Establishment of stable synthetic mutualism without co-evolution between microalgae and bacteria demonstrated by mutual transfer of metabolites (NanoSIMS isotopic imaging) and persistent physical association (Fluorescent in situ hybridization)

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The demonstration of a mutualistic interaction requires evidence of benefits for both partners as well as stability of the association over multiple generations. A synthetic mutualism between the freshwater microalga Chlorella sorokiniana and the soil-derived plant growth-promoting bacterium (PGPB) Azospirillum brasilense was created when both microorganisms were co-immobilized in alginate beads. Using stable isotope enrichment experiments followed by high-resolution secondary ion mass spectrometry (SIMS) imaging of single cells, we demonstrated transfer of carbon and nitrogen compounds between the two partners. Further, using fluorescent in situ hybridization (FISH), mechanical disruption and scanning electron microscopy, we demonstrated the stability of their physical association for a period of 10 days after the aggregated cells were released from the beads. The bacteria significantly enhanced the growth of the microalgae while the microalgae supported growth of the bacteria in a medium where it could not otherwise grow. We propose that this microalga-bacterium association is a true synthetic mutualism independent of co-evolution.

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1. Introduction

Mutualism, an interaction where two organisms benefit from an association, is ubiquitous in nature [61]. Natural mutualism usually evolves over a prolonged association between the partners and is very stable [6], spanning multiple generations. Most mutualistic interactions are inherently complicated by multiple variables and, therefore, it can be difficult to isolate their components for specific studies and determine if they are truly mutualistic [24]. Synthetic biology proposes to increase the mechanistic understanding of biological systems by artificially constructing them. Theoretically, experiments using the synthetic biology approach conducted under highly controlled conditions contain less variables than experiments conducted under natural settings, and thus can be more straight-forward to interpret. Most studies employing this approach focus on molecular mechanisms, using gene modules as their building blocks [39,48].

An extension of synthetic biology is synthetic ecology, which mixes discrete populations of cells to generate defined systems with reduced complexity as compared to natural systems. This approach provides increased control and defined platforms to address many questions regarding natural systems. Engineered symbiotic co-cultures having multiple microorganisms may be a means of assembling a novel combination of metabolic capabilities that have potential biotechnological advantages. Such artificial microbial communities can perform complex tasks and endure more variable environments than monocultures. An important feature of artificial microbial consortia is their ability to perform functions requiring multiple steps [5,28,30,45,57]. All of these are central characteristics for potential biotechnological innovations. The specific aim of synthetic ecology is to develop a cooperative and steady-state microbial community that performs a desirable biotechnological function [7–9,17,23,36,47,50,55,64]. In constructing a synthetic mutualism, the goal is to construct artificial associations that are beneficial to both organisms [37,42,63]. Most artificially engineered
microalgae–bacteria consortia show benefits only to the bacteria (i.e., O_2 and organic carbon production by the microalgae is beneficial to the bacterial partner) [5,30,45], and see additional references therein. Demonstration of mutual benefits and self-maintained duration of association over several consecutive generations are fundamental to the validation this approach [62].

Unicellular green microalgae Chlorella spp. are known to maintain stable natural symbiotic associations, for example with ciliates [59], hydra [51], fungi and bacteria [27,31,60]. There is only one example of artificially created association with common bacteria, Chlorella sorokiniana–Microbacterium spp. [35]. All other synthetic associations were demonstrated only with PGPB such as: Chlorella spp.–Azospirillum spp. [18,26], Chlorella spp.–Bacillus pumilus [33], and the cyanobacteria Synechococcus elongatus–Azospirillum brasilense [52]. A main factor to maintain the latter mutualistic associations is close physical proximity of the two microorganisms [13,20]. For example, close proximity leading to colonization of roots is a pre-requisite to ensure plant growth promotion by many species of plant growth-promoting bacteria (PGPB; [2,29]).

One of the fundamental questions arising from studies of mutualistic associations is whether an artificial mix of different microorganisms that originated from completely different environments can develop into a stable mutualism in a short period of time without lengthy co-evolution [45,53]. This is critical if the mutualism is constructed for a biotechnological purpose because an unstable mutualism would quickly lose its utility. This question arises because the birth of a novel mutualism has not been observed. Consequently, it is not yet been determined if co-evolution is a necessary prerequisite to mutualism or if ecologically changing conditions yield new mutualisms [46]. Demonstrating that a co-culture exhibits traits of synthetic mutualism through measuring direct transfer of metabolites between partners is not trivial. Thus far, evidence of metabolite transfer and mutual benefits between Chlorella and their partners has been circumstantial, involving enhanced growth and longevity [21] and major changes in the metabolic activities of both partners caused by the synthetic association [9,22,41,43,44].

The goals of the work described here were to test the hypothesis that synthetic mutualism could be created if strategically well-matched partners are selected and appropriate growth conditions are provided, and further to demonstrate the exchange of metabolites between the partners. To test this hypothesis, we created an association between two microorganisms with very different evolutionary histories that had not previously been in contact. The first is the freshwater microalga C. sorokiniana and the second is a terrestrial, generalist PGPB used in many agricultural applications, A. brasilense. These organisms were co-immobilized in polymeric beads to enhance physical interaction. Even though these two species did not previously share a habitat and so had never been in contact with each other, they established an association that has previously been demonstrated to benefit the algal partner [13]. The general question that we addressed in the current study is whether this synthetic association, having many biotechnological implications, is indeed a mutualistic one, where both microorganisms benefit. Our experiments were aimed at specifically answering three questions. First, is the physical interaction between algal and bacterial cells maintained for an extended period of time after the beads are removed? To address this, we used mild mechanical disruption of aggregates and fluorescent in situ hybridization (FISH) followed by imaging with confocal microscopy and scanning electron microscopy. Second, do the partners exchange C and N compounds, and, third, does physical attachment play a role in this exchange? To address these questions, we carried out isolate labeling experiments where one partner was labeled with stable isotopes and incubated with its unlabeled partner, and then vice-versa. We then analyzed the transfer of C and N between individual cells of each microorganism using high-resolution secondary ion mass spectrometry (SIMS).

2. Materials and methods

2.1. Microorganisms and cultivation methods

Co-culture experiments utilized the unicellular microalgae C. sorokiniana Shih. et Krauss (UTEX 2714, University of Texas, Austin, TX, formerly C. vulgaris UTEX 2714, [3]) and the bacterium A. brasilense Cd (DMS 1843, Leibniz-Institut DMSZ, Braunschweig, Germany). For routine cultivation of the microalgae, 10 mL axenic culture of C. sorokiniana was inoculated into 90 mL sterile mineral 847 medium (ATCC; http://www.atcc.org/~media/BF319BAA63DE4EA6B65428349CAF766E.shtml), composed of 10 mL of each of the following stock solutions (g·L⁻¹): NaNO₃ (10), CaCl₂ (1), MgSO₄·7H₂O (3), K₂HPO₄ (3), KH₂PO₄ (7), NaCl (1); 1 g·L⁻¹ of proteose peptone, and a drop of 1% FeCl₃ solution to a final volume of 1 L. To obtain ¹³C enriched algal cells, the medium was supplemented with 0.2 g·L⁻¹ NaH¹³CO₃ (99%¹³C, cat # CLM-441-1, Cambridge Isotope Laboratories). The microalgae were incubated at 27 ± 2°C with stirring at 140 rpm under fluorescent lighting (cool white, irradiance = 60 μmol photon·m⁻²·s⁻¹) for 7 days [25]. For cultivation of A. brasilense Cd, BTB-1 medium was used [4], composed of (in g·L⁻¹): NaCl (1.2), MgSO₄·7H₂O (0.25), K₂HPO₄ (0.13), CaCl₂ (0.22), K₂SO₄ (0.17), Na₂SO₄ (2.4), NaHCO₃ (0.5), Na₂CO₃ (0.09), Fe₃O₄·EDTA (0.07), tryptone (5), yeast extract (5), and Na-glucanate (5). To obtain ¹³C and ¹⁵N enriched bacterial cells, the medium was supplemented with ¹⁵NH₄Cl (0.010 g L⁻¹), 95%¹³C, cat # 29,925 Aldrich) and ¹³C-acetate (5 g L⁻¹, 99%¹³C, cat # CLM-381-1, Cambridge Isotope Laboratories). Added stable isotope carbon and nitrogen sources were sterilized by filtration through a 0.2 μm syringe filter (cat # 194-2520, Thermo Scientific). The medium was adjusted to pH 7 with 1 M KOH and incubated at 32 ± 2°C with stirring at 120 rpm for 16 h.

2.2. Immobilization of microorganisms in polymeric beads

Algal and bacterial cells were co-immobilized in alginate, employing the procedure described in [17]. Briefly, after washing the axenic cultures (C. sorokiniana or A. brasilense) to remove unincorporated isotonically enriched substrates, cells were re-suspended in 10 mL 0.85% saline solution. Suspensions of both organisms were mixed with 2% alginate solution and then mixed together to create co-cultures before forming the beads. Beads (3–4 mm diameter) were formed by dropping the alginate solution into a 2% calcium chloride solution using a syringe and 18 gauge needle [14].

2.3. Experimental culture conditions

For experiments, 4 g of beads with immobilized microorganisms were inoculated in 100 mL synthetic growth medium (SGM, [20]) and incubated in C-free, minimal SGM medium (in mg·L⁻¹): NaCl (7), CaCl₂ (4), MgSO₄·7H₂O (2), K₂HPO₄ (21.7), KH₂PO₄ (8.5), Na₂HPO₄ (33.4), and NH₄Cl (89) [20] under light conditions as described above for 4 days. Two independent experiments were carried out for a duration of three days, each with 3 replicates. In the first, A. brasilense cells enriched with both ¹³C and ¹⁵N were co-immobilized with non-enriched C. sorokiniana. In the second, C. sorokiniana cells enriched with ¹³C were co-immobilized with non-enriched A. brasilense. Samples for NanoSIMS analysis of isotope abundance were prepared from beads at the time of immobilization (T = 0) and after 3 days of co-incubation in the beads.

2.4. Fixation and preparation of samples for scanning electron microscopy, NanoSIMS, and fluorescent in situ hybridization

The alginate matrix of 10 beads per replicate incubation was dissolved in 10 mL 4% sodium bicarbonate (Sigma Aldrich, St Louis, MO) for 30 min. One mL of dissolved beads was centrifuged (14,000 x g), the
pellet was washed twice in 1 × PBS (15% v/v 200 mM sodium phosphate buffer/130 mM NaCl at pH 7.4), and fixed with 4% paraformaldehyde (Sigma Aldrich, St Louis, MO) for 1 h at 4 °C. The pellet was washed twice with 1 × PBS and stored in a mix of 1 × PBS/96% ethanol (1:1 v/v) at −20 °C until used [20].

2.4.1. Scanning electron microscopy

For scanning electron microscopy, samples were dehydrated by successive 20, 40, and 60% ethanol solutions for 40 min each, and then for 18 h in 100% ethanol. Dehydrated samples were dried with CO2 in a critical point dryer (Samdri-PVT-3B, Tousimis Research, Rockville, MD). The samples were mounted on a stub, submitted to osmium vapor and additional drying for 4 d and coated with palladium foil for 35 min at 40 mA in a sputter coater (Vacuum Desk II, Denton, Scotia, NY). Visualization was performed with a scanning electron microscope (S-3000N, Hitachi, Tokyo, Japan) at 15 kV, using a 45° angle of the slide to the electron beams. The photomicrographs were processed with software (Quartz PCI 5.5, Quartz Imaging, Vancouver, BC, Canada).

2.4.2. In situ hybridization

All procedures were performed according to [20]. Briefly, prior to hybridization, 10 μL of each sample was added to a gelatin (0.1% w/v) and chromium potassium sulphate (0.01% w/v)-coated microscope slide, air-dried, and dehydrated by successive 50, 80, and 96% ethanol washes (3 min each). Samples were then air-dried again [11]. An equimolar mixture of probes EUB-338 [1], II, and III [12], for the domain Bacteria, and the specific probe Abrams 1420 [58] for A. brasilense were used. The EUB-338 I, II, and III probes were labeled with the fluorochrome fluorescein isothiocyanate (FITC), and Abras 1420 was labeled with the fluorochrome Cy3 (Thermo Electron, Ulm, Germany). Hybridization was performed at 35% formamide stringency at 46 °C for 2 h. The final concentration of the probes was 3 ng·L−1. Samples were then washed at 48 °C for 5 min with 50 mL pre-warmed washing buffer. The slides were rinsed for a few seconds with ice-cold, deionized water and then air dried. Slides were stored at −20 °C in the dark until visualization. Before visualization, the slides were mounted in AF1 antifading reagent (Citifluor, Electron Microscopy Sciences, Hatfield, PA, USA). The hybridized samples were observed in an LSM 510 META system with an Axiosvert 100 M inverted microscope (Zeiss) [56]. A helium neon laser provided the excitation wavelength of 543 nm (Cy3), and an argon ion laser provided the excitation wavelength of 488 nm (FITC). To distinguish between the fluorescence from Cy3 and FITC-labeled oligonucleotide probes, the specific signals were detected in red and green, respectively. The third color channel (helium laser, 633 nm singular wavelengths) was used to visualize autofluorescence of the microalgae and was assigned a blue color. The three signals were combined and depicted as a red–green–blue (RGB) image. An Achromat 63 × /1.2 water immersion lens was used for all analyses and acquisition of images. Images were analyzed using the software LSM 510 v4.2 (Zeiss). For epifluorescence microscopy, an Axiosplan 2 (Zeiss), equipped with a mercury lamp (HXP120, Osram, Munich, Germany) and Zeiss filter sets for FITC/GFP (Emitter BP 525/50, Beam splitter FT 495, Exciter BP 470/40), Cy3 (Emitter BP 605/70, Beam splitter FT 570, Exciter BP 545/25), and Cy5 (Emitter BP 690/50, Beam splitter FT 660, Exciter BP 640/30) excitation were used. An Achromat 63 × /1.2 water immersion lens (Zeiss) was used for all observations. Images were recorded with the CCD camera AxioCam MRm controlled by the software AxioVision Rel. v4.6 (Zeiss) and further processed with Adobe Photoshop v6.0 (Adobe Systems, Mountain View, CA, USA).

2.4.3. NanoSIMS single cell analyses

Single cell analyses were performed using the Lawrence Livermore National Laboratory NanoSIMS 50 high-resolution SIMS instrument (Cameca, Gennevilliers, France). Samples were prepared by spotting fixed cells onto 5 × 5 mm silicon wafers, air drying, coating with ~5 nm of gold, and then imaging with an FEI Inspect F SEM (Hillsboro, OR) to identify regions for analysis. For NanoSIMS imaging, a focused 2 pA, 16 keV 13C12N− primary ion beam was rastered over (5 × 5 µm2 to 15 × 15 µm2) analysis areas with 256 × 256 pixels and a dwell time of 1 ms/pixel for 10 to 20 scans (cycles). The beam was focused to a nominal spot size of ~150 nm. Before analysis, samples were pre-sputtered to reach sputtering equilibrium and to remove non-cellular surface material. Serial quantitative secondary ion images (maps) were simultaneously collected for 12C2−, 13C12N−, 12C14N−, and 13C12N− using electron multipliers in pulse counting mode (13C12C−/12C2− = 213C/12C and 12C15N−/12C14N− = 15N/14N). Secondary electrons were also simultaneously collected for sample visualization. A mass resolving power of ~7000 was used to separate isotopic interferences from the isotopes of interest, e.g., 13C12C− from 13C12H− at mass 25. NanoSIMS ion image data were processed with the software package LIIMAGE (L.R. Nittler, Carnegie Institution of Washington) run with PV-Wave (Rogue Wave Software, Boulder, Colorado). Quantitative ion ratio images were generated from the summed ion images. The isotope data are expressed in delta notation, δ = [(Rmeas / Rref) − 1] × 1000‰, where Rmeas is the measured ratio, and Rref is the corresponding reference ratio (0.011237 for 13C12C and 0.00367 for 15N/14N), or in Atom Percent Excess (APE), which is the percentage enrichment of the rare isotope (15N or 13C) above the natural abundance percentage. Regions of interest (ROIs) for quantification of isotopic ratios were selected based on secondary electron and 12C15N− ion images, which allowed cells to be specifically selected. The 13C or 15N enrichment image was used to help identify the cell type. The isotopic ratios were extracted for each cycle and averaged. Uncertainties are expressed as 2 standard errors (2SE) of the mean based on cycle to cycle ratio reproducibility.

2.4.4. Disruption of microalgae–bacteria aggregates

To avoid damage to cells from aggressive mechanical disruption, such as extended vortex or mild cell sonication, we used a mechanical separation procedure. After dissolving the beads in citrate buffer (55 mM sodium citrate, 30 mM EDTA, 150 mM NaCl, 10 mL beads per 40 mL buffer), the solution was centrifuged at 10,732 g for 5 min. The supernatant was discarded and the pellet was resuspended in citrate buffer and centrifuged again. This procedure was repeated three times. The last pellet was suspended in saline (0.85% NaCl) and was centrifuged at 429 g for 2 min. This created a density gradient where mostly microalgae were sedimented and bacteria were left in the supernatant phase or as a layer on the sedimented microalgae. Using a 100 μL pipette, the supernatant layer was removed and discarded. The pellet containing mostly microalgae combined with fewer bacteria was suspended in 1 mL saline and passed through a glass filter (Whatman #52; [38]) trapping mostly the microalgae aggregates allowing additional free bacteria to be filtered. This filter was vacuum-rinsed twice, as described by [32], which includes reverse filtering to allow recovery of the microalgae aggregates. A. brasilense cells were counted by the fluorescein diacetate method described by [10] under a fluorescent microscope (BX41, Olympus, Tokyo, C. sorokiniana was counted under a light microscope, using a Neubauer hemocytometer connected to an image analyzer (Image ProPlus 4.5, Media Cybernetics, Silver Spring, MD). The microagal aggregates were then processed for Fluorescent in Situ Hybridization and examined by FISH.

2.5. Experimental design and statistical analysis

Batch cultures were used for all experiments. Each experimental treatment was performed in triplicate, where a single 250 mL Erlenmeyer flask containing 100 mL SGM served as a replicate. Experiments for NanoSIMS analysis were performed for 4 d, with samplings at day 0 and 4. Experiments for FISH were performed for up to 10 days. Natural abundance stable isotope measurements
correspond to $\delta^{15}\text{N} = -10\%$ and $\delta^{13}\text{C} = -15\%$. The rare stable isotope enrichment ($^{13}\text{C}$ or $^{15}\text{N}$) is considered to be incorporated by a cell if its isotopic composition is more than 3 SE higher than these values.

3. Results

3.1. Transfer of carbon and nitrogen compounds between A. brasilense and C. sorokiniana

As has been observed previously [13,18], the presence of A. brasilense as a bacterial partner significantly enhanced the growth of the microalgae (growth rate at 7 days ($\mu = 0.16$ for C. sorokiniana alone and 0.34 for C. sorokiniana immobilized with A. brasilense). To document the transfer of C and N from the bacteria to the algae, we immobilized and incubated enriched ($^{13}\text{C}$ and $^{15}\text{N}$) A. brasilense cells for four days with unenriched C. sorokiniana cells and subsequently analyzed the isotopic composition of both bacteria and algae, including cells physically attached to one another. Initial scanning electron microscopy guided us in these analyses, especially to identify cell aggregates (Fig. 1A). We carried out isotope imaging analysis of a total of 23 bacterial and 41 algal cells, including 13 algae attached to bacteria (including aggregates) and 13 bacteria attached to algae (not including $T_0$ controls). We checked for relatively high $^{13}\text{C}^{14}\text{N}^{-}$ counts (Fig. 1B) to ensure that the putative cells identified with the SEM were indeed cellular in nature rather than mineral. Since the bacterial cells were highly enriched with both $^{15}\text{N}$ and $^{13}\text{C}$, A. brasilense cells attached to C. sorokiniana cells were easily identified in isotope ratio images (Fig. 1C, D). We point out that in few cases (including in Fig. 1), the morphological identity (bacteria or algae) of cells in attached clusters was not definitive by SEM because of the orientation of the cells in the images, but isotope ratio images clearly showed which cells were the highly enriched A. brasilense, and which were newly enriched C. sorokiniana. In this case, the difference is several orders of magnitude. Thus we used isotope ratio image post-analysis to confirm which cells were algae, which were bacteria, and whether they were attached to one another or not. The isotopic data show that A. brasilense cells were highly enriched in $^{13}\text{C}$ and $^{15}\text{N}$ when added to the C. sorokiniana (Fig. 1I). They then became less enriched between days 0 and 4, at least in part due to the addition of non-enriched medium at the start of the co-incubation with C. sorokiniana. Growth on organic matter produced by C. sorokiniana is quantified in the other experiment below.

This first experiment showed that C. sorokiniana received both C and N from the A. brasilense. Some C. sorokiniana cells became enriched with both $^{15}\text{N}$ and $^{13}\text{C}$, while others were enriched with only $^{13}\text{C}$ or $^{15}\text{N}$ (Fig. 1J). C. sorokiniana cells enriched with $^{13}\text{C}$ but not $^{15}\text{N}$ (an example is shown on Fig. 1E-H) were most likely actively growing algal cells fixing carbon using remineralized $^{13}\text{CO}_2$ produced by enriched A. brasilense cells, but may have received organic carbon from the bacteria. Evidence for $^{15}\text{N}$ uptake without $^{13}\text{C}$ enrichment suggests that the bacteria released ammonium or nitrate, which C. sorokiniana incorporated, or that the quantity of $^{13}\text{C}$ received with the N was negligible relative to the rate of carbon fixation using natural HCO$_3^-$. Finding C. sorokiniana enriched with both $^{15}\text{N}$ and $^{13}\text{C}$, on the other hand, suggests that direct transfer of C- and N-containing molecules from bacteria to algae likely occurred. The most highly enriched algal cells (Fig. 1J) were those with an attached bacterium, suggesting that bacterial attachment promoted metabolite transfer. We point out, however, that bacterial attachment was not required for C and N transfer to occur, and algal cells with attached bacteria were not always more heavily enriched than algal cells without attached bacteria (Fig. 1J).

To document metabolite transfer from algae to bacteria, the reverse experiment was also performed (isotopically enriched algae were incubated with non-enriched bacteria); in this experiment, we only examined the transfer of C. For the four day time point, we identified 3 algal cells attached to algal cells, 8 algal cells with no obvious attached bacteria, and 8 unattached bacteria (e.g., Fig. 2). The magnitude of algal cell isotopic enrichment declined from day 0 to day 4, though cells after 4 days, with or without attached bacteria, were still highly enriched (Fig. 2G). The

![Fig. 1](image-url)
decreasing magnitude of $^{13}$C enrichment was most likely due to algal cell growth and photosynthetic carbon fixation of non-enriched CO$_2$. After three days, both attached and free-living _A. brasilense_ cells were $^{13}$C enriched, but several attached _A. brasilense_ were not enriched and were presumably either not actively growing or dead (Fig. 2H). The growth rate ($\mu$) in this synthetic medium of _A. brasilense_ in the absence of the microalgae is $-0.08$ and in the presence of _C. sorokiniana_ is $0.23$.

### 3.2. Stability of the synthetic mutualism between _A. brasilense_ and _C. sorokiniana_

A multi-step mild mechanical disruption procedure did not disassociate the microalgal–bacteria aggregates after 3, 7 and 10 days. We found populations of _A. brasilense_ always remained associated with or attached to the microalgal cells (Fig. 3 single and double arrows). Once formed,
the aggregates were stable for at least 10 days containing both species (Fig. 3). The FISH technique we used allows precise identification of *A. brasilense* interacting with *C. sorokiniana*, as the bacterial cells were specifically labeled by four independent fluorescent probes [20]. Positive fluorescence signals from *A. brasilense* varied in color from yellow-green to orange depending on the fluorophore used. Microalgae exhibited some autofluorescence in all channels used for detection, but could be nonetheless differentiated from bacterial cells because *Chlorella* cells were not probe-labeled. An additional differentiation criterion was the size of the microbial cells, the algal cells being, in general, larger (1–3 μm for *A. brasilense* and 5–10 μm for *C. sorokiniana*). Within 1 day of encapsulating cells of both microorganisms in the beads to intensify their physical interaction, a potential association was being created, but without any visible connection among cells or creation of cell clusters (Fig. 4A, B). After 3 to 7 days of association, clusters of the two microorganisms emerged. Within a cluster, both microorganisms were connected to each other by fibrils and sheath materials of an unknown nature (Fig. 4D, F). After 10 days, large clusters of both microorganisms were connected to each other by these connective materials, forming solid structures. These attachment structures persisted even when the alginate bead was dissolved, and bacteria and algae remained attached to each other for at least 10 days (equivalent to approximately 9–10 generations) implying a stable physical interaction (Fig. 4).

4. Discussion

Theory on mutualism asserts that co-evolution is a prerequisite for a functioning mutualistic interaction, where the benefits of mutualism outweigh the benefits of parasitism [45]. Synthetically-created microbial communities may be used as models that allow detailed study and analysis of these theories. There are only a few examples of synthetic associations involving *Chlorella* spp. All claiming to be mutualisms and were created specifically to obtain biotechnological gains, such as for fatty acid biosynthesis, wastewater treatment and starch accumulation [7,8,16,17,19,41,47]. This biotechnological approach is similar to that used to create other synthetic associations between other microalgal species and bacteria and fungi [34,37,40].

In previous work, we showed that *Chlorella* sp. populations greatly benefitted from the association with *A. brasilense* and *Bacillus pumilus* and the bacteria in these associations survived in the presence of the microalgae within polymeric beads [9,26,33]. Until now, the evidence for metabolic exchange was indirect and circumstantial. As an explanation for mutual benefits, it was hypothesized that there is an exchange of beneficial metabolites between the two species, creating a true synthetic mutualism. For example, a phytohormone, such as indole-3-acetic acid (IAA) was proposed to be transferred from the bacteria to the microalgae [21,43,44]. Concomitantly, the microalgal cells exuded tryptophan and thiamine, which are obligatory precursor and co-factor, respectively of IAA production in *Azospirillum* spp. [49,54] and other carbon sources needed for bacterial growth. IAA and tryptophan are independently produced and IAA-attenuated bacterial mutants have lower growth-promoting phenotypes [21,43,44]. Yet, the defining characteristics of mutualism in this synthetic association, namely the beneficial exchange of metabolites between the two partners and stable association over a long period of time, were not solidly demonstrated.

The present study is proposing that co-evolution is not a prerequisite for a functioning synthetic mutualism between a microalga and a PGPB. Using highly specific analytical tools capable of analyzing single cells within the association such as NanoSIMS isotopic imaging...
and FISH, combined with enforcing initial proximity between cells of the two species in alginate beads, we directly showed that C and N containing compounds were exchanged during interaction and association, which is beneficial to both microorganisms as demonstrated by their mutually enhanced growth. Furthermore, because this association was man-made [13] and created almost spontaneously without lengthy co-evolution, it challenges a basic paradigm of mutualism. This association arises relatively fast and forms a stable association lasting for at least 10 days. Considering that the generation time of the bacteria is ~30 h when immobilized in beads and that of the microalgae is ~20 h at least 10 days. Considering that the generation time of the bacteria is normally exudes labile carbon compounds that 

C. sorokiniana normally exudes labile carbon compounds that partners can use, and A. brasilense normally promotes the growth of its photosynthetic partners. The significant difference is that these two microbes come from completely different environments.

Author contributions


Conflict of interest

The authors declare no conflict of interest.

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