Title: Converting Methoxy Groups on Lignin-Derived Aromatics from a Toxic Hurdle to a Useful Resource: A Systems-Driven Approach

Authors: Alexander B. Alleman,[1] Nkrumah A. Grant,[1] Alekhya Govindaraju,[2] Akorede L. Seriki,[1] Isaiah D. Jordan,[3] Jeremy A Draghi,[3] N. Cecilia Martinez-Gomez,[2] Andreas E. Vasdekis,[1] and **Christopher J. Marx[1]* (cmarx@uidaho.edu)**

Institutions: [1]University of Idaho, Moscow, ID; [2]University of California, Berkeley, CA; [3]Virginia Institute of Technology, Blacksburg, VA

Project Goals: In order to convert methoxylated aromatics that originate from lignin hydrolysis into bioproducts it is critical to manage the toxicity of formaldehyde that is generated internally from these methoxy groups. We have discovered both novel genetic factors in *Methylobacterium extorquens* involved in managing formaldehyde toxicity and a key role for cell-to-cell variability in key phenotypes. As such, our goals are to (1) identify physiological hurdles to growth/viability and production, (2) assess the environmental influence upon single cell heterogeneity in these traits, and then (3) generate genetic combinations from targets uncovered in *1* and use a model-driven approach to synthesize knowledge regarding heterogeneous phenotypes uncovered in *2* to generate strains with improved butanol production and optimized combinations of environmental variations.

Abstract Text: Lignin-derived compounds from plant biomass are amongst the most recalcitrant for microbial conversion. Hydrolysates contain a wide variety of aromatic molecules, which are often toxic. Furthermore, a particular issue with these molecules is that many of them are methoxylated: these methoxy groups are released as formaldehyde during degradation, which creates a second source of toxicity that can challenge standard heterotrophs. In an earlier DOE project, we discovered that some *Methylobacterium* strains grow exceptionally well on aromatics, and do not release formaldehyde into the medium from the methoxy groups present, unlike classic systems for aromatic degradation (*e.g.*, *Pseudomonas putida*). One reason methylotrophic bacteria may be well suited for utilizing methoxylated aromatics is their high capacity to produce and consume formaldehyde internally during growth on single-carbon compounds like methanol. Beyond this, however, both Marx and Martinez-Gomez have recently discovered that methylotrophs also have a complex formaldehyde stress response involving multiple sensors and response proteins, as well as the unexpected involvement of lanthanide-dependent dehydrogenases.

We have been developing *Methylorubrum* (formerly *Methylobacterium*) *extorquens* into a model system for degradation of methoxylated aromatics. Recently we have uncovered that both formaldehyde stress response sensors and the lanthanide-dependent systems are needed for effective vanillic acid utilization. In our current project, we discovered that the aromatic backbone converts acetyl-CoA and β-ketoadipate into acetoacetate and succinyl-CoA, this allows carbon to flow efficiently into the species' high-flux glyoxylate-regeneration pathway from

which our target product, butanol, is produced. An advantage of targeting butanol is that the same glyoxylate-regeneration pathway is naturally used to generate the internally-accumulated compound, poly-β-hydroxybutyrate (PHB). As such, we leverage PHB as a reporter, a visualizable single-cell proxy for production capacity through this pathway that can guide our design process.

We have found that a fundamental challenge to developing *M. extorquens* into a catalyst for conversion of lignin-derived methoxylated aromatics into butanol is that populations exhibit tremendous cell-to-cell variability in key phenotypes. We have discovered significant heterogeneity in growth, viability, PHB production, and even internal accumulation of lanthanide granules. We hypothesize that the physiological thresholds that lead to cell-to-cell variability will also exist for mutations or environmental variables that affect the same processes. Indeed, we have observed examples of both genetic interactions between beneficial mutations and with environmental parameters such as substrate concentration consistent with this hypothesis. This variability amongst cells and the fundamental role of stress responses indicate that stability of growth and production, and not just catalytic capacity, is paramount to develop effective growth and production from these difficult feedstocks.

Our project combines genome-scale approaches that will broadly identify the physiological hurdles cells face in growth and production from vanillic acid with a suite of single-cell approaches to examine phenotypic heterogeneity. In particular, a new optical method developed by Vasdekis to assay PHB in single, live cells will permit us to examine the correlations between growth, stress response, and production in an unprecedented manner. Using a model of heterogeneity as a guide, we can then combine our learnings from genetic underpinnings and their impact on single-cell physiology to develop improved strains of *M. extorquens* for conversion of vanillic acid to butanol. In the process, we will develop and demonstrate a novel approach that embraces phenotypic heterogeneity as a major source for future innovation in DOE-relevant biosystems design.

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