Systems Biology to Enable Modular Metabolic Engineering of Fatty Acid Production in Cyanobacteria

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Abstract

Fatty acids are synthesized solely from the sequential condensation of acetyl-CoA subunits in the photosynthetic cyanobacteria *Synechococcus* sp. PCC 7002. Feeding acetyl-CoA precursors, such as acetate, can increase acetyl-CoA availability and enhance photosynthetic fatty acid production in the eukaryotic algae Chlamydomonas reinhardtii.[1] However, we found that the same strategy failed to increase fatty acid production in a strain of the cyanobacteria PCC 7002 engineered to produce the free fatty acid (FFA) octanoic acid. We supplemented PCC 7002 cultures with ^{13}C acetyl-CoA precursors acetate and pyruvate simultaneously to determine if these were utilized for fatty acid synthesis and to reveal potential bottlenecks limiting fatty acid synthesis from acetyl-CoA precursors. ^{13}C labeling data showed that the supplements, taken together, incorporated into the acetyl-CoA pool at a rate of roughly 40 – 50%, but failed to increase fatty acid production. Additionally, acetyl-CoA labeling from acetate saturated at the lowest supplement concentration, while acetyl-CoA labeling from pyruvate did not. These data appear to suggest that precursors can increase acetyl-CoA availability without increasing fatty acid synthesis and point to bottlenecks in acetate uptake or acetate conversion to acetyl-CoA.

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1. Introduction

This work focuses on a variant of the fast-growing halotolerant strain PCC 7002, bearing the high expression, lac inducible 143 CupTE expression construct. The heterologous expression of the Cuphea-derived thioesterase CupTE enables the cyanobacteria *Synechococcus* sp. PCC 7002 to produce octanoic acid by cleaving fatty acid chains once they are eight carbons long. This medium-chain free fatty acid is a direct precursor to the biodiesel methyl octanoate. There are many benefits of using cyanobacteria for energy production. Unlike traditional methods, cyanobacterial free fatty acid production is carbon negative. These organisms are genetically tractable, require few nutrients for growth, and do not compete with crops for arable land. Finally, free fatty acid can be recovered directly from the media, unlike cellular lipids like triacyclglycerols.

Our collaborators have shown that the addition of an overlay of isopropyl myristate (IPM) can increase FFA titers. The organic overlay and aqueous media form two liquid phases. When octanoic acid is synthesized by the cells, it escapes into the nonpolar overlay. The removal of toxic octanoic acid from the aqueous solution detoxifies the growth media and drives the equilibrium of the reaction forward by Le Chatelier's principle.

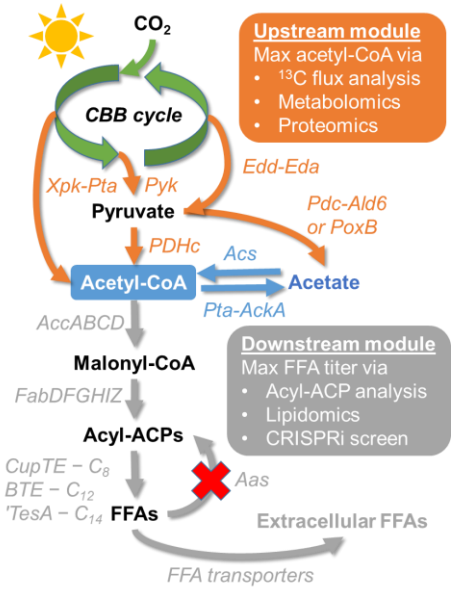

Figure 1. Cyanobacterial metabolism is split into two modules, one upstream and the other downstream of acetyl-CoA, which is the "hub metabolite" that connects fatty acid synthesis to photosynthesis.

This experiment focused on metabolism upstream of acetyl-CoA (**Figure 1**). Since pyruvate and acetate are precursors to acetyl-CoA, we hypothesized that supplementing these two metabolites should increase acetyl-CoA availability, thereby boosting FFA production. Additionally, if these precursors substantially contributed to the acetyl-CoA pool, but failed to increase fatty acid production, it would suggest that acetyl-CoA

availability may not be a limiting factor for fatty acid production under these conditions.

To evaluate our hypothesis, we measured cellular lipid titers with GCMS, as well as the incorporation of the supplements into acetyl-CoA, octanoic acid, and long-chain fatty acids with ^{13}C metabolic tracer analysis.

2. Materials and Methods

2.1 Cultures

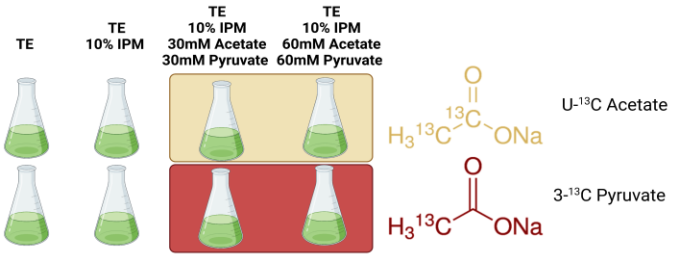

Figure 2. The eight cultures used in this experiment along with the structures of the two labeling agents.

All eight cultures in the experiment were grown in MAD media and inoculated from a single starter, which was itself inoculated from a single colony. Each of the cultures were composed of 50 mL of the MAD media cultures plus 6 mL of a treatment mix. All treatment mixes contained 1 mM IPTG (Isopropyl ß-D-1-thiogalactopyranoside, to induce thioesterase expression) and MAD media; some also contained carbon supplements, as described below.

The first two cultures were given neither overlay nor carbon supplement as shown in **Figure 2**. Their treatment mix consisted of just the overlay and media. The next two cultures were given the same treatment mix plus a 10% IPM overlay. The fifth culture was given a treatment mix with 30 mM uniformly labeled acetate and 30mM pyruvate, plus the overlay. The sixth culture was given a treatment mix with 60 mM uniformly labeled acetate and 60mM pyruvate, plus the overlay. The seventh culture was given a treatment mix with 30 mM acetate and 30 mM labeled pyruvate, plus overlay. The eighth culture was given a treatment mix with 60 mM acetate and 60 mM labeled pyruvate, plus overlay.

2.2 Isotopomer Spectral Analysis

This technique is used to evaluate biosynthesis pools and pathways using stable isotopes.[2] Nonlinear regression is used on the mass isotopomer distributions (MID) of cellular lipid pools to estimate the two unknown parameters D and g(t) (**Figure 3**). D is the concentration of acetyl-CoA in the pool that has been labeled with tracer; g(t) is the fraction of fatty acid that has been produced since the addition of the tracer (utilizing the labeled acetyl-CoA) as a function of time. Our lab's proprietary software, INCA, was used to perform ISA.

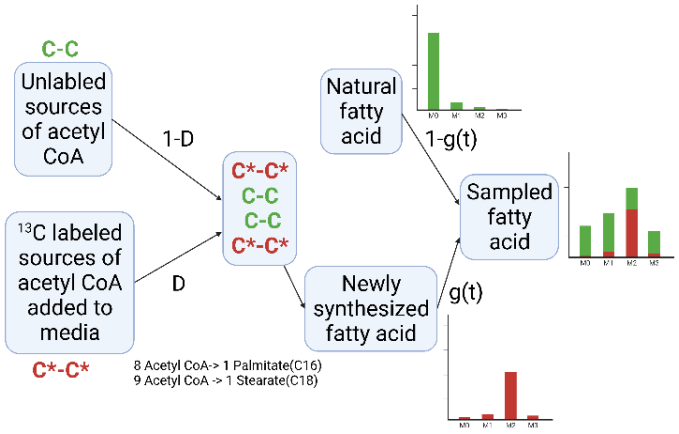

Figure 3. The model used in ISA. Unlabeled and labeled sources make up the acetyl-CoA pool. The parameter "D" represents the contribution of labeled sources to the acetyl CoA pool. The fatty acid pool is composed of preexisting and newly synthesized fatty acid. The second parameter "g(t)" is the portion of "new" fatty acids synthesized after the introduction of labeled tracer.

2.3 Methyl chloroformate (MCF) Derivatization

Analytes must readily enter the gas phase to be analyzed by GCMS. Consequently, we volatilized our samples by converting the fatty acids' carboxylic acid groups to methyl esters (FAME) using this MCF-based derivatization technique.

First, the corresponding overlay was added back to the broth samples since the overlay and broth were sampled separately. The two samples that were not grown in overlay were given fresh IPM overlay. In this way, the total volume and concentration of overlay were standardized across the experimental samples. Next, in octanoic acid quantification samples, 25 µL of 10M HCl was added to lyse cells without freeing the bound fatty acids via saponification. NaOH was used for cellular lipid quantification samples to free the palmitic and steric acids from their glycerol heads prior to derivatization. The samples were then vortexed for 10 minutes.

The next few steps were time sensitive and were performed in rapid succession. 200 µL of freshly made 166:34 methanol/pyridine solution was added to each sample. Then, 40 µL of MCF was added and the sample was vortexed for 30 seconds. We repeated the MCF addition and vortexing steps before quenching the reaction with 800 µL of chloroform and vortexing the mixture for 10 seconds.

Lastly, 400 µL of 100 g/L $NaHCO_3$ was added to neutralize the acid and the samples were centrifuged for 20 minutes at 4000 g. 250 µL of the organic (bottom) layer was reverse pipetted into a GC vial for analysis.

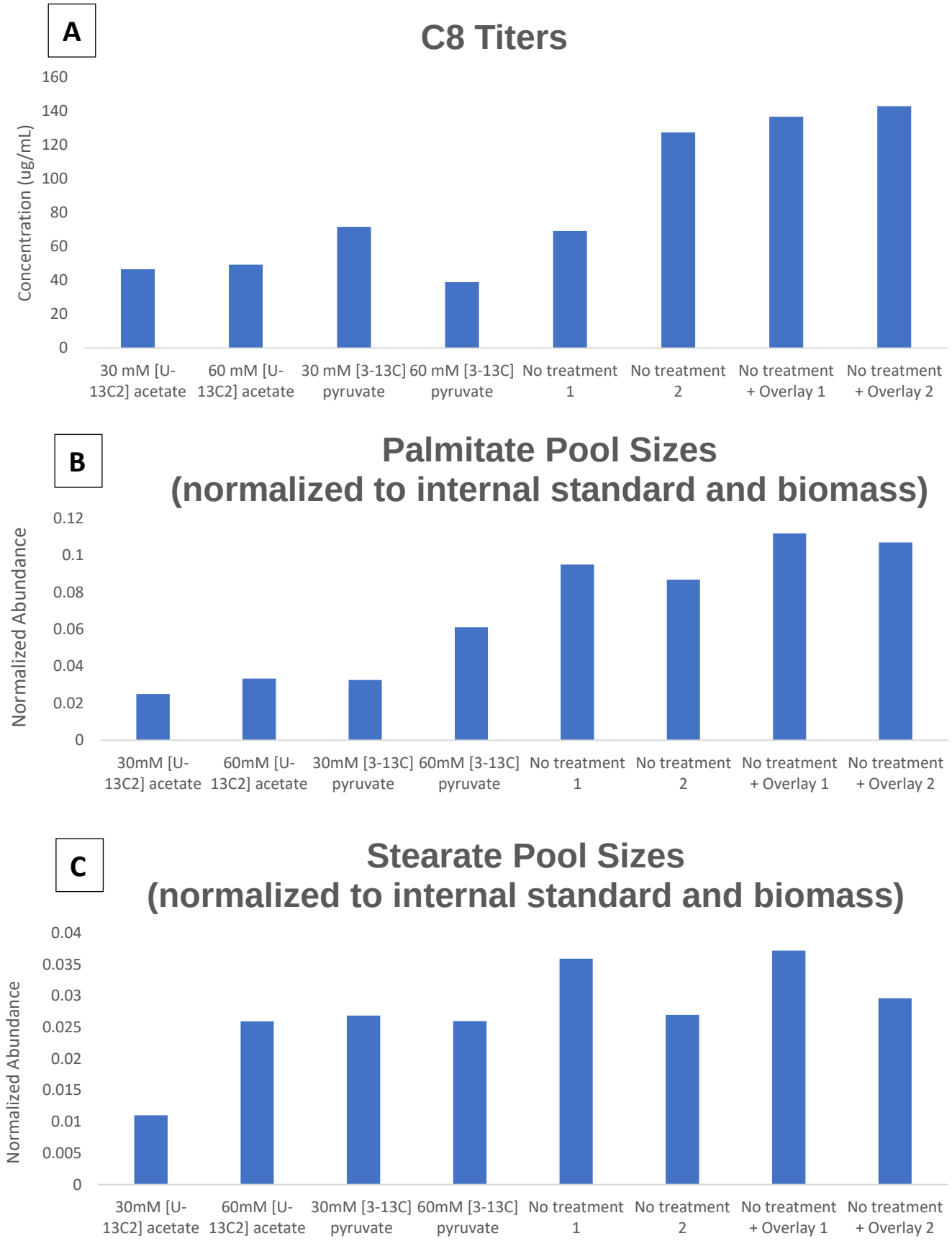

Figure 4. Lipid abundance for product: (A) Octanoate and cellular lipids: (B) Palmitate and (C) Stearate.

3. Results and Discussion

3.1 Cellular Lipid Titers

Figure 4 shows the quantity of methyl octanoate (**A**), methyl palmitate (**B**), and methyl stearate (**C**) produced in each of the eight samples, normalized to internal standard and biomass. No rigorous statistics were performed on the results. Curiously, however, the samples without carbon supplementation appear to have produced more octanoate and palmitate, contradicting our expected results. There was no clear trend in the distribution for stearate. Another notable result is the large discrepancy in octanoate titers for the two samples without overlay.

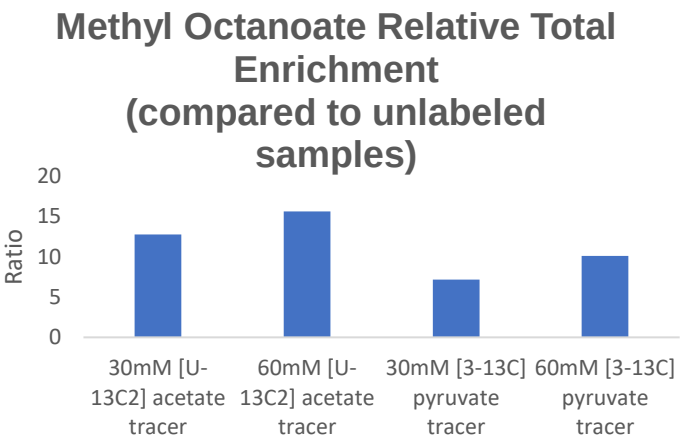

Figure 5. C8 Relative Total Enrichment. The ratios of total tracer incorporation (weighted sum of isotopomers) in C8 over the average incorporation in unlabeled samples, for each labeled sample.

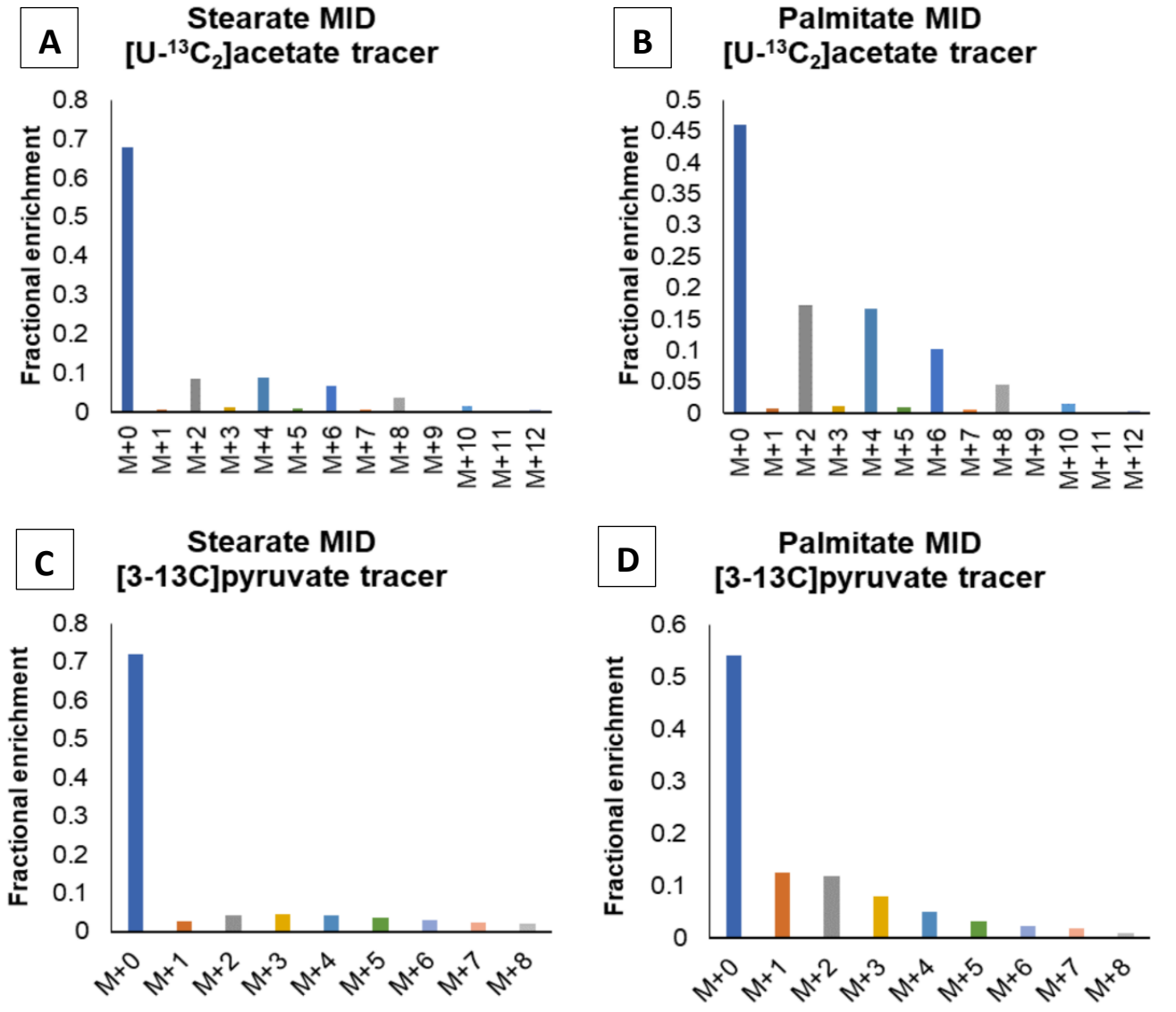

Figure 6. Mass Isotope Distributions of (A) Stearate in the 30 mM labeled acetate sample, (B) Palmitate in the 30 mM labeled acetate sample, (C) Stearate in the 30 mM labeled pyruvate sample, and (D) Palmitate in the 30 mM labeled pyruvate sample.

3.2 Tracer Incorporation

Figure 5 shows the relative total enrichment of methyl octanoate in each of the four labeled samples, relative to the average total incorporation in the four unlabeled samples. Since the acetate supplement was double labeled, whereas the pyruvate supplement was only single labeled, the acetate samples exhibit a greater total enrichment. For both acetate and pyruvate, however, the 60 mM concentrations give a greater total enrichment than the 30 mM concentrations.

Figure 6 shows the extent of labeled ^{13}C incorporation into two long chain fatty acids for the two 30 mM labeled samples, corrected for natural abundance. Panels (**A**) and (**B**) show the MIDs of the 30 mM labeled acetate sample for methyl stearate and methyl palmitate, respectively. They both show that the tracer incorporated into the even isotopologues up to M10, since the acetate was double labeled. The figure suggests that more of the tracer is incorporated into the heavier isotopologues of palmitate than those of stearate.

Panels (**C**) and (**D**) show the MIDs of the 30 mM labeled pyruvate sample for stearate and palmitate, respectively. The tracer was incorporated into up to the M8 isotopologue in both long chain fatty acids. The lower isotopologues (i.e., M1 – M5) seemed to show greater relative abundance in palmitate.

To investigate these results further, we employed ISA on the data from the two labeled acetate samples. Each panel of **Figure 7** shows that roughly 20 – 30% of the acetyl-CoA pool was labeled with acetate/pyruvate (D in the ISA model). Taken together, however, the carbon supplementation may be contributing up to 50% to the pool size, in other words, doubling acetyl-CoA availability. More direct measurements are needed to confirm this observation, since a large contribution to pool size and a decreased pool size are not mutually exclusive.

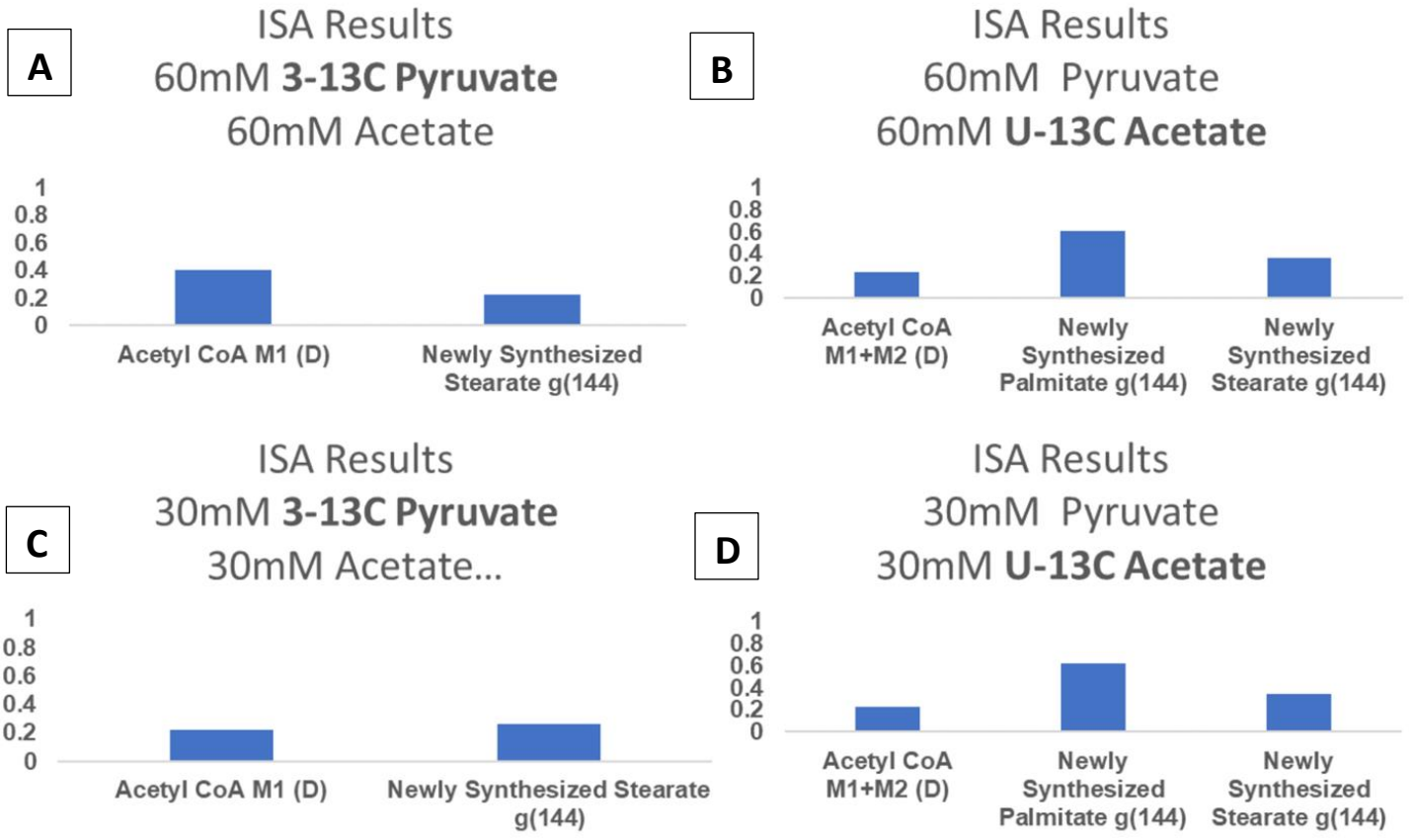

Figure 7. ISA Results, which estimate the two parameters D and g(t) from the model for the (A) 60 mM labeled pyruvate sample, (B) 60 mM labeled acetate sample, (C) 30 mM labeled pyruvate sample, and (D) 30 mM labeled acetate sample.

Another key observation here is that when the concentration of labeled pyruvate was increased, the parameter D increased as well. On the other hand, when labeled acetate concentration was increased, D stayed constant at around 0.2. This seems to suggest that there is a cap on the contribution of acetate to the acetyl-CoA pool. As stated previously, the parameter g(t) is the proportion of fatty acids sampled at time t that are

labeled. This is essentially the turnover rate for fatty acids and is less useful to us than D.

When ISA was run on the data from the two labeled pyruvate samples, the data did not fit. This was with the palmitate and stearate data, so we tried running ISA on each data set independently. When we included only the palmitate data, the model did not fit; however, when we included only the stearate data, the model did fit. This could either mean that the biological model is incomplete or that measurement error was inaccurate/underestimated because it was calculated from too few GCMS runs. Since the samples are still in the freezer, these calculations could be redone using more replicates of the unlabeled samples.

4. Conclusion

This experiment showed that acetate and pyruvate contributed roughly 40 – 50% to PCC 7002 143 CupTE's acetyl-CoA pool under tandem supplementation, and that acetate/pyruvate derived carbon was incorporated into lipid biomass. However, the supplementation did not increase titers; in fact, it may have decreased them. Furthermore, the relatively low level of flux from acetate to acetyl-CoA points to potential bottlenecks in acetate uptake or acetate conversion to acetyl-CoA under the studied experimental conditions. Future work will seek to identify and eliminate the flux bottlenecks limiting the conversion of acetate to acetyl-CoA by overexpressing acetate transport genes (e.g., E. coli ActP) and genes responsible for acetate conversion (e.g., acetyl-CoA synthetase).

Additionally, we will examine the effects of replacing the enzyme FabH, which has previously been shown to be the sole rate-limiting step for PCC 7002 fatty acid biosynthesis *in vivo,* with a kinetically superior FabH enzyme derived from E. coli*.* If FabH overexpression increases the rate of fatty acid synthesis and, therefore, acetyl-CoA consumption, increasing acetyl-CoA availability may boost FFA titers in this new strain.

References

1. Therien, J.B., Zadvornyy, O.A., Posewitz, M.C. *et al.* Growth of *Chlamydomonas reinhardtii* in acetate-free medium when co-cultured with alginate-encapsulated, acetate-producing strains of *Synechococcus* sp. PCC 7002. *Biotechnol Biofuels* **7,** 154 (2014). https://doi.org/10.1186/s13068-014-0154-2

2. Kelleher, J. (1994). Isotopomer Spectral Analysis: A Nonlinear Modeling Method for Estimating Biosynthesis. *IFAC Proceedings Volumes*, *27*(1), 371-372. doi: 10.1016/s1474-6670(17)46266-5