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Genomic deletions disrupt nitrogen metabolism pathways of a cyanobacterial diatom symbiont

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Diatoms with symbiotic N₂-fixing cyanobacteria are often abundant in the oligotrophic open ocean gyres. The most abundant cyanobacterial symbionts form heterocysts (specialized cells for N₂ fixation) and provide nitrogen (N) to their hosts, but their morphology, cellular locations and abundances differ depending on the host. Here we show that the location of the symbiont and its dependency on the host are linked to the evolution of the symbiont genome. The genome of *Richelia* (found inside the siliceous frustule of *Hemiaulus*) is reduced and lacks ammonium transporters, nitrate/nitrite reductases and glutamine:2-oxoglutarate aminotransferase. In contrast, the genome of the closely related *Calothrix* (found outside the frustule of *Chaetoceros*) is more similar to those of free-living heterocyst-forming cyanobacteria. The genome of *Richelia* is an example of metabolic streamlining that has implications for the evolution of N₂-fixing symbiosis and potentially for manipulating plant–cyanobacterial interactions.

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Cyanobacteria form partnerships with taxonomically diverse hosts that are usually multicellular, and these symbioses are ubiquitous in terrestrial and aquatic environments¹. Cyanobacteria are autotrophic microorganisms and some can convert dinitrogen (N₂) gas to ammonium. Two groups of understudied planktonic symbioses are the partnerships between marine diatoms and the heterocyst-forming cyanobacteria, *Richelia intracellularen* and *Calothrix rhizosoleniae* (Fig. 1a–c).

Richelia and *Calothrix* species convert N₂ and transfer the fixed N to their host². *Richelia* and *Calothrix* associate with different hosts and also differ in cellular location (internal versus external), implying different life histories and mechanisms for nutrient exchanges with their partners. The *Richelia* symbionts of the diatom genera *Rhizosolenia* and *Hemiaulus* reside inside the diatom cell wall and are passed on to the next generation of the host³. The *Rhizosolenia* symbiont is outside the plasmalemma in the periplasmic space³; the *Hemiaulus* symbiont's location is unknown. In contrast, *Calothrix* attaches externally to *Chaetoceros* spp. and can be cultured without the host diatom in N-deplete media⁴. Reports of free-living *Richelia* may be a result of broken diatoms^{5,6}, whereas *Calothrix* have been observed as individual trichomes in the plankton^{7,8}. The mechanism of formation of a *Calothrix*–*Chaetoceros* association and whether the symbiont is transmitted to the next generation is unknown.

We compared the genomes of two of the *Richelia* internal symbiont strains (*R. intracellularen* HH01, RintHH, symbiont of *Hemiaulus hauckii* and *R. intracellularen* HM01, RintHM, symbiont of *H. membranaceus*) with that of the external symbiont *Calothrix rhizosoleniae* SC01 (CalSC). We found that genome size and content, especially N metabolism genes, differed substantially, suggesting the cellular location (intracellular versus extracellular) has dictated varying evolutionary paths and resulted in different mechanisms involved in maintaining the symbiosis (Table 1).

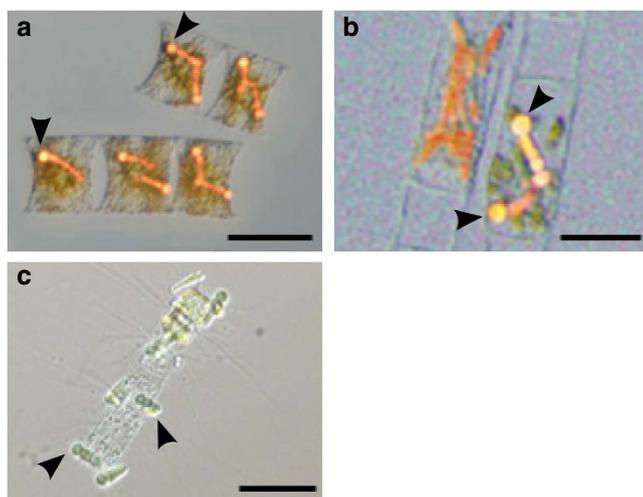


Figure 1 | Cyanobacteria in symbiosis with diatoms. Photomicrographs of cyanobacterial symbionts (denoted by arrows) representative of those sequenced in this study with host diatoms. Differential interference contrast bright field overlaid with blue light epifluorescence images of the diatoms *H. membranaceus* (a) and *H. hauckii* (b), with intracellular cyanobacterial symbionts. Bright-field microscopy image of epiphytic cyanobacterial symbiont *C. rhizosoleniae* SC01 attached to the host diatom *Chaetoceros* sp. (c). Scale bars, 50 μ m.

Results

General features of the diatom symbiont genomes. On the basis of the 16 rRNA and *ntcA* gene sequences, the diatom symbionts cluster within the cyanobacterial Order Nostocales (Fig. 2), but their genome sizes vary greatly (RintHH, 3.2 Mb; CalSC, 6.0 Mb; Table 1). The percent coding information of the CalSC genome is only slightly lower than the free-living Nostocales members, whereas the RintHH genome percent coding is further reduced, similar to '*Nostoc azollae*' 0708 (Table 1). Similarly, the RintHH genome GC content and transporter count are lower than any other genome in the Order, whereas the CalSC genome is a more characteristic Nostocales genome in each respect (Table 1).

The genome of RintHM, the symbiont of *H. membranaceus*, a diatom that is closely related to *H. hauckii*, is only 2.2 Mb and is lacking a number of sequences expected of a full genome (including transfer RNAs for four amino acids and several nitrogenase genes). Therefore, we believe it is a partial genome, likely due to low-sequencing coverage (average depth of coverage 13 \times). However, 16S rRNA and *ntcA* sequences confirm the morphologically similar symbionts are also related genetically (Fig. 2), as previously demonstrated by *nifH* and *hetR* sequences^{9,10}. In addition, analysis of the contigs showed that there are no evident gene insertions/deletions or genome rearrangements between the two *Hemiaulus* sp. symbiont genomes. The 1,671 shared genes of the symbionts average 97.5% sequence identity (DNA) (Supplementary Fig. S1) and show no significant difference in the GC content of the genes sequenced.

Nitrogen metabolism of the diatom symbionts. Given its small size, the RintHH genome is highlighted by many gene deletions, including numerous genes important in N metabolism, such as the transporters for ammonium and nitrate, and the genes encoding nitrate and nitrite reductases (Fig. 3). The diatom symbiont genomes are each missing genes that encode urea transporters and urease, which are functional in all previously sequenced Nostocales genomes, except for the genome of '*N. azollae*' 0708 (ref. 11).

The most unusual gene deletion in RintHH is the gene for an important enzyme in C and N metabolism, glutamate synthase, also known as glutamine:2-oxoglutarate aminotransferase (GOGAT). This enzyme is part of glutamine synthetase (GS)-GOGAT (GS-GOGAT), a generally universal pathway for high-affinity N assimilation (found in all other sequenced cyanobacterial genomes¹², including CalSC and '*N. azollae*' 0708), which uses glutamine, synthesized by GS, and a C skeleton, 2-oxoglutarate, to produce two glutamate molecules. The glutamate produced by GOGAT is then recycled for further ammonium assimilation by GS. The gene encoding GS is present and functional in each symbiont genome; however, they are each lacking a gene that encodes a GS-inactivating factor that is found in all previously sequenced Nostocales genomes (asl2329 in *Nostoc* sp. PCC 7120).

The multiple N metabolism genes missing from the RintHH genome are common to, and widely dispersed across, the genomes of all closed Nostocales genomes (Supplementary Fig. S2). Given this, and the high-sequencing coverage of the RintHH draft genome (average depth of coverage >40 \times), it is unlikely that the missing genes are actually present in the RintHH genome. The RintHH genome does contain a tRNA for each of the 20 amino acids, as expected from a complete genome. Other sequences expected to be present are also in the assembly, such as the previously studied genes *hetR* and *nifH*^{9,10}, and genes responsible for known characteristics of RintHH, such as nitrogen fixation, heterocyst formation, and phycoerythrin and chlorophyll

Table 1 | Nostocales genomes statistics.

| Cyanobacterium | Accession number | Symbiotic state | Size (Mb) | Percent GC | Percent coding | TCs |
|---|------------------|-----------------|-----------|------------|----------------|-----|
| <i>Anabaena variabilis</i> ATCC 29413 | PRJNA10642 | Free-living | 7.1 | 41 | 82 | 570 |
| <i>Nostoc punctiforme</i> PCC 73102 | PRJNA216 | Facultative | 9.1 | 41 | 77 | 575 |
| <i>Nostoc</i> sp. PCC 7120 | PRJNA244 | Free-living | 7.2 | 41 | 82 | 559 |
| ' <i>Nostoc azollae</i> ' 0708 | PRJNA30807 | Obligate | 5.5 | 38 | 52 | 286 |
| <i>Raphidiopsis brookii</i> D9 ^a | PRJNA40111 | Free-living | 3.2 | 40 | 86 | 300 |
| <i>Cylindrospermopsis raciborskii</i> CS-505 ^a | PRJNA40109 | Free-living | 3.9 | 40 | 85 | 344 |
| <i>Nodularia spumigena</i> CCY 9414 ^a | PRJNA13447 | Free-living | 5.3 | 41 | 82 | 428 |
| <i>Richelia intracellularis</i> HH01 ^a | PRJEA104979 | Obligate | 3.2 | 34 | 56 | 190 |
| <i>Calothrix rhizosoleniae</i> SCO1 ^a | PRJNA19291 | Facultative | 6.0 | 39 | 76 | 400 |

TC, transporter classification.
^aGenome is in a draft state.
 Available genomes of the Order Nostocales.

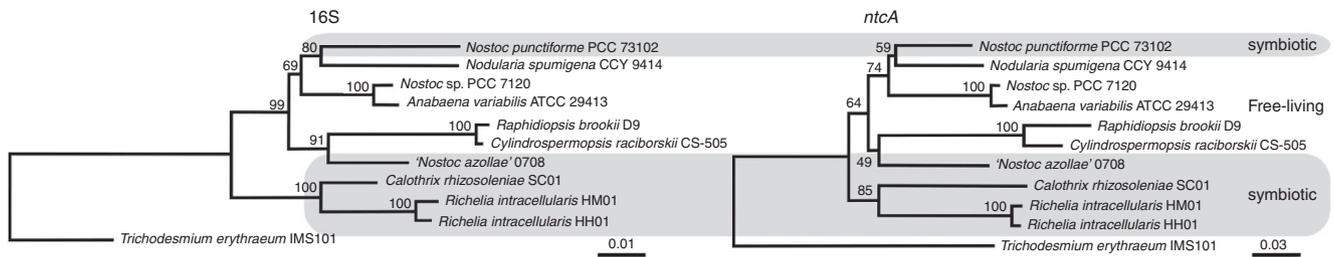


Figure 2 | Nostocales phylogeny. Neighbor-joining phylogenetic trees of 16S rRNA and *ntcA* sequences from seven previously sequenced cyanobacteria and three additional diatom symbionts from this study. The organisms observed in symbiotic relationships are shaded in grey. Both trees are rooted with *T. erythraeum* IMS101. Locus tags (16S, *ntcA*): '*N. azollae*' 0708 (Aazo_R0008, Aazo_1065), *R. brookii* (CRD_01297,CRD_00550), *C. raciborskii* CS-505 (CRC_01246, CRC_00858), *N. sp.* PCC 7120 (allrr01, alr4392), *N. spumigena* CCY9414 (N9414_r17988, N9414_19492), *N. punctiforme* PCC 73102 (Npun_r020, Npun_F5511), *A. variabilis* ATCC 29413 (Ava_R0006, Ava_3283), *T. erythraeum* IMS101 (Tery_R0014, Tery_2023), *C. rhizosoleniae* SCO1 (CSC01_11477, CSC01_6586), *R. intracellularis* HH01 (RintHH_r10, RintHH_12150), *R. intracellularis* HM01 (RintHM_3660, RintHM_9700).

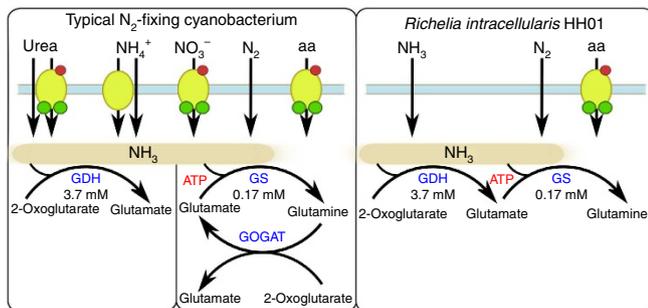


Figure 3 | Overview of the limited N pathways of *Richelia*. Nitrogen metabolism pathways common in N_2 -fixing cyanobacteria compared with *R. intracellularis* HH01. Redrawn from refs 33,34. K_m -values for GDH and GS are for ammonia in each reaction from the N_2 -fixing cyanobacterium *Synechocystis* PCC 6803 (refs 18,35).

pigments. Moreover, to decrease any possible bias during the process, two RintHH samples were separately sorted, amplified and sequenced.

The majority of the N metabolism genes show no similarity to the RintHH genome. However, an intergenic sequence, which contains a small predicted hypothetical protein, has a top hit to the *Raphidiopsis brookii* GOGAT-encoding gene in the NCBI database of non-redundant (nr) protein sequences (BLASTx, E -value = $3e^{-14}$; Fig. 4). The intergenic sequence covers less than 20% of the GOGAT gene and aligns in all three unidirectional frames. This intergenic region is found downstream of two genes that are part of a conserved region downstream of the GOGAT

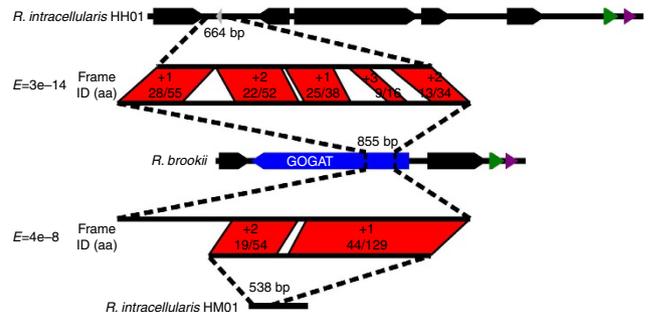


Figure 4 | GOGAT gene remnants in *Richelia*. The intergenic space between two *R. intracellularis* HH01 ORFs, including an annotated 120 bp hypothetical protein (in grey, RintHH_15420) compared with the gene encoding GOGAT in *Raphidiopsis brookii* D9 (in blue, CRD_00957) and the genomic context of each. The middle alignment represents the top BLAST hit of the *R. intracellularis* HH01 intergenic space (1561 bp) in the nr database (BLASTx, E -value = $3e^{-14}$). The green and purple genes encode a mannose-6-phosphate isomerase and a probable iron binding protein, respectively.

gene in Nostocales genomes. A single contig from the RintHM genome also shows similarity to the GOGAT gene in the same manner (Fig. 4).

Gene interruptions on the diatom symbiont *nif* operon. The similarities with other heterocyst-forming cyanobacteria include the presence of insertion sequences in the middle of RintHH and CalSC N_2 -fixation genes^{13,14}. The RintHH *nifH* gene is

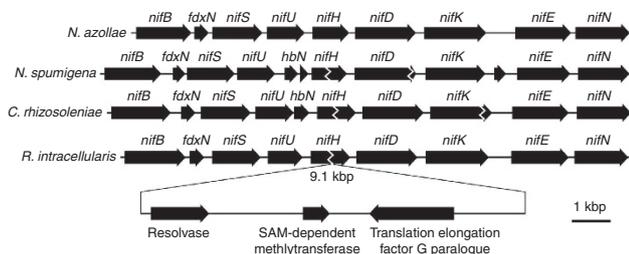


Figure 5 | Nitrogenase gene organization. The *nif* operon and surrounding genes of *R. intracellularis* HH01 and *C. rhizosoleniae* SCO1 compared with other representative Nostocales cyanobacteria ‘*Nostoc azollae*’ 0708 and *Nodularia spumigena* CCY9414, and the genes along the insertion element interrupting the *R. intracellularis* HH01 *nifH* gene.

interrupted in this manner by a 9.1-kb sequence (Fig. 5). The CalSC *nifH* and *nifK* genes are each interrupted in the same manner by longer sequences (each >20 kb). The *nifH* interruptions in RintHH and CalSC appear to occur at the same location within the *nifH* gene; however, the CalSC *nifH* element is at least twice as long as that in the RintHH genome. Recombination genes found on each *nifH* elements show high similarity to each other (71% ID, protein) and are presumably the mechanisms for excision of the element during heterocyst formation.

Discussion

To date, the intracellular RintHH genome is the smallest N_2 -fixing, heterocyst-forming cyanobacteria genome sequenced. Within Nostocales, the *R. brookii* D9 genome is slightly smaller than that of RintHH, but it is unable to form heterocysts or fix N_2 (ref. 15). In contrast, the CalSC genome is similar in size and content to the genomes of free-living organisms in this Order and *N. punctiforme*, a facultative, or opportunistic, symbiont.

The genome reduction in RintHH, marked by its size, percent coding and GC content, is similar to that of ‘*N. azollae*’ 0708, the obligate, or host-dependent, symbiont of the water fern *Azolla filiculoides*¹¹. These features are commonly exhibited by genomes of obligate symbionts, indicating that RintHH is also dependent on its host. Obligate symbionts have more unnecessary genes than free-living or facultative symbiotic organisms due to metabolic redundancy encoded by the host genome and the lack of full exposure to the environment¹⁶. Examples of genes dispensable to obligate symbionts may be those absent or non-functional in both RintHH and ‘*N. azollae*’ 0708, but present in other heterocyst-forming cyanobacteria genomes (Supplementary Table S1). Decreased evolutionary pressure to keep functional genes leads to a lower percent coding and eventually to genome size reduction as non-functioning genes are deleted. The smaller genome leads to accelerated sequence evolution, increasing AT bias¹⁶. The lack of CalSC genome reduction may be taken as evidence that this organism is an opportunistic partner. This is consistent with the external location of CalSC on the diatom setae (spine-like projections) and the ability to maintain it in culture independent of the host diatom in filtered seawater-based media⁴. In contrast, RintHH lives inside the host diatom cell wall, and possibly even within the cytoplasm, with little or no exposure to the external environment, and thus the genome reduction is consistent with that of an obligate symbiont.

The numerous absent N metabolism genes appear to have been selectively deleted from multiple regions throughout the RintHH genome. The lack of ammonium transporters and enzymes required to take up and assimilate urea or nitrate limits the possible N sources for RintHH to amino acids, N_2 , and passive

diffusion of ammonia in oceanic environments, where concentrations of amino acids and ammonium are extremely low. Therefore, deletions in N metabolism genes ensure N_2 fixation within the partner diatom persists, and is likely important for maintaining the symbiotic partnership.

The lack of GOGAT, on the other hand, likely streamlines host–symbiont interactions and seems to be a more recent deletion than the other N metabolism genes, given the similarity between GOGAT genes and intergenic space in the RintHH genome. Without GOGAT, RintHH must use an alternate pathway for assimilation of N_2 -derived ammonium with glutamate dehydrogenase (GDH; Fig. 3), unless the host diatom provides glutamate for the symbiont. In contrast, GS-GOGAT is the main N assimilation pathway used by *Anabaena azollae* in obligate symbiosis with host *Azolla caroliniana*, and very little N is assimilated through GDH¹⁷. Given the high N_2 fixation rates by the cyanobacterial symbiont when associated with the host diatom², it is feasible that intracellular ammonium concentrations are elevated and facilitate assimilation by the low-affinity GDH enzyme¹⁸. However, an adequate concentration of 2-oxoglutarate would also be needed to support ammonium assimilation. If these C skeletons are provided by the host, as in the *Nostoc–Gunnnera* symbiosis¹⁹, the symbiont may perceive the increase of intracellular C:N as N starvation²⁰, causing continued N_2 fixation by the cyanobacterium. Thus, the lack of GOGAT eliminates a common metabolic pathway and creates an N exchange pathway between host and symbiont that provides the host with a way to regulate the symbiont’s growth and activity.

The lack of a GS-inactivating factor streamlines N metabolism further in RintHH. GS catalyses the conversion of glutamate to glutamine, and without an inactivating factor it will maintain low intracellular glutamate concentrations. The subsequent increasing glutamine pool may indicate this amino acid is the form of N passed to the host. The absence of this regulator shows parallels between the *Richelia–Hemiaulus* and *Calothrix–Chaetoceros* associations, and separates the diatom symbionts from other heterocyst-forming cyanobacteria.

However, with regard to N metabolism, the similarities are minimal and the fundamental differences between the RintHH and CalSC genomes reflect the evolutionary selection of their metabolic interactions and cellular locations with the partner diatom. The extracellular CalSC symbiont is exposed to the open ocean environment at all times, and can therefore use a suite of dissolved inorganic nitrogen sources, albeit at low concentrations. Furthermore, the CalSC genome possesses a gene to encode GOGAT and, thus, the symbiont is capable of assimilating N through the high-affinity GS-GOGAT, in addition to GDH. However, a scenario for enhancing N_2 fixation by C transfer from the diatom to the external symbiont CalSC, as hypothesized for the *Richelia–Hemiaulus* association, would require a direct host–symbiont transport system. Otherwise, the extracellular C would likely be diluted immediately and available to other microorganisms. Thus, the extracellular location of CalSC on *Chaetoceros* spp. likely requires different mechanisms for N metabolism and exchange than intracellular RintHH. The differences in genome content and metabolic potential reflect the differences between obligate and facultative symbionts.

Many heterocyst-forming cyanobacteria have DNA sequences interrupting N_2 fixation-related genes in vegetative cells, which are excised during genome rearrangements coincident with heterocyst development²¹, but the functional significance and evolutionary origin of these elements are unknown. These interrupting sequences have been seen previously in several genes^{13,14,22}, but the CalSC genome is the first example of a *nifK* element. The location of elements within *nifH* and high similarity between the genes likely responsible for the excision of the

interrupting sequence are the only apparent similarities between the two *nifH* elements in these closely related cyanobacteria. Although their similarities indicate the *nifH* elements in each organism have the same evolutionary origin, there seems to be little evolutionary pressure on the contents and length of the element.

The characteristics of the genomes of symbiotic heterocyst-forming cyanobacteria reflect the differences in cellular location and host dependency. The absence of basic N metabolism enzymes and transporters in the RintHH genome streamline it, while maintaining the association and providing a mechanism for host regulation of the symbiont. In contrast, the genome of CalSC has few deletions relative to free-living heterocyst-forming cyanobacteria. The differences between genomes suggest mechanisms that may be important in defining facultative or obligate symbioses, with implications for the biology and ecology of these widespread symbiotic associations in the sea. Furthermore, differences in the genomic composition of morphologically and taxonomically similar microorganisms provides an important example of how one partner's metabolic capabilities can evolve with a symbiosis. Finally, the genomes reported in this study, in addition to other recent discoveries of extensive metabolic streamlining in N_2 -fixing cyanobacteria²³, yield the possibility of yet undiscovered plants or algae containing N_2 -fixing organelles.

Methods

***H. hauckii* and *H. membranaceus* symbiont DNA preparation.** Stable *Hemiaulus-Richelia* cultures, isolated from the western Gulf of Mexico, were grown in N-free YBC-II medium at 25 °C (ref. 24), filtered on to a 3- μ m pore size, 25-mm diameter polyester filter (Sterlitech) and frozen for storage. TE buffer (1 \times) was added, and once the filter thawed the cells were resuspended by vortexing for 1 min. The majority of diatoms were broken at this stage, releasing the symbionts in the process. Samples were then analysed on the Influx flow cytometer and cell sorter (BD Biosciences), and cyanobacteria cells were distinguished from other cells by their phycoerythrin pigmentation (Fig. 6). For the *H. hauckii* symbionts, the vegetative trichomes and heterocysts had separated during the sample preparation and the cells formed separate populations on the flow cytometer based on slightly different chlorophyll and phycoerythrin signatures (Fig. 6). Sorting gates, defined by relative pigment values, allowed for the isolation of vegetative cells from the rest of the sample. Two replicate sorts of 5,000 symbiont vegetative trichomes (3–5 cells

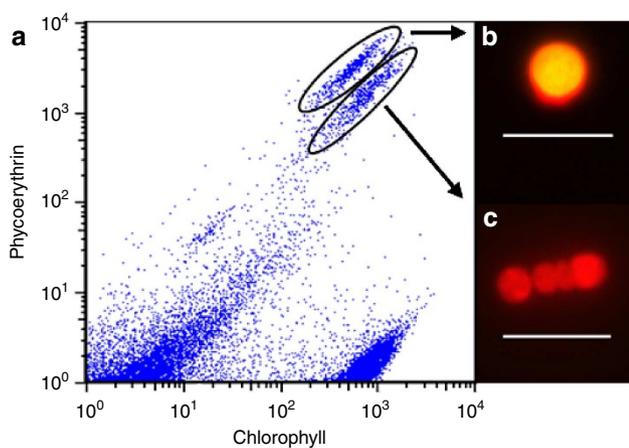


Figure 6 | Flow cytometric analysis of *R. intracellularis*-*H. hauckii* culture samples. A cytogram displaying events gated based upon chlorophyll (692/40 nm) and phycoerythrin (572/27 nm) detection channels with the cyanobacteria symbiont populations easily separated (a). Insets are microscopy images under blue excitation of a vegetative trichome (b) and a heterocyst cell (c) representative of circled populations. Scale bars, 10 μ m. The vegetative trichome population was sorted for genome sequencing samples.

per trichome) were sorted. Genomic DNA in each sample was amplified by multiple displacement amplification using the Repli-g Midi kit (Qiagen). The manufacturer's protocol for 0.5 μ l of cell material was followed with one exception: after buffer D2 was added, the samples were incubated for 5 min at 65 °C and then put on ice for 1 min, instead of 10 min on ice without a 65 °C incubation.

To ensure uncontaminated samples, each amplified DNA sample was PCR-amplified using universal 16S rRNA primers 27F (5'-AGAGTTTGATCMTGGCTCAG-3') and 1492R (5'-GGTTACCTGTTCACGACTT-3')²⁵. The PCR was carried out in 50 μ l reactions consisting of 1 \times PCR buffer, 2 mM MgCl₂, 200 μ M dNTPs, 0.2 μ M of each primer and 1.5 U of Platinum Taq DNA polymerase (Invitrogen). A touchdown PCR was performed as follows: an initial denaturing step at 94 °C for 5 min, followed by 30 cycles of three 1-min steps (denaturation at 94 °C, annealing at 53–41 °C and elongation at 72 °C) and a final elongation step at 72 °C for 10 min. The first cycle annealing took place at 53 °C and was lowered by 0.4 °C for each cycle to reach 41 °C for the final cycle. Resulting products were run on a 1.2% agarose gel, the distinct bands of ~1,500 bp were excised and then recovered using the Zymoclean DNA Recovery Kit (Zymo Research). The recovered DNA was then ligated and plated for blue/white screening using the pGem-T and pGem-T Easy Vectors Systems (Promega). Twenty-four colonies per sample were picked and grown overnight at 37 °C in 2 \times LB media with carbenicillin (200 μ g ml⁻¹). The Montage Plasmid Miniprep_{HTS} Kit (Millipore) was used following the manufacturer's instructions for the full lysate protocol for plasmid DNA miniprep. Samples were sequenced at UC-Berkeley DNA Sequencing Facility and each sequence was subject to BLAST analysis against the nt database (BLASTn). All sequences were identical and had top hits to 16S rRNA sequences of heterocyst-forming cyanobacteria, confirming no contaminant genomes were present in the samples.

DNA concentration and quality were checked (Agilent 2100 Bioanalyzer, Agilent Technologies) before submission for 454 Titanium sequencing (Roche) at the UCSF Genome Technology Center.

The symbionts of *H. membranaceus* were processed in the same manner, but heterocysts and vegetative cells did not separate during sample preparation, and both cell types were present in the sorted samples. Moreover, we were confident from flow cytometry that the cell preparation was pure enough to determine the comparative features we were looking for, and that we would be able to distinguish between the closely related symbiont and the few bacteria that could be carried through by flow cytometry. Therefore, no contamination or DNA quality checks were performed in preparation of the RintHM samples.

***H. hauckii* and *H. membranaceus* symbiont genome assembly.** A total of 433,028 reads were sequenced from RintHM samples. The reads assembled to nearly 8 Mb and the assembly contained four 16S rRNA sequences with low similarity to each other (<83% ID), indicating multiple DNA sources in the data. RintHM contigs were defined as those which had a better BLAST hit to RintHH than to any other organism in the nt database. The resulting 2,212,909 bp (941 contigs, coverage depth 13 \times) were made up of 77,324 reads averaging 380 bp each. An additional 31 contigs, totalling 97,821 bp, had a top hit in the nt database to a cyanobacterium other than RintHH, but none of those contigs contained any of the N metabolism genes of interest.

The two RintHH samples yielded a total 409,035 reads, averaging 344 bp each. The read data were pooled and assembled into 3,243,759 bp in 90 contigs (coverage depth 43 \times) and appeared to be non-contaminated. There are seven contigs longer than 100 kbp, an additional 32 contigs longer than 25 kbp (Supplementary Fig. S3) and 91% of bases with 15 \times coverage or greater.

CalSC DNA preparation. CalSC genomic DNA was extracted from pelleted cells using a sucrose lysis protocol, including the optional back extraction²⁶. The exceptions to this protocol were our use of 10% SDS in the lysate for Fraction B instead of 20% SDS and the 1-h incubation of Fraction B after adding the lysate was at 37 °C rather than 55 °C. The genomic DNAs from Fraction B were pooled and divided into three equal volume samples. The three genomic extracts were checked for purity and quantity (Agilent 2100 Bioanalyzer, Agilent Technologies), and the DNA concentrations ranged between 38.37 and 66.38 ng μ l⁻¹. Samples were then submitted to JCVI for 454 sequencing.

CalSC genome assembly. Once the read data from JCVI (2,477,040 reads, 968 MB) were assembled, the number of contigs (69,919) and size of the assembly (81.4 Mb) immediately suggested that more than one organism was in the sequencing samples. The longest contig of 1.2 MB in length contained a full-length rRNA operon predicted by RDP (Ribosomal Database Project) to be a Planctomycete, confirming the presence of organisms other than CalSC. A plot of the number of reads on each contig against the length of the contig showed strong linear relationships (Supplementary Fig. S4), representing defined clusters of coverage depth, based on the relative abundance of the each organism's genome in the sample. Spot-checking the phylogeny of BLASTn results for long open reading frames (ORFs) on long contigs revealed that the contigs lying along the line marked in red (representing a coverage depth of 30 \times) were those that came from CalSC (Supplementary Fig. S4). Each predicted ORF >450 bp on contigs with depth of coverage 15–45 \times was subject to BLAST analysis against the nt database. A contig

was considered to be part of the CalSC genome if at least one of these ORFs on the contig had a top hit to a cyanobacterial sequence, and 471 contigs met this criterion (5,967,587 bp). One additional contig (5,416 bp) containing the rRNA operon was added. It had been overlooked initially due to its lack of ORFs and its relatively higher coverage depth ($71\times$, indicating it is present in two copies in the genome). The end result was a 5,973,003 bp genome composed of 472 contigs ($30\times$ coverage depth).

Genomic analysis. After assembly and contamination screening, the genomes were submitted to RAST (Rapid Annotation using Subsystem Technology)²⁷ for annotation.

The nitrogen metabolism genes not found in the RintHH genome were pulled from each Nostocales genome, and each gene was subject to BLAST analysis against a database of all 409,035 reads (tBLASTn, $e < 10$). Two thousand seven hundred and twenty reads had hits at least 25% identical (AA) across at least 50% the length of the read or gene, whichever was shorter. A BLAST analysis of each of these reads against the nr database was performed (BLASTx, $e < 10$). Twenty-one reads had a top hit to a GOGAT-encoding gene, and each of these reads is assembled into the intergenic region discussed below as likely GOGAT remnants in the RintHH genome. No other reads had a top hit in the nr database of a nitrogen metabolism-related gene.

Predicted ORFs in each genome with a BLAST hit in the Transporter Classification database²⁸ (BLASTp, E -value $< 1E-19$) were counted as transporter genes.

For the 16S rRNA and the *ntcA* phylogenetic trees, nucleotide sequences were acquired from DOE Joint Genome Institute for each of the seven previously sequenced Nostocales genomes and *Trichodesmium erythraeum* IMS101, and were aligned with the sequences from the three diatom symbiont genomes using Clustal W²⁹ (1,421 bp, 16S rRNA; 646 bp, *ntcA*). Phylogenetic analyses were rendered in Mega5 (ref. 30) using the Neighbor-Joining method³¹. The Tamura–Nei test was run to detect the best models. Statistical support for nodes was based on 1,000 bootstrap replicates³².

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Author contributions

J.A.H., R.A.F., J.P.Z. and T.A.V. designed the study and wrote the paper; T.A.V. and R.A.F. grew and collected cultures; J.A.H. and B.J.C. sorted symbiont cells; J.A.H. and H.J.T. assembled and analysed the genomes.

Additional information

Accession codes: The genomes described in this study have been deposited at the European Nucleotide Archive (ENA) under accession numbers CAIY01000001 to CAIY01000090 (RintHH), CAIS01000001 to CAIS01000941 (RintHM) and SRX023670 (CalSC).

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