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Minireview

Predicting substrate exchange in marine diatom-heterocystous cyanobacteria symbioses

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Summary

In the open ocean, some phytoplankton establish symbiosis with cyanobacteria. Some partnerships involve diatoms as hosts and heterocystous cyanobacteria as symbionts. Heterocysts are specialized cells for nitrogen fixation, and a function of the symbiotic cyanobacteria is to provide the host with nitrogen. However, both partners are photosynthetic and capable of carbon fixation, and the possible metabolites exchanged and mechanisms of transfer are poorly understood. The symbiont cellular location varies from internal to partial to fully external, and this is reflected in the symbiont genome size and content. In order to identify the membrane transporters potentially involved in metabolite exchange, we compare the draft genomes of three differently located symbionts with known transporters mainly from model free-living heterocystous cyanobacteria. The types and numbers of transporters are directly related to the symbiont cellular location: restricted in the endosymbionts and wider in the external symbiont. Three proposed models of metabolite exchange are suggested which take into account the type of transporters in the symbionts and the influence of their cellular location on the available nutrient pools. These models provide a basis for several hypotheses that given the importance of these symbioses in global N and C budgets, warrant future testing.

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Introduction

In vast expanses of open ocean environments concentrations of dissolved inorganic nitrogen are below analytical detection. Here, microorganisms capable of reducing dinitrogen (N_2), which comprises 78% of the atmosphere, are at an advantage and typically dominate. The process of N_2 fixation, or the reduction of N_2 to ammonium, is performed by a small and diverse group of bacteria and archaea (Young, 1992). Some N_2 fixing (diazotrophic) populations in the open ocean are symbiotic, with a few genera of microalgae, specifically diatoms as hosts and heterocystous cyanobacteria as symbionts (Foster and O'Mullan, 2008) (Fig. 1A–C). Collectively these symbioses are referred to as diatom diazotrophic associations (DDAs). DDAs are globally distributed and are considered major contributors to both N and carbon (C) cycles due to seasonal blooms with high N_2 and C fixation rates and rapid sinking (Mague *et al*., 1974; Venrick, 1974; Carpenter *et al*., 1999; Subramaniam *et al*., 2008; Karl *et al*., 2016). Despite their recognition as globally significant, there still remain large gaps in our understanding of DDAs, especially how the partners interact and acquire (and share) the elements necessary for metabolism and growth. Here, we analysed the available DDA symbiont genomes to identify membrane transporters potentially involved in interactions with the host partners. This allowed us to recognize target proteins for further investigation by genetic approaches, for instance by the heterologous expression and analysis of genes from the symbionts.

The diatom diazotrophic associations

The heterocystous cyanobacterial symbionts

The heterocystous cyanobacterial symbionts of DDAs have been characterized by several genetic markers, including 16S rRNA, the *nifH* gene, which encodes the nitrogenase reductase component (Fe protein) of the nitrogenase complex for N_2 fixation, and the *hetR* gene,

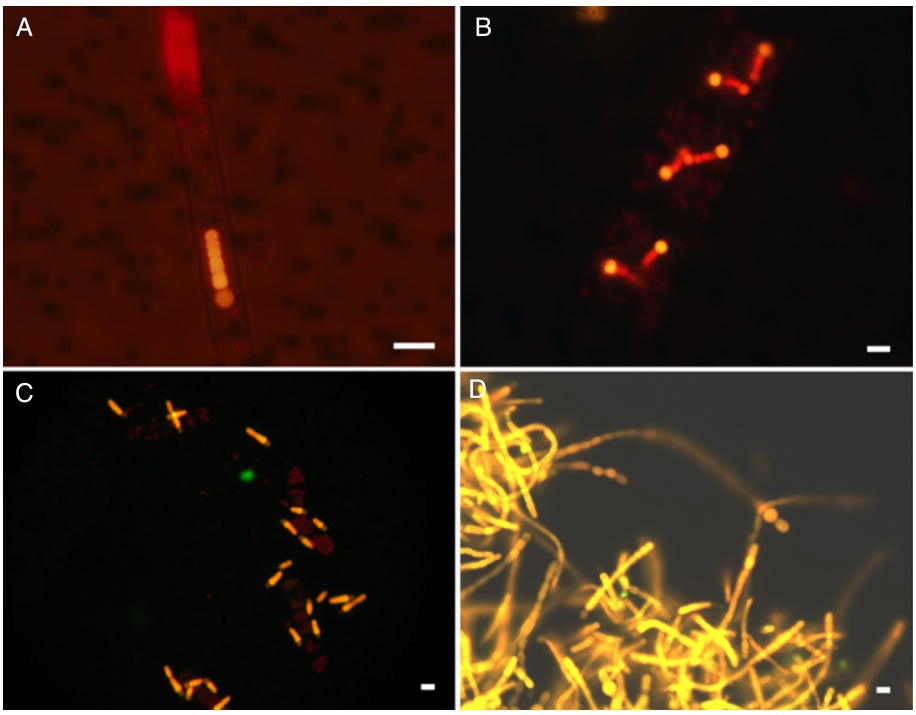

Fig. 1. Epifluorescent images taken of wild populations (A–C) of the various Diatom Diazotroph Associations (DDAs) and the external symbiont *Calothrix* SC01 after isolation (D). Cells imaged in the field used a blue excitation filter (450–490 nm) to distinguish the heterocystous cyanobacterial symbionts (yellow-orange) from the diatom chloroplasts (red). A. One *Rhizosolenia clevei-R. intracellularis* (RintRC01) symbiosis. Note the longer filament of *Richelia* when associated with *Rhizosolenia*. B. A chain of three *Hemiaulus hauckii* diatoms associated with *Richelia intracellularis* (RintHH01). C. A chain of >10 *Chaetoceros compressus* diatoms with *Calothrix rhizosoleniae* (CalSC01) attached to the outside. Note that the spines are not visible for the diatom and the taper of the *Calothrix* symbiont differs from that of *Richelia*. D. The CalSC01 isolate several months after isolation (imaged with blue excitation). Note the longer filaments when growing freely of its host diatom. Scale bars are approximately 10 μm. [Color figure can be viewed at wileyonlinelibrary.com]

which encodes a key regulator of cell differentiation in heterocystous cyanobacteria (Janson *et al*., 1999; Foster and Zehr, 2006). Based on these phylogenies the symbionts are related to other heterocystous cyanobacteria in the order Nostocales (e.g. *Anabaena* sp., *Nostoc* sp. and *Calothrix* sp.). Notably, the symbionts are more related between them than to any other known heterocystous cyanobacterium (Caputo *et al*., 2019). In spite of that, symbiont *hetR* and *nifH* sequences are relatively divergent (84% and 91% identity respectively) suggesting a high specificity in the partnerships (Janson *et al*., 1999; Foster and Zehr, 2006). In other words, one particular symbiont strain associates with one particular host genus, and the driver of the specificity is currently unknown (Janson *et al*., 1999; Foster and Zehr, 2006). Morphologically, the symbionts vary in terms of filament length and taper, and all possess terminal heterocysts.

The symbionts vary in their cellular location: internal, partial and external. 'Internal' symbionts have penetrated the diatom's cytoplasm; 'partial' (or 'periplasmic') refers to symbionts that reside between the diatom's cytoplasmic membrane and frustule (outer silicified cell wall of diatoms); and 'external' are symbionts that are attached to the surface of the diatom (Villareal, 1989, 1990; Caputo *et al*., 2019). Importantly, the external symbiont (*Calothrix rhizosoleniae*, CalSC01) can be grown independently of its host in the laboratory (Foster *et al*., 2010) (see Fig. 1D). Draft genomes are available for the heterocystous symbionts: *Richelia intracellularis* RintHH01 (internal, associated with *Hemiaulus hauckii*), 3.24 Mbp; *R. intracellularis* RintHM01 (internal, associated with *H. membranaceus*), 2.21 Mbp; *R. intracellularis* RintRC01 (partial, associated with *Rhizosolenia clevei*), 5.4 Mbp; and *Calothrix rhizosoleniae* CalSC01 (external, associated with *Chaetoceros compresus*), 5.97 Mbp (Hilton *et al*., 2013; Hilton, 2014). This shows that genome size is directly related to the cellular location of the symbionts. General features (size, GC content and percent coding) of the symbiont genomes are similar to other Nostocales, including free-living and facultative and obligate symbiotic strains of multicellular plants (e.g. *Anabaena* sp. PCC 7120, *Nostoc punctiforme* PCC 73102 and *Nostoc azollae* 0708 respectively; PRJNA244, PRJNA216 and PRJNA30807 respectively) (Meeks *et al*., 2001; Hilton *et al*., 2013). The *R. intracellularis* RintHM01 draft genome lacks several sequences expected of a full genome due to low sequencing coverage (Hilton *et al*., 2013), and therefore it was not compared further.

The symbiotic diatoms

Diatoms are single-celled eukaryotic plankton widely distributed in aquatic environments that contribute significantly (20%) to global primary production (Field *et al*., 1998). Most diatoms dominate coastal environments, where dissolved nutrients are high, and like other microalgae, diatoms can utilize nitrate and ammonium (Guillard and Kilham, 1977; Armbrust, 2009). In addition, diatoms possess a complete urea cycle (Allen *et al*., 2011). On the other hand, in oligotrophic marine

environments, some diatoms form a symbiosis with cyanobacteria (Villareal, 1992; Foster and O'Mullan, 2008). In general, we know far less about the symbiotic host diatoms compared with their respective symbionts. The host diatoms differ dramatically in cell size (e.g. *Hemiaulus hauckii*, 12–35 μm; *H. membranaceus*, 30–70 μm; *Rhizosolenia clevei*, 7–250 μm; *Chaetoceros compressus*, 7–40 μm). The *Hemiaulus* spp. and *Chaetoceros* spp. hosts are capable of forming long chains (>50 cells), while *R. clevei* tend to be solitary. These observations raise questions on how/if symbiont metabolism (e.g. N_2 fixation) is influenced by host cell size, and whether/how substrates are also exchanged between symbiotic cells. The genetic identity of the hosts was only recently characterized for a few genetic markers and resulted in congruent phylogenies with the respective symbiont phylogenies suggesting co-evolution (Caputo *et al*., 2019).

The symbiosis

An important, interesting, and often challenging characteristic of any symbiosis is defining the function of each partner. In the DDAs the symbiont function is obvious, since only the symbiont can reduce N_2, and the provision of fixed N to the diatom hosts has been shown on the cellular level (Foster *et al*., 2011), but the mechanism of N transfer is unstudied. In terrestrial symbioses involving heterocystous cyanobacteria, the symbiotic populations often reside in darkened cavities and rely (heterotrophically) on their host plants for reduced C substrates (Söderbäck and Bergman, 1993). In DDAs, both partners are photosynthetic, and hence capable of C fixation, but the possible exchange and transfer of C substrates is unknown. Nonetheless, interestingly, a recent cellular model for DDAs estimates that 25% of C fixed by the host is transferred to the symbiont, since the C requirement by the symbiont for N_2 fixation is higher than the fixation of C predicted from its own photosynthesis (Inomura *et al*., 2020).

Since all DDAs have evaded long-term isolation (Villareal, 1989, 1990), it is difficult to study experimentally how the partners interact, share and potentially compete for substrates. Here, we have identified various candidate transporters in the symbiotic *R. intracellularis*/*C. rhizosoleniae* (hereafter *Richelia*/*Calothrix*) draft genomes by comparison mainly to those of model heterocystous cyanobacteria. We have focused on transporters for C, N and some other elements (e.g. iron, phosphorus and sulfur) that are important for the basis of the partnership.

Note on methodology

To identify particular gene products in the DDA symbionts, we have performed BLASTp analyses (Altschul *et al*., 1997). We generally used proteins of known function from *Anabaena* sp. PCC 7120 (hereafter *Anabaena*) as queries, although in some particular cases well-characterized proteins from other sources, mainly filamentous cyanobacteria (*Nostoc punctiforme* ATCC 29133, *Trichodesmium erythraeum* IMS101) were used. The symbionts are also predicted to have some membrane proteins not found in other cyanobacteria, and such membrane proteins were compared with transporters from other biological sources.

To define orthologues, we have followed conservative criteria. Thus, generally, we checked that the symbiont's protein was of approximately the same length as the query protein, which, combined with significant similarity, gave very low Expect values (e.g. $<10^{-50}$ to $<10^{-150}$ [indicated as <e-50 or <e-150] for proteins of 150–500 amino acid residues approximately). On the other hand, comparisons with Expect values of, e.g. $>10^{-20}$ (indicated as >e-20) were considered to denote similar but not necessarily orthologous proteins. This is especially common in membrane proteins that can belong to the same family of transporters but recognize different substrates. The alignments, using Clustal O (Madeira *et al*., 2019), of some examples of proteins that give significant similarity to be considered orthologues are presented in Figs. S1–S5.

Outer membrane translocators

Cyanobacteria are diderm bacteria, i.e. they contain an outer membrane (OM) outside of the cytoplasmic membrane (Hahn and Schleiff, 2014). The OM characteristically contains numerous proteins that take a β-barrel conformation, and the OM outer leaflet contains lipopolysaccharide (LPS) as a characteristic component. Although cyanobacterial LPS is not identical to that of the best-studied Gram-negative bacteria (Hahn and Schleiff, 2014), the genome of the heterocystous symbionts of DDAs encode a number of OM insertion proteins (BamA family proteins) as well as proteins involved in LPS synthesis and transport that collectively suggest the presence of a mature OM in the symbionts (Table S1). Hence, materials transferred between the diatom and the symbiont in DDAs must traverse the OM. Substrate translocation across the OM generally takes place through porins, which are trimeric β-barrel proteins (Yamashita and Buchanan, 2010).

There are currently about 90 recognized families of β-barrel porins in the Transporter Classification Database [TCDB (Saier Jr *et al*., 2016); http://www.tcdb.org/], and cyanobacteria possess characteristic porins that constitute one of those families (TCDB #1.B.23). A characteristic feature of the cyanobacterial porins is the presence of an N-terminal domain with similarity to 'S-layer homology' domains that may connect the OM to the peptidoglycan

layer. These cyanobacterial porins show similarity to the OprB-type porins that mediate transfer of saccharides in bacteria such as *Pseudomonas aeruginosa* (van den Berg, 2012). Indeed, one of these porins from the facultative symbiont *N. punctiforme* (Npun_R5320) has been shown to facilitate uptake of glucose and fructose into the cyanobacterium (Ekman *et al*., 2013). Nonetheless, it appears that these porins fulfil classical porin function generally permitting the transfer of small molecules and ions through the OM (Hahn and Schleiff, 2014). The *Anabaena* genome encodes seven OprB-type porins, of which All4499 and Alr4550 appear to be particularly abundant (Moslavac *et al*., 2007b; Nicolaisen *et al*., 2009).

Using All4499 and Npun_R5320 as queries, we found four homologues in CalSC01 and two in each of the RintRC01 and RintHH01 genomes (Table 1). Thus, the external, facultative symbiont CalSC01 is more similar to *Anabaena* in number of porins than the partial and internal symbionts (RintRC01 and RintHH01, respectively). In the latter two, one of the porins (RintRC01_7172, RintHH01_240) may be additionally subjected to regulation by zinc (Zn), since the DNA sequence upstream of the encoding gene contains a possible binding site for the Zur transcription factor, which is involved in Zn homeostasis (Sein-Echaluce *et al*., 2015). The other OM porin (RintRC01_1265, RintHH01_6530), which shows the highest similarity to All4499 and Npun_R5320 (Table 1), may represent, therefore, the general porin of the endosymbionts.

The *Anabaena* genome encodes an unusually high number of TonB-dependent OM transporters (Hahn and Schleiff, 2014), which mediate uptake through the OM of iron (Fe) complexes or vitamin B_{12}. This uptake is energized by the interaction of a periplasmic domain of the TonB-dependent OM transporter with the inner membrane protein TonB (Yamashita and Buchanan, 2010). Neither RintRC01 nor RintHH01 appear to bear any TonB-dependent transporter, whereas CalSC01 shows two TonB-dependent Fe complex transporters (see Table 4 below) and one TonB-dependent vitamin B_{12} transporter (Table 1). This points to a different strategy for trace element uptake in the symbiont strains (see below) that is likely influenced by the symbiont location: the nutrient pool of the external symbiont (CalSC01) is that of the

Table 1. Outer membrane (OM) proteins encoded in the DDA symbiont genomes.

Query	CalSC01 (external)	RintRC01 (partial)	RintHH01 (internal)	Protein/function
All4499	2790721588 Ga0265390_12245 (1.5e-78)	RintRC_1265 (0.0)	RintHH _6530 (0.0)	OM porin OprB
	2790720937 Ga0265390_11703 (2.3e-81) 2790720522 Ga0265390_113824 (6.5e-85) 2790722855 Ga0265390_13252 (6.5e-95)	RintRC_7172 Zn regulated (?) (1e-60)	RintHH _240 Zn regulated (?) (1e-112)	
Npun_R5320	2790721588 Ga0265390_12245 (1e-129)	RintRC_1265 (1e-126)	RintHH_6530 (1e-128)	OM porin OprB
	2790720937 Ga0265390_11703 (1e-133) 2790720522 Ga0265390_113824 (9e-134) 2790722855 Ga0265390_13252 (5e-127)	RintRC_7172 Zn regulated (?) (2e-54)	RintHH_240 Zn regulated (?) (1e-107)	
Alr4028-Alr4029	2790719984 Ga0265390_110111 (2e-122; 2e-119)	nd	nd	Vitamin B_{12} transporter
Alr2887 (HgdD)	2790721775 Ga0265390_124212 (0.0)	RintRC_2765 (0.0)	RintHH_21590* (0.0)	TolC-like OM exporter

ORFs from the symbionts (CalSC01, RintRC01, RintHH01) identified in BLASTp analysis (Expect values indicated in parenthesis) using the indicated protein from *Anabaena* (All, Alr) or *Nostoc punctiforme* (Npun) as a query. An asterisk indicates a possibly incomplete sequence; nd, not detected; (?), Zn-regulation is possible but not yet demonstrated.

surround, while the endosymbionts lack direct contact with the outside water column and are limited (if not reliant) to the host cytoplasm (RintHH01) or region between frustule and the host cytoplasmic membrane (RintRC01).

TolC-type exporters are trimeric proteins that make an OM channel in the form of a β-barrel and a periplasmic channel formed by α-helixes (Yamashita and Buchanan, 2010). TolC exporters are commonly able to function together with several different plasma membrane exporters thus mediating the export of different substances from the cell. The *Anabaena* genome encodes only one TolC-like protein, HgdD (Alr2887), which can participate in the export of different substances including heterocyst-specific glycolipids (Moslavac *et al*., 2007a) and toxic compounds such as ethidium bromide (Hahn *et al*., 2012). Like *Anabaena*, each of the three symbionts has only one TolC-like protein (Table 1), which likely is involved in multiple export functions.

Cytoplasmic membrane transporters: ABC and MFS transporters

Cytoplasmic membrane transporters are currently classified into numerous phylogenetically distinct superfamilies and families (see TCDB [Saier Jr *et al*., 2016]; http://www.tcdb.org/). Two such superfamilies with numerous protein members commonly referred to in this article are the ATP-Binding Cassette transporter superfamily (ABC; TCDB #3.A.1) and the Major Facilitator Superfamily (MFS; TCDB #2.A.1). The ABC transporter superfamily is one of the largest families among transport systems with a wide distribution in all three domains of life. ABC transporters can be divided into exporters, found in both eukaryotes and prokaryotes, and importers, which, with a few eukaryotic exceptions, are largely found in prokaryotes mediating the uptake of nutrients (Wilkens, 2015). ABC importers generally contain one periplasmic solute-binding protein (SBP), which binds the ligand in the periplasm for delivery to the appropriate membrane transporter complex; two integral membrane proteins (transmembrane domains [TMDs]) that form the solute-translocation pathway; and two nucleotide-binding proteins or domains (NBD) that hydrolyze ATP in the cytoplasm (Cui and Davidson, 2011). MFS proteins are the largest family of secondary transporters and allow the transport of a large variety of ions and solutes across membranes (Reddy *et al*., 2012). They comprise facilitators, symporters and antiporters, which move substrates across membranes via facilitated diffusion, co-transport or exchange respectively (Yan, 2015). MFS proteins are integral membrane proteins that generally possess 12 or 14 transmembrane segments (TMSs) (Reddy *et al*., 2012).

C-compound transporters

Cyanobacteria are mainly photoautotrophic, fixing carbon dioxide (CO_2), but many strains can also assimilate some organic compounds including sugars, mainly glucose, fructose and sucrose (Rippka *et al*., 1979). Diatoms are also predominantly photosynthetic and many are facultative heterotrophs (Hellebust and Lewin, 1977). In both diatoms and cyanobacteria, CO_2 fixation is catalysed by ribulose-1,5-bisphosphate carboxylase/oxygenase (RubisCO). Since both partners of DDAs possess RubisCO, a key question for the symbionts (especially for the internal and partial symbionts: RintHH01 and RintRC01) is how they obtain and/or compete for C, especially to cope with the high rate of N_2 fixation.

Inorganic carbon

Similar to many other autotrophs, both cyanobacteria and diatoms have developed C Concentration Mechanisms (CCMs) to increase the concentration of CO_2 around RubisCO (Raven *et al*., 2008). The CCMs of diatoms are considered genetically diverse and include high interspecies variations (Young *et al*., 2016; Shen *et al*., 2017). In general, cyanobacterial and diatom CCMs show functional and compositional similarities, and both possess subcellular compartments for housing RubisCO, i.e. carboxysomes in cyanobacteria and pyrenoids in diatoms, but their CCM genes are not homologous (Young and Hopkinson, 2017). The CCMs of diatoms and cyanobacteria are composed of bicarbonate (HCO_3^-) transporters and carbonic anhydrases (CAs) that mediate the interconversion of CO_2 and HCO_3^-, and cyanobacteria possess, in addition, CO_2 transporters (Kaplan and Reinhold, 1999; Badger *et al*., 2006; Cameron *et al*., 2014). CAs vary in number, subtype (α, ß, γ, others) and localization in model diatoms such as *Phaeodactylum tricornutum* and *Thalassiosira pseudonana* (Hopkinson *et al*., 2016).

The DDA symbionts contain the *ccmKLMNO* operon (with two copies of *ccmK* in each of RintHH01, RintRC01 and CalSC01) for β-carboxysomes and the gene for carboxysomal CA, which are essential for carboxysome functionality. The uptake systems for CO_2 and HCO_3^- species (inorganic C, Ci) in cyanobacteria include ABC and MFS transporters for HCO_3^- and a specialized type of NAD(P)H dehydrogenase (NDH-1; photosynthetic complex I) that functions in trapping CO_2 (Price, 2011; Schuller *et al*., 2020). In diatoms both CO_2 and HCO_3^- enter the cell from the environment. HCO_3^- is actively transported by membrane-embedded transporters, while CO_2 relies on a diffusive flux mediated by the 'chloroplast pump', the active pumping of HCO_3^- to the chloroplast (Hopkinson *et al*., 2011).

The *Anabaena* genome encodes two CO_2 uptake systems of the NDH-1 type, the NDH-1_3 and NDH-1_4 protein complexes, and three HCO_3^- importers, including one ABC transporter (high affinity) and two sodium-dependent transporters (SbtA, high affinity; BicA, low affinity) (Herrero and Flores, 2019), whereas the symbiont's Ci uptake systems appear to be influenced by the diatom host. The chloroplast pump mechanism and subsequent distribution of Ci is particularly interesting in relation to symbiont cellular location and their respective genome content for Ci transport. Thus, none of the endosymbionts has genes encoding high-affinity HCO_3^- transporters, whereas the external symbiont CalSC01 has a gene encoding an MFS high-affinity HCO_3^- transporter, SbtA (Table 2; Fig. 2A). The absence of the high-affinity HCO_3^- transporters in the internal symbionts may reflect the influence of the 'chloroplast pump' and CA mechanisms of its host, which drive high HCO_3^- concentrations within the vicinity of RintRC01 and RintHH01 symbionts. Low HCO_3^- concentrations are common in the surface ocean and could drive the retention of the high-affinity HCO_3^- transporter (SbtA) in the external symbiont, CalSC01. Thus, an open and important question in each symbiosis is the uptake kinetics for Ci, and how the symbiont cellular location influences the activity and partner interaction.

Interestingly, the three symbionts bear genes encoding proteins similar to the SulP-family HCO_3^- transporter BicA. CalSC01 and RintRC01 each shows two genes encoding BicA-like proteins, whereas RintHH01 shows the possible *bicA* gene fragmented in four consecutive sequences, perhaps reflecting incomplete sequencing coverage or, alternatively, that it is a gene in the process of degeneration (Table 2, see also Table 5 below). Because SulP transporters for HCO_3^- and sulfate are very similar to each other, experimental tests are needed to define the actual substrate of those proteins. Additionally, each of the three symbionts encodes the components of an NDH-1 type (NDH-1_3) of CO_2 uptake system. Whereas CalSC01 is expected to fix CO_2 (at least when growing independently of the diatom), the presence of RubisCO, carboxysome genes, an NDH-1 complex and, likely, a BicA transporter in the internal and partial symbionts support the idea that they can also fix CO_2, which is consistent with high rates of C fixation measured in field populations (Carpenter *et al*., 1999; Subramaniam *et al*., 2008).

Organic carbon

Despite the fact that the main trophic mode of cyanobacteria is photoautotrophy, some strains, including some heterocystous types that engage in symbioses with terrestrial plants, have the capacity of sugar uptake supporting heterotrophic growth. Thus, GlcP (MFS transporter for glucose) and Frt (ABC transporter for fructose) in symbiotically competent *N. punctiforme* (Ekman *et al*., 2013) and Frt in *A. variabilis* (Ungerer *et al*., 2008) are well-known examples of transporters for some kind of heterotrophic growth (Wolk and Shaffer, 1976; Summers *et al*., 1995). Additionally, mixotrophic growth with different C sources (Malatinszky *et al*., 2017) and the identification of components of ABC glucoside transporters involved in sugar-stimulated growth in *Anabaena* have been recently reported (Nieves-Morión and Flores, 2018). Surprisingly, no homologues to GlcP or Frt proteins are encoded in any of the diatom symbionts. However, the three symbionts may encode the ABC transporter Gls (Table 2; Fig. 2A), which in *Anabaena* functions in the uptake of glucosides (Nieves-Morión and Flores, 2018). CalSC01 bears two homologous proteins to each GlsQ and GlsP (TMDs), which might suggest the function of two ABC-glucoside transporters (Fig. 2A) as it has been reported in *Anabaena* (Nieves-Morión and Flores, 2018). In contrast, due to the low similarity of the putative SBP of either *Richelia* strain to the *Anabaena* SBP GlsR (Table 2), experimental confirmation of their substrates is required. The *Anabaena* Alr3705 MFS symporter is a possible glycoside transporter, although it could not experimentally transport glucosides in *Anabaena* (Nieves-Morión *et al*., 2017). Whereas RintHH01 and RintRC01 bear a homologous protein, CalSC01 contains two possible homologues for Alr3705 (Table 2; Fig. 2A). The presence of these transporters raises the possibility that the symbionts can assimilate a sugar such as sucrose, which in *Anabaena* has a principal role in the transfer of reduced C from vegetative cells to heterocysts (López-Igual *et al*., 2010; Nürnberg *et al*., 2015). Consistently, the three symbionts encode invertases, which are enzymes that irreversibly split sucrose into glucose and fructose (Table 2).

In cyanobacteria, sucrose is synthesized by the combined action of sucrose-phosphate synthase (Sps) and sucrose-phosphate phosphatase (Spp), which constitute an irreversible pathway of sucrose biosynthesis, or by the reversible enzyme sucrose synthase (Sus) (Salerno and Curatti, 2003). Whereas RintRC01 and CalSC01 bear genes encoding Sps and Spp, RintHH01 lacks these enzymes or the alternative sucrose synthase. Hence, RintHH01 has the capacity to hydrolyze but not synthesize sucrose. Therefore, a mixotrophic growth, fixing CO_2 and assimilating sugars provided by the host, is possible for the symbionts, and this could be especially relevant in the case of the internal symbiont RintHH01. While CalSC01 and RintRC01 encode invertases InvA and InvB, the only invertase present in RintHH01 is most similar to *Anabaena* InvB, which is heterocyst-specific (López-Igual *et al*., 2010). It would, therefore, be of great

Table 2. Carbon uptake transporters and related proteins encoded in the DDA symbiont genomes.

Query	CalSC01 (external)	RintRC01 (partial)	RintHH01 (internal)	Protein/function
Alr2877 (CmpA) Alr2878 (CmpB) Alr2879 (CmpC) Alr2880 (CmpD)	nd	nd	nd	ABC Bicarbonate transporter: CmpA (SBP) CmpB (TMD) CmpC (NBD) CmpD (NBD)
All2134	2790721,442 Ga0265390_12075 (3e-178)	nd	nd	High-affinity bicarbonate:Na^+ symporter, SbtA
BicA from *Synechococcus* sp. PCC 7002	2790719465 Ga0265390_10577 (0.0) 2790720482 Ga0265390_11353 (0.0)	RintRC_3892 (0.0) RintRC_4851 (0.0)	RintHH_3960# (4e-12) RintHH_3970# (1e-103) RintHH_3980# (3e-21) RintHH_3990# (5e-32)	Low-affinity, SulP-family bicarbonate: Na^+ symporter, BicA (two similar proteins in *Anabaena*: All1304, Alr1635)
Alr4156 (NdhF)	2790722617 Ga0265390_130615 (0.0) Downstream genes: 2790722616 2790722615	RintRC_4170 (0.0) Downstream genes: RintRC_4169 RintRC_4168	RintHH_18810 (0.0) Downstream genes: RintHH_18800 RintHH_18780	Three-gene clusters that encode an NDH-1 type (NDH-1_3) of CO_2 uptake complex
Alr2532 (GlsQ)	2790723713 Ga0265390_139111 (TMD, 3e-61) 2790721923 Ga0265390_12555 (TMD, 6.9e-56)	RintRC_0814 (TMD, 9e-62)	RintHH_290 (TMD, 8e-52) RintHH_15070 (TMD, 9e-47)	Glucoside ABC transporter Gls: GlsQ (TMD) GlsP (TMD) GlsR (SBP) GlsC (NBD) GlsD (NBD)
All0261 (GlsP)	2790722162 Ga0265390_127217 (TMD, 2e-55) 2,790721031 Ga0265390_117612 (TMD, 3.95 e-61)			
All1916 (GlsR)	2790721653 Ga0265390_12342 (SBP, 2e-52)	RintRC_6152 (SBP, 4e-11)	RintHH_17430 (SBP, 9e-21)	
Alr4781 (GlsC)	2790719311 Ga0265390_10456 (NBD, 8e-118)	RintRC_6050 (NBD, 1e-92)	RintHH_19540 (NBD, 1e-166)	
All1823 (GlsD)	2790721447 Ga0265390_12083 (NBD, 2e-117)			
Alr3705	2790723960 Ga0265390_14405 (1.3e-71) 2790720797 Ga0265390_11606 (3.18e-169)	RintRC_2529 (0.0)	RintHH_3250** (1e-39) RintHH_3260** (1e-174)	Glycoside-Pentoside-Hexuronide: Cation MFS symporter
Alr1521	2790722378 Ga0265390_12903 (0.0)	RintRC_4014 (0.0)	nd	Invertase InvA
Alr0819	2790722061 Ga0265390_12672 (0.0)	RintRC_3156 (0.0)	RintHH_3860 (0.0)	Invertase InvB (heterocyst-specific in *Anabaena*)
All3028 (SBP)	2790720991 Ga0265390_11733 (SBP, 0.0)	RintRC_2147 (SPB, 1e-134)	RintHH_12690 (SBP, 0.0)	TRAP carboxylate transporter. Consists of SBP, small TMD (DctQ), and large TMD (DctM)
Alr3026 (small TMD)	2790722400 Ga0265390_12928 (TMD small, 8e-59)	RintRC_3100 (TMD small, 8e-93)	RintHH_15260 (TMD small, 1e-83)	
Alr3027 (large TMD)	2790722399 Ga0265390_12927 (TMD large, 0.0)	RintRC_3101 (TMD large, 0.0)	RintHH_15250 (TMD large, 0.0)	

(Continues)

Table 2. Continued

Query	CalSC01 (external)	RintRC01 (partial)	RintHH01 (internal)	Protein/function
ActP of *Rh*. *capsulatus*[a]	2790722390 Ga0265390_129110 (e-45)	RintRC_5151 (e-49)	nd	Cation/acetate permease
PutP of *B*. *subtilis*[b]	2790719613 Ga0265390_10674 (4e-61)	nd	RintHH_18700 (e-67)	Nutritional high-affinity sodium/proline permease PutP

a. *Rh*., *Rhodobacter*.
b. *Bacillus*.
ORFs from the symbionts (CalSC01, RintRC01, RintHH01) identified in BLASTp analysis (Expect values indicated in parenthesis) using the indicated protein from *Anabaena* (unless indicated otherwise) as a query. Double asterisks designate genes that are split; Hash indicates genes that are fragmented; nd, not detected.

interest to locate and determine the functionality of InvB in the endosymbiont strains RintRC01 and RintHH01.

Tripartite ATP-independent periplasmic (TRAP) transporters are generally carboxylate transporters (Mulligan *et al*., 2011). *Anabaena* contains three genes encoding a TRAP transporter that mediates the uptake of pyruvate and other monocarboxylate 2-oxoacids (Pernil *et al*., 2010). Genes encoding homologues to the *Anabaena* TRAP transporter proteins can be detected in the three symbionts (Table 2; Fig. 2A). A recent field investigation reported the co-expression of the RintRC01 gene for the TRAP solute receptor and EAMA-like transporters in the diatom host *Rhizosolenia* (Harke *et al*., 2018). EAMA transporters belong to the Drug/Metabolite Transporter superfamily (TCDB #2.A.7), are associated with triose-phosphate translocators located on the plastid membrane, and function in the model diatom *P*. *tricornutum* to export carbohydrates derived from photosynthesis (Moog *et al*., 2015). Hence, it was suggested that the diatom host was supplying sugar substrates to the symbiont (Harke *et al*., 2018). However, considering that the RintRC01 resides outside the host diatom cytoplasm in the periplasmic space, an additional transport mechanism across the host cell membrane is required. Finally, if indeed reduced C substrates are transported from the host, other candidates for simple organic molecule transport are a predicted acetate permease present in CalSC01 and RintRC01, and a predicted proline transporter in CalSC01 and RintHH01 (Table 2). Identifying the substrate(s) of these transporters would be of great interest to understand the physiology of the DDAs, especially in the case of the internal symbiont (RintHH01), in which substrate(s) can be taken up directly from the host diatom's cytoplasm.

N-compound transporters

Cyanobacteria and diatoms have the ability to assimilate inorganic and simple organic N compounds, and some cyanobacteria fix atmospheric N_2. However, the environments where the DDAs are reported are notoriously N deplete, since concentrations of inorganic and organic N are below analytical detection. Hence, the host diatoms are dependent on the symbionts for N.

Primary N sources

Nitrogenase, the enzyme responsible for N_2 fixation, is inactivated in the presence of O_2, and therefore N_2-fixing cyanobacteria have to separate spatially or temporally N_2 fixation and oxygenic photosynthesis (Flores *et al*., 2015). Under conditions of combined-N deprivation, cyanobacteria of the order Nostocales (including, e.g. *Anabaena*, *Calothrix*, *Nostoc* and *Richelia*) produce differentiated cells called heterocysts where N_2 fixation takes place. A common observation in terrestrial-based symbioses with heterocystous cyanobacteria is that heterocyst frequency (normally about 7%-10%) increases (e.g. to 17%–60%) when the symbionts are living in symbioses rather than in a free-living state (Meeks, 2009). The *Richelia*/*Calothrix* symbionts, however, are unique symbionts in that they possess terminal heterocysts, and therefore cannot increase the number of heterocysts, but rather maintain a high ratio of heterocysts to vegetative cells if the number of vegetative cells is small. In RintHH01 and CalSC01 filaments the latter is true, since they typically have 1–3 vegetative cells, however, filaments tend to be longer in the case of RintRC01 (Fig. 1; see also Villareal, 1992). Longer filaments in RintRC01 and notably in free-living CalSC01 imply more vegetative cells performing C fixation and hence a higher C supply from their own photosynthesis. It is currently unknown how filament length is regulated in these organisms.

Heterocyst formation involves morphological and metabolic changes that allow the expression and function of nitrogenase (Flores *et al*., 2019b). One of these morphological changes consists in the deposition of two envelope layers outside of the OM: the glycolipid layer (HGL)

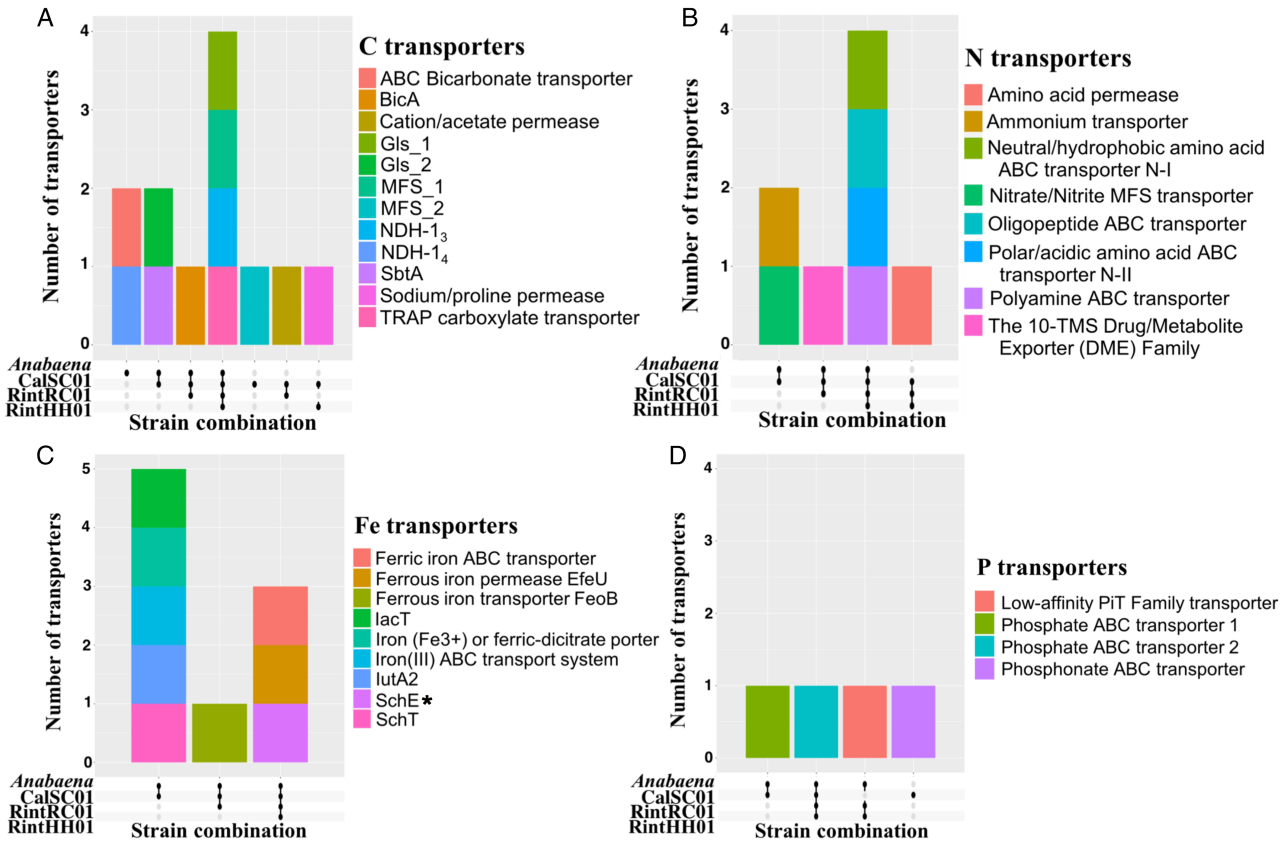

Fig. 2. Upset diagram showing the intersection of each set of transporters in the model cyanobacterium *Anabaena* sp. PCC 7120 and the DDA symbionts *Calothrix* CalSC01, *Richelia* RintRC01, and *Richelia* RintHH01 for (A) carbon, (B) nitrogen, (C) iron and (D) phosphorus. The C, N, Fe and P transporter content in CalSC01 is similar to *Anabaena* and higher in number than transporters present in the endosymbionts RintRC01 and RintHH01. A. Note the presence of SbtA, Gls_2 and MFS_2 in the external symbiont and their absence in the endosymbionts. TRAP carboxylate transporter, NDH-1$_3$ and the Gls_1 are present in the three symbionts. CalSC01 bears two homologous proteins each to GlsQ and GlsP (Table 2) suggesting the presence of two glucoside ABC transporters (Gls_1 and Gls_2) as in *Anabaena*. We refer to the second transporter as that containing Ga0265390_12555 and _117612. Two possible MFS transporters for glycosides are identified in CalSC01: MFS_1 (Ga0265390_14405) and MFS_2 (Ga0265390_11606) (see Table 2). B. Note the ammonium transporter (Amt) and nitrate/nitrite MFS transporter are only present in the external symbiont. The three symbionts bear a possible amino acid permease (APC superfamily), which is not present in *Anabaena*. C. The three symbionts bear the ferric iron ABC transporter and the ferrous iron permease EfeU, as in *Anabaena*, whereas uncertainty of SchE in the symbionts is designated with an asterisk (see the text). D. Note the presence of the phosphonate ABC transporter and a second phosphate ABC transporter (Phosphate ABC transporter 2) in the external symbiont CalSC01. We refer to the second phosphate ABC transporter 2 as that containing Ga0265390_11512, _12441, _12442, _12443, _11047 (see Table 5).

that reduces the permeation of O_2, and the polysaccharide layer (HEP) that provides protection (Nicolaisen *et al*., 2009). The HGL is composed of fatty alcohols glycosidically linked to sugar residues. Whereas HGLs made of C6 sugar have been found in freshwater free-living cyanobacteria and some strains of benthic *Calothrix* (Bauersachs *et al*., 2009), *Richelia* (RintHH01, RintRC01) contains novel HGLs with a C5 sugar, ribose, rather than a C6 sugar (Schouten *et al*., 2013; Bale *et al*., 2015). Recently, a novel HGL was characterized for a new *Calothrix* sp. CCY1611 isolated from the North Atlantic (Bale *et al*., 2018); it is unknown if CCY1611 is similar and/or genetically related to CalSC01, and the HGLs of CalSC01 are uncharacterized. The C5 sugar found in RintRC01 and RintHH01 might be explained by an adaptation of the endosymbiont to the high O_2 concentration within the diatom host (Walsby, 1985). This adaptation illustrates the importance of N_2 fixation in the DDAs, and details of the production of the heterocyst envelope in the symbionts will merit specific research in the future.

In addition to N_2, heterocystous cyanobacteria can assimilate from the environment various sources of N including ammonium, nitrate, nitrite, urea and some amino acids (Herrero and Flores, 2019). The intracellular conversion of these compounds to ammonium is required for N incorporation into carbon skeletons to produce organic N compounds. Ammonium is a preferred N source for many organisms and its uptake in environments with low external concentrations involves the Amt

Table 3. Nitrogen-compound uptake transporters encoded in the DDA symbiont genomes.

Query	CalSC01 (external)	RintRC01 (partial)	RintHH01 (internal)	Protein/function
Alr0990 (Amt4) Alr0991 (Amt1) Alr0992 (AmtB)	2790720545 Ga0265390_113847 Amt1-like (e-161) 2790720547 Ga0265390_113849 (2e-15) AmtB-like (N-terminal)*/ 2790722393 Ga0265390_12921 (4e-93) AmtB-like (C-terminal)*	nd	nd	Ammonium transporter
Npun_R1527	2790722858 Ga0265390_13255 (0.0)	nd	nd	Nitrate/nitrite MFS transporter *Nostoc punctiforme*
All1046 (NatA)	2790721047 Ga0265390_11777 (NBD, e-155)	RintRC_2443 (NBD, e-158)	RintHH_6690 (2.0 e-85)	Neutral/hydrophobic amino acid ABC transporter N-I (TCDB 3.A.1.4.1): NatA (NBD)
All1047 (NatC)	2790721046 Ga0265390_11776 (TMD, 0.0)	RintRC_2444 (TMD, 0.0)	RintHH_6700 (TMD, 0.0)	NatB (SBP) NatC (TMD) NatD (TMD)
Alr1834 (NatB)	2790719962 Ga0265390_110012 (SBP, 0.0)	RintRC_3291 (SBP, 0.0)	RintHH_11820 (SPB, 0.0)	NatE (NBD)
All1284 (NatD)	2790723726 Ga0265390_139211 (TMD, 2e-177)	RintRC_1047 (TMD, e-170)	RintHH_12020 (TMD, e-158)	
All2912 (NatE)	2790719321 Ga0265390_104516 (NBD, 8e-152)	RintRC_2490 (NBD, e-144)	RintHH_22020 (NBD, e-121)	
Alr4164 (NatF)	2790719035 Ga0265390_102315 (SBP, 0.0)	RintRC_5050 (SBP, e-180)	RintHH_12770 (SBP, e-166)	Polar/acidic amino acid ABC transporter N-II (TCDB 3.A.1.3.18): NatF (SBP)
Alr4165 (NatG)	2790719036 Ga0265390_102316 (TMD, 2e-179)	RintRC_7437 (TMD, e-132)	RintHH_12760 (TMD, e-155)	NatG (TMD) NatH (TMD) BgtA (NBD)
Alr4166 (NatH)	2790719037 Ga0265390_102317 (TMD, 3e-90)	RintRC_4447 (TMD, 9e-48)*	RintHH_12750 (TMD, e-163)	
Alr4167 (BgtA)	2790722348 Ga0265390_128835 (NBD, 3e-164)	RintRC_6091 (NBD, 2.0 e-90)	RintHH_6220 (NBD, e-154)	
RintHH_4450	2790720375 Ga0265390_112922 (0.0)	RintRC_1364 (0.0)	RinTHH_4450 (used as query)	Possible APC superfamily amino acid transporter/antiporter with cytoplasmic regulatory domain. May transport ammonium
Alr3884 (OppA)	2790723586 Ga0265390_13829 (SBP, 0.0) 2,790,721,558 Ga0265390_12199 (SBP, 0.0)	RintRC_3281 (SBP, 0.0)	RintHH_13100 (SPB, 0.0)	Oligopeptide ABC transporter (TCDB 3.A.1.5.1): OppA (SBP) OppB (TMD) OppC (TMD) OppD (NBD)
Alr4583 (OppB)	2790723692 Ga0265390_138815 (TMD, 0.0)	RintRC_1799 (TMD, 0.0)	RintHH_1460 (TMD, 0.0)	
Alr1556 (OppC)	2790723542 Ga0265390_13797 (TMD, 0.0)	RintRC_6207 (TMD, 0.0)	RintHH_18690 (TMD, 0.0)	
All4778 (OppD)	2790719309 Ga0265390_10454 (NBD, 0.0)	RintRC_2702 (NBD, e-126)	RintHH_7150 (NBD, 0.0)	
All3551	2790719557 Ga0265390_106142 (e-170)	RintRC_4379 (e-162)	RinTHH_13930 (e-80)*	The 10-TMS Drug/Metabolite Exporter (DME) Family (TCDB 2.A.7.3)
All5044 (PotA)	2790721024 Ga0265390_11765 (NBD, 0.0)	RintRC_2177 (NBD, 0.0)	RintHH_17600 (NBD, 2e-79)	Polyamine ABC transporter (TCDB 3.A.1.11.1): PotA (NBD)

(Continues)

Table 3. Continued

Query	CalSC01 (external)	RintRC01 (partial)	RintHH01 (internal)	Protein/function
All5043 (PotD)	2790721025 Ga0265390_11766 (SBP, 0.0)	RintRC_2176 (SBP, 0.0)	RintHH_7180 (SBP, 3e-26)	PotD (SBP) PotB (TMD)
All5042 (PotB)	2790722175 Ga0265390_12742 (TMD, 6e-134)	RintRC_4310 (TMD, e-125)	RintHH_17090 (TMD, 3e-13)	RintHH (TCDB 3.A.1.11.7)

ORFs from the symbionts (CalSC01, RintRC01, RintHH01) identified in BLASTp analysis (Expect values indicated in parenthesis) using the indicated protein from *Anabaena* (unless indicated otherwise) as a query. An asterisk indicates a possibly incomplete sequence; nd, not detected.

proteins (Paz-Yepes *et al*., 2008). Whereas RintRC01 and RintHH01 do not bear genes encoding Amt transporters, CalSC01 has retained two *amt* genes (Table 3; Fig. 2B). One of these genes (Ga0265390_113847) is homologous to Amt1, which is the cyanobacterial Amt protein most active in uptake assessed by ^{14}C-labelled methylamine assays (Montesinos *et al*., 1998; Vázquez-Bermúdez *et al*., 2002). Nitrate/nitrite uptake is mediated by an ABC transporter (*nrtABCD*) in *Anabaena* or by an MFS protein (*nrtP*) in *N. punctiforme*. These transporters concentrate nitrate or nitrite inside the cells that are then sequentially reduced by nitrate and nitrite reductase (NarB and NirA respectively) to produce ammonium (Cai and Wolk, 1997; Flores *et al*., 2005; Aichi *et al*., 2006). The CalSC01 genome is the only of those considered here encoding a nitrate transporter, which is an NrtP-like permease (Table 3; Fig. 2B), and possesses the enzymes for nitrate reduction.

Urea is the simplest organic N compound and the main component of dissolved organic nitrogen in the oceans (Luque and Forchhammer, 2008), albeit at low concentrations in the regions where DDAs thrive. Urea is taken up in *Anabaena* by an ABC transporter (*urtABCDE*), and once inside the cells it is hydrolyzed to CO_2 and ammonium by urease (Valladares *et al*., 2002). Since neither *Richelia* nor *Calothrix* possesses this type of transporter or urease-encoding genes and diatoms contain a complete urea cycle with an active urease (Allen *et al*., 2011), the symbionts appear not to exchange urea with the host.

Ammonium is incorporated into C skeletons through the glutamine synthetase/glutamate synthase (GS/GOGAT) pathway. GS catalyses the production of glutamine from ammonium and glutamate in an ATP-dependent reaction, and GOGAT catalyses the synthesis of glutamate from glutamine and 2-oxoglutarate (2-OG; derived from CO_2 fixation) using electrons frequently provided by reduced ferredoxin (Muro-Pastor *et al*., 2005). Interestingly, the GS/GOGAT pathway links the C and N metabolisms, and 2-OG is the compound that reflects the C/N ratio in cyanobacteria (Muro-Pastor *et al*., 2001). In heterocystous cyanobacteria, ammonium resulting from N_2 fixation is immediately incorporated into glutamate by GS producing glutamine (Wolk *et al*., 1976). Incorporation of N_2-derived ammonium takes place in the heterocysts, but heterocysts lack GOGAT, implying that glutamate has to be received from the adjacent vegetative cells to provide the substrate for GS (Thomas *et al*., 1977; Martín-Figueroa *et al*., 2000). Hence, an exchange of glutamine for glutamate is a possible mechanism of N transfer from heterocysts to vegetative cells (Wolk *et al*., 1976; Thomas *et al*., 1977). The three DDA symbionts have GS, whereas only CalSC01 and RintRC01 contain GOGAT (Hilton, 2014). Based on these observations, another pathway involving glutamate dehydrogenase (GDH) has been proposed for ammonium assimilation in RintHH01 (Hilton *et al*., 2013). However, since GDH is normally a catabolic enzyme (Herrero *et al*., 2019), we propose an alternative hypothesis consisting of uptake of glutamate from the diatom into RintHH01 mediated by amino acid transporter(s). This could be at the basis of a mechanism of glutamine for glutamate exchange operating between the symbiont and host in the DDAs. Furthermore, the increased supply of C, in the form of glutamate, could provide the host with a strategic mechanism to alter the cellular C/N ratio favouring an increase in N_2 fixation by their respective symbionts (see also next paragraph). These suggestions remain to be tested.

In cyanobacteria, the expression of N assimilation genes is regulated by the global transcription factor NtcA. This protein belongs to the CRP (or CAP) family of bacterial transcriptional regulators and orchestrates C/N-regulated processes such as the different N assimilation pathways and heterocyst differentiation and function (Herrero and Flores, 2019). As in the genomes of other planktonic N_2-fixing symbionts (UCYN-A1 and UCYN-A2), RintHH01 and RintRC01 have retained NtcA (RintRC_3237, RintHH_12150) but lack the inorganic N or urea transporters. This may force the *ntcA* gene to be constitutively upregulated, stimulating N_2 fixation in response to the availability of C likely supplied by the host (Zehr *et al*., 2016). Since NtcA is activated by 2-OG (Valladares *et al*., 2008), the possibility that the symbiont is provided with 2-OG by the diatom is appealing.

Because TRAP transporters generally transport carboxylates, uptake of 2-OG could be a function of the symbiont TRAP transporter. In contrast, the external symbiont CalSC01 resembles the model heterocystous cyanobacterium *Anabaena* with the presence of ammonium and nitrate/nitrite transporters (Table 3; Fig. 2B).

Amino acids and peptides

Studies of amino acid uptake in *Anabaena* have shown its capacity to take up amino acids of different chemical groups (Pernil *et al*., 2015). Three ABC transporters are together responsible for most (>96%) of the amino acid uptake activity in *Anabaena*: the N-I transporter for neutral/hydrophobic amino acids (which can also transport other amino acids including glutamate), the N-II transporter for neutral and acidic amino acids, and the Bgt transporter for basic amino acids (the two latter transporters share the NBD component, BgtA; Pernil *et al*., 2008). Of interest for our discussion here, the N-I and N-II systems together are responsible for most of the glutamate import activity in *Anabaena* (Pernil *et al*., 2008). The three symbionts bear genes encoding transporters specifically homologous to N-I and N-II (Table 3; Fig. 2B), suggesting that they can take up glutamate and some other amino acids.

The three symbiont genomes encode a membrane protein with an amino acid permease-associated region (RintHH_4450, RintRC_1364, Ga0265360_112922; see Table 3) that, according to its 3D structure predicted by Phyre2 (http://www.sbg.bio.ic.ac.uk/~phyre2/), contains two domains (Fig. S6). The N-terminal domain shows homology to bacterial amino acid transporters of the APC superfamily (TCDB #2.A.3) including glutamate-γ-aminobutyrate and arginine-agmatine antiporters, and the C-terminal domain is homologous to the cytoplasmic regulatory domain of a cation (Na^+ or K^+)-chloride co-transporter (Warmuth *et al*., 2009) that could exert a regulatory function on the N-terminal transporter. However, recently available protein structures identify a global similarity of these DDA symbiont proteins to the metazoan K^+-Na^+-Cl^- transporter NKCC1 (TCBD #2.A.30.1.4; see data for RintRC_1364 in Fig. S6). Interestingly, NKCC1 may also transport ammonium (Worrell *et al*., 2008), raising the question of whether the symbiont proteins could also have this activity. Similar proteins are not widely distributed in cyanobacteria, but they can be found in a heterocystous strain such as *Rivularia* sp. PCC 7116 or in marine strains such as *Synechococcus* sp. PCC 7002 and the diazotrophs *Cyanothece* sp. ATCC 51142 and *T. erythraeum* IMS101. Of much interest is the presence of a homologue in UCYN-A (Zehr *et al*., 2008; Tripp *et al*., 2010). UCYN-A, or *Candidatus* Atelocyanobacterium thalassa, are marine N_2-fixing unicellular cyanobacteria, of which three strains (UCYNA-1, UCYNA-2 and UCYNA-3) are known to be symbiotic with another single-celled marine eukaryotic microalga (prymnesiophyte) (Thompson *et al*., 2012; Turk-Kubo *et al*., 2017; Foster and Zehr, 2019).

The three heterocystous DDA symbionts have an ABC system annotated as an oligopeptide transporter (Table 3; Fig. 2B). Interestingly, the NBD protein of this transporter (which is a double NBD protein) is most similar to that of a glutathione transporter from *E. coli* (Suzuki *et al*., 2005). Glutathione (GSH) is an antioxidant and functions to prevent damage (Day and Suzuki, 2005). The presence of an oligopeptide transporter that may have GSH as substrate suggests a possible uptake by the symbionts of this protective molecule provided by the host.

Proteins in the drug and metabolite exporter (DME) family are involved in the export of diverse compounds in different organisms, including the export of amino acids in *E. coli* (Jack *et al*., 2001). Each of the three symbionts has at least one DME protein (although the sequence in RintHH01 appears incomplete) that is most similar to *Anabaena* All3551 (Table 3; Fig. 2B). Although this *Anabaena* protein has not been characterized, inactivation of another DME family protein in *Synechococcus elongatus* resulted in altered transport of several amino acids (Escudero *et al*., 2015). It will be of interest to investigate whether the symbiont DME proteins are involved in transport (import or export) of amino acids or other N-containing metabolites.

In summary, whereas the CalSC01 genome encodes transporters such as Amt1 (ammonium) and NrtP (nitrate/nitrite) that should be useful for thriving independently of the diatom, the three symbionts have transporters that may be involved in the import and/or export of N-containing organic compounds (including amino acids and peptides) and may be relevant in the exchange of materials between the two partners in the symbiosis.

Cyanophycin, arginine and polyamines

Polyamines are generally found in most organisms and are essential for heterocyst differentiation in cyanobacteria (Michael, 2016; Burnat *et al*., 2018). Long-chain polyamines are derived from arginine and ornithine precursors of the urea cycle and are necessary for diatoms to mineralize their siliceous frustules (Kröger and Poulsen, 2008; Prihoda *et al*., 2012). It is, therefore, a possibility that polyamines are acquired by the symbionts from the host diatom and/or competed for as a shared necessary resource. Cyanophycin (multi-L-arginyl-poly[L-aspartic acid]) is a reserve material produced by many cyanobacteria and generally found in heterocystous strains, in which cyanophycin granules accumulate at the heterocyst poles (Flores *et al*., 2019a). Nitrogen fixed by

nitrogenase is incorporated into amino acids (Wolk *et al*., 1976), and then into cyanophycin that appears to have a role as a nitrogen buffer (Finzi-Hart *et al*., 2009).

Cyanophycin is synthesized by cyanophycin synthetase (CphA), which adds aspartate and arginine to a cyanophycin primer, and degraded stepwise by (i) cyanophycinase (CphB), which releases β-aspartyl-arginine dipeptide, and (ii) isoaspartyl dipeptidase (IadC), which cleaves the dipeptide into the free amino acids aspartate and arginine (reviewed in Flores *et al*., 2019a). In *Anabaena*, it has been shown that the dipeptidase is produced at significantly higher levels in vegetative cells than in heterocysts, implying the transfer of β-aspartyl-arginine from heterocysts to vegetative cells as an N vehicle; the dipeptide is then split in the vegetative cells, in which aspartate and arginine are used in metabolism (Burnat *et al*., 2014). In cyanobacteria, arginine can be catabolized through a pathway involving arginine-guanidine removing enzyme (AgrE) and proline oxidase (PutA), which globally releases three molecules of ammonium and one of glutamate that can be used in anabolism (Burnat *et al*., 2019). On the other hand, arginine is used for the biosynthesis of *sym*-homospermidine that is the characteristic polyamine of heterocystous cyanobacteria (Hamana *et al*., 1983). The polyamine biosynthesis pathway in *Anabaena* consists of arginine decarboxylase (SpeA), agmatinase (SpeB) and a deoxyhypusine synthase-like homospermidine synthase (SpeY) (Burnat *et al*., 2018).

The external (CalSC01) and internal (RintHH01) DDA symbionts have genes encoding the enzymes of cyanophycin biosynthesis and degradation, whereas the partial DDA symbiont (RintRC01) lacks such genes (CphA, CphB, IadC; Table S2). Lack of cyanophycin in a *R. intracellularis* strain that is a partial DDA symbiont has been noted previously in a transmission electron microscopy study (Janson *et al*., 1995). To the best of our knowledge, this is the first known example of a heterocystous cyanobacterium that does not produce cyanophycin. Consistently, RintRC01 lacks the genes for arginine catabolism (other than *putA* that may have a direct role in proline utilization) and homospermidine biosynthesis (Table S2). In contrast, CalSC01 has a complete arginine catabolism pathway and a possible homospermidine biosynthesis pathway (although it lacks an evident agmatinase, which may reflect incomplete sequencing coverage or imply a variation of the pathway). On the other hand, RintHH01, which can produce and mobilize cyanophycin, does not bear genes encoding the arginine catabolism pathway or a complete homospermidine biosynthesis pathway (Table S2). These observations suggest that the internal endosymbiont (RintHH01) may transfer arginine to its diatom host, from which it may obtain polyamines. The partial symbiont (RintRC01) should also obtain polyamines from the host, whereas the external symbiont (CalSC01) is likely able to produce its own polyamines.

Anabaena has an operon, *potADB*, encoding an ABC polyamine transporter (Burnat *et al*., 2018). A possible *potAD* operon with a *potB* gene found somewhere else in the genome is present in both RintRC01 and CalSC01, whereas RintHH01 has dispersed genes that may encode a somewhat different ABC polyamine transporter (Table 3; Fig. 2B). (Note the low similarity of the putative RintHH01 TMD protein to the *Anabaena* TMD protein [Table 3].) It is interesting that the genes encoding the ABC transporters are congruently configured in RintRC01 and CalSC01 while they are different and dispersed in RintHH01. This may reflect that both RintRC01 and CalSC01 reside external to the potential source of polyamines derived from the host's urea cycle, while RintHH01 has direct access residing within the cytoplasm, and therefore has got a specialized transporter. Interestingly, CalSC01 may be able to obtain polyamines from the host when living in symbiosis and to synthesize its own polyamines when living autonomously.

Other transporters: Fe, S and P transporters

Cyanobacteria have evolved certain transport mechanisms for the uptake of other crucial nutrients such as sources (mainly inorganic) of iron (Fe), sulfur (S) and phosphorus (P), which (at least in the case of Fe and P) are considered as limiting nutrients because of the low concentration or availability in environments where DDAs live. Here, we looked for relevant transporters encoded in the genomes of the three symbionts and discuss their possible role in metabolite exchange with their respective diatom hosts (Tables 4 and 5; Fig. 2C and D).

Iron transporters

Fe is a limiting nutrient for phytoplanktonic growth (Martin *et al*., 1991; Moore *et al*., 2001), in particular for diazotrophs, because of the low solubility of ferric iron (Fe (III) or Fe^{3+}) in aquatic systems and the high Fe requirement for photosynthesis and N_2 fixation (Kustka *et al*., 2003; Chappell *et al*., 2012; Sunda and Huntsman, 2015). Additionally, Fe is vital for the photosynthetic machinery of diatoms, which thrive in some of the most Fe limited regions of the oceans (Moore *et al*., 2001; Armbrust, 2009). Ferrous iron (Fe (II) or Fe^{2+}) also occurs in the surface ocean and is more soluble than Fe^{3+}. Fe acquisition and transport in eukaryotes and prokaryotes is highly complex and largely uncharacterized with the exception of a few model systems. Cyanobacteria have evolved transport mechanisms for the uptake of Fe to cope with the low availability in the environment.

Table 4. Iron uptake transporters encoded in the DDA symbiont genomes.

Query	CalSC01 (external)	RintRC01 (partial)	RintHH01 (internal)	Protein/function
All4025	2790720260 Ga0265390_112310 (8e-17)	RintRC_1461 (2e-36)	RintHH_15140 (1e-34)	SchE, schizokinen exporter
Alr0397	2790721133 Ga0265390_11858 (5e-79) 2790721126 Ga0265390_11851 (2e-73)	nd	nd	SchT, TonB-dependent schizokinen transporter
Alr2581	2790721133 Ga0265390_11858 (5e-79)	nd	nd	TonB-dependent receptor, IutA2
All4026	2790721126 Ga0265390_11851 (2e-73)	nd	nd	TonB-dependent receptor; iron and copper transporter, IacT
All0387 (TMD)	2790721127 Ga0265390_11852 (TMD, 5e-56) 2790721128	RintRC_6052 (NBD, 4e-21)‡	RintHH_5360 (NBD, 2e-22)‡	Iron(III)-siderophore ABC transport system FhuBCD: FhuB (TMD) FhuD (SBP)
All0389 (NBD)	Ga0265390_11853 (NBD, 7e-80)			FhuC (NBD)
All0388 (SBP)	2790721129 Ga0265390_11854 (SBP, 5e-29)			
Alr1382 (SBP)	2790720115 Ga0265390_111426 (SBP, 2e-153)	RintRC_5566 (SBP, 9e-29)	RintHH_17100 (SBP, 1e-139)	Ferric iron ABC transporter FutABC: FutA (SBP) FutB (TMD)
Alr1383 (TMD)	2790720114 Ga0265390_111425 (TMD, 6e-82)	RintRC_5565 (TMD, 5e-59)	RintHH_17080 (TMD, 2e-82) RintHH_17090 (TMD, 1e-134)	FutC (NBD)
Alr1384 (NBD)	2790720113 Ga0265390_111424 (NBD, 3e-103)	RintRC_5564 (NBD, 1e-149)	RintHH_17070 (NBD, 1e-138)	
Alr2583: FecB (SBP, 7e-21)	2790721134 Ga0265390_11859 (SBP, 4e-57)	nd	nd	Iron (Fe^{3+}) or ferric-dicitrate porter *Anabaena* transporter compared with FecBCDE of *E. coli*
All2586: FecC (TMD, 2e-56)	2790721473 Ga0265390_12101 (TMD, 9e-146)			
All2585: FecD (TMD, 3e-74)	2790721127 Ga0265390_11852 (TMD, 5.7e-54)			
All2584: FecE (NBD, 1e-78)	2790721128 Ga0265390_11853 (NBD, 1e-60)			
All3939	2790719355 Ga0265390_10489 (1e-42)	RintRC_2155 (1e-170)	RintHH_17850 (1e-147)	Ferrous iron permease EfeU; Iron/lead transporter (TCDB 2.A.108.2.3)
Alr2118 (4e-24) Alr2119 (1e-22)	2,790,724,058 Ga0265390_14666 (4e-18)	RintRC_5533 (7e-32)	nd	Ferrous iron transporter *Anabaena* transporter compared with FeoB of *E. coli*

ORFs from the symbionts (CalSC01, RintRC01, RintHH01) identified in BLASTp analysis (Expect values indicated in parenthesis) using the indicated protein from *Anabaena* (unless indicated otherwise) as a query. Expect values in *Anabaena* that correspond to comparison to the *E. coli* proteins are provided in parenthesis. Double dagger indicates unlikely identification; nd, not detected.

Siderophores are organic compounds only found in prokaryotes that chelate Fe(III) (Vraspir and Butler, 2009). *Anabaena* is a model of siderophore-secreting cyanobacteria. This cyanobacterium produces schizokinen, an α-carboxylate-hydroxamate siderophore similar in structure to aerobactin secreted by *E. coli* (Simpson and Neilands, 1976), and appears to produce additional siderophores (Jeanjean *et al*., 2008). The secretion of siderophores in *Anabaena* is mediated by the MFS protein SchE and the OM TolC-like protein HgdD (Nicolaisen *et al*., 2010;

Table 5. Sulfur (S) and phosphorus (P) uptake transporters in the DDA symbiont genomes.

Query	CalSC01 (external)	RintRC01 (partial)	RintHH01 (internal)	Protein/function
S transporters				
All0322: Sbp/CysP (SBP, 2e-78)	2790720073 Ga0265390_11105 (SBP, 3e-165)	RintRC_1654 (SBP, 1e-160)	RINTHH_15970 (SBP; 1e-148)	Sulfate ABC transporter *Anabaena* transporter compared with Sbp/CysPTWA of *E. coli*
All0321: CysT (TMD, 1e-73)	2790720072 Ga0265390_11104 (TMD, 2e-158)	RintRC_1652 (TMD, 1e-117)	RINTHH_15950 (TMD, 2e-83)	
All0320: CysW (TMD, 4e-84)	2790720071 Ga0265390_11103 (TMD, 8e-136)	RintRC_1651 (TMD, 1e-147)	RINTHH_15960 (TMD, 3e-48)	
All0126: CysA (NBD, 1e-100)	2790720074 Ga0265390_11106 (NBD, 8e-98) 2790720370 Ga0265390_112917 (NBD, 0.0)	RintRC_0507 (NBD, 0.0)	RintHH_17600 (NBD, 0.0)	
Alr1635 All1304	2790720482 Ga0265390_11353 (0.0) 2790719465 Ga0265390_10577 (0.0)	RintRC_4851 (0.0) RintRC_3892 (0.0)	RINTHH_3970 (6e-77)* [partial sequence, likely completed with RintHH_3990, RintHH_3980, RintHH_3960]	The sulfate permease (SulP) family (TC 2.A.53), possible BicA
Alr1633	Ga0265390_13451 (0.0)	RintRC_3409 (0.0)	RintHH_20770 (0.0)	The Sulfate Permease (SulP) family (TCDB 2.A.53)
P transporters				
All4575: PstS/PhoS (SBP, 4e-80)	2790721358 Ga0265390_12015 (SBP, 0.0)	RintRC_5856 (SBP, 1e-175) RintRC_3236 (SBP, 2e-69)	RintHH_1380 (SBP, 1e-167)	Phosphate ABC transporter PhoT (TCDB 3.A.1.7.1) *Anabaena* transporter compared with Pst system of *E. coli*
All4574: PstA (TMD, 1e-73)	2790721357 Ga0265390_12014 (TMD, 4e-71)	RintRC_5857 (TMD, 8e-94)	RintHH_1370 (TMD, 3e-95)	
All4573: PstC (TMD, 2e-65)	2790721356 Ga0265390_12013 (TMD, 5e-65)	RintRC_5858 (TMD, 1e-125)	RintHH_1360 (TMD, 1e-129)	
All4572: PstB (NBD, 1e-108)	2790721355 Ga0265390_12012 (NBD, 3e-173)	RintRC_1806 (NBD, 1e-169)	RintHH_1350 (NBD, 1e-163)	
All0911 (SBP)	2790720643 Ga0265390_11512 (SBP, 3e-170)	RintRC_1535 (SBP, 1.76e-119)	nd	Phosphate ABC transporter PhoT (TCDB 3.A.1.7.1)
All0910 (TMD)	2790721789 Ga0265390_12441 (TMD, 1e-123)	RintRC_1540 (TMD, 3e-50)*		
All0909 (TMD)	2790721790 Ga0265390_12442 (TMD, 1e-128)			
All0908 (NBD)	2790721791 Ga0265390_12443 (NBD, 6e-142)			
All0907 (NDB)	2790721791 Ga0265390_12443 (NBD, 5e-129)			
Alr1094 (SBP)	2790720020 Ga0265390_11047 (SBP, 6e-173)			
Alr2336	nd	RintRC_5791 (e-178)	RintHH_17980 (e-166)	Low-affinity PiT family transporter (TCDB 2.A.20)
T. erythraeum Ga0074568_ _113154 (SPB) _113153 (NBD)	2790720877 Ga0265390_11656 (SPB, 0.0) 2790720876 Ga0265390_11655	nd	nd	Phosphonate ABC transporter (TCDB 3.A.1.9.1 for *E. coli*)

(Continues)

Table 5. Continued

Query	CalSC01 (external)	RintRC01 (partial)	RintHH01 (internal)	Protein/function
	(NBD, 7e-169)			
_113152 (TMD)	2790720875			
	Ga0265390_11654			
	(TMD, 4e-153)			
_113151 (TMD)	2790720874			
	Ga0265390_11653			
	(TMD, 1e-165)			

ORFs from the symbionts (CalSC01, RintRC01, RintHH01) identified in BLASTp analysis (Expect values indicated in parenthesis) using the indicated protein from *Anabaena* (unless indicated otherwise) as a query. Expect values in *Anabaena* that correspond to comparison to the *E. coli* proteins are provided in parenthesis. An asterisk indicates a possibly incomplete sequence; nd, not detected.

Stevanovic *et al*., 2011). Once Fe(III) is bound to the siderophore in the medium, the complex is taken up by the OM protein SchT (Alr0397), a TonB-dependent transporter (Nicolaisen *et al*., 2008), and then translocated into the cytoplasm by the ABC transporter FhuBCD (Stevanovic *et al*., 2011; Rudolf *et al*., 2016). Additionally, *Anabaena* expresses other TonB-dependent transporters involved in Fe and copper (Cu) uptake including IutA2 (Alr2581; Rudolf *et al*., 2016) and IacT (All4026; Nicolaisen *et al*., 2010). Only the external symbiont CalSC01 contains homologues to siderophore biosynthesis genes (see Table S3), although its genome encodes a protein with only low similarity to the SchE exporter (Table 4; Fig. 2C). Because bacteria often express receptors for siderophores that they do not produce or for Fe contained in their hosts (Braun and Killman, 1998; Ratledge and Dover, 2000), CalSC01 homologues to schizokenin receptor SchT and TonB-dependent transporters IutA2 and IacT (Table 4; Fig. 2C) might be involved in the uptake of siderophores secreted by CalSC01 itself as well as by other bacteria. CalSC01 bears also the ABC-transporter FhuBCD required for Fe^{3+}-siderophore acquisition (Table 4; Fig. 2C). Because siderophores are secreted in response to low dissolved Fe concentration (Wilhelm and Trick, 1994), these transporters may allow CalSC01 to maintain Fe homeostasis in a low Fe concentration environment. Moreover, CalSC01 is expected to acquire Fe through other pathways, as supported by the presence of another Fe^{+3} ABC transporter (homologue to *E. coli* FutABC), a Fe^{3+} or ferric-dicitrate ABC transporter (homologue to *E. coli* FecBCDE), a high-affinity Fe^{2+}/Pb^{2+} permease, and (possibly) a FeoB-like ferrous iron transporter (Table 4; Fig. 2C). Finally, although eukaryotes cannot produce siderophores, some, including diatoms, can access the bound Fe from siderophores (Amin *et al*., 2009; Kazamia *et al*., 2018); perhaps a similar strategy occurs in the *Chaetoceros-Calothrix* symbiosis and the host diatom uses its symbiont's siderophore synthesis for its own Fe acquisition.

RintHH01 and RintRC01 symbionts lack any TonB-dependent transport system and the ABC transporter FhuBCD required for ferric-siderophore acquisition, but their genomes encode homologues of a ferric iron ABC transporter (FutABC homologue), the high-affinity Fe^{2+}/Pb^{2+} permease and only in RintRC01, possibly the FeoB-like transporter (Table 4; Fig. 2C). Residing inside the diatom, the concentration of Fe might be sufficient and apparently, both RintHH01 and RintRC01 symbionts evolved to incorporate inorganic or organic forms of Fe(III) and Fe(II) by pathway(s) different to the siderophore uptake systems, as also reported for *Synechocystis* sp. PCC 6803 and *T. erythraeum* IMS101 (Roe and Barbeau, 2014; Jiang *et al*., 2015). The presence of proteins similar to schizokinen exporter SchE in the three DDM symbionts (Table 4) should be taken with caution, since those proteins are indeed most similar to bacterial AmpG (Expect values <e-100), which is involved in the uptake of cell wall degradation products. In summary, whereas endosymbionts RintRC01 and RintHH01 appear to be non-siderophore-utilizing cyanobacteria having other pathways for Fe homeostasis, CalSC01, living externally attached, appears to be able to use siderophores and have more strategies for Fe uptake.

Sulfate ABC transporter and sulfate permease

Sulfate is a macronutrient, which is required for photosynthetic organisms. Sulfur is present in proteins, lipids, electron transport components and many cellular metabolites. The limited intracellular storage of S implies the uptake, mainly in the form of sulfate anion, from the environment through specific transporters. The main prokaryotic transporter involved in sulfate uptake is in the SulT group of ABC transporters (TCDB #3.A.1.6). This transporter, Sbp/CysPTWA, is responsible for sulfate and thiosulfate uptake in prokaryotes (Saier Jr *et al*., 1999). It is composed of two TMD proteins, CysT and CysW, the NBD CysA protein (homodimer-forming CysA) and the sulfate-binding (Sbp) and thiosulfate-binding (CysP) periplasmic SBPs (Hryniewicz *et al*., 1990; Sirko *et al*., 1990).

Anabaena and the three symbionts bear the genes that encode the components of this transporter (Table 5), which supports the idea that they can take up sulfate.

Proteins of the SulP family are permeases that can transport several different anions including sulfate (TCDB #2.A.53). *Anabaena* and the three symbionts contain several proteins of this family, some of which could be sulfate transporters, whereas others could be bicarbonate transporters as they are most similar to the bicarbonate transporter BicA from oceanic cyanobacteria (Price *et al*., 2004) mentioned earlier (Tables 2 and 5). The identification of the substrate transported by each particular SulP-family protein will be of much interest and requires further investigation.

Phosphate and phosphonate transporters

Similar to Fe, phosphorus (P) is often a limiting nutrient in marine systems since it can be found at low concentrations or even in forms not biologically available (Schindler, 1977; Karl *et al*., 2001). The phosphorus compounds and strategies for acquisition under P limitation have been characterized in some model systems, and some progress has recently been made on marine phytoplankton (reviewed in Lin *et al*., 2016). The preferred form of P for phytoplankton is inorganic phosphate (Pi). However, under the chronically low Pi conditions typical of the oligotrophic ocean where DDAs reside, some phytoplankton utilize dissolved organic phosphorus (DOP) substrates (Cui *et al*., 2015) and/or reduce their cellular P quotas by using non-P lipids in their lipid membranes (Van Mooy *et al*., 2009). DOP utilization has been less studied in diatoms, and most work to date has used alkaline phosphatase activity assays as indicative of DOP utilization or, recently, quantitative profiling of transcripts and proteins under P deficient conditions (Perry, 1976; Dyhrman *et al*., 2006, 2012). In the DDA hosts, it is not known how or which P substrates are utilized.

Cyanobacteria have evolved mechanisms and regulatory adaptations to acquire Pi since it is a crucial requirement to fix N_2 (Bardin *et al*., 1996) and maintain growth. Two major transporters for Pi acquisition in bacteria are the PhoT and PiT systems. The PhoT transporters (Phosphate Uptake Transporter Family; TCDB #3.A.1.7) are high-affinity ABC transporters that are normally expressed in cells growing under low-Pi concentrations (Rao and Torriani, 1990). The *E. coli* PhoT system Pst comprises a periplasmic SBP (PstS), two TMD proteins (PstA and PstC) and the NBD protein (PstB). On the other hand, proteins in the PiT (Inorganic Phosphate Transporter) family (TCDB #2.A.20) normally consist of 10–12 TMSs and mediate transport of Pi—complexed with a metal divalent cation—in a symport mechanism with H^+ or Na^+ ions (Jackson *et al*., 2008). In *E. coli*, PitA and PitB are low-affinity transport systems that function when the external Pi concentration is higher than 20 μM, which represses the Pst system (Rao and Torriani, 1990). *Anabaena* contains two PhoT (All4575 to All4572 and All0911 to All0907) and two PiT (Alr2336 and Alr3096) transporters. Each of the three symbionts contains the components of at least one PhoT ABC transporter that is most similar to the *Anabaena* All4575 to All4572 transporter (Table 5; Fig. 2D). Whereas RintRC01 and RintHH01 bear homologues to the *Anabaena* PiT transporter All2336, CalSC01 lacks this Pi transporter, as do several freshwater strains and most marine picocyanobacteria (Su *et al*., 2007; Scanlan *et al*., 2009) (Table 5; Fig. 2D). Nevertheless, only the CalSC01 symbiont contains an extra gene cluster and two independent genes that together may encode another PhoT family ABC transporter (Table 5; Fig. 2D), resembling the duplicate or multiple Pst systems found in some freshwater and terrestrial cyanobacterial strains (Pitt *et al*., 2010; Hudek *et al*., 2016).

Some cyanobacteria can also utilize phosphonates, as first demonstrated for the marine N_2-fixing cyanobacterium *T. erythraeum* IMS101 (Dyhrman *et al*., 2006). Phosphonates are organic molecules containing a covalent bond between atoms of P and C, which are derived from the degradation of glycolipids, glycoproteins, antibiotics or phosphonolipids (Kolowith *et al*., 2001). Bacteria, some eukaryotes, and plants are known to synthesize phosphonates (Horigushi, 1984; Kugler *et al*., 1990), however, only prokaryotes and some fungi are capable of acquiring phosphonates as a source of P, N or C (Kononova and Nesmeyanova, 2002). In *E. coli*, the ABC transporter PhnCDE mediates the uptake of phosphonates. Importantly, assimilation of phosphonates also requires the hydrolysis of the C—P bond, which in *E. coli* and many other bacteria is carried out by the enzymes of the C-P lyase pathway (Hove-Jensen *et al*., 2014). *Trichodesmium erythraeum* contains the genes encoding the phosphonate ABC transporter (Table 5; Fig. 2D) and the *phnG* to *phnM* genes encoding the C-P lyase pathway (Dyhrman *et al*., 2006). We, therefore, used the *T. erythraeum* genes to look for phosphonate utilization genes in the DDA symbionts [although *Anabaena* also seems to contain the phosphonate utilization pathway (Hove-Jensen *et al*., 2014), it has not been experimentally characterized]. Among the DDA symbionts, only CalSC01 bears the phosphonate transporter (Table 5; Fig. 2D) and some homologues for genes encoding the C-P lyase pathway (although the pathway gene cluster appears to be incompletely sequenced).

In summary, the endosymbionts RintHH01 and RintRC01 contain high-affinity and low-affinity phosphate transporters, whereas the external symbiont CalSC01 contains high-affinity phosphate transporters and a phosphonate utilization pathway. Concentrations of Pi are

often low in the regions in which the DDAs thrive (i.e. 10-year average, 25-m depth in North Pacific subtropical gyre: 70 $\pm$ 39 nmol L^{-1}; Björkman *et al*., 2018); hence the absence of the low-affinity PiT transporter in the external symbiont (CalSC01) is not surprising.

Micronutrient transporters in RintHH01

Given the cytoplasmic location of RintHH01 within the diatom, we checked the presence of possible transporters for micronutrients in this endosymbiont. RintHH01 contains possible transporters for soft metals, including a Na^+:H^+ exchanger (RintHH_10180; TCDB #2.A.36), a K^+ channel (RintHH_22210; TCBD #1.A.1), the KtrAB transporter for K^+ (RintHH_14160, _14170; TCBD #2.A.38.4) and a possible transporter for Mg^{2+} (RintHH_14380; TCDB #1. A.26). RintHH01 also contains possible ABC transporters for Mn^{2+} and/or Zn^{2+} (RintHH_220, _230, _21250 and RintHH_5870, _5880, _5890; both belonging to TCDB #3. A.1.15), Ni^{2+} and/or Co^{2+} (RintHH_19750, _19760, _19770; TCDB #3.A.1.23) and molybdate (RintHH_20340, _20350; TCDB #3.A.1.8). Finally, possible folate-biopterin transporters (RintHH_9790, _1390; TCDB#2.A.71) are also present. The presence of these transporters in RintHH01 identifies some essential micronutrients that the endosymbiont can take up actively from the diatom's cytoplasm.

Proposed C and N fixation and metabolite exchange (C, N, Fe, S and P) model in the diatom-cyanobacterium symbioses

Based on the main knowledge of the symbiosis between diatoms and diazotrophic cyanobacteria and the new insights introduced in this article regarding different transport mechanisms in three symbionts, we propose the following models. In each model, the symbiont cellular location and how this might favour or limit the symbiont acquisition and transport of a particular substrate is considered.

Model for RintHH01

Although RintHH01 resides inside its host, it is expected that the symbiont also performs CO_2 fixation because of the presence of RubisCO, carboxysome genes and an NDH-1 complex (Fig. 3A). (The presence of a bicarbonate transporter, BicA, in RintHH01 is unsure). Of particular interest is the expected concentration gradient of Ci within the host diatom cytoplasm, in which HCO_3^- concentration is predicted to be several-fold higher nearer to the chloroplast (Hopkinson *et al*., 2016; Young and Hopkinson, 2017). RintHH01 is commonly observed surrounded by the host chloroplast (Caputo *et al*., 2019). Interestingly, a competition for C uptake, based on an extra C requirement for N_2 fixation in *Richelia*, could exist between the partners. This competition might rely on the uptake of C compounds from the host mediated by *Richelia* transporters, including the uptake of glucosides by ABC (Gls) or MFS transporters and the uptake of carboxylates such as 2-OG by the TRAP transporter. Moreover, since RintHH01 appears to have the capability of hydrolyzing but not synthesizing sucrose, this disaccharide would provide reduced C to the symbiont. Indeed, an invertase (InvB) is present in RintHH01 that potentially functions in the cleavage into glucose and fructose of sucrose taken up from the host (Fig. 3A).

RintHH01 contains the complete suite of genes to carry out N_2 fixation but lacks any Amt ammonium transporter and the nitrate/nitrite and urea utilization pathways, which defaults the exchange of N-related compounds to the diatom. Therefore, the reduced N compounds must be exchanged by other transporters, such as amino acid ABC transporters N-I and N-II (taking up glutamate and other amino acids), an oligopeptide transporter (possible glutathione transporter), the polyamine ABC transporter, a proline permease, the possible ammonium/amino acid permease RintHH_4450, and the DME permease (with an incomplete sequence) (Tables 2 and 3; Fig. 3A). Due to the lack of GOGAT in RintHH01, we propose the uptake of glutamate from the diatom with the participation of amino acid transporter(s) such as N-I and N-II. Glutamate could be exchanged with glutamine in a process resembling the Gln for Glu exchange between heterocysts and vegetative cells in free-living heterocystous cyanobacteria. Additionally, the presence of cyanophycin metabolism genes but absence of arginine catabolism genes in RintHH01 strongly supports the transfer of arginine from the symbiont to the host. Hence, for N supply to the diatom, glutamine and arginine are candidate vehicles. Regarding Fe, S and P acquisition, RintHH01 presents a low number of transporters with respect to *Anabaena* and CalSC01 (Fig. 2). RintHH01 might acquire Fe through the Fe^{3+} ABC transporter FutABC and the Fe^{2+} permease EfeU. Sulfate transport is identical in all three symbionts and *Anabaena* and characterized by the Sbp/CysPTWA ABC transporter and a SulP permease. RintHH01 is limited to Pi, using a high-affinity Pst (PhoT) ABC transporter and the low-affinity PiT transporter.

Model for RintRC01

Although RintRC01 resides between the cytoplasmic membrane and the frustule of the diatom, it has C and N compound uptake capabilities similar to those in RintHH01. For example, genes encoding Amt ammonium transporters or nitrate/nitrite and urea assimilation pathways are also missing. On the other hand, genes

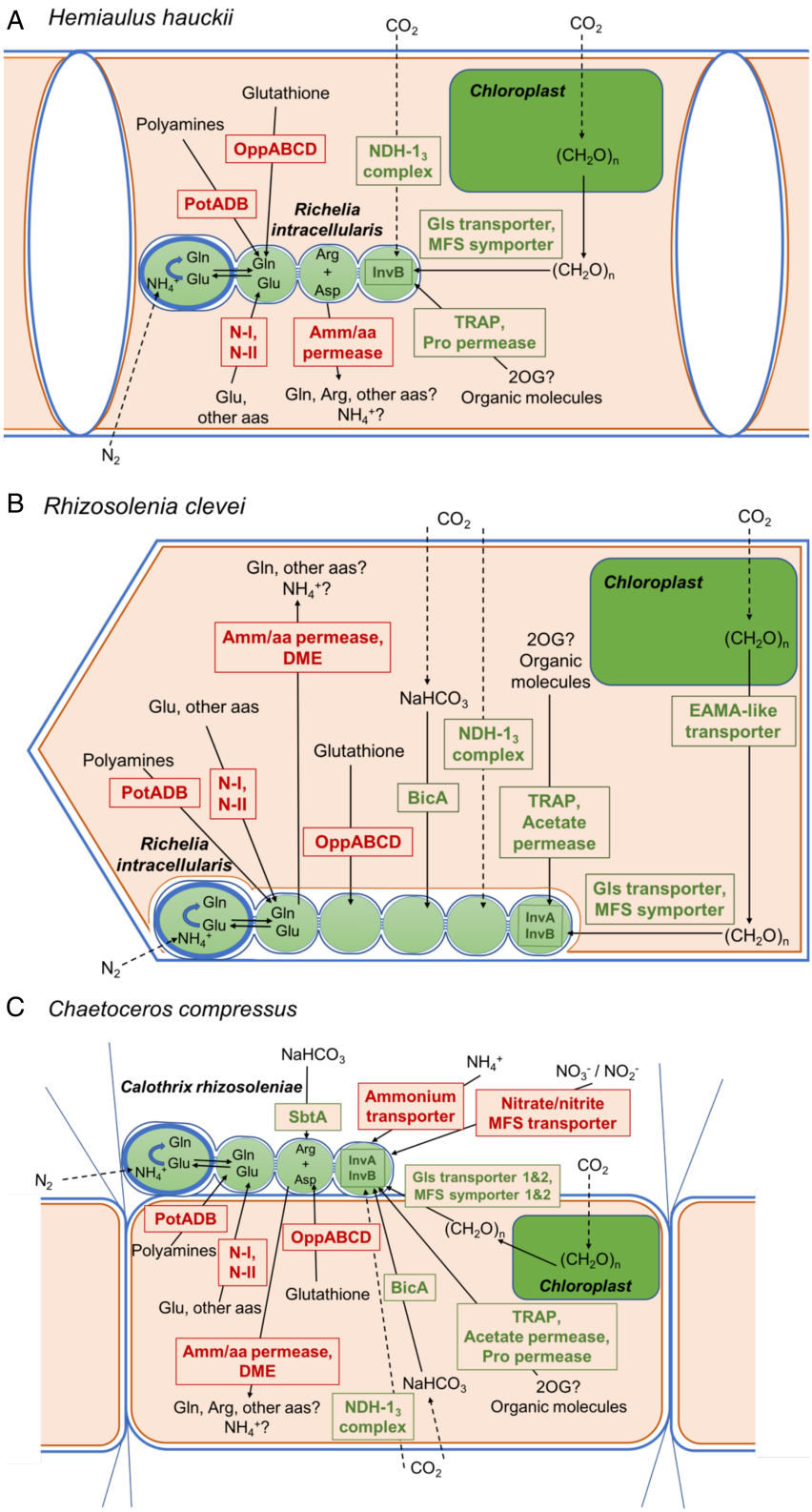

Fig. 3. Proposed models of metabolite exchange and transport mechanisms in the DDAs. The filament of the symbionts is composed of a single terminal heterocyst and a variable number of vegetative cells. Some diatom hosts develop chains. A. Model for the *Hemiaulus hauckii-Richelia intracellularis* (RintHH01) symbiosis. From two to four filaments of *Richelia* per diatom can reside in the host cytoplasm (Bustos-Díaz *et al*., 2019; Caputo *et al*., 2019). Note the white areas showing the spaces between two single diatoms in a chain. B. Model for the *Rhizosolenia clevei-Richelia intracellularis* (RintRC01) symbiosis. The symbiont is located between the diatom plasma membrane and the frustule with the terminal heterocyst close to the diatom valve (Taylor, 1982). Note that the number of vegetative cells in the symbiont is higher than in the two other symbionts. C. Model for *Chaetoceros compressus-Calothrix rhizosoleniae* (CalSC01) symbiosis. The diatom host contains spines to which the symbiont attaches transversely with the heterocyst (Norris, 1961). Symbiont transporters and related proteins are shown framed. 2OG, 2-oxoglutarate; aa(s), amino acid(s); Amm/aa permease, RintHH_4450/ RintRC_1364/ Ga0265390_112922 proteins; DME, Drug/Metabolite Exporter; EAMA, Drug/Metabolite Transporter superfamily protein; Gls, ABC glucoside transporter; MFS, Major Facilitator Superfamily; N-I and N-II, amino acid ABC transporters; TRAP, Tripartite ATP-independent periplasmic Transporter. Other transporters are indicated by their formal names: BicA, NDH-1_3, OppABCD, PotADB and SbtA.

encoding transporters for the uptake of glucosides, amino acids, peptides (possibly glutathione) and polyamines are present, as well as genes encoding possible amino acid exporters including a DME protein and RintRC_1364 that might also transport ammonium. Significant differences with respect to RintHH01 are the presence in RintRC01 of the genes encoding sucrose biosynthesis proteins, a second invertase (InvA) and an acetate permease instead of the proline permease (Fig. 3B). Additionally, the presence of a BicA transporter in RintRC01 is more likely than in RintHH01. On the other hand, RintRC01 lacks cyanophycin making the exchange of arginine less likely than in RintHH01. Iron, S and Pi uptake capabilities are also similar between RintRC01 and RintHH01. Thus, other than some specific metabolites that may be exchanged (e.g. arginine and proline in RintHH01/*H. hauckii*; bicarbonate and acetate in RintRC01/*R. clevei*), the main difference between the symbiotic associations involving RintRC01 and RintHH01 must reside in the physiology of and/or dependency on the host diatom. Whereas RintHH01 takes up nutrients directly from the diatom's cytoplasm, RintRC01 takes up nutrients from the 'periplasmic space', implying that materials that the cyanobacterium obtains from the diatom are exported through the diatom's cytoplasmic membrane. The specific study of export mechanisms in the diatom will be therefore of much interest. The possibility that RintRC01 takes up nutrients more from the external medium (ocean) than from the diatom is not supported by our findings, which show a remarkably similar array of transporters in RintRC01 and RintHH01.

Model for CalSC01

CalSC01 resides external to the frustule of the diatom and can grow as a free-living organism, thus it is a facultative symbiont (Foster *et al*., 2010). Consistently, the membrane transporter repertoire of this symbiont is more similar to that of typical free-living heterocystous cyanobacteria, including high-affinity bicarbonate transporter (SbtA) and ammonium (Amt) and nitrate/nitrite permeases (Fig. 3C). Additionally, in contrast to the endosymbionts, CalSC01 contains a phosphonate utilization pathway and, notably, has the capability of incorporating Fe with a complete concourse of siderophores. Thus, ammonium, nitrite/nitrate, phosphonate and ferric iron-siderophore complexes are most likely obtained from the surrounding marine medium. On the other hand, the external symbiont (CalSC01) has several transporters similar to those of the endosymbionts (Fig. 3), suggesting that it may facultatively use resources obtained from the host's phycosphere (nutrient-rich area surrounding phytoplankton cells), thus broadening its growth options. Compounds obtained from the diatom host could include glucosides, amino acids, peptides (possibly glutathione), polyamines and carboxylates. Regarding N nutrition of the host, CalSC01 might behave similarly to the endosymbionts providing arginine and glutamine (as we hypothesize for RintHH01) or glutamine (which is possible for RintRC01). Additionally, the presence of Amt proteins in CalSC01 suggests the possibility of an exchange of ammonium, which would be more likely in CalSC01 than in the endosymbionts. Given that CalSC01 is capable of growth in the laboratory with reported evidence on solid growth medium (Foster *et al*., 2010), it could potentially be a model system to test the functionality of several of the transporters mentioned here.

Concluding remarks

In this article, we have focused on the membrane transporters encoded in the genomes of globally distributed and biogeochemically significant N_2-fixing symbionts of diatoms with special emphasis on the acquisition of macronutrients. Our findings show that the endosymbionts, either internal or partial (RintHH01 and RintRC01 respectively), contain a similar array of transporters, suggesting a similar dependence on the diatom host physiology, whereas the external symbiont (CalSC01) has transporters similar to those of the endosymbionts and additionally other transporters useful for life in a dilute ocean.

Based on current knowledge, the only role of the cyanobacterial symbionts is to provide their host diatoms with fixed N. Heterocystous cyanobacteria that engage in terrestrial symbioses such as *Nostoc azollae* and *N. punctiforme* release N in the form of ammonium to their plant partners, whereas another *Nostoc* symbiont releases organic N, mainly citrulline and glutamine (reviewed in Meeks and Elhai, 2002). The amount of N_2-derived ammonium released to terrestrial host plants varies tremendously (40%–90% of fixed N) and is largely controlled by the GS activity of the respective symbionts (Meeks, 2009). Currently, the amount of N and the chemical form in which N is transferred in the DDAs is unknown. GS and N_2 fixation activities in the various DDAs are expected to vary given the absence of GOGAT in RintHH01 and retention in the other symbionts. It will be of great interest to determine which form of N (ammonium, amino acids) is transferred from the symbiont and the role of the host in potentially providing glutamate or other C skeletons to influence the extent of N_2 fixation in the symbionts. A possible role of Amt transporters has been discussed in the transfer of ammonium in various symbioses including the *N. azollae* – *Azolla* symbiosis (Roy *et al*., 2020). Here, we have hypothesized transfer of glutamine and arginine in DDAs, but the presence of Amt proteins specifically in CalSC01 and of an NKCC1-type cation transporter that might transport

ammonium in the three symbionts make ammonium a possible N vehicle in at least some DDAs.

In this article, we have proposed a number of transporters encoded in the DDA genomes as responsible for the transfer of specific compounds between symbiont and host. The substrates of many of those transporters can be predicted with reasonable certainty from sequence analysis, but for some other transporters, only the general chemical nature of the substrate can be anticipated. In addition to performing experimental research to corroborate the activity of those transporters whose substrates cannot be defined with certainty, further research is needed. In particular, future work should also address the composition of the diatom's membrane transporter systems, which will be important to understand its nutritional physiology in the marine environment as well as its peculiar symbiosis with the partial and external symbionts, which are likely provided with nutrients by the diatom host.

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Supporting Information

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

Figure S1-S6 Supporting information

Table S1 Presence in the DDA symbionts of some proteins involved in OM protein insertion and LPS synthesis and transport.

Table S2 Proteins of cyanophycin and arginine metabolism encoded in the DDA symbionts.

Table S3 Siderophore biosynthesis proteins encoded in the DDA symbionts.