Enhancing metabolic flux to photosynthetic biofuels
DOE Early Career Award (DE-SC0008118)

Jamey D. Young (PI)
Vanderbilt University

Abstract
The overall objective of this project was to test new metabolic engineering and systems biology approaches for maximizing metabolic flux to biofuels in cyanobacterial hosts. Our central hypothesis was that combining directed metabolic engineering approaches with global rewiring of circadian transcriptional programs would lead to enhanced biofuel productivities. As proof of principle, we applied our novel toolset to optimize cyanobacterial production of isobutyraldehyde (IBA), which is a direct precursor of isobutanol, starting from a parental IBA-producing strain of *Synechococcus elongatus* PCC 7942.

Keywords
Synechococcus elongatus PCC7942, isobutyraldehyde, photosynthesis, biofuels, metabolic flux analysis

Major Accomplishments

Development of novel metabolic flux analysis (MFA) technologies for photosynthesis research
Dr. Young previously pioneered a novel MFA approach, termed isotopically nonstationary MFA (INST-MFA), which uses transient isotope labeling measurements to determine metabolic fluxes. One key benefit of the INST-MFA approach is that it can be used to determine metabolic fluxes in photosynthetic organisms using $^{13}CO_2$ as an isotopic tracer, an application that is not possible using conventional steady-state MFA. Funds from this award were used to develop the first publicly available software package (INCA) capable of both isotopic steady-state MFA and INST-MFA [1]. The software provides a framework for comprehensive analysis of metabolic networks using mass balances and isotopomer balances. The generation of balance equations and their computational solution is completely automated and can be performed on networks of arbitrary size and complexity. INCA has been licensed for academic use over 100 times annually since 2014, and commercial licenses have been granted to 10 different companies. The Young lab has successfully applied INCA to achieve several breakthroughs, including the first comprehensive ^{13}C flux studies of photosynthetic bacteria and plant leaves. All software packages developed in the Young lab are freely available to the scientific community for academic use. Several review articles have been published describing the application of INCA and MFA to studies of photosynthetic metabolism [2-5].

Optimal design of ^{13}C labeling studies for flux analysis of cyanobacterial metabolism
We performed a series of simulation studies to assess how selection of isotope labeling measurements and sample time points impacts the precision of flux estimates obtained from ^{13}C INST-MFA. Further details can be found in [6].

Isotopically nonstationary ^{13}C flux analysis of cyanobacterial isobutyraldehyde (IBA) production
We applied ^{13}C INST-MFA to compare the pathway fluxes of wild-type (WT) *Synechococcus elongatus* PCC 7942 to an engineered strain (SA590) that produces IBA. The flux maps revealed a potential bottleneck at the pyruvate kinase (PK) reaction step that was associated with diversion of flux into a three-step PK bypass pathway involving the enzymes PEP carboxylase (PEPC), malate dehydrogenase (MDH), and malic enzyme (ME). Overexpression of *pk* in SA590 led to a significant improvement in IBA

specific productivity. Single-gene overexpression of the three enzymes in the proposed PK bypass pathway also led to improvements in IBA production, although to a lesser extent than *pk* overexpression. Combinatorial overexpression of two of the three genes in the proposed PK bypass pathway (*mdh* and *me*) led to improvements in specific productivity that were similar to those achieved by single-gene *pk* overexpression. This research demonstrated how ^{13}C flux analysis can be used to identify potential metabolic bottlenecks and novel metabolic routes, and how these findings can guide rational metabolic engineering of cyanobacteria for increased production of desired molecules. Further details can be found in [7].

Elucidating the metabolic impact of increased IBA production

In addition to PK overexpression in SA590, we found that the addition of thiamin hydrochloride, a cofactor for the synthetic IBA-producing pathway, significantly boosted IBA production (**Fig 1**). Hence, to understand the metabolic impact of increased product formation, we applied ^{13}C INST-MFA to independently assess the effects of PK overexpression and thiamin hydrochloride supplementation. Our results indicate that IBA flux directly correlates with flux through PK and inversely correlates with flux through pyruvate dehydrogenase (PDH, **Fig 2**). Since the gene for PK was already overexpressed, we chose to focus on attenuating PDH flux as a means to further increase IBA production. By supplementing cultures of SA590-PK with a PDH chemical inhibitor (fluoropyruvate), we observed a significant decrease in growth rate (**Fig 3A**). However, the IBA production rates (**Fig 3B**) and titers (**Fig 3C**) were surprisingly unaffected, which suggest that primary metabolites could be accumulating in the media. We are actively pursuing studies to shed light on this phenomenon.

Modeling method for increased precision and scope of directly measurable fluxes at the genome-scale

The majority of work using MFA has been limited to core models of metabolism due to challenges in implementing genome-scale MFA and the undesirable trade-off between increased scope and decreased precision in flux estimations. Therefore, we sought to develop a tunable workflow for expanding the scope of MFA to the genome-scale without trade-offs in flux precision. In collaboration with the Palsson lab at UCSD, we developed and tested a genome-scale MFA model of *E. coli*, iDM2014. The model contained 537 net reactions, which included the core pathways of traditional MFA models and also covered the additional pathways of purine, pyrimidine, isoprenoid, methionine, riboflavin, coenzyme A, and folate, as well as other biosynthetic pathways [8]. We also developed a novel LC-MS/MS workflow, termed MID Max, that takes advantage of additional scan types that maximize the number of mass isotopomer distributions (MIDs) that can be acquired in a given experiment [9]. The analytical method was found to measure the MIDs of 97 metabolites, corresponding to 74 unique metabolite-fragment pairs (32 precursor spectra and 42 product spectra) with good accuracy and precision. The compounds measured included metabolic intermediates in central carbohydrate metabolism and cofactors of peripheral metabolism (e.g., ATP). When evaluating iDM2014 using the MID Max measurement set, it was found that a total of 232 net fluxes of central and peripheral metabolism could be resolved in the *E. coli* network. The increase in scope was shown to cover the full biosynthetic route to an expanded set of bioproduction pathways, which should facilitate applications such as the design of more complex bioprocessing strains and aid in identifying new antimicrobials. Importantly, it was found that there was no loss in precision of core fluxes when compared to a traditional core model, and additionally there was an overall increase in precision when considering all observable reactions.

Training accomplishments

Two chemical engineering graduate students, Ms. Adeola Adebiyi and Dr. Lara Jeline Jazmin, have received valuable training through this project. In Fall of 2015, Ms. Adebiyi successfully defended her

master's thesis with the results and expertise she gained on this project. Similarly, Dr. Jazmin defended her Ph.D. dissertation in May 2016. Dr. Jazmin is currently employed in the biotechnology industry. This project has also given Dr. Yi Ern Cheah the opportunity for postdoctoral training that will be valuable for his future research career in academia or industry. Dr. Cheah is currently focusing his job search on the microbial biotechnology industry.

Products

1. Young JD: **INCA: a computational platform for isotopically non-stationary metabolic flux analysis**. *Bioinformatics* 2014, **30**:1333-1335.
2. Cheah YE, Hasenour CM, Young JD: **13C Flux Analysis in Biotechnology and Medicine**. *Systems Biology* 2017, **6**:25-70.
3. Cheah YE: **Isotopically nonstationary metabolic flux analysis (INST-MFA): putting theory into practice**. 2018, **54**:80-87.
4. Jazmin LJ, O'Grady JP, Ma F, Allen DK, Morgan JA, Young JD: **Isotopically nonstationary MFA (INST-MFA) of autotrophic metabolism**. *Methods Mol Biol* 2014, **1090**:181-210.
5. McAtee AG, Jazmin LJ, Young JD: **Application of isotope labeling experiments and (13)C flux analysis to enable rational pathway engineering**. *Curr Opin Biotechnol* 2015, **36**:50-56.
6. Adebiyi AO, Jazmin LJ, Young JD: **13 C flux analysis of cyanobacterial metabolism**. *Photosynth Res* 2015, **126**:19-32.
7. Jazmin LJ, Xu Y, Cheah YE, Adebiyi AO, Johnson CH, Young JD: **Isotopically nonstationary 13C flux analysis of cyanobacterial isobutyraldehyde production**. *Metab Eng* 2017, **42**:9-18.
8. McCloskey D, Young JD, Xu S, Palsson BO, Feist AM: **Modeling Method for Increased Precision and Scope of Directly Measurable Fluxes at a Genome-Scale**. *Anal Chem* 2016, **88**:3844-3852.
9. McCloskey D, Young JD, Xu S, Palsson BO, Feist AM: **MID Max: LC-MS/MS Method for Measuring the Precursor and Product Mass Isotopomer Distributions of Metabolic Intermediates and Cofactors for Metabolic Flux Analysis Applications**. *Anal Chem* 2016, **88**:1362-1370.

Figures

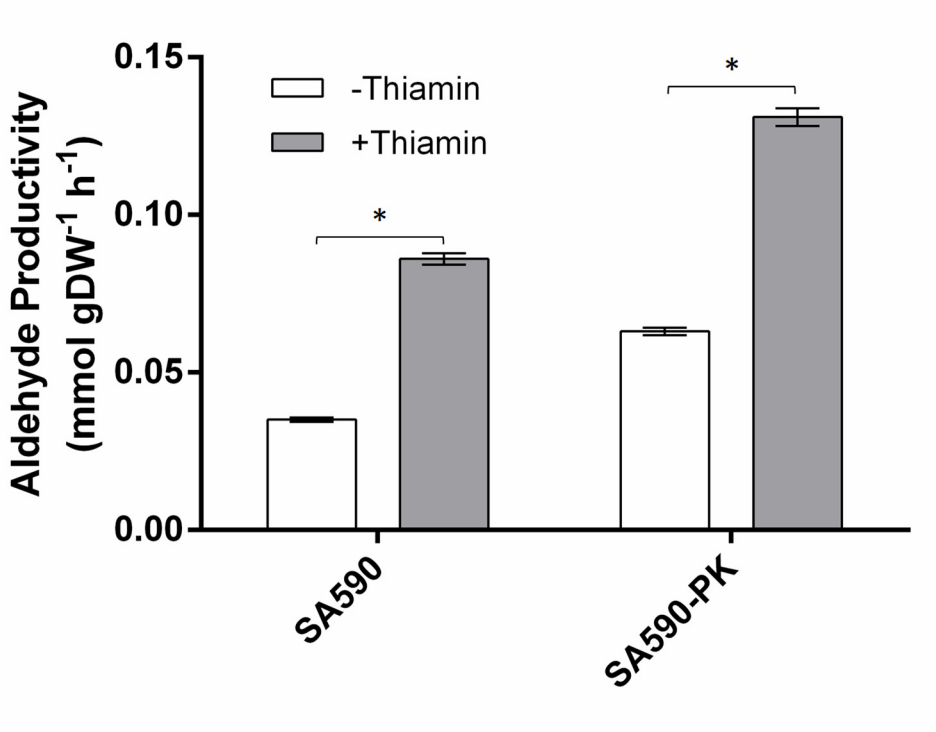

Fig 1. Effect of supplementing thiamin on aldehyde productivity. Supplementing 5mg/L thiamin hydrochloride into cultures of SA590 and SA590-PK increased their aldehyde productivities by 148% and 107% respectively. Aldehyde productivities were calculated by regressing aldehyde concentration and cell density. Data ± SEM, n = 3. *$p < 0.05$.

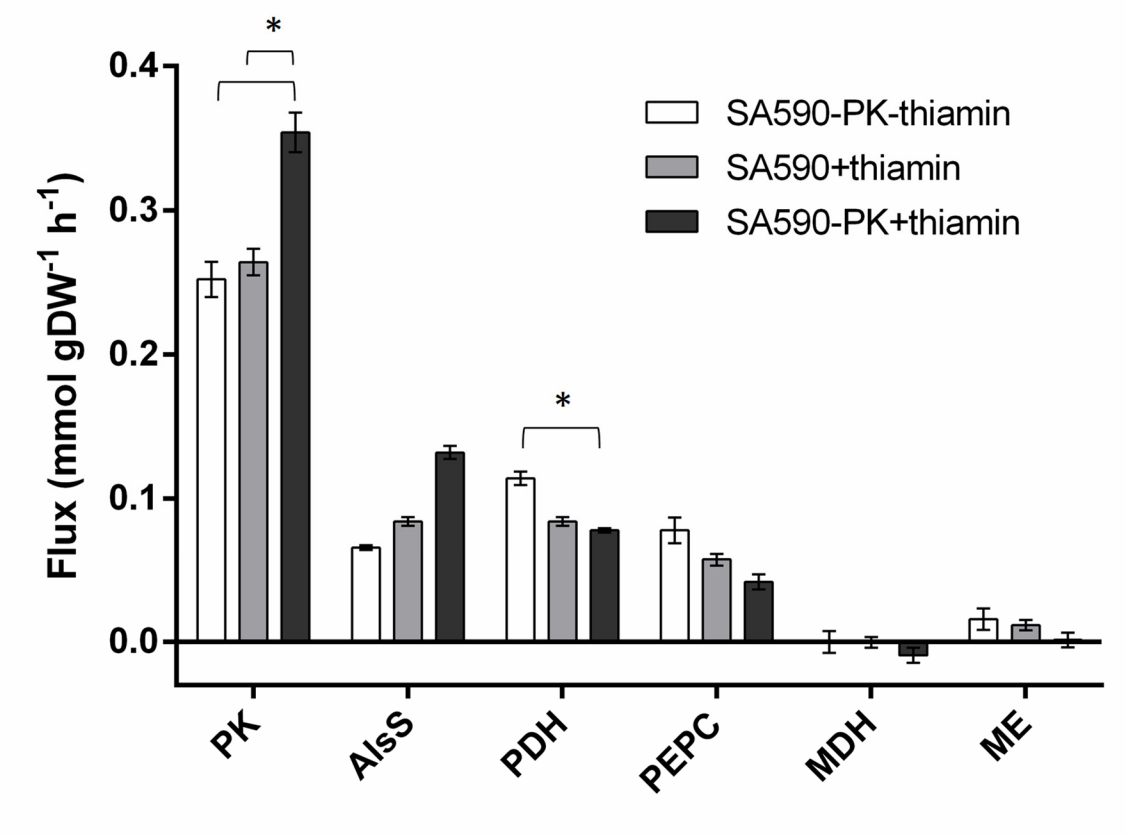

Fig 2. Comparison of fluxes around the pyruvate node as determined by INST-MFA. In these 3 cases, fluxes through pyruvate kinase (PK), acetolactate synthase (AlsS), pyruvate dehydrogenase (PDH), PEP carboxylase (PEPC), malate dehydrogenase (MDH), and malic enzyme (ME) are shown. As all flux through AlsS is directed toward IBA production, the AlsS flux is representative of IBA flux. AlsS flux directly correlates to PK flux and inversely correlates to PDH and PEPC flux. Data ± SE, where SE represents the standard error of the best-fit flux estimate. *$p < 0.05$.

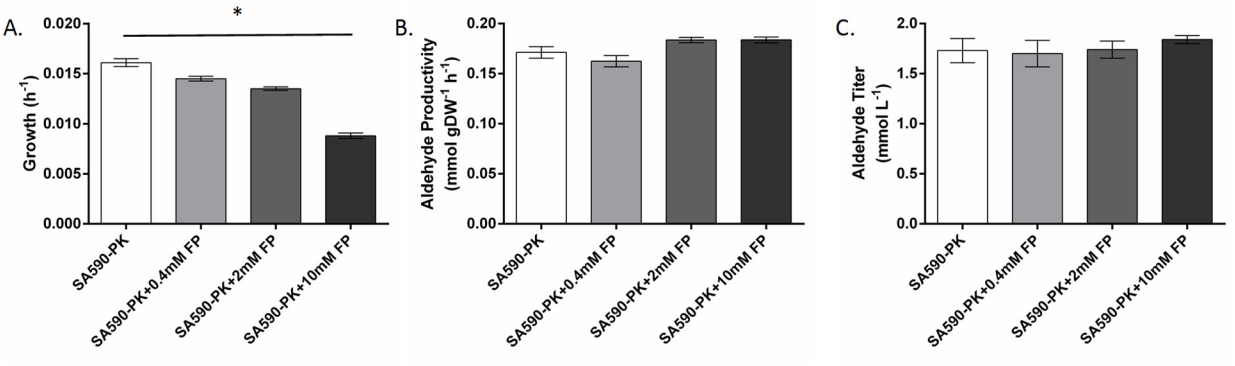

Fig 3. Effect of titrating fluoropyruvate (FP) on the growth, aldehyde productivity, and aldehyde titers of SA590-PK cultures. A) Growth rates were significantly impaired by FP, B) aldehyde productivities were not affected, and C) aldehyde titers at 36h were not affected. No significant differences in titers were observed at previous times points (6 h, 12 h, 24 h, and 30 h, data not shown). Data ± SEM, n =3. *$p < 0.05$.