Final Technical Report

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"Metabolomics of Clostridial Biofuel Production"

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Abstract

Members of the genus Clostridium collectively have the ideal set of the metabolic capabilities for fermentative biofuel production: cellulose degradation, hydrogen production, and solvent excretion. No single organism, however, can effectively convert cellulose into biofuels. Here we developed, using metabolomics and isotope tracers, basic science knowledge of Clostridial metabolism of utility for future efforts to engineer such an organism. In glucose fermentation carried out by the biofuel producer *Clostridium acetobutylicum*, we observed a remarkably ordered series of metabolite concentration changes as the fermentation progressed from acidogenesis to solventogenesis. In general, high-energy compounds decreased while low-energy species increased during solventogenesis. These changes in metabolite concentrations were accompanied by large changes in intracellular metabolic fluxes, with pyruvate directed towards acetyl-CoA and solvents instead of oxaloacetate and amino acids. Thus, the solventogenic transition involves global remodeling of metabolism to redirect resources from biomass production into solvent production. In contrast to *C. acetobutylicum,* which is an avid fermenter, *C. cellulolyticum* metabolizes glucose only slowly. We find that glycolytic intermediate concentrations are radically different from fast fermenting organisms. Associated thermodynamic and isotope tracer analysis revealed that the full glycolytic pathway in *C. cellulolyticum* is reversible. This arises from changes in cofactor utilization for phosphofructokinase and an alternative pathway from phosphoenolpyruvate to pyruvate. The net effect is to increase the high-energy phosphate bond yield of glycolysis by 150% (from 2 to 5) at the expense of lower net flux. Thus, *C. cellulolyticum* prioritizes glycolytic energy efficiency over speed. Degradation of cellulose results in other sugars in addition to glucose. Simultaneous feeding of stable isotope-labeled glucose and unlabeled pentose sugars (xylose or arabinose) to *C. acetobutylicum* revealed that, as expected, glucose was preferred, with the pentose sugar selectively assimilated into the pentose phosphate pathway (PPP). Simultaneous feeding of xylose and arabinose revealed an unexpected hierarchy among these pentose sugars, with arabinose utilized preferentially over xylose. Pentose catabolism occurred via the phosphoketolase pathway (PKP), an alternative route of pentose catabolism that directly converts xylulose-5-phosphate into acetyl-phosphate and glyceraldehyde-3-phosphate. Taken collectively, these findings reveal two hierarchies in Clostridial pentose metabolism: xylose is subordinate to arabinose, and the PPP is used less than the PKP. Thus, in addition to massively expanding the available data on Clostridial metabolism, we identified three key regulatory points suitable for targeting in future bioengineering efforts: phosphofructokinase for enhancing fermentation, the pyruvate-oxaloacetate node for controlling solventogenesis, and the phosphoketolase reaction for driving pentose catabolism.

1. Acidogenic-solventogenic switch

The anaerobic bacterium *Clostridium acetobutylicum* is a promising biofuel producer due to its capacity to ferment a variety of carbohydrates into acetone, butanol and ethanol. The metabolism of this organism is characterized by two sequential phases. The first, acidogenic phase, occurs during exponential growth and involves the rapid production of acids (acetate and butyrate). The second, solventogenic phase, occurs as cell growth slows down and involves the production of solvents (butanol, acetone, and ethanol) and the partial re-assimilation of previously produced acids (Dürre, 2005; Monot *et al*, 1982). A comprehensive understanding of the mechanisms that control the transition into the solventogenic state would be an important step towards the commercial production of solvents using this anaerobic bacterium. Despite numerous research efforts, however, they remain incompletely understood (Alsaker and Papoutsakis, 2005; Durre *et al*, 2002; Grupe and Gottschalk, 1992; Monot *et al*, 1984).

We conducted the first metabolomics analysis of the switch into solventogenesis (Fig. 1). In this experiment, the onset of solventogenesis occurred at around 12 h. Our analysis revealed an orderly progression of changes in the levels of intracellular metabolites throughout the entire time course analyzed (Figure 1). Rather than being restricted to metabolites in the acid/solvent producing pathways, these alterations encompassed all analyzed pathways. In general, higher energy compounds such as amino acids, upper glycolytic intermediates, acetyl-CoA, ATP, and NADH decrease in abundance upon entry into solventogenesis, whereas lower energy compounds such nucleoside monophosphate and bases increase. Butyryl-CoA, acetyl-CoA, acetyl-phosphate and butyryl-phosphate maintain relatively high levels during exponential growth and early solventogenesis but they all decrease significantly at the onset of acid re-assimilation (mid-solventogenic phase). This observation appears to be counterintuitive since it is expected that during acid reutilization large amounts of butyryl-CoA and acetyl-CoA would be produced. The fact that they instead decrease, implies their consumption increases yet more than their production. This suggests that acid re-uptake, rather than conversion of acetyl-CoA and butyryl-CoA into solvents, is likely to have greater control of solvent production flux.

In addition to metabolite concentrations, in vivo reaction rates are among the most important characteristics of a biochemical network. The widespread changes in metabolite concentrations during the acidogenic-solventogenic transition suggested that core metabolic fluxes are affected. Although metabolic fluxes are not directly observable, they can be measured based on nutrient uptake and waste excretion rates and based on the assimilation of stable isotope tracers into intracellular metabolites. During solventogenesis the specific rate of glucose consumption decreases over three fold, going from ~12 mM•h-1•gCDW-1 during mid-exponential phase to ~3.5 mM•h-1•gCDW-1 during mid-solventogenic phase. About 70% of the consumed glucose is excreted as acids during acidogenesis. During solventogenesis, about 85% of the glucose consumed is converted into solvents while additional solvent production occurs from acid reutilization. Since glucose consumption decreases during solventogenesis, overall intracellular metabolic flux must also decrease. However, we do not expect all metabolic pathways to be equally (proportionally) affected. To investigate the remodeling of central metabolic fluxes during solventogenesis, we followed the dynamics of incorporation of labeled glucose into intracellular metabolites during acidogenesis and solventogenesis. While glycolysis was rapidly labeled from glucose in both acidogenesis and solventogenesis, labeling into the TCA cycle and amino acid was dramatically decreased during solventogenesis. Quantitative interpretation of the labeling data via a differential equation model revealed strongly decreased flux from pyruvate into oxaloacetate (catalyzed by pyruvate carboxylase). Inhibition of this flux impairs amino acid synthesis and growth while augmenting flux into acetyl-CoA, NAD(P)H, and accordingly solvents. Therefore, pyruvate carboxylase is a particularly appealing target for controlling solventogenesis via metabolic engineering.

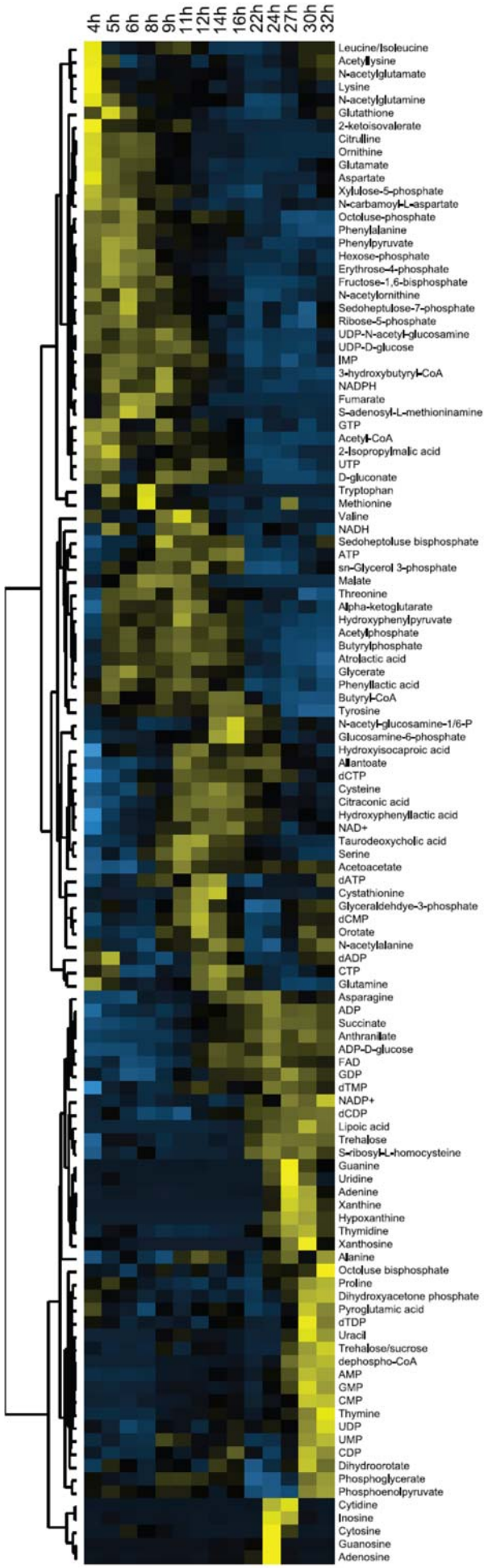
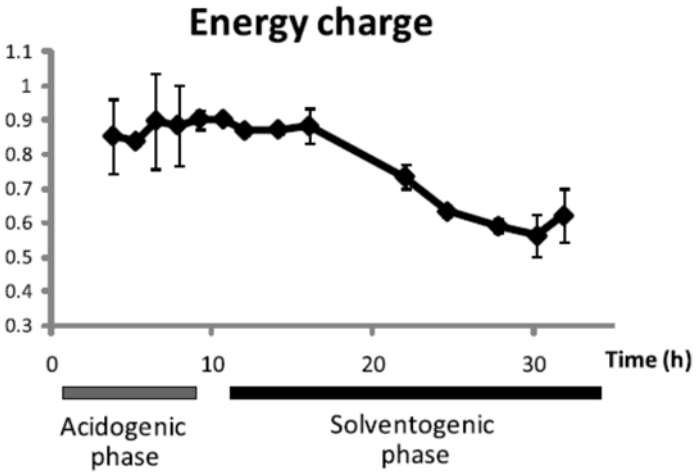


Figure 1. Metabolomics of the switch into solventogenesis in *C. acetobutylicum.* In the heat map, yellow indicates high concentrations and blue indicates low concentrations. There is a steady progression in metabolite concentrations, from initiation of fermentation onwards, generally towards fewer high-energy species and more low-energy species. This is highlighted by the change in adenylate energy charge.



2. Hierarchy in pentose sugar metabolism

The most common polysaccharides in plant detritus are cellulose and hemicellulose. Cellulose is a polymer of glucose whereas hemicellulose, the second most abundant component of plant cell walls, is composed primarily of the pentose sugars xylose and arabinose (Sun and Cheng, 2002). Efficient utilization of plant biomass thus requires the metabolism of both hexose and pentose sugars. Previous findings indicated that C. acetobutylicum metabolizes hexose sugars preferentially over pentose sugars (Gu *et al,* 2009; Xin *et al,* 2014; Xiao *et al,* 2011). The metabolic fate of carbons from mixed-sugar sources has, however, not been determined. Understanding mixed sugar fermentation is critical for engineering effective conversion of the full scope of plant sugars into biofuels.

To explore mixed sugar catabolism, C. acetobutylicum were fed glucose, glucose:arabinose, glucose:xylose, or arabinose:xylose. Each of the sugar mixtures, including the pentose sugars alone (arabinose:xylose) resulted in a cell doubling time between 1.8 and 2.5 h. Thus, pentose sugars alone were sufficient to support reasonably rapid cell growth. Measurement of nutrient uptake via NMR revealed, unsurprisingly, a strong preference for glucose over either of the pentose sugars. Intriguingly, in the pentose sugar mixture, arabinose was consumed > 3-fold faster than xylose.

To explore the metabolic fates of these sugars, we used isotope labeling. Feeding of mixtures of labeled glucose and labeled arabinose or xylose resulted in extensive labeling of pentose phosphate pathway compounds but essentially no labeling into glycolysis. Moreover, concentrations of pentose phosphate compounds were increased > 5-fold relative to glucose-fed cells. Thus, the pentose sugars are assimilated but their catabolism via the pentose phosphate pathway is too slow to contribute significantly to downstream glycolytic pools; accordingly, they accumulate as pentose phosphate pathway intermediates. Feeding of labeled arabinose and unlabeled xylose revealed extensive labeling throughout metabolism; in contrast, minimal labeling was observed when arabinose was unlabeled and xylose was labeled. This confirms the hierarchical preference for arabinose over xylose.

Intriguingly, levels of acetyl-phosphate increased with pentose feeding (in the presence of glucose, and yet more in its absence). Acetyl-phosphate can be produced from the pentose phosphate pathway intermediate xyulose-5-phosphate by the phosphoketolase reaction. To evaluate whether this reaction was carrying substantial flux, we fed positionally labeled arabinose such that phosphoketolase flux would produce 1-^{13}C-acetyl-phosphate without producing labeled triose-phosphates; all other pathways would label either both or neither. We observed nearly 50% labeling of acetyl-phosphate with < 5% labeling of triose-phosphates. Quantitative flux analysis (Figure 2) confirmed that arabinose was used predominantly over xylose and that the phosphoketolase pathway was the primary mode of pentose catabolism. This is evident for the arabinose and xylose-fed cells in the greater absolute magnitude *pk1* flux than transketolase flux, and the minimal flux through glycolytic reactions upstream of GAP, which is made by the *pk1* reaction.

From a metabolic engineering perspective, a potential downside of phosphoketolase flux is production of acetyl-phosphate which is then excreted as acetate (an undesired acid) as opposed to an electron-rich biofuel. Indeed, we observed increased acetate production when phosphoketolase flux was high. This was accompanied, however, by increases also production of acetone, a desired product. Thus, our data argue for a two-pronged approach to converting pentoses into solvents: stimulation of the phosphoketolase pathway, the naturally preferred way of catabolizing these sugars, with further engineering to drive acetyl-phosphate into acetone.

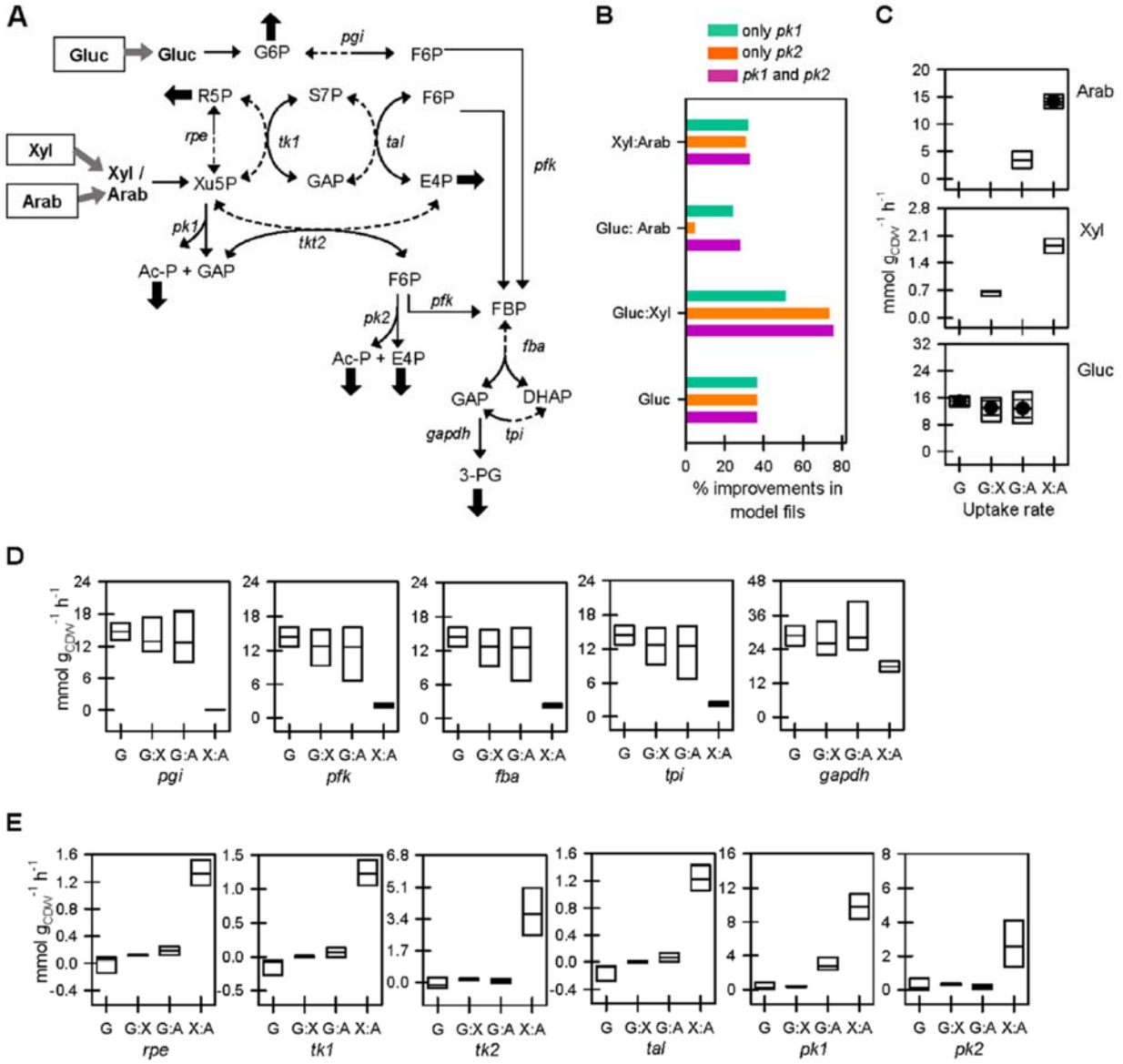


Figure 2. Quantitative flux analysis of mixed sugar utilization. (A) Model metabolic network, (B) model improvements following incorporation of both or one of the phosphoketolase reactions relative to the absence of these reactions, (C) fluxes estimated for uptake rate, (D) reactions in glycolysis, and (E) reactions in pentose phosphate and phosphoketolase pathways during C. acetobutylicum growth on glucose (G), glucose:xylose (G:X), glucose:arabinose (G:A), and xylose:arabinose (X:A). Note that the absolute magnitude of the *pk1* flux far exceeds that of transketolase, indicating the predominance of the phosphoketolase pathway of pentose catabolism.

3. Energy efficiency versus speed in glycolysis

C. cellulolyticum is an obligate anaerobe capable of degrading cellulose into simple sugars and convert them into useful biofuels such as ethanol. However, conversion yields and production rates are too low to allow its use for commercial production of biofuels. For example, compared to the typical biofuel producer *C. acetobutylicum*, *C. cellulolyticum* has a very slow sugar catabolism, even when growing on simple sugars such as glucose or cellobiose. We utilized metabolomic tools, in combination with isotope tracers and quantitative flux modeling, to gain a quantitative understanding of the sugar catabolic pathways in this bacterium.

We compared intracellular metabolite concentrations in *C. cellulolyticum* with those previously reported in the anaerobic biofuel producer *C. acetobutylicum* and the model organism *E. coli.* This comparison revealed major differences in the intracellular concentrations of glycolytic intermediates, amino acids, and cofactors, most strikingly, extremely low concentration of the upper glycolytic intermediate fructose-1,6-bisphosphate (FBP) (Figure 3). This is noteworthy because typically, when glucose is available, FBP is one of the metabolites with the highest intracellular concentration to provide thermodynamic driving force for glycolysis. Steady-state labeling experiments using equimolar amounts of universally labeled ^{13}C-glucose and non-labeled glucose revealed unique labeling patterns in *C. cellulolyticum*'s upper glycolytic intermediates: a large fraction of partially labeled fructose-6-phosphate (Figure 3). This labeling pattern indicated significant back flux from glycolysis and the pentose phosphate pathway. In particular, the large fraction of fructose-6-phosphate containing three ^{13}C-carbons indicated that the conversion of fructose-6-phosphate into FBP was, unexpectedly, highly reversible in *C. cellulolyticum*.

In the canonical EMP glycolytic pathway, the conversion of fructose-6-phosphate to FBP, catalyzed by an ATP-dependent phosphofructokinase (PFK), is generally regarded as irreversible. Indeed, a high reversibility in this particular reaction would create a futile cycle that wastes ATP. By performing enzymatic assays on cell free extracts we discovered that *C. cellulolyticum* does not have an active ATP-dependent PFK but instead utilizes a pyrophosphate-dependent PFK. By using pyrophosphate instead of ATP as a cofactor, this enzyme operates near equilibrium while saving an ATP.

Another major thermodynamically driven reaction in canonical glycolysis is the conversion of phosphoenolpyruvate to pyruvate. Surprisingly, isotopic tracer experiments using ^{13}C-acetate showed that conversion of conversion of phosphoenolpyruvate to pyruvate was also reversible. Interestingly, we were unable to find any enzymatic activity (pyruvate kinase, pyruvate phosphate dikinase, or PEP synthase) for the direct interconversion between PEP and pyruvate in a cell free extract from *C. cellulolyticum.* Isotopic tracer experiments using 50% ^{13}C-glucose corroborated the lack of direct interconversion between PEP and pyruvate and suggested instead an indirect conversion route via oxaloacetate or oxaloacetate and malate as depicted in Figure 4. Using enzymatic assays we found enzymatic activity for all of the proposed steps in this interconversion route: PEP carboxykinase (GDP dependent), malate dehydrogenase (NADH-linked), and malic enzyme (NADP-linked). No PEP carboxytransphosphorylase (PEP + Pi <--> OAA + PPi) or PEP carboxylase were found. Figure 4 depicts a model of *C. cellulolyticum*'s sugar catabolism that is consistent with all of our experimental observations. This pathway is highly energy efficient and can produce as many as 5 ATP equivalents per molecule of glucose, as opposed to 2 ATPs produced by the canonical EMP pathway. Using our intracellular metabolite concentration measurements and flux ratios (backward-to-forward flux) obtained from our steady-state labeling experiments we calculated the changes in free energy (ΔG) associated with this unique glycolytic pathway (Figure 4). The low thermodynamic driving force for the pathway results in it being slow but energy-efficient.

In summary, we discovered that, unlike in most bacteria, glycolysis in *C. cellulolyticum* is fully reversible, with all of its reactions taking place near equilibrium. Such reversibility is achieved by replacing the cofactor ATP with pyrophosphate or GTP as the high-energy phosphate donors/acceptors as well as by using canonically anaplerotic and gluconeogenic reactions for glucose catabolism. This results in a highly energy-efficient sugar catabolism that generates more energy per glucose than the canonical EMP glycolytic pathway used by most bacteria. The unique glycolysis in *C. cellulolyticum* reflects the evolution of the metabolic pathway to cope with the low energy availability that can be attributed to anaerobiosis on cellulose. Our findings suggest metabolic engineering strategies that could be used to improve glycolytic flux in *C. cellulolyticum*. For example, replacing the pyrophosphate-dependent PKF with an ATP-dependent PFK or adding a pyruvate kinase to directly convert PEP to pyruvate would result in a more thermodynamically forward driven and thus faster fermentation pathway.

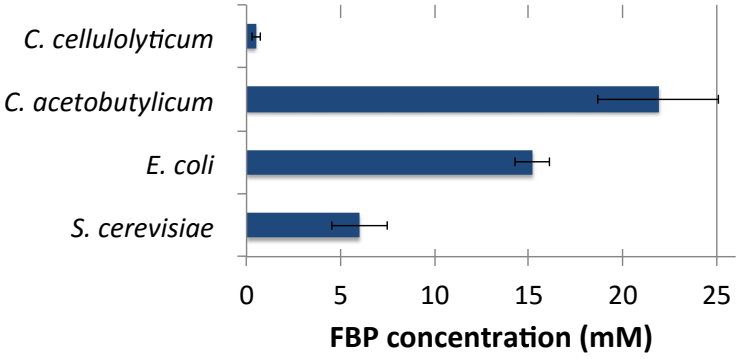


Figure 3. Low concentration of fructose-1, 6-bisphosphate (FBP) in *C. acetobutylicum* results from its pyrophosphate-dependent phosphofructokinase and leads to upper glycolysis being reversible, as indicated by the partial labeling of fructose-6-phosphate (F6P) in cells fed 50% U-^{13}C-glucose.

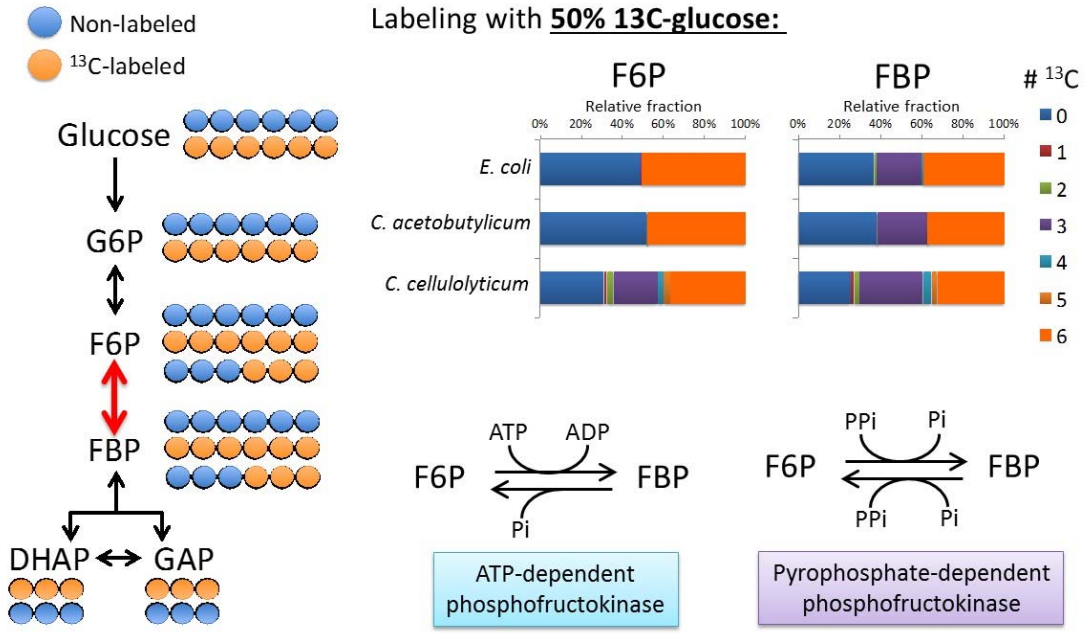
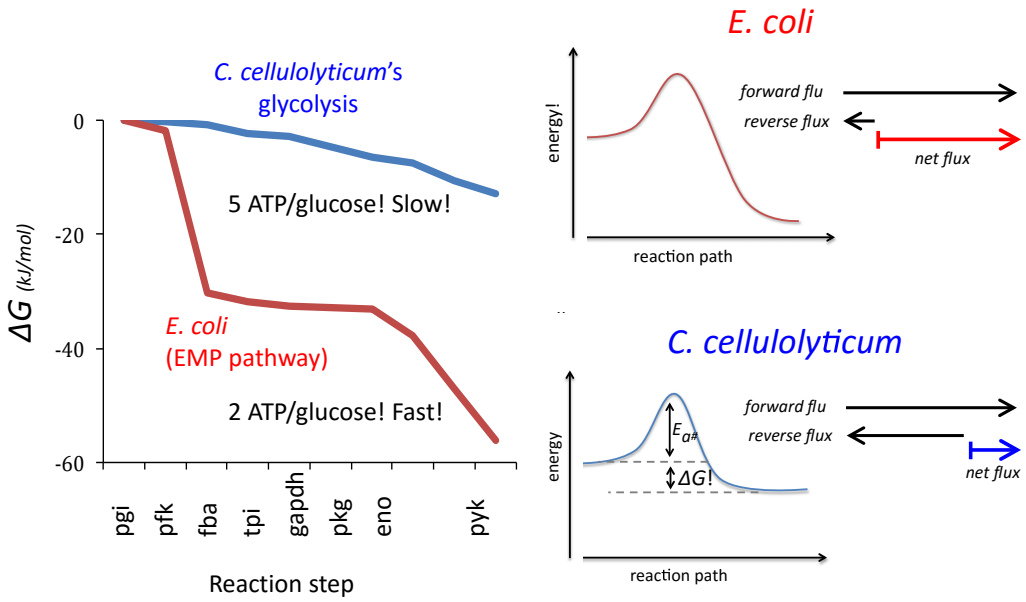
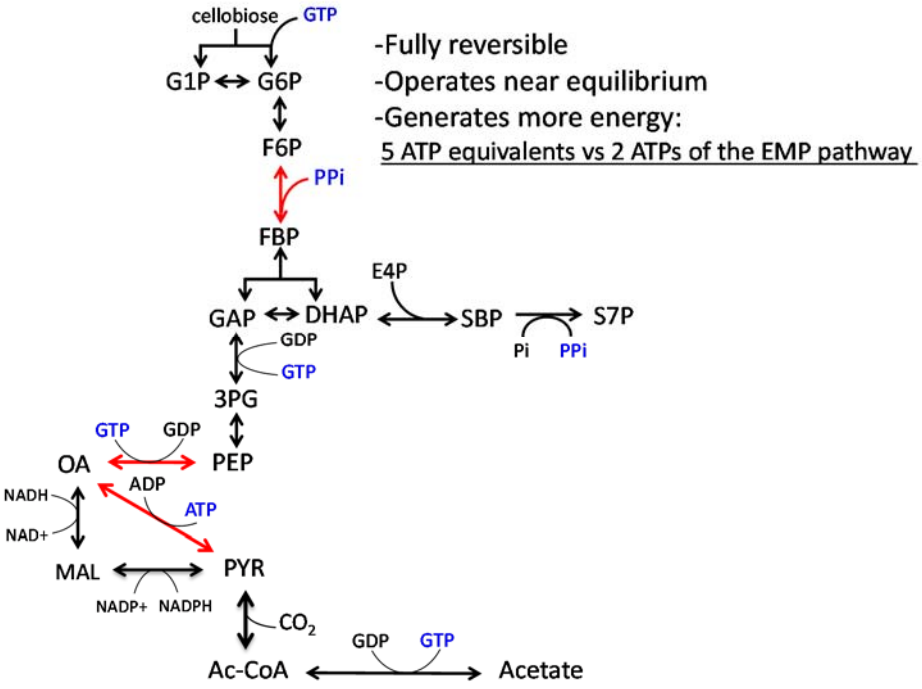


Figure 4. Metabolic pathways involve a fundamental trade-off between energy efficiency and speed. Energy efficiency requires reactions to run near equilibrium, which results in large reverse fluxes and thus little net flux per unit enzyme. *C. cellulolyticum*, presumably because of its natural growth in a glucose-poor anaerobic environment, differs from other organisms studied to date (including *C. acetobutylicum*), in prioritizing energy efficiency over speed. This is achieved by use of the alternative reactions highlighted in red in the pathway diagram.





4. Training impact

This award resulted in substantial impact via training of undergraduates and graduate students. These include undergraduate Sara Rubin (now pursuing her MD/PhD at Harvard), Jun Park (soon to receive his PhD and planning to pursue a bioengineering-relevant postdoc), and Yifan Xu, PhD (currently a research group leader in biofuels at DuPont). **Most distinctively, it resulted in postdoctoral training for two stellar underrepresented minority scientists** (the co-authors of this report, Daniel Amador-Noguez and Ludmilla Aristilde). **Daniel is now a professor at University of Wisconsin and Ludmilla is a professor at Cornell University.** Both are running their own groups, engaged in DOE relevant research that builds on the ideas and skills developed in this grant.

5. Key publications

Amador-Noguez, D., Brasg, I. A., Feng, X. J., Roquet, N., Rabinowitz, J. D. (2011). Metabolome remodeling during the acidogenic-solventogenic transition in Clostridium acetobutylicum. Appl. Environ. Microbiol., 77(22): 7984-97.

Aristilde, L., Lewis, I. A., Park, J. O., Rabinowitz, J. D. (2015). Hierarchy in pentose sugar metabolism in clostridium acetobutylicum. Appl. Environ. Microbiol., 81(4):1452-62.