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Comparative genomics, transcriptomics, and physiology distinguish symbiotic from free-living *Chlorella* strains

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ABSTRACT

Most animal–microbe symbiotic interactions must be advantageous to the host and provide nutritional benefits to the endosymbiont. When the host provides nutrients, it can gain the capacity to control the interaction, promote self-growth, and increase its fitness. Chlorella-like green algae engage in symbiotic relationships with certain protozoans, a partnership that significantly impacts the physiology of both organisms. Consequently, it is often challenging to grow axenic *Chlorella* cultures after isolation from the host because they are nutrient fastidious and often susceptible to virus infection. We hypothesize that the establishment of a symbiotic relationship resulted in natural selection for nutritional and metabolic traits that differentiate symbiotic algae from their free-living counterparts. Here, we compare metabolic capabilities of 5 symbiotic and 4 free-living *Chlorella* strains by determining growth levels on combinations of nitrogen and carbon sources. Data analysis by hierarchical clustering revealed clear separation of the symbiotic and free-living *Chlorella* into two distinct clades. Symbiotic algae did not grow on nitrate but did grow on two symbiont-specific amino acids (Asn and Ser) on which the free-living strains did not grow. The use of these amino acids was exclusively affected by the presence/absence of Ca^{2+} in the medium, and the differences were magnified if galactose was provided rather than sucrose or glucose. In addition, *Chlorella variabilis* NC64A genomic and differential expression analysis confirmed the presence of abundant amino acid transporter protein motifs, some of which were expressed constitutively both axenically and within the host. Significantly, all 5 symbiotic strains exhibited similar physiological phenotypes even though they were isolated as symbionts from different host organisms. Such similarities indicate a parallel coevolution of shared metabolic pathways across multiple independent symbiotic events. Collectively, our results suggest that physiological changes drive the *Chlorella* symbiotic phenotype and contribute to their natural fitness.

1. Introduction

Successful endosymbiosis provides advantages to both the host and the endosymbiont. Benefits may include better adaptation to limited nutrients or reduction of mortality via protection against damage by UV light or pathogens (e.g., viruses). In such scenarios, symbionts increase their reproductive capacity and fitness within their hosts relative to possible non-host environments [3,14,18,19]. For example, some protozoans harbor intracellular green algae in an inherited mutually beneficial symbiotic relationship, which serves as a well-recognized model for studying endosymbiotic relationships [27,28,37,45]. These unicellular *Chlorella*-like green algae, often referred to as zoochlorellae, inhabit the gastrodermal symbiosomes (perialgal vacuoles) of different protozoans, and transfer a significant amount of their photosynthetically fixed carbon (e.g., maltose, fructose) to their non-photosynthetic partners [7,18,31]. In this context, symbiotic *Chlorella* spp. still require nutrients such as nitrogen, which they obtain from the host and then assimilate into the algal metabolome [33,50]. The mechanisms involved in these interactions have not been completely elucidated; however, the metabolic pathways involved in nitrogen (N) and carbon (C) utilization could be crucial physiological signatures of the endosymbiotic existence [32]. Therefore, elucidating how such processes work would open new avenues of research in the understanding of the molecular, cellular, and organismal adaptations that allow successful mutualism.

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Protozoa–*Chlorella* interactions can be disrupted, and some attempts to isolate intact algae free of the host have been successful. These include algae that associate with several species of protozoans, including *Paramecium bursaria* [23,45], *Acanthocystis turfacea* [23], and *Hydra viridis* [32]. Another approach for identifying ex-symbiotic algal strains has relied on their susceptibility to large DNA virus infections after disruption of the host–*Chlorella* interaction [22,30,34,47]. The only documented symbiotic, virus-susceptible *Chlorella* strains that have been cultured axenically include *Chlorella variabilis NC64A 1* [49], *C. variabilis Syngen 2-3* [47], *C. variabilis* F36-ZK [11,16,38], *C. variabilis* OK1-ZK ([11, 38], Quispe et al., manuscript in preparation), and *C. heliozoae* SAG 3.83 [6]. For the purpose of this paper, these symbiotic virus-susceptible algal strains will be referred to as symbiotic algae.

We have studied the *Chlorella*–virus interaction for the past 35 years and have been aware of the fastidious nutrient requirements possessed by symbiotic algae [1,20,33]. For example, unlike most *Chlorella* species, the symbiotic algae do not grow on Bolds' Basal Medium (BBM), which has nitrate (NO_3) as its sole N source. Consequently, 0.1% peptone is added to BBM for axenic growth of these symbiotic strains [15,16,25, 47,49]. We hypothesize that this requirement reflects a past symbiotic relationship that spurred selection for specific nutritional and metabolic features present in symbiotic algae. In this study, we test this idea by analyzing some physiological traits and growth requirements in 4 free-living and 5 symbiotic *Chlorella* species.

Our physiological evaluation focused on alternative N and C sources. The results indicate that symbiotic algae are better able to assimilate N and C sources not normally available to the free-living strains. Significantly, they prefer organic N sources rather than the inorganic N sources (e.g., NO_3 or NH_4), which are the primary N sources in the environment. Importantly, all symbiotic strains tested exhibit similar metabolic phenotypes even though they are polyphyletic and may have arisen as protozoan symbionts from several independent symbiotic events [13,38]. Importantly, these similarities denote a parallel coevolution of similar metabolic pathways across multiple independent symbiotic events. Taken together, this evolutionary genome plasticity and metabolic regulatory rewiring could come at a cost in the form of the inability of symbiotic *Chlorella* to survive as free-living organisms in virus replete and/or nutrient limiting environments [39].

2. Materials and methods

2.1. Algal strains

Symbiotic *C. variabilis* ATCC 50258 (NC64A), *C. variabilis* ATCC 30562 (Syngen 2-3), and *C. heliozoae* SAG 3.83 (SAG 3.83) were maintained as slant stocks at 4 °C. Symbiotic *C. variabilis* NIES-2540 (F36-ZK) and *C. variabilis* NIES-2541 (OK1-ZK) were obtained from the Japanese Culture Collection of the National Institute for Environmental Studies (http://mcc.nies.go.jp). Stock samples of free-living *Chlorella* strains *Chlorella sorokoniana* UTEX-1230 (UTEX-1230), *Cyamus kessleri* UTEX-2228 (B228), and *Chlorella protothecoides* UTEX-29 (CP-29) were obtained from the Culture Collection of Algae at the University of Texas at Austin (https://utex.org), and *C. sorokoniana* CCTCC M209220 (CS-01) was obtained from Minxi Wan at Johns Hopkins University. The selection for free-living strains was based on the proposed phylogeny of *Chlorella* species published by Rosenberg et al. [41]), from which we chose four representative strains.

2.2. Cell cultures

Symbiotic and free-living strains were grown on BBM [3] supplemented with 0.1% (*w*/*v*) peptone, 0.5% (*w*/*v*) sucrose, and 0.001% (*w*/*v*) thiamine (complete MBBM). All of the BBM modifications had NO_3 and sucrose omitted (N-/C-BBM) and being replaced by the labeled N and C sources. Where indicated, 0.1% peptone was replaced with 0.1% (*w*/*v*) casamino acids. The ability of algae to use different N and C sources was tested by adding them to unsupplemented BBM (N-/C-BBM). Thus, 0.22 μm filter-sterilized stock solutions of N and C sources were added to a final concentration of 10 mM. All flasks were supplemented with 0.001% (*w*/*v*) thiamine. To test the effect of Ca^{2+} deprivation on algal growth, we used a C-, N-, and Ca^{2+}-deficient BBM (N-/C-/Ca^{2+}-BBM), i.e., Ca^{2+} was not included in the BBM.

The algae were grown in 125 ml narrow mouth Erlenmeyer flasks with 30 ml of supplemented BBM. For the inoculum, MBBM log-phase actively growing cells were pelleted and washed 3 times with either N-/C-BBM or N-/C-/Ca^{2+}-BBM medium. Flasks were inoculated to a final low cell density of 3–5 × 10^4 cells/ml and shaken at 26 °C and 180 rpm in continuous light for variable time periods because the symbiotic growth rates were slower than their free-living counterparts. Free-living strains were grown for 9 days on BBM with added N or for 7 days when both N and C were added. Similarly, symbiotic strains were grown for 12 days on BBM with added N or for 9 days when both N and C were included. MBBM and un-supplemented BBM were used as controls. Triplicate samples were used for the symbiotic algae, and duplicate samples were used for the free-living strains. Photographs of the flasks were taken with a 12.1 M pixel Sony Cyber-shot digital camera and organized using Adobe Photoshop CS5.1. They are shown in Supplementary Figs. 1–6.

2.3. Hierarchical clustering analysis

Cluster 3.0 for Mac OS X (http://rana.lbl.gov/EisenSoftware.htm) and JavaTreeView Version 1.1.6r4 (http://jtreeview.sourceforge.net/) programs were used to analyze and quantify the growth experiments. The hierarchical clustering algorithm was performed using the average-linkage method applied to the data set. This algorithm produced a dendrogram that assembled all elements into a single tree, which arranged the strains and treatments according to similarities in their growth patterns. The data set consisted of rows and columns representing the 9 algal strains and the numerical score for each media condition. Analyses were performed both on the bulk data and as subsets by treatment. Numerical scores were assessed for individual flasks using a 0 to 5 scale, with 5 representing the best growth and 0 the absence of visible growth. The data sets were represented graphically in hierarchical clusters by coloring each cell on the basis of the numerical flask score. Flasks with scores of 0 were colored black while the higher scores were reds of increasing intensity to denote growth. The dendrogram is attached on both axes to the colored graph to indicate the computed relationships among both growth conditions and *Chlorella* species.

2.4. Comparative genomics

Members of a collection of characterized AA transporters from *Arabidopsis thaliana* [46] were used to perform reciprocal BLAST searches [2] against *C. variabilis* NC64A and *C. sorokoniana* UTEX-1230 (UNL algal consortium, in preparation) genomes, using a value of 1×10^{-10} as a cutoff. Each algal protein identified an *A. thaliana* AA transporter, and the gene designations and *E*-values for each gene are presented in Supplementary Tables 1 and 2, respectively. Similarly, 35 putative AA transporters from NC64A [4] were used to perform a reciprocal BLAST search against the UTEX 1230 proteome using a value of 1×10^{-10} as a cutoff (Table 1). The orthology of candidate sequences was verified using the KEGG database [17].

2.5. RNAseq analysis

Data sets from RNAseq experiments were downloaded to the public Galaxy platform server (www.usegalaxy.org) and manipulated with data analysis tools as described below. Tophat settings were as defined by the usegalaxy.org defaults (Galaxy Version 0.9) except that the maximum intron length was set at 1500 bp. For axenic *C. variabilis* NC64A