Metabolomics of Carbon Fixing Mutants of Cyanobacteria by GC/Q-TOF

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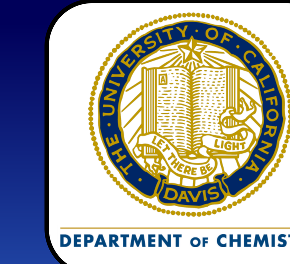

Overview

Mutants of the model cyanobacterium *Synechococcus elongatus* PCC7942 (Figure 1) were evaluated by untargeted metabolomics to identify possible phenotypic changes that allow more efficient growth. Wild-type *S. elongatus* was mutagenized and mutants that grow more efficiently were enriched. The mutants were analyzed by GC-QTOF for changes in metabolite profiles. This approach identified that a possible source of changes causing increased growth efficiency to be the carbon fixation pathway, a known limitation in cyanobacterial growth that is notoriously difficult to improve.

Figure 1. *S. elongatus*. Photo by C.R-Deere

Introduction

Due to the finite availability of fossil fuels, as well as detrimental accumulation of CO_2 emissions, there is great interest in engineering microorganisms to generate sustainable fuel alternatives. Cyanobacteria, photosynthetic CO_2–fixing microorganisms, are especially attractive for their ability to utilize the greenhouse gas as a carbon source, and light as an energy source. However, for cyanobacteria-based biofuels to be economically feasible, cellular growth rates must be improved. Previous studies have shown that carbon fixation is a key limitation in cyanobacterial growth. However, many past attempts to increase the rate of carbon fixation by directed changes have yielded only minimal improvements. Thus, a directed evolution approach may allow identification of beneficial metabolic changes that had not previously been considered.

Methods

Cyanobacteria Preparation

Wild-type *S. elongatus* PCC7942 was mutagenized using ethyl methanesulfonate (EMS), or nitrosoguanidine (NTG). After recovery, cells were grown in the presence of high concentrations of CO_2. Dense cultures were back-diluted over several rounds to enrich for cells with faster growth rates. Promising isolates were later confirmed in triplicate using growth assays. Of sixteen candidates, four mutants were chosen for further analysis based on growth rates compared to wild type (Table 1).

S. elongatus strain	Growth rate
Wild type	1.0251
M2	1.0510
M3	1.0257
M4	0.8205
M10	1.0891
M12	1.1569

Table 1. Growth rates of *S. elongatus* strains

Metabolite Preparation

Metabolites were extracted using methanol/chloroform extraction. Aqueous fractions (containing central carbon metabolites) were collected, dried, and consecutively derivatized by methoximation using a saturated solution of hydroxylamine HCl in pyridine and by silylation with N-methyl-N-(trimethylsilyl) trifluoroacetamide (MSTFA) and 1 % trimethylchlorosilane (TMCS), respectively. The samples were analyzed by GC/Q-TOF. The data were processed by chromatographic peak deconvolution software for peak finding, followed by compound identification using a mass spectral library. Metabolite identities were further confirmed using accurate mass information.

Figure 2. 7200 series GC/Q-TOF system

Analytical Conditions

This study was performed using an Agilent 7890 GC coupled to an Agilent 7200 series Quadrupole-Time-of-Flight MS (Figure 2). GC and MS conditions are described in Table 2.

GC and MS Conditions	
Column	DB-5 MS UI, 30 meter, 0.25 mm ID, 0.25 µm film
Injection volume	1 µL
Split ratio	10:1
Split/Splitless inlet temperature	250 °C
Oven temperature program	60 °C for 1 min 10 °C/min to 325 °C, 3.5 min hold
Carrier gas	Helium at 1 mL/min constant flow
Transfer line temperature	290 °C
Ionization mode	EI
Source temperature	230°C
Quadrupole temperature	150°C
Mass range	50 to 600 m/z
Spectral acquisition rate	5 Hz, collecting both in centroid and profile modes

Table 2. GC-MS conditions used in the study.

Results

Data Processing: Chromatographic Deconvolution and Library Searches

The data were processed by deconvolution software using the Unknowns Analysis tool in MassHunter Quantitative Analysis software package. Approximately 400-500 components were detected in each sample (Figure 3). Specific compounds were then identified by comparison with the Agilent-Fiehn GC/MS Metabolomics Retention Time Locked (RTL) Library. Statistical evaluation of the data was performed by Mass Profiler Professional (MPP), a multivariate statistical analysis package. Metabolite identification was further confirmed using accurate mass information.

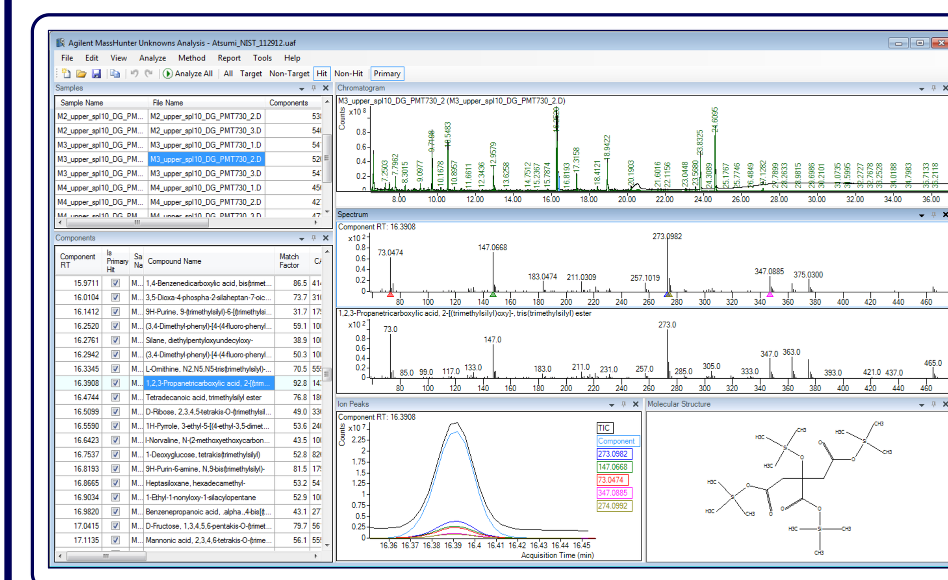

Figure 3. Representative results and analysis from the Unknowns Analysis tool. The lower middle panel shows deconvoluted ions of the component. The ions have the same apex and peak shape, thus confirming that they all belong to the same component.

Identification of Significant Metabolomic Differences: Principal Component Analysis (PCA)

PCA was employed to evaluate clustering of the samples that belong to the same strain (either wild type or a mutant). Distinct clustering of each group of samples suggested good repeatability of sample replicates and significant differences between the mutants (Figure 4).

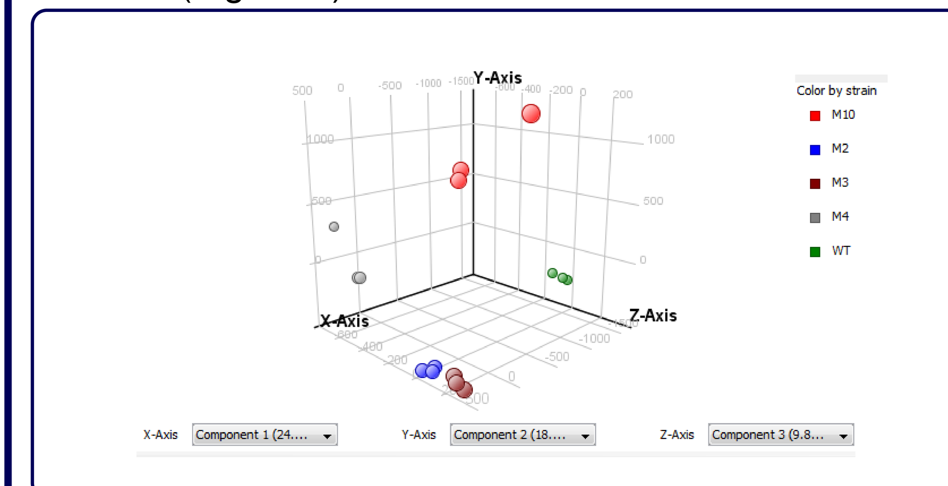

Figure 4. PCA plot of a comparison between wild type and mutants confirms the existence of distinct clusters.

Identification of Significant Metabolomic Differences: Fold Change Analysis (FCA)

Significant metabolic changes were further identified using FCA, where the fold change in the concentration of any given compound, along with the statistical significance of this change, was visualized on a Volcano plot (Figure 5).

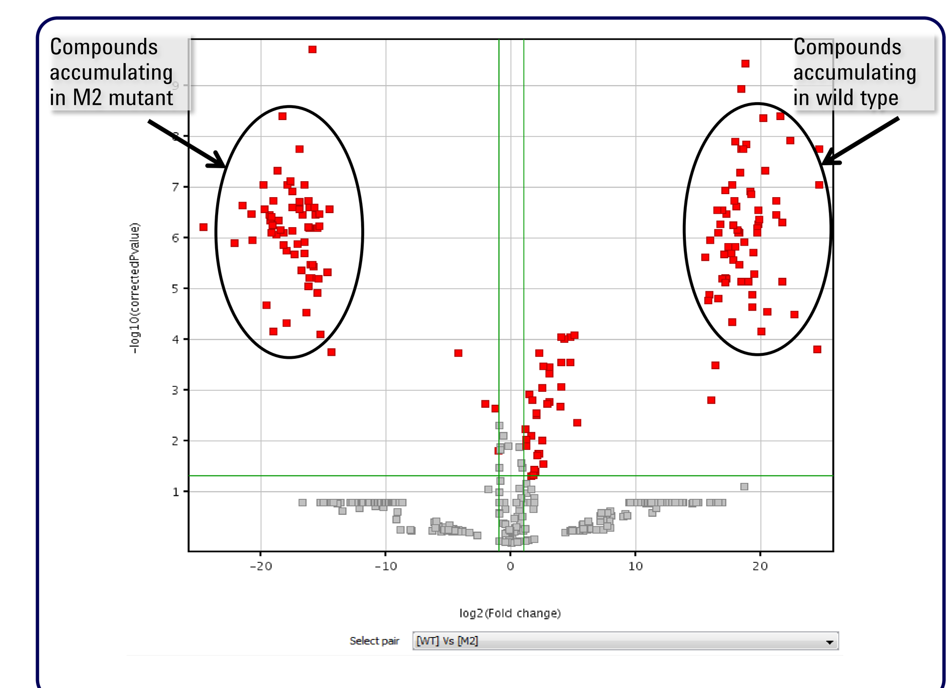

Figure 5. Volcano plot of fold change in concentration versus probability value for wild type vs M2 mutant.

Visualization of Metabolomic Differences

Differences in metabolite abundance levels between strains were visualized using direct comparison (Figure 6), and included organic acids, amino acids, sugar monophosphates. Metabolites with a role in key metabolic pathways are of special interest.

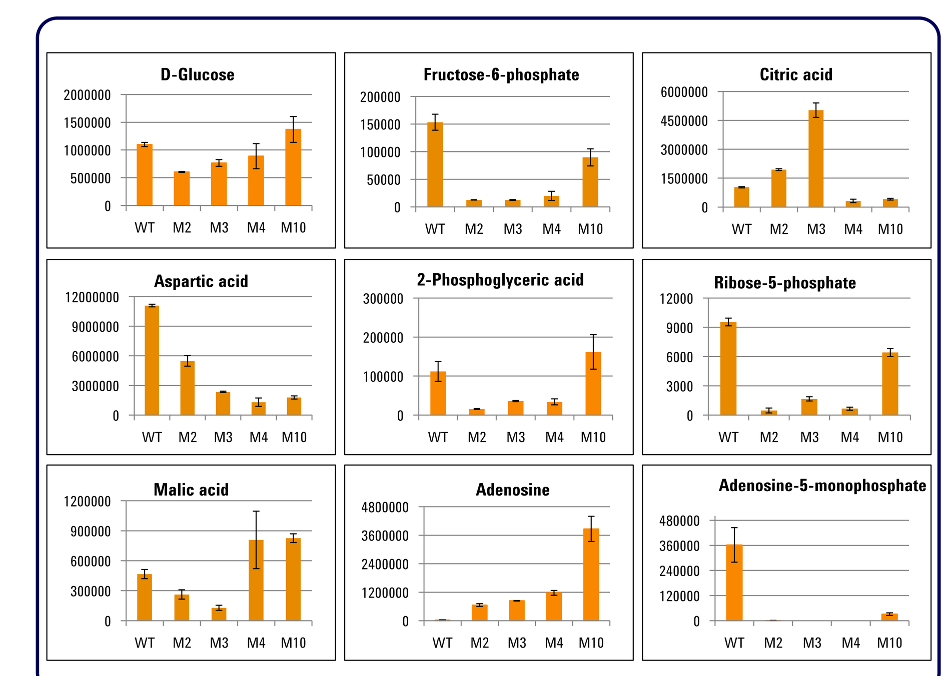

Figure 6. Selected results of metabolomics analysis. While levels of the central metabolite D-glucose are similar for all strains, other metabolites, including those key to the TCA and CBB cycles, show significant differences between wild type and mutants. Adenosine, but not adenosine-5-monophosphate, accumulation in all mutants vs wild type suggests a common metabolic bottleneck.

Discussion

Metabolomic Differences Suggest Genotypic Changes: Pathway Analysis

S. elongatus mutants M2 and M12 were investigated in further detail to understand how a change in levels of specific metabolites may be caused by changes to specific metabolic pathways. Untargeted pathway analysis was performed in MPP for M2 (Figure 7) and targeted analysis was performed for M12 (Figure 8). Results suggest that beneficial genotypic changes may have occurred in several key metabolic pathways, including the carbon fixation (CBB) and tricarboxylic acid (TCA) cycles, glycolysis, and fatty acid biosynthesis.

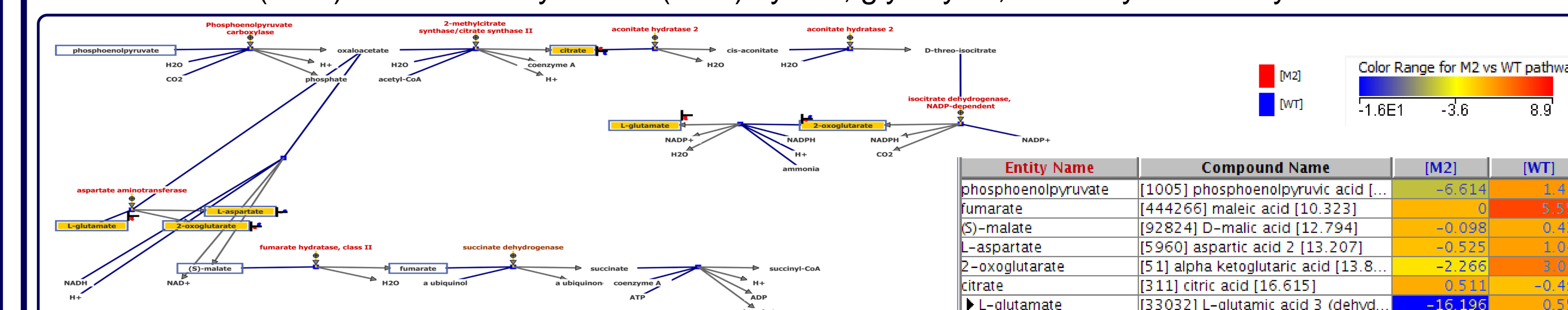

Figure 7. MPP Pathway Analysis: A detailed look at pathway (in this case, TCA cycle) differences in M2 vs WT.

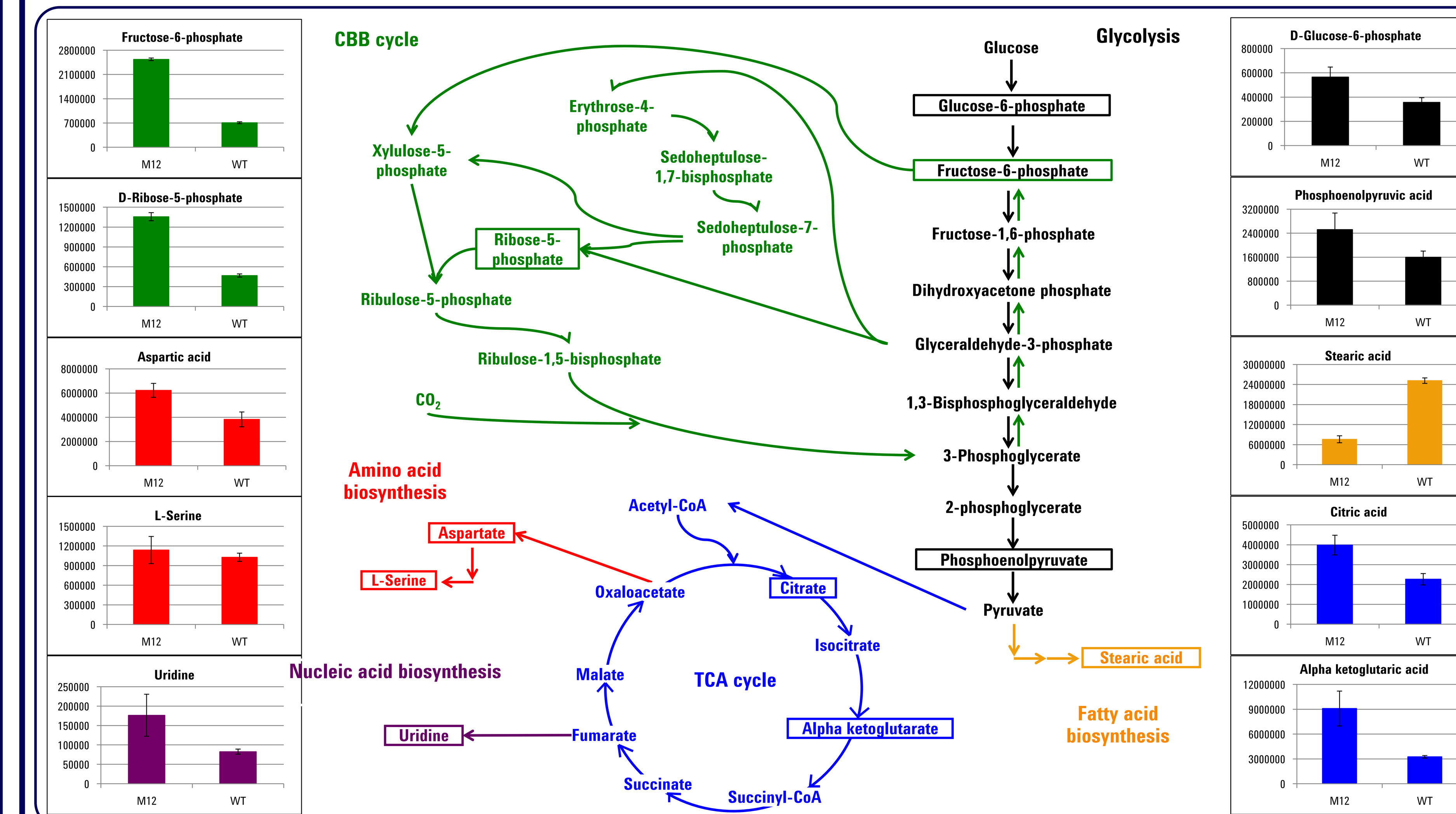

Figure 8. An integrated metabolic schema illustrates that there are likely genetic differences involving several fundamental metabolic pathways between M12 and wild-type *S. elongatus* strains.

Conclusions

- All five *S. elongatus* mutants selected for untargeted metabolomics analysis displayed differences in their metabolite profiles when compared to each other and to wild type.
- Differences were observed for various classes of metabolites including:
  - Organic acids
  - Amino acids
  - Sugar monophosphates
  - Central carbon metabolites (i.e.: Ribose-5-phosphate [R5P]).
- Altered R5P levels suggest that enhancement of the M12 growth rate may be at least partially due to beneficial mutations directly affecting carbon fixation.
- It is possible that a combination of mutations, affecting more than one metabolic pathway (i.e.: the TCA and CBB cycles), has allowed these mutants' total improvement of growth rate.
- Detected metabolomics changes suggest specific metabolic bottlenecks, and provide a direction for further strain development.

A metabolomics approach can help identify specific metabolomic changes that highlight or narrow multiple possible genetic changes in mutants that display improved characteristics useful for a range of applications including biofuel production.