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

Application Note

Biofuel

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Abstract

Mutants of the model cyanobacterium *Synechococcus elongatus* were evaluated by untargeted metabolomics to identify possible phenotypic changes that allow more efficient growth. The mutants were analyzed using the Agilent 7200 Series GC/Q-TOF, and changes in metabolite profiles were evaluated using Agilent Pathway Architect Software in Mass Profiler Professional. This approach identified a number of metabolic pathways as possible targets that could explain the improved phenotype of the mutants.

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 carbon dioxide–fixing microorganisms, are especially attractive for their ability to use 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, as the major obstacle to industrial-scale production are low yields [1]. Previous studies have shown that carbon fixation is a key limitation in cyanobacterial growth [2]. 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.

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This application note describes a study aimed at overcoming the growth limitations of cyanobacteria by randomly mutagenizing and screening the *S. elongatus* PCC7942 wild type cyanobacterium for mutants that grow and fix CO_2 more efficiently. The resulting mutants were further evaluated using an untargeted metabolomics approach on the accurate mass high resolution 7200 GC/Q-TOF system. The accurate mass data were processed using multiple software tools, including Pathway Architect, in order to identify metabolic pathways that might be involved in beneficial changes. This study revealed a number of metabolic pathways that might be responsible for the improved characteristics of the cyanobacteria mutants.

Experimental

Instruments

This study was performed using an Agilent 7890B GC system, coupled to an Agilent 7200 GC/Q-TOF system. The instrument conditions are listed in Table 1.

Table 1. GC and Mass Spectrometer Conditions

GC run conditions	
Column	Agilent J&W DB-5 MS Ultra Inert, 30 m × 0.25 mm, 0.25 µm (p/n 122-5532UI)
Injection volume	1 µL
Split ratio	10:1
Split/splitless inlet temperature	250 °C
Oven temperature program	60 °C for 1 minute, 10 °C/min to 325 °C, 3.5 minutes hold
Carrier gas	Helium at 1 mL/min constant flow
Transfer line temperature	290 °C
MS conditions	
Ionization mode	EI
Source temperature	230 °C
Quadrupole temperature	150 °C
Mass range	50 to 800 *m/z*
Spectra acquisition rate	5 Hz, collecting both in centroid and profile modes

Sample 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 cells with faster growth rates. Promising isolates were later confirmed in triplicate using growth assays. Of 16 candidates, four mutants were chosen for further analysis based on growth rates compared to wild type.

Metabolites were extracted by methanol/chloroform extraction. Aqueous fractions 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.

Data processing and statistical analysis

The data were processed by chromatographic peak deconvolution using the Unknowns Analysis tool from MassHunter Quantitative Analysis Software package (B.07), followed by compound identification by comparison to the Agilent Fiehn GC/MS Metabolomics Retention Time Locked (RTL) Library. Metabolite identities were further confirmed using accurate mass information and the accurate mass tools in the MassHunter Qualitative Analysis Software package (B.07).

Statistical analysis was performed by Mass Profiler Professional (MPP) (12.6), a multivariate statistical analysis package, to identify metabolomic differences between the mutants and the wild type strain as well as differences between the mutants. The relative levels of metabolites were further quantified using MassHunter Quantitative Analysis Software. Finally, biochemical pathways potentially involved in beneficial changes in the mutants were identified using Pathway Architect in MPP.

Results and Discussion

Chromatographic peak deconvolution and library search

The data were processed by chromatogram deconvolution using the Unknowns Analysis tool (Figure 1) with a 100 ppm accurate mass setting and variable Retention Time Window Size Factor (75–200) in order to find the highest number of true components (Figure 1). Compounds were identified by comparison with the Agilent-Fiehn GC/MS Metabolomics Retention Time Locked (RTL) Library. The deconvolution process took approximately 4–5 minutes for each sample, and the library search took less than a minute per sample using default screening parameters.

A number of compounds were identified only when a mass extraction window of ± 100 ppm, but not a parameter of + 0.3/–0.7 Da, was used for deconvolution. Thus, using accurate mass settings allowed more real components to be found (Figure 2). The identity of each compound found by deconvolution was confirmed using accurate mass information, the Molecular Formula Generator tool, and the Fragment Formula Annotation tool in MassHunter Software (Figure 3).

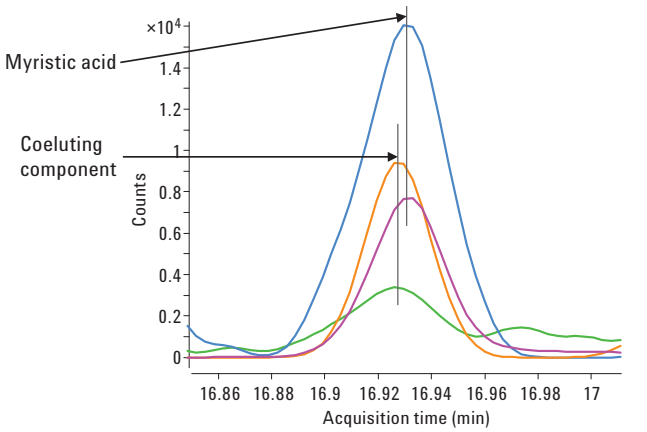

Figure 2. Myristic acid (blue and pink peaks) was distinguished from a coeluting component (yellow and green peaks) and hence identified only when an RT Window Size Factor deconvolution parameter of 100 ppm, but not + 0.3/– 0.7 Da, was selected

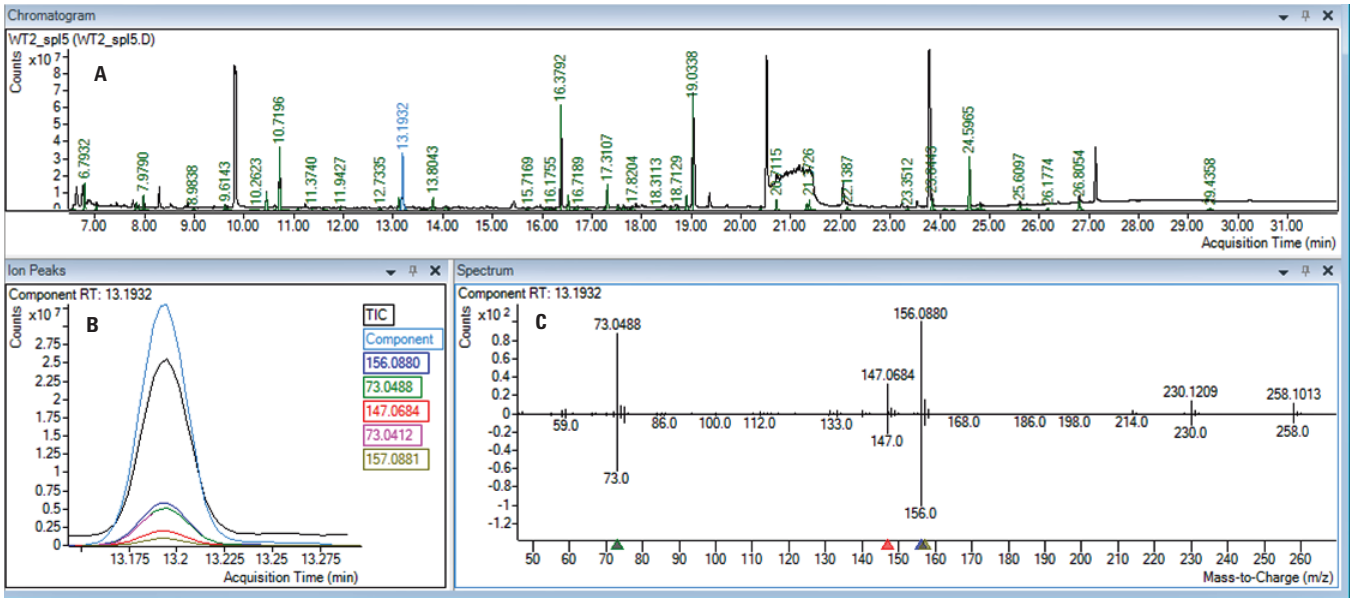

Figure 1. An unknowns analysis software tool was used to perform chromatogram deconvolution. The chromatogram is shown in A, while the overlaid EICs of the component are shown in B, and the mirror plot of the component and library hit spectra is shown in C.

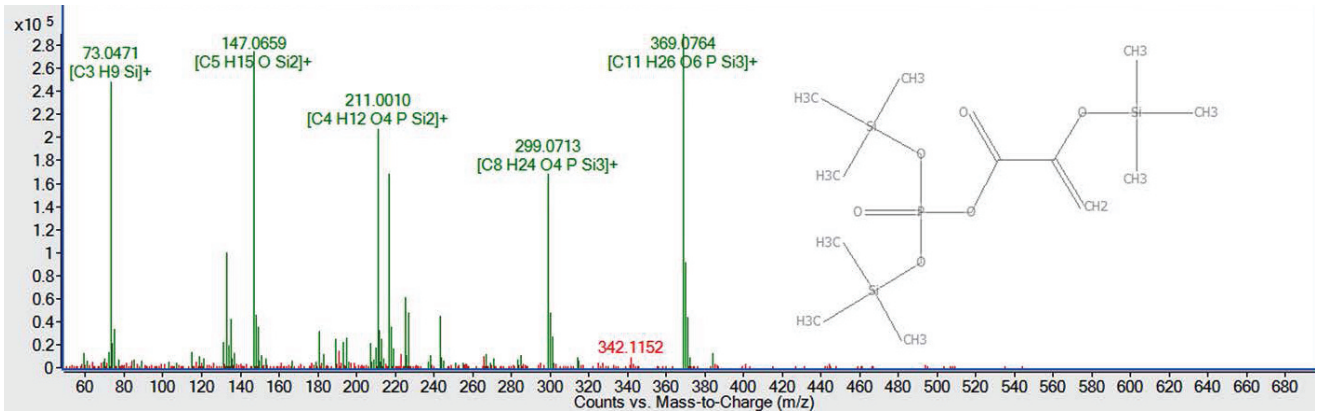

Figure 3. Formula Generator tool of Agilent MassHunter Qualitative Software package was used together with library search and Fragment Formula Annotation to automatically assign fragments of the compound to confirm compound identification. A fragment is annotated and colored green when the formula of the fragment is a subset of the molecular formula identified by a library search. Verification was performed using the NIST11.L library.

Identification of significant metabolomic differences

Significant differences in metabolite production between the cyanobacteria wild type and mutant strains were determined using statistical tools in MPP. First, Principal Component Analysis (PCA) was used to visualize clustering of the data. Distinct PCA clusters of each group of replicate samples indicated good repeatability of data and significant metabolomics differences between the five cyanobacteria strains (Figure 4).

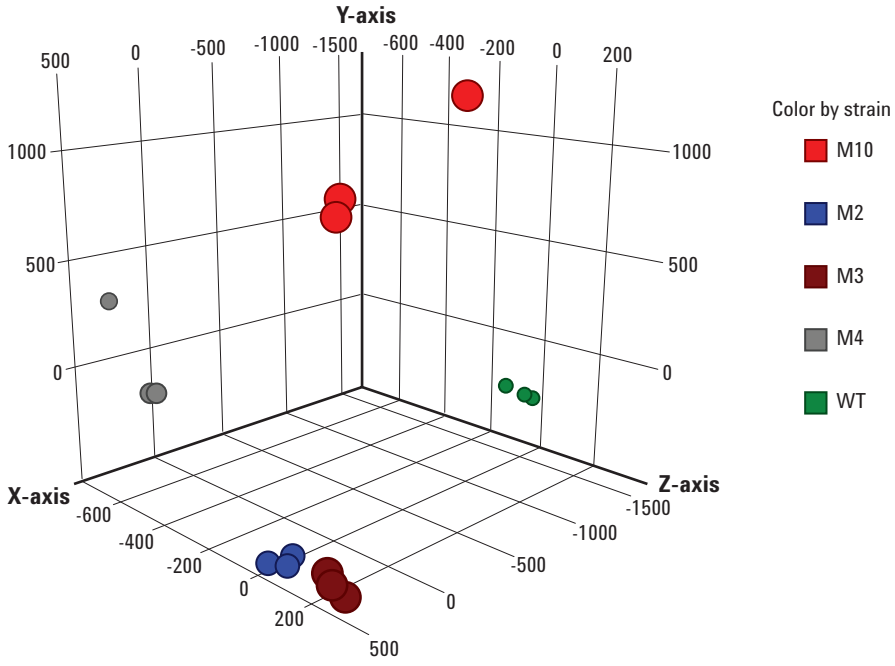

Figure 4. The PCA plot of wild type and mutant samples confirms the existence of distinct clusters due to variability between each strain.

Significant differences in metabolite levels between strains were determined using Fold Change Analysis (FCA) and seen on a Volcano plot where the Fold Change of metabolite concentration was plotted against the statistical significance of this change (Figure 5). Compounds with high Fold Change in their levels and low p-values between a pair of the compared strains (conditions) are colored in red and thus can be easily identified. Compounds on the right side of the plot are those in higher abundance in the wild type, while compounds on the left side of the plot are those in higher abundance in the mutant, relative to the wild type (Figure 5).

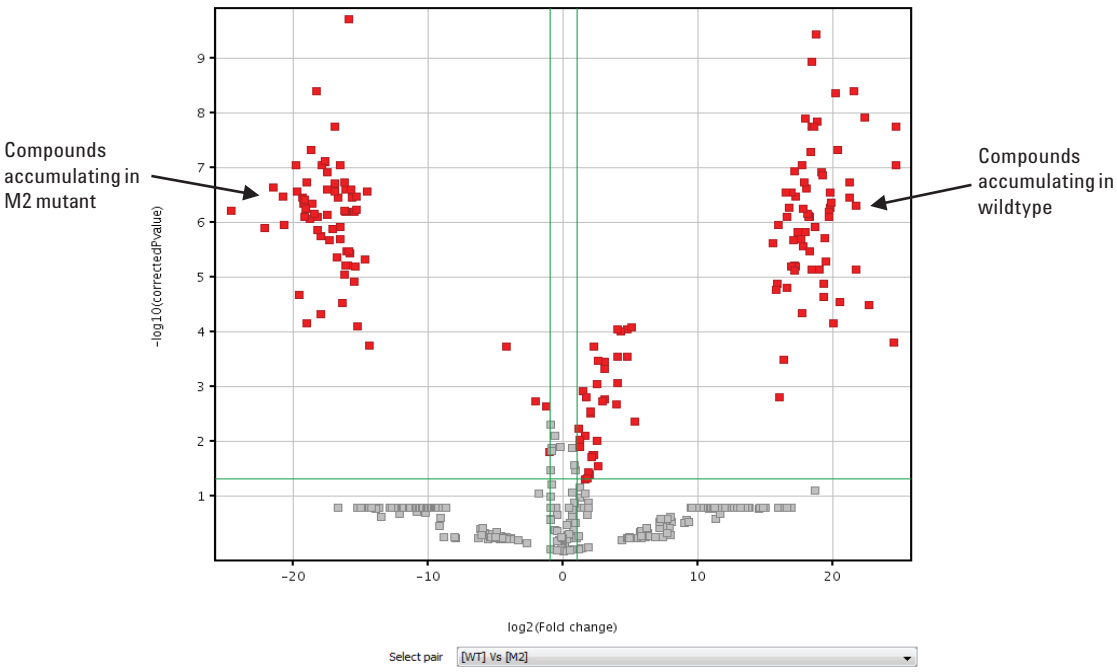

Figure 5. Volcano plot of Fold Change showing negative log10 of p-value versus log2 of Fold Change in metabolite levels for wild type versus. the M2 mutant. The data points that satisfy p-value cut-off of 0.05 and Fold Change cut-off of 2 are colored in red.

MassHunter Quantitative Analysis Software was also used to make a semiquantitative comparison of metabolite concentrations in the mutants versus the wild type cyanobacteria strain. The software automatically creates a quantitation method for all metabolites using the acquired data and the information from the library search. It is optimized to select for both abundance and specificity of quantifier and qualifier ions. Selection of a narrow mass extraction window of 100 ppm for the quantifier ions took advantage of accurate mass information and increased selectivity to ensure superior quantitation (Figure 6).

The important differences in metabolite abundance levels between strains included organic acids, amino acids, and sugar monophosphates among others (Figure 7). While levels of the central metabolite glucose are similar for all strains, other metabolites, including those key to the TCA (citric acid, malic acid, and so forth) and carbon fixation cycles (2-phosphoglyceric acid, ribose -6-phosphate), show significant differences between wild type and mutants. Accumulation of adenosine, but not adenosine-5-monophosphate, in all mutants versus the wild type suggests a common metabolic bottleneck (Figure 7).

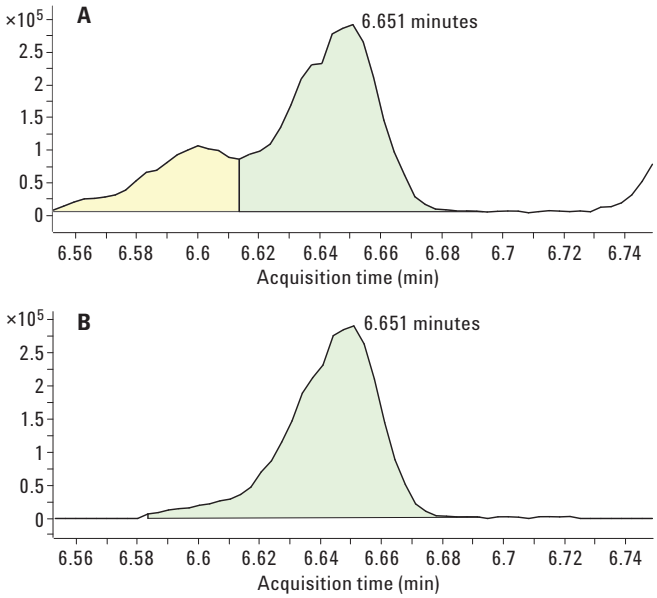

Figure 6. Extracted ion chromatogram (EIC) of a quantifier ion for pyruvic acid extracted with the mass window of +0.3/–0.7 Da (A) and ±100 ppm (B). Using the narrow 100 ppm mass window increases the accuracy of quantitation by eliminating interferences included in the quantitation using the less selective +0.3/–0.7 window setting.

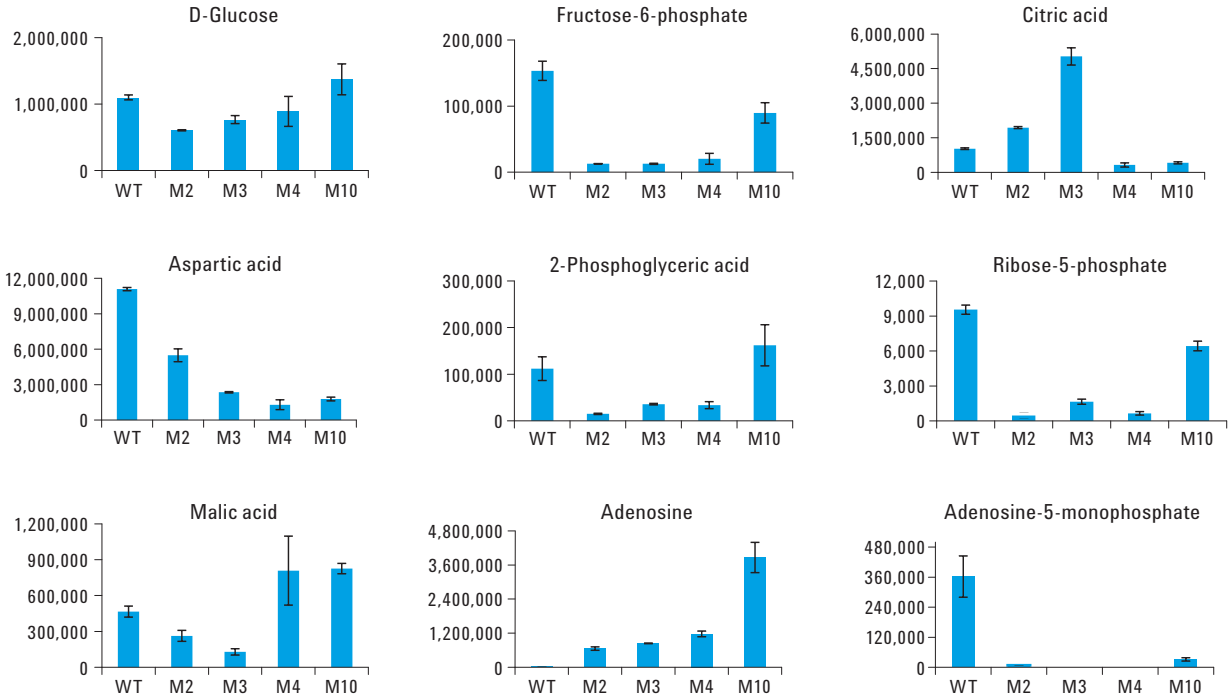

Figure 7. Semiquantitative comparison of selected metabolite levels in the wild type versus the mutants.

Pathway Analysis of Metabolomic Differences

The *S. elongatus* mutants were investigated in further detail to understand how a change in levels of specific metabolites may be caused by changes to specific metabolic pathways. Pathway analysis was performed using Pathway Architect, and some of the results are shown for M2 as an example (Figure 8). The results suggested that beneficial genotypic changes may have occurred in several key metabolic pathways, including the carbon fixation and tricarboxylic acid (TCA) cycles, glycolysis, and fatty acid biosynthesis.

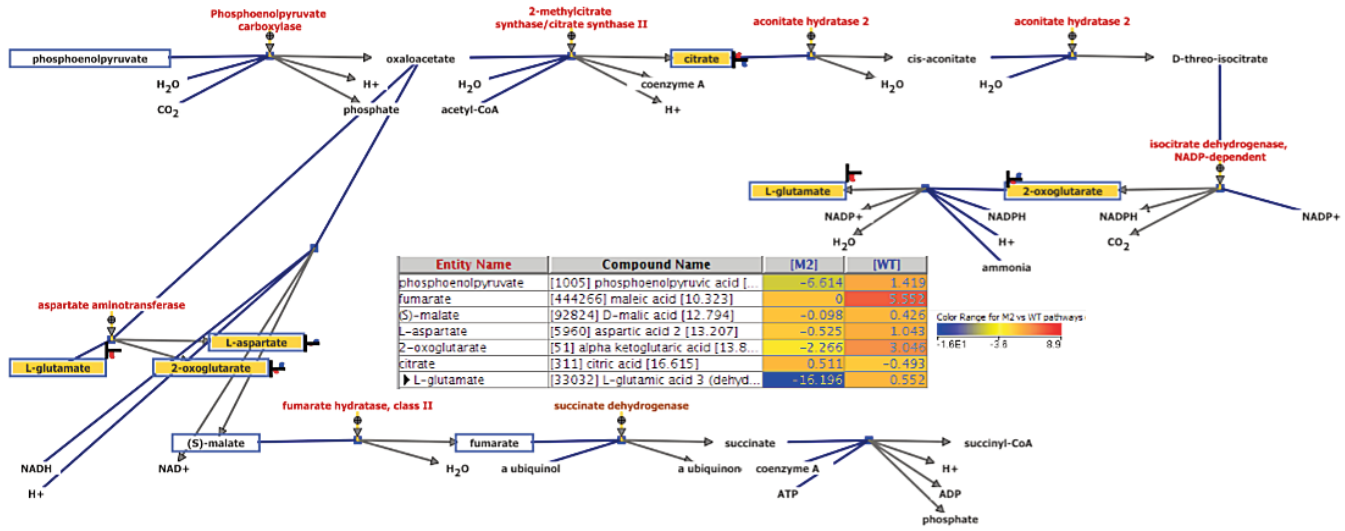

Figure 8. Example pathway analysis results for the M2 mutant strain suggest changes to the TCA cycle, versus the wild type strain. Highlighted metabolites mapped on the pathway are the ones that belong to the pathway and identified in the experiment. The heat map in the table indicates relative levels of metabolites between two compared strains.

Conclusions

An untargeted metabolomics approach has been used to identify metabolomics changes in mutants that display improved characteristics useful for a range of applications, including biofuel production. Accurate mass information provided by the Agilent 7200 GC/Q-TOF was crucial for specific metabolite identification and quantitation. Several software tools, including Unknowns Analysis in MassHunter Quantitative Analysis, as well as Principal Component Analysis, Fold Change analysis, and Pathway Architect in Mass Profiler Professional were essential for identification of metabolic changes in specific pathways responsible for the improved characteristics observed in the mutants.

References

1. D.N. Juurlink and I.A. Dhalla "Bioengineering of carbon fixation, biofuels, and biochemicals in cyanobacteria and plants" *J Biotechnol*. **162**, 134-147 (2012).
2. G.G. Tcherkez, G.D. Farquhar, T.J. Andrews "Despite slow catalysis and confused substrate specificity, all ribulose bisphosphate carboxylases may be nearly perfectly optimized" *PNAS* **103**, 7246-7251 (2006).

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