**Engineering ethylmalonyl-CoA pathway in *Methylobacterium extorquens* AM1 for butanol production: Identification of a regulator activating the expression of a key gene**

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https://depts.washington.edu/mllab/

**Project Goals: The main goal of this project is to characterize control points for assimilatory metabolism in a facultative methylotroph, *Methylobacterium extorquens* AM1. Methylotrophs have potential for conversion of reduced one-carbon compounds to value-added chemicals, but a more detailed understanding of assimilatory metabolism is necessary for that potential to be reached. This project will generate systems-level datasets to inform optimization approaches for a new platform for biofuels synthesis, tools for systems approaches via multi-level datasets, and starting strains for biofuels strain development using methanol + $CO_2$ as biofeedstocks.**

Our initial work focused on potential regulatory genes for the ethylmalonyl-CoA (EMC) pathway, the part of assimilatory metabolism that involves crotonyl-CoA as an intermediate. We have identified CcrR, a TetR-type activator, which has been shown to regulate the expression of crotonyl-CoA reductase/carboxylase, a key enzyme of EMC pathway. The *ccrR* mutant is impaired in its ability to grow on C1 and C2 compounds, correlating with the reduced activity of crotonyl-CoA reductase/carboxylase. The *ccr* gene was found to be cotranscribed with an upstream gene (*katA)* and the promoter strength was found to reduce as much as 50% in the absence of CcrR compared to that in wild type *M. extorquens* AM1. Gel retardation assays with purified His-tagged CcrR showed that Ccr binds to the promoter-regulatory region of *ccr*. A palindromic sequence upstream of *katA* at position -334 to -321 with respect to the translational start site was found to be the binding site and mutations in this region eliminated gel retardation with CcrR. These results show that CcrR stimulates expression of the *katA-ccr* promoter on the order of two-fold but is not required for this expression. The identification of a specific activator protein regulating expression of one of the genes of the EMC pathway is a first step in understanding how the pathway as a whole is regulated, and this work also generates information for manipulating flux to end products that use crotonyl-CoA as precursor, such as butanol.

As a proof of principle example for manipulation of carbon flux through crotonyl-CoA, we have initiated studies to introduce a two-step pathway into *M. extorquens* AM1 to convert crotonyl-CoA into butanol. The initial step would be reduction of crotonyl-CoA to butyryl-CoA by a crotonyl-CoA specific trans-enoyl-CoA reductase from *Treponema denticola* (Ter), coupled with another reduction step catalyzed by alcohol dehydrogenase from *Clostridium acetobutylicum* ATCC8244 (AdhE2) to produce butanol. Genes encoding Ter and AdhE2 were synthesized with codon usage optimized using a codon usage table derived from highly expressed genes of *M. extorquens* AM1 grown on methanol. Each gene was subcloned into *M. extorquens* AM1-adaptable expression vectors for expression. Enzyme assays showed that both Ter and AdhE2 activities were detected in recombinant *M. extorquens* AM1 strains containing plasmids with *ter*

or *adhE2*. The immediate future plan is to integrate both genes into a single operon with various expression capabilities to develop a strain with high butanol productivity.

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