Discovery of Phenylacetate Decarboxylase, a New Glycyl Radical Enzyme Enabling First-Time Biochemical Production of Toluene

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Project Goals: The Joint BioEnergy Institute (JBEI) aims to produce a chemically diverse suite of biofuels from lignocellulosic biomass. Our objective in this project was enzyme discovery to enable first-time biochemical production of toluene, an important octane booster and petrochemical with a global annual market of 29 million tons, from cellulosic sugars.

Toluene is an important fuel additive and chemical feedstock with a global market of 29 million tons per year. It would be desirable to offset the enormous volume of petroleum-derived toluene with biochemically produced toluene made from a renewable resource (such as cellulosic biomass) using engineered microbes. Microbial toluene biosynthesis was reported in anoxic lake sediments more than 3 decades ago, however the enzyme(s) catalyzing this reaction have never been elucidated. Here we report the discovery of a toluene synthase (phenylacetate decarboxylase) from an anaerobic, sewage-derived enrichment culture that stoichiometrically produced toluene from phenylacetate. The discovery process (Zargar et al. 2016) included metagenome sequencing of the culture (which included more than 340,000 protein-coding genes), anaerobic FPLC (Fast Protein Liquid Chromatography) of cell-free extracts of the culture, and differential metaproteomic analyses to identify proteins present in active (toluene-producing) FPLC fractions but absent in adjacent inactive FPLC fractions (i.e., toluene synthase candidates). Toluene synthase candidates included a novel glycyl radical enzyme (GRE) of bacterial origin and its cognate activating enzyme [AE; a radical SAM (*S*-adenosyl-L-methionine) enzyme]. For functional confirmation, recombinant, N-terminally tagged, codon-optimized versions of the GRE and AE genes were expressed in *E. coli* and purified under anaerobic conditions. After *in vitro* reconstitution of the AE to restore its [4Fe-4S] cluster, its activity was confirmed *in vitro* by measuring conversion of SAM to methionine. *In vitro* assays with purified GRE, AE, and SAM were shown to successfully convert ^{13}C-labeled phenylacetate to ^{13}C-labeled toluene, whereas no toluene was produced in control assays lacking SAM or in assays containing mutant versions of GRE in which the site of the glycine radical was converted to alanine. Notably, metagenomic analysis of anoxic, toluene-producing lake sediment enrichment cultures contained nearly identical versions of the GRE and AE (>85% protein sequence identity) even though the lake sediment and sewage cultures had very different community composition. We have begun to further characterize the GRE (e.g., with respect to its activity on substituted phenylacetic acid analogs). Overall, this first-time identification of a

phenylacetate decarboxylase will ultimately enable bio-based toluene production *via* engineered microbial hosts.

References:

1. Zargar, K., R. Saville, R. Phelan, S. G. Tringe, C. J. Petzold, J. D. Keasling, and H. R. Beller. 2016. *In vitro* characterization of phenylacetate decarboxylase, a novel enzyme catalyzing toluene biosynthesis in an anaerobic microbial community. *Scientific Reports* (*Nature*) **6**, 31362 doi: 10.1038/srep31362.

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