Pathway and Protein Engineering for Improved Glucaric Acid Production in *Escherichia coli*

by

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Technical Summary

Microbial fermentation is an attractive method for the renewable production of chemicals. Glucaric acid was identified as a "top value added chemical from biomass" by the Department of Energy in 2004, and a biological route for its production from glucose in *E. coli* was developed in the Prather lab in 2009. Two pathway enzymes, *myo*-inositol phosphate synthase (MIPS) and *myo*-inositol oxygenase (MIOX), have been shown to control flux under various conditions. This work addressed several limitations of these reactions.

One approach was the relief of reactive oxygen species (ROS) to improve MIOX performance. MIOX converts *myo*-inositol (MI) to glucuronic acid. The enzyme is sensitive to hydrogen peroxide, and its mechanism involves superoxo and hydroperoxo and intermediates, but the relevance of ROS to *in vivo* glucaric acid production was unknown. We found that the addition of purified catalase to crude lysates increased MIOX activity. In addition, overexpression of *E. coli* catalase and superoxide dismutases *in vivo* led to significantly higher titers of glucuronic acid from MI. This result corresponded to improved maintenance of MIOX activity and expression over the course of the fermentation. A reduction in labile iron levels, which are linked to ROS formation via the Fenton reaction, was also shown to improve glucuronic acid titers. These methods can likely be extended to alleviate elevated ROS levels in other engineered systems.

A second approach was the examination of natural MIPS diversity. MIPS converts glucose-6-phosphate to *myo*-inositol-1-phosphate, and it competes with central carbon metabolism for its substrate. With the aid of a sequence similarity network, thirty-one representative MIPS homologs were selected from the MIPS Pfam family (PF01658). Nineteen variants produced detectible *myo*-inositol (MI) from glucose, and active variants spanned all domains of life. *H. contortus* MIPS performed equally well or better than the *S. cerevisiae* MIPS currently used in the glucaric acid pathway. Substantial differences in stability were identified between even closely related variants, and further work to explore the network may yield more information about important sequence features.

A third approach was the evaluation of screening methods for glucuronic and glucaric acid to support protein engineering. A screen based on growth from MI was previously attempted, but MI import was found to control flux. We endeavored to extend this screen to growth from glucose by engineering a strain with *pgi* and *zwf* gene knockouts. However, while growth was achieved from MI in this strain, low flux appeared to prevent growth from glucose. In addition to

the growth screen, we also tested a previously-developed biosensor based on the regulator CdaR. We discovered that the biosensor does not respond to glucaric acid but instead to a downstream metabolite, likely glycerate. In addition, we found that the biosensor is affected by catabolite repression in the presence of glucose. Neither of these findings about the biosensor's behavior had been previously reported. While a reliable screen was not realized for the glucaric acid pathway, our improved understanding of native regulation aids in the identification of alternative strategies.

This work overall produced significant improvements in the glucaric acid pathway and helped to identify opportunities for further development.

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