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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.

Keywords: *Chlorella variabilis,* Alga-paramecium symbiosis, Algal nitrogen metabolism, Amino acid transporters

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.

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 Figures 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

Table 1. Accession and scaffold numbers of putative *C. variabilis* NC64A orthologs to *Chlorella sorokiniana* UTEX-1230 proteins involved in AA transport.

	NC64A gene ID	Protein ID	Scaffold in *C. sorokonian*	e-value	Bit score	Identity	Protein length (aa) *C. variabilis*	*C. sorokoniana*
1	138810	EFN52376	18.g93.iso1	3E-073	231 bits (590)	126/181 (70%)	183	504
2	50436	EFN58616	4.g163.iso1	5E-088	284 bits (726)	186/451 (41%)	410	690
3	138809	EFN52375	18.g93.iso1	2E-113	340 bits (871)	190/306 (62%)	287	504
4	144770[b]	EFN56324	56.g4.iso2	7E-128	380 bits (977)	193/361 (53%)	471	405
5	144819	EFN56345	4.g37.iso3	2E-120	364 bits (935)	213/389 (55%)	489	469
6	142340[a]	EFN58068	34.g94.iso1	1E-134	426 bits (1096)	219/305 (72%)	695	1070
7	37093[a,b]	EFN51991	172.g106.iso1	2E-141	431 bits (1108)	244/446 (55%)	519	815
8	135113[a]	EFN54610	170.g37.iso1	3E-134	414 bits (1063)	246/309 (80%)	932	461
9	145403	EFN55845	4.g163.iso1	2E-134	410 bits (1053)	255/547 (47%)	535	690
10	133029	EFN59609	28.g135.iso1	5E-139	409 bits (1052)	257/443 (58%)	431	449
11	134730	EFN54961	57.g52.iso1	2E-156	455 bits (1171)	261/424 (62%)	453	469
12	134234[a]	EFN55146	33.g185.iso1	4E-142	420 bits (1079)	263/484 (54%)	473	480
13	142091	EFN57962	8.g116.iso2	2E-158	461 bits (1186)	266/417 (64%)	471	466
14	135437[a]	EFN54731	3.g27.iso1	4E-161	475 bits (1222)	272/399 (68%)	518	606
15	51413	EFN57306	84.g138.iso1	1E-174	503 bits (1294)	282/452 (62%)	452	490
16	49669	EFN60144	4.g158.iso1	8E-179	533 bits (1372)	294/470 (63%)	726	742
17	133360[a]	EFN60071	174.g65.iso1	0	552 bits (1422)	296/405 (73%)	692	685
18	56488[a]	EFN59984	53.g43.iso1	0	585 bits (1509)	300/495 (61%)	973	515
19	138717	EFN52627	21.g78.iso1	0	547 bits (1410)	317/540 (59%)	498	547
20	133351	EFN60067	27.g54.iso1	0	600 bits (1548)	331/494 (67%)	489	473
21	138133[a,b]	EFN59501	110.g43.iso1	0	594 bits (1532)	336/587 (57%)	576	512
22	58128[a,b]	EFN54604	15.g150.iso1	0	637 bits (1644)	337/516 (65%)	522	484
23	57473[a,b]	EFN56726	91.g67.iso3	0	681 bits (1758)	346/471 (73%)	476	468
24	138505	EFN52920	43.g21.iso1	0	659 bits (1701)	353/546 (65%	544	560
25	32765[a,b,c]	EFN51990	35.g117.iso2	0	659 bits (1701)	366/650 (56%)	605	635
26	59057[a,b]	EFN51898	35.g117.iso2	0	645 bits (1664)	373/698 (53%)	742	635
27	133392	EFN60084	39.g82.iso1	0	688 bits (1775)	378/516 (73%)	516	496
28	59479[a,b,c]	EFN50622	172.g106.iso1	3E-056	194 bits (492)	97/158 (61%)	270	815
29	133952	EFN55688	28.g105.iso1	2E-023	101 bits (251)	50/88 (57%)	336	674
30	133953	EFN55689	28.g102.iso1	7E-053	172 bits (435)	93/183 (51%)	197	238
31	24724[a,b]	EFN54340	76.g4.iso1	1E-051	177 bits (450)	107/269 (40%)	227	560
32	53357[b]	EFN53996	34.g191.iso1	4E-066	224 bits (572)	166/486 (34%)	385	689
33	137496[a]	EFN53131	27.g54.iso1	7E-047	176 bits (446)	144/415 (35%)	1010	473
34	138482	EFN52813	177.g11.iso1	1E-065	220 bits (560)	215/445 (48%)	425	489
35	17797[b]	EFN50713	172.g67.iso1	4E-031	115 bits (287)	54/87 (62%)	92	815
36	142334[b]	EFN58316.1	13.g249.iso3	0	661 bits (1706)	365/420 (87%)	535	487
37	140447[b]	EFN58455.1	106.g215.iso2	1.00E-96	302 bits (774)	142/179 (79%)	376	576
38	7483[b]	EFN50706.1	92.g116.iso2	0	667 bits (1720)	373/497 (75%)	493	624
39	58448[b]	EFN53780.1	136.g46.iso1	0	560 bits (1442)	316/671 (47%)	639	1388
40	36103[b]	EFN54400.1	139.g141.iso1	2E-130	400 bits (1028)	247/427 (58%)	444	832

a. *C. variabilis* gene ID of expressed AA transporter genes during axenic growth ([4] and [42]); see Figure 7.
b. *C. variabilis* gene ID of differentially expressed AA transporter genes during axenic and endosymbiont states ([42] and [26]); see Table 2.
c. Genes (32765 and 59479) that accounted for the majority of the mapped differentially expressed reads in axenic and symbiont growth.

(Figure 7), we used an uninfected control sample (NCBI SRA accession SRX316780) from a published viral infection experiment conducted in our lab [42]. For *C. variabilis* growing endosymbiotically within *P. bursaria*, we downloaded RNAseq data sets (NCBI SRA accessions DRX003053, DRX003054, and DRX003055) [26]. These sequence files were reported to contain RNAseq reads mapping to the NC64A genome, thus providing potential information regarding algal genes that are differentially expressed when grown axenically on MBBM versus its natural endosymbiont stage within *P. bursaria*.

The FASTQ files were converted to FASTQSANGER format with the FASTQ Groomer tool (default settings) [5] and then Tophat [24] was used to align these data sets to the NC64A genome assembly [4] with minimum and maximum intron lengths of 50 and 5000, respectively. Around 1% (~970,000) of the *P. bursaria* derived reads aligned to the *C. variabilis* NC64A genome, and these reads were taken to represent a snapshot of gene expression in the endosymbiont cells. The same analysis pipeline was applied to the axenic *C. variabilis* NC64A data. Reads that mapped to the genomic intervals for each putative AA transporter in Supplementary Table 1 were counted using the Integrated Genome Browser software package [36] and normalized as total mapped reads per gene in each condition per million mapped reads.

3. Results

3.1. An overview of nitrogen metabolism in symbiotic algae

This study addresses difficulties in the isolation and growth of symbiotic algae. For example, *C. variabilis* NC64A grows well on BBM with 0.1% peptone and 0.5% sucrose (MBBM) but does not grow on unsupplemented BBM [15,16,25,47,49]. Additionally, previous reports established that peptone supplemented BBM was sufficient for growth of *C. variabilis* NC64A and *C. variabilis* F36-ZK [16]. We have now included three more symbiotic strains (*C. variabilis* OK1-ZK and Syngen 2-3 as well as *C. heliozoae* SAG 3.83), and these 5 *Chlorella* strains comprise the symbiotic algae used in this study. Our focus on N metabolism in the symbiotic algae was prompted by our observation that NC64A grew almost as well in a defined galactose-urea medium (BBM with 10 mM galactose and 10 mM urea) as it did in MBBM. Two of the other symbiotic algae (SAG 3.83 and Syngen 2-3) also grew well when NO_3 and sucrose in BBM were replaced with 10 mM galactose and 10 mM urea, while F36-ZK and OK1-ZK grew well with the addition of 0.001% thiamine. Since galactose-urea-BBM is a chemically defined growth medium, we conclude that three of the symbiotic algae have no vitamin requirements while two

require thiamine. In particular, we did not find evidence that symbiotic *Chlorella* required biotin or cobalamin/vitamin B_{12} [9]. It is quite possible that these strains would grow better with added vitamin B_{12} [23]. For convenience, 0.001% thiamine was added to all subsequent test media.

3.2. Nutritional analyses identified physiologic signatures for symbiotic and free-living chlorella species

The 5 symbiotic and 4 free-living *Chlorella* strains [(*C. sorokoniana* (UTEX-1230), *C. sorokoniana* (CS-01), *C. kessleri* (B228), and *C. protothecoides* (CP-29)] were compared with regard to their ability to utilize 64 different N and C sources. For each combination, a low cell density of *Chlorella* (3–5×10^4 cells/ml)was inoculated into the indicated medium and cell growth was assessed by the intensity of the green color after 7–12 days (Supplementary Figures 1–6). Triplicate and duplicate samples were used for the symbiotic and free-living algae, respectively. The matrix of 64 growth conditions and 9 *Chlorella* strains was analyzed by two-way heat maps (Figure 1). These average-linkage maps display differences in the metabolic capabilities of the 5 symbiotic and 4 free-living *Chlorella*. The organic and inorganic N sources were tested with or without one of three sugars (sucrose, glucose, or galactose) as a C source. Both the C and N sources were routinely added at 10 mM. The rows in Figure 1 represent combinations of 3C and 12 N sources (2 complex mixtures, 7 organic, and 3 inorganic) or the N sources alone, while the columns represent the growth levels achieved by the 9 *Chlorella* strains on the 64 media combinations. The 9 *Chlorella* strains clearly separated into two clusters based on their nutritional capabilities: a symbiotic clade and a free-living clade (Figure 1). Purple labels identify inorganic N sources present at 1 mM instead of 10 mM while orange labels represent media prepared without Ca^{2+}. As a major difference, 4 out of the 5 symbiotic *Chlorella* did not grow on NO_3 as the sole N source, either with or without added sugar, whereas the 4 free-living *Chlorella* grew well on NO_3 with sugar and poorly on NO_3 without sugar (Figure 1).

3.3. Casamino acids and peptone supply all the C and N needed by symbiotic algae

The heat map presented in Figure 1 is cumbersome to read due to its large size. Therefore, we prepared 41 smaller analyses reflecting metabolic subsets of the data and of these the 6 most relevant subsets are presented here. In each subdivision, the 5 symbiotic and 4 free-living *Chlorella* were compared based on their growth on the respective supplemented BBMs. In the first case (Figure 2), we analyzed peptone and casamino acids with and without sucrose. The symbiotic and free-living strains formed two distinct clades (Figure 2) while two media clusters appeared, one for casamino acids alone and another for peptone alone, peptone and sucrose (MBBM), and casamino acids and sucrose. The free-living strains did not grow as robustly as the symbiotic *Chlorella* did on casamino acids alone (Figure 2).

All the symbiotic *Chlorella* grew better on 0.1% casamino acids than on 0.1% peptone and, in all cases removal of sucrose and NO_3 from the control MBBM had no effect on their growth (Figure 2). Although the differences were slight, the results suggest that organic N sources rich in AAs (casamino acids)were better assimilated by the symbiotic *Chlorella* than the free-living strains. By contrast, the free-living *Chlorella* responded better to the addition of sucrose, suggesting that they were better adapted to the presence of a sugar source.

3.4. Asn and Ser were better assimilated by symbiotic Chlorella

The results in Figure 2 suggested that free AAs were better assimilated by the symbiotic strains. Therefore, we tested the 9 *Chlorella* strains on 10 organic and inorganic N sources. The dendrogram (Figure 3) shows a clear separation between the symbiotic and free-living *Chlorella* based on their N assimilation patterns. All of the *Chlorella* grew with Arg, urea, Gln, and Gly as the sole N source but the symbiotic grew slightly better. Additionally, a separate cluster was formed by Asn and Ser (Figure 3). Again, the symbiotic algae grew better on these AAs, with Asn being better than Ser (Figure 3). Thus, Asn and Ser appeared to be symbiont-specific in that they were used poorly, if at all, by free-living *Chlorella*. Hence, Asn and Ser might be important N sources during symbiotic growth. Pro also clustered with Asn and Ser (Figure 3), but it did not support growth of the symbiotic *Chlorella* strains as well as Asn and Ser. It is important to note that these AAs were not simply fulfilling auxotrophic requirements because all symbiotic *Chlorella* were fully prototrophic for AA biosynthesis. That is, they all grew on minimal defined media with urea as the sole N source.

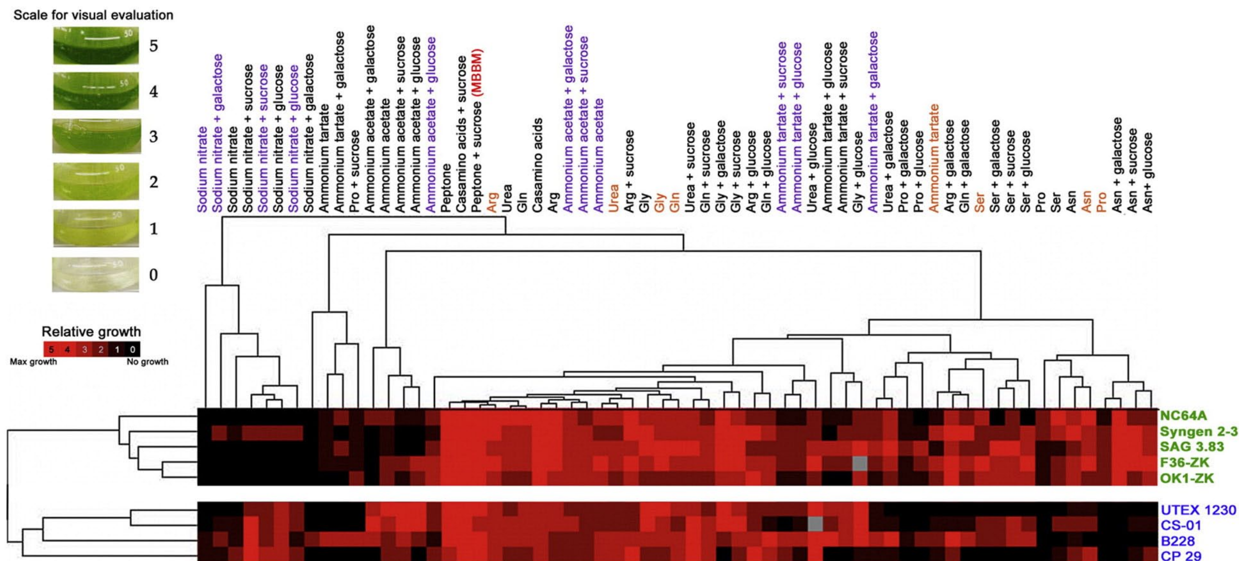

Figure 1. Hierarchical heat map (average-linkage) clusters of symbiotic and free-living *Chlorella* strains based on their metabolic capabilities. Columns represent combinations of organic and inorganic nitrogen (N) sources with or without the addition of a carbon (C) source. All were added at 10 mM concentrations except purple labels identify inorganic N sources at 1 mM concentration, and orange labels represent media without Ca^{2+}. MBBM medium was the control. Rows represent the 5 symbiotic strains (green) and 4 free-living strains (blue). Tree diagrams indicate the nature of the computed relationship among growth conditions and among *Chlorella* species. A color scale indicates relative growth: red represents robust growth and black represents absence of growth. Flask tests were performed for 9–12 days for the symbiotic and 7–9 days for the free-living strains. Subsequent heat map Figures 2–6 follow a similar layout.

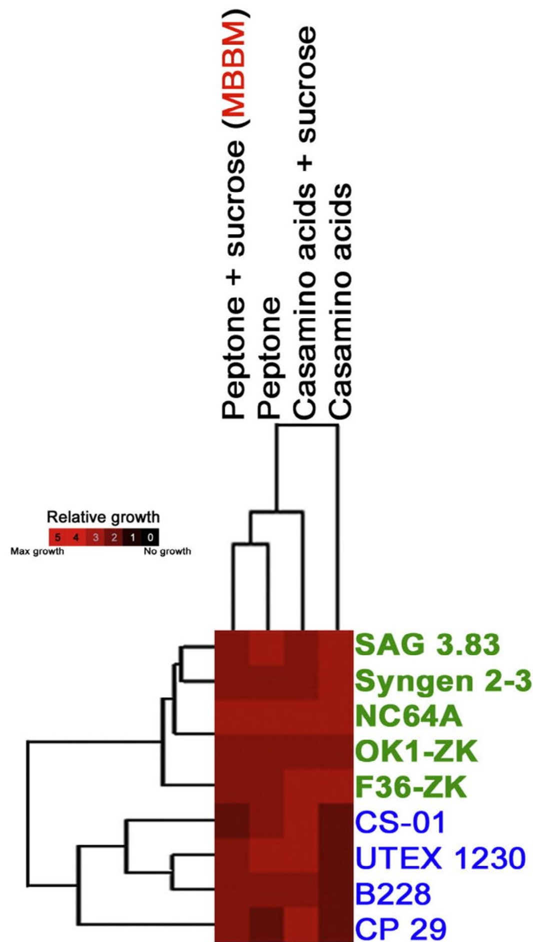

Figure 2. Heat map subgroup from Figure 1 displays variations of MBBM (sucrose + peptone). Peptone is replaced by 1% casamino acids. A color scale indicates relative growth. Flask tests were performed for 9 days for the symbiotic and 7 days for the free-living strains.

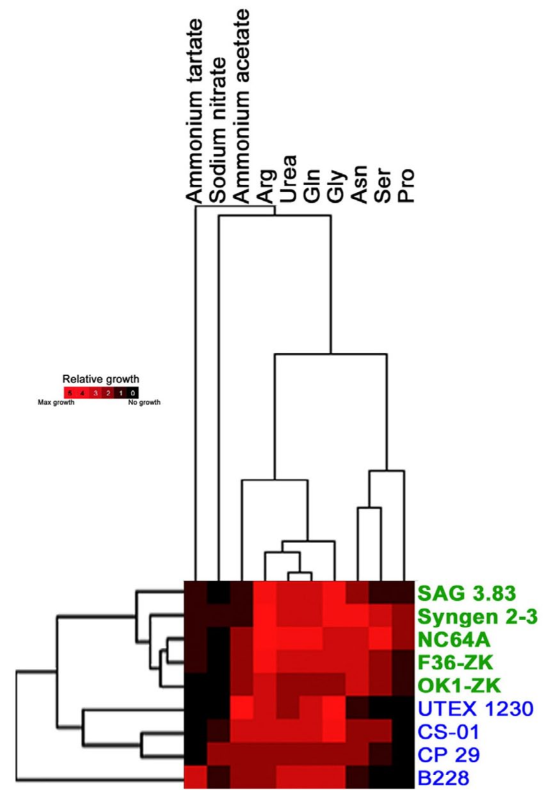

Figure 3. Heat map subgroup from Figure 1 compares inorganic and organic N sources at 10 mM concentrations as the sole N source. Inorganic sources include NH_4 tartrate, sodium NO_3, and NH_4 acetate. Organic sources include arginine (Arg), urea, glutamine (Gln), glycine (Gly), asparagine (Asn), serine (Ser), and proline (Pro). A color scale indicates relative growth. Flask tests were performed for 12 days for the symbiotic and 9 days for the free-living strains.

AA transport is coupled to movement of ions, including Na^+, H^+, K^+, Ca^{2+}, and/or Cl^- as well as movement of sugars. Additionally, Kato and Imamura [21]) showed that the presence of divalent but not monovalent cations decreased AA uptake in *C. variabilis* F36-ZK. We also noticed minimal growth of 3 symbiotic strains when Ca^{2+} was present in the media. Therefore, the influence of Ca^{2+} on AA transport and possible differences between symbiotic and free-living *Chlorella* species was also examined. We compared the effectiveness of organic N sources both with (Figure 4, black color) and without Ca^{2+} (Figure 4, orange color). Ca^{2+} is one of the salts normally present in BBM so the Ca^{2+}-free medium was made by not adding Ca^{2+}. The method ensures that trace levels of Ca^{2+} will be presented from the water, inoculum, and other media components. The effect of Ca^{2+} on the assimilation of organic N sources was variable. However, we observed that Ca^{2+} influenced the assimilation of Asn, Ser, and Pro in symbiotic growth but it was strain specific. By contrast, the absence of Ca^{2+} had no appreciable effects on Asn, Ser, and Pro uptake in the free-living *Chlorella*, but strain-specific differences were observed for urea, Arg, Gly, and Gln. We concluded that Ca^{2+} ions influenced the assimilation of Asn, Ser, and Pro in symbiotic algae and the assimilation of Arg, Gly, and Gln in free-living *Chlorella*.

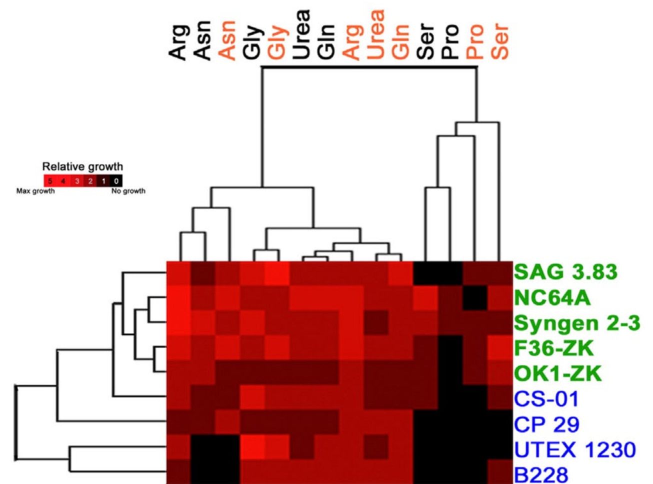

Figure 4. Heat map subgroup from Figure 1 compares removal of Ca^{2+} (orange) from media with organic N sources. Flask tests were performed for 12 days for the symbiotic and 9 days for the free-living strains.

3.5. Inorganic nitrogen sources

The only inorganic N source that clustered within the organic group was NH_4 acetate (Figure 3). NH_4 tartrate and sodium NO_3 clustered in a different clade which, without added sugar, gave poor or no growth for all strains (Figure 3). This finding was surprising because NH_4 and NO_3 are the primary sources of N in most aquatic and marine environments [43]. The symbiotic algae exhibited a remarkable duality in that they were unable to use extracellular NH_4 or NO_3 but were able to use the intracellular NH_4 formed after Arg, Gln, Asn, and urea uptake. These observations confirm reports on *C. variabilis* F36-ZK regarding the loss of NO_3 assimilation coupled with an enhanced ability to take up certain AAs [20]. F36-ZK uses a pH-dependent proton symport for general AA transport [16].

3.6. Low NH_4 concentrations are better assimilated by symbiotic strains

Fungal growth media commonly include a 10 mM N source. However, 10 mM NH_4 could be inhibitory or toxic to some *Chlorella* strains (Figure 3) and thus, we compared NH_4 and NO_3 at 1 and 10 mM in both the absence (Figure 5) and in the presence of sugars (Figure 6A). NH_4 tartrate was chosen because it does not acidify fungal growth media as much as $(NH_4)_2SO_4$ or NH_4Cl [29]. Lower levels of NH_4 acetate and NH_4 tartrate (1mM) supported growth of all the symbiotic strains while higher concentrations (10 mM) were inhibitory (Figure 5). Similarly, Minaeva and Ermilova [35] demonstrated that in NH_4-supplemented medium, *C. variabilis* NC64A showed significantly lower growth rates than in the peptone-containing MBBM. Thus, we conclude that the symbiotic *Chlorella* exhibit NH_4 toxicity Both symbiotic and free-living strains grew better on NH_4 acetate (1 mM) than NH_4 tartrate (1 mM). This inability to utilize NH_4 efficiently might be different if we had included vitamin B_{12} in our basal medium since Kessler and Huss [23]) reported that related *Chlorella* strains grow normally on NH_4 in a B_{12}- containing medium. However, our current understanding of the metabolic function of B_{12} in algae focuses on its role in methionine biosynthesis, not NH_4 utilization [9].

The lower NO_3 level (1mM) did not support any algal growth while 3 of the free-living and Syngen 2-3 (symbiotic group) had minimal growth at the higher level (10mM) (Figure 5). This is the only cluster analysis where a symbiotic strain did not group within the symbiotic cluster. A separate question concerns why the 4 free-living *Chlorella* only exhibit minimal growth on NO_3 (Figure 5). The answer is likely a combination of inoculating at low cell density ($3–5\times10^4$ cells/ml) and only following growth for the first 9 days. Additionally, all free-living Chlorella grew well on NO_3 when either glucose or sucrose was added (Figure 6A). Presumably, the sugars provided extra reducing power needed for the NO_3 to NH_4 conversion. In summary, symbiotic algae possess an efficient system to import and metabolize many AAs and small oligopeptides but they cannot efficiently utilize NO_3 or NH_4 as sole N sources.

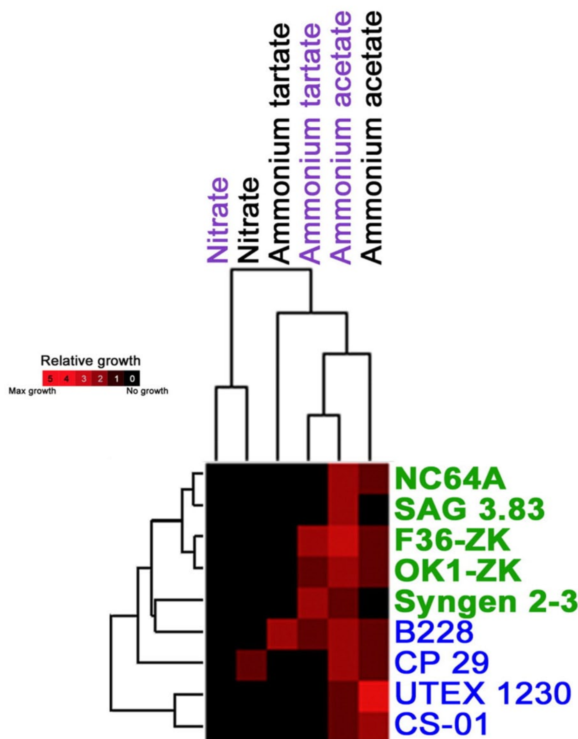

Figure 5. Heat map subgroup from Figure 1 displays growth on NO_3 and ammonium salts at 1 mM (purple) and 10 mM concentrations. Inorganic N sources are listed in the columns. A color scale indicates relative growth. Flask tests were performed for 12 days for the symbiotic and 9 days for the free-living strains.

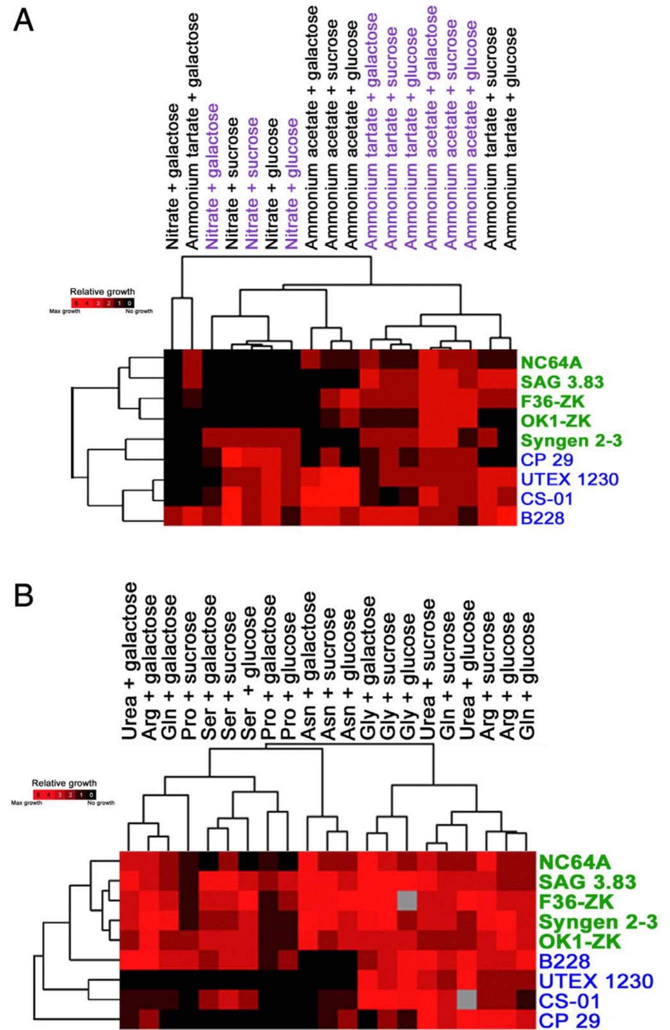

Figure 6. Heat map subgroups from Figure 1. Inorganic (A) and organic (B) N sources supplemented with glucose, sucrose or galactose. Purple labels identify N sources at 1 mM concentration. A color scale indicates relative growth. Flask tests were performed for 9 days for the symbiotic and 7 days for the free-living strains.

3.7. Galactose is a better C source than glucose or sucrose for symbiotic Chlorella but not for free-living Chlorella

All of the algal growth levels in Figures 3 and 5 were lower than those observed on MBBM, which contains both sucrose and peptone. Thus, the presence or absence of sugars is important for an analysis of N metabolism. In this regard, Schlee and Komor [44]) showed that *Chlorella* had a single high-affinity NH_4 transporter whose synthesis was repressed by NH_4 but stimulated by glucose. Similarly, Cho and Komor [8]) showed that *Chlorella vulgaris* expressed multiple AA transport systems, the synthesis of which was not repressed by NH_4 but most were stimulated by glucose. Accordingly, we supplemented all N sources with 3C sources: sucrose (present in MBBM), glucose, or galactose. Galactose was chosen because in other microbes it does not exert catabolite repression [12]. Results for the inorganic and organic N sources are reported in Figures 6A and 6B, respectively. They should be compared with Figures 3 and 5, which use the same N sources but without added sugars. None of the symbiotic strains grew with any combination of sugar and NO_3 (Figure 6A) except for Syngen 2-3. Thus, even after addition of sugars, 4 of the 5 symbiotic strains were unable to utilize NO_3 as a sole N source (Figure 6A). Similarly, as had been observed in Figure 5, 1 mM NH_4 salts gave better growth than 10 mM regardless of the sugar used,

and better growth was achieved with 1 mM NH_4 acetate than with 1 mM NH_4 tartrate.

In all cases, the symbiotic *Chlorella* grew with galactose or sucrose while they grew poorly with glucose (Figures 6A and 6B). The symbiotic *Chlorella* grew as well in galactose and organic N source as they did in MBBM (Figure 6B). NC64A appears to have more enzymes involved in carbohydrate metabolism than other sequenced chlorophytes [4], including many which are related to galactose metabolism. Interestingly, added glucose inhibited the symbiotic *Chlorella* on most organic N sources, with the most glucose-sensitive strain being NC64A (Figure 6B). By contrast, the free-living strains had similar or better growth with sugars than on MBBM, and they preferred sucrose and glucose (Figure 6A). Galactose had inhibitory effects on the growth of most free-living strains with all of the organic N sources except Gly. Strain B228 was the only free-living strain able to utilize multiple organic N sources in the presence of galactose (Figure 6B).

3.8. Bioinformatic and transcriptomic analysis of amino acid transporter orthologs in Chlorella species

AA transporters act as extracellular and intracellular nutrient sensors as well as transporters in all domains of life [10]. The five symbionts studied must have efficient systems for importing AAs from the *P. bursaria* host, which is reflected in their continued ability to use some AAs as a source of N instead of inorganic N sources (Figures 2, 3, and 6B as well as [1,4,18–20]). Thus, the physiological observations depicted in Figures 1–6 led us to hypothesize that in nature the protozoan host regulates the population of symbiotic *Chlorella* by using AAs to restrict their N supply. Keeping the N supply low and the chlorophyll content high (5- to 10-fold higher) is consistent with the symbiont functioning to provide excess photosynthate to the host in the form of secreted maltose [40]. It seemed likely that the function of symbiont-specific AA transporters might persist following their release from symbiosis. In order to evaluate potential contributions of endosymbiosis on the evolution and expression of the AA transporters identified, we compared the genes for AA transporters in the symbiont NC64A and the free-living alga *C. sorokoniana* UTEX-1230 (Table 1), as well as analyzing gene expression profiles of NC64A growing both in axenic culture and within *P. bursaria* (Table 2).

C. variabilis NC64A encodes at least 40 putative AA transporters (Table 1). Genes 1 to 35 in Table 1 were identified by Blanc et al. [4] on the basis of their significant homology with the AA transporter protein family profile (PfamPF01490.9). Of these 35 genes, 15 of them were expressed at significant levels as judged by read counts from RNAseq experiments shown in Figure 7 [4,42]. Each of these 35 genes had an apparent ortholog in *C. sorokoniana* UTEX-1230 (Table 1), and in 5 cases, two genes from NC64A identified the same gene from UTEX-1230 (Table 1).

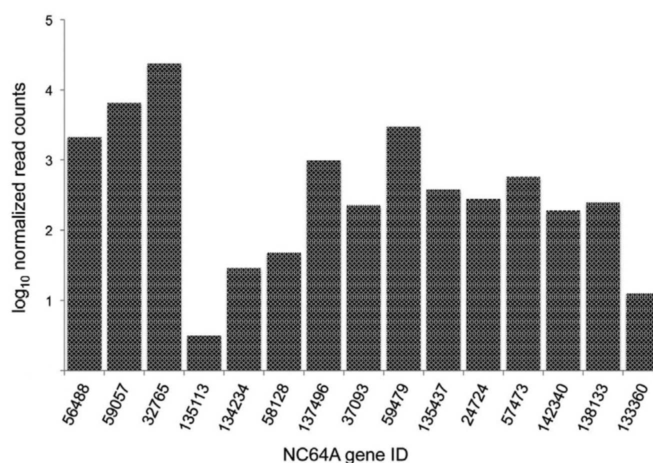

Figure 7. *C. variabilis* NC64A mRNAs coded by AA transporter genes during axenic growth. Normalized mRNA abundance of 15 AA transporter genes.

In a separate approach, the characterized AA transporters in the plant *Arabidopsis thaliana* (Table 1 in [46]) were used to perform reciprocal BLAST searches against NC64A [4] and UTEX-1230 (UNL algal consortium, in preparation) genomes, using an expected value of 1×10^{-10} as a cutoff. The major predicted isoform for each identified locus was selected and used to perform a BLAST search against the *Arabidopsis* genome, again with an expected value of 1×10^{-10} as a cutoff. Each algal protein returned an *Arabidopsis* AA transporter, and the gene designations and *E*-values for NC64A and UTEX 1230 are presented in Supplementary Tables 1 and 2, respectively. Results of the initial and reciprocal BLAST searches identified 16 putative orthologs for *Arabidopsis* AA transporters in NC64A (Supplementary Table 1) and 11 of these putative orthologs coincided with genes, which had been identified by [4], see Table 1, while 5 of them had not been identified previously. These 5 are indicated by genes 36 to 40 in Table 1. By contrast, 25 putative orthologs were identified in UTEX 1230 (Supplementary Table 2). Due to the highly interrupted nature of the *C. sorokoniana* UTEX-1230 genome, there remain some discrepancies in the annotation. However, we are confident that even the partial nature of some UTEX-1230 AA transporter orthologs (Table 1) reflects AA transporters

Table 2. Expression summary of predicted AA transporter genes in *C. variabilis* NC64A grown in culture or as a symbiont in *Paramecium bursaria*. Expression data for each gene represent manual counts of reads aligning to the corresponding genomic interval normalized per million mapped reads.

NC64A Gene ID	Protein ID	Scaffold	Axenic	Symbiont	Fold change	Log2 FC
58128	EFN54604	13:78,798–84,676 (−)	7	99	13.46	3.7
24724	EFN54340	14:418,634–420,275 (−)	30	8	0.27	−1.8
36103	EFN54400.1	14:833,000–835,270 (−)	3	17	6.01	2.5
53357	EFN53996	15:290,406–294,216 (−)	30	8	0.27	−1.8
58448	EFN53780.1	16:626,917–630,844 (−)	52	6	0.11	−3
138133	EFN59501	2:2,150,538–2,155,669 (−)	16	9	0.57	−0.8
32765	EFN51990	25:222,544–226,977 (−)	2635	2794	1.06	0.08
37093	EFN51991	25:227,152–230,758 (−)	35	42	1.19	0.26
59057	EFN51898	25:231,079–235,117 (+)			No Exp	
140447	EFN58455.1	3:553,777–556,173 (+)	95	32	0.33	−1.57
142334	EFN58316.1	4:1,840,877–1,844,482 (−)	48	12	0.25	−1.95
7483	EFN50706.1	43:40,210–42,680 (+)	8	8	0.97	−0.04
17797	EFN50713	43:87,189–87,664 (+)			No Exp	
57473	EFN56726	7:287,118–291,257 (−)	83	84	1	0
59479	EFN50622	79:1541–9952 (+)	1124	4846	4.31	2.1
144770	EFN56324	8:392,270–395,765 (−)	25	29	1.14	0.19
AAT-All		Sum of all reads	4193	7996	1.9	0.93

3.9. Comparison of in symbiont vs. axenic expression

The 16 AA transporters identified in NC64A (Supplementary Table 1) were examined for their expression levels in the axenic and endosymbiont states (Table 2). Fourteen of the AA transporter orthologs were expressed at detectable levels in the 2 *P. bursaria* assemblies (Table 2), and of these expressed isoforms, 3 were down regulated in axenic culture, 6 were up regulated, and 5 remained roughly constant (Table 2). The sample size and differences in sequencing platform precluded a formal statistical analysis of the significance of these differential gene expressions. However, 2 genes (32765 and 59479) accounted for 90 and 95% of the mapped reads in axenic and symbiont growth, respectively; indicating that these transporters likely provide the majority of AA uptake capabilities in NC64A and other symbiotic *Chlorella*. The expression of 32765 is equivalently high in the axenic and symbiotic states while 59479 is elevated 4.3-fold in the symbiont (Table 2). Thus, mining the genomes and transcriptomes available for *Chlorella* has led us to identify AA transporter genes that might play important roles in symbiotic N metabolism. It will be of interest to learn the AA specificity of the 32765 and 59479 AAT genes.

4. Discussion

Nine *Chlorella* strains (5 symbiotic and 4 free-living) were compared with regard to their abilities to grow on organic and inorganic N sources. The 9 strains separated into two clusters, one containing all the symbiotic strains and the other all the free-living strains. This analysis confirms the robust nutritional/metabolic differences between symbiotic and free-living *Chlorella* strains as well as the generalization that the free-living *Chlorella* are better adapted for inorganic N sources while the symbiotic *Chlorella* are adapted for organic N sources. In particular, symbiotic *Chlorella* strains (a) could not use NO_3; (b) exhibited some NH_4 toxicity in that the cells grew slightly on 1 mM NH_4 but poorly or not at all on 10 mM NH_4; (c) in general preferred urea or amino acids; and (d) used two amino acids (Asn and Ser) that were not utilized by the free-living strains. We conclude that the symbiotic algae have physiological signatures that are conserved after they are separated from their symbiotic hosts and that one such signature concerns the constitutive expression of their AA transporters. For NC64A, it seems likely that these AA transporters reflect the mechanism by which *P. bursaria* might control the growth rate and population of its photosynthetic algal symbiont.

Thus, we have physiological, genomic, and transcriptomic data showing that the symbiotic and free-living *Chlorella* strains differ significantly in their N metabolism. The biological implications of this conclusion are three-fold.

i) The inability of 4 symbiotic *Chlorella* to use NO_3 as the N source occurs despite the fact that recent work by Sanz-Luque et al. [43]) showed that *C. variabilis* NC64A has a complete set of genes needed for NO_3 assimilation (transporters, reductases, and synthesis of the molybdenum cofactor Moco). They concluded that the mechanism responsible for silencing NO_3 utilization is unknown [43].

ii) The 5 symbiotic *Chlorella* strains may be polyphyletic [13,38]; namely, they arose from multiple independent symbiotic events. However, a competing hypothesis invokes a common ancestor with a specific genotype/genome flexible enough to enable symbiotic evolution with several hosts. The symbiotic *Chlorella* we have studied are similar in their inability to use NO_3 and their rapid uptake and utilization of certain AAs as sole N sources. Additionally, their growth rates were slower compared to their free-living counterparts, and they all are susceptible to dsDNA chlorovirus infections ([48]; Quispe et al. manuscript in preparation). These phenotypic differences reflect major cellular and metabolic reprogramming at the structural and molecular levels. Our results provide a possible connection between the endosymbiotic life style, AA transporters, and virus susceptibility, illustrating the trade-offs endosymbiotic *Chlorella* must make in nature.

iii) Although the scope of this paper focuses mainly on N metabolism, the galactose preference exhibited by all symbiotic algae tested is also relevant. For instance, [4], suggested that NC64A has more enzymes involved in galactose metabolism than other sequenced chlorophytes. They looked for 2 protein families of galactose related carbohydrate esterases, 18 families of glycosyl hydrolases, and 8 families of glycosyl transferases present in the 6 algal genomes which had been sequenced at that time. NC64A had a total of 149 galactose related genes, whereas *Chlamydomonas reinhardtii*, *Micromonas pusilla* RCC299, *Micromonas* sp. CCMP, *Ostreococcus lucimarinus*, and *O. tauri* had 84, 36, 30, 36, and 29 respectively [4]. Thus genetic and physiological changes in the metabolic capabilities of *Chlorella* symbiotic strains include major changes in their ability to assimilate both N and C sources. Future studies should improve our knowledge about the entirety of nutrient acquisition in *Chlorella*, including the assimilation of different N and C sources and its regulation, their relationship to the rest of metabolism, how metabolism is altered during symbiosis, and how these aspects of symbiosis are tied to the viral susceptibility of some *Chlorella* strains.

Supplementary data to this article follows the **References**.

Disclosure statement — The authors declare no conflict of interest.

Author contributions — C.F.Q., K.W.N., and J.L.V.E. designed research; C.F.Q. and O.S., performed the experiments; C.F.Q., M.K,W.R., K.W.N., and J.L.V.E. analyzed data; and C.F.Q., K.W.N., and J.L.V.E. wrote the paper.

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References

[1] D. Albers, W. Reisser, W. Wiessner, Studies on the nitrogen supply of endosymbiotic *Chlorellae* in green *Paramecium bursaria*, Plant Sci. Lett. 25 (1) (1982) 85–90.

[2] S.F. Altschul, T.L. Madden, A.A. Schaffer, J. Zhang, Z. Zhang, W. Miller, D.J. Lipman, Gapped BLAST and PSI-BLAST: a new generation of protein database search programs, Nucleic Acids Res. 25 (17) (1997) 3389–3402.

[3] Bischoff, H. W., Bold, H. C. 1963. Phycological Studies IV. Some Soil Algae from Enchanted Rock and Related Algal Species. No. 6318 ed. Austin, Texas. University of Texas Publication.

[4] G. Blanc, G. Duncan, I. Agarkova, M. Borodovsky, J. Gurnon, A. Kuo, E. Lindquist, S. Lucas, J. Pangilinan, J. Polle, et al., The *Chlorella variabilis* NC64A genome reveals adaptation to photosymbiosis, coevolution with viruses, and cryptic sex, Plant Cell 22 (9) (2010) 2943–2955.

[5] D. Blankenberg, A. Gordon, G. Von Kuster, N. Coraor, J. Taylor, A. Nekrutenko, G. Team, Manipulation of FASTQ data with galaxy, Bioinformatics 26 (14) (2010) 1783–1785.

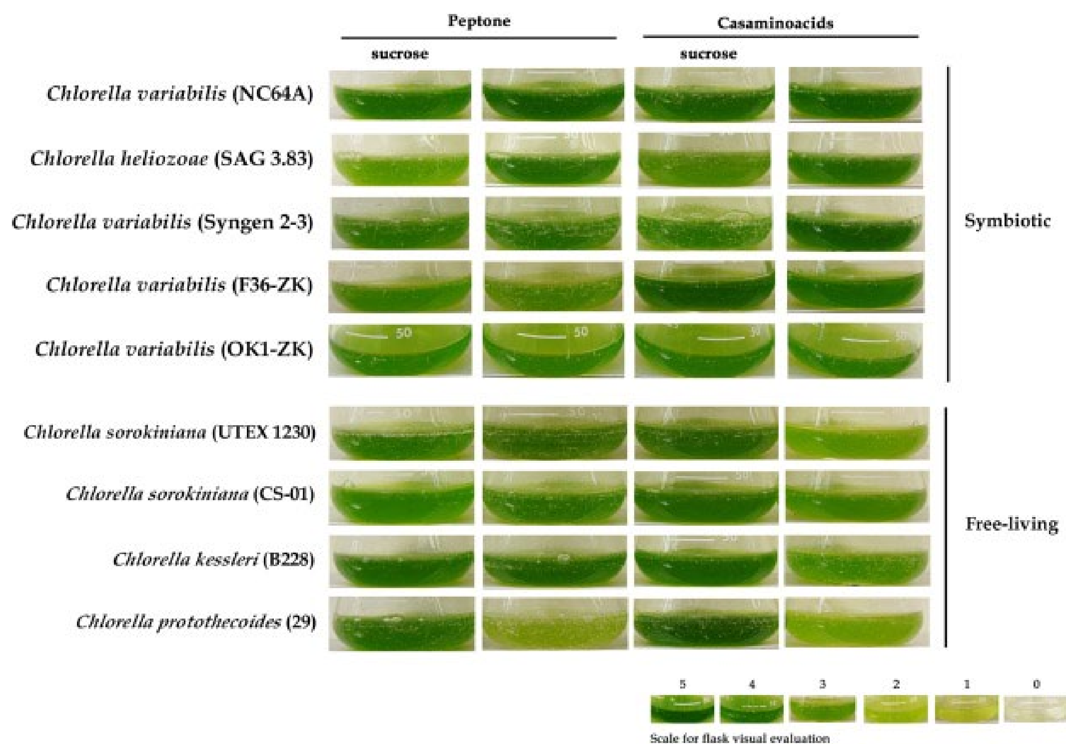
[6] J.A. Bubeck, A.J. Pfitzner, Isolation and characterization of a new type of chlorovirus that infects an endosymbiotic *Chlorella* strain of the heliozoon *Acanthocystis turfacea*, J. Gen. Virol. 86 (Pt 10) (2005) 2871–2877.
[7] E. Cernichiari, L. Muscatine, D. Smith, Maltose excretion by the symbiotic algae of *Hydra viridis*, Proc. R. Soc. Lond. Ser. B Biol. Sci. 173 (1033) (1969) 557–576.
[8] B. Cho, E. Komor, The amino acid transport systems of the autotrophically grown green alga *Chlorella*, Biochim. Biophys. Acta Biomembr. 821 (3) (1985) 384–392.
[9] M.T. Croft, M.J. Warren, A.G. Smith, Algae need their vitamins, Eukaryot. Cell 5 (8) (2006) 1175–1183.
[10] W.N. Fischer, M. Kwart, S. Hummel, W.B. Frommer, Substrate specificity and expression profile of amino acid transporters (AAPs) in *Arabidopsis*, J. Biol. Chem. 270 (27) (1995) 16315–16320.
[11] M. Fujishima (Ed.), Endosymbionts in Paramecium, Springer-Verlag, Berlin, 2010.
[12] J.M. Gancedo, Yeast carbon catabolite repression, Microbiol. Mol. Biol. Rev. 62 (2) (1998) 334–361.
[13] R. Hoshina, N. Imamura, Multiple origins of the symbioses in *Paramecium bursaria*, Protist 159 (2008) 53–63.
[14] M.D. Johnson, The acquisition of phototrophy: adaptive strategies of hosting endosymbionts and organelles, Photosynth. Res. 107 (1) (2011) 117–132.
[15] E. Jolley, D.C. Smith, The green hydra symbiosis. I. Isolation, culture and characteristics of the *Chlorella* symbiont of 'European' *Hydra viridis*, New Phytol. 81 (3) (1978) 637–645.
[16] S. Kamako, R. Hoshina, S. Ueno, N. Imamura, Establishment of axenic endosymbiotic strains of Japanese *Paramecium bursaria* and the utilization of carbohydrate and nitrogen compounds by the isolated algae, Eur. J. Protistol. 41 (3) (2005) 193–202.
[17] M. Kanehisa, S. Goto, KEGG: kyoto encyclopedia of genes and genomes, Nucleic Acids Res. 28 (1) (2000) 27–30.
[18] M.W. Karakashian, Symbiosis in *Paramecium bursaria*, Symp. Soc. Exp. Biol. 29 (1975) 145–173.
[19] S.J. Karakashian, M.W. Karakashian, Evolution and symbiosis in the genus *Chlorella* and related algae, Evolution 19 (3) (1965) 368–377.
[20] Y. Kato, S. Ueno, N. Imamura, Studies on the nitrogen utilization of endosymbiotic algae isolated from Japanese *Paramecium bursaria*, Plant Sci. 170 (3) (2006) 481–486.
[21] Y. Kato, N. Imamura, Effect of calcium ion on uptake of amino acids by symbiotic *Chlorella* F36-ZK isolated from Japanese *Paramecium bursaria*, Plant Sci. 174 (1) (2008) 88–96.
[22] H. Kawakami, N. Kawakami, Behavior of a virus in a symbiotic system, *Paramecium bursaria* zoochlorella, J. Protozool. 25 (2) (1978) 217–225.
[23] E. Kessler, V. Huss, Biochemical taxonomy of symbiotic *Chlorella* strains from *Paramecium* and *Acanthocystis*, Botanica Acta 103 (2) (1990) 140–142.
[24] D. Kim, G. Pertea, C. Trapnell, H. Pimentel, R. Kelley, S.L. Salzberg, TopHat2: accurate alignment of transcriptomes in the presence of insertions, deletions and gene fusions, Genome Biol. 14 (4) (2013) (R36,2013-14-4-r36).
[25] Y. Kodama, M. Fujishima, Differences in infectivity between endosymbiotic *Chlorella variabilis* cultivated outside host *Paramecium bursaria* for 50 years and those immediately isolated from host cells after one year of reendosymbiosis, Biol. Open 5 (1) (2015) 55–61.
[26] Y. Kodama, H. Suzuki, H. Dohra, M. Sugii, T. Kitazume, K. Yamaguchi, S. Shigenobu, M. Fujishima, Comparison of gene expression of *Paramecium bursaria* with and without *Chlorella variabilis* symbionts, BMC Genomics 15 (2014) (183,2164-15- 183).
[27] G. Kovacevic, Value of the hydra model system for studying symbiosis, Int. J. Dev. Biol. 56 (6–8) (2012) 627–635.
[28] G. Kovacevic, M. Kalafatic, N. Ljubesic, New observations on green hydra symbiosis, Folia Biol. (Krakow) 55 (1–2) (2007) 77–79.
[29] R.K. Kulkarni, K.W. Nickerson, Nutritional control of dimorphism in *Ceratocystis ulmi*, Exp. Mycol. 5 (2) (1981) 148–154.
[30] B.V.G. Kvitko, New finding of a titratable infectious zoochlorella virus, Dokl. Akad. Nauk SSSR (279) (1984) 998–999.
[31] B. Matzke, E. Schwarzmeier, E. Loos, Erratum: maltose excretion by the symbiotic *Chlorella* of the heliozoan *Acanthocystis turfacea*, Planta 182 (2) (1990) 312.
[32] P.J. McAuley, Isolation of viable uncontaminated *Chlorella* from green hydra, Limnol. Oceanogr. 31 (1) (1986) 222–224.
[33] P. McAuley, Nitrogen limitation and amino-acid metabolism of *Chlorella* symbiotic with green hydra, Planta 171 (4) (1987) 532–538.
[34] R.H. Meints, J.L. Van Etten, D. Kuczmarski, K. Lee, B. Ang, Viral infection of the symbiotic *Chlorella*-like alga present in *Hydra viridis*, Virology 113 (2) (1981) 698–703.
[35] E. Minaeva, E. Ermilova, Sequencing and expression analysis of the gene encoding PII signal protein in *Chlorella variabilis* NC64A, J. Plant Biochem. Physiol. 2 (2015) 142.
[36] J.W. Nicol, G.A. Helt, S.G. Blanchard Jr., A. Raja, A.E. Loraine, The integrated genome browser: free software for distribution and exploration of genome-scale datasets, Bioinformatics 25 (20) (2009) 2730–2731.
[37] H.D. Park, C.L. Greenblatt, C.F.T. Mattern, C.R. Merril, Some relationships between chlorohydra, its symbionts and some other chlorophyllous forms, J. Exp. Zool. 164 (2) (1967) 141–161.
[38] T. Pröschold, T. Darienko, P.C. Silva, W. Reisser, L. Krienitz, The systematics of zoochlorella revisited employing an integrative approach, Environ. Microbiol. 13 (2) (2011) 350–364.
[39] C.F. Quispe, O. Sonderman, A. Seng, B. Rasmussen, G. Weber, C. Mueller, D.D. Dunigan, J.L. Van Etten, Three-year survey of abundance, prevalence and genetic diversity of chlorovirus populations in a small urban lake, Arch. Virol. 1–9 (2016).
[40] T. Rees, Are symbiotic algae nutrient deficient? Proc. R. Soc. B Biol. Sci. 243 (1308) (1991) 227.
[41] J.N. Rosenberg, N. Kobayashi, A. Barnes, E.A. Noel, M.J. Betenbaugh, G.A. Oyler, Comparative analyses of three chlorella species in response to light and sugar reveal distinctive lipid accumulation patterns in the microalga *C. sorokiniana*, PLoS One 9 (4) (2014), e92460.
[42] J.M. Rowe, D.D. Dunigan, G. Blanc, J.R. Gurnon, Y. Xia, J.L. Van Etten, Evaluation of higher plant virus resistance genes in the green alga, *Chlorella variabilis* NC64A, during the early phase of infection with *Paramecium bursaria Chlorella* virus-1, Virology 442 (2) (2013) 101–113.
[43] E. Sanz-Luque, A. Chamizo-Ampudia, A. Llamas, A. Galvan, E. Fernandez, Understanding nitrate assimilation and its regulation in microalgae, Front. Plant Sci. 6 (2015) 899.
[44] J. Schlee, E. Komor, Ammonium uptake by *Chlorella*, Planta 168 (2) (1986) 232–238.
[45] R.W. Siegel, Hereditary endosymbiosis in *Paramecium bursaria*, Exp. Cell Res. 19 (2) (1960) 239–252.
[46] M. Tegeder, Transporters for amino acids in plant cells: some functions and many unknowns, Curr. Opin. Plant Biol. 15 (3) (2012) 315–321.
[47] J.L. Van Etten, D.E. Burbank, D. Kuczmarski, R.H. Meints, Virus infection of culturable *Chlorella*-like algae and development of a plaque assay, Science 219 (4587) (1983) 994–996.
[48] J.L. Van Etten, D.D. Dunigan, Chloroviruses: not your everyday plant virus, Trends Plant Sci. 17 (1) (2012) 1–8.
[49] J.L. Van Etten, D.E. Burbank, Y. Xia, R.H. Meints, Growth cycle of a virus, PBCV-1, that infects *Chlorella*-like algae, Virology 126 (1) (1983) 117–125.
[50] Yellowlees, Metabolic interactions between algal symbionts and invertebrate hosts, Plant Cell Environ. 31 (5) (2008) 679.

Supplementary Table 1. Accession numbers of putative *C. variabilis* NC64A orthologs to *A. thaliana* proteins involved in AA transport. AAP = amino acid permeases, AAT = amino acid transporters, LHT = lysine histidine transporter.

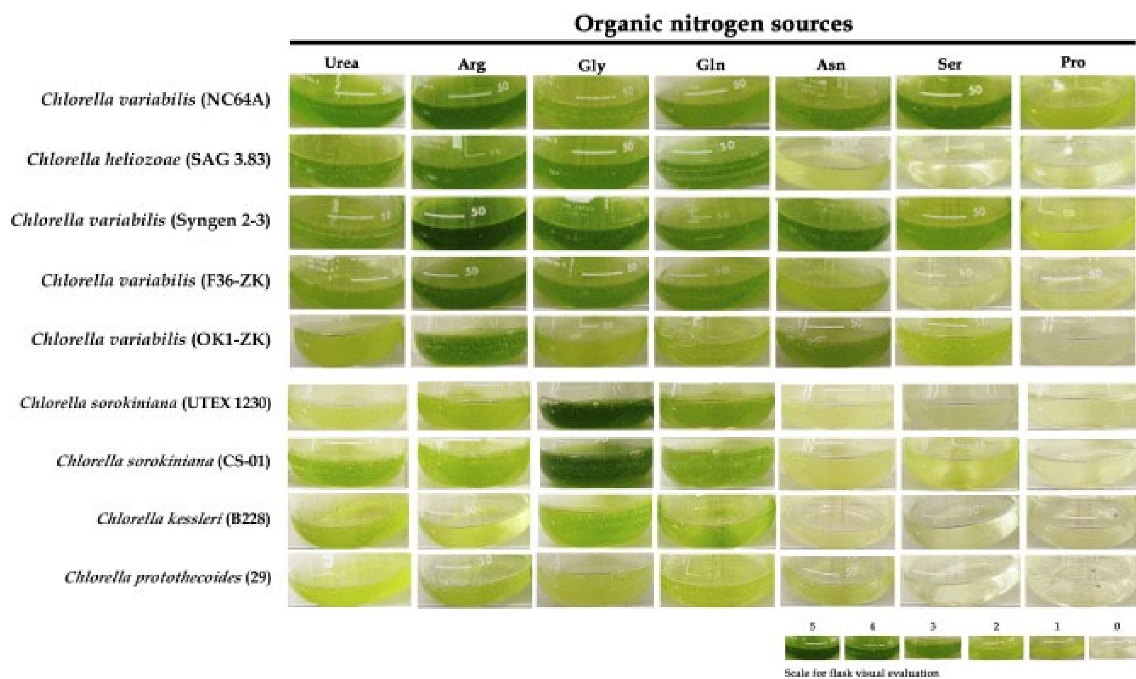
AA transporter ortholog in NC64A	Protein ID	A. thaliana best hit	e-value
37093	EFN51991	AAP2	2E-76
58128	EFN54604	AAP2	9E-72
32765	EFN51990	AAP2	1E-52
57473	EFN56726	LHT 1	2E-83
138133	EFN59501	AAP2	2E-49
59057	EFN51898	AAP2	2E-45
53357	EFN53996	AAP	3E-45
59479	EFN50622	AAP2	3E-29
24724	EFN54340	AAP2	1E-21
144770	EFN56324	GABA transporter 1	7E-31
17797	EFN50713	AAP8	1E-19
142334	EFN58316.1	AAP or GABA permease	4E-158
140447	EFN58455.1	AAT1	1E-34
7483	EFN50706.1	AAT1	5E-112
58448	EFN53780.1	AAT1	2E-61
36103	EFN54400.1	AAT1	2E-54

Supplementary Table 2. Scaffold numbers of putative *C. sorokiniana* UTEX-1230 orthologs to *A. thaliana* proteins involved in AA transport. AAP = amino acid permeases, AAT = amino acid transporters, LHT = lysine histidine transporter.

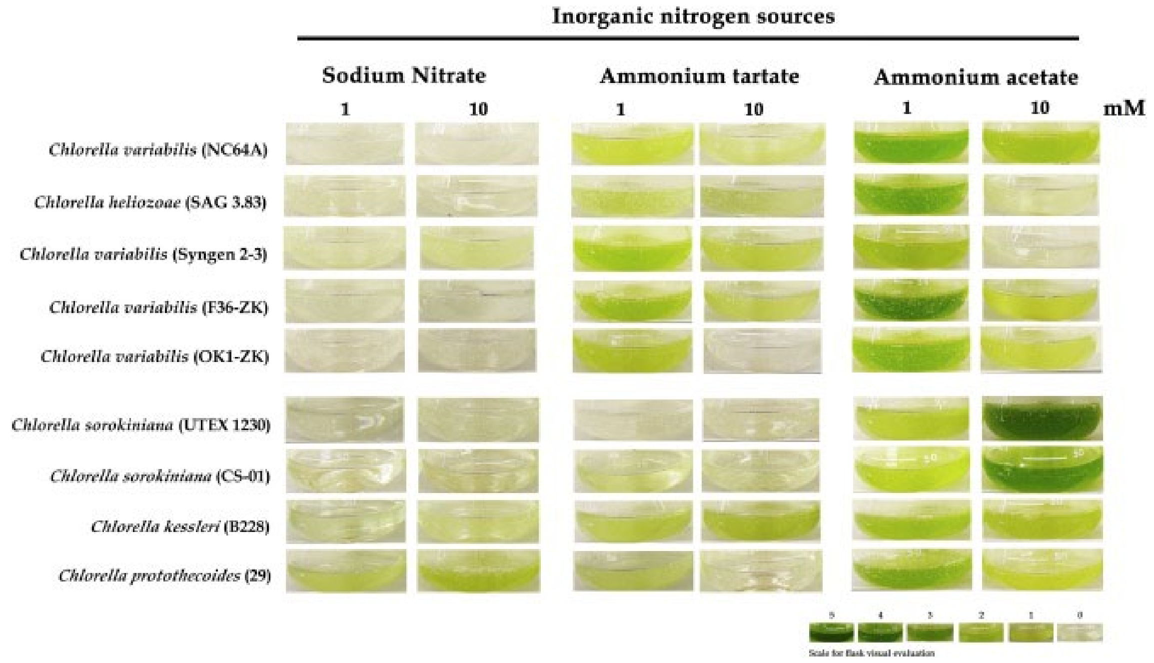
AA transporter ortholog in UTEX-1230	A. thaliana best hit	e-value
scaffold 82.g49.iso1	AAP2	4E-81
scaffold 181.g27.iso1	AAP5	1E-73
scaffold 172.g106.iso1	AAP3	1E-76
scaffold 15.g150.iso1	AAP2	4E-69
scaffold 99.g53.iso4	AAP2	1E-67
scaffold 106.g243.iso1	AAP2	4E-63
scaffold 34.g191.iso1	AAP CAA54632.1	1E-63
scaffold 91.g67.iso3	LHT1	6E-83
scaffold 35.g114.iso1	AAP2	2E-51
scaffold 13.g237.iso1	AAP2	2E-58
scaffold 35.g117.iso2	AAP2	2E-43
scaffold 6.g13.iso1	AAP2	5E-54
scaffold 270.g17.iso1	LHT1	6.5E-60
scaffold 56.g4.iso2	GABA transporter 1	3E-34
scaffold 124.g21.iso1	AAP8	1E-34
scaffold 57.g99.iso1	AAT	9E-64
scaffold 76.g4.iso1	AAP2	2E-45
scaffold 132.g58.iso1	AAP AAB71468.1	9E-29
scaffold 1.g418.iso1	GABA transporter 1	1E-87
scaffold 110.g43.iso1	AAP2	4E-36
scaffold 6.g14.iso2	AAP3	2E-11
scaffold 13.g234.iso1	AAP4	9E-13
scaffold 92.g116.iso1	AAP1	3E-117
scaffold 98.g9.iso1	AAP1	2E-60
scaffold 13.g249.iso1	AAT1	8E-125



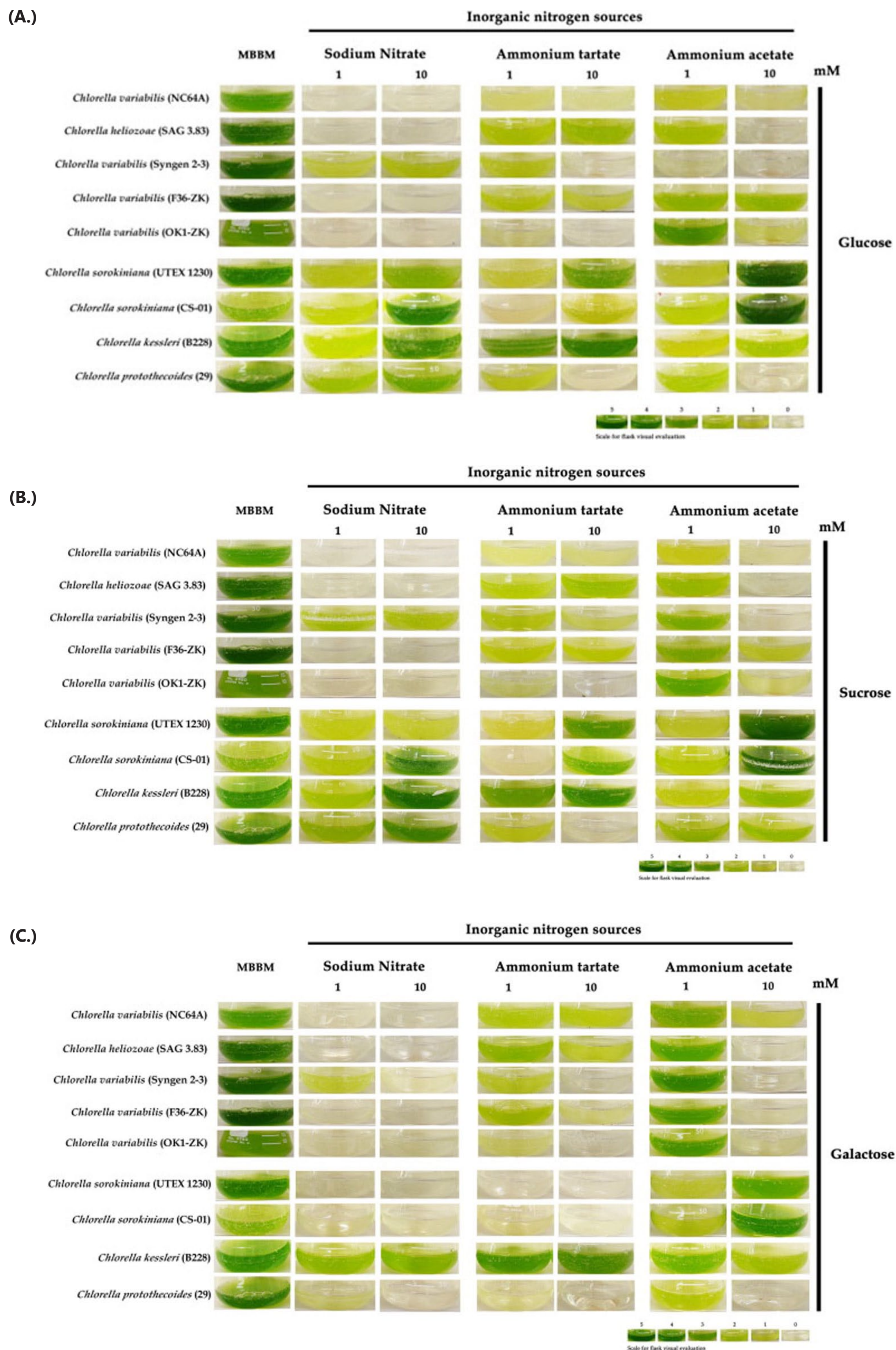
Supplementary Figure 1. In vitro flask test identifies metabolic differences between symbiotic and free-living *Chlorella* strains grown on variations of MBBM (sucrose + peptone). Columns represent combinations of complex N (0.1% peptone or 0.1% casamino acids) with or without the addition of sucrose (10 mM). MBBM is the control. Rows represent the 9 strains. The 5 symbiotic strains include *Chlorella variabilis* NC64A, *C. heliozoae* SAG 3.83, *C. variabilis* Syngen 2-3, *C. variabilis* F36-ZK, and *C. variabilis* OK1-ZK. The 4 free-living strains are *C. sorokiniana* UTEX 1230, *C. sorokiniana* CS-01, *C. kessleri* B228, and *C. protothecoides* 29. Flasks were shaken at 200 rpm and 26 °C in constant light. Symbiotic strains were incubated for 9 days and free-living strains were incubated for 7 days. For each growth medium, triplicates were performed for the symbiotic *Chlorella* strains and duplicates for the free-living *Chlorella* species. Flasks were evaluated based on the color scale included. Subsequent supplementary figures have similar layouts.



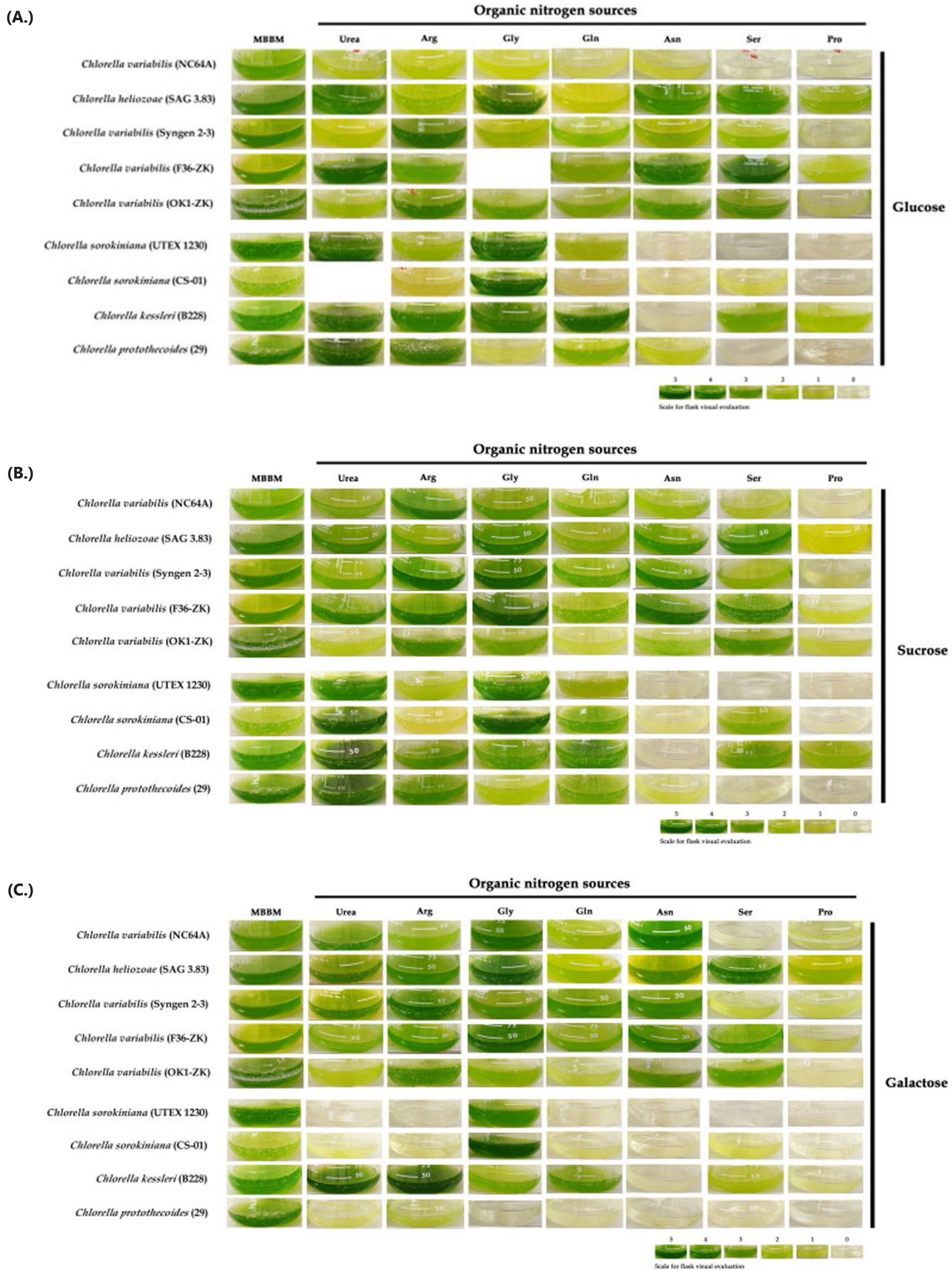
Supplementary Figure 2. In vitro flask test of organic N (10 mM). Nitrogen sources include urea, Arg, Gly, Gln, Asn, Ser, and Pro. Photographs were taken after 12 days for the symbiotic and after 9 days for the free-living strains.



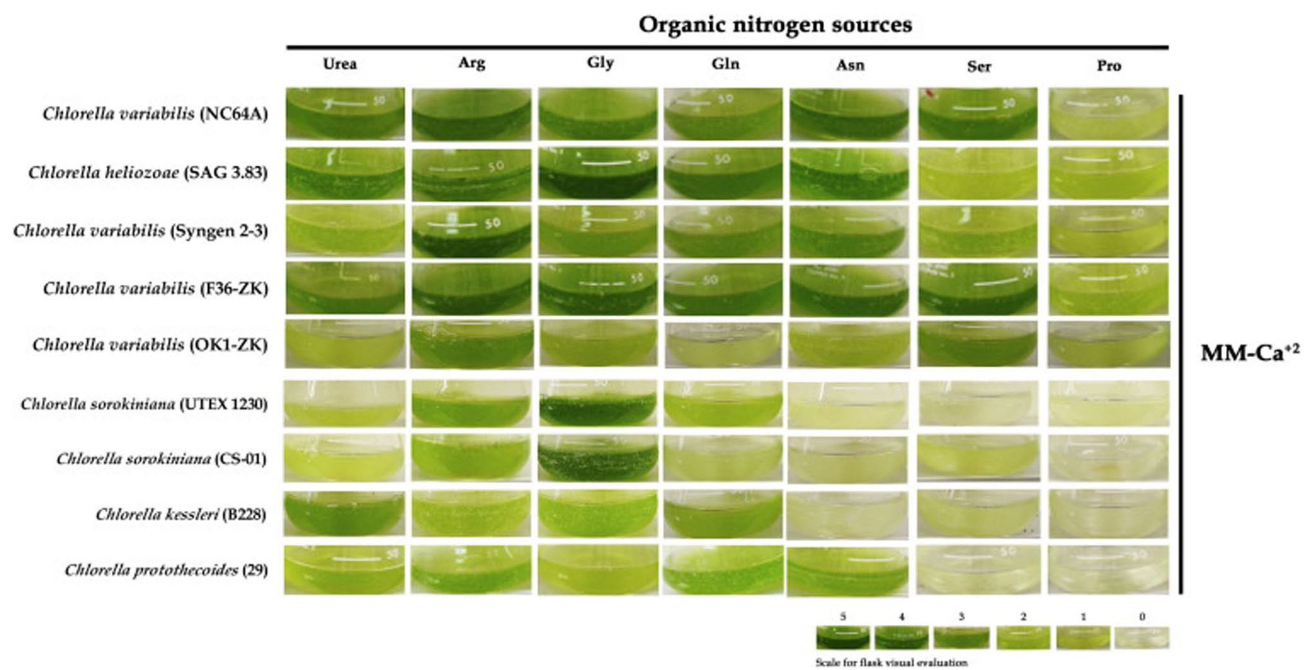
Supplementary Figure 3. In vitro flask test of inorganic N (1 or 10 mM). Nitrogen sources include sodium NO_3, NH_4 tartrate, and NH_4 acetate. Photographs were taken after 12 days for the symbiotic and after 9 days for the free-living strains.



Supplementary Fig. 4. In vitro flask test of inorganic N (1 or 10 mM) supplemented with C source (10 mM). Carbon sources include **(A)** glucose, **(B)** sucrose, and **(C)** galactose. Nitrogen sources include sodium NO3, NH4 tartrate, and NH4 acetate. Photographs were taken after 9 days for the symbiotic and after 7 days for the free-living strains.



Supplementary Fig. 5. In vitro flask test of organic N (10 mM) supplemented with C source (10 mM). Carbon sources include **(A)** glucose, **(B)** sucrose, and **(C)** galactose. Nitrogen sources include urea, Arg, Gly, Gln, Asn, Ser, and Pro. Photographs were taken after 9 days for the symbiotic and after 7 days for the free-living strains.



Supplementary Fig. 6. In vitro flask test displays removal of Ca^{2+} from media with organic N sources (10 mM). Nitrogen sources include Urea, Arg, Gly, Gln, Asn, Ser, and Pro. Photographs were taken after 12 days for the symbiotic and after 9 days for the free-living strains.