junctions (Mariscal, 2014), of which the proteins SepJ, FraC and FraD are putative components (Flores *et al*., 2007; Merino-Puerto *et al*., 2010). These form two types of structures (SepJ and FraC/FraD) for the transfer of the artificial tracer dyes calcein and 5-carboxyfluorescein diacetate (hereafter mentioned as 5-CFDA), respectively (Mullineaux *et al*., 2008; Mariscal *et al*., 2011; Merino-Puerto *et al*., 2011). The tracers have different masses (622 and 374 Da, for calcein and 5-CFDA, respectively) and can be loaded into the cytoplasm of the cells in order to follow their transfer in, *e.g.* filamentous cyanobacteria. In this technique (termed FRAP), fluorescence recovery after photobleaching is assayed. SepJ

and FraC/FraD may be also involved in the stabilization of trichome integrity as deletion of *sepJ*, *fraC* or *fraD* significantly decreases trichome length (Flores *et al*., 2007; Merino-Puerto *et al*., 2010; Mariscal *et al*., 2011). Transfer of substrates along *Anabaena* PCC7120, might also take place within the continuous periplasm that covers its trichomes in conjunction with amino-acid transporters (Flores *et al*., 2006; Mariscal *et al*., 2007; Pernil *et al*., 2008).

*Cylindrospermopsis raciborskii* CS-505 (hereafter *C. raciborskii*) is an ecologically successful filamentous cyanobacterium, as reflected in its today often invasive behavior in fresh-water aquatic environments (Wiedner *et al*., 2007). In contrast to the majority of other heterocyst forming cyanobacteria (Section IV and V), including the model cyanobacterium *Anabaena* PCC7120, *C. raciborskii* has a minimal frequency of heterocysts and these are exclusively differentiated at the ends of the trichomes (terminal heterocysts) (Reddy & Talpasayi, 1974; Stucken *et al*., 2010). As shown recently, these terminal heterocysts are the exclusive sites for $N_2$ fixation in *C. raciborskii* (Plominsky *et al*., 2013). Still, this cyanobacterium is able to develop long trichomes (up to 100 cells) when grown diazotrophically (Plominsky *et al*., 2013). Thus, *C. raciborskii* is challenged to transfer the $N_2$ fixed in their two terminal heterocysts to the up to 50 associated vegetative cells. In contrast, *Anabaena* PCC7120 develops a higher heterocyst frequency, including regularly spaced intercalary heterocysts, supporting merely 7-15 vegetative cells each with fixed nitrogen (Neunuebel & Golden, 2008; Corrales-Guerrero *et al*., 2013).

To get a deeper insight into the physiology and cellular mechanisms operative in the low-frequency-heterocyst phenotype of the unexpectedly ecologically successful cyanobacterium *C. raciborskii*, we analyzed and compared heterocyst differentiation time spans, diazotrophic growth rates and intercellular transfer capacities to that of the heterocystous cyanobacterium *Anabaena* PCC7120 differentiating intercalary heterocysts.

## Materials and Methods

### Cyanobacterial strains, media and growth conditions

Non-axenic culture of *C. raciborskii* was obtained from the CSIRO Collection of Living Microalgae. *Anabaena* PCC7120 was obtained from the Pasteur Culture Collection. *Anabaena* PCC7120 *patA* deletion mutant UHM101 (hereafter UHM101) was kindly donated by Dr. Sean Callahan, University of Hawaii (Orozco *et al*., 2006). Steady state liquid cultures of *C. raciborskii* were grown in MLA medium (Castro *et al*., 2004), at 25 °C under continuous light (50 μE $m^{-2}$ $s^{-1}$). Liquid cultures of *Anabaena* PCC7120 and UHM101 were grown in BG11 medium under continuous light as described in Merino-Puerto *et al*. (2011). For nitrogen depletion experiments, all cultures were grown to an $OD_{750}$ of 0.25-0.3 in MLA or BG11 with 2mM $NH_4Cl$ as their sole combined nitrogen source and 1mM of HEPES buffer (pH 7.5) ($MLA_N$ and $BG11_N$). Then, experimental cultures were washed with 2 volumes of their corresponding medium without any combined nitrogen source ($MLA_0$ and $BG11_0$) by vacuum filtration through a nitrocellulose filter (8μm pore size, Millipore), and resuspended in their corresponding nitrogen free medium.

**Growth analysis, protein extraction and nitrogenase activity measurements.**

Liquid cultures of *C. raciborskii* and *Anabaena* PCC7120 were grown 14 days with or without combined nitrogen sources under their corresponding optimum growth conditions (see above). Then, they were subcultured to an $OD_{750}$ of 0.02 (~ 0.1μg of protein $mL^{-1}$). Samples were taken and washed carefully through a 0.8 μm nitrocellulose filter to avoid contaminants every 24 h during their exponential growth phase, which under these conditions correspond to $OD_{750}$ 0.02-0.4 (~ 0.1–15 mg of protein $mL^{-1}$). To calculate their growth rate constants ($\boldsymbol{\mu} = \ln 2 / t_d$; where $t_d$ is the doubling time for each culture), protein concentrations were determined for each sample through a modified Lowry procedure (Markwell *et al*., 1978). Nitrogenase activity was determined by acethylene reduction assay, and normalized to protein content as described previously (Plominsky *et al*., 2013). For trichome length determination, *C. raciborskii* cultures were diazotrophically induced (see above) and their number of cells was counted in triplicate at 0, 4, and 8 days after the change of medium for 100 trichomes each time-point. The number of heterocysts per vegetative cells in these induced cultures was recorded 4, 8 and 12 days after the change of

medium.

## Transmission electron microscopy

*C. raciborskii* and *Anabaena* PCC7120 cell cultures were concentrated by centrifugation, fixed for 4 h in 3 % glutaraldehyde in a 0.134 M sodium cacodylate buffer (pH 7.2) at room temperature, washed overnight with sodium cacodylate buffer at 4 ºC, and post-stained 1 h with $OsO_4$ 1 % (w/v). The cells were then washed 3 × 10 min with distilled water, stained 1 h with uranyl acetate 1 % (w/v) and washed again. Samples were dehydrated in an acetone series (30-100 % [v/v]), embedded in LR White, sectioned and visualized by electron microscopy as described previously (Plominsky *et al*., 2013). To better expose the phospho-lipoproteins over the peptidoglycan, cells were in addition stained with $KMnO_4$.

## Calcein and 5-CFDA labeling, and FRAP assays

The cultures were grown at 30 °C, harvested at an $OD_{750}$ of 0.25-0.3 (~7 mg of protein $mL^{-1}$) and diazotrophically induced (see above). All other procedures for calcein and 5-CFDA labeling, FRAP measurements, and the quantification of calcein diffusion (***E***) or 5-CFDA fluorescence recovery (***R***) rates were performed as described previously (Merino-Puerto *et al*., 2011). However, *C. raciborskii* was incubated 1.5 h in 40 μg $mL^{-1}$ of calcein-AM. For valid comparison of 'terminal' vegetative cells and heterocysts (with one cell junction) with cells in the middle of filaments (with two cell junctions), R-values were divided by 2 in the latter case. Statistical analyses were performed with Statistica v7.0 (Statsoft).

## Genetic procedures and bioinformatic analysis

*Anabaena* PCC7120 NatABCDEFGH, BgtAB, FraCD and SepJ homologs were used as queries to retrieved *C. raciborskii* and other cyanobacterial homologs (Table S1 and S2) from the NCBI protein database (v08-28-2012). The primers Cr-sepJ_1F (AGCTTAGTAGTAACATAGTAGC) and CR-sepJ_1R (TAGAGGTGAGAAACTAATGG)

or CR-sepJ_2F (GAACAGCCAACATAAAAGC) and CR-sepJ_2R (CTCTAATCTTCATTATTATCC) were designed and synthesized according to the known genomic sequence of the *C. raciborskii sepJ* homolog and its flanking genes (Fig. S1). Genomic DNA of *C. raciborskii* was extracted (Wilson, 1990), and the region covering *Cr-sepJ* was amplified by PCR using Platinum-Pfx proof reading polymerase (Invitrogen) and the primers described above. Both PCR products were sequenced after amplification. All sequences were aligned using Clustal Omega (Sievers *et. al*., 2011) and edited with Jalview (Waterhouse *et. al*. 2009).

## Results and Discussion

### Diazotrophic growth and heterocyst differentiation in *C. raciborskii*.

The time period required for *C. raciborskii* to differentiate mature nitrogen-fixing terminal heterocysts was first determined, by following the onset of nitrogenase activity after combined nitrogen removal. In parallel, this event was compared to that required by the well-examined cyanobacterium *Anabaena* PCC7120. Since *C. raciborskii* suffers extensive chlorosis during the induction of diazotrophic growth (N-depletion; data not shown), nitrogenase activity rates were normalized to total protein. About 72 h of diazotrophic induction was required before nitrogenase activity was detected in *C. raciborskii*, a sign of the presence of mature heterocysts. Consistent rates were registered after 96 h (6.01 [SD ± 2.12] nmol $C_2H_4$ mg of protein$^{-1}$ h$^{-1}$) (Fig. 1a). In contrast, cultures of *Anabaena* PCC7120, subjected to the same diazotrophic induction procedure, nitrogenase activity was apparent already after 24 h, and consistent rates were observed after 36 h (9.68 [SD ± 1.39] nmol $C_2H_4$ mg of protein$^{-1}$ h$^{-1}$) (Fig. 1b).

Notably, although the lengthening of the *C. raciborskii* trichomes seemed arrested during the first 4 days after the diazotrophic induction, 8 days later they exhibited similar lengths as the non-diazotrophic cultures (Fig. 1c). At this stage the majority of trichomes were composed of at the most 50 cells, although the frequency of longer trichomes steadily increased (Fig. 1c). Thus, once their heterocysts have matured, these *C. raciborskii* trichomes can reach the same lengths as those grown with a combined nitrogen source. Notably, 12 days after the diazotrophic induction, *C. raciborskii* trichomes were on average equipped with one terminal heterocysts per every 27.5 (SD ± 1.75) vegetative cells (Fig. 1d), *i.e*. almost double the 7-15 vegetative cells per heterocyst seen in *Anabaena* PCC7120 (Neunuebel & Golden, 2008; Corrales-Guerrero *et al*., 2013). To compensate for the long trichomes differentiating only terminal heterocysts with capacity to deliver fixed nitrogen, these few heterocysts would have to efficiently supply a greater number of vegetative cells compared to cyanobacteria with intercalary heterocysts such as *Anabaena* PCC7120. This in turn would require highly efficient transport mechanisms from the terminal heterocysts to all vegetative cells of the trichome. To determine whether this low frequency of heterocysts fully supports diazotrophic growth in *C. raciborskii*, the growth rates (**μ**) under non-diazotrophic (grown on 2 mM $NH_4^+$ ) and under diazotrophic conditions were determined and compared to that of *Anabaena* PCC7120 subjected to the same growth conditions. Although, the two cyanobacteria exhibited similar non-diazotrophic growth (**μ**,Table 1) and nitrogen fixation activities, growth was more than 4 times slower in *C. raciborskii* in the absence of combined nitrogen compared to that of *Anabaena* PCC7120 (Table 1). The slower growth in *C. raciborskii* suggests that although the vegetative cells in the vicinity of the end heterocyst are efficiently supplied with fixed nitrogen, and therefore divide more rapidly, a diminished transfer of nitrogen to more distantly located mid-

trichome vegetative cells could take place and that this would explain their reduced growth rate.

**Structures and proteins involved in intercellular transfer**

Since *C. raciborskii* has a significantly reduced genome (3.9 Mbp) compared to other heterocystous cyanobacteria (Stucken *et al*., 2010), we next examined whether any known structural and genetic elements involved in intercellular transfer are negatively impaired. Ultrastructural analysis showed that outer membrane in *C. raciborskii* surrounds the trichomes in a continuous fashion, and that the outer membrane does not enter the septa between two vegetative cells (Fig. 2a and b), nor between vegetative cells and heterocysts (Fig 2c and d). Thus, a common periplasmic space exists in *C. raciborskii,* which may function as a conduit for the transfer of solutes between cells (Flores *et al*., 2006; Mariscal *et al*., 2007; Mariscal and Flores, 2010). The utilization of this transfer pathway requires both the existence of transporters that release the nitrogen fixed into the periplasmic space of the heterocysts and that takes up this combined nitrogen into the non-fixing vegetative cells. In *Anabaena* PCC7120 three ABC-type amino acid transporters, N-I (NatABCDE), N-II (NatFGH-BgtA) and Bgt (BgtAB), have been characterized (Herrero & Flores 1990; Montesinos *et al*., 1995; Picossi *et al*., 2005; Pernil *et al*., 2008). Whereas Bgt is dispensable for diazotrophic growth, N-I appears to contribute together with N-II to the diazotrophic physiology of *Anabaena* PCC7120 (Pernil *et al*., 2008). Although yet undetermined, it is likely that the N-I and N-II transporters might allow the vegetative cells to uptake the amino acids produced by the heterocysts which could move through the continuous periplasmic space (Flores *et al*., 2006 ; Pernil *et al*., 2008). Examining the

existence of these genes in the *C. raciborskii* genome showed that those needed to synthesize complete N-I and N-II transporters are present, but that it lacks a *bgtB* homolog (Table S1). However, since *bgtB* seems to be dispensable for diazotrophic growth in *Anabaena* PCC7120 (Pernil *et al*., 2008), the genomic background for the uptake of amino acids from the periplasm exists in *C. raciborskii*.

Next, analysis of the septa between vegetative cells in *C. raciborskii* were performed by electron microscopy and $KMnO_4$ staining, which highlights septal junctions (Lang & Fray, 1971; Flores *et al*, 2006; Wilk *et al*., 2011). These analyses clearly demonstrated the presence of septal junctions connecting cells in *C. raciborskii* (Fig. 2e). Not unexpectedly, the genes encoding the FraC/FraD and SepJ proteins, components of septal junction complexes (Flores *et al*, 2007; Merino-Puerto *et al*., 2011; Mariscal, 2014), were also identified in the *C. raciborskii* genome (Table S2). These proteins are known to have key roles in trichome integrity and intercellular connectivity in *Anabaena* PCC7120 (Bauer *et al*., 1995; Flores *et al*., 2007; Mullineaux *et al*., 2008; Merino-Puerto *et al*., 2010; Merino-Puerto *et al*., 2011). However, the predicted protein sequence encoded by the *C. raciborskii sepJ* homolog (*CRC_03186*) was 222 residues shorter than that of *Anabaena* PCC7120 (Table S2). Further analysis showed that the predicted protein of *CRC_03186* and its neighboring open reading frame *CRC_03185,* aligned with residues 1 to 628 and 628 to 746 of the SepJ protein in *Anabaena* PCC7120, respectively (Table S3). The genomic region of *C. raciborskii sepJ* was therefore re-sequenced (see Materials and Methods), which confirmed that it is split in two parts, *CRC_03185* and *CRC_03186* (Fig. S1). Canonical SepJ from most section IV cyanobacteria holds three distinct domains (Flores *et al*., 2007; Mariscal *et al*., 2011): an N-terminal 200-residue coiled-coiled domain, an internal

connectin/extensin linker domain of variable size, and a 340-residue C-terminal integral membrane domain. The predicted sequence of *C. raciborskii* SepJ (Fig. S2; Table S2), has a shorter linker domain and, even when considering the sequences of *CRC_03185* and *CRC_03186*, it lacks several transmembrane domains (Fig. 3). Notably, the deletion of these transmembrane domains in *Anabaena* PCC7120 SepJ, were critical for its insertion in the cytoplasmic membrane and thus affected the calcein transference capacities of this mutant (Fig. S2; Mariscal *et al*., 2011).

Furthermore, we recently showed that *C. raciborskii* has a hypothetically truncated PatA pathway, *i.e*. it lacks a *patL* homologue involved in heterocyst differentiation, which might be responsible for its lack of the classical pattern formation, developing a terminal heterocyst phenotype only (Plominsky *et al*., 2013). Additionally, Young-Robbins *et al*. (2010) suggested that the PatA pathway is involved in the formation of the cell division ring in vegetative cells of cyanobacteria, and thus the formation of septa. Thus, to determine how the loss of this pathway would impact intercellular transfer in cyanobacteria, the dyes calcein and 5-CFDA were used to determine transfer rates between cells in the *patA* mutant UHM101 of *Anabaena* PCC7120, able to develop terminal heterocyst only (Orozco *et al*., 2006). However, after 24 hours of the diazotrophic induction, the transfer rates for both dyes were within the same range as those reported for wild type *Anabaena* PCC7120 (***E*** = 0.070 [SE ± 0.007; n = 6] and ***R*** = 0.068 [SE ± 0.016; n = 6]; Mariscal *et al*., 2011; Merino-Puerto *et al*., 2011). Thus, the lack of the *patA* pathway in *C. raciborskii* may not interfere with the transfer of these tracers.

**Intercellular transfer of calcein and 5-CFDA in *C. raciborskii***

Calcein transfer is assumed to take place through SepJ channels, as a Δ*sepJ* mutant is negatively affected in calcein transfer in *Anabaena* PCC7120, but only partially affected in 5-CFDA transfer (Merino-Puerto *et al*., 2011). FraC and FraD may form a complex that allows the transfer of 5-CFDA, since Δ*fraC*, Δ*fraD* and Δ*fraC*/*fraD* mutants show a strong reduction in 5-CFDA transfer rates in *Anabaena* PCC7120 (Merino-Puerto *et al*., 2011).

As the *sepJ* gene in *C. raciborskii* is truncated, the calcein as well as the 5-CFDA transfer rates were next examined. First, transfer rates between vegetative cells were followed in cultures grown in the presence of ammonium followed by nitrogen deprivation for 5, 10 and 30 days (Fig. 4). The exchange rate of calcein in centrally located vegetative cells of *C. raciborskii* trichomes radically dropped to about half the rate after N step down. As seen in Figure 4a, the ***E*** values were significantly lower at 10 and 30 days after diazotrophic induction ($F_{(3, 61)} = 6.2358$; Pvalue < 0.001; Tukey's test). This is contrary to the situation in *Anabaena* PCC7120, in which the calcein exchange rates increase under diazotrophic conditions (Mullineaux *et al*., 2008; Mariscal *et al*., 2011). However, the 5-CFDA exchange rates between vegetative cells in *C. raciborskii* were not significantly different after diazotrophic induction (Fig. 4b; two-way ANOVA).

The calcein and 5-CFDA transfer rates were next examined between specific cells in *C. raciborskii* trichomes. Additionally, the transfer of these tracers was quantified at 10 days after the onset of diazotrophic induction to assure full maturity of the heterocysts (Fig. 1). Notably, the rate of calcein exchange was similar both between heterocysts and their adjacent vegetative cells, and between 'terminal' vegetative cells and their neighbors (Fig.

4a). However, the transfer of calcein was almost 4 times higher between such end cells than the transfer rates recorded between vegetative cells located in the center of the trichome and therefore further away from any heterocyst (Fig. 4a). Interestingly, this finding is contrary to the fact that diazotrophically grown vegetative cells have calcein transference rates 10 times higher compared to intercalary heterocysts and their adjacent vegetative cells in the intercalary heterocystous cyanobacteria *Anabaena cylindrica* (Mullineaux *et al*., 2008). As the exchange of calcein potentially takes place through SepJ channels (Mullineaux *et al*., 2008; Mariscal *et al*., 2011), it is notable that *C. raciborskii* seems to have overcome the split of its *sepJ* gene, along with the loss of several of its key transmembrane domains (Fig. 3; Fig. S2). However, mechanisms involved in the regulation of differential transference rates (of calcein or solutes) between cells, depending on their position within the trichomes, now remains to be determined in *C. raciborskii*.

In contrast to in *Anabaena* PCC7120 (Merino-Puerto *et al*., 2011), the 5-CFDA dye also rapidly stained the *C. raciborskii* heterocysts and thus allowed quantification of the transference rates towards the adjacent vegetative cells (Fig. 4b). The transference of 5-CFDA between 'terminal' vegetative cells and adjacent vegetative cells in *C. raciborskii* was similar to that among centrally located vegetative cells under the same N regime (Fig. 4b). This rate was also similar to that detected between terminal heterocysts and their adjacent vegetative cells, and among diazotrophically grown centrally located vegetative cells (Fig. 4b). This is the first time that a transfer of 5-CFDA from heterocysts to adjacent cells has been shown. An exchange of 5-CFDA through the FraC/FraD channels may be the case in *C. raciborskii* as postulated for *Anabaena* PCC7120.

The heterocysts and 'terminal' vegetative cells exhibit similar calcein intercellular transfer

rates in *C. raciborskii* (Fig. 4), although the heterocysts form distinct cyanophycin polar 'nodules', filling up the narrow connection to the adjacent vegetative cell (Fig. 2C & D). It has been shown that the presence of cyanophycin polar nodules reduces calcein transference rates (approximately by 3 times) between heterocysts and vegetative cells in *Anabaena variabilis* ATCC 29413 (Mullineaux *et al*., 2008). This is apparently not the case in *C. raciborskii*.

Taken together, our data not unexpectedly reveal that cells within the trichome of *C. raciborskii* efficiently mobilize transport of compounds (such as fixed nitrogen) between its cells. However, we also show that transfer rates differ depending on both cell types involved and on their physical location within the trichome. In order to fulfill nitrogen demands of growing vegetative cells, the very limited number of heterocysts differentiating in *C. raciborski*i by necessity have to support a considerably higher number of vegetative cells (with fixed nitrogen) than cyanobacteria with several-fold higher frequencies of heterocysts (*e.g. Anabaena* PCC7120). As shown here, rates of transfer between cells located at the end of the trichomes, *i.e*. between a mature heterocyst or 'terminal' vegetative cell and their adjacent vegetative cell, are higher than rates observed between vegetative cells in central parts of the trichomes. This suggests that cells close to the ends of the trichomes might contribute to the trichome expansion to a much higher degree than cells in central areas, likely the result of the proximity to the $N_2$-fixing heterocysts. To what extent the split *sepJ* gene (encoding channels) lacking several transmembrane domains, is involved in the unusual transfer characteristic of *C. raciborskii* is a possibility that now needs to be addressed.

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## References

Bauer CC, Buikema WJ, Black K & Haselkorn R (1995) A short-filament mutant of *Anabaena* sp. strain PCC 7120 that fragments in nitrogen-deficient medium. *J Bacteriol* **177**:1520-1526.

Bergman B, Gallon JR, Rai AN & Stal LJ (1997) N2 fixation by non-heterocystous cyanobacteria. *FEMS Microbiol Rev* **19**:193-185.

Castro D, Vera D, Lagos N, Garcia C & Vasquez M (2004) The effect of temperature on growth and production of paralytic shellfish poisoning toxins by the cyanobacterium *Cylindrospermopsis raciborskii* C10. *Toxicon* **44**:483-489.

Corrales-Guerrero L, Mariscal V, Flores E & Herrero A (2013) Functional dissection and evidence of intercellular transfer of the heterocyst-differentiation *patS* morphogen. *Mol Microbiol* **88**:1093-1105.

Flores E, Herrero A, Wolk CP & Maldener I (2006) Is the periplasm continuous in filamentous multicellular cyanobacteria? *Trends Microbiol* **14**:439-443.

Flores E, Pernil R, Muro-Pastor AM, Mariscal V, Maldener I, Lechno-Yossef S, Fan Q, Wolk CP & Herrero A. (2007) Septum-localized protein required for filament integrity and diazotrophy in the heterocyst-forming cyanobacterium *Anabaena* sp. strain PCC 7120. *J Bacteriol* **189**:3884-3890.

Flores E & Herrero A (2010) Compartmentalized function through cell differentiation in filamentous cyanobacteria. *Nat Rev Microbiol* **8**:39-50.

Herrero A & Flores E (1990) Transport of basic amino acids by the dinitrogen-fixing cyanobacterium *Anabaena* PCC 7120. J Biol Chem **265**:3931-3935.

Higa KC, Rajagopalan R, Risser DD, Rivers OS, Tom SK, Videau P & Callahan SM (2012) The RGSGR amino acid motif of the intercellular signaling protein, HetN, is required for patterning of heterocysts in *Anabaena* sp. strain PCC 7120. *Mol Microbiol* **83**:682-693.

Lang NJ, & Fay P (1971) The heterocysts of blue-green algae II. Details of ultrastructure.

394 *Proc R Soc Lond B* **178**: 193-203.

395

396 Markwell MA, Haas SM, Bieber LL & Tolbert NE (1978) A modification of the Lowry
397 procedure to simplify protein determination in membrane and lipoprotein samples. *Anal*
398 *Biochem* **87**:206-210.

399

400 Mariscal V, Herrero A & Flores E (2007) Continuous periplasm in a filamentous,
401 heterocyst-forming cyanobacterium. *Mol Microbiol* **65**:1139-1145.

402

403 Mariscal V, & Flores E (2010) Multicellularity in a heterocyst-forming cyanobacterium:
404 pathways for intercellularcommunication. *Adv Exp Med Biol* **675**: 123-135.

405

406 Mariscal V, Herrero A, Nenninger A, Mullineaux CW & Flores E (2011) Functional
407 dissection of the three-domain SepJ protein joining the cells in cyanobacterial trichomes.
408 *Mol Microbiol* **79**:1077-1088.

409

410 Mariscal V (2014) Cell-Cell joining proteins in heterocyst-forming cyanobacteria. *In:*
411 Flores, E. and Herrero, A. (eds) The Cell Biology of Cyanobacteria. Caister Academic
412 Press. pp. 293-304.

413

414 Merino-Puerto V, Mariscal V, Mullineaux CW, Herrero A & Flores E (2010) Fra proteins
415 influencing filament integrity, diazotrophy and localization of septal protein SepJ in the
416 heterocyst-forming cyanobacterium *Anabaena* sp. *Mol Microbiol* **75**:1159-1170.

417

418 Merino-Puerto V, Schwarz H, Maldener I, Mariscal V, Mullineaux CW, Herrero A & Flores
419 E (2011) FraC/FraD-dependent intercellular molecular exchange in the filaments of a
420 heterocyst-forming cyanobacterium, *Anabaena* sp. *Mol Microbiol* **82**:87-98.
421
422 Montesinos ML, Herrero A & Flores E (1995) Amino acid transport systems required for
423 diazotrophic growth in the cyanobacterium *Anabaena* sp. strain PCC 7120. *J Bacteriol*
424 **177**:3150-3157.
425
426 Mullineaux CW, Mariscal V, Nenninger A, Khanum H, Herrero A, Flores E & Adams DG
427 (2008) Mechanism of intercellular molecular exchange in heterocyst-forming
428 cyanobacteria. *EMBO J* **27**:1299-1308.
429
430 Neunuebel MR & Golden JW (2008) The *Anabaena* sp. strain PCC 7120 gene all2874
431 encodes a diguanylate cyclase and is required for normal heterocyst development under
432 high-light growth conditions. J Bacteriol 190: 6829-6836.
433
434 Orozco CC, Risser DD & Callahan SM (2006) Epistasis analysis of four genes from
435 *Anabaena* sp. strain PCC 7120 suggests a connection between PatA and PatS in heterocyst
436 pattern formation. *J Bacteriol* **188**:1808-1816.
437
438 Pernil R, Picossi S, Mariscal V, Herrero A & Flores E (2008) ABC-type amino acid uptake
439 transporters Bgt and N-II of *Anabaena* sp. strain PCC 7120 share an ATPase subunit and
440 are expressed in vegetative cells and heterocysts. Mol Microbiol **67**:1067-1080.
441

Picossi S, Montesinos ML, Pernil R, Lichtlé C, Herrero A & Flores E (2005) ABC-type neutral amino acid permease N-I is required for optimal diazotrophic growth and is repressed in the heterocysts of *Anabaena* sp. strain PCC 7120. Mol Microbiol **57**:1582-1592.

Plominsky AM, Larsson J, Delherbe N, Bergman B & Vásquez M (2013) Dinitrogen fixation is restricted to the terminal heterocysts in the invasive cyanobacterium *Cylindrospermopsis raciborskii* CS-505. *PLoS One* **8**: e51682.

Popa R, Weber PK, Pett-Ridge J, Finzi JA, Fallon SJ, Hutcheon ID, Nealson KH & Capone DG (2007) Carbon and nitrogen fixation and metabolite exchange in and between individual cells of *Anabaena oscillarioides*. *ISME J* **1:**354-360.

Reddy PM & Talpasayi ERS (1974) Heterocyst formation in a blue-green alga, *Cylindrospermum*. *Nature* **249**:493-494.

Risser DD & Callahan SM (2009) Genetic and cytological evidence that heterocyst patterning is regulated by inhibitor gradients that promote activator decay. *Proc Natl Acad Sci U S A* **106**:19884-19888.

Sievers F, Wilm A, Dineen D, *et al*. (2011) Fast, scalable generation of high-quality protein multiple sequence alignments using Clustal Omega. *Mol Syst Biol* **7**:539.

Smith RV & Evans MCW (1971) Nitrogenase activity in cell-free extracts of the blue-green

alga, *Anabaena cylindrical*. *J Bacteriol* **105**:913-917.

Stucken K, John U, Cembella A, *et al.* (2010) The smallest known genomes of multicellular and toxic cyanobacteria: comparison, minimal gene sets for linked traits and the evolutionary implications. *PLoS One* **5**:e9235.

Valladares A, Maldener I, Muro-Pastor AM, Flores E & Herrero A (2007) Heterocyst development and diazotrophic metabolism in terminal respiratory oxidase mutants of the cyanobacterium *Anabaena* sp. strain PCC 7120. *J Bacteriol* **189**:4425-4430.

Waterhouse AM, Procter JB, Martin DMA, Clamp M & Barton GJ (2009) Jalview Version 2--a multiple sequence alignment editor and analysis workbench. *Bioinformatics* **25**:1189-1191.

Wiedner C, Rücker J, Brüggemann R & Nixdorf B (2007) Climate change affects timing and size of populations of an invasive cyanobacterium in temperate regions. *Oecologia* **152**:473-484.

Wilk L, Strauss M, Rudolf M, Nicolaisen K, Flores E, Kühlbrandt W & Schleiff E (2011) Outer membrane continuity and septosome formation between vegetative cells in the filaments of *Anabaena* sp. PCC 7120. *Cell Microbiol* **13**:1744-1754.

Wilson K (1990) Preparation of Genomic DNA from Bacteria. Current protocols in molecular biology (Ausubel F, Brent R, Kingston R, Seidman J, Smith J & Struhl K, eds),

pp. 2.4.1–2.4.5. Wiley Interscience, New York, NY.

Wolk CP (1968) Movement of carbon from vegetative cells to heterocysts in *Anabaena cylindrica*. *J Bacteriol* **96**:2138-2143.

Yoon HS & Golden JW (1998) Heterocyst pattern formation controlled by a diffusible peptide. *Science* **282**:935-938.

Young-Robbins SS, Risser DD, Moran JR, Haselkorn R & Callahan SM (2010) Transcriptional regulation of the heterocyst patterning gene *pat*A from *Anabaena* sp. strain PCC 7120. *J Bacteriol* **192**:4732-4740.

## Figure legends

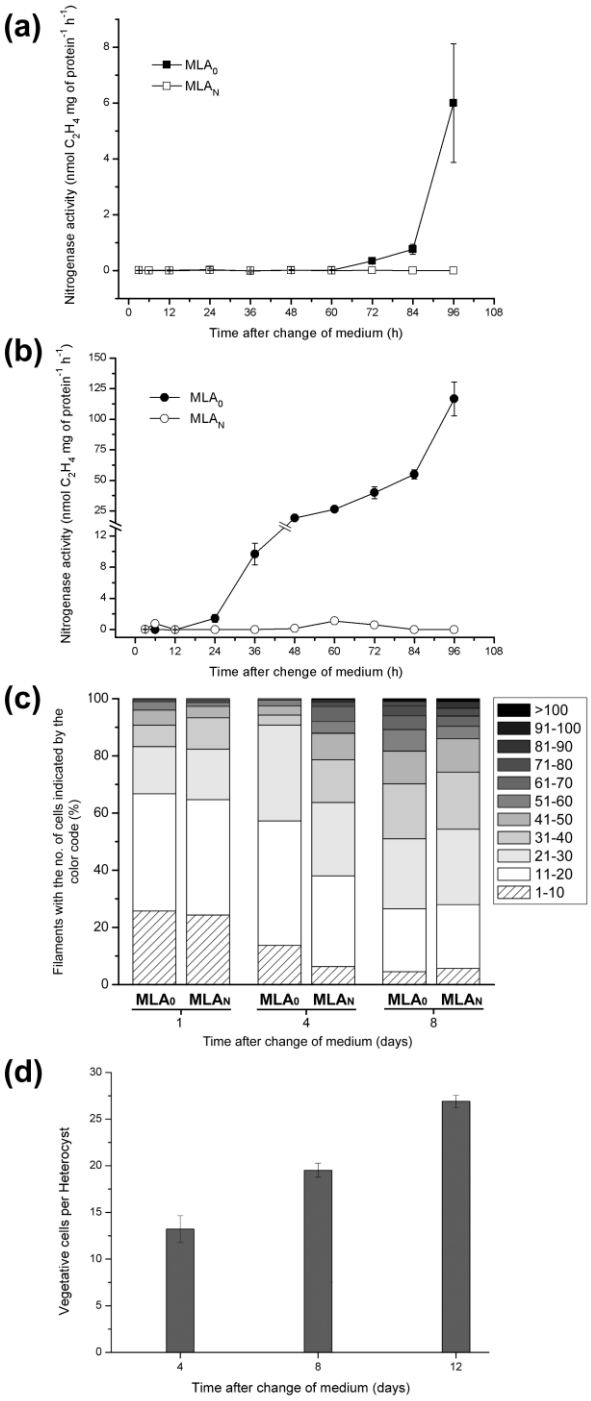



**Fig. 1.** Nitrogenase activity in (a) *C. raciborskii* and (b) *Anabaena* PCC7120 after diazotrophic induction. Each number (analyzed by ARA) represents the mean of 3 biological replicates, where error bars denote the standard deviation. (c) The mean length of

100 trichomes visualized and counted in 4 diazotrophically induced ($MLA_0$) and 3 non-diazotrophic control cultures ($MLA_N$) using light microscopy. (d) The number of vegetative cells per each terminal heterocyst in *C. raciborskii* trichomes after diazotrophic induction. Error bars denote the standard deviation.

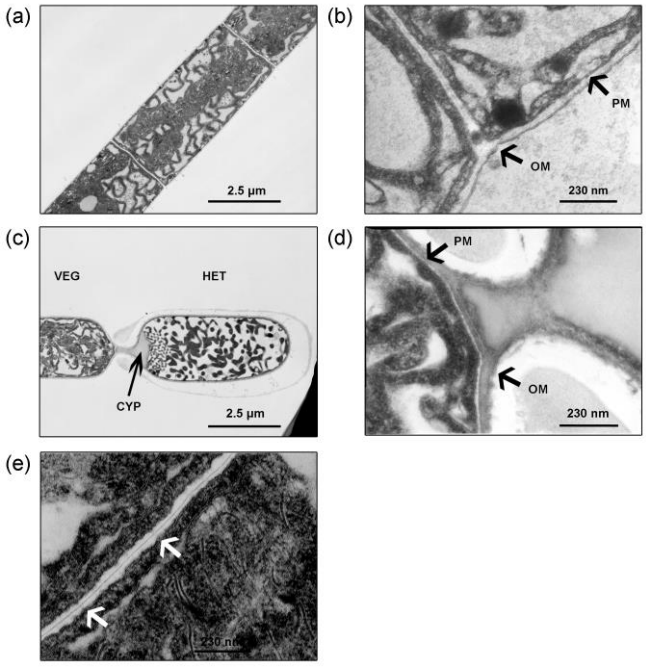



**Figure 2.** Transmission electron microscopy micrographs of *C. raciborskii*. (a, b) High magnification of septa between vegetative cells in trichomes stained with $KMnO_4$ to highlight the outer and inner membrane proteins located on each side of the peptidoglycan layer. The outer (OM) and plasma membrane (PM) are indicated with black arrows. (c, d) Illustrates the septum between a vegetative cell (VEG) and a heterocyst (HET), and a cyanophycin (CYP) granule (black arrow) at the narrow junction in the heterocyst. (e) Multiple septal junction-like structures between two vegetative cells of *C. raciborskii* (some denoted with black arrows).

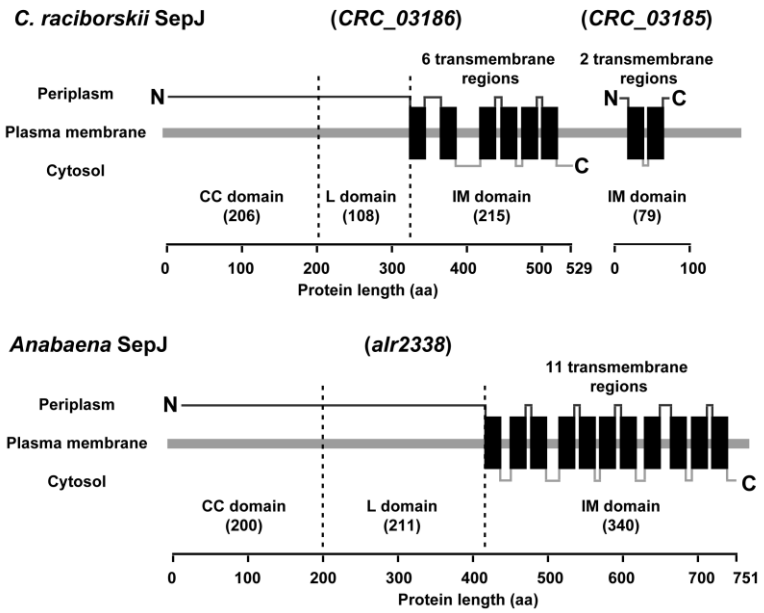
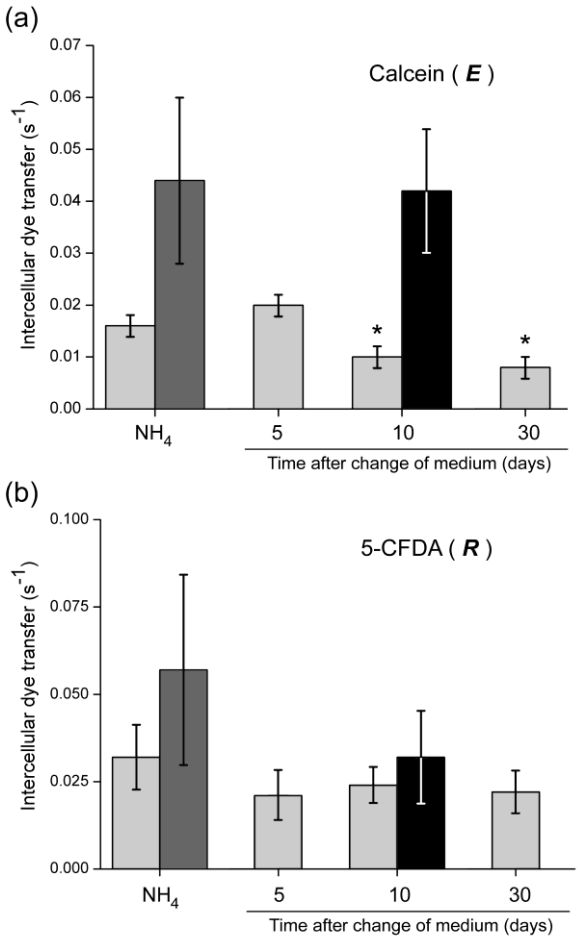



**Figure 3.** Structure of (a) *C. raciborskii* SepJ and (b) the canonical SepJ in *Anabaena* PCC 7120. The illustrations show the length and predicted secondary structure of the SepJ proteins. Due to its high identity to the C-terminal region of canonical SepJ sequences (Table S3; Fig. S2) *CRC_03185* was included to denote that even if both protein products form a split SepJ, it still lacks several transmembrane domains compared to canonical proteins. The "CC", "L" and "IM" denote the Coiled-Coiled, Linker and Integral-Membrane domains, respectively. The numbers of amino-acid residues of each domain are showed in parenthesis.




530

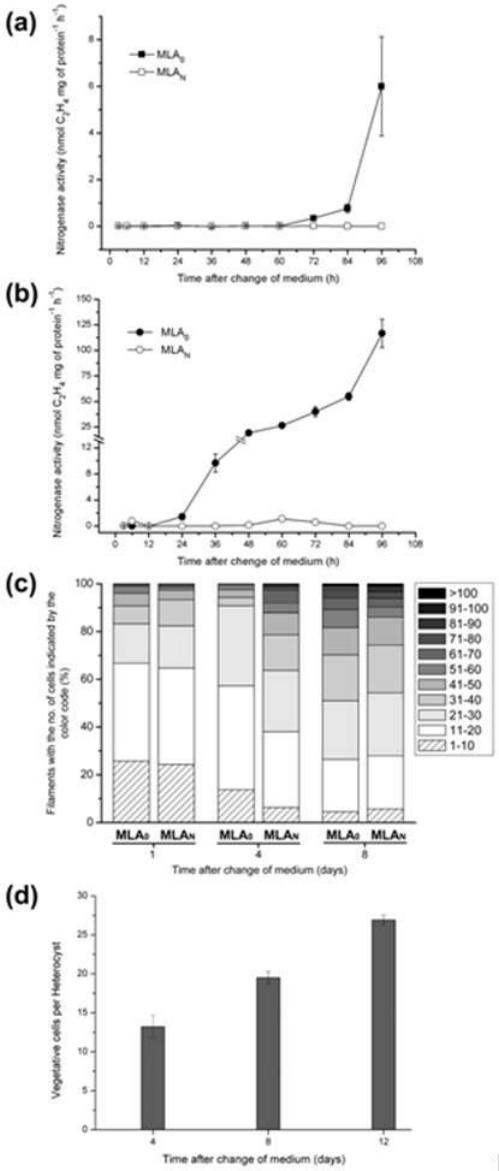
531 **Fig. 4.** The exchange of (a) calcein (***E***) and (b) 5-CFDA (***R***) in *C. raciborskii* between
532 centrally located vegetative cells (light grey), between 'terminal' vegetative cells (dark grey)
533 and terminal heterocysts and their adjacent vegetative cells (black). These cultures were
534 grown with 2 mM $NH_4$ prior to being subjected to nitrogen deprivation. '*' Denotes that the
535 rates are significantly different from that recorded for centrally located vegetative cells
536 before inducing diazotrophic growth ($F_{(3, 61)} = 6.2358$; Pvalue < 0.001; Tukey's test). Error
537 bars denote the standard error of the mean. The transference of these dyes between
538 'terminal' vegetative cells and terminal heterocysts, and their adjacent vegetative cells were
539 quantified only before the removal of combined nitrogen sources (*i.e.* before any
540 heterocysts had been differentiated) and 10 days after their removal, respectively.

**Table 1.** Diazotrophic and non-diazotrophic growth of *C. raciborskii* and *Anabaena* PCC7120.

| Culture | Medium | **μ** ($day^{-1}$)* |
|---|---|---|
| *Anabaena* PCC7120 | $BG11_N$ | **0.690** ± 0.142 |
| | $BG11_0$ | **0.700** ± 0.105 |
| *C. raciborskii* | $MLA_N$ | **0.688** ± 0.083 |
| | $MLA_0$ | **0.154** ± 0.025§ |

* Growth rate constant was determined as described in materials and methods, values correspond to the mean of 4 cultures grown at continuous light, ± SD is shown. § This value is significantly different from the other condition examined in *C. raciborskii* and the **μ** under both culture conditions in *Anabaena* PCC7120 (Pvalue < 0.001).

549 **Graphical_abstract**




*Cylindrospermopsis raciborskii* CS-505 is an invasive freshwater filamentous cyanobacterium that when grown diazotrophically may develop trichomes of up to 100 vegetative cells and differentiating at each end one heterocyst, the sole sites for their $N_2$ fixation process. Thus, in this cyanobacterium each heterocyst supplies assimilated nitrogen for a mean of 27 vegetative cells, which compared to *Anabaena* sp. strain PCC7120 almost doubles the vegetative cells that intercalary heterocysts feed. The structures and proteins for intercellular communication in *C. raciborskii* CS-505 were analyzed, and its transfer of calcein and 5-carboxyfluorescein was quantified in its vegetative cells and heterocysts. These dyes were mobilized along both cell types, but the terminal vegetative cells and heterocysts exhibited greater transfer rates than its intercalary vegetative cells.

Nitrogenase activity in (a) *C. raciborskii* and (b) *Anabaena* PCC7120, analyzed by ARA. Each value represents the mean of 3 biological replicates. (c) The mean length of 100 trichomes visualized and counted in 4 diazotrophically induced ($MLA_0$) and 3 non-diazotrophic control cultures ($MLA_N$) using light microscopy. (d) The number of vegetative cells supported by each terminal heterocyst in *C. raciborskii* trichomes

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551

## Supplementary Information

## Figures Legends

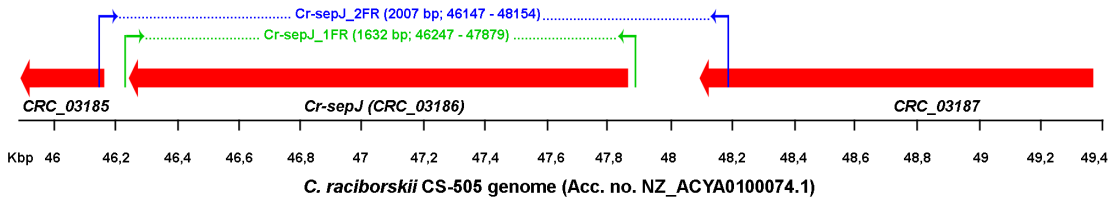


**Figure S1.** Localization of primer pairs along the genomic region of *Cr-sepJ*.

Schematic diagram showing *Cr-sepJ* (*CRC_03186*) and its flanking genes *CRC_03185* and *CRC_03187*. Bent lines with arrowheads denote the position of both primer pairs Cr-sepJ-1FR (green) and Cr-sepJ-2FR (blue). The size and the exact amplified region are shown. The sequence below the scheme corresponds to a zoom into the re-sequenced region comprising the stop (red) and start (blue) codons of *CRC_03186* and *CRC_03185*, respectively (numbers denote the exact position of this sequence along the corresponding genomic scaffold).


565

566 **Figure S2**. SepJ alignment.

567 Alignment of various cyanobacterial SepJ sequences. The abbreviations and protein
568 accession numbers are given in Table S1. The three main domains of the SepJ proteins are
569 denoted above each section of the alignment. Predicted protein sequences of *CRC_03186*
570 are highlighted in white letters within a black background, while that of *CRC_03185* is
571 highlighted in white letters within a grey background. The amino-acid residues deleted in
572 the CSVM36 mutant (Mariscal *et al*., 2011), are denoted with a dark-grey bar above the
573 corresponding region of the SepJ sequences.

574 **Table S1.** Components of the N-I, Bgt and N-II amino acid permease/transporter in

575 *Anabaena* PCC7120 and *C. raciborskii.*

| N-I neutral amino acid permease | | | | | | |
|---|---|---|---|---|---|---|
| Gene | *Anabaena* PCC7120 (locus tag) | Protein length (aa) | *C. raciborskii* (locus tag) | Protein length (aa) | Similarity (%) | E-value |
| NatA | *all1046* | 260 | *CRC_01670* | 261 | 81 | $5e^{-156}$ |
| NatB | *alr1834* | 441 | *CRC_01900* | 451 | 74 | 0 |
| NatC | *all1047* | 377 | *CRC_01503* | 368 | 80 | 0 |
| NatD* | *all1284* | 288 | *CRC_00618/ CRC_00619* | 126 / 151 | 75 / 87 | $3e^{-57}$ / $2e^{-75}$ |
| NatE | *all2912* | 247 | *CRC_01531* | 255 | 85 | $7e^{-136}$ |

576

| Bgt and N-II transport systems | | | | | | |
|---|---|---|---|---|---|---|
| Gene | *Anabaena* PCC7120 (locus tag) | Protein length (aa) | *C. raciborskii* (locus tag) | Protein length (aa) | Similarity (%) | E-value |
| BgtA | *alr4167* | 248 | *CRC_01297* | 246 | 85 | $5e^{-157}$ |
| BgtB | *alr3187* | 501 | - | - | - | - |

| | | | | | | |
|---|---|---|---|---|---|---|
| NatF | *alr4164* | 369 | *CRC_01293* | 378 | 80 | 0 |
| NatG | *alr4165* | 308 | *CRC_01294* | 313 | 72 | $9e^{-153}$ |
| NatH | *alr4166* | 381 | *CRC_01295* | 340 | 61 | $7e^{-150}$ |

*C. raciborskii* homologues were retrieved by using the corresponding sequences of *Anabaena* PCC7120 as queries in a protein BLAST search against the non-redundant protein database (nr, v2012-11-15). * Denotes that the *C. raciborskii* homolog is separated in two contiguous ORFs.

**Table S2.** Filament integrity and/or metabolite transfer related proteins of *Anabaena* PCC7120 and *C. raciborskii*

| | *Anabaena* PCC7120 (locus tag) | Protein length (aa) | *C. raciborskii* (locus tag)* | Protein length (aa) | Similarity (%) | E-value |
|---|---|---|---|---|---|---|
| FraC | *alr2392* | 179 | *CRC_01281* | 181 | 46 | $1e^{-54}$ |
| FraD | *alr2393* | 343 | *CRC_01280* | 336 | 57 | $3e^{-144}$ |
| SepJ | *alr2338* | 751 | *CRC_03186* | 529 (1-628)§ | 52 | $4e^{-61}$ |
| | | | *CRC_03185* | 77 (670-746)§ | 39 | $6e^{-7}$ |

Similarity and E-value correspond to the values obtained when utilizing the protein sequence of the *Anabaena* PCC7120 homolog as a query against the *C. raciborskii* predicted proteins. § Denotes that this homolog is divided in two ORFs. The specific regions where each one of these ORFs align to the nucleic acid sequence of the *Anabaena* PCC7120 *sepJ* is shown in parenthesis.

**Table S3.** Organism abbreviation, accession number, gene locus tag and putative protein length of cyanobacterial SepJ homologs aligned. Heterocystous cyanobacteria are denoted with underlined fonts.

| **Organism** | **Organism abbreviation** | **Locus tag** | **Protein accession** | **Protein GI number** | **Length (aa)** |
|---|---|---|---|---|---|
| *Anabaena* sp. PCC 7120 | *Anabaena* | alr2338 | BAB74037 | 17131430 | 751 |
| *Anabaena variabilis* ATCC 29413 | *Avariabilis* | Ava_0157 | YP_320678 | 75906382 | 751 |
| *Nodularia spumigena* CCY9414 | *Nodularia* | N9414_12528 | ZP_01631104 | 119512008 | 853 |
| *Nostoc punctiforme* PCC 73102 | *Npunctiforme* | Npun_R1723 | YP_001865335 | 186682139 | 779 |
| '*Nostoc azollae*' 0708 | *Nazollae* | Aazo_3725 | YP_003722392 | 298492215 | 728 |
| *C. raciborskii* CS-505 | *Craciborskii* | CRC_03186 | ZP_06309680 | 282901765 | 529 |
| *Raphidiopsis brookii* D9 | *Rbrookii* | CRD_01464 | ZP_06304767 | 282896761 | 677 |
| *Trichodesmium erythraeum* IMS101 | *Tricodesmium* | Tery_0269 | YP_720225 | 113474164 | 583 |