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PAPER

Untargeted metabolic footprinting reveals a surprising breadth of metabolite uptake and release by *Synechococcus sp. PCC 7002*†

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Cyanobacteria are important primary producers in diverse ecosystems, yet little is known about the extent of their metabolic interactions with the environment. We have used an integrated, untargeted metabolic footprinting approach to systematically evaluate the uptake and release of metabolites between a model marine cyanobacterium *Synechococcus sp. PCC 7002* and different growth media. It was found that 47 out of 202 detected metabolites were consumed, and an additional 55 metabolites were released by the cells. Surprisingly, *Synechococcus* was found to uptake a great diversity of metabolites dominant in and specific to its own metabolite extract including histidine betaine (hercynine), γ-glutamyl phenylalanine and a hexosamine-based trisaccharide. This provides *Synechococcus* a mechanism to benefit from the lysis of part of their population (*i.e.* due to environmental stress or predation). Additionally, stable isotope probing was used to show that adenine and glutamate are actively metabolized following uptake. A significant turnover of glucosylglycerol, a cyanobacterial compatible solute, as opposed to a negligible turnover of the hexosamine-based trisaccharide were also observed using stable isotope probing. The untargeted metabolic footprinting approach used in this study is generally applicable to investigate metabolic interactions of microorganisms with the environment and may prove useful to construct microbial community foodwebs.

Introduction

Microorganisms are dominant determinants of global carbon cycling and exist in complex, interdependent, microbial communities. Little is understood of the interconnections of metabolic networks occurring between cells in pure culture much less in microbial communities.[1] Cyanobacteria are prominent primary producers in a wide diversity of environments[2] and are important members of marine phytoplankton.[3] As part of marine phytoplankton, cyanobacteria contribute to the formation of dissolved organic matter (by exudation or lysis) which is considered to be consumed almost exclusively by heterotrophs.[4] However, cyanobacteria have been reported to utilize specific metabolites photoheterotrophically. Previous studies have focused primarily on utilization of specific metabolite classes including amino acids,[5–8] glycerol, glucose,[9] glucosylglycerol, trehalose, and sucrose.[10] However, the extent of cyanobacterial photoheterotrophic metabolite utilization has never been studied systematically using an untargeted approach.

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Untargeted mass spectrometry-based metabolomics allows comprehensive profiling of metabolites in complex biological samples[11] and has been used in various contexts for microbial functional genomics.[12,13] Previously, we used untargeted metabolite profiling to identify a number of unexpected metabolites in extracts of *Synechococcus sp. PCC 7002*.[14] We also identified metabolites in spent media extracts such as methylated nucleosides or nonpolar amino acids. Release of nonpolar amino acids has previously been reported for cyanobacteria.[5] However, metabolites with the highest ion counts in metabolite profiles of cell extracts, such as glucosylglycerol, hexosamine-based trisaccharide (trisaccharide), histidine betaine, or γ-glutamyl dipeptides, were not detected in the media extracts.[14] Lack of these metabolites in the media extracts suggests that the release of metabolites is not due to cell lysis which would lead to a release of all intracellular metabolites.

Analysis of metabolite profiles of growth media from microbial cultures, referred to as metabolic footprinting,[15] has proved a valuable approach for the characterization of cellular physiology and genetic perturbations[16] or investigation of selectivity of substrate utilization.[17] More recently, metabolic footprinting was used to study pleiotropic effects of specific mutations,[18] investigation of metabolic impact of heterologous protein production,[19] and optimization of amino acid composition of growth media to support microbial growth.[20]

Here we present a systematic study of metabolite uptake or release between the model marine non-filamentous cyanobacterium *Synechococcus sp. PCC 7002* and its environment using an untargeted metabolic footprinting approach. These studies were performed using minimal media and the minimal media with the addition of yeast extract, *Synechococcus* extract, or MEBM media. The fate of selected metabolites was determined using stable isotopic labeling of *Synechocccus* and allowed differentiation of the degree of turnover of uptaken metabolites.

Materials and methods

Chemicals and strains

HPLC grade water, methanol, acetonitrile (Honeywell), inorganic salts and vitamin B_{12} for culture media preparation, adenine, glutamate, methionine sulfone (Sigma-Aldrich), [^{13}C]sodium bicarbonate, (Cambridge Isotope Laboratories), Bacto Yeast Extract (BD), Mammary Epithelium Basal Medium (MEBM; Lonza). *Synechococcus sp. PCC 7002* (American Type Culture Collection, ATCC number 27264).

Culture conditions

The culture of *Synechococcus* obtained from ATCC was subcultured two times in 1047 MN Marine medium (ATCC Medium 957), cells were pelleted, resuspended in one tenth of the original culture volume in 1047 MN Marine media containing 10 mM glycerol and 5% (v/v) methanol and aliquoted into vials to be stored under cryogenic conditions. Cells from a cryogenic vial were subcultured eight times in A+ media[21,22] containing 10 μg L^{-1} of vitamin B_{12} (pH was adjusted to 8.2 prior to autoclaving). Trizma base buffer was not included in the media to prevent large peaks of this compound and potential suppression of peaks of coeluting metabolites in LC-MS datasets. This A+ media was used in both metabolic footprinting and stable isotope probing experiments.

For metabolic footprinting experiments, cells were grown as 3.7 ml cultures (3.5 ml of media + 200 μl inoculum) in 14 ml polypropylene round-bottom culture tubes placed in a vertical rotating wheel in a model 1925 Shel Lab incubator at 34 °C. The optical density at 730 nm (OD_{730}) of cultures after inoculation was below 0.03. Cultures were subjected to a 14/10 h light/dark cycle (fluorescent lights) with a photon flux rate at the top of the rotating wheel of approximately 63 μmol m^{-2} s^{-1}. Four media were used for metabolic footprinting experiments: A+ media, A+ media supplemented with MEBM (1 : 1 v/v), A+ media supplemented with yeast extract (0.1% w/v), and A+ media supplemented with metabolite extract of *Synechococcus* (as described below). Four replicates were used for each culture. Additional four replicates of each media were incubated under the same conditions without cells as controls. An additional culture for each media was covered with aluminum foil and incubated in the dark under the same conditions to test for the possible presence of contaminating heterotrophs. Metabolites were extracted from the media after five days of incubation as described below.

For stable isotope probing experiments, cells were grown in A+ media without Trizma base containing 1 g L^{-1} [^{13}C]sodium bicarbonate as four 49.5 ml cultures in 50 ml centrifuge tubes as described previously.[14] After four days of incubation, the cultures were distributed into twelve 15 ml centrifuge tubes, cetrifuged for 10 min at 4500 g, and the supernatant was discarded. Cells were resuspended in the same A+ media containing 1 g L^{-1} [^{13}C]sodium bicarbonate. Three replicates did not contain any additional metabolites (controls), three replicates contained 100 μM adenine, three replicates contained 100 μM glutamate, and three replicates contained metabolite extract of *Synechococcus* (as described below).

Metabolite extraction

1.8 ml of culture media supernatant was dried down with a Savant SpeedVac Plus SC110A and redissolved in 500 μl of methanol. Redissolved samples were stored overnight at 4 °C, centrifuged for 5 min at 2300 × g, supernatant was dried down again, redissolved in 100 μl of methanol, stored overnight at 4 °C, filtered using 0.20 μm PVDF membrane micro-centrifugal filters (National Scientific) and analyzed using LC-MS. Metabolite extraction of cells was performed as described previously.[14] Metabolites were extracted from 600 ml of different *Synechococcus* cultures (OD_{730} 0.5–2) to supplement A+ media in footprinting experiments. The final metabolite extract was dried down and redissolved in water before being added to the media *via* a 0.2 μm polyethersulfone membrane sterile syringe filter (VWR). Similarly, 160 ml of *Synechococcus* cultures was used to extract metabolites for supplementing the A+ media in stable isotope probing experiment.

LC-MS analysis

Metabolite extracts of media from metabolic footprinting experiments and of media and cells from stable isotope probing experiments were analyzed using normal phase liquid chromatography with a ZIC-HILIC column (150 × 1 mm, 3.5 μm 100 Å, Merck Sequant) using an Agilent 1200 capillary LC system with an Agilent 6520 dual-ESI-Q-TOF mass spectrometer as described previously.[14] MS acquisition was performed in fast polarity switching mode.

Data analysis

Raw metabolite profile datasets from the footprinting experiment were preprocessed by MathDAMP package[23] into matrix format with unit m/z resolution. This was performed for four replicates of control and spent culture media for four different medias – a total of 32 datasets. Differences among the datasets were found by calculating F-ratios (one-way ANOVA) across all corresponding data points in the datasets. Overlaid chromatograms from analyzed datasets in the vicinities of most significant differences were generated (Fig. S1). These were manually curated to remove false positives (*e.g.* tails of peaks already highlighted as different). Chromatographic peaks correlated ($r \geq 0.9$) with a highlighted peak in one of the datasets were generated (Fig. S1). These peaks were then manually analyzed using Agilent MassHunter Workstation Software Qualitative Analysis (Version B.03.01). Putative mass spectral peaks correspoding to $[M + H]^+$ (or $[M - H]^-$ in negative mode) were identified based on the presence of specific adduct ions (*e.g.* $[M + Na]^+$ or $[2M + H]^+$; Fig. S1).

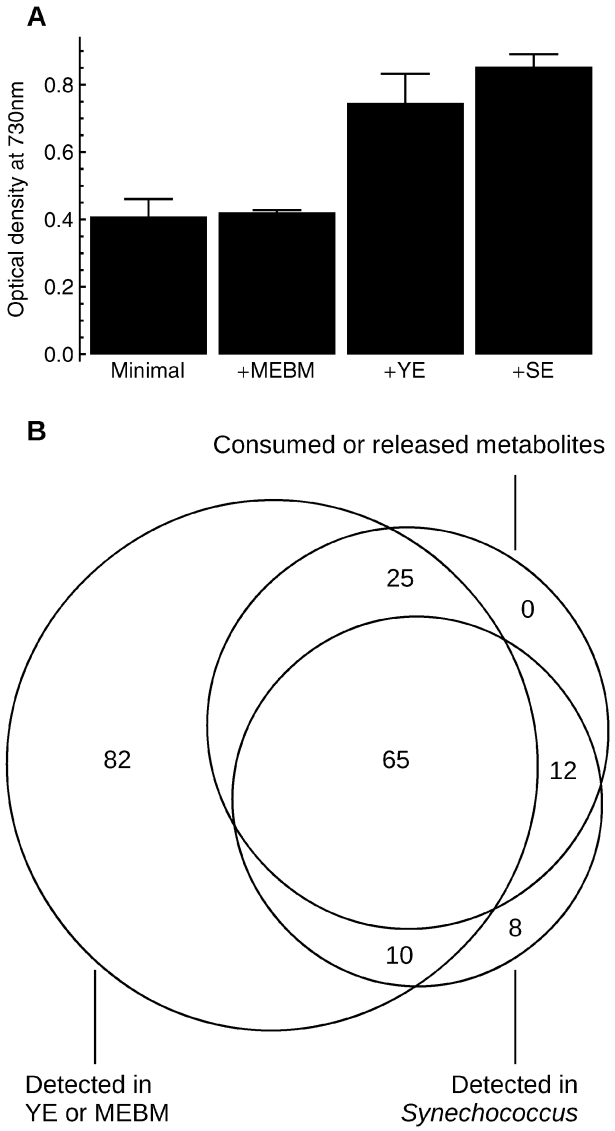

Fig. 1 (A) Optical densities (730 nm) of *Synechococcus* cultures in minimal A+ media supplemented with yeast extract (+YE) or its own metabolite extract (+SE) compared to minimal A+ media alone (Minimal) or a mixture (1 : 1 v/v) of minimal A+ media with mammary epithelium basal media (+MEBM). Optical densities of replicate cultures (n = 4) were measured at the point of metabolite extraction for the assessment of metabolite uptake or release. (B) Overlap of metabolites consumed or released by *Synechococcus* with metabolites detected in metabolite extracts of *Synechococcus* or with metabolites detected in YE or MEBM.

Putative chemical formulas were then calculated using MassHunter software and stored. Redundant chromatographic peaks for the same metabolite (isotopic, fragment, or adduct peaks) from the F-ratio result were identified during this curation process. F-ratio result highlighted differences among peaks with reproducible retention times. To highlight additional differences between peaks of metabolites with drifting retention times, direct absolute × relative comparisons[23] between replicates of spent minimal media *vs.* control minimal media were performed. Additionally, three-way comparisons[24] between replicates of spent complex media *vs.* control complex media *vs.* control minimal media were performed for all three complex medias. In addition to the identification of peaks with drifting retention times, three-way comparisons also allowed the discrimination between background signals (such as plasticizers and other contaminants), which would have similar intensities in the control minimal media dataset. Overlaid chromatograms in the vicinities of candidate differences were generated and further processed as for the F-ratio results. The raw datasets acquired with fast polarity switching were first split into positive mode and negative mode datasets and analyzed along the workflow described above. Signals corresponding to the same metabolite in positive and negative modes were identified based on similarities in retention time and correspondence of the mass spectra. Start and end times of peaks were identified manually and peak areas were integrated. A ± 20 ppm mass accuracy window was selected for the purpose of peak area integration performed using both positive and negative mode datasets. Integration was performed only for those replicate media datasets, where the corresponding peaks were detected. Identifications and putative identifications were based on our previous work (ref. 14) and manual curation of the data which included accurate mass, isotopic profile and MS/MS data. Accurate mass measurements were almost in all cases within 5ppm of the predicted exact mass (Table S1 shows average ppm errors for both positive and negative modes from all replicate datasets). The peak areas from positive and negative mode datasets were summed and these areas were used for relative comparisons (Table S1). Centroids of the peaks were calculated for the assignment of retention times. The $[M + H]^+$ and $[M - H]^-$ peaks of detected metabolites were subjected to MS/MS analysis and metabolites were identified or putatively identified based on MS/MS spectra as described previously[14] using collision energy 10 V and comparison of characteristic fragments with mass spectral databases Metlin[25] and MassBank.[26]

For the stable isotope probing experiments, chromatographic peaks of isotopomers of selected metabolites were integrated as described above. These metabolites of *Synechococcus* were identified previously using uniform stable isotope labeling of cells and MS/MS analysis.[14]

Results and discussion

Synechococcus was cultured in A+ minimal media and three different complex media. Cultures grown on minimal media supplemented with either yeast extract (YE) or metabolite extract of *Synechococcus* (SE) reached higher optical densities than cultures grown on media supplemented with mammary epithelium basal media (MEBM) or minimal media (Fig. 1A). This is consistent with previous observations of faster photoheterotrophic growth with added glycerol.[27] Minor growth exhibited as a weak green tint was also observed in control cultures with YE or SE incubated under the same conditions in the dark showing purely heterotrophic growth of *Synechococcus*. However, optical densities of these cultures (0.043 and 0.063) were significantly lower than corresponding cultures incubated under a 14/10 h light/dark cycle (Fig. 1A). We have previously validated the axenic state of our *Synechococcus* cultures using biomarker sequencing.[14] Negligible growth in control cultures

incubated in the dark as well as the presence of nutrients such as glucose, amino acids and dipeptides in assay culture media following growth of *Synechococcus* (as described below) indicates an absence of potential heterotrophic contaminants.

Metabolites were extracted from growth media of the cultures and from growth media alone (control) incubated under the same conditions (n = 4). Metabolite extracts were analyzed using normal phase liquid chromatography–time-of-flight mass spectrometry and data were processed along the workflow outlined in Fig. S1 and described in Materials and methods section. A total of 202 distinct metabolites were detected in the datasets (Table S1). We had previously identified a number of *Synechococcus* metabolites.[14] Additional metabolites were identified using tandem mass spectrometry (MS/MS; Fig. S2). A total of 92 metabolites were identified or putatively identified (Table S1).

Levels of 102 out of the 202 detected metabolites were significantly different in at least one pair of control and culture media extracts (two-sided student *t*-test *p*-value < 0.05; Fig. 1B and Table S1). It is interesting to note that within different classes of metabolites, such as amino acids, nucleobases, or nucleosides, there are instances of metabolites that were only consumed or others that were only released. For example, glutamate, adenine, or adenosine were consumed from complex media, while phenylalanine, cytosine, uridine, or guanosine were released into the minimal media as well as complex media irrespective of the level of the metabolite already present in the media (Fig. 2). Other metabolites, such as methionine or tryptophan, were released into minimal and some of the complex media, but consumed in other complex media with higher levels of the metabolite (Fig. 2). In some cases, such as for leucine in media with YE (Fig. 2), the level of the metabolite present may have been too high to observe a significant difference due to only a small fraction of the metabolite being taken up or released by the cells. Accumulation of nonpolar amino acids in the media is probably due to leaking of these metabolites from the cells.[28] We previously identified a series of γ-glutamyl dipeptides of these amino acids in *Synechococcus*[14] suggesting a mechanism to retain these amino acids inside cells as γ-glutamylation increases solubility of these amino acids preventing leakage through cell membranes.[29]

Synechococcus was found to consume metabolites having the most intense peaks in metabolite profiles of its own extract. A total of 29 metabolites from SE were found to be significantly consumed (*p*-value < 0.05; Table S1) including metabolites we previously classified as ''unexpected'' as they were not present in the draft metabolic network of *Synechococcus* reconstructed based on genome annotation.[14] Examples of these metabolites, which were detected only in SE but not in the remaining complex media, are histidine betaine (hercynine), γ-glutamyl phenylalanine, or the trisaccharide (Fig. 2). For dipeptides without a γ-glutamyl linkage, which were detected in YE but not in SE, no significant uptake was found (Fig. 2, Table S1). An additional 18 metabolites detected in YE or MEBM but not in SE were significantly consumed as well (Table S1). Examples of these metabolites are the vitamin thiamine or the nucleobase uracil (Fig. 2, Table S1).

The existence of photoheterotrophic capabilities indicates some advantage for *Synechococcus*. The uptake of metabolites from the media lifts the burden of their biosynthesis by the cells and facilitates the reaching of higher cell densities over the same period of time (Fig. 1A). The ability of *Synechococcus* to consume its dominant soluble intracellular metabolites may thus be highly beneficial for cells surviving lysis of a part of the population (due to environmental stress or predation). Utilization of extracellular metabolites may cause a competition for these compounds between *Synechococcus* (and perhaps other photoautotrophs) and heterotrophs in different environments. The apparent hard-wiring of uptake *vs.* release of a number metabolites found in this study highlights metabolic inefficiencies, yet may provide *Synechococcus* with straightforward means to participate in mutualistic relationships.[30]

Some metabolites were released by *Synechococcus* in only one of the tested media. For example, unidentified metabolite 15 was released only in media with MEBM or unidentified metabolite 133 was released only in the media with SE (Table S1). These released metabolites may be a result of overflow metabolism[15,17] due to large levels of specific metabolites taken up from the media modifying thermodynamic driving forces or imposing bottlenecks on reactions in the metabolic network of *Synechococcus*. Thermodynamic driving forces may also be responsible for the release of some metabolites to a certain level or their consumption if their levels cross a certain threshold (*e.g.* methionine; Fig. 2). The ability to detect increased biosynthesis of metabolites induced by high levels of extracellular metabolites offers the possibility for systematic probing of capabilities of microbial metabolic networks.[15] High levels of specific metabolites may have also had toxic effects – probably one of the causes of no significant improvement of growth on media supplemented with MEBM in spite of uptake of specific metabolites (Fig. 1A and 2). Cyanobacteria are known to synthesize a wide range of bioactive compounds either constitutively or following specific environmental stimuli.[31] Such secondary metabolites may be among the unidentified released metabolites found in this study.

To investigate if consumed metabolites are metabolized or kept by the cells ''as they are'' after uptake, we performed uniform labeling of the cells with a stable isotope of carbon (^{13}C) followed by probing of metabolism with unlabeled (^{12}C) metabolites of interest. Labeled cultures of *Synechococcus* were probed with adenine, glutamate, or SE. Relative intensities of mass spectral peaks of selected cellular metabolites with different numbers of incorporated ^{13}C isotope were evaluated (Fig. 3, Fig. S3). Incorporation of ^{13}C isotope into metabolites in the control cultures (without any added unlabeled metabolites) was not complete as indicated by the presence of smaller peaks corresponding to compounds with one or few ^{12}C isotopes (Fig. 3, Fig. S5). This is due to sub-100% isotope purity of the [^{13}C]bicarbonate used as a carbon source and due to the incorporation of ^{12}C isotope from CO_2 in the headspace of the cultures. Addition of [^{12}C]adenine to labeled cultures did not significantly influence the isotopic distribution of glucosylglycerol, the trisaccharide, glutamate (Fig. 3) or additional metabolites (Fig. S3). However, the isotopic distribution of adenine shifted dramatically towards a fully unlabeled (^{12}C) form (Fig. 3A) along with a small peak corresponding to an ion with a single ^{13}C due to natural occurrence of this isotope. Part of this shift may be due to the contamination of cells with media prior to metabolite

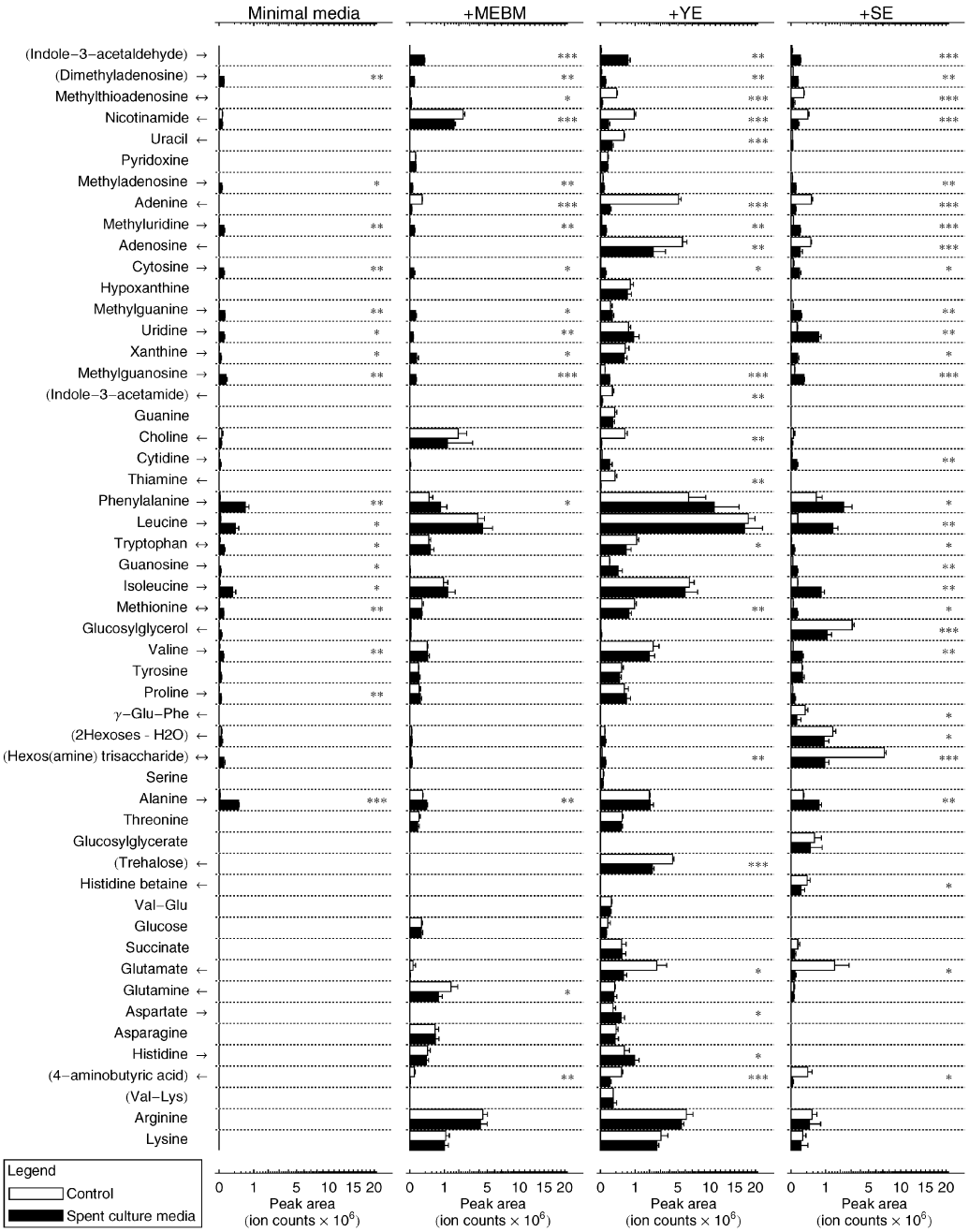

Fig. 2 Comparison of levels of selected metabolites in the growth media following growth of *Synechococcus* (full bars) against their levels in control media (open bars, n = 4). The peak areas axis was scaled with a square root to improve the visualization of smaller peaks. Statistically significant differences are indicated as "*" ($p < 0.05$), "**" ($p < 0.01$), or "***" ($p < 0.001$). An arrow is shown next to the name of a metabolite if it was found to be significantly consumed (←), released (→), or both consumed and released (↔). Table S1 shows this comparison for all detected metabolites.

extraction, as a significant amount of adenine was still left in the media after cultivation (Fig. S4). However, the isotopic profile of adenosine (Fig. 3B), with a significant shift towards a condensation product of [^{12}C]adenine with [^{13}C]ribose, shows that adenine was consumed and metabolized. Additional compounds with an affected isotopic profile following probing with adenine include histidine betaine (Fig. S3A) and a putative condensation product of histidine and glycerate (Fig. S3B), which we identified previously.[14] These shifts are not as dramatic as for adenosine, but rather consistent with known biosynthesis of histidine, a single carbon of which originates from the adenine moiety of ATP.[32] Similarly, probing of labeled cells with unlabeled glutamate did not affect the isotopic profile of adenine or adenosine, however, shifts in isotopic distribution were observed for intracellular glutamate or γ-glutamyl phenylalanine (Fig. 3C and D). Only a small amount of remaining glutamate was detected in the media in this case (Fig. S4).

An overall shift towards an increased enrichment of the ^{12}C isotope in all analyzed metabolites was observed in cultures

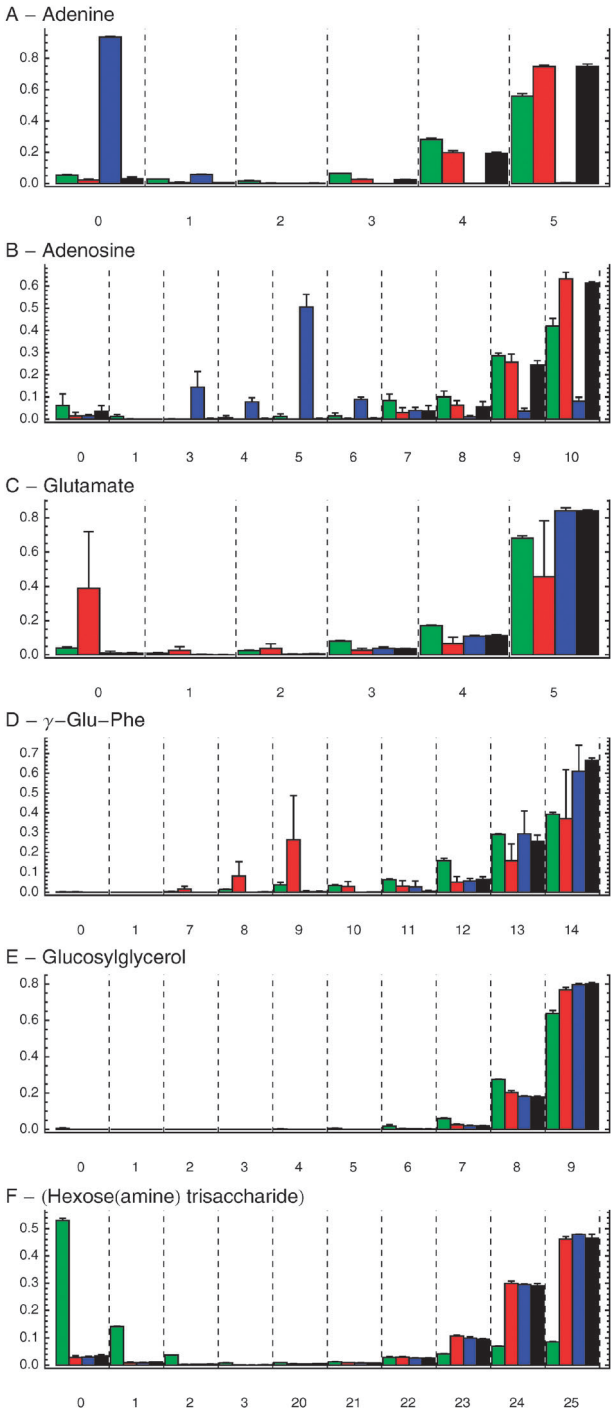

Fig. 3 Relative intensities of mass spectral peaks of selected metabolites containing different numbers of a ^{13}C stable isotope (shown below the abscissa). Intensities were normalized to the sum of intensities of all isotopomers. Intermediate numbers of incorporated ^{13}C isotopes for compounds containing larger number of carbons are not shown for clarity. Cells were grown in media containing $NaH^{13}CO_3$ as the carbon source (black) supplemented with unlabeled [^{12}C]adenine (blue), [^{12}C]glutamate (red), or [^{12}C]SE (green, n = 3). Shifts towards higher peaks with a smaller number of ^{13}C isotopes show incorporation of carbons from unlabeled metabolites. Fig. S3 in the electronic supplementary information shows this comparison for additional metabolites.

with added [^{12}C]SE (Fig. 3, Fig. S3) indicating turnover of some consumed metabolites and incorporation of their carbons into other metabolites. For some metabolites, such as for the trisaccharide, a high proportion of peaks corresponding to unlabeled compounds originating from the media was detected in cell extracts (Fig. 3F). Only a negligible amount of the trisaccharide was detected in the media after cultivation (Fig. S4). Thus, it is unlikely that these compounds undergo significant turnover after uptake. However, only a negligible amount of the completely unlabeled form of glucosylglycerol was detected pointing to complete metabolic turnover of uptaken glucosylglycerol (Fig. 3E, Fig. S4). Rapid changes in levels of glucosylglycerol following exposure to hyperosmotic or hypoosmotic stress were reported previously,[33,34] but no significant turnover of glucosylglycerol was observed in salt-acclimated cells.[35,36] Our results suggest that in addition to serving as a compatible solute glucosylglycerol may also have a function of an energy or carbon store.

In conclusion, we have used an integrated, untargeted metabolic footprinting approach to systematically evaluate the uptake and release of metabolites between a model marine cyanobacterium *Synechococcus sp. PCC 7002* and different growth media. We found a surprising breadth of metabolites being uptaken including many intracellular *Synechococcus* metabolites. Using a novel stable isotope approach of labeling the cell and probing with native ^{12}C isotopes we found that adenine, glutamate and glucosylglycerol are actively turned over as opposed to the hexosamine-based trisaccharide which is uptaken and maintained in its native state. This study has highlighted the utilization of a number of metabolites and can enable the search for corresponding transport proteins and enzymes. Overall, our results point to a broader utilization of dissolved organic matter by primary producers. The untargeted metabolic footprinting coupled to stable isotope probing approach we used in this work is generally applicable to other microorganisms and can be further extended to study interactions between multiple organisms.

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