Second Revision: Algal Research

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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Abstract

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. (155 words)

Keywords: microalgae; NanoSIMS; plant growth-promoting bacteria; synthetic mutualism

1. Introduction

Mutualism, an interaction where two organisms benefit from an association, is ubiquitous in nature (Wernegreen 2004). Natural mutualism usually evolves over a prolonged association between the partners and is very stable (Brucker and Bordenstein 2012), 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 (Dunham 2007). 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 straightforward to interpret. Most studies employing this approach focus on molecular mechanisms, using gene modules as their building blocks (Kubo et al, 2013; Ortiz-Marquez et al, 2013).

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 (Brenner et al, 2008; Gotelli et al, 2012; Großkopf and Soyer 2014; Momeni et al, 2011; Shou et al, 2007). 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 (Choix et al, 2012 a,b, 2014; de-Bashan et al, 2004; Do Nascimento et al, 2013; Jagmann and Philipp 2014; Ortiz-Marquez et al, 2014; Pandhal and Noirel 2014; Santos and Reis 2014; Zuroff and Curtis 2012). In constructing a synthetic mutualism, the goal is to construct artificial associations that are beneficial to both organisms (Kim et al, 2014; Lőrincz et al, 2010; Zhang and Reed 2014). Most artificially engineered microalgae-bacteria consortia show benefits only to the bacteria (i.e., O₂ and organic carbon production by the microalgae is beneficial to the bacterial partner) (Brenner et al, 2008; Großkopf and Soyer 2014; Momeni et al, 2011, 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 (Wintermute and Silver 2010).

Unicellular green microalgae *Chlorella* spp. are known to maintain stable natural symbiotic associations, for example with ciliates (Summerer et al, 2007), hydra (Rahat and Reich 1986), fungi and bacteria (Gonzalez-Bashan et al, 2000; Guo and Tong 2014; Watanabe et al, 2005). There is only one example of artificially created association with common bacteria, *Chlorella sorokiniana*–*Microbacterium* spp. (Imase et al, 2008). All other synthetic associations were demonstrated only with PGPB such as: *Chlorella* spp.–*Azospirillum* spp. (de-Bashan et al, 2015; Gonzalez and Bashan 2000), *Chlorella* spp.–*Bacillus pumilus* (Hernandez et al, 2009), and the cyanobacteria *Synechococcus elongates*- *A. brasilense* (Ruiz-Güereca and Sánchez-Saavedra 2015). A main factor to maintain the latter mutualistic associations is close physical proximity of the two microorganisms (de-Bashan and Bashan 2008; de-Bashan et al, 2011). For example, close proximity leading to colonization of roots is a pre-requisite to ensure plant growth

88 promotion by many species of plant growth-promoting bacteria (PGPB; Bashan et al, 2004; Gray
89 and Smith, 2005).

90 One of the fundamental questions arising from studies of mutualistic associations is
91 whether an artificial mix of different microorganisms that originated from completely different
92 environments can develop into a stable mutualism in a short period of time without lengthy co-
93 evolution (Momeni et al, 2011 Sachs et al, 2004). This is critical if the mutualism is constructed
94 for a biotechnological purpose because an unstable mutualism would quickly lose its utility. This
95 question arises because the birth of a novel mutualism has not been observed. Consequently, it is
96 not yet been determined if co-evolution is a necessary prerequisite to mutualism or if
97 ecologically changing conditions yield new mutualisms (Morris et al, 2012). Demonstrating that
98 a co-culture exhibits traits of synthetic mutualism through measuring direct transfer of
99 metabolites between partners is not trivial. Thus far, evidence of metabolite transfer and mutual
100 benefits between *Chlorella* and their partners has been circumstantial, involving enhanced
101 growth and longevity (de-Bashan et al, 2008a) and major changes in the metabolic activities of
102 both partners caused by the synthetic association (de-Bashan et al, 2008 b; Choix et al, 2014;
103 Leyva et al, 2014; Meza et al, 2015a,b).

104 The goals of the work described here were to test the hypothesis that synthetic mutualism
105 could be created if strategically well-matched partners are selected and appropriate growth
106 conditions are provided, and further to demonstrate the exchange of metabolites between the
107 partners. To test this hypothesis, we created an association between two microorganisms with
108 very different evolutionary histories that had not previously been in contact. The first is the
109 freshwater microalga *Chlorella sorokiniana* and the second is a terrestrial, generalist PGPB used
110 in many agricultural applications, *Azospirillum 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 (de-Bashan and Bashan 2008). 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 isotope 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 microalga *Chlorella sorokiniana* Shih. et Krauss (UTEX 2714, University of Texas, Austin, TX, formerly *C. vulgaris* UTEX 2714, Bashan et al, 2015) and the bacterium *Azospirillum brasilense* Cd (DMS 1843, Leibniz-Institut

DMSZ, Braunschewegi, 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/BF31F98AA63D4EA6B65428349CAF766E.ashx>), composed of 10 ml of each of the following stock solutions ($\text{g} \cdot 0.4 \text{ L}^{-1}$): NaNO_3 (10), CaCl_2 (1), $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ (3), K_2HPO_4 (3), KH_2PO_4 (7), NaCl (1); $1 \text{ g} \cdot \text{L}^{-1}$ of proteose peptone, and a drop of 1% FeCl_3 solution to a final volume of 1 L. To obtain ^{13}C enriched algal cells, the medium was supplemented with $0.2 \text{ g} \cdot \text{L}^{-1}$ $\text{NaH}^{13}\text{CO}_3$ (99 at % ^{13}C , cat # CLM-441-1, Cambridge Isotope Laboratories). The microalgae were incubated at $27 \pm 2 \text{ }^\circ\text{C}$ with stirring at 140 rpm under fluorescent lighting (cool white, irradiance = $60 \mu\text{mol photon} \cdot \text{m}^{-2} \cdot \text{s}^{-1}$) for 7 days (Gonzalez et al, 1997). For cultivation of *A. brasilense* Cd, BTB-1 medium was used (Bashan et al. 2011), composed of (in $\text{g} \cdot \text{L}^{-1}$): NaCl (1.2), $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ (0.25), K_2HPO_4 (0.13), CaCl_2 (0.22), K_2SO_4 (0.17), Na_2SO_4 (2.4), NaHCO_3 (0.5), Na_2CO_3 (0.09), $\text{Fe}_{\text{III}}\text{EDTA}$ (0.07), tryptone (5), yeast extract (5), Na-gluconate (5). To obtain ^{13}C and ^{15}N enriched bacterial cells, the medium was supplemented with $^{15}\text{NH}_4\text{Cl}$ (0.010 g L^{-1} 99 at % ^{15}N , cat # 29,925 Aldrich,) and ^{13}C -acetate (5 g L^{-1} 99% ^{13}C , cat # CLM-381-1, Cambridge Isotope Laboratories). Added stable isotope carbon and nitrogen sources were sterilized by filtration through a $0.2 \mu\text{m}$ syringe filter (cat # 194-2520, Thermo Scientific). The medium was adjusted to pH 7 with 1 M KOH and incubated at $32 \pm 2 \text{ }^\circ\text{C}$ 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 de- Bashan et al, (2004). Briefly, after washing the axenic cultures (*C. sorokiniana* or *A.*

brasilense) to remove unincorporated isotopically 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 (de-Bashan and Bashan 2010).

2.3 Experimental culture conditions

For experiments, 4 g of beads with immobilized microorganisms were inoculated in 100 mL synthetic growth medium (SGM, de-Bashan et al, 2011) 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) (de-Bashan et al. 2011) 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 ($14,000 \times g$), the pellet was washed twice in $1 \times$ 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 $1 \times$ PBS and stored in a mix of $1 \times$ PBS/96% ethanol (1:1 v/v) at -20 °C until used (de-Bashan et al, 2011).

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 CO_2 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 de-Bashan et al, (2011). Briefly, prior to hybridization, 10 μL of each sample was added to a gelatin (0.1% w/v,) and chromium

potassium sulfate (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 (Daims et al, 2005). An equimolar mixture of probes EUB-338 I (Amann et al, 1990), II, and III (Daims et al, 1999), for the domain Bacteria, and the specific probe Abras 1420 (Stoffels et al, 2001) 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·μL⁻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 Axiovert 100 M inverted microscope (Zeiss) (Schmid et al, 2009). 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 depicted 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 Apochromat 63 X/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 Axioplan 2 (Zeiss), equipped with a mercury lamp (HXP120, Osram, Munich, Germany) and Zeiss filter sets for FITC/GFP (Emitter BP 525/50, Beamsplitter FT 495,

Exciter BP 470/40), Cy3 (Emitter BP 605/70, Beamsplitter FT 570, Exciter BP 545/25), and Cy5 (Emitter BP 690/50, Beamsplitter FT 660, Exciter BP 640/30) excitation were used. An Apochromat 63 X/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 v8.0 (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 $^{133}\text{Cs}^+$ primary ion beam was rastered over (5 × 5 μm^2 to 15 × 15 μm^2) 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 $^{12}\text{C}_2^-$, $^{13}\text{C}^{12}\text{C}^-$, $^{12}\text{C}^{14}\text{N}^-$, and $^{12}\text{C}^{15}\text{N}^-$ using electron multipliers in pulse counting mode ($^{13}\text{C}^{12}\text{C}^-/^{12}\text{C}_2^- = 2 \cdot ^{13}\text{C}/^{12}\text{C}$ and $^{12}\text{C}^{15}\text{N}^-/^{12}\text{C}^{14}\text{N}^- = ^{15}\text{N}/^{14}\text{N}$). Secondary electrons were also simultaneously collected for sample visualization. A mass resolving power of ~7,000 was used to separate isobaric interferences from the isotopes of interest, e.g., $^{13}\text{C}^{12}\text{C}^-$ from $^{12}\text{C}_2\text{H}^-$ at mass 25. NanoSIMS ion image data were processed with the software package LIMAGE (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, $\delta = [(R_{\text{meas}}/R_{\text{ref}}) - 1] \times 1000\text{‰}$, where R_{meas} is the measured ratio, and R_{ref} is the corresponding reference ratio (0.011237 for $^{13}\text{C}/^{12}\text{C}$ and 0.00367 for $^{15}\text{N}/^{14}\text{N}$), or in Atom Percent Excess (APE), which is the percentage enrichment of the rare isotope (^{15}N or ^{13}C) above the natural abundance percentage. Regions of interest (ROIs) for quantification of isotopic ratios were selected based on secondary electron and $^{12}\text{C}^{14}\text{N}^-$ ion images, which allowed cells to be specifically selected. The ^{13}C or ^{15}N 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_2 , 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 one mL saline and passed through a glass filter (Whatman #52; Kruskopf and Plessis 2004) trapping mostly the microalgae aggregates allowing additional free bacteria to be filtered. This filter was vacuum-rinsed twice, as described by Haney and Jaworski (1977), which includes reverse filtering to allow recovery of the microalgae aggregates. *A. brasilense* cells were counted by the fluorescein diacetate method described by Chrzanowski et al. (1984) 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 microalgal 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 $\delta^{15}\text{N} \sim 10 \text{ ‰}$ and $\delta^{13}\text{C} \sim -15 \text{ ‰}$. The rare stable isotope enrichment (^{13}C or ^{15}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 (de-Bashan and Bashan 2008; de-Bashan et al, 2015), the presence of *A. brasilense* as a bacterial partner significantly enhanced the growth of the microalgae (growth rate at 7 days (μ) = 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}C and ^{15}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 $^{12}\text{C}^{14}\text{N}^-$ 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}N and ^{13}C , *A. brasilense* cells attached to *C. sorokiniana* cells were easily identified in isotope ratio images (Figs. 1 C, 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 images post-analysis to confirm which cells were algae,

which were bacteria, and whether they were attached to one another or not. The isotope data show that *A. brasilense* cells were highly enriched in ^{13}C and ^{15}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}N and ^{13}C , while others were enriched with only ^{13}C or ^{15}N (Fig. 1J). *C. sorokiniana* cells enriched with ^{13}C but not ^{15}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}N uptake without ^{13}C enrichment suggests that the bacteria released ammonium or nitrate, which *C. sorokiniana* incorporated, or that the quantity of ^{13}C received with the N was negligible relative to the rate of carbon fixation using natural HCO_3^- . Finding *C. sorokiniana* enriched with both ^{13}C and ^{15}N , 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 bacterial 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 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 (μ) 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 microalgae-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 (de-Bashan et al 2011). 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 one 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 (Figs. 4 A,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 (Figs. 4 D, F). After 10 days, large clusters of both microorganisms were connected to the inert alginate and 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 (Figs. 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 (Momeni et al, 2011). 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 claimed to be mutualisms and were created specifically to obtain biotechnological gains, such as for fatty acid biosynthesis, wastewater treatment and starch accumulation (de-Bashan et al, 2002, 2004; Leyva et al, 2014; Choix et al, 2012; Ortiz-Marquez et al, 2014). This biotechnological approach is similar to that used to create other

synthetic associations between other microalgal species and bacteria and fungi (Hom and Murray 2014; Kim et al, 2014; Le Chevanton et al, 2013).

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 (Gonzalez and Bashan 2000; Choix et al, 2014; Hernandez et al, 2009). 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 (de-Bashan et al, 2008a; Meza et al, 2015 a,b). Concomitantly, the microalgal cells exuded tryptophan and thiamine, which are obligatory precursor and co-factor, respectively of IAA production in *Azospirillum* spp. (Spaepen et al, 2007; Palacios et al. 2016) and other carbon sources needed for bacterial growth. IAA and tryptophan are independently produced and IAA-attenuated bacterial mutants have lower growth-promoting phenotypes (de-Bashan et al, 2008a; Meza et al, 2015 a, b). 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 pre-requisite 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 (de-Bashan and Bashan 2008) 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 (de-Bashan et al, 2005; Bashan et al, 2011), and bearing in mind that artificial (experimental) and natural systems (evolution) operate on drastically different time scales, this should be considered to be a moderately long-term association. These results, therefore, demonstrate the basic characteristics of true mutualism (Momeni et al, 2011). Our observations are in agreement with the hypothesis that when specific conditions are artificially created, they can induce free-living species, a microalga and a bacterium, to become mutualists in order to survive. This establishes a synthetic system for studying the evolution of mutualism on short time scales and emergent biochemical properties not found in monocultures (Wintermute and Silver 2010).

This study complements earlier observations by providing evidence that in the microalgae-PGPB association, the microorganisms have the intrinsic phenotypic plasticity to adjust to new partners and create new, truly mutualistic relationships, even though the partners never previously shared the same habitat. Attachment of the two microorganisms to each other favors mutualism (more bacterial cells were C labeled) but is not a crucial factor (similar level of C labeling). Based on our findings, we propose that the simplest explanation for the success of this synthetic mutualism is that the two microbes are inherently compatible and inclined towards mutualism. *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:

Y.B., B.M.B., X.M. and L.E.dB. designed research; B.M.B., P.K.W. and L.P.B. supervised research; L.E.dB., X.M., A.M.D. and J.P.H. performed research; X.M., B.M.B, Y.B., P.K.W., and L.E.dB., analyzed data; and Y.B., B.M.B., X.M., P.K.W., L.E.dB. and L.P.B. wrote the paper.

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Conflict of interest

The authors declare no conflict of interest.

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Legends to figures

Fig 1. NanoSIMS data summary and example images of isotopically enriched *A. brasilense* (Az) incubated with initially nonenriched *C. sorokiniana* (Cs) for 4 days. The first four panels show an aggregate of 3 algal and 4 bacterial cells, and the next four panels an aggregate of 3 algal cells. Panels include SEM images (A, E), $^{12}\text{C}^{14}\text{N}^-$ images to identify cellular material (B, F) and isotope ratio images $^{13}\text{C}/^{12}\text{C}$ (C and G) and $^{15}\text{N}/^{14}\text{N}$ (D and H). Plots below show data from all analyzed cells, including *A. brasilense* (I) that were all isotopically enriched, as well as *C. sorokiniana* (J) that included both enriched and non-enriched cells. APE-Atom Percent Excess.

Fig 2. Data summary and examples of initially non-enriched *A. brasilense* incubated with isotopically enriched *C. sorokiniana*. The first three panels show an aggregate of 1 algal and 1 bacterial cell, and the next three panels a free-living bacterial cell. Panels include SEM images (A, D), $^{12}\text{C}^{14}\text{N}^-$ images to identify cellular material (B, E) and isotope ratio images $^{13}\text{C}/^{12}\text{C}$ (C and F). Plots below show data from all analyzed cells, including *C. sorokiniana* (G) that were all isotopically enriched, as well as *A. brasilense* (H) that included both enriched and unlabeled cells. APE-Atom Percent Excess.

Fig 3. Mild mechanical disruption of microalgae-bacteria aggregates. A and C- light microscopy of the synthetic mutualism; B and D- FISH analysis of the same sample under fluorescent microscopy according to the procedure of FISH described in Bashan et al. (2011) using an equimolar mixture of probes EUB-338 I, II, and III, for the domain Bacteria, and the specific probe Abras 1420 for *A. brasilense*. Bars represent 1 μm . Arrows indicate *A. brasilense* cells attached to the microalgae; E- counting of bacteria left in the association with the microalgae

654 after the mechanical disruption, n=22, bars represent standard error. F- Visualization of partial
655 separation of *A. brasilense* (Az, pink color) and *C. sorokiniana* (Cs, green color) during the
656 mechanical disruption of the aggregates.

657

658 Fig 4. Fluorescence *in situ* hybridization (FISH) and scanning electron microscopy SEM) of the
659 synthetic mutualism over time. A,G,I (FISH by Axioplan); C,E (FISH by CLSM). Bar represents
660 1 μ m. Procedure of FISH described in de-Bashan et al. (2011) using an equimolar mixture of
661 probes EUB-338 I, II, and III, for the domain Bacteria, and the specific probe Abras 1420 for *A.*
662 *brasilense*. The images demonstrate attachment between bacteria and algae as long as 10 days
663 after dissolution of the beads.

Figure

Figure

Figure

Before separation

After separation

Figure

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