Quantitative Tools for Dissection of Hydrogen-Producing Metabolic Networks

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DOE award number: DE-FG02-07ER64488

Period: June 1, 2008 to Dec 31 2011

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Funding Opportunity Announcement Number: DE-PS02-07ER07-13

Funding Opportunity Title: Quantitative Microbial Biochemistry and Metabolic Engineering for Biological Hydrogen Production

DOE/Office of Science Program Office: Office of Biological and Environmental Research

DOE/Office of Science Program Office Technical Contact: Dr. Sharlene Weatherwax

Abstract

During this project we have pioneered the development of integrated experimental-computational technologies for the quantitative dissection of metabolism in hydrogen and biofuel producing microorganisms (i.e. *C. acetobutylicum* and various cyanobacteria species). The application of these new methodologies resulted in many significant advances in the understanding of the metabolic networks and metabolism of these organisms, and has provided new strategies to enhance their hydrogen or biofuel producing capabilities. As an example, using mass spectrometry, isotope tracers, and quantitative flux-modeling we mapped the metabolic network structure in *C. acetobutylicum*. This resulted in a comprehensive and quantitative understanding of central carbon metabolism that could not have been obtained using genomic data alone. We discovered that biofuel production in this bacterium, which only occurs during stationary phase, requires a global remodeling of central metabolism (involving large changes in metabolite concentrations and fluxes) that has the effect of redirecting resources (carbon and reducing power) from biomass production into solvent production. This new holistic, quantitative understanding of metabolism is now being used as the basis for metabolic engineering strategies to improve solvent production in this bacterium. In another example, making use of newly developed technologies for monitoring hydrogen and NAD(P)H levels *in vivo*, we dissected the metabolic pathways for photobiological hydrogen production by cyanobacteria *Cyanothece* sp. This investigation led to the identification of multiple targets for improving hydrogen production. Importantly, the quantitative tools and approaches that we have developed are broadly applicable and we are now using them to investigate other important biofuel producers, such as cellulolytic bacteria.

Aims summary

Aim 1: Develop quantitative experimental tools for simultaneous measurements of multiple metabolite concentrations and fluxes.

As proposed initially in the application, we developed a comprehensive set of experimental tools for measuring multiple intermediate metabolites (e.g., those of central carbon and biosynthetic metabolism) and gases (H₂, O₂ and CO₂) implicated in energy metabolism. Our initial target was to enable quantitation of at least 200 different metabolites by LC-MS/MS, and our current LC-MS/MS methods achieve that target [4, 6, 9], although not every metabolite is detectable in every sample (typically we see about 150 metabolites in a given sample type). We also aimed to enable quantitation (using isotopic tracers) of a wide spectrum of metabolic fluxes, and this has also been achieved [1, 2, 7, 8].

Our initial plan was to use complementary electrochemical, fluorescence, or NMR assays for compounds that cannot be readily measured by LC-MS. To this end, we have successfully built the proposed unique combination electrochemical/fluorescence instrument for simultaneously monitoring the kinetics of extracellular dissolved H₂ and intracellular reduced pyridine nucleotide concentrations [12]. In addition, we have developed NMR methods for measuring important excreted metabolites such as acids (e.g., acetate, butyrate) and solvents (e.g., acetone, butanol) [1, 5].

Aim 2: Map the organisms' dynamic metabolic responses to changing environmental conditions.

We proposed to experimentally modulate both nutrient availability and environmental stresses, while monitoring the resulting time-dependent changes (including H₂ production rates) in the cellular metabolome and fluxome, in both *Clostridium acetobutylicum* and cyanobacterial species. In addition, we proposed to investigate the acidogenic-solventogenic transition in *C. acetobutylicum*, as understanding this metabolic transition is key to efforts to improve solvent production in this bacterium. These experiments have been successfully conducted, and have led to important insights into metabolic regulation in these organisms [1, 3, 12]. For example, we found that flux through pyruvate carboxylase shuts off as *C. acetobutylicum* initiates solventogenesis [1].

Aim 3. Develop predictive models of H₂-metabolism.

We aimed to integrate high quality, dynamic metabolomic data to (a) better understand metabolic regulation, and (b) develop predictive, quantitative metabolic models. Our proposed approach to model building was progression from descriptive qualitative models to predictive quantitative ones. We proposed to first identify the main functional connections among the metabolites, and subsequently develop structural kinetic models of linear causal relationships between these metabolites, and finally to engage in nonlinear ordinary differential equation (ODE) modeling of the detailed temporal behavior.

We have made substantial progress developing the relevant modeling framework [10, 11]. Through this framework, we have been able to successfully model isotope tracer experiments [1, 2]. These efforts include each of the proposed modeling steps, and result in detailed ODE models of labeling dynamics. In addition, we have identified important longer-distance regulatory connections, e.g., between pyruvate carboxylase and solventogenesis [1]. Due to their complexity, however, involving both genetic and metabolic regulation with numerous unknown molecular steps, we have yet to develop a complete predictive model of them. Nevertheless, our current level of understanding has already had profound engineering implications, enabling genetic and small molecule tools to control solventogenesis.

Research Achievements

Our research in this project has resulted in the development of novel experimental and computational approaches for the quantitative study of metabolism in hydrogen and biofuel producing microorganisms. Our efforts concentrated in hydrogen producing cyanobacterial species such as *Cyanothece* sp and *Synechococcus* sp, as well as in the hydrogen and solvent (butanol and acetone) producer *C. acetobutylicum*. Our investigation yielded many significant advances in the understanding of the metabolic networks and metabolism of these organisms, and has provided new strategies to enhance their hydrogen or biofuel producing capabilities.

Metabolomic analysis via reversed-phase ion-pairing liquid chromatography coupled to a stand alone orbitrap mass spectrometer [9].

Addressing our objective of creating a comprehensive set of experimental tools for measuring multiple intermediate metabolites, we developed a liquid chromatography-mass spectrometry (LC-MS) method that capitalizes on the mass-resolving power of the orbitrap to enable sensitive and specific measurement of known and unanticipated metabolites in parallel, with a focus on water-soluble species involved in core metabolism. The reversed phase LC method, with a cycle time 25 min, involves a water-methanol gradient on a C18 column with tributylamine as the ion pairing agent. The MS portion involves full scans from 85 to 1000 m/z at 1 Hz and 100,000 resolution in negative ion mode on a stand alone orbitrap ("Exactive"). The median limit of detection, across 80 metabolite standards, was 5 ng/mL with the linear range typically ≥ 100 -fold. For both standards and a cellular extract from *Saccharomyces cerevisiae* (Baker's yeast), the median inter-run relative standard deviation in peak intensity was 8%. In yeast exact, we detected 137 known compounds, whose ^{13}C -labeling patterns could also be tracked to probe metabolic flux. These examples demonstrate the suitability of the present method for quantitative metabolomics, fluxomics, and discovery metabolite profiling.

Overlay of extracted ion chromatographs of selected metabolites from *C. acetobutylicum*.

Identification and quantification of water-soluble metabolites by cryoprobe-assisted nuclear magnetic resonance spectroscopy applied to microbial fermentation [5].

Following our objective of developing complementary assays for compounds of particular importance that cannot be reliably measured by LCMS/MS, we developed a range of cryoprobe-assisted NMR methods for studying metabolite production by cyanobacteria and clostridial species, which should be valuable for a wide range of biological applications requiring ultrasensitivity and precise concentration determination over a large dynamic range. Fermentative end products were identified and quantified in concentrations ranging from 50 to 3000 μM in cell-free media with less than 5.5% relative error in under 10 min of acquisition per sample with the assistance of an efficient water-suppression protocol.

Metabolic pathways for photobiological hydrogen production by nitrogenase- and hydrogenase-containing unicellular cyanobacteria *Cyanothece* [12].

We quantified both dark- and light-induced H_2 effluxes by *Cyanothece* sp. Miami BG 043511 and established their respective origins. Dark, anoxic H_2 production occurs via hydrogenase utilizing reductant from glycolytic catabolism of carbohydrates (auto-fermentation). Photo- H_2 is shown to occur via nitrogenase and requires illumination of PSI, whereas production of O_2 by co-illumination of PSII is inhibitory to nitrogenase above a threshold pO_2 . Carbohydrate also serves as the major source of reductant for the PSI pathway mediated via non-photochemical reduction of the plastoquinone pool by NADH dehydrogenases type-1 and type-2 (NDH-1 and NDH-2). Redirection of this reductant flux exclusively through the proton-coupled NDH-1 by inhibition of NDH-2 with flavone increases the photo- H_2 production rate by 2-fold (at the expense of the dark- H_2 rate), due to production of additional ATP (via the proton gradient). Comparison of photo-biological hydrogen rates, yields, and energy conversion efficiencies reveals opportunities for improvement. This research highlights our use of unique electrochemical and fluorescence instruments for monitoring the kinetics of extracellular dissolved H_2 and intracellular reduced pyridine nucleotide concentrations.

Proposed model for coupling of carbohydrate catabolism to photo- H_2 production in *Cyanothece* where nonphotochemical reduction of PQ by catabolically derived NAD(P)H is the primary source of reductant for PSI-dependent, nitrogenase-mediated photo- H_2 .

Systems-level metabolic flux profiling elucidates a complete, bifurcated tricarboxylic acid cycle in *Clostridium acetobutylicum* [2].

Addressing our objective of advancing our quantitative understanding of the metabolic networks of hydrogen and biofuel producing microorganisms, we used isotope tracers, mass spectrometry, and quantitative flux modeling, to directly map the metabolic pathways of *Clostridium acetobutylicum*. While genome annotation suggested the absence of most tricarboxylic acid (TCA) cycle enzymes, our results demonstrated that this bacterium has a complete, albeit bifurcated, TCA cycle; oxaloacetate flows to succinate both through citrate/alpha-ketoglutarate and via malate/fumarate. Our investigations also yielded insights into the pathways utilized for glucose catabolism and amino acid biosynthesis and revealed that the organism's one-carbon metabolism is distinct from that of model microbes, involving reversible pyruvate decarboxylation and the use of pyruvate as the one-carbon donor for biosynthetic reactions. This study represented the first in vivo characterization of the TCA cycle and central metabolism of *C. acetobutylicum*. Our results established a role for the full TCA cycle in an obligatory anaerobic organism and demonstrated the importance of complementing genome annotation with isotope tracer studies for determining the metabolic pathways of biofuel producing microorganisms.

Isotope-tracer experiments reveal a complete, bifurcated TCA cycle. Cells were switched from non-labeled glucose to universally labeled ^{13}C -glucose at $t=0$. In each small graph, the y axis represents the fraction of the observed compound of the indicated isotopic form. The labeling patterns in citrate are consistent with its production from acetyl-CoA and oxaloacetate via citrate synthase. α -ketoglutarate is produced oxidatively from citrate. Malate and fumarate are produced from pyruvate via pyruvate carboxylase and oxaloacetate. Succinate is produced both via fumarate and via α -ketoglutarate. All compounds were measured by LC-MS.

Metabolome remodeling during the acidogenic-solventogenic transition in *Clostridium acetobutylicum* [1].

In line with our objective of mapping dynamic metabolic responses critical for hydrogen and biofuel production, we used metabolomics, isotope tracers, and quantitative flux modeling, we have mapped the metabolic changes associated with the acidogenic-solventogenic transition in *C. acetobutylicum*. We observed a remarkably ordered series of metabolite concentration changes, involving almost all of the 114 measured metabolites, as the fermentation progresses from acidogenesis to solventogenesis. The intracellular levels of highly abundant amino acids and upper glycolytic intermediates decrease sharply during this transition. NAD(P)H and nucleotide triphosphates levels also decrease during solventogenesis, while low-energy nucleotides accumulate. These changes in metabolite concentrations are accompanied by large changes in intracellular metabolic fluxes. During solventogenesis, carbon flux into amino acids, as well as flux from pyruvate (the last metabolite in glycolysis) into oxaloacetate, decreases by more than 10-fold. This redirects carbon into acetyl coenzyme A, which cascades into solventogenesis. In addition, the electron-consuming reductive tricarboxylic acid (TCA) cycle is shutdown, while the electron-producing oxidative (clockwise) right side of the TCA cycle remains active. Thus, the solventogenic transition involves global remodeling of metabolism to redirect resources (carbon and reducing power) from biomass production into solvent production.

Alterations in metabolic fluxes during solventogenesis. We used kinetic flux profiling (KFP) to obtain a quantitative estimate of metabolic fluxes from the kinetics of incorporation of ¹³C-labeled glucose into downstream metabolites during acidogenesis and solventogenesis.

LC-MS data processing with MAVEN: a metabolomic analysis and visualization engine [6].

MAVEN is an open-source software program for interactive processing of LC-MS-based metabolomics data. MAVEN enables rapid and reliable metabolite quantitation from multiple reaction monitoring data or high-resolution full-scan mass spectrometry data. It automatically detects and reports peak intensities for isotope-labeled metabolites. Menu-driven, click-based navigation allows visualization of raw and analyzed data. Here we provide a User Guide for MAVEN. Step-by-step instructions are provided for data import, peak alignment across samples, identification of metabolites that differ strongly between biological conditions, quantitation and visualization of isotope-labeling patterns, and export of tables of metabolite-specific peak intensities. Together, these instructions describe a workflow that allows efficient processing of raw LC-MS data into a form ready for biological analysis.

The software developed in this paper contributes to our objective of creating tools that aids the analysis of metabolomic data and facilitates its integration with computational models.

Regulatory and metabolic rewiring during laboratory evolution of ethanol tolerance in *E. coli* [8].

Increasing solvent (e.g. ethanol, butanol) tolerance in biofuel producing strains is critical for achieving high yields. To provide insight into this problem, we developed a combined experimental/computational framework to understand the genetic basis of ethanol tolerance in *Escherichia coli*. We used fitness profiling to measure the consequences of single-locus perturbations in the context of ethanol exposure. A module-level computational analysis was then used to reveal the organization of the contributing loci into cellular processes and regulatory pathways (e.g. osmoregulation and cell-wall biogenesis) whose modifications significantly affect ethanol tolerance. Strikingly, we discovered that a dominant component of adaptation involves metabolic rewiring that boosts intracellular ethanol degradation and assimilation. Through phenotypic and metabolomic analysis of laboratory-evolved ethanol-tolerant strains, we investigated naturally accessible pathways of ethanol tolerance. Remarkably, these laboratory-evolved strains, by and large, follow the same adaptive paths as inferred from our coarse-grained search of the fitness landscape.

Riboneogenesis in yeast [7].

Glucose is catabolized in yeast via two fundamental routes, glycolysis and the oxidative pentose phosphate pathway, which produces NADPH and the essential nucleotide component ribose-5-phosphate. Here, we describe riboneogenesis, a thermodynamically driven pathway that converts glycolytic intermediates into ribose-5-phosphate without production of NADPH. Riboneogenesis begins with synthesis, by the combined action of transketolase and aldolase, of the seven-carbon bisphosphorylated sugar sedoheptulose-1,7-bisphosphate. In the pathway's committed step, sedoheptulose bisphosphate is hydrolyzed to sedoheptulose-7-phosphate by the enzyme sedoheptulose-1,7-bisphosphatase (SHB17), whose activity we identified based on metabolomic analysis of the corresponding knockout strain. The crystal structure of Shb17 in complex with sedoheptulose-1,7-bisphosphate reveals that the substrate binds in the closed furan form in the active site. Sedoheptulose-7-phosphate is ultimately converted by known enzymes of the nonoxidative pentose phosphate pathway to ribose-5-phosphate. Flux through SHB17 increases when ribose demand is high relative to demand for NADPH, including during ribosome biogenesis in metabolically synchronized yeast cells.

While this paper does not directly investigate hydrogen production, it maps a new metabolic pathway in an important industrial biofuel producing organism, and this new pathway modulates production of NADPH, the key cofactor impacting H_2 production.

Ribogenesis in Yeast

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Publication submitted or in preparation

Nicholas J. Skizim, Elizabeth H. Burrows, Michael Abrams, Diana Chien, Derrick R.J. Kolling, Susan L. Brown, and G. Charles Dismukes. (2012) Development and application of a DOP-PCR based genetic screen for [NiFe] hydrogenases: bioprospecting for efficient hydrogen producing organisms. *Submitted, in revision.*

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Nicholas J. Skizim, Gennady M. Ananyev, Yu Xu, Donald A. Bryant, and G. Charles Dismukes. (2012) Methods to Increase Catabolic Rate in *Cyanothece* for Increased Biological H₂ and Ethanol Production. *In preparation.*