Title: Engineering *Novosphingobium aromaticivorans* to Stoichiometrically Convert S-type Aromatics into PDC

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Project Goals: The overall project aims to valorize the lignin fraction of plant biomass via chemical fractionation and depolymerization followed by conversion of the resulting mixtures of aromatic compounds into single valuable chemicals by genetically engineered bacteria. The goal of this study was to identify the key O-demethylases and ring-opening dioxygenases involved in the degradation of plant-derived aromatic compounds in *Novosphingobium aromaticivorans*.

One of the major components of plant biomass is lignin, a heterogeneous and recalcitrant aromatic polymer. A strategy to make value from lignin is to use chemical techniques to depolymerize it into mixtures of phenolic compounds and then funnel these mixtures into a single product using engineered bacteria. *Novosphingobium aromaticivorans* DSM12444 can naturally degrade multiple lignin-derived phenolic compounds and we have previously engineered it to produce 2-pyrone-4,6-dicarboxylic acid (PDC) from a variety of compounds that contain S-, G-, or H-type aromatic structures (two, one, or no methoxy groups attached to the aromatic ring, respectively). However, conversion of S-type aromatics into PDC by this engineered strain is below stoichiometric, suggesting the existence of competing metabolic routes that do not involve the production of the target product. One potential competing pathway involves aromatic O-demethylation followed by oxidative aromatic ring opening, with gallic acid as an intermediate metabolite. Since enzymes performing these type of reactions commonly have broad substrate specificity and their inactivation can potentially affect the conversion of other aromatic compounds, we investigated enzymes hypothesized to be involved in O-demethylation of syringic acid, vanillic acid, and 3-methoxygallic acid (3-MGA), and enzymes hypothesized to participate in ring opening of 3-MGA, gallic acid, and protocatechuic acid (PCA). Our results confirmed the existence of an alternative pathway for the degradation of S-type aromatics and the generally broad substrate specificity of the tested enzymes. For instance, the O-demethylase DesA was active on syringic and vanillic acids, whereas LigM was involved in O-demethylation of vanillic acid and 3-MGA. We also found evidence of a new O-demethylase transforming 3-MGA into gallic acid. Among aromatic ring opening dioxygenases, LigAB had activity with 3-MGA, gallic acid, and PCA. In addition, LigAB2 was identified as a new aromatic ring opening dioxygenase active with the same substrates.

The data obtained in this study revealed a previously uncharacterized route for metabolism of S-type aromatic compounds in *N. aromaticivorans* that involves O-demethylation of 3-MGA into gallic acid followed by aromatic ring opening. We predict that in wild-type *N. aromaticivorans* this pathway carries ~15% of the carbon flux from syringic acid, explaining the below stoichiometric transformation of S-type aromatics to PDC in engineered strains that did not block this pathway. By further inactivating O-demethylation of 3-MGA in the PDC-producing strain, we created a new engineered strain (PDC2) that stoichiometrically converts S-, G-, and H-type aromatics to PDC.

1. Jose M. Perez et al. (2019) Funneling aromatic products of chemically depolymerized lignin into 2-pyrone-4-6-dicarboxylic acid with *Novosphingobium aromaticivorans*. *Green Chemistry*, DOI:10.1039/c8gc03504k.
2. Jose M. Perez et al. (2021) Redundancy in aromatic O-demethylation and ring opening reactions in *Novosphingobium aromaticivorans* and their impact in the metabolism of plant derived phenolics. *Applied and Environmental Microbiology*. DOI:10.1128/aem.02794-20

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