Discovery, characterization, and metabolic engineering of Rieske non-heme iron monooxygenases for guaiacol O-demethylation

Alissa Bleem*[1,2] (alissa.bleem@nrel.gov), Eugene Kuatsjah*[1,2] (eugene.kuatsjah@nrel.gov), Gerald N. Presley[2,4,6], Daniel J. Hinchen[3], David C. Garcia[5], William E. Michener[1], Marco N. Allemann[4], John E. McGeehan[3], Richard J. Giannone[2,4], Gregg T. Beckham[1,2], Joshua K. Michener[2,4], and **Gerald A. Tuskan**[2,4]

[1]Renewable Resources and Enabling Sciences Center, National Renewable Energy Laboratory, Golden, CO; [2]Center for Bioenergy Innovation, Oak Ridge National Laboratory, TN.; [3]Center for Enzyme Innovation, School of Biological Sciences, Institute of Biological and Biomedical Sciences, University of Portsmouth, UK; [4]Biosciences Division, Oak Ridge National Laboratory, TN; [5]The Bredesen Center for Interdisciplinary Research and Graduate Education, University of Tennessee, Knoxville, TN; [6]Department of Wood Science and Engineering, Oregon State University, Corvallis, OR.

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Project Goals: The Center for Bioenergy Innovation (CBI) vision is *to accelerate domestication of bioenergy-relevant, non-model plants and microbes to enable high-impact innovations at multiple points in the bioenergy supply chain.* CBI addresses strategic barriers to the current bioeconomy in the areas of 1) high-yielding, robust feedstocks, 2) lower capital and processing costs via consolidated bioprocessing (CBP) to specialty biofuels, and 3) methods to create valuable byproducts from the lignin. CBI will identify and utilize key plant genes for growth, composition, and sustainability phenotypes as a means of achieving lower feedstock costs, focusing on poplar and switchgrass. We will convert these feedstocks to biofuels using CBP with cotreatment at high rates, titers and yield in combination with catalytic upgrading into drop-in hydrocarbon fuel blendstocks.

Lignin is the second most abundant renewable biopolymer on earth after cellulose, and aerobic bacteria offer a promising approach to convert mixtures of lignin-related aromatics to a single, value-added product. Aryl-*O*-demethylation represents a critical reaction in the catabolism of lignin-related phenols such as guaiacol, and it is often a bottleneck for both native and engineered bioconversion pathways in these organisms. Here, we utilized randomly barcoded transposon insertion sequencing (RB-TnSeq) to identify a novel Rieske-type guaiacol *O*-demethylase, GdmA, in the aromatic-catabolic bacterium *Novosphingobium aromaticivorans*. Similarity searches identified GdmA homologs in related bacteria, as well as candidate reductase partners, GdmB. Various GdmAB combinations were characterized biochemically for activity on lignin-related substrates, including guaiacol. GdmAB combinations were also evaluated in *Pseudomonas putida* KT2440, a common microbial chassis for bioconversion of aromatic compounds that does not natively utilize guaiacol. The GdmAB pair from *Cupriavidus necator* N-1 demonstrated the highest rate of guaiacol turnover both *in vitro* and in engineered strains of *P. putida*. Additionally, comparison of the GdmAB Rieske-type mechanism with a previously described cytochrome P450 system (GcoAB) demonstrated superior performance of GdmAB for guaiacol catabolism in *P. putida*. The novel GdmAB *O*-demethylases described here represent a new mechanism for

biological assimilation of guaiacol, and they offer a suite of options for microbial conversion of a common lignin-derived substrate.

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