Engineering *Escherichia coli* for growth on methanol through dynamic regulation and protein engineering

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Monday, August 26th, 2019 - 12:30 PM - CCM 106

Industrial microbial fermentation is frequently used to produce a wide array of desirable products, including amino acids, vitamins, recombinant proteins, pharmaceuticals, and alternative renewable fuels. Production of these compounds is most commonly accomplished utilizing glucose or other sugar substrates. Methanol has emerged in recent years as an attractive non-food feedstock option due to the increased production of natural gas. Methanol is produced from methane, the main component of natural gas, or can be produced from renewable sources at a higher cost. Methanol offers a high degree of reduction, or more electrons per carbon than sugar substrates, which translates to improved product titers, as well as a low contamination risk for large-scale fermentations. Native methylotrophic bacteria, capable of utilizing single-carbon substrates for their carbon and energy needs, have limited genetic tools to engineer the production of heterologous products. *Escherichia coli* is a model organism that has been extensively researched, has a well-developed genetic toolbox, and has been previously engineered to produce a wide array of products. For these reasons, *E. coli* is an appropriate host organism for the implementation of synthetic methylotrophy.

Attempts to engineer synthetic methylotrophy in *E. coli* have struggled with various bottlenecks. Most implementations rely on the expression of at least three heterologous enzymes for methanol assimilation. An NAD-dependent methanol dehydrogenase (Mdh) oxidizes methanol to formaldehyde, a cytotoxic compound, which is fixed to ribulose 5-phosphate (Ru5P) by 3-hexulose-6-phosphate synthase (Hps) to form hexulose 6-phosphate. Subsequent isomerization by 6-phospho-3-hexuloisomerase (Phi) generates fructose 6-phosphate, an intermediate in central carbon pathways. We target several points of weakness to improve methanol assimilation, focusing on instituting formaldehyde-based regulation in *E. coli* to avoid accumulation of the toxic intermediate, and driving methanol oxidation through redox perturbation and protein

engineering. Characterization of an *E. coli* formaldehyde-inducible promoter, P_{frm}, allows for its utilization in implementing dynamic formaldehyde regulation, driving the expression of genes involved in methanol and formaldehyde assimilation directly in response to intracellular formaldehyde concentrations. Specific genes from native methylotroph *Bacillus methanolicus* involved with the regeneration of Ru5P, necessary for formaldehyde fixation, were also expressed and placed under formaldehyde control to improve formaldehyde assimilation. Redox perturbation achieved by knocking out malate dehydrogenase, an NAD-dependent enzyme, to drive NAD-dependent methanol oxidation, resulted in a dramatically higher growth benefit with methanol on a yeast extract co-substrate. Lastly, the Mdh sourced from *Geobacillus stearothermophilus* was engineered for higher methanol activity and selectivity to drive methanol oxidation.

Together, we show these approaches to significantly improve the utilization of methanol carbon and energy in *E. coli*. Future attempts to create a pure synthetic methylotrophy will likely need to continue focusing on regulation and the adequate expression of relevant enzymes for sustained methanol assimilation and growth.