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Research Letter

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

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#Each of these authors led a section of the work and made an equal contribution.
One Sentence Summary: C. raciborskii CS-505 is an invasive freshwater filamentous cyanobacterium with terminal heterocyst. The terminal vegetative cells and heterocysts exhibited greater calcein and 5-carboxyfluorescein transfer rates than its intercalary vegetative cells.
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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 5-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).

Key words: *Cylindrospermopsis raciborskii*; intercellular transfer; diazotrophy

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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 and Herrero 2010). Since the N_2-fixing enzyme nitrogenase is irreversibly inactivated by atmospheric concentrations of oxygen (Smith and 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 and Herrero 2010). Heterocysts in turn rely on carbon supplied from their neighboring vegetative cells to function (Wolk 1968). Most cyanobacteria of the Nostocales clade (Subsection IV) differentiate heterocysts in a regular pattern among the dominating vegetative cells (intercalary heterocysts) (Flores and 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 and Golden 1998; Risser and 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, Herrero and Flores 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 (Sections 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 and 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 and 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, Risser and Callahan 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 (8 μm 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 (~ 0.1 μg 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 ($\mu = \ln 2/t_d$, 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 acetylene 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 timepoint. 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

Cylindrospermopsis 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°C, 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 phospholipoproteins 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 (Tables S1 and S2, Supporting Information) 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, Supporting Information). Genomic DNA of *C. raciborskii* was extracted (Wilson 1990), and the region covering *Cr-sepJ* was amplified by PCR using Platinum-Pfx proofreading 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 from 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

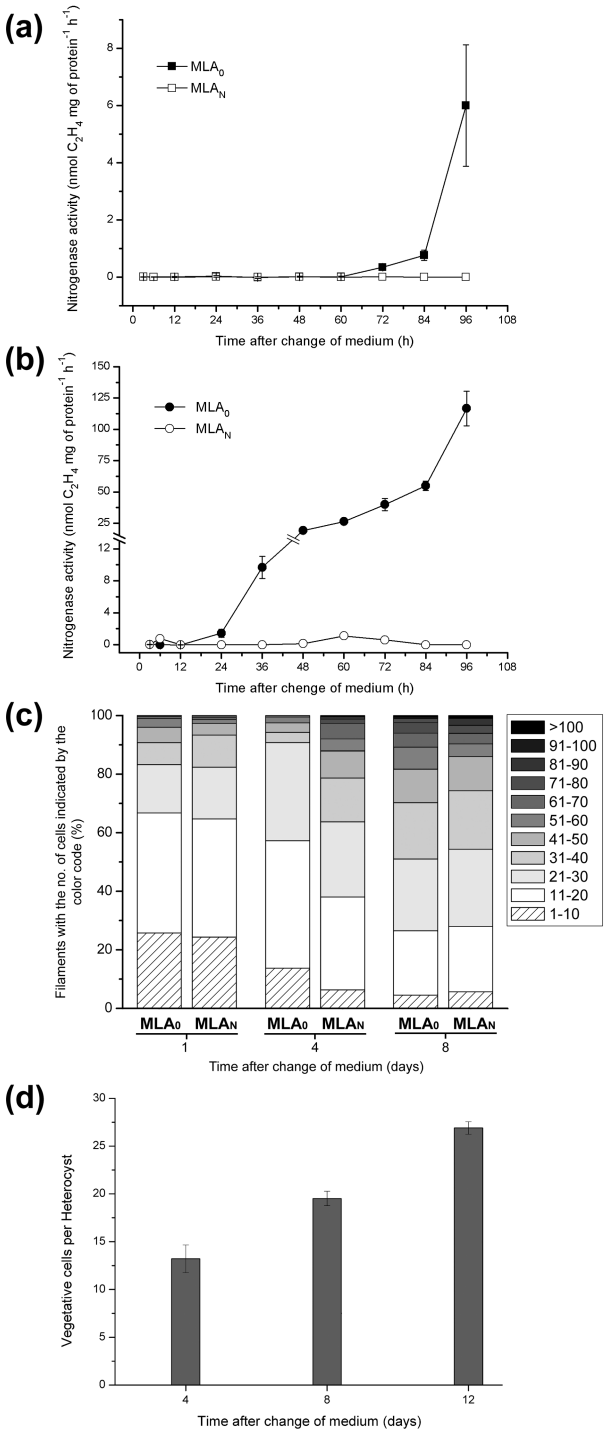

Figure 1. Nitrogenase activity in (**a**) *C. raciborskii* and (**b**) *Anabaena* PCC7120 after diazotrophic induction. Each number (analyzed by ARA) represents the mean of three biological replicates, where error bars denote the standard deviation. (**c**) The mean length of 100 trichomes visualized and counted in four diazotrophically induced (MLA_0) and three 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.

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
	MLA0	0.154 ± 0.025§

* Growth rate constant was determined as described in 'materials and methods', values correspond to the mean of four 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 (*P*-value < 0.001).

± 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 and 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 two 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 fixed nitrogen 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 and Flores 1990; Montesinos, Herrero and Flores 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, Supporting Information). 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* was performed by electron microscopy and $KMnO_4$ staining, which highlights septal junctions (Lang and Fay 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, Supporting Information). 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, 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, Supporting Information). 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, Supporting Information). The genomic region of *C. raciborskii sepJ* was therefore re-sequenced (see the section 'Materials and Methods'), which confirmed that it is split in two parts, *CRC˙03185* and *CRC˙03186* (Fig. S1, Supporting Information). 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, Supporting Information) 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 was critical for its insertion in the cytoplasmic membrane and thus

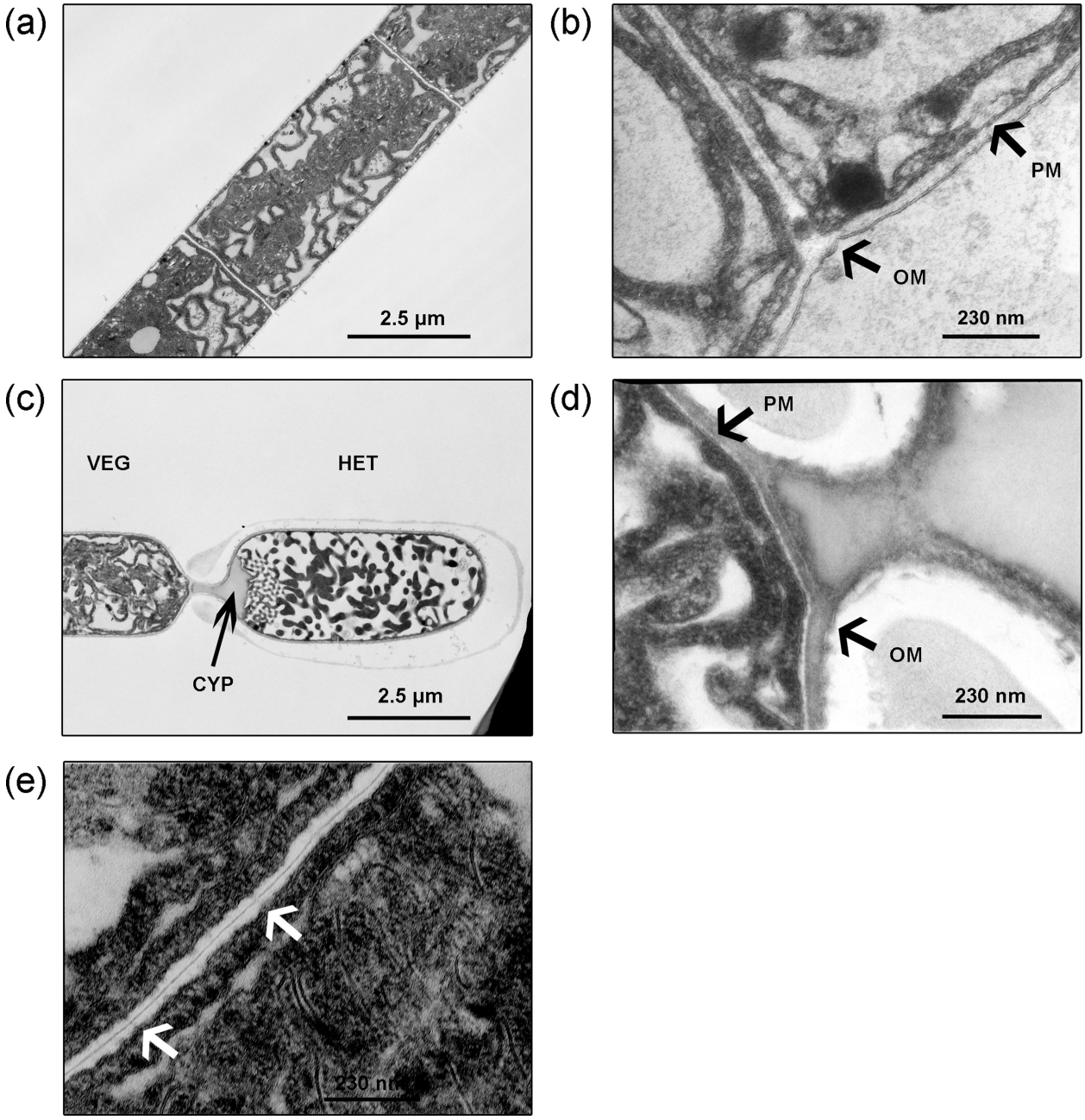

Figure 2. Transmission electron microscopy micrographs of *C. raciborskii*. (**a** and **b**) High magnification of septa between vegetative cells in trichomes stained with $KMnO_4$ to highlight the outer and inner membrane proteins located on each side of the peptidoglycan layer. The outer membrane (OM) and plasma membrane (PM) are indicated with black arrows. (**c** and **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).

affected the calcein transference capacities of this mutant (Fig. S2, Supporting Information; Mariscal *et al.*, 2011).

Furthermore, we recently showed that *C. raciborskii* has a hypothetically truncated PatA pathway, i.e. it lacks a *patL* homolog 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 h 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 $\pm$ 0.007; $n = 6$) and $R = 0.068$ (SE $\pm$ 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.

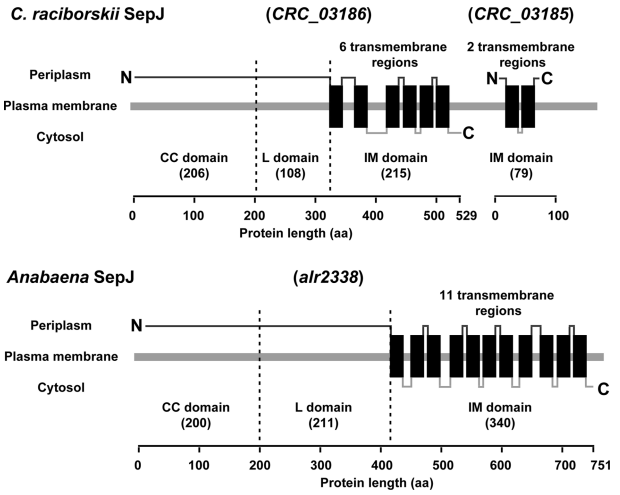

Figure 3. Structure of (**a**) *C. raciborskii* SepJ and (**b**) the canonical SepJ in *Anabaena* PCC 7120. The illustrations show the length and predicted secondary structure of the SepJ proteins. Due to its high identity to the C-terminal region of canonical SepJ sequences (Table S3 and Fig. S2, Supporting Information), *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.

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 Fig. 4a, the *E*-values were significantly lower at 10 and 30 days after diazotrophic induction ($F_{(3,\ 61)} = 6.2358$, *P*-value < 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 four 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 di-

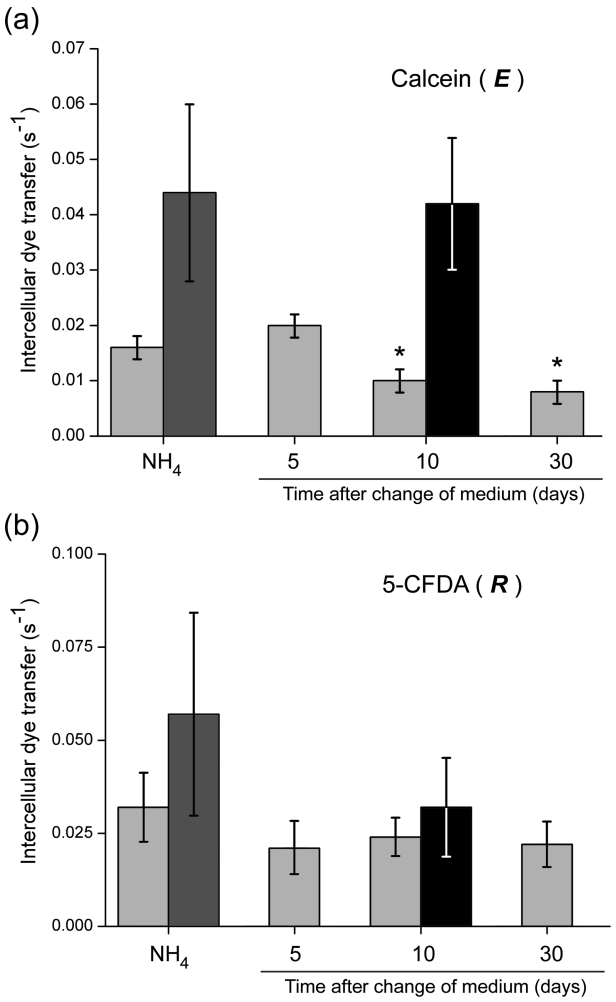

Figure 4. The exchange of (**a**) calcein (*E*) and (**b**) 5-CFDA (*R*) in *C. raciborskii* between centrally located vegetative cells (light grey), between 'terminal' vegetative cells (dark grey) and terminal heterocysts and their adjacent vegetative cells (black). These cultures were grown with 2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

azotrophically 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, Supporting Information). 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 and D). It has been shown that the presence of cyanophycin polar nodules reduces calcein transference rates (approximately by three 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. raciborskii* 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.

SUPPLEMENTARY DATA

Supplementary data is available at FEMSLE online.

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