Title: Catabolism of Methyl-3-(4-hydroxyphenyl)propionate (MHPP), a Model Substrate for Metabolic Addiction with Unexpected Implications for *p*-Coumaric Acid Catabolism in *Pseudomonas flourescens*

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Project Goals: The Pacific Northwest National Laboratory Persistence Control Scientific Focus Area is focused on developing fundamental understanding of factors governing the persistence of engineered microbial functions in rhizosphere environments. From this understanding, we will establish design principles to control the environmental niche of native rhizosphere microbes. In our first funding period, we are examining the efficacy of genome reduction and metabolic addiction to plant root exudates in environmental isolates as persistence control strategies using the bioenergy crop sorghum and defined microbial communities as a model ecosystem. Effective persistence control will lead to secure plant–microbe biosystems that promote stress-tolerant and highly productive biomass crops.

Abstract: Metabolic addiction is a tool in which survival of engineered microorganisms is restricted in the absence of an essential metabolite. Engineered addiction to bioenergy crop-specific root exudate compounds has great potential to enable the responsible use of engineered plant growth-promoting rhizobacteria in the environment, while preventing uncontrolled spread of such organisms in the environment. To date, methyl-3-(4-hydroxyphenyl)propionate (MHPP) has only been reported to be present in root exudates produced by *Sorghum bicolor* and *Brachiara pasturis*, where they are produced as biological nitrification inhibitors, and thus is an excellent model carbon source for demonstrating metabolic addiction. *Pseudomonas fluorescens* SBW25 can utilize MHPP as a sole carbon source, but the pathway for its catabolism is currently unknown. We performed a combination of RB-TnSeq, RNA-Seq, and individual gene deletions to elucidate a catabolic pathway for MHPP. We identified enzymes involved in MHPP catabolism, and now propose a catabolic pathway that funnels MHPP into the pathway responsible catabolism of *p*-coumaric acid. The *p*-coumaric acid catabolic pathway in *Pseudomonas putida* KT2440 is exceptionally similar to its analog in SBW25 at the levels of protein sequence, gene sequence, and even gene synteny. Thus, it was highly surprising that we found that several enzymes that appear to be essential for phenylpropanoid catabolism in *Pseudomonas putida* KT2440 are non-essential in SBW25. In fact, at least one appears to equally share its function with a second enzyme – with deletion of the genes encoding either enzyme resulting in a similar moderate growth defect when *p*-coumaric acid is the sole carbon source. This underscores the challenges with using genomic and phenotypic information from even

closely related organisms alone to predict the roles of orthologs in other organisms. Ultimately, the approach we piloted to identify the MHPP pathway will be used to discover pathways for additional root exudates. This will lead to identification of catabolic pathways for compounds that are fully unique to sorghum (e.g. *s*orgoleone), and thus will enable the assessment of metabolic addiction as a persistence control strategy in soil and plant ecosystems.

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