214. Overcoming barriers for the conversion of lignocellulose to biofuels by *E. coli*

Eduardo H. Morales[1]* (emorales2@wisc.edu), Yaoping Zhang[1], David H. Keating[1], Irene M. Ong[1], Sean McIlwain[1], Jeffrey A. Grass[1], Donna Bates[1], Mary Tremaine[1], William Bothfeld[1], Alan Higbee[1], Arne Ulbrich[1], Allison Balloon[1], Emma E. Coughlin[1], Michael S. Westphall[1], Cameron Cotton[1], Leonardo da Costa Sousa[2], Mingjie Jin[2], Venkatesh Balan[2], Joonhoon Kim[1], Jennifer L. Reed[1], James Ellinger[1], Oleg Moskvin[1], Yury Bukhman[1], Joshua Coon[1], John Markley[1], Bruce Dale[2], **Patricia J. Kiley[1], Robert Landick[1].**

[1] DOE Great Lakes Bioenergy Research Center, University of Wisconsin, Madison, WI; [2] DOE Great Lakes Bioenergy Research Center, Michigan State University, East Lansing, MI

https://www.glbrc.org/research/conversion

Project goals: Our goal is to understand and overcome barriers to development of an economically viable and sustainable conversion of lignocellulose to biofuels by bacteria. Currently, two major barriers are targeted. First, most microbes and chemical processes cannot simultaneously produce fuels from both C_6 (e.g., glucose) and C_5 (e.g., xylose) sugars, thereby limiting their ability to utilize rapidly all of the sugars in the hydrolysate. Second, most microbes are intolerant to stresses experienced during biofuel fermentation, including high ethanol or biofuel concentrations, toxic pretreatment byproducts, and high osmotic strengths experienced in the hydrolysate.

Multiomic and computational analyses define effects of (lignotoxins) LTs on metabolic and regulatory networks. Study of *E. coli* ethanologenesis in AFEX-treated corn stover hydrolysate (ACSH) coupled with analyses of its chemical composition allowed the development of a next generation synthetic medium (SynHv2) containing a cocktail of lignocellulose-derived inhibitors (referred to here as lignotoxins, LTs) that replicates the ACSH growth, sugar utilization, and gene expression profiles of an *E. coli* ethanologen (GLBRCE1). Major stress responses are caused by aromatic aldehyde, amide, and carboxylate LTs derived from lignocellulose. Gene expression patterns in the ethanologen were similar for ACSH and SynH containing the LT cocktail, indicating that the new SynH closely mimics the stresses caused by ACSH. Using this system as a model for ACSH, the effects of LTs on conversion and *E. coli* physiology were studied in detail.

Lignotoxins significantly perturbed metabolism, causing dramatic elevations of pyruvate and acetaldehyde with concomitant depletion of ATP, NADPH, and NADH. Transcriptomic, proteomic, and bioinformatic analyses revealed 5 major regulatory responses consistent with LT- induced energy stress in *E. coli*. One, mediated by elevated levels of RpoS, reflects a general stress response. Four others, mediated by YqhC, FrmR, AaeR, and MarA/Rob/SoxS, reflect specific responses to LTs. YqhC and FrmR, known regulators of aldehyde detoxification pathways, showed early induction that was ameliorated in stationary phase. Additionally, a decline in hydrolysate aromatic aldehyde levels paralleled by an accumulation of their alcohol forms in the extracellular medium was observed, consistent with detoxification of this class of LTs and the observed down regulation of *yqhC* and *frmR* in stationary phase. AaeR and MarR/MarA/Rob control efflux pumps and metabolic detoxification pathways. Both aromatic carboxylate-specific (AaeAB) and more general "multidrug resistance" pumps were activated; notably, these pumps consume ATP in each efflux cycle and their expression remained elevated throughout all stages of fermentation. Unlike the aromatic aldehydes, aromatic acid and amide LT levels remained constant throughout fermentation, suggesting that they were not

metabolized and might create an energetic challenge for cells that limits their ability to generate adequate reducing equivalents for conversion of pyruvate to ethanol, particularly when catabolizing C5 sugars (eg, xylose) where the energetics are less favorable. In agreement, directed evolution of *E. coli* in glucose-depleted ACSH resulted in a *rob* knockout mutation that improved xylose conversion, consistent with the hypothesis that Rob-mediated stress responses divert energy that can otherwise drive xylose to ethanol conversion.

Identification of strains capable of xylose utilization during growth in lignocellulose hydrolysates. It is known that xylose transport is subject to inducer exclusion, and that this is the predominant mechanism that prevents pentose sugar uptake when glucose is present. To test whether removing a component of the inducer exclusion pathway improved xylose utilization in the presence of glucose, we deleted the gene for the main glucose transporter *ptsG*. Although elimination of PtsG resulted in glucose/xylose co-utilization in SynHv2, xylose consumption was not complete, and only 50% the glucose was consumed. We also examined altered function mutants of Crr (or $EIIA^{GLC}$), a second component of the inducer exclusion pathway that is required for glucose phosphorylation, activation of adenylate cyclase, and inhibits transport of non-PTS sugars by directly binding the relevant transporters. We analyzed mutant variants that we predicted would separately inactivate these various functions and tested whether any of these variants improved xylose utilization. Of the Crr mutants tested, only substitution of His 90 to Ala showed slightly increased xylose utilization, suggesting that this residue plays a role in inhibiting xylose uptake in the presence of glucose, in addition to its expected role in transfer of phosphoryl groups to the glucose transporter PtsG. We are currently testing if mutating His 90 to aspartic acid or glutamic acid, which is expected to mimic the phosphorylated form of Crr and to abolish its interaction with secondary sugar transporters, results in glucose/xylose co-utilization.

This work was funded by the DOE Great Lakes Bioenergy Research Center (DOE BER Office of Science DE-FC02-07ER64494).