Algal Research 15 (2016) 179–186

Contents lists available at ScienceDirect

Algal Research

journal homepage: www.elsevier.com/locate/algal

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)

Luz E. de-Bashan [a,b,c], Xavier Mayali [d], Brad M. Bebout [e], Peter K. Weber [d], Angela M. Detweiler [e,f], Juan- Pablo Hernandez [a,c], Leslie Prufert-Bebout [e], Yoav Bashan [a,b,c,*]

[a] The Bashan Institute of Science, 1730 Post Oak Court, Auburn, AL 36830, USA
[b] Dept. of Entomology and Plant Pathology, 301 Funchess Hall, Auburn University, Auburn, AL 36849, USA
[c] Environmental Microbiology Group, Northwestern Center for Biological Research (CIBNOR), Calle IPN 195, La Paz 23096, B.C.S., Mexico
[d] Nuclear and Chemical Sciences Division, Lawrence Livermore National Laboratory, Livermore, CA 94551, USA
[e] Exobiology Branch, NASA Ames Research Center, Moffett Field, CA 94035, USA
[f] Bay Area Environmental Research Institute, Petaluma, CA 94952, USA

ARTICLE INFO

Article history:
Received 20 August 2015
Received in revised form 22 December 2015
Accepted 20 February 2016
Available online xxxx

Keywords:
Microalgae
NanoSIMS
Plant growth-promoting bacteria
Synthetic mutualism

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.

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

* Corresponding author at: The Bashan Institute of Science, 1730 Post Oak Court, Auburn, AL 36830, USA.
E-mail address: ybb0001@auburn.edu (Y. Bashan).

http://dx.doi.org/10.1016/j.algal.2016.02.019
2211-9264/

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 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 *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, 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 ($g \cdot 0.4\ L^{-1}$): $NaNO_3$ (10), $CaCl_2$ (1), $MgSO_4 \cdot 7H_2O$ (3), K_2HPO_4 (3), KH_2PO_4 (7), NaCl (1); 1 g⊕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 $0.2\ g \cdot L^{-1}$ $NaH^{13}CO_3$ (99% ^{13}C, 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 $\cdot m^{-2} \cdot s^{-1}$) for 7 days [25]. For cultivation of *A. brasilense* Cd, BTB-1 medium was used [4], composed of (in $g \cdot L^{-1}$): NaCl (1.2), $MgSO_4 \cdot 7H_2O$ (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), Fe_{III}EDTA (0.07), tryptone (5), yeast extract (5), and Na-gluconate (5). To obtain ^{13}C and ^{15}N enriched bacterial cells, the medium was supplemented with $^{15}NH_4Cl$ (0.010 g L^{-1} 99% ^{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 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 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 [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 $mg \cdot L^{-1}$): NaCl (7), $CaCl_2$ (4), $MgSO_4 \cdot 7H_2O$ (2), K_2HPO_4 (21.7), KH_2PO_4 (8.5), Na_2HPO_4 (33.4), and NH_4Cl (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 ^{13}C and ^{15}N were co-immobilized with non-enriched *C. sorokiniana*. In the second, *C. sorokiniana* cells enriched with ^{13}C 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 × *g*), the