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Original Research Article

Engineering the biological conversion of methanol to specialty chemicals in *Escherichia coli*

W. Brian Whitaker[d,e,1], J. Andrew Jones[a,c,1], R. Kyle Bennett[d,e,1], Jacqueline E. Gonzalez[d], Victoria R. Vernacchio[a], Shannon M. Collins[a], Michael A. Palmer[d], Samuel Schmidt[d], Maciek R. Antoniewicz[d], Mattheos A. Koffas[a,b], Eleftherios T. Papoutsakis[d,e,*]

[a] *Department of Chemical and Biological Engineering, Center for Biotechnology and Interdisciplinary Studies, Rensselaer Polytechnic Institute, 110 8th St., Troy, NY 12180, USA*
[b] *Department of Biological Sciences, Center for Biotechnology and Interdisciplinary Studies, Rensselaer Polytechnic Institute, 110 8th St., Troy, NY 12180, USA*
[c] *Department of Chemistry, Hamilton College, 198 College Hill Rd., Clinton, NY 13323, USA*
[d] *Department of Chemical and Biomolecular Engineering, University of Delaware, 150 Academy St., Newark, DE 19716, USA*
[e] *The Delaware Biotechnology Institute, University of Delaware, 15 Innovation Way, Newark, DE 19711, USA*

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ABSTRACT

Methanol is an attractive substrate for biological production of chemicals and fuels. Engineering methylotrophic *Escherichia coli* as a platform organism for converting methanol to metabolites is desirable. Prior efforts to engineer methylotrophic *E. coli* were limited by methanol dehydrogenases (Mdhs) with unfavorable enzyme kinetics. We engineered *E. coli* to utilize methanol using a superior NAD-dependent Mdh from *Bacillus stearothermophilus* and ribulose monophosphate (RuMP) pathway enzymes from *B. methanolicus*. Using ^{13}C-labeling, we demonstrate this *E. coli* strain converts methanol into biomass components. For example, the key TCA cycle intermediates, succinate and malate, exhibit labeling up to 39%, while the lower glycolytic intermediate, 3-phosphoglycerate, up to 53%. Multiple carbons are labeled for each compound, demonstrating a cycling RuMP pathway for methanol assimilation to support growth. By incorporating the pathway to synthesize the flavanone naringenin, we demonstrate the first example of *in vivo* conversion of methanol into a specialty chemical in *E. coli*.

1. Introduction

Methylotrophs are organisms capable of using C1 compounds such as methane and methanol as a carbon and energy source (Whitaker et al., 2015). They represent a polyphyletic microbial group consisting of both Gram negative and Gram positive bacteria as well as methylotrophic yeasts (Anthony, 1982; Lidstrom and Stirling, 1990). Bacteria can be further categorized by the type of enzyme used to oxidize methanol: pyrroloquinoline quinone (PQQ)-dependent methanol dehydrogenase (Mdh) for Gram$^-$ bacteria and NAD-dependent Mdh for Gram$^+$ species (Whitaker et al., 2015). In both cases, oxidation of methanol yields the toxic product formaldehyde, which must then be assimilated into central metabolism. Native methylotrophs can be further categorized by the means in which they assimilate formaldehyde: either via the serine pathway, the ribulose monophosphate (RuMP) pathway or the Calvin-Benson-Bassham (CBB) cycle (Anthony, 1982; Chistoserdova et al., 2009; Lidstrom and Stirling, 1990; Whitaker et al., 2015). Both the serine and CBB pathways require an input of energy (ATP), and as such are inferior candidates for engineering synthetic methylotrophy to produce metabolites (Muller et al., 2015; Whitaker et al., 2015). The RuMP pathway consists of two core enzymes: 3-hexulose-6-phosphate synthase (Hps), which fixes formaldehyde to ribulose 5-phosphate (Ru5P) to yield hexulose 6-phosphate (Hu6P), and 6-phospho-3-hexuloisomerase (Phi), which isomerizes Hu6P to fructose 6-phosphate (F6P) (Kato et al., 2006; Yurimoto et al., 2009). There are several variations of this pathway based on how Ru5P is regenerated. Some variations generate 1 ATP and all variations generate 1 NADH per every 3 formaldehyde

* Correspondence to: University of Delaware, 15 Innovation Way Room 284, Newark, DE 19711, USA.
E-mail addresses: wbwhit@udel.edu (W.B. Whitaker), jajones@hamilton.edu (J.A. Jones), rkbenne@udel.edu (R.K. Bennett), jgonz@udel.edu (J.E. Gonzalez), vernav@rpi.edu (V.R. Vernacchio), collis6@rpi.edu (S.M. Collins), mpalmer@udel.edu (M.A. Palmer), schm@udel.edu (S. Schmidt), mranton@udel.edu (M.R. Antoniewicz), koffam@rpi.edu (M.A. Koffas), papoutsakis@dbi.udel.edu, epaps@udel.edu (E.T. Papoutsakis).
[1] These authors contributed equally to this work.

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assimilated (Kato et al., 2006; Whitaker et al., 2015; Yurimoto et al., 2009), making this the most energetically favorable pathway for heterologous hosts such as *E. coli*.

The ability to produce commodity and specialty chemicals and biofuels from methanol is hindered by the fact that most natural methylotrophs lack well-developed genetic tools for the implementation of extensive synthetic production pathways. At the same time, platform organisms such as *Escherichia coli* have been extensively engineered for superior industrial growth and production of an enormous range of useful metabolites. Biosynthesis of most useful metabolites produced by industrial organisms requires electrons in the form of NADH, and culture conditions that are largely microaerobic or anaerobic (Papoutsakis, 2015; Whitaker et al., 2015). As such, native methylotrophs are primarily confined to the production of amino acids and polyhydroxyalkanoates (Schrader et al., 2009). Recently, the serine pathway methylotroph, *Methylobacterium extorquens,* has been engineered to produce butanol from ethylamine, but not from methanol, though the butanol yields achieved by this strain are significantly lower than recombinant strains of *E. coli* or native clostridial butanol producers (Hu and Lidstrom, 2014). Thus, there is an impetus to engineer a non-native methanol utilization pathway into industrial microorganisms that would be capable of producing simple and complex metabolites using methanol as a substrate.

Efforts have recently been reported to engineer methanol utilization in *Corynebacterium glutamicum* (Leßmeier et al., 2015; Witthoff et al., 2015) and *E. coli* (Muller et al., 2015). For the *C. glutamicum* strains, methanol utilization was reported only in the presence of glucose. Using Mdh and RuMP genes from *B. methanolicus*, Muller et al. (2015) demonstrated labeling in glycolytic intermediates in *E. coli*. However, it should be noted that in their study, growth of the recombinant strain in methanol or quantifiable methanol consumption was not presented. Similarly, labeling in TCA cycle intermediates, amino acids and biomass was not presented.

Here we show that expressing a suitable NAD-dependent Mdh along with RuMP enzymes enables an engineered *E. coli* strain to convert methanol into biomass components and high-value specialty chemicals (Fig. 1). Specifically, we show that expressing the Mdh from *Bacillus stearothermophilus* in combination with the Hps and Phi from *B. methanolicus* enables the engineered *E. coli* strain to utilize methanol in the presence of low concentrations of yeast extract, resulting in superior growth with improved biomass yields. Importantly, we demonstrate, for the first time, that our methylotrophic *E. coli* utilizes methanol as a growth substrate, which leads to a 30% improvement in biomass when grown on a mixture of yeast extract and methanol compared to yeast extract alone. We show that ^{13}C from methanol is present in glycolytic and TCA cycle intermediates, free intracellular amino acids and biomass. To demonstrate the usefulness of this strain for metabolite production, we have also expressed the biosynthetic pathway for the flavonoid naringenin and show methanol-derived naringenin production by the engineered *E. coli* strain.

2. Methods

2.1. Strains and plasmids

All strains and plasmids used in this study are listed in Supplementary table 1. Characterization of the genes required for methanol assimilation in *E.* coli was performed in a BW25113 Δ*frmA* host strain. Methanol assimilation genes were expressed and characterized using the pETM6 vector that has been modified to employ the *tac* promoter upstream of the multiple cloning site. *E. coli* DH5α was used to propagate all plasmids, while the BL21star™(DE3) was used as the host for flavonoid production. The ePathBrick vectors, pETM6, pCDM4 and pACM4 were used as the basis for all plasmid construction and pathway expression (Xu et al., 2012). Appropriate antibiotics were added at the following concentrations: Ampicillin, 80 μg/mL; Streptomycin, 50 μg/mL; Chloramphenicol, 25 μg/mL.

2.2. Genetic manipulations

To insert the *tac* promoter into the pETM6 and pACM4 vectors, inverse primers (Supplementary Table 2, primers 8 and 9) containing the *tac* promoter sequence were designed to amplify the vectors, omitting the existing T7 promoter. The resulting PCR products were restriction endonuclease digested and re-circularized, yielding pM6tac. The methanol utilization pathway genes and their transcriptionally varied mutants were constructed using standard ePathBrick and ePathOptimize cloning procedures (Jones et al., 2015b; Xu et al., 2012). The flavonoid pathway was cloned from pETM6-At4CL-PhCHS to pCDM4 and pACM4 by digestion with ApaI and NheI (FastDigest, Thermo Scientific), gel purification (E.Z.N.A. MicroElute Gel Extraction Kit, Omega Bio-tek), and ligation (Rapid DNA Ligation, Thermo Scientific). Colonies were screened by restriction digest. Transcriptionally varied ePathOptimize mutants were sequenced (GENEWIZ, Inc.) to determine the specific promoters controlling expression of each gene in the pathway using primers 1–3, Supplementary Table 2. Sequencing results for ePathOptimize mutants are shown in Supplementary Table 1.

2.3. Media and growth conditions

E. coli strains were cultured in Luria Bertani (LB) medium or M9 minimal medium for methanol consumption and/or metabolite analysis. *Methylomonas* L3 was cultured as described previously (Chu and Papoutsakis, 1987). Pre-culture conditions for the methanol-assimilating strains were as follows: a single colony was picked from a plate and grown in M9 minimal medium supplemented with 5 g/L of growth substrate (glucose, xylose, tryptone or yeast extract). After overnight growth, these cultures were pelleted, washed and re-suspended in 100 mL M9 minimal medium supplemented with 60 mM methanol plus 1 g/L of an additional growth substrate and incubated in a 500 mL baffled flask at 37 °C with shaking (225 rpm). The optimal induction point was determined to be 4.5 h post inoculation for all growth experiments in BL21star™(DE3). Scale-up batch cultures were performed as above, except that cultures were grown in 1.5 L in a 4 L bioreactor (Bioflow II and 110, New Brunswick Scientific, Edison, NJ, USA). The pH was monitored and adjusted to 7 using 2 N NaOH, temperature was maintained at 37 °C, agitation was maintained at 200 rpm and airflow was maintained at ca. 0.67 vvm. Methanol was maintained at ca. 80 mM by feeding methanol every 24 h. The methanol evaporation rate, along with any native methanol oxidation, was determined from cultures of the empty vector control strain (Δ*frmA* pM6tac), and the data were used to adjust the biomass yield and uptake rate of methanol in the methanol-assimilating strain in both flask cultures and bioreactors.

To quantify the effect of methanol on the recombinant *E. coli* strain, the following growth parameters were calculated. To calculate the biomass yield on a substrate, S, (e.g., YE) (Y_{SX}; gCDW/gS), Eq. (1) was employed, where X and S represent the concentrations of biomass and substrate, respectively. Cell dry weight (CDW; in g/L) was determined using the conversion factor (Soini et al., 2008): 1 OD_{600} unit=0.33 CDW. For biomass yield on methanol (Y_{MX}; gCDW/gMeOH), Eq. (2) was employed, where M represents the concentration of methanol. Y_{MX} was based on the assumption that the total methanol consumption accounted for all of the additional biomass in co-substrate cultures. For the specific methanol uptake rate (q_M; gMeOH/gCDW·h), Eq. (3) was employed, which determined methanol uptake of resting cells.

$$Y_{SX}=\frac{dX/dt}{dS/dt}=\frac{X}{S}(gCDW/gS) \quad (1)$$